Functional characterisation of a fungal endonuclease effector and regulated host cell death

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Zusammenfassung

Der mutualistische Wurzelendophyt *Serendipita indica* fördert das Pflanzenwachstum und führt zu erhöhter Resistenz gegen abiotische und biotische Stressfaktoren in vielen experimentellen Wirtspflanzen. *S. indica* hat sich durch seine Anpassungsfähigkeit, sein großes Wirtsspektrum und die Fähigkeit auch in axenischen Kulturen wachsen zu können, zum Modellorganismus der Pilzordnung Sebacinales entwickelt. Zusätzlich ist das Genom sequenziert und der Pilz ist transformierbar. Um die molekularen Werkzeuge für die funktionelle Charakterisierung von Effektorproteinen weiterzuentwickeln, wurde ein Proteinproduktionssystem mit Hilfe des induzierbaren *Si*FGB1 Promotors zur Aufreinigung von sekretierten Proteinen in *S. indica* etabliert. Modulare Vektoren und Kulturkonditionen wurden für die Expression und Sekretion von homologen und heterologen Proteinen verbessert. Außerdem wurde ein Gendeletionssystem basierend auf homologer Rekombination mit einer geteilten Resistenzkassette in *S. indica* entwickelt.

S. indica löst nach einer anfänglichen biotrophen Phase Zelltod während der kompatiblen Pflanzenbesiedlung aus. Die pilzlichen Induktoren und Effektoren, welche diesen Wurzelzelltod initiieren und regulieren sind unbekannt. Um den vom Pilz verursachten Wurzelzelltodmechanismus besser zu charakterisieren, wurde die Auflösung der Nuklei in kolonisierten Arabidopsiswurzeln mittels verschiedener Färbungen unter dem konfokalen Laserrastermikroskop analysiert. Außerdem wurde eine pilzliche, sekretierte Endonuklease, genannt *Si*NUCA, im apoplastischen Fluid kolonisierter Gerstenwurzeln gefunden. *Si*NUCA wurde funktionell charakterisiert und es wurde überprüft, ob das Protein in der Zelltodreaktion involviert ist. *Si*NUCA ist ein konserviertes, kleines sekretiertes Protein, welches zur Superfamilie der His-Me Finger Endonukleasen gehört. Das Protein wurde über heterologe Expression in *Ustilago maydis* und über Proteinaufreinigung aus dem Kulturfiltrat von *S. indica* charakterisiert und es wurde gezeigt, dass *Si*NUCA zuckerunspezifische Nukleaseaktivität hat. Ektopische Expression in Arabidopsis führte zu einem erhöhten Kolonisierungslevel von *S. indica* und mehr Wurzelzelltod. *Si*NUCA lokalisierte in späten Kolonisierungsphasen im Pflanzennukleus, wo das Protein Zelltod ausführen könnte. Im Apoplast könnte *Si*NUCA an der Inaktivierung der extrazellulären DNA, welche ein sogenanntes «damage-associated molecular pattern» ist, beteiligt sein.

Abstract

The mutualistic root endophyte *Serendipita indica* leads to growth promotion and enhanced resistance to abiotic and biotic stresses in many experimental hosts. Due to its versatility, broad host range and ability to grow in axenic culture *S. indica* emerged as the model species in the fungal order Sebacinales. Furthermore, its genome is available and the fungus is genetically tractable. To further develop the molecular tools for functional characterisation of effector proteins a protein production system for purification of secreted proteins was established in *S. indica* using the inducible promoter *Si*FGB1. Modular vectors and culture conditions were improved for expression and secretion of homologous and heterologous proteins. In addition, gene deletion via homologous recombination using a split resistance cassette was developed in *S. indica*.

After an initial biotrophic phase, *S. indica* triggers cell death during compatible root colonisation. The fungal elicitors and effectors, which initiate and regulate this root cell death are unknown. To better characterise the mechanisms involved in fungal-mediated root cell death nuclei disappearance was analysed in colonised Arabidopsis roots with different stainings by confocal laser scanning microscopy. Additionally, a fungal-secreted endonuclease named *Si*NUCA was found in the apoplastic fluid of colonised barley roots, functionally characterised and its involvement in cell death examined. *Si*NUCA revealed to be a conserved small secreted protein belonging to the His-Me finger endonucleases. Characterisation of *Si*NUCA by heterologous expression in *Ustilago maydis* and by purification of the protein from *S. indica* culture filtrate showed that *Si*NUCA has sugar non-specific nuclease activity. Ectopic expression in Arabidopsis led to higher colonisation levels and more root cell death. *Si*NUCA localised to the plant nucleus at later colonisation stages where it could function as an executor of plant cell death. In the apoplast, *Si*NUCA could be involved in inactivation of the damage-associated molecular pattern extracellular DNA.

List of Abbreviations

ADP	Adenosine diphosphate
AF	Alexa fluor
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
APF	Apoplastic fluid
ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
BLC	Border- like cells
bp	base pair
c.v.	cultivar
ca.	circa
Cas 9	CRISPR associated protein 9
cDNA	complementary DNA
CLSM	Confocal laser scanning microscopy
CM	Complete medium
CRISPR	Clustered Regularly Interspaced Short Palindromic Repeats
dAdo	2'-deoxyadenosine
DAMP	Damage-associated molecular pattern
dAMP	2'-deoxyadenosine monophosphate
DAPI	4',6-Diamidine-2'-phenylindole dihydrochloride
dd	double distilled
DEPC	Diethyl pyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
dNTP	nucleoside triphosphate
dpi	days post inoculation
DTT	dithiothreitol
DUF	Domain of unknown function
eATP	extracellular ATP
EDTA	Ethylenediaminetetraacetic acid
ER	Endoplasmic reticulum
ETI	Effector-triggered immunity
exDNA	extracellular DNA
FGB1	Fungal Glucan-Binding 1
FITC	fluorescein isothiocyanate
GA	Gibberellic acid
gDNA	genomic DNA
GFP	Green fluorescent protein
GPD	glyceraldehyde-3-phosphate dehydrogenase
kDa	kilodalton
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
MAMP	Microbe-associated molecular pattern
MES	2-(*N*-morpholino)ethanesulfonic acid
mRFP	monomeric red fluorescent protein
MTI	MAMP-triggered immunity
NET	Nucleic acid extracellular trap
NUCA	Nuclease A
OD_{600}	Optical density at a wavelength of 600 nm
PBS	phosphate-buffered saline
PCR	Polymerase Chain Reaction
PEG	polyethylene glycol
PRR	Pattern-recognition receptor
qPCR	quantitative PCR
RACE-PCR	Rapid amplification of cDNA-ends with PCR
RCD	Regulated cell death
RNA	Ribonucleic acid
RNAi	RNA interference
RNase	Ribonuclease
ROS	Reactive oxygen species
RT	Room temperature
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SMART	Simple modular architecture research tool
SNP	Single nucleotide polymorphism
SP	Signal peptide
sp.	species
SSP	Small secreted protein
ssp.	subspecies
TEF	Translation elongation factor 1-a
UBI	Ubiquitin
UV	ultraviolet
v/v	volume per volume
VPE	Vacuolar processing enzyme
w/o	without
w/v	weight per volume
WGA	Wheat Germ Agglutinin
wt	wildtype
YNB	Yeast nitrogen base

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1 Introduction

1.1 The fungal order Sebacinales-from saprotrophic to mycorrhizal fungi

Organisms do not live on their own but rather in constant exchange with each other in a dynamic environment. The symbiosis between two species can range from antagonism (negative effect for both partners) over parasitism (one-sided profit) to mutualism (beneficial for both sides) (Martin & Schwab, 2013). Around 85 % of the land plants live in constant symbiosis with mycorrhizal fungi (Brundrett, 2009). Mycorrhizal fungi facultatively associate with their plant host or live as obligate biotrophs meaning that fungal growth and proliferation is plant host-dependent (Brundrett & Tedersoo, 2018). They make specialised structures for nutrient exchange termed arbuscules (arbuscular mycorrhiza), the hartig net (ectomycorrhiza) or hyphal coils (ericoid and orchid mycorrhiza) (Brundrett & Tedersoo, 2018).

In general, inorganic phosphate and nitrogen in form of ammonium, nitrate, urea or amino acids is taken up from the fungus and given to the plant through specific phosphate and ammonium/nitrate transporters at the plant-fungus interface (Bonfante & Genre, 2010; Parniske, 2008). In exchange, the fungus receives photosynthetically fixed carbon via hexose transporters (Bonfante & Genre, 2010; Parniske, 2008). The plant does not only benefit from nutrients but also from an increased tolerance to abiotic stresses like drought and heat and increased resistance to soil-borne pathogens, nematodes and feeding insects (Cameron et al., 2013; Jung et al., 2012; Latef et al., 2016).

The fungal order Sebacinales (Agaricomycetes, Basidiomycota) contains fungi with saprotrophic, endophytic and mycorrhizal lifestyles (Weiss et al., 2016). In the Basidiomycota, the Sebacinales is the most basal branch with mycorrhizae and the more derived forms are obligate biotrophs (Weiss et al., 2004; Weiss et al., 2011). Therefore, a nutritional shift from saprotrophy to obligate biotrophy was suggested in this group (Weiss et al., 2004; Weiss et al., 2011). Fungi of the order Sebacinales can be found in different habitats worldwide, are highly abundant, and have a broad host range (Garnica et al., 2013; Oberwinkler et al., 2013; Weiss et al., 2004; Weiss et al., 2011). This suggests that they play a hidden but important role in natural and cultivated ecosystems (Weiss et al., 2011). The root endophyte *Serendipita indica* (basionym *Piriformospora indica*) is

the best characterised species of the Sebacinales because it is genetically tractable and thus, represents a model for this order (Gill et al., 2016; Weiss et al., 2016).

1.2 The root endophyte *Serendipita indica*

S. indica was originally isolated with an arbuscular mycorrhizal spore of *Glomus mosseae* from the woody shrubs *Prosopis juliflora* and *Ziziphus nummularia* in the Indian desert (Verma et al., 1998). This fungus is able to colonise a broad range of experimental plant hosts as an endophyte but can also saprotrophically grow in the absence of a living host (Franken, 2012; Varma et al., 1999). Among the tested plant hosts are the crops wheat, barley, maize and tomato, medicinal plants like *Spilanthes calva* and *Withania somnifera,* and the non-mycorrhizal model plant *Arabidopsis thaliana* (Fakhro et al., 2010; Peškan-Berghöfer et al., 2004; Rai et al., 2001; Serfling et al., 2007; Varma et al., 1999; Waller et al., 2005). *S. indica* plant colonisation results in plant growth promotion, improved development and seed germination as shown for various plant hosts (Franken, 2012). Moreover, protection against abiotic stresses such as drought and salt in addition to increased local and systemic resistance to pathogens can be observed (Molitor et al., 2011; Saddique et al., 2018; Sherameti et al., 2008; Sun et al., 2014; Waller et al., 2005).

S. indica-colonised Chickpea and rice show elevated nitrogen, phosphate and potassium respectively phosphate and zinc levels (Nautiyal et al., 2010; Saddique et al., 2018). Furthermore, the phosphate amount is higher in shoots of colonised maize compared to non-colonised plants and plants colonised with a *S. indica* knock-down mutant of a high affinity phosphate transporter (Yadav et al., 2010). *S. indica* colonisation also leads to higher biomass and nitrogen levels in tobacco seedlings where *S. indica* induces a nitrate reductase (Sherameti et al., 2005). Direct phosphate uptake was shown for Arabidopsis and maize: higher levels of radioactively labelled phosphate were detected in colonised plants (Bakshi et al., 2015; Shahollari et al., 2005; Yadav et al., 2010). However, the phosphate or nitrogen content was not enhanced with *S. indica* colonisation in *Nicotiana attenuata* and barley (Achatz et al., 2010; Barazani et al., 2005). Dependency of phosphate uptake on phosphate concentration in the medium was demonstrated in maize but could not be confirmed in Arabidopsis (Bakshi et al.,

2015; Kumar et al., 2011). Thus, it was hypothesised that nutrient uptake is dependent on the plant host and development stage (Gill et al., 2016).

S. indica colonises the plant root extracellularly and intra- and intercellularly the epidermal cells, cortex cells and root hairs but not the vascular tissue or endodermis (Deshmukh et al., 2006; Jacobs et al., 2011). Therefore, fungal growth is restricted to the roots and is not systemic. In barley and Arabidopsis, colonisation mainly occurs at the maturation zone but rarely at the elongation and meristematic zone (Deshmukh et al., 2006; Jacobs et al., 2011). Cells are directly penetrated without specific structures and colonisation increases with root maturation, which is in contrast to mycorrhizal colonisation (Deshmukh et al., 2006; Gill et al., 2016; Jacobs et al., 2011; Qiang et al., 2012a) (Fig. 1.1).

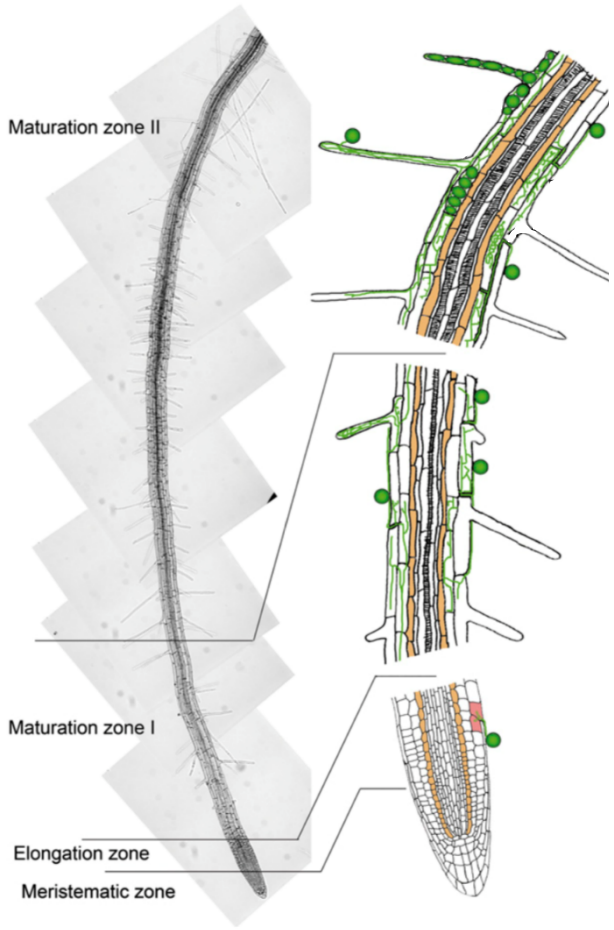

Fig.1.1: *S. indica* colonisation pattern in Arabidopsis roots.
S. indica (green) colonises root hairs, epidermal and cortex cells but cannot enter the endodermal layer (brown) and the vasculature. Mainly the maturation zones I and II and not the elongation and meristematic zones are colonised.
(adapted from Jacobs et al., 2011)

Zuccaro et al. established a protoplast-mediated *S. indica* transformation system in 2009 for stable random genome integration of a linearised plasmid (Zuccaro et al., 2009). For selection of the transformants the Hygromycin B or Geneticin resistance cassette driven by the *S. indica* translation elongation factor 1-a (TEF) or glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter were used. Gene silencing based on RNA interference (RNAi) was introduced in 2012 by Hilbert et al. The genome was sequenced in 2011 and transcriptomic analysis of fungal growth *in planta* was performed (Lahrmann et al., 2013; Lahrmann et al., 2015; Zuccaro et al., 2011).

S. indica harbours a heterokaryotic mycelium with two nuclei (Zuccaro et al., 2011). Only the asexual lifecycle with chlamydospore formation but never karyogamy, followed by meiosis to obtain basidiospores with germinating homokaryotic mycelium, has been observed so far. However, homokaryotic mycelium formation can be artificially achieved by fungal transformation because in some cases one of the two nuclei gets lost during the protoplast formation and regeneration process (Wawra et al., 2016). Homokaryotic and dikaryotic strains harbouring a random integrated Geneticin resistance cassette in the genome were analysed for their saprotrophic and endophytic growth behaviour (Bachelor thesis Griebel, 2016). Interestingly, in all obtained homokaryotic strains the same mating type is detected meaning that always protoplasts with the same nuclear type are regenerating after transformation. Homokaryotic strains are impaired in growth on plates but not in colonising barley roots seven days post inoculation (dpi) (Bachelor thesis Griebel, 2016). The genome of the homokaryotic strain used as reference strain in Wawra et al. 2016 is sequenced. Comparison to the dikaryotic wildtype genome from Zuccaro et al. 2011 reveals the absence of ca. 40 genes in this homokaryotic strain (personal communication Dr. G. Langen). The missing genes code for hypothetical proteins without functional domains, which could be interesting candidates for characterisation. These genes could be responsible for the inability of one of the nuclear types to regenerate or for the homokaryotic saprotrophic growth phenotype.

1.3 *S. indica* requires regulated host cell death for development *in planta*

S. indica has a biphasic lifestyle with an early biotrophic and a later cell death-associated phase (Deshmukh et al., 2006; Jacobs et al., 2011; Zuccaro et al., 2011). In the first stage, the invasive hyphae are embedded by the host plasma membrane and the plant cell stays alive (Jacobs et al., 2011). In the second stage, *S. indica* grows and makes chlamydospores in dead cells (Deshmukh et al., 2006; Lahrmann et al., 2013). However, this host cell death is restricted to colonised cells and necrosis or tissue browning has never been observed (Deshmukh et al., 2006; Jacobs et al., 2011; Lahrmann et al., 2013). Plant development is not harmed in the long-lasting symbiosis (Deshmukh et al., 2006; Jacobs et al., 2011; Lahrmann et al., 2013).

At the transition from the biotrophic to the cell death- associated phase *S. indica* undergoes a transcriptional and nutritional reprogramming (Lahrmann et al., 2013; Zuccaro et al., 2011). This reprogramming is activated by nitrogen depletion and leads to induction of fungal hydrolytic enzymes and nutrient transporters around four dpi (Lahrmann et al., 2013; Zuccaro et al., 2011) (Fig. 1.2). The fungal high-affinity ammonium transporter Amt1 might work as nitrogen sensor because down regulation of *Amt1* leads to a prolonged biotrophic phase in barley (Lahrmann et al., 2013). BAX inhibitor-1 (BI-1) is a conserved suppressor of cell death, which regulates endoplasmic reticulum (ER) stress supporting cell integrity under stress conditions (Gill et al., 2016; Hückelhoven, 2004). *S. indica* suppresses BI-1 in barley from five dpi on and colonisation is reduced in plants overexpressing BI-1 (Deshmukh et al., 2006). Therefore, Deshmukh et al. proposed that *S. indica* needs to interact with host cell death for its development (Deshmukh et al., 2006).

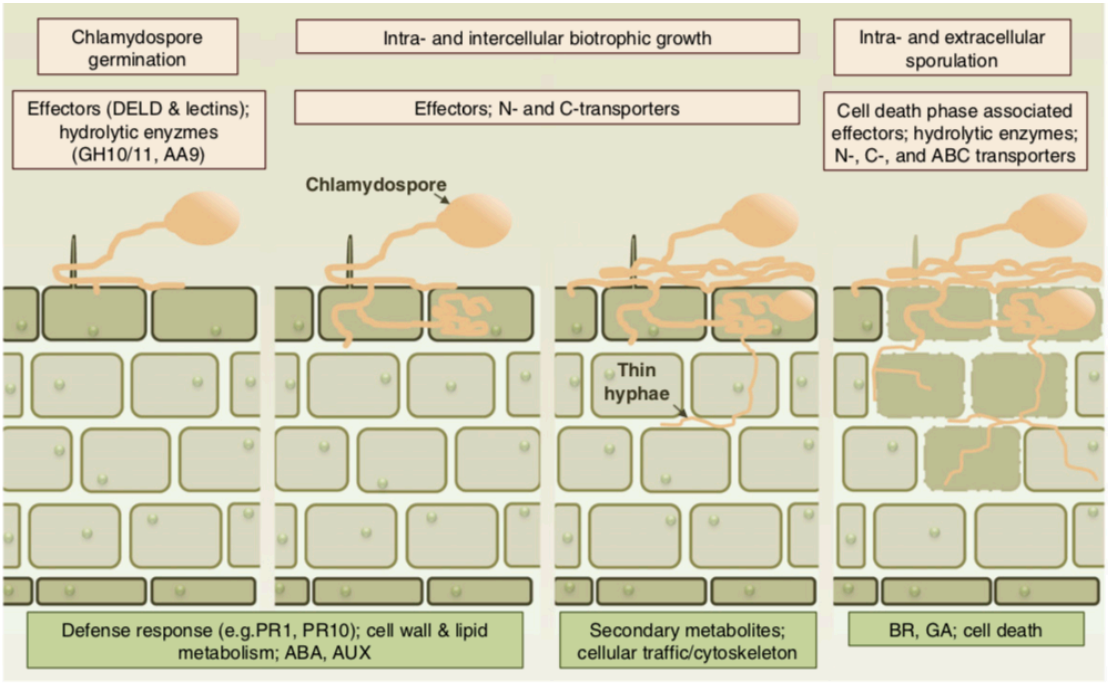

Fig.1.2: Lifestyle of *S. indica* in the host barley.

Chlamydospores germinate and emerging hyphae attach to and enter an epidermal root cell without forming specific structures. Effectors and hydrolytic enzymes are needed in this phase to penetrate the root cell. The plant reacts with defence responses including pathogenesis- related (PR) proteins and the phytohormones abscisic acid (ABA) and auxin (AUX). Consequently, *S. indica* suppresses the plant immune response secreting specific effectors and grows biotrophically in and between epidermal and cortex cells. Moreover, fungal nitrogen (N) and carbohydrate (C) transporters are upregulated. After a few days, a switch to the cell death-associated phase occurs where the fungus secretes effectors and hydrolytic enzymes. This leads to changes in brassinosteroide (BR) and gibberellic acid (GA) metabolism on the plant side. Subsequently, colonised cells are dying and the fungus produces chlamydospores intra- or extracellularly to fulfil its lifecycle.
(adapted from Zuccaro and Lahrmann, 2014)

In Arabidopsis, cell death is observed from three dpi onwards (Jacobs et al., 2011). Gibberellic acid (GA) levels negatively correlate with the expression levels of BOI (Botrytis Susceptible1 Interactor), which negatively regulates cell death (Jacobs et al., 2011; Luo et al., 2010). Mutants deficient in GA biosynthesis are less colonised by *S. indica* at later colonisation stages and in accordance with this Arabidopsis mutants with constant GA signalling have a higher colonisation rate (Jacobs et al., 2011). Therefore, Jacobs et al. hypothesised that *S. indica* recruits GA, which leads to lower BOI levels and a reduced threshold for cell death (Jacobs et al., 2011). Cytological studies in Arabidopsis

roots revealed disintegration of the ER net and globular ER structures at the end of the biotrophic phase of colonised cells, which leads to vacuole collapse and consequently cell death (Qiang et al., 2012b). A marker for cell death in roots is the activity of the vacuolar processing enzyme (VPE) (Qiang et al., 2012b). VPE is a cysteine proteinase processing vacuolar proteins that arouses vacuolar rupture and cell death (Hatsugai et al., 2015). Its enzymatic activity resembles caspase 1- a key cysteine protease in animal cell death (Hatsugai et al., 2015). At 7 dpi (but not at 3 dpi) VPE and caspase 1-like activity is enhanced in *S. indica*-colonised Arabidopsis roots, which coincides with less cell viability detected by fluorescein diacetate (Lahrmann et al., 2013; Qiang et al., 2012b). In accordance, a *vpe-null* mutant is better colonised at 3 dpi and less colonised at 7 dpi (Qiang et al., 2012b). Qiang et al. showed that mainly γVPE of the four VPEs in Arabidopsis is involved in *S. indica* colonisation (Qiang et al., 2012b). Furthermore, colonised barley roots also have enhanced VPE activity (Lahrmann et al., 2013). Qiang et al. suggested that *S. indica* enhances ER stress and concurrently suppresses the adaptive unfolded protein response, which is supposed to support the ER under stress conditions (Bao & Howell, 2017; Qiang et al., 2012b). As a consequence, a VPE-dependent cell death is conducted, which is essential for later colonisation stages (Qiang et al., 2012b) (Fig. 1.3).

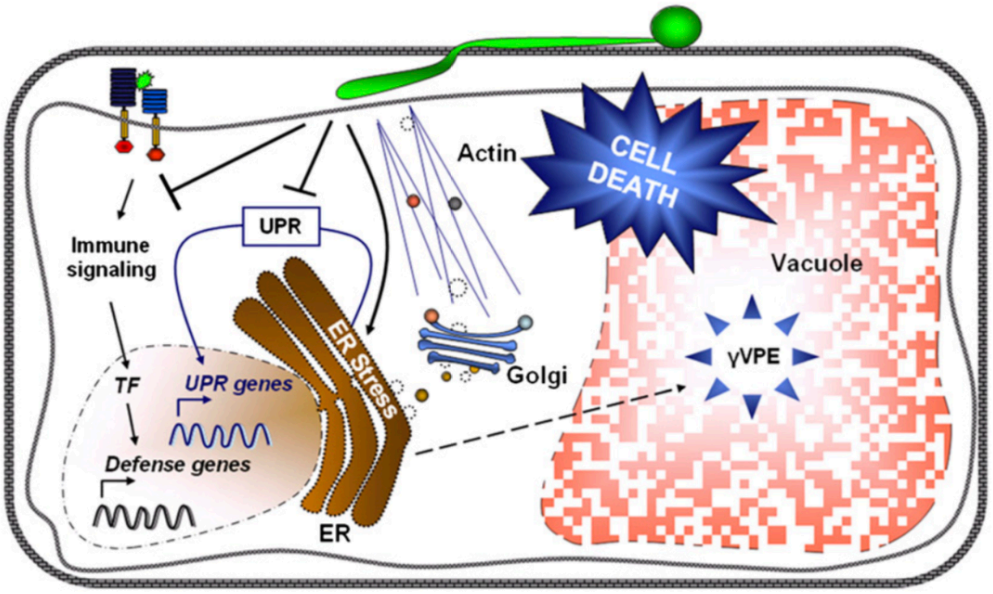

Fig.1.3: Model for cell death-associated Arabidopsis root colonisation by *S. indica.*

S. indica interacts with the immune system for example by interacting with the GA signalling to prepare for root cell death. *S. indica* enhances ER stress and inhibits the adaptive unfolded protein response (UPR) at the same time. This leads to ER swelling followed by vacuolar collapse and local cell death where VPE and caspase 1-like enzymes are active. Mainly γVPE of the four VPEs in Arabidopsis is involved.

TF= transcription factor.

(adapted from Qiang et al. 2012b)

There are several types of cell death described in plants. Regulated cell death (RCD) has important functions in plant development, homeostasis and in coping with (a)biotic stresses (Galluzzi et al., 2018; Kabbage et al., 2017). In many papers programmed cell death is referred to apoptotic-like cell death, which shows characteristics like chromatin condensation, DNA laddering, cell shrinkage and maintenance of plasma membrane integrity. (Dickman & Fluhr, 2013; Galluzzi et al., 2018). Recently, Distefano et al. described an iron-dependent cell death in plants called ferroptosis-like cell death (Distefano et al., 2017). Ferroptosis-like cell death is induced in plant roots by heat shock and leads to reduction of glutathione and ascorbic acid and an increase in reactive oxygen species (ROS) (Distefano et al., 2017).

Autophagy is described as a process where proteins or whole organelles are sequestered in autophagosomes and are finally degraded in vacuoles (Kabbage et al., 2017). It is an effective mechanism to gain biosynthetic building blocks under stress conditions and as a consequence to maintain energy homeostasis (Kabbage et al., 2017). Emerging data suggest that autophagy is a key regulator in the tightly controlled RCD, which can result in cell survival or cell death (Üstün et al., 2017). A set of conserved genes named autophagy related genes (Atg) are involved in this regulation (Üstün et al., 2017). Moreover, autophagy regulates basal immunity and hypersensitive response (HR) thus, connecting autophagy and plant immunity (Leary et al., 2017; Y. Liu et al., 2005). Autophagy can suppress RCD induced by necrotrophic pathogens and promotes locally restricted HR triggered by biotrophic pathogens (Üstün et al., 2017). On the other side, the RCD machinery can be hijacked by necrotrophic pathogens, which feed on dead host cells (Kabbage et al., 2017). *Sclerotinia sclerotiorum* is a necrotrophic pathogen that produces oxalic acid as a key virulence toxin, which leads to apoptotic-like cell death in

the hosts (Kim et al., 2008). Kabbage et al. showed that an oxalic acid deficient mutant is not pathogenic and exhibits a RCD type proved to be autophagy (Kabbage et al., 2013). Therefore, different types of RCD can lead to disease susceptibility or resistance and it also highly depends on whether the plant or the pathogen is controlling RCD (Bagniewska-Zadworna & Arasimowicz-Jelonek, 2016; Kabbage et al., 2017; Leary et al., 2017).

S. indica-colonised dead cells do not show accumulation of ROS or enhanced autofluorescence caused by phenolic compounds proposing that the RCD type is not HR (Deshmukh et al., 2006; Jacobs et al., 2011). Moreover, *S. indica* was able to better colonise Arabidopsis mutants compromised in autophagy suggesting that autophagy contains *S. indica* and its triggered RCD (Fig. 1.4). Deshmukh et al. observed DNA laddering and rarely DNA double strand breaks giving evidence for an apoptosis-like cell death (Deshmukh et al., 2006). It is unknown if the same kind of RCD is induced in different plant hosts. Furthermore, we do not know the *S. indica* elicitors and effectors that initiate and regulate RCD and if the metabolic status of the host effects the *S. indica* cell death-associated phase.

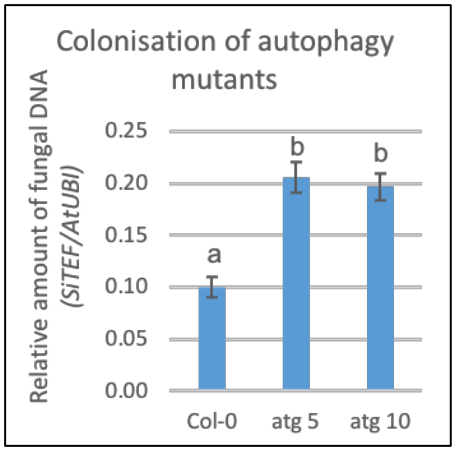

Fig.1.4: Colonisation of Arabidopsis autophagy mutant lines with *S. indica.*

Roots of Arabidopsis Col-0, *atg 5* and *atg 10* lines inoculated with *S. indica* wt and analysed 14 dpi. Colonisation rate detected as relative amount of fungal to plant DNA (measured as ratio of *SiTEF* to *AtUBI)*. ANOVA with posthoc Tukey test $p < 0.01$.

(data provided by Lisa Leson)

1.4 The orchid mycorrhizal fungus *Serendipita vermifera*

Serendipita vermifera (MAFF 305830), basionym *Sebacina vermifera*, is an orchid mycorrhizal fungus isolated from the Australian orchid *Cyrtostylis reniformis* and is the closest known homologue of *S. indica* (Lahrmann et al., 2015; Warcup, 1988). *S. vermifera* can grow as saprotroph and as endophyte in various experimental hosts and

analysis of its genome and transcriptome in Arabidopsis revealed a similar lifestyle to *S. indica* (Kohler et al., 2015; Lahrmann et al., 2015). Barley colonisation with *S. vermifera* isolates increases shoot length, shoot weight and biotic resistance (Deshmukh et al., 2006). Inoculation of *N. attenuata* seeds results in growth promotion, earlier flowering and higher seed production (Barazani et al., 2005; Barazani et al., 2007). Moreover, inoculation of switchgrass under drought stress and Arabidopsis roots with *S. vermifera* leads to growth promotion (Ghimire et al., 2009; Ghimire & Craven, 2011; Lahrmann et al., 2015). On top, the genome was sequenced and the *S. indica* transformation protocol was adapted for *S. vermifera* in 2015 (Kohler et al., 2015; Lahrmann et al., 2015).

1.5 Effector proteins and mutualism

Microbes are recognised by conserved molecules called microbe-associated molecular patterns (MAMPs) whereby chitin for fungi and flagellin for bacteria are the best studied examples (Jones & Dangl, 2006; Yu et al., 2017). Moreover, damage-associated molecular patterns (DAMPs) are released by the host upon cellular damage (Choi & Klessig, 2016). MAMPs and DAMPs are specifically perceived by the plant through pattern-recognition receptors (PRRs) and as a consequence a fast plant response including calcium influx into the cytoplasm and production of extracellular ROS is activated (Choi & Klessig, 2016; Couto & Zipfel, 2016). Signal transduction cascades are followed, which lead to transcriptional changes of defence-related genes and to hormone signalling (Couto & Zipfel, 2016; Yu et al., 2017). This basal and undirected immune response is successful against non-adapted microbes and called MAMP-triggered immunity (MTI) (Jones & Dangl, 2006).

In order to overcome MTI, microbes secrete effectors into the apoplast (apoplastic effectors) or are alternatively translocated into the host cell (cytoplasmic effectors) (Win et al., 2012). In general, effectors bind host targets to alter plant processes, which results in propagation in the host (called effector-triggered susceptibility) (Jones & Dangl, 2006). Traditionally, effectors are characterised as small secreted proteins (SSP) with a size of less than 300 amino acids that possess a signal peptide (SP) but lack conserved domains (Lo Presti et al., 2015). Moreover, many SSP are cysteine rich for the formation of stabilising disulphide bridges (Lo Presti et al., 2015). Effectors are highly variable and dispensable, and can be host, colonisation stage and organ specific (Lo Presti et al., 2015;

Rövenich et al., 2014; Toruno et al., 2016). If apoplastic effectors are recognised by receptor-like proteins on the cell surface a plant response is triggered, which ends in effector-triggered defence (Stotz et al., 2014). Cytoplasmic effectors might be intracellularly sensed by nucleotide binding-leucine rich repeat receptors, which leads to effector-triggered immunity (ETI). ETI typically culminates in HR with local cell death (Selin et al., 2016; Win et al., 2012).

Under natural conditions a plant is not interacting with one pathogen but with a whole bunch of different microbes with various lifestyles - its microbiome (Hacquard et al., 2017; Vandenkoornhuyse et al., 2015). The plant can dynamically shape its microbial community, which results in higher stress resistance (Vandenkoornhuyse et al., 2015). Along with this, MTI is needed to limit microbial growth and to control long term colonisation (Fesel & Zuccaro, 2016; Hacquard et al., 2017). It was shown that indolic glucosinolates, as part of plant innate immunity, are necessary to maintain a mutualistic interaction of the root endophytes *S. indica, S. vermifera* and *Colletotrichum tofialdiae* with Arabidopsis (Hiruma et al., 2016; Lahrmann et al., 2015; Nongbri et al., 2012). Therefore, not only pathogens but also mutualists need to interact with the plant immune system to establish their symbiosis with the host (Rövenich et al., 2014; Zipfel & Oldroyd, 2017). *S. indica* can actively suppress the plant immune system for example by reduction of the MAMP-induced oxidative burst (Jacobs et al., 2011; Schafer et al., 2009).

Therefore, the question arose how the plant immune system can distinguish between beneficial and pathogenic invaders (Plett & Martin, 2015). Little is known but it was suggested that PRRs for MAMP/ DAMP perception and nutrient monitoring on the plant side and symbiotic factors, effector proteins and small interfering RNAs on the microbe side might play key roles (Plett & Martin, 2018; Zipfel & Oldroyd, 2017). Banhara et al. showed that *S. indica* plant colonisation is independent of plant-common symbiosis genes, which are required for arbuscular mycorrhizae and root nodule formation of nitrogen-fixing bacteria (Banhara et al., 2015). Compared to pathogenic fungi only few effector proteins of mycorrhizal fungi have been characterised so far (Casarrubia et al., 2018; Kloppholz et al., 2011; Plett et al., 2011; Tsuzuki et al., 2016; Zhang et al., 2018). Two functionally characterised effector proteins are published for the root endophyte *S. indica*. PIIN_08944 was suggested to be involved in root colonisation by suppressing

MTI responses (Akum et al., 2015). The fungal specific lectin FGB1 was shown to modify its cell wall and to suppress MTI too (Wawra et al., 2016).

The ability of *S. indica* to colonise various hosts requires adaptation and specific response to different plant signals (Jacobs et al., 2011; Lahrmann et al., 2013; Lahrmann et al., 2015; Zuccaro et al., 2011). Effector proteins are proposed to have a key role in these processes (Lahrmann et al., 2013; Lahrmann et al., 2015; Lahrmann & Zuccaro, 2012; Zuccaro et al., 2011). Lahrmann et al. showed that different fungal genes are upregulated depending on the colonisation stage and plant host (Lahrmann et al., 2013). 463 of the genes induced *in planta* are putative secreted proteins with 216 of them are SSPs (Lahrmann et al., 2013) (Fig. 1.5). 562 putative effectors were detected in *S. vermifera* with 112 being upregulated in Arabidopsis at different colonisation time points (Lahrmann et al., 2015). 104 orthologous SSPs were found in *S. indica* with 17 of them transcriptionally induced in Arabidopsis suggesting that these are conserved core effectors (Lahrmann et al., 2015). Moreover, fungal proteins in the apoplastic fluid (APF) of inoculated barley roots were analysed by a proteomics approach (Wawra et al., 2016). 102 fungal proteins are specifically found in the APF and are not secreted in axenic culture (Nizam et al., 2018). There are different proteins present depending on the colonisation stage. The combination of transcriptomic and proteomic data offers good effector candidates for further functional analysis in *S. indica* plant colonisation.

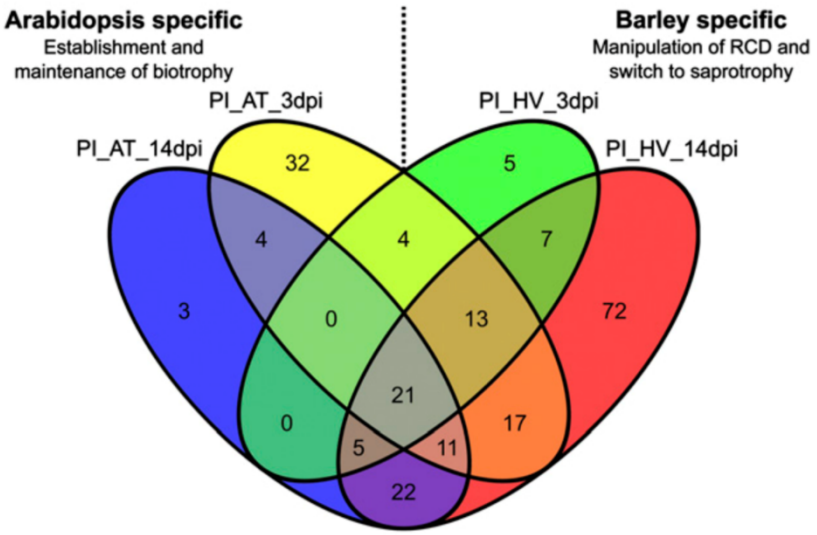

Fig.1.5: *S. indica* expression of SSPs is host and colonisation phase dependent.
Arabidopsis (PI_AT) and barley (PI_HV) colonised with *S. indica.* Number of SSPs induced *in planta* at 3 and 14 dpi detected by a microarray chip.
(adapted from Lahrmann et al. 2013)

1.6 Fungal protein production systems

Heterologous protein expression and purification is routinely applied to characterise proteins of interest in fundamental science and is also the method of choice in industrial protein production. Biopharmaceuticals like vaccines, antibodies and hormones, and enzymes for many industrial processes are produced in heterologous protein production systems (Adrio & Demain, 2010; Berlec & Strukelj, 2013; Corchero et al., 2013; Demain & Vaishnav, 2009). The most utilised organism is *Escherichia coli*, which is well-studied and detailed information about genomics, transcriptomics and metabolomics are available (Corchero et al., 2013). In addition, this bacterium is very suitable for genetic engineering (Corchero et al., 2013). *E. coli* is fast-growing, can produce high protein yields and is cheap in production (Berlec & Strukelj, 2013; Demain & Vaishnav, 2009). Major drawbacks of this system are the inability to carry out eukaryotic post-translational modifications and no or improper disulphide bridge formation (Berlec & Strukelj, 2013; Demain & Vaishnav, 2009). Therefore, the production of eukaryotic proteins in this system often leads to non-native protein folding and accumulation of protein aggregates in inclusion bodies (Berlec & Strukelj, 2013; Demain & Vaishnav, 2009). Bacteria like *Bacillus subtilis* can overcome the inclusion body problem as proteins can be secreted into the culture medium (Demain & Vaishnav, 2009; Schmidt, 2004). Generally, purification of secreted proteins is preferred as the culture medium contains less host proteins and downstream protein purification is faster and easier (Demain & Vaishnav, 2009; Schmidt, 2004).

The most widespread eukaryotic protein expression systems are yeasts like *Saccharomyces cerevisiae* or *Pichia pastoris* (Berlec & Strukelj, 2013; Demain & Vaishnav, 2009). The main advantages over prokaryotic systems are glycosylation of proteins, disulphide bridge formation and efficient protein secretion (Berlec & Strukelj, 2013; Demain & Vaishnav, 2009). Protein expression systems are also established for filamentous fungi mainly of the Ascomycetes *Aspergillus* sp. and *Trichoderma* sp.

(Nevalainen & Peterson, 2014). They are preferentially used for the production of industrial enzymes, metabolites and antibiotics (Adrio & Demain, 2010; Schmidt, 2004). The main advantages of filamentous fungi are the secretion of very high protein yields and post-translational modifications (Corchero et al., 2013; Demain & Vaishnav, 2009; Nevalainen & Peterson, 2014).

Fungi of the Basidiomycetes are less established for protein production probably due to less sequenced organisms (Joint Genome Institute (JGI) (http://jgi.doe.gov/fungi)) and few established transformation protocols for these fungi. For example, a laccase was produced in high amounts in the white rot fungus *Pycnoporus cinnabarinus* (Alves et al., 2004). A laccase was also overexpressed in the model organism *Coprinopsis cinerea* (Kilaru et al., 2006) and *C. cinerea* was established for heterologous expression of various enzymes like a xylanase from *Aspergillus oryzae* (Kikuchi et al., 2004) or a immunomodulatory protein from the medicinal fungus *Ganoderma sinense* (Han et al., 2010). Moreover, a lectin from *Pleurocybella porrigens* was produced in *Phanerochaete sordida* that was not possible to express in *E. coli* and the yeast *Kluyveromyces lactis* (Suzuki et al., 2014). Basidiomycetes secrete unique lignin-degrading enzymes, (hemi)cellulases and proteases which are highly valuable in industrial processes like food and textile production (Erjavec et al., 2012; D. M. Mate & Alcalde, 2017; Ward, 2012). Some proteins were successfully produced in heterologous systems like for example a laccase from *Cyathus bulleri* in *P. pastoris* with high activity levels (Garg et al., 2012), a laccase from *P. cinnabarinus* in *Aspergillus niger* (Record et al., 2002) or a pyranose dehydrogenase from *Agaricus meleagris* in *P. pastoris* (Sygmund et al., 2012). However, there is still lots of potential for the exploration of valuable Basidiomycete proteins and high yield homologous or heterologous production (Erjavec et al., 2012).

Mainly wood-degrading and edible Basidiomycete species have been characterised for biotechnological use so far (Erjavec et al., 2012). One of the best characterised Basidiomycete protein expression system of a plant-interacting fungi is *Ustilago maydis* (Feldbrugge et al., 2013). The maize pathogen *U. maydis* can grow in a yeast-like state in liquid cultures and also fermenters (Feldbrugge et al., 2013). This model Basidiomycete is genetically well-characterised (Kämper et al., 2006) and tools for high protein expression and secretion are established (Terfruchte et al., 2017). Proteins are fused to the chitinase Cts1 for unconventional secretion, which leads to non-

glycosylated secreted proteins (Sarkari et al., 2014; Stock et al., 2012; Terfruchte et al., 2017). This is an advantage for the production of biopharmaceutics because improper glycosylation might lead to immune reactions (Gerngross, 2004). However, many eukaryotic proteins need specific glycosylation patterns for correct folding and protein activity (Corchero et al., 2013; Nevalainen & Peterson, 2014).

Depending on the properties of the protein and the required modifications different organisms must be tested for optimal protein production, purification and activity (Corchero et al., 2013). The establishment of a protein production system in an endophytic fungus of the Agaricomycota might bring complementing properties which can be advantageous not only for the production of homologous but also heterologous proteins especially of the Basidiomycetes. *S. indica* has been established as the model organism of the Sebacinales (Agaricomycota) over the last years (Gill et al., 2016; Weiss et al., 2016). Its genome is sequenced, the fungus is transformable and can easily grow in axenic culture (Verma et al., 1998; Zuccaro et al., 2009; Zuccaro et al., 2011). Vassilev et al. showed that *S. indica* can be used in a repeated-batch fermenter (Vassilev et al., 2017). *S. indica* has the potential to obtain the "Generally Recognised As Safe" status because of its non-pathogenic nature and since there were no genes for the production of toxic secondary metabolites and cyclic peptides identified in its genome (Zuccaro et al., 2011). This fact is important for the production of proteins in the food and biopharmaceutical industry (Ward, 2012). Moreover, *S. indica* naturally produces many cell wall-degrading enzymes (Lahrmann et al., 2015; Zuccaro et al., 2011) whose production could be optimised.

1.7 Aim of the thesis

The aim of this thesis was to shed light on how Sebacinoid core effectors are involved in establishing and maintaining root symbiosis. In order to functionally characterise the Sebacinoid effectors, the toolbox of the model fungus and root endophyte *S. indica* of the order Sebacinales was further developed. A protein production system was established by optimising vectors and conditions for expression and secretion of homologous and heterologous proteins in *S. indica*. A gene deletion system using homologous recombination with a split resistance cassette was developed. Besides, the colonisation abilities of the homokaryotic versus the dikaryotic *S. indica* strains were studied.

The cell death-associated colonisation phase of *S. indica* was further analysed by cytological studies and by functional characterisation of a fungal effector protein with nuclease features named *Si*NUCA. The protein domains, phylogeny and gene expression patterns of *Si*NUCA were depicted. *Si*NUCA was heterologously expressed in the model Basidiomycete *U. maydis* to analyse protein secretion and enzyme activity. Enzymatic specifications were further determined by purification of the native protein with a C-terminal HA:His tag from culture filtrate. *Si*NUCA was overexpressed and deleted in *S. indica* to study the effect on Arabidopsis and barley colonisation. On top, *Si*NUCA was ectopically expressed in Arabidopsis for protein localisation studies and to analyse its effect on colonisation and its involvement in host cell death.

The tools developed in this thesis will help to further characterise fungal key factors in plant-microbe interactions. These data provide a deeper insight into how cell death is involved in symbiotic plant-microbe interactions.

2 Results

2.1 *S. indica* as a protein production system

2.1.1 The *Si*FGB1 promoter is strong and inducible in axenic culture

For gene expression in *S. indica* the *S. indica* constitutive promoters GPD or TEF were routinely used (Hilbert et al., 2012; Zuccaro et al., 2009). This led for example to good expression of *S. indica* codon-optimised green fluorescent protein (oGFP, Hilbert et al., 2012) but only to weak expression of endogenous genes like FGB1 probably due to gene silencing (Wawra et al., 2016). Therefore, inducible or heterologous promoters for gene expression in *S. indica* were tested. First of all, the promoter of *S. vermifera* TEF was cloned in front of GFP. Transformation with random integration of the plasmid led to strong cytoplasmic GFP expression in *S. indica* (Fig. 2.1) indicating that this promoter is a good alternative for constitutive gene expression in *S. indica*.

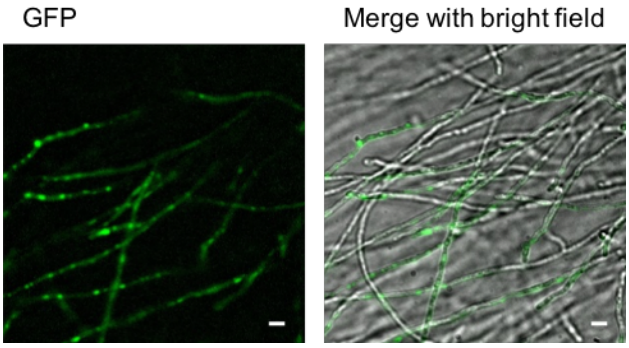

Fig. 2.1: GFP expression driven by the *S. vermifera* TEF promoter in *S. indica*.
S. indica strain expressing cytosolic GFP under the control of the constitutive *S. vermifera* TEF promoter. Fluorescence microscopy of living *S. indica* grown for 6 days in liquid CM. Bars = 100 µm.

In a next step, the *S. indica* promoter of FGB1 was tested as an inducible promoter in axenic culture. FGB1 is one of the most abundant secreted proteins in complete medium (CM) but not in yeast nitrogen base (YNB) medium (Wawra et al., 2016). In accordance, *S. indica* strains expressing FGB1 with a C-terminal GFP driven by its own promoter had a strong GFP signal in CM medium whereas only a weak signal was visible in fungal mycelium grown in YNB medium (Fig. 2.2A). An anti-GFP Western blot confirmed a strong FGB1 promoter activity in cultures growing in CM but not in YNB and secretion of the fusion protein (Fig. 2.2B). Notably, proteolytic stability of the fusion protein was higher in YNB than in CM medium. In CM medium, the presence of a GFP fluorescence signal in vesicles moving towards hyphal tips was detected by confocal laser scanning microscopy (CLSM) showing protein secretion for this fusion protein (Fig. 2.2C).

Moreover, the FGB1 promoter was active in germinating spores and in fungal hyphae colonising Arabidopsis and barley roots in both extra- and intracellularly growing hyphae (Fig. 2.2C/D).

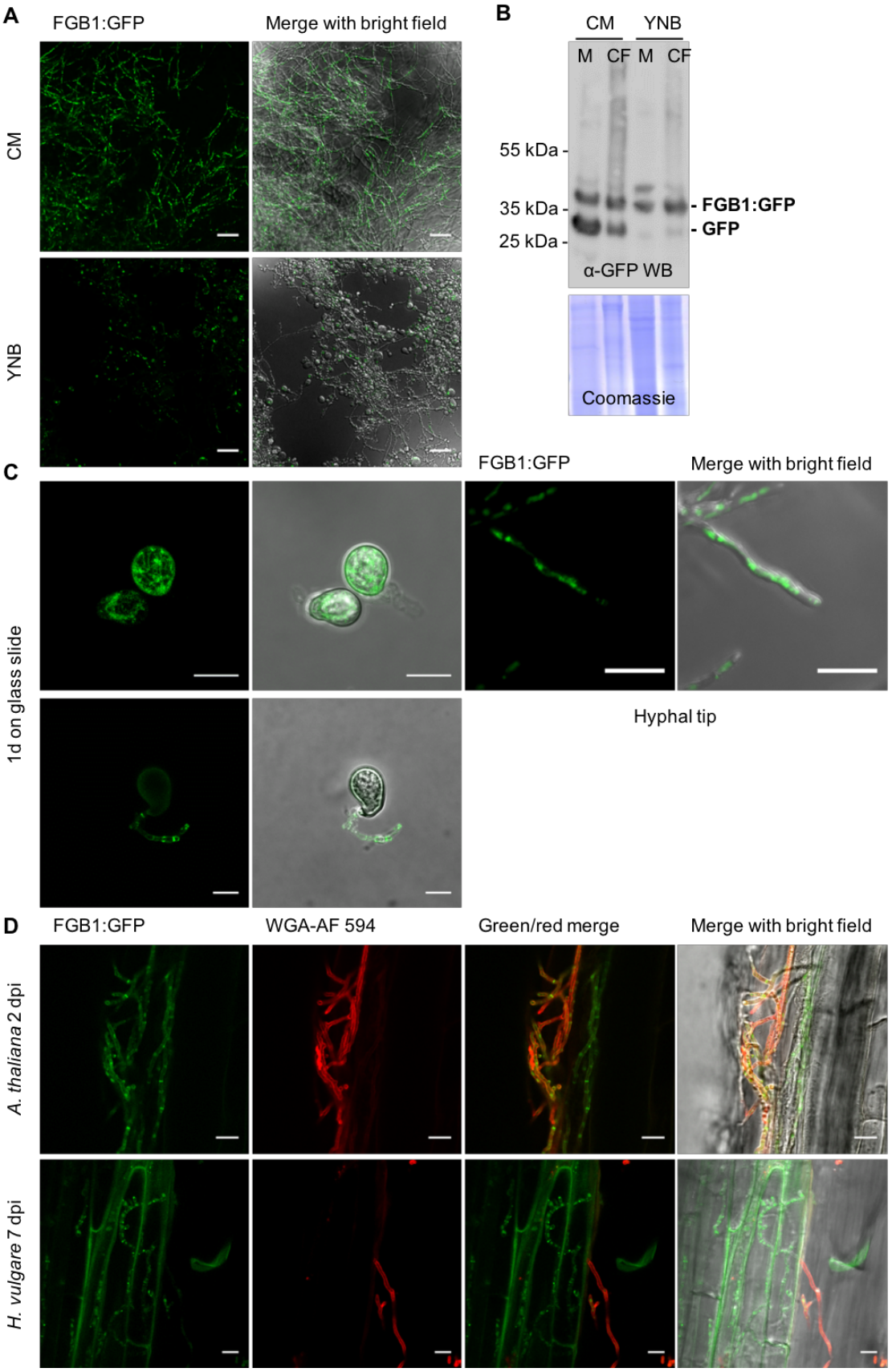

Fig. 2.2: Comparison of FGB1:GFP expression in *S. indica* in axenic culture and *in planta.*

A) FGB1:GFP fusion protein is strongly expressed in *S. indica* grown in CM medium compared to YNB medium. CLSM live cell images of cultures grown for 7 days in liquid CM medium, crushed and grown in CM or YNB for 3 days. Bars= 25 µm. **B)** Anti-GFP Western blot of mycelium (M) and culture filtrate (CF). Cultures used in A) after 5 days of growth in CM or YNB. Coomassie stained SDS-PAGE of the same samples as loading control. **C)** CLSM live cell images of FGB1:GFP expression in *S. indica* spores germinated on glass slide and a hyphal tip in CM. Maximum projection of z-stacks. Bars= 10 µm. **D)** *S. indica* FGB1:GFP strain colonising Arabidopsis and barley roots. The cell wall of extracellular hyphae were counterstained with the chitin dye Wheat Germ Agglutinin Alexa Fluor 594 conjugate (WGA-AF 594), which cannot enter living plant cells (Lahrmann et al. 2013). Bars= 10 µm.

2.1.2 Optimisation of media conditions and tags for fusion protein stability and secretion

To optimise protein production and secretion the following fungal growth media were tested: CM, MYP, YEPS light and MEP. Fungal plugs of a *S. indica* FGB1:GFP strain were grown in liquid CM medium, subsequently crushed, regenerated in CM medium and then switched to different media. Differences in protein secretion were observed depending on the media composition (Fig. 2.3 A/B). In MYP and YEPS light GFP signal was strongest at the cell wall, septa and in the culture filtrate fraction on the SDS-PAGE implying that the fusion protein was secreted. At the same time, native FGB1 production was inhibited according to the SDS-PAGE. In contrast, in CM and MEP medium GFP signal was mainly detected in the ER. Since also native FGB1 was detected on the SDS-PAGE in large amounts under these conditions one can speculate that the native protein and the fusion protein compete for the same binding sites at the cell wall. Therefore, a GFP signal could not be detected there. In contrast to MEP medium, less secreted GFP was observed in CM medium. In a next step, we tested if a pre-cultivation step in CM followed by cultivation in MYP medium is a prerequisite for high protein secretion. Only growth, crushing and regeneration in CM medium followed by growth in MYP medium led to vast amounts of secreted FGB1:GFP (Fig. 2.3C). However, most of the secreted fusion protein was cleaved and mainly free GFP was detected indicating that under these conditions also a large number of proteases were produced.

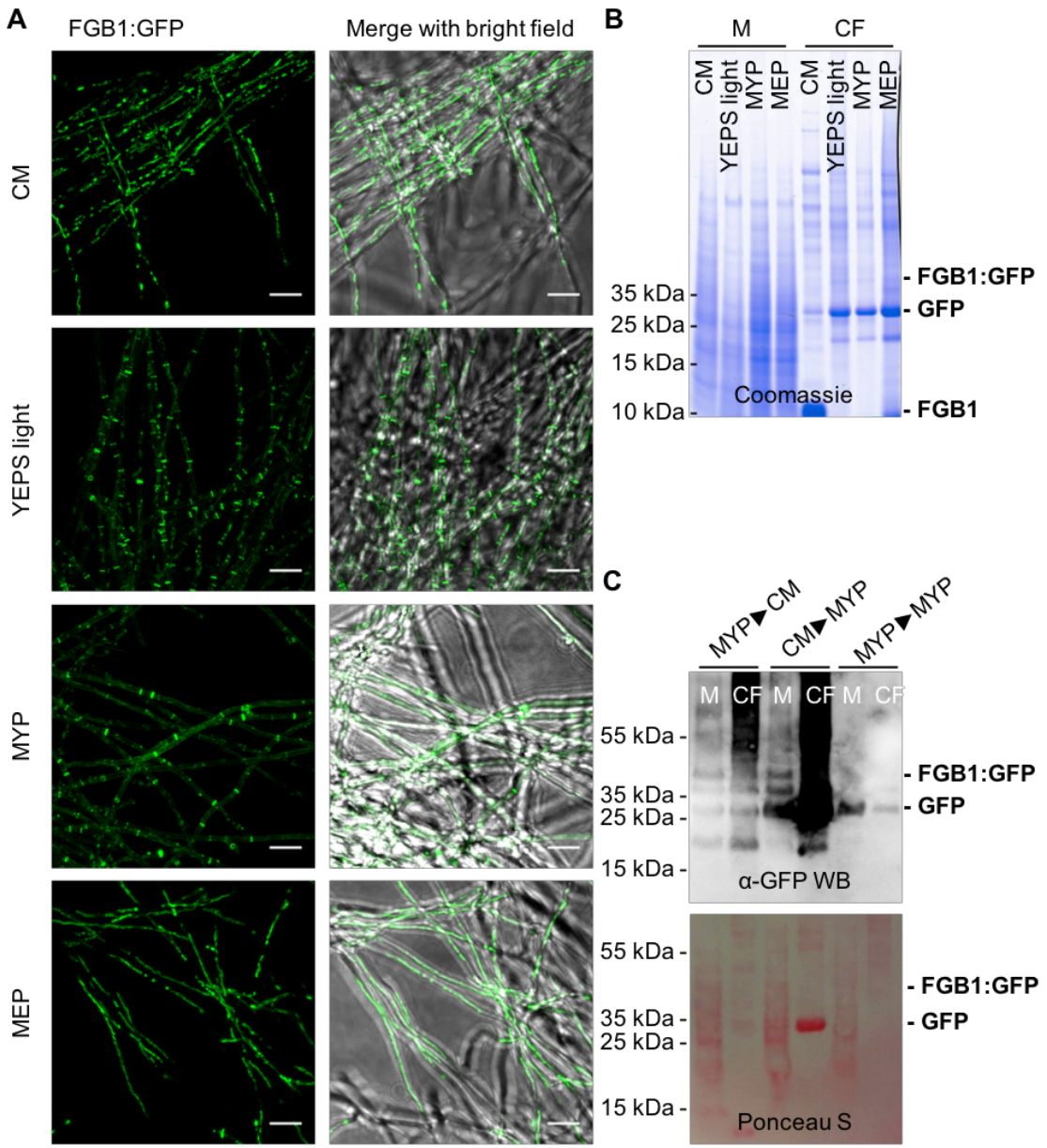

Fig. 2.3: FGB1:GFP secretion depends on medium composition.

A) FGB1:GFP expressing *S. indica* grown in CM medium, crushed, regenerated for 2 days in CM and grown for 1 day in CM, YEPS light, MYP or MEP medium. Depending on the medium GFP signal was mainly detected at the cell wall and septa (YEPS light, MYP) or in the ER (CM, MEP). Live cell imaging by CLSM. Maximum projection of z- stacks. Bars = 10 µm. **B)** Coomassie-stained SDS-PAGE of mycelium (M) and culture filtrate (CF) of cultures in A) after 5 days in the respective medium. **C)** anti-GFP Western blot of FGB1:GFP expressing *S. indica* grown for 7 days in the first medium, crushed, regenerated for 2 days in the same medium and grown for 3 days in the second medium. Ponceau S stained nitrocellulose membrane as loading control below.

To increase fusion protein stability of secreted proteins the modular vector pFGB1, based on pGoGFP from Hilbert et al. (2012), was generated to facilitate cloning and testing of different constructs (Fig. 2.4). Each module was designed to be exchangeable by the use of unique restriction sites. The FGB1 promoter is followed by the gene of interest and if necessary the SP can be individually exchanged using the ClaI restriction site between the SP and the rest of the gene. Therefore, a SP can be easily added to genes without SP for protein secretion. The PreScission site, a selective proteolytic cleavage site for the HRV 3C protease, was added between the gene of interest and the tags (Waugh, 2011). Consequently, tags can be specifically removed after protein purification by digestion with the HRV 3C protease. For protein purification a 2x hemagglutinin (HA) and a hexahistidine tag were added after oGFP followed by a Stop codon and the tnos terminator. For selection in *S. indica* the Hygromycin B resistance cassette driven by the *S. indica* TEF promoter and the Ampicillin resistance for selection in *E.coli* were used. The Hygromycin B resistance cassette is also interchangeable due to the flanking SfiI sites. As alternative a version with N-terminal HA:His:GFP was constructed too.

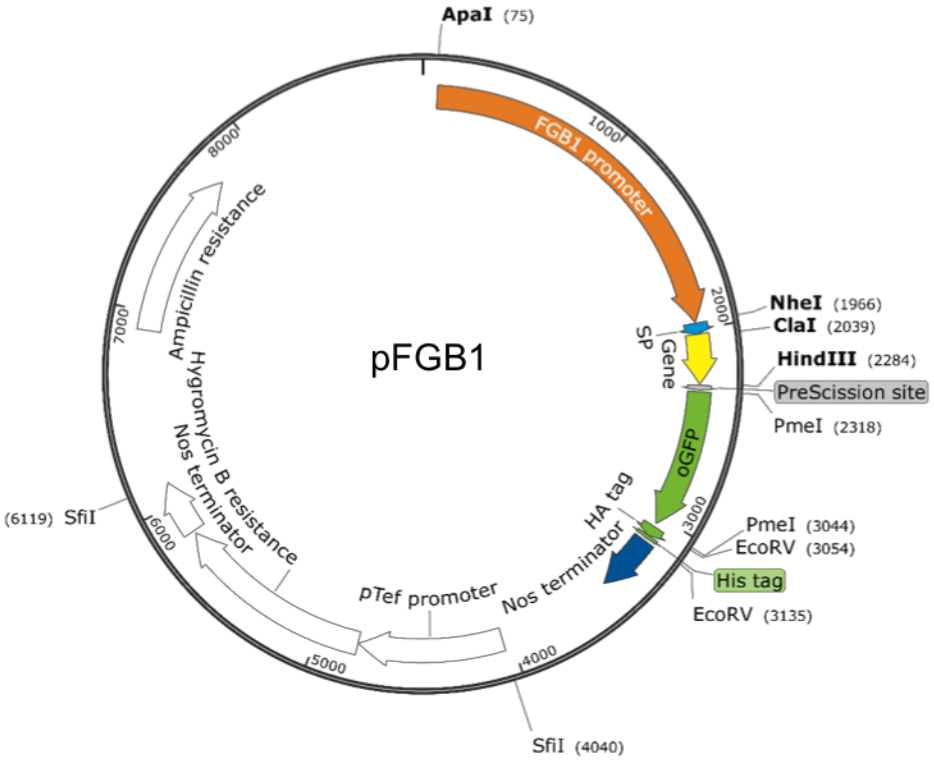

Fig. 2.4: Map of the modular vector pFGB1 used for expression of secreted proteins in *S. indica*.
The gene of interest is driven by the strong and inducible *S. indica* promoter FGB1. The SP for protein secretion might be cloned separately. A C-terminal GFP (codon-optimised for *S. indica),* a 2x HA and His_6 tags can be cleaved after protein production and purification using the PreScission site after the gene. A stop codon and the tnos terminator were added after the tags. The selection markers are the Hygromycin B resistance cassette driven by the *S. indica* TEF promoter for *S. indica* and Ampicillin for *E. coli*.

Besides the PreScission site as linker between the gene of interest and the tags also vector variants without any added linker (restriction sites HindIII and EcoRV translating to KLDI) and a flexible linker (KLYSSGSGSSAQSLN including restriction sites HindIII and PmeI) were tested. However, no significant improvements were achieved regarding linker cleavage. Comparing Western blots of the same samples probed with either anti-HA or anti-GFP antibodies suggested that cleavage occurred not only between the gene of interest and GFP but also between GFP and HA:His. Therefore, GFP was replaced by the monomeric red fluorescent protein (mRFP) because the beginning and end of the protein sequence are highly different. However, we did not manage to get high amounts of intact secreted protein despite strong signals were detected in the ER in *S. indica* expressing FGB1:mRFP:HA:His and FGB1:GFP:HA:His (data not shown). The reason might be that FGB1 was binding to a matrix surrounding the cell wall and therefore was not detected in the culture filtrate fraction by Western blots. To test this hypothesis the native purified FGB1 labelled with fluorescein isothiocyanate (FITC) was added to the mycelium for localisation (labelled protein was kindly provided by Dr. S. Wawra). Using FGB1-FITC a matrix surrounding the cell wall of different fungi growing in axenic culture and *in planta* could be visualised (Fig. 2.5).

A handful of other proteins were tested for protein expression using this system. Expression attempts of a secreted E3 ubiquitin ligase from *S. vermifera (Sebve1_14646*) in *E. Coli* and *P. Pastoris* were not successful. A reason could be the 20 cysteines present in the protein sequence that might form a complex pattern of disulphide bridges and incorrect or no disulphide bridge formation could lead to protein degradation. In contrast, expression of this protein with a C-terminal GFP fusion in *S. indica* was successful (data not shown) suggesting that heterologous proteins can be produced in this system too. However, secretion into the culture filtrate was not observed. The

homologues protein of FGB1 from the plant pathogenic fungus *Bipolaris sorokiniana* was expressed in *S. indica* with the C-terminal tags GFP:HA:His, mRPF:HA:His and HA:His. Similar to FGB1 production of *Bs*FGB1 was more stable in YNB compared to CM and MYP medium (data not shown). Other successfully overexpressed proteins from *S. indica* were the SSPs WSC3 (a glucan-binding lectin), DLD1 (a metal ion-binding zipper protein) and *Si*NUCA (an endonuclease). The expression of DLD1 and WSC3 in *S. indica* was not successful under the GPD promoter and WSC3 was not possible to express in *U. maydis* (personal communication Dr. P. Fesel and Dr. S. Nizam).

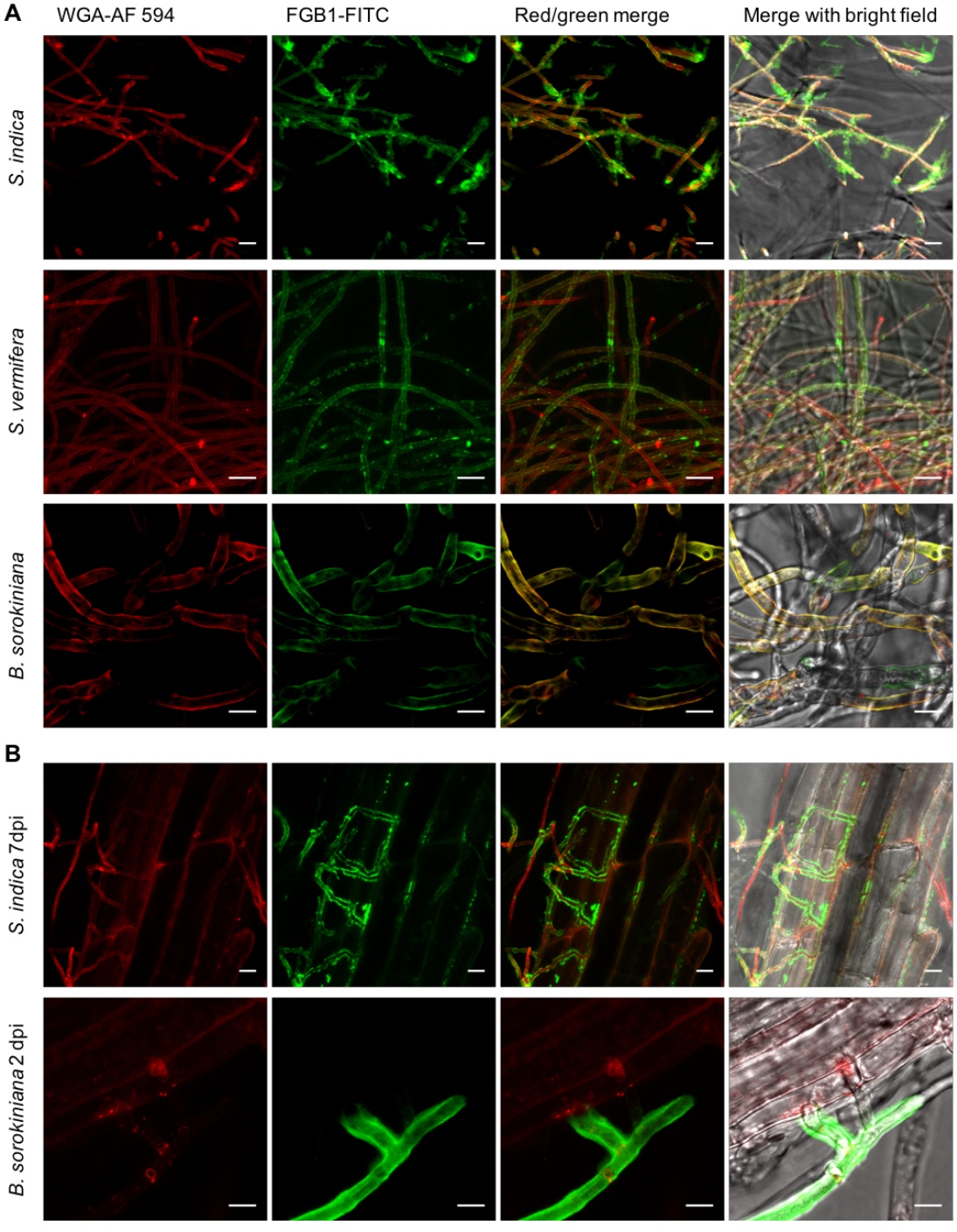

Fig. 2.5: FITC-labelled native FGB1 localises to the fungal cell wall and matrix surrounding it in different fungi.

CLSM live cell imaging of FITC-FGB1 staining compared to the chitin staining WGA-AF 594 of **A)** different fungi grown in axenic liquid cultures. *S. indica* grown for 7 days in CM, crushed and regenerated for 2 days in CM. *S. vermifera* was growing for 5 days in MYP, crushed and regenerated for 1 day in MYP. The plant pathogenic Ascomycete *Bipolaris sorokiniana* was growing for 3 days in MYP, crushed and regenerated for 1 day in MYP. **B)** *S. indica* and *B. sorokiniana* colonising Arabidopsis roots. Arabidopsis is a non-host of *B. sorokiniana*.

Maximum projection of z-stacks. Bars= 10 µm.

2.1.3 *Si*NUCA protein production and purification

The *S. indica* 21 kDa protein *Si*NUCA (see 2.2.2) was tested in this system because heterologous expression *in E. coli* led to protein accumulation in inclusion bodies. *Si*NUCA C-terminally fused with an HA:His tag and driven by the FGB1 promoter was secreted into the culture filtrate (Fig. 2.6A). A *S. indica* strain expressing cytosolic GFP (from Hilbert et al., 2012) was processed in parallel to confirm that the detected protein originated from active secretion and not from mycelium leakage. Inhibition of the FGB1 promoter activity by ammonium sulphate was shown as reduced protein levels were detected in YNB with ammonium sulphate compared to YNB without ammonium sulphate but supplemented with asparagine as nitrogen source (Fig. 2.6B). In contrast to FGB1, the largest amount of secreted fusion protein was detected in CM medium. Therefore, the culture filtrate of cultures grown in CM was further used for *Si*NUCA:HA:His purification.

As the His tag was cleaved for most of the fusion protein in all tested conditions, an alternative method to protein purification by Nickel Nitrilotriacetic acid columns was chosen. Therefore, the supernatant of dense 500 ml cultures in CM medium was precipitated with 80 % ammonium sulphate and the resulting protein pellet was resuspended in 20 mM Tris pH 8/ 150 mM NaCl buffer. The soluble protein was further purified by size exclusion chromatography (Fig. 2.6C). *Si*NUCA:HA:His was enriched in fractions corresponding to a retention volume of 90- 100 ml on a Sephadex G 200 column. The fractions containing the target protein were concentrated, desalted and loaded on an SDS-PAGE to estimate yield and purity. With this purification method a fusion protein purity of circa 70 % and a protein yield of 13 mg/L was obtained. In

collaboration with Dr. S. Metzger (Mass spectrometry platform CEPLAS, University of Cologne), liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of the sample confirmed the identity of *Si*NUCA by matching of four unique proteins. The activity of the protein was assayed and confirmed in chapter 2.2.4.

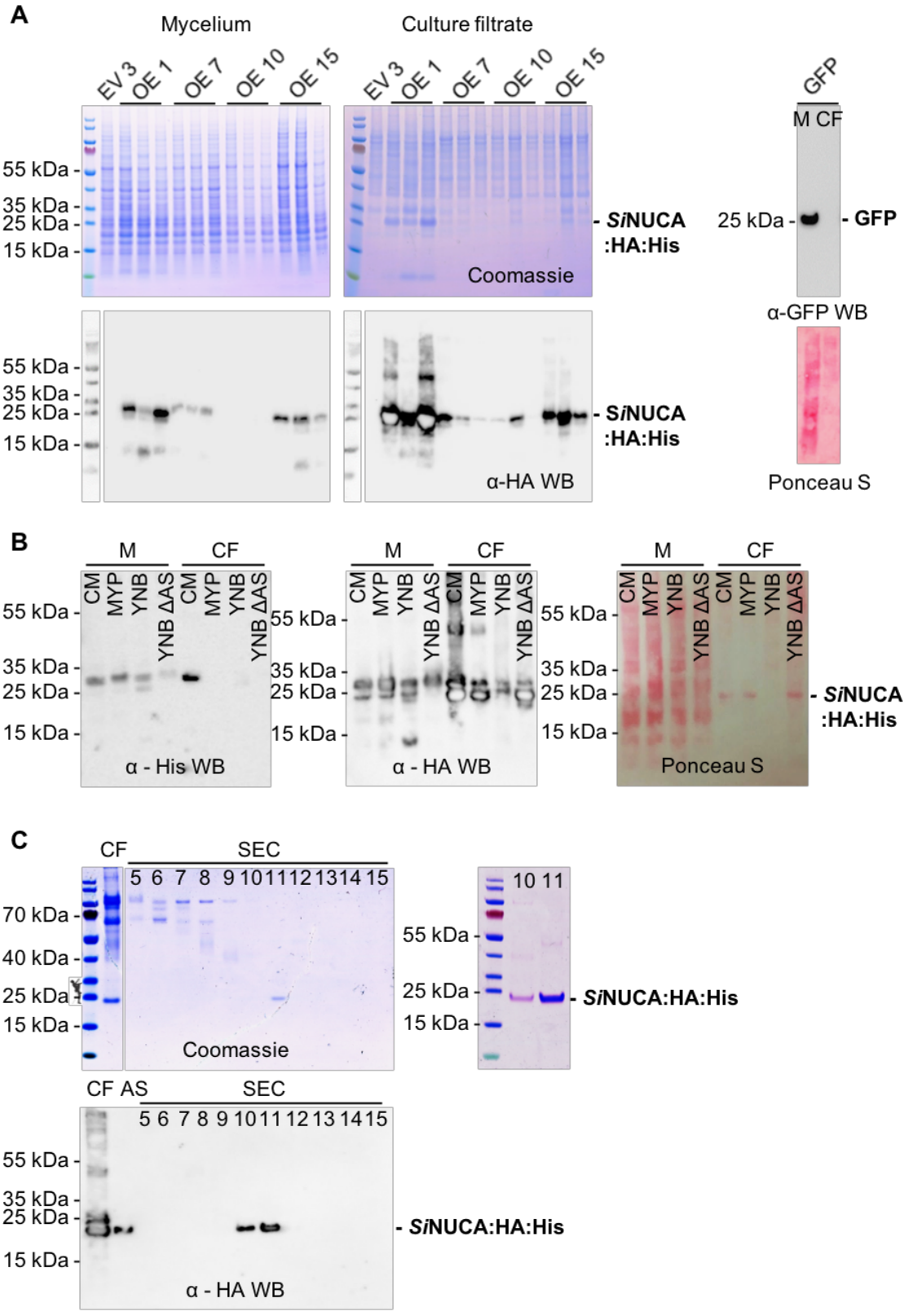

Fig. 2.6: *Si*NUCA:HA:His expression and secretion in different media.

A) *S. indica Si*NUCA:HA:His (OE 1, 7, 10, 15) and the empty vector strain 3 (EV 3) were grown for 5 days in CM medium followed by 2 days in MYP medium. Protein expression and secretion was analysed with mycelium (M) and culture filtrate (CF) on coomassie-stained SDS-PAGEs and anti-HA Western blots. Right side: control for protein leakage from M into CF with *S. indica* strain expressing cytosolic GFP processed in parallel. **B)** Anti-His and anti-HA Western blots of *S. indica Si*NUCA:HA:His strain 15 grown in CM for 5 days, crushed, grown for 1 day in CM with YNB and subsequently for 1 day in different media (CM, MYP, YNB or YNB without ammonium sulphate (ΔAS) plus asparagine). Right side: Ponceau S stained nitrocellulose membrane as loading control. **C)** *Si*NUCA:HA:His protein enrichment from CF precipitated with 80% ammonium sulphate (AS) and separated by size exclusion chromatography (SEC, fractions 5- 15 shown). Fractions 10 and 11 were confirmed to contain *Si*NUCA:HA:His by an anti-HA Western blot, were concentrated and loaded on an SDS-PAGE gel again (right side) to analyse its purity and yield.

2.2 Molecular characterisation of the *S. indica* effector protein *Si*NUCA

2.2.1 Cell death in *S. indica* colonised Arabidopsis roots

S. indica has a biphasic lifestyle with an early biotrophic phase and a later cell death-associated phase (Deshmukh et al., 2006; Jacobs et al., 2011; Zuccaro et al., 2011). Deshmukh et al. showed that nuclei of barley root cells disappear with the ongoing of colonisation by *S. indica* indicating cell death of the colonised cells (Deshmukh et al., 2006). In Arabidopsis, Jacobs et al. observed the lack of ER and nucleus in colonised cells (Jacobs et al., 2011). Cell death in colonised Arabidopsis roots was further investigated by looking at plant nuclei by CLSM over time. The nucleic acid dyes 4',6-Diamidine-2'-phenylindole dihydrochloride (DAPI) and SYTOX Orange and Arabidopsis lines expressing Histone H2B:mCherry were used to visualise plant nuclei. Disappearance of plant nuclei with progression of *S. indica* colonisation in epidermal and cortex cells was confirmed with all three nucleus-visualisation methods (Fig. 2.7). Nuclei stained with SYTOX Orange were visible five to six dpi suggesting that the plasma membrane was permeable enough for the dye to enter the cell. Nuclei were absent in heavy colonised root parts. The nuclei were often stretched, faded and finally disappeared (Fig. 2.7B). Moreover, *S. indica* hyphae embedded within the plant nuclei were observed.

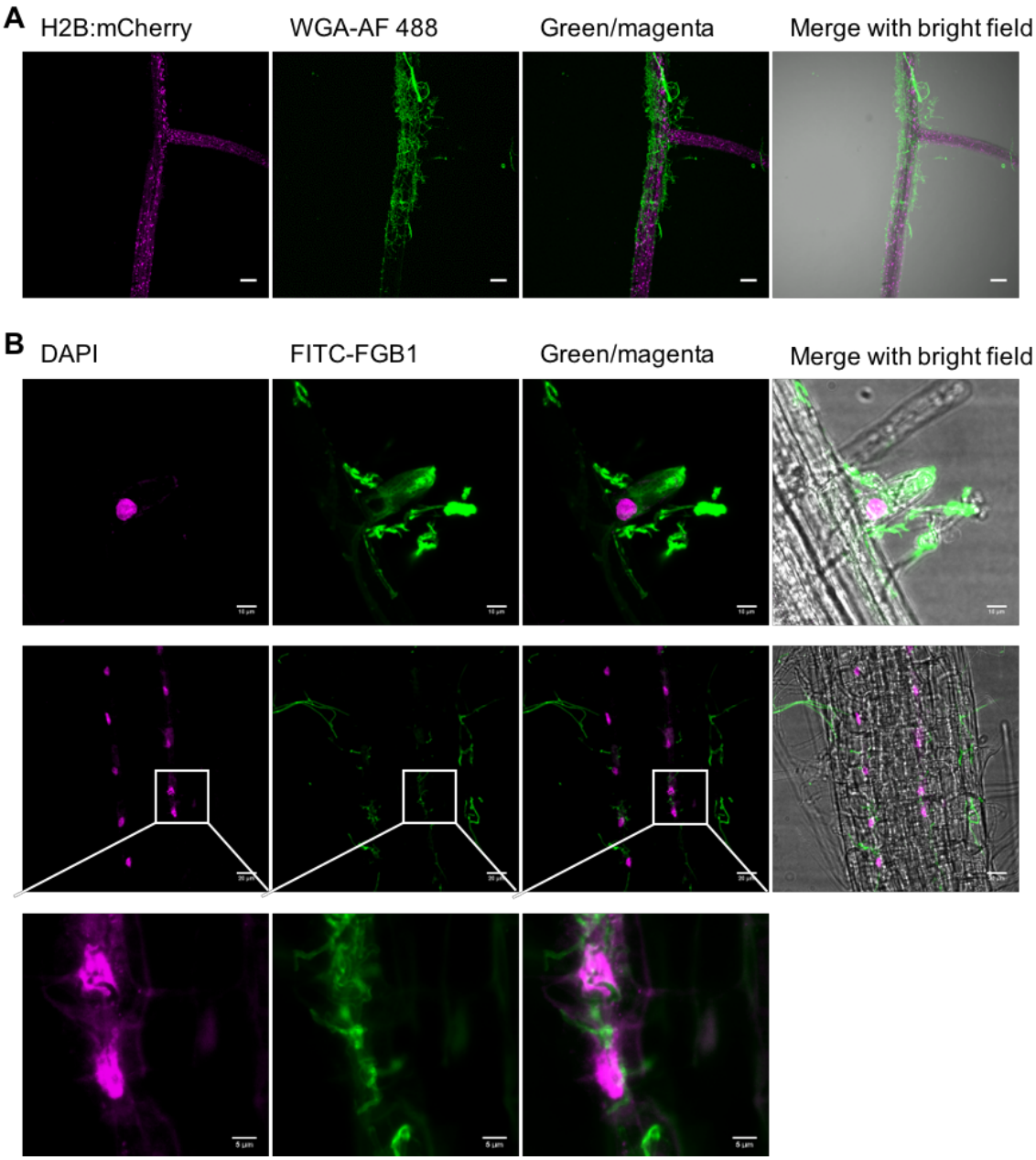

Fig. 2.7: Arabidopsis root nuclei disappear with progression of *S. indica* colonisation.

CLSM live cell imaging of *S. indica*-colonised Arabidopsis roots. **A)** Arabidopsis UBQ10::H2B:mCherry expressing root 10 dpi. Fungal hyphae outside living plant cells stained with WGA-AF 488. Nuclei in epidermal and cortex cells of *S. indica*-colonised root parts are almost completely absent. Bars = 100 µm. Maximum projection of z-stacks. **B)** Nuclei stained with DAPI and fungal cell wall and matrix with FITC-FGB1. With progression of *S. indica* colonisation nuclei get stretched and fade. *S. indica* hyphae are embedded in the plant nucleus. 6 dpi. Pictures by S. Wawra. Bars = 10 µm, 20 µm respectively 5 µm.

Jacobs et al. showed that *S. indica* is colonising the maturation but not elongation and meristematic root zones (Jacobs et al., 2011). We noticed by direct imaging on the plate where colonised Arabidopsis roots were growing that *S. indica* extracellularly colonised

the whole root including root tips. (Fig. 2.8). However, intracellular growth in the root cap cells or border- like cells (BLC), which are loosely attached to the root tip, was never observed. Moreover, fading nuclei were also detected in BLC of root tips in contact with *S. indica* mycelium.

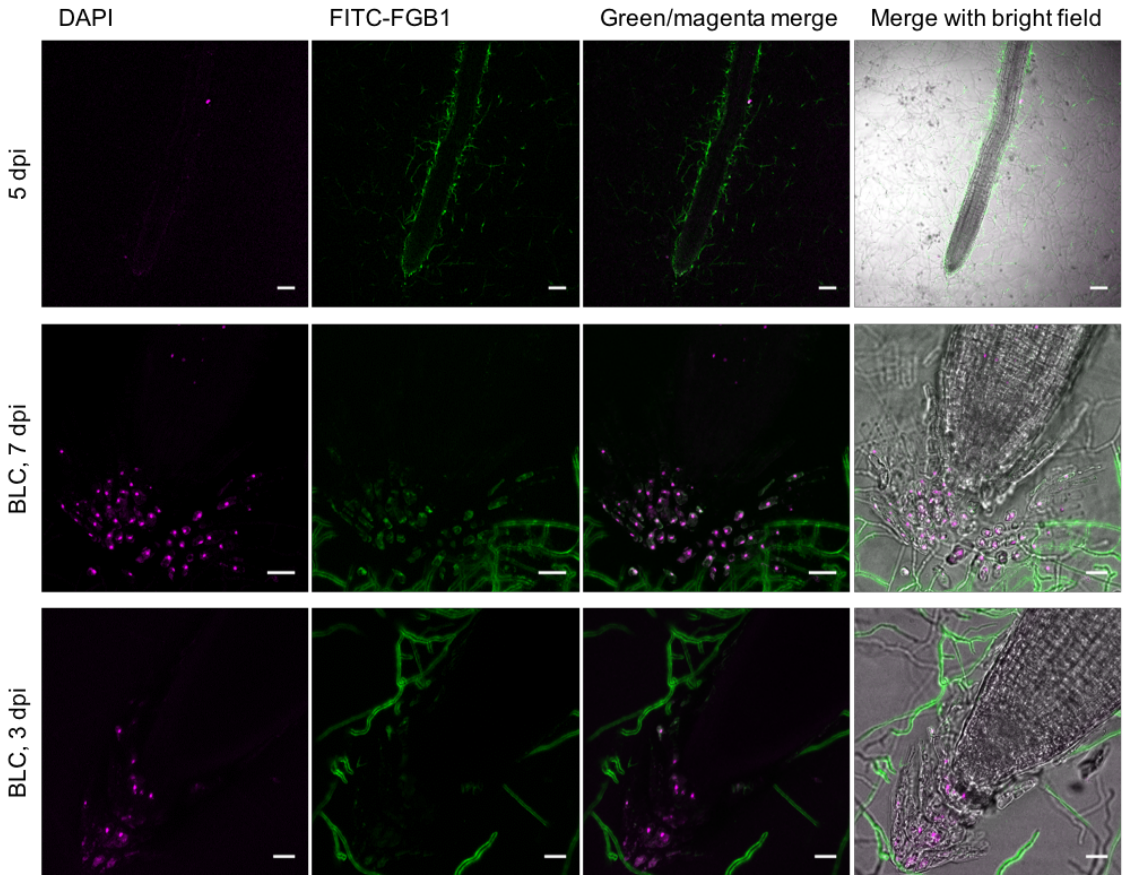

Fig. 2.8: *S. indica* growth around Arabidopsis BLC.

CLSM live cell imaging of *S. indica* colonised Arabidopsis roots. Nuclei stained with DAPI and fungal cell wall and matrix with FITC-FGB1. Roots directly stained and imaged on plate where the root was growing. Therefore, only nuclei of BLC are visible. *S. indica* is growing around the root tip and BLC of the root cap. Last picture: The root was growing towards and just reaching a layer of germinating *S. indica* spores on the plate. Nuclei of the outer BLC layer are blurred. Bars = 100 µm (First picture), Bars = 20 µm

2.2.2 An apoplastic *S. indica* nuclease

In order to find fungal factors which trigger cell death the apoplast proteome of colonised barley roots was analysed. At 5 dpi, where the transition from the biotrophic to the cell-death associated colonisation stage happens, a predicted nuclease (PIIN_02121, named *S. indica* Nuclease A (*Si*NUCA)) was found. The open reading frame

of *SiNUCA* was verified by rapid amplification of cDNA-ends with polymerase chain reaction (RACE-PCR). This revealed a 30 bp earlier start and a single nucleotide polymorphism (SNP) at base pair number 33 compared to the genome sequence derived from a consensus sequence from the dikaryotic *S. indica* wt strain. Therefore, the protein is 211 amino acids long. According to simple modular architecture research tool (SMART) analysis *Si*NUCA has a predicted SP and Pfam DUF 1524 domain, which belongs to the His- Me finger endonuclease superfamily (Fig. 2.9A). Characteristics of these endonucleases are a histidine in their catalytic side, binding of one divalent metal ion and having a HNH or HNN sequence motif (Jablonska et al., 2017). Moreover, *Si*NUCA has four cysteines suggesting the presence of two disulphide bridges. A homologous protein of *Si*NUCA was found in the closely related fungus *S. vermifera* with 89 % protein sequence identity. Further homologs were mainly detected in the bacterial class Actinobacteria, in the Basidiomycetes of the class Agaricomycetes and in the classes Dothideomycetes, Sordariomycetes, Leotiomycetes and Eurotimycetes belonging to the Ascomycetes. A multiple alignment with the top Basic Local Alignment Search Tool (BLAST) hits on protein level for Actinobacteria, Agaricomycetes and Ascomycetes showed that not only the DUF 1524 domain but also the first part of the protein (except the SP) is conserved (Fig. 2.9B). Additionally, the HNN sequence motif was highly conserved.

SiNUCA expression was induced in *S. indica* colonising Arabidopsis and barley roots compared to axenic growth according to microarray data (Lahrmann et al., 2013). This data was verified by quantitative PCR (qPCR) analysing *SiNUCA* expression relative to *TEF* expression of *S. indica* colonising Arabidopsis or barley roots over time (Fig. 2.10A/B). *SiNUCA* expression peaked in barley at 7 dpi whereas in Arabidopsis *SiNUCA* expression was highest at 14 dpi. The expression of the homologous gene in *S. vermifera*, *Sebve1_12299,* was highest in barley at 3 dpi and in Arabidopsis at 7 dpi (Fig. 2.10C/D). In summary, *Si*NUCA shows typical effector characteristics as it is a small secreted protein and its expression is induced *in planta.* Therefore, *Si*NUCA was chosen for further molecular characterisation and its involvement in plant cell death was investigated.

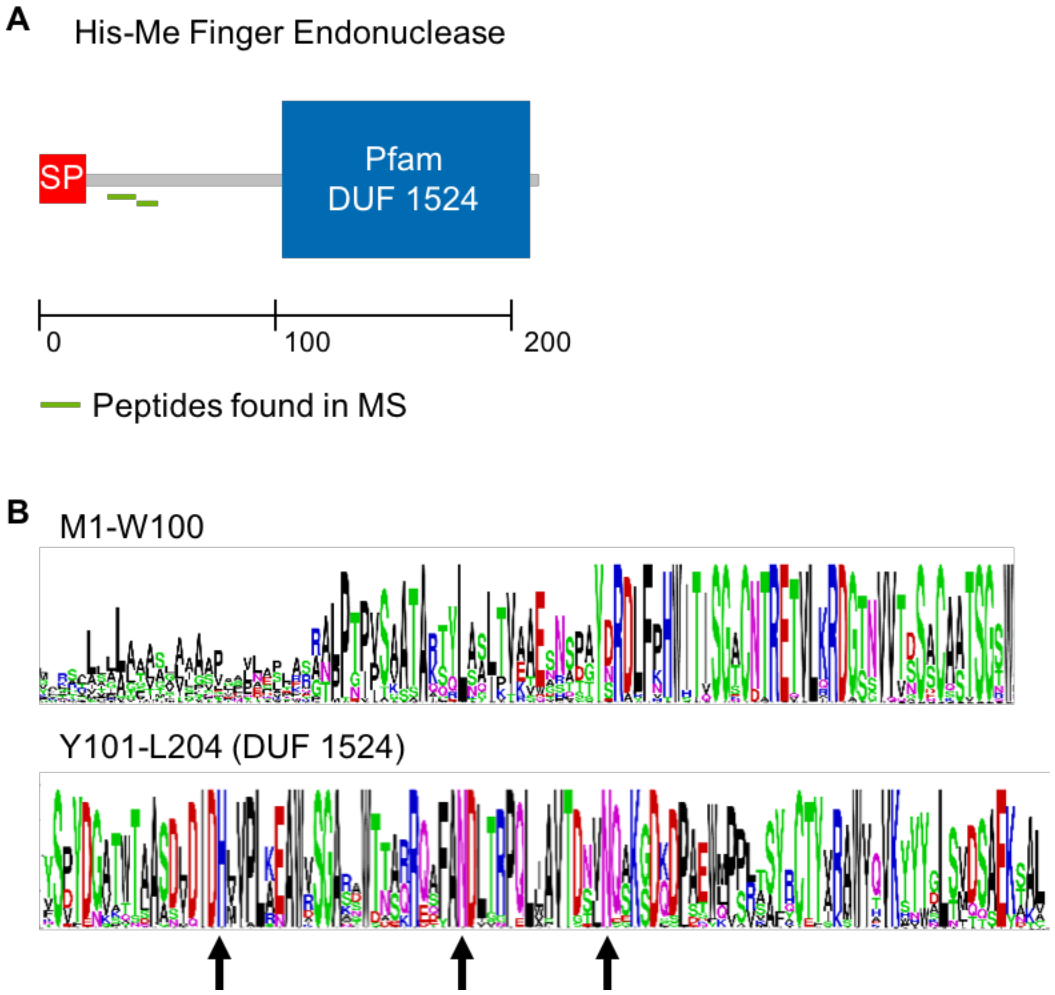

Fig. 2.9: *Si*NUCA is predicted to be a secreted endonuclease.

A) *Si*NUCA (PIIN_02121) is 211 amino acids long, has a predicted SP (first 20 amino acids) and the Pfam domain DUF 1524 belonging to the His-Me finger endonuclease superfamily. Two unique peptides were found in the APF of inoculated barley roots at 5 dpi by LC-MS/MS **B)** Protein logo of multiple alignment of *Si*NUCA and ten best protein BLAST hits for bacteria, Basidiomycetes and Ascomycetes each. Amino acids M1-W100 and Y101-L204 (DUF 1524 domain) shown. With the exception of the SP, the protein sequence is highly conserved. Arrows show the conserved HNN motif.

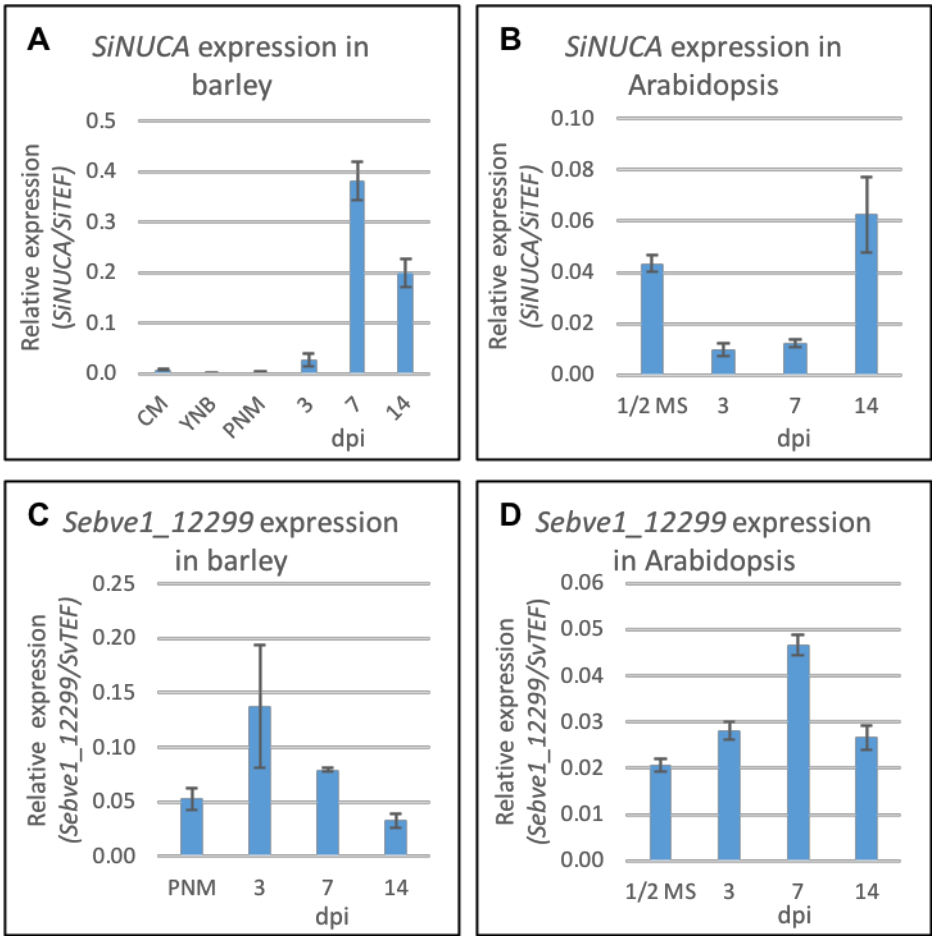

Fig. 2.10: *SiNUCA* and *Sebve1_12299* are expressed *in planta*.

A) *SiNUCA* expression levels of *S. indica* grown in barley roots on PNM 3, 7 and 14 dpi compared to growth in axenic culture after 7 days (CM, YNB and PNM medium). **B)** *SiNUCA* expression of *S. indica* grown in Arabidopsis roots on ½ MS medium 3- 14 dpi and in ½ MS for 7 days. **C)** *Sebve1_12299* expression levels of *S. vermifera*-inoculated barley roots grown on PNM medium 3, 7 and 14 dpi and *S. vermifera* grown on PNM medium for 7 days. **D)** *Sebve1_12299* expression levels of *S. vermifera*-inoculated Arabidopsis roots grown on ½ MS 3, 7 and 14 dpi and *S. vermifera* grown on ½ MS for 7 days.

The expression levels of *SiNUCA* and *Sebve1_12299* were normalised to TEF. Error bars represent the standard error of the mean (SEM) of three biological replicates.

2.2.3 *SiNUCA* heterologously expressed in *U. maydis* is secreted and has nuclease activity

SiNUCA was heterologously expressed in the model Basidiomycete *U. maydis* to study its function. *U. maydis* has no homologue of *SiNUCA* and *SiNUCA* was constitutively expressed using the *U. maydis* otef promoter (Aichinger et al., 2003). *Si*NUCA:mCherry was expressed and efficiently secreted in axenic culture as detected by an anti- mCherry Western blot of pellet and supernatant samples (Fig. 2.11A). Correct prediction of the SP length was confirmed using the construct SP^{SiNUCA}:mCherry as mCherry was detected in the supernatant fraction on the Western blot. An *U. maydis* strain expressing cytosolic mCherry was processed in parallel to exclude protein detection in the supernatant fraction due to leakage and not active protein secretion. In order to test nuclease activity, linearised plasmid, genomic DNA or RNA from *S. indica* was added to the supernatant of the cultures, incubated and the samples loaded on an agarose gel. Nucleic acids were only digested in supernatant of the strains secreting *Si*NUCA thus showing nuclease activity of *Si*NUCA (Fig. 2.11B). Culture supernatants of *Si*NUCA expressing strains were also able to digest barley and Arabidopsis gDNA and RNA therefore giving evidence for an unspecific nuclease activity (Fig. 2.11C). Addition of ethylenediaminetetraacetic acid (EDTA) inhibited gDNA digestion exhibiting a metal ion-dependent catalytic activity. Moreover, RiboLock, a non-competitive RNase inhibitor, also partially inhibited *Si*NUCA activity.

2.2.4 Native *Si*NUCA activity

*Si*NUCA:HA:His driven by the FGB1 promoter was overexpressed in *S. indica* and shown to be secreted into axenic culture medium (see 2.1.3). First, nuclease activity was directly confirmed in the culture filtrate of the overexpressing strains growing in axenic conditions. Linearised plasmid was incubated in culture filtrate of the *Si*NUCA:HA:His strains 7 and 15 compared to the empty vector strains 3 and 6 (Fig. 2.12A). Second, the fusion protein was purified from culture filtrate (see 2.1.3) and used for further biochemical characterisation.

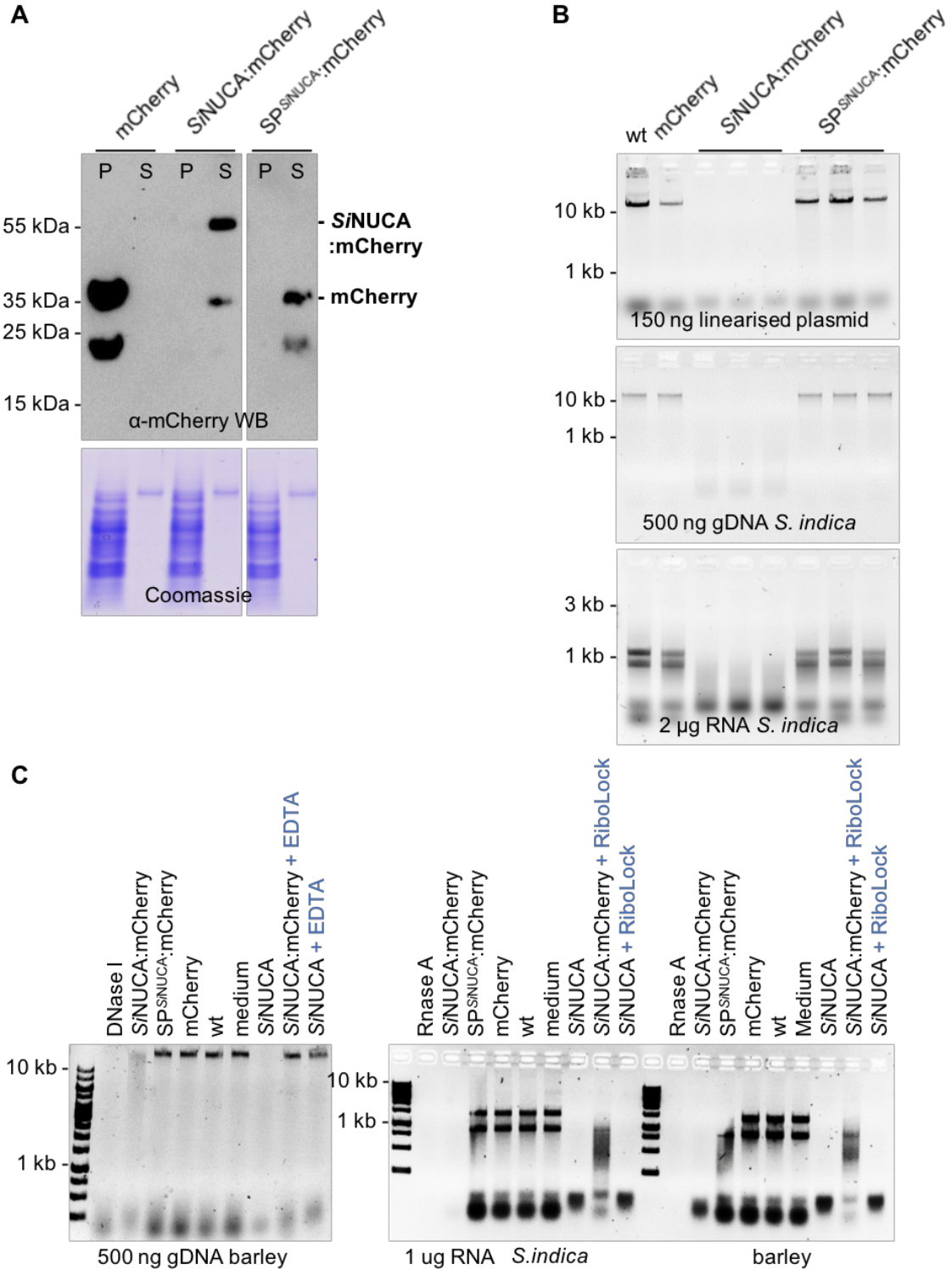
A
mCherry
SiNUCA:mCherry
SPSiNUCA:mCherry
P S P S P S
55 kDa
35 kDa
25 kDa
15 kDa
SiNUCA :mCherry
mCherry
α-mCherry WB
Coomassie
B
wt mCherry
SiNUCA:mCherry
SPSiNUCA:mCherry
10 kb
1 kb
150 ng linearised plasmid
500 ng gDNA S. indica
3 kb
2 µg RNA S. indica
C
DNase I
SiNUCA:mCherry
SPSiNUCA:mCherry
mCherry
wt
medium
SiNUCA
SiNUCA:mCherry + EDTA
SiNUCA + EDTA
Rnase A
SiNUCA:mCherry + RiboLock
SiNUCA + RiboLock
Medium
500 ng gDNA barley
1 ug RNA S.indica
barley

Fig. 2.11: *Si*NUCA heterologously expressed in *U. maydis* is secreted into the medium and is able to digest fungal and plant gDNA and RNA.

A) Anti-mCherry Western blot of pellet (P) and supernatant (S) of liquid cultures. *U. maydis* SG200 expressing mCherry, *Si*NUCA:mCherry or SP^{SiNUCA}:mCherry. Coomassie-stained SDS-PAGE as loading control below. **B)** gDNA or RNA of *S. indica* or linearised plasmid incubated in supernatants used in A) and subsequently loaded on an agarose gel. 1-3 independent transformants per strain were tested. **C)** gDNA and RNA of barley and RNA of *S. indica* were inoculated with supernatants used in A). Additionally, the strain *Si*NUCA was tested. *U. maydis* SG200 (wt), YEPS light only (medium) and 1 U DNase I or 10 µg RNase A were used as controls. Addition of 1 mM EDTA or 20 U RiboLock RNase inhibitor partially inhibited digestion by *Si*NUCA:mCherry and *Si*NUCA.

For optimal *Si*NUCA enzyme activity, different pH and salt conditions were tested with linearised plasmid. *Si*NUCA was more active at pH 8 than 5 and addition of microelements, 1mM $CaCl_2$ and 1mM $MgCl_2$ increased its activity (Fig. 2.12B). In contrast, 25 mM of $MgSO_4$, $MgCl_2$, NaCl or $NaPO_4$, EDTA or RiboLock RNase inhibitor blocked enzyme activity. The unspecific nuclease activity, as seen by heterologous expression in *U. maydis,* was confirmed by digestion of *S. indica* and Arabidopsis gDNA and RNA (Fig. 2.12C).

2.2.5 Establishment of gene deletion via homologous recombination in *S. indica*

In order to test if *SiNUCA* downregulation has an effect on plant colonisation, *S. indica* RNAi strains were produced using the protocol in Hilbert et al., 2012. A 550 bp fragment of *SiNUCA* was expressed from both sides by the constitutive *S. indica* promoters of GPD and TEF to obtain a double stranded *SiNUCA* fragment for gene silencing. *SiNUCA* expression was verified in six transformed *S. indica* strains compared to six empty vector strains. Neither growth of the different strains in axenic culture nor in barley roots showed significant lower *SiNUCA* expression levels in RNAi strains indicating that gene silencing did not occur (data not shown).

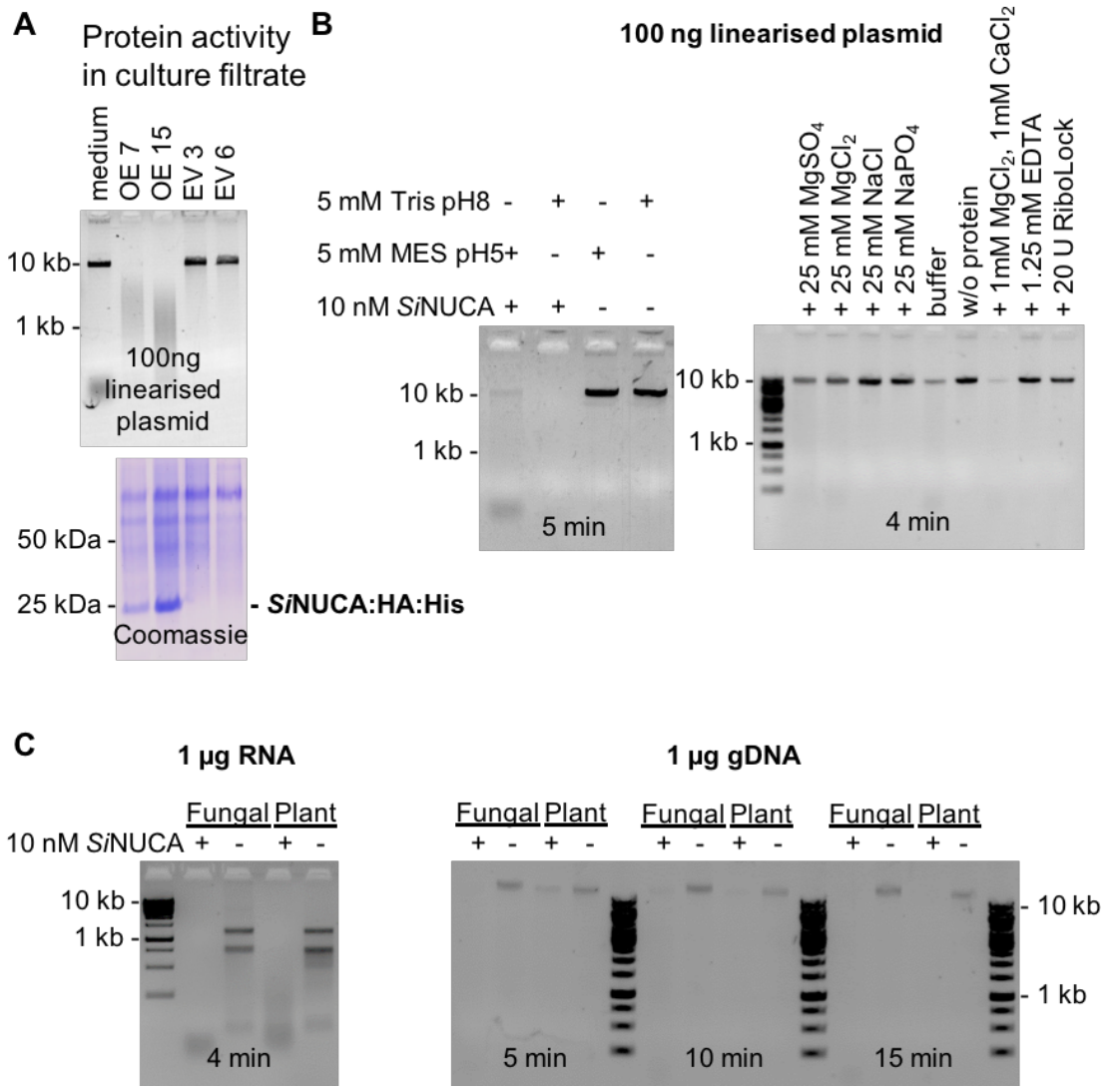

Fig. 2.12: *Si*NUCA:HA:His protein activity in culture filtrate and of enriched protein fraction.

A) Culture filtrate (CF) of *S. indica Si*NUCA:HA:His overexpression strains OE7 and OE15 and empty vector strains EV3 and EV6 incubated with 100 ng linearised plasmid for 30 min and loaded on an agarose gel. Cultures grown in CM medium for 5 days, crushed and grown for 3 days. 20 ml CF precipitated with trichloroacetic acid and loaded on SDS-PAGE as control for total protein amount in CF. **B)** 100ng linearised plasmid added to 10 nM *Si*NUCA in 5 mM Tris pH 8 or 5 mM MES pH 5 with 1mM $MgCl_2$, 1mM $CaCl_2$ and microelements incubated at RT for 5 min and loaded on an agarose gel. Right side: 100 ng linearised plasmid added to 10 nM *Si*NUCA in buffer 5 mM Tris pH8 supplemented with microelements. The influence of the addition of different salts, EDTA and RiboLock RNase inhibitor on protein activity was tested. The linearised plasmid in the different solutions with *Si*NUCA was incubated for 4 min at RT and loaded on an agarose gel. **C)** Specificity of protein activity tested with *S. indica* (fungal) and Arabidopsis (plant) RNA and gDNA. 10 nM *Si*NUCA in buffer 5 mM Tris pH8 with 1mM $MgCl_2$, 1mM $CaCl_2$ and microelements incubated with RNA or gDNA for the indicated time points at RT and loaded on agarose gel.

Next, gene deletion in *S. indica* via homologous recombination was established to produce a *SiNUCA* gene deletion strain for studying its effects on plant colonisation. A homokaryotic *S. indica* strain was used to improve deletion efficiency. The *S. indica* wt strain is dikaryotic and therefore has two gene copies to target. The homokaryotic strain GenR C2 16/3 (GenR C2) was used as a reference homokaryotic *S. indica* strain because its genome was sequenced (Wawra et al., 2016 and unpublished data G. Jeena and Dr. G. Langen).

For homologous recombination a fragment of 1-2 kb up- and downstream of the *SiNUCA* open reading frame with the Hygromycin B resistance cassette in between was constructed and transformed. In order to lower the chance of random fragment integration into the genome the fragment was cut into two pieces with an overlapping region of 410 bp in the Hygromycin B resistance cassette and both fragments were mixed for transformation (Fig. 2.13A). One gene deletion strain was obtained after screening of 447 transformants in total (transformed with one or two fragments of different lengths). Using at least 1.8 kb of the up- and downstream regions, splitting the fragment and adding more than 10 µg of each fragment for the transformation appeared to be the critical points for successful homologous recombination reaching a frequency of approximately 3-10 %. The obtained *SiNUCA* gene deletion strain (*ΔsinucA)* was confirmed by PCR, sequencing of the whole region and Southern blot using one probe on the left border (Fig. 2.13A) and one on the right border of *SiNUCA*. There was no ectopic fragment integration or additional SNPs in *ΔsinucA*. Therefore, *ΔsinucA* was used for *in planta* studies.

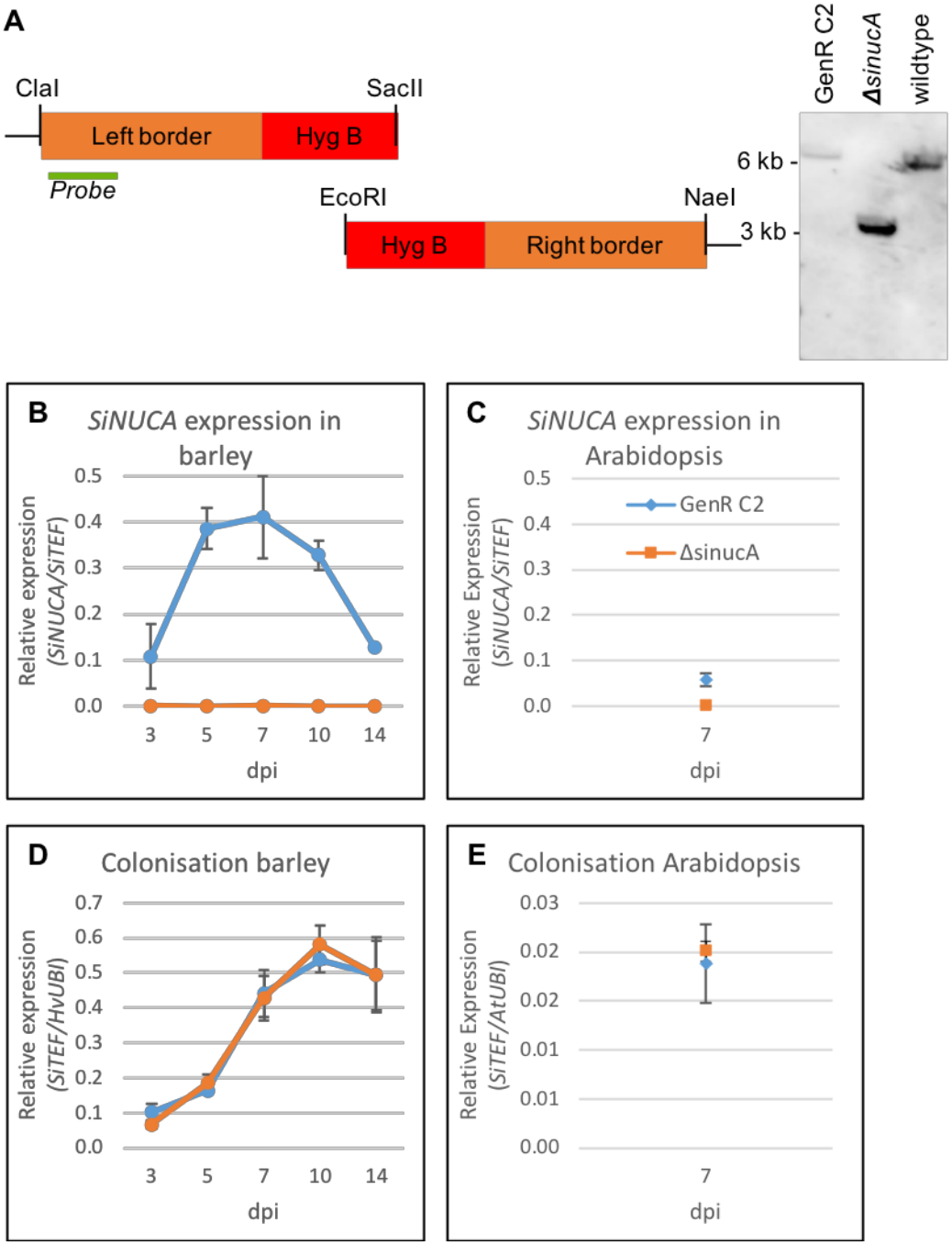

Fig. 2.13: Colonisation of barley and Arabidopsis roots with *S. indica* GenR C2 and *ΔsinucA* strains.

A) Scheme of the two fragments used for *SiNUCA* deletion in *S. indica* homokaryotic strain GenR C2 via homologues recombination. 1.8 kb left and right border of *SiNUCA* fused to a split Hygromycin B cassette with 410 bp overlap. Right side: Southern blot to confirm gene deletion with probe on left border of *SiNUCA*. **B)** and **C)** Expression of *SiNUCA* in *S. indica* GenR C2 and *ΔsinucA* strains in colonised barley (B) or Arabidopsis (C) roots. Expression normalised to *SiTEF*. **D)** and **E)** Colonisation of barley (D) or Arabidopsis (E) roots with *S. indica* GenR C2 and Δ*sinucA* strains measured as relative expression of *SiTEF* to *ubiquitin* (*UBI)*. Paired t-tests: no significant differences between colonisation of GenR C2 and Δ*sinucA*.

SEM of 3-6 biological replicates for barley colonisation and 3 replicates for Arabidopsis colonisation.

2.2.6 *SiNUCA* gene deletion does not alter barley or Arabidopsis colonisation

The *ΔsinucA* strain was compared to its background GenR C2 strain in plant colonisation over time. The absence of *SiNUCA* expression in the *ΔsinucA* strain was confirmed by qPCR (Fig. 2.13B/C). There was no significant difference in barley and Arabidopsis colonisation between the *ΔsinucA* and GenR C2 strain at the different evaluated time points (Fig. 2.13D/E). *S. indica PIIN_05889*, a xylanase used as marker gene for saprotrophic growth in barley (Lahrmann et al., 2013), was induced in the *ΔsinucA* compared to the GenR C2 strain in barley (Fig. 2.14A). No significant differences were observed in the expression of the pathogenesis- related gene *PR10*, a marker for plant immunity activation by *S. indica* in barley, and VPE activity, used as marker for root cell death (Lahrmann et al., 2013) (Fig. 2.14B/C).

*Si*NUCA has neither homologous proteins nor proteins with a homologous *S. indica* DUF 1524 domain in *S. indica*. Bioinformatic analysis using the predicted secretome of *S. indica* (Lahrmann et al., 2013) depicted at least four additional secreted nucleases (*PIIN_00073, PIIN_03873, PIIN_06794* and *PIIN_09504)* unrelated to *SiNUCA*. Among them, *PIIN_00073* and *PIIN_09504* were induced *in planta*. *PIIN_00073* was additionally found in the APF of inoculated barley roots at 10 and 14 dpi but not at 5 dpi like *Si*NUCA. This indicates that gene redundancy might play a role in the colonisation of *S. indica* with altered *SiNUCA* expression levels. However, *PIIN_00073* and *PIIN_09504* were not significantly differently regulated in *ΔsinucA in planta* to compensate for *SiNUCA* gene deletion (Fig. 2.14D-G).

The capability of *ΔsinucA* to digest DNA was tested by growing the *S. indica* strains on plates with salmon testis DNA as sole nitrogen source. The *SiNUCA* overexpression strain was growing faster compared to the empty vector control strain or wt (Fig. 2.15). Staining of the plates with ethidium bromide for visualisation of the DNA under UV light showed digestion of the DNA where the strains were growing with the biggest hallow for the *SiNUCA* overexpression strain. The diameter of the *ΔsinucA* strain was not smaller but the mycelium was thinner compared to the GenR C2 strain. However, DNA digestion was not completely absent in the *ΔsinucA* strain. These results suggest the presence of other secreted nucleases in the *ΔsinucA* strain in axenic culture, which might be active *in planta* too.

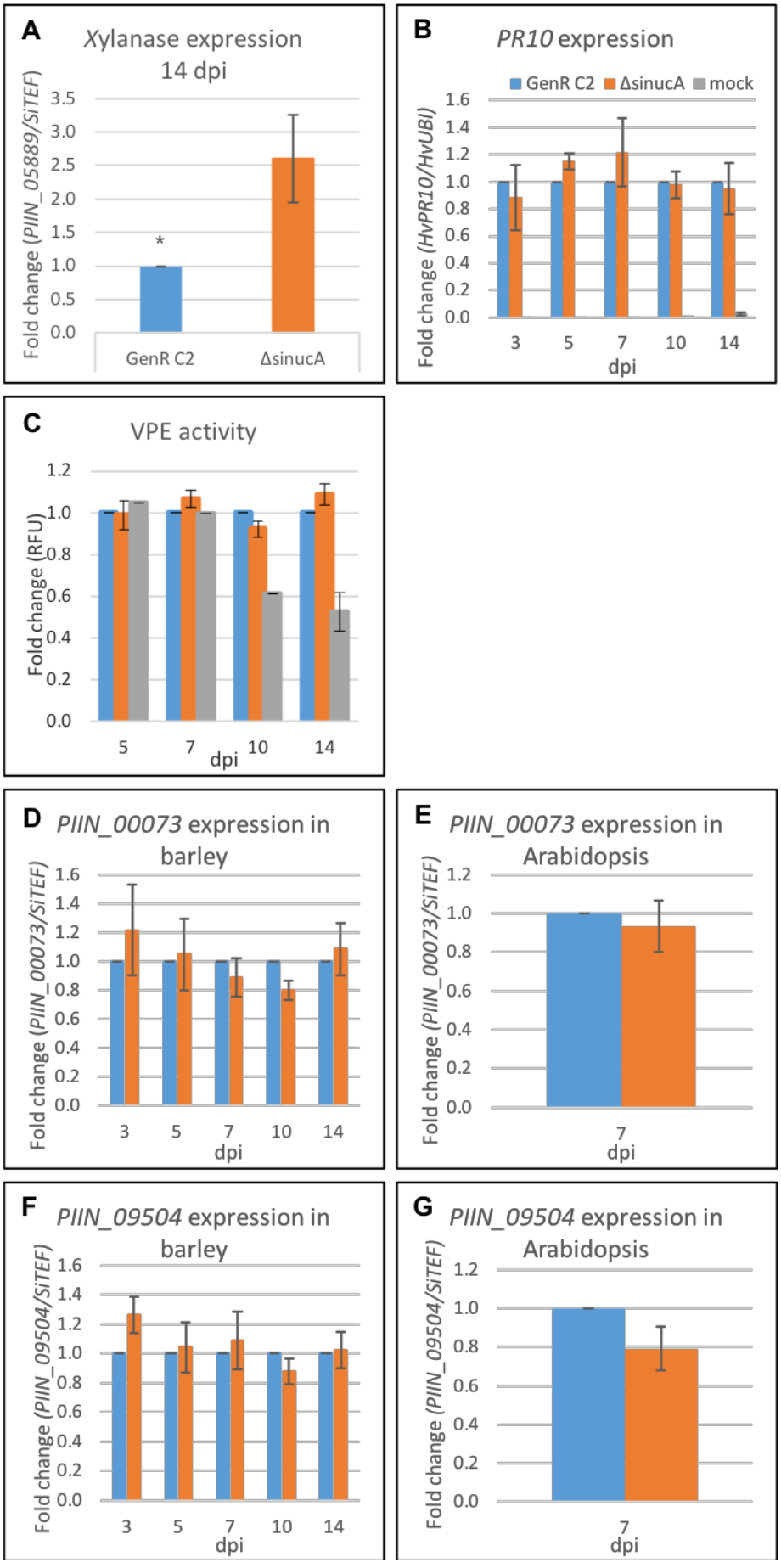
A
Xylanase expression
14 dpi
Fold change (PIIN_05889/SiTEF)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
GenR C2
ΔsinucA
B
PR10 expression
GenR C2
ΔsinucA
mock
Fold change (HvPR10/HvUBI)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3
5
7
10
14
dpi
C
VPE activity
Fold change (RFU)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
5
7
10
14
dpi
D
PIIN_00073 expression in barley
Fold change (PIIN_00073/SiTEF)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3
5
7
10
14
dpi
E
PIIN_00073 expression in Arabidopsis
Fold change (PIIN_00073/SiTEF)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
7
dpi
F
PIIN_09504 expression in barley
Fold change (PIIN_09504/SiTEF)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0.0
3
5
7
10
14
dpi
G
PIIN_09504 expression in Arabidopsis
Fold change (PIIN_09504/SiTEF)
1.2
1.0
0.8
0.6
0.4
0.2
0.0
7
dpi

Fig. 2.14: Gene expression of marker genes and other secreted nucleases in *S. indica* GenR C2 compared to *ΔsinucA* strains *in planta*.

A and B) Marker gene expression in *S. indica* GenR C2 and Δ*sinucA* strains colonising barley roots 3- 14 dpi. SEM of 3- 6 biological replicates. A) *S. indica PIIN_05889* (xylanase) expression as marker for saprotrophic growth. *PIIN_05889* expression relative to *SiTEF* and normalised to GenR C2 B) barley *PR10* expression, a marker for plant immunity activation by *S. indica*, measured relative to *HvUBI* and normalised to GenR C2 **C)** VPE activity in colonised barley roots indicates root cell death. Spectrophotometric measurement of fluorescent substrate for VPE (Ac-ESEN-MCA), which was added to the root extracts. Relative fluorescence units (RFU) normalised to GenR C2 **D to G)** Gene expression of two predicted *S. indica* secreted nucleases (*PIIN_00073* and *PIIN_09504*). Expression relative to *SiTEF* and normalised to GenR C2 D) and F) Comparison of *S. indica* GenR C2 and Δ*sinucA* strain colonising barley roots 3-14 dpi. SEM of 3-6 biological replicates. E) and G) Comparison of *S. indica* GenR C2 and Δ*sinucA* strain colonising Arabidopsis roots 7 dpi. SEM of 3 biological replicates.
Paired t-tests with * p< 0.05.

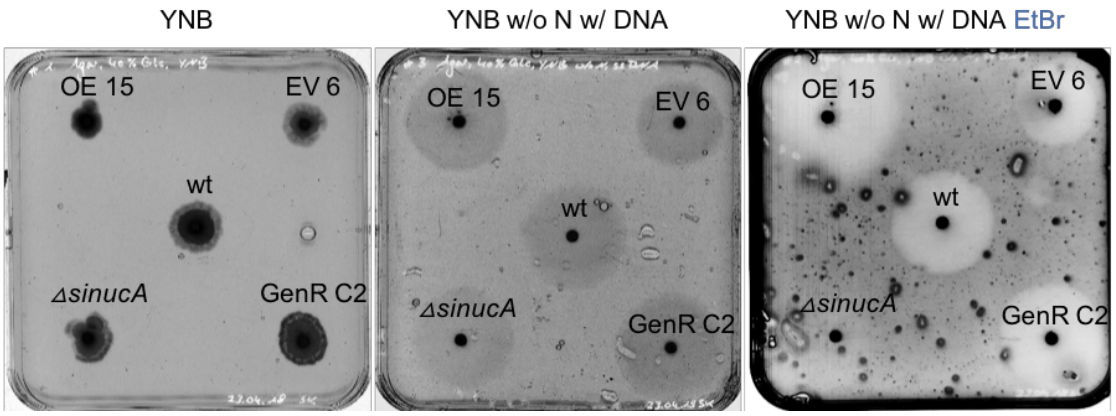

Fig. 2.15: Nuclease activity of *S. indica* strains.
Growth of *S. indica* wt*, SiNUCA* overexpression strain 15, empty vector strain EV6, *ΔsinucA* and GenR C2 strain on DNA as nitrogen source. *S. indica* grown for 5 days on YNB medium or YNB without ammonium sulphate supplemented with 100 µg/ml salmon testis DNA as nitrogen (N) source. Right side: Plate in middle stained with Ethidium bromide (EtBr) to visualise DNA in plate (grey).

2.2.7 Characterisation of homokaryotic and dikaryotic *S. indica* strains *in planta*

The homokaryotic GenR C2 strain, which was used as background for *SiNUCA* gene deletion, was further characterised to evaluate its suitability for deletion of SSPs. Its behaviour in barley colonisation was compared to wildtype. The colonisation rate was lower in the GenR C2 strain compared to wt in an early phase, then reaching the same level und later having a lower colonisation rate again (Fig. 2.16A). Interestingly, at the

same time points where colonisation by the GenR C2 strain was impaired *SiNUCA* expression levels were higher (Fig. 2.16B). No significant differences were detected for xylanase expression speaking for no differences in saprotrophic growth until 14 dpi (Fig. 2.16C). *PR10* expression was increased at 7 dpi and VPE activity was tendentially higher at later time points in the GenR C2 strain (Fig. 2.16D/E). Whether a gene was hit by random integration of the fragment with the Geneticin resistance cassette was checked in the genome sequence of the GenR C2 strain (analysis provided by Dr. G. Langen). *PIIN_08972*, a hypothetical secreted protein, was disrupted. This was confirmed by qPCR showing that in wt but not in GenR C2 *PIIN_08972* was induced over time in colonised barley roots (Fig. 2.16F). The fact that the GenR C2 strain was impaired in colonisation, showed higher *PR10* expression levels at 7 dpi and disruption of a putative secreted protein induced in barley made it difficult to dissect the effects derived by deletion of *SiNUCA.* Thus, this strain is not suitable for characterisation of *SiNUCA* gene deletion.

Other homokaryotic and dikaryotic strains were used to verify if the differences in the GenR C2 compared to the wt strain were specific or a general difference between homokaryotic and dikaryotic strains. Therefore, two homokaryotic and two dikaryotic strains with an average growth behaviour on plate were selected (data from bachelor thesis B. Griebel). Single integration of the Geneticin resistance cassette into the genome was confirmed by Southern blot and the behaviour of this strains during barley colonisation was analysed over time (Fig. 2.16G). The differences observed between wt and GenR C2 could not be detected in other dikaryotic and homokaryotic strains colonising barley.

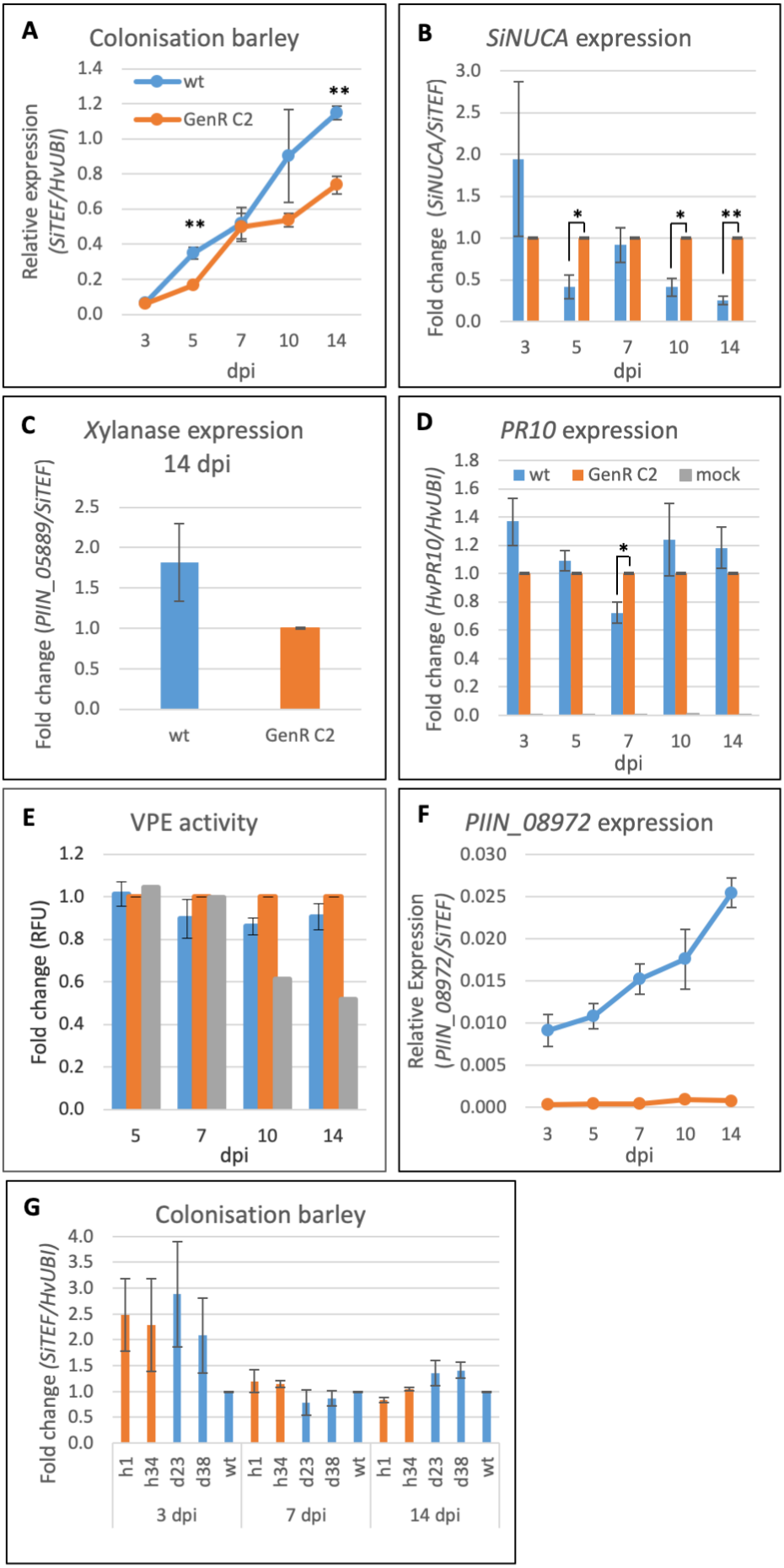
A
Colonisation barley
Relative expression (SiTEF/HvUBI)
wt
GenR C2
**
**
dpi
B
SiNUCA expression
Fold change (SiNUCA/SiTEF)
*
*
**
dpi
C
Xylanase expression
14 dpi
Fold change (PIIN_05889/SiTEF)
wt
GenR C2
D
PR10 expression
Fold change (HvPR10/HvUBI)
wt
GenR C2
mock
*
dpi
E
VPE activity
Fold change (RFU)
dpi
F
PIIN_08972 expression
Relative Expression (PIIN_08972/SiTEF)
dpi
G
Colonisation barley
Fold change (SiTEF/HvUBI)
h1
h34
d23
d38
wt
3 dpi
7 dpi
14 dpi

Fig. 2.16: Behaviour of dikaryotic versus homokaryotic *S. indica* strains in barley.
A)-F) Colonised barley roots by *S. indica* wt and GenR C2 strain 3- 14 dpi. Paired t-tests with * p< 0.05, ** p<0.01. SEM of 3 biological replicates (mock 1 biological replicate). **A)** Colonisation rate measured as relative expression of the genes *SiTEF* to *HvUBI*. **B)** *SiNUCA* expression relative to *SiTEF* and normalised to GenR C2 for each biological replicate. **C)** *S. indica PIIN_05889* (xylanase) expression as marker for saprotrophic growth. *PIIN_05889* expression relative to *SiTEF* and normalised to GenR C2 **D)** barley *PR10* expression, a marker for plant immunity activation by *S. indica*, relative to *HvUBI* and normalised to GenR C2. Expression compared to mock-treated roots. **E)** VPE activity of colonised roots in A) as indication for cell death. Spectrophotometric measurement of fluorescent substrate for VPE (Ac-ESEN-MCA), which was added to the root extracts. **F)** Relative expression of *PIIN_08972* relative to *SiTEF*. **G)** Colonisation of barley roots 3, 7 and 14 dpi of two homokaryotic strains (h1, h34) and three dikaryotic strains (d23, d38 and wt) harbouring the Geneticin resistance cassette. Relative expression of *SiTEF* to *HvUBI* and normalised to wt. Statistical analysis done with ANOVA. SEM of three biological replicates.

2.2.8 *SiNUCA* overexpression has no influence on Arabidopsis colonisation

Three *SiNUCA* overexpression (*Si*NUCA:HA:His) and empty vector control strains were selected for plant colonisation. All strains were homokaryotic as verified by PCR on mating type genes. In Arabidopsis a 7-19- fold increase in *SiNUCA* expression was reached in the *SiNUCA* overexpression strains compared to the empty vector strains and GenR C2 strain 7 dpi (Fig. 2.17B). Nevertheless, *SiNUCA* overexpression did not alter Arabidopsis colonisation levels (Fig. 2.17A). Therefore, *SiNUCA* was heterologously expressed in Arabidopsis to reach higher *SiNUCA* levels and avoid effects due to be homokaryotic.

2.2.9 *SiNUCA* heterologously expressed in Arabidopsis increases colonisation by *S. indica*

SiNUCA was heterologously expressed in Arabidopsis using the 35S promoter and homozygous T3 lines with high *SiNUCA* expression levels were selected. From segregating T2 seedlings neither growing on Hygromycin B nor having the *SiNUCA* gene (PCR verified) were selected and its T3 progeny used as control lines. *SiNUCA* expression levels were up to 6800 times higher compared to the control lines at 7 dpi (Fig. 2.17C). The lines expressing *SiNUCA* showed a higher *S. indica* wt colonisation rate at 7 dpi but

not at 14 dpi compared to the control lines (data not shown). The better colonisation at 7 dpi was confirmed with the two best *SiNUCA* expressing lines 53 and 56 compared to the control lines 4, 18 and wt (Fig. 2.18A/B). However, seedling growth was impaired in the lines expressing *SiNUCA.* They showed shorter root length and lower shoot fresh weight in non-inoculated and inoculated seedlings (Fig. 2.18C). Therefore, an effect of the delayed development of *SiNUCA* expressing lines compared to control lines on colonisation cannot be ruled out. VPE activity was measured in roots to unravel the influence of *SiNUCA* expression on cell death. Induction of VPE activity in Arabidopsis roots colonised by *S. indica* compared to mock inoculation was confirmed (Fig. 2.18D). Additionally, VPE activity was induced in colonised *SiNUCA* expressing line 53 compared to the control line 4 calculated relative to mock-inoculated roots. Therefore, expression of *SiNUCA* and inoculation with *S. indica* led to more cell death in Arabidopsis roots.

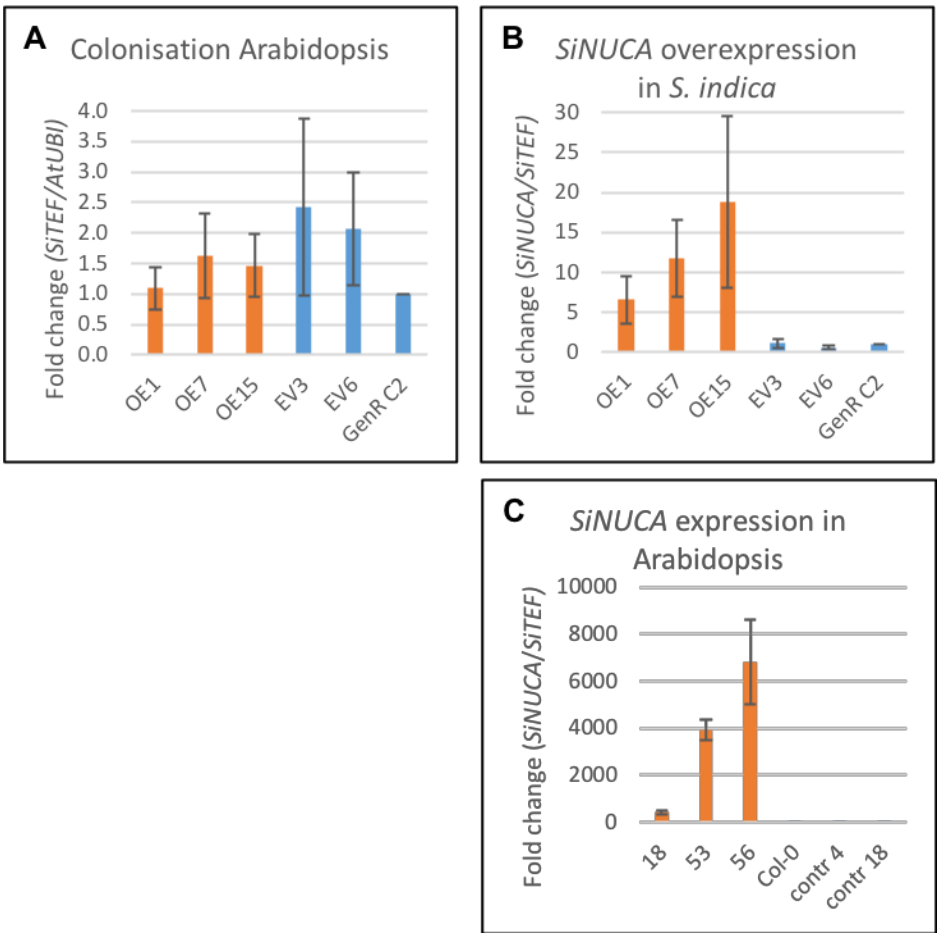

Fig. 2.17: Colonisation of Arabidopsis roots with *S. indica SiNUCA* overexpression strains compared to *S. indica* empty vector strains and *SiNUCA* expression levels in *S. indica* versus in Arabidopsis.

A) Colonisation of Arabidopsis roots with *Si*NUCA:HA:His expressing (OE1, OE7 and OE15) compared to the empty vector (EV3 and EV6) and the GenR C2 strains 7 dpi. Measured as relative expression of *SiTEF* to *AtUBI* and each biological replicate normalised to GenR C2. **B)** *SiNUCA* expression relative to *SiTEF* and normalised to GenR C2 of strains used in A) 7 dpi. **C)** *SiNUCA* expression relative to *SiTEF* and normalised to Col-0 of *SiNUCA* expressing Arabidopsis lines (18, 53, 56) and control lines (Col-0, contr 4, contr 18) inoculated with *S. indica* wt 7 dpi.

SEM of three biological replicates.

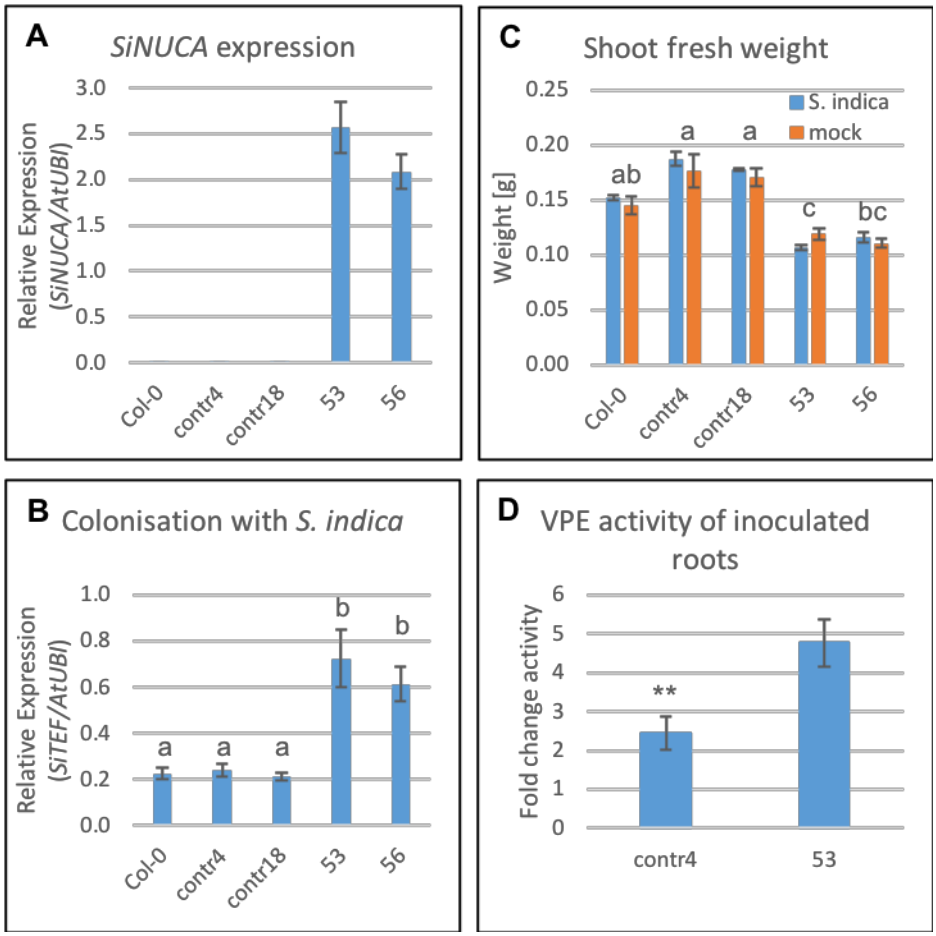

Fig. 2.18: Arabidopsis lines expressing *Si*NUCA colonised by *S. indica* wt.

Arabidopsis lines expressing *SiNUCA* driven by the 35S promoter (lines 53 and 56) compared to control lines (lines contr 4 and contr 18 segregating from T2 generation and wt (Col-0)). Roots inoculated with *S. indica* wt and analysed 7 dpi. **A)** *SiNUCA* expression normalised to *AtUBI*. **B)** Colonisation rate measured as relative expression of *SiTEF* to *AtUBI*. ANOVA with posthoc Tukey test $p < 0.001$ **C)** Shoot fresh weight of colonised and mock-treated roots. Two-way ANOVA with posthoc Tukey test $p < 0.05$ **D)** VPE activity of colonised relative to mock-treated roots. Spectrophotometric measurement of

fluorescent substrate for VPE (Ac-ESEN-MCA), which was added to the root extracts. Paired t-test with ** p< 0.01.

SEM of 5- 6 biological replicates (mock-inoculated plants three biological replicates)

2.2.10 *Si*NUCA localisation in Arabidopsis

In order to localise *Si*NUCA in plant colonisation a *S. indica* strain expressing *Si*NUCA:GFP:HA:His was constructed. Weak expression and only free GFP was detected on the Western blot (data not shown). Next, the high expressing strain *Si*NUCA:HA:His 15 (see 2.1.3) was inoculated with Arabidopsis roots and *Si*NUCA visualised by immunolocalization. Whole- mount Arabidopsis seedlings were labelled with Anti-His-AF 488 or Anti-HA/Anti-mouse-AF 488 antibodies and analysed by CLSM. Signals were only detected with Anti-His-AF 488 in hyphae and not *in planta* probably due to too much dilution in the plant cell or cleavage of the His tag *in planta*. Therefore, heterologous expression of a *Si*NUCA:mCherry fusion in Arabidopsis was applied for *Si*NUCA localisation.

*Si*NUCA:mCherry driven by the 35S promoter was expressed in Arabidopsis and the plant roots were analysed by CLSM. mCherry signal was mainly found surrounding the plant cell (Fig. 2.19A). Plasmolysis was performed to distinguish between cell wall and plasma membrane localisation. Interestingly, *Si*NUCA:mCherry localised at the cell wall but also accumulated in the whole protoplast. To confirm that the localisation outside the plant cell was not an artefact of the fusion protein unable to re-enter the plant cell *Si*NUCA without SP and C-terminally fused to mCherry was analysed as well. *Si*NUCA(w/o SP):mCherry localised to the cytoplasm (Fig. 2.19B). Therefore, we concluded that *Si*NUCA localised outside the plant cell and not the plant nucleus. However, *Si*NUCA:mCherry rearranged around the penetrating hyphae when *Si*NUCA:mCherry expressing roots were inoculated with *S. indica* wt (Fig. 2.20A). At later colonisation stages, *Si*NUCA:mCherry was found at disintegrated nuclei of colonised cells but not at intact nuclei of adjacent cells (Fig. 2.20B).

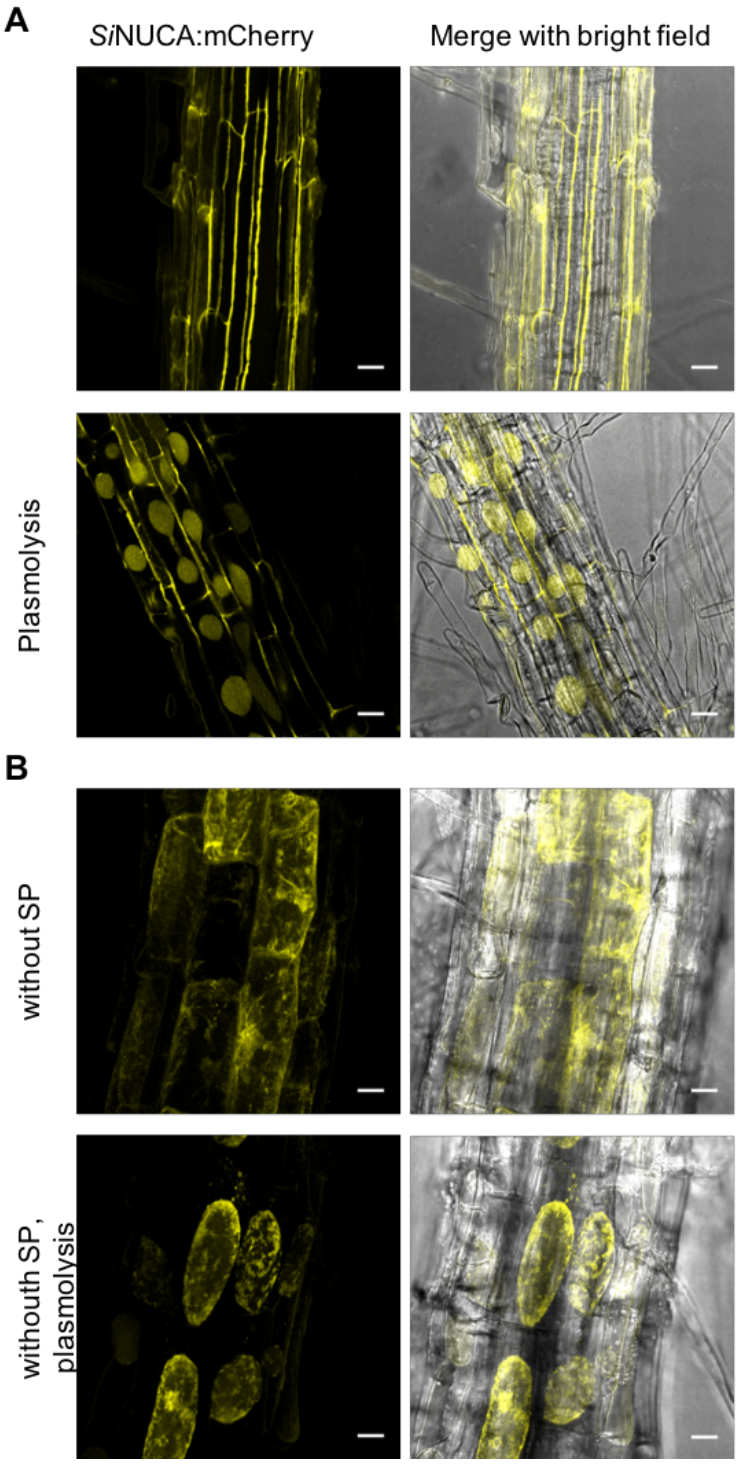

Fig. 2.19: Localisation of *Si*NUCA in Arabidopsis heterologously expressing *Si*NUCA:mCherry.

CLSM live cell imaging of Arabidopsis roots. **A)** Arabidopsis root expressing *Si*NUCA:mCherry. Plasmolysis with 1M NaCl. **B)** Arabidopsis root expressing *Si*NUCA (w/o SP):mCherry. Plasmolysis with 1 M sorbitol. Maximum projection of z-stacks.

Bars = 20 µm.

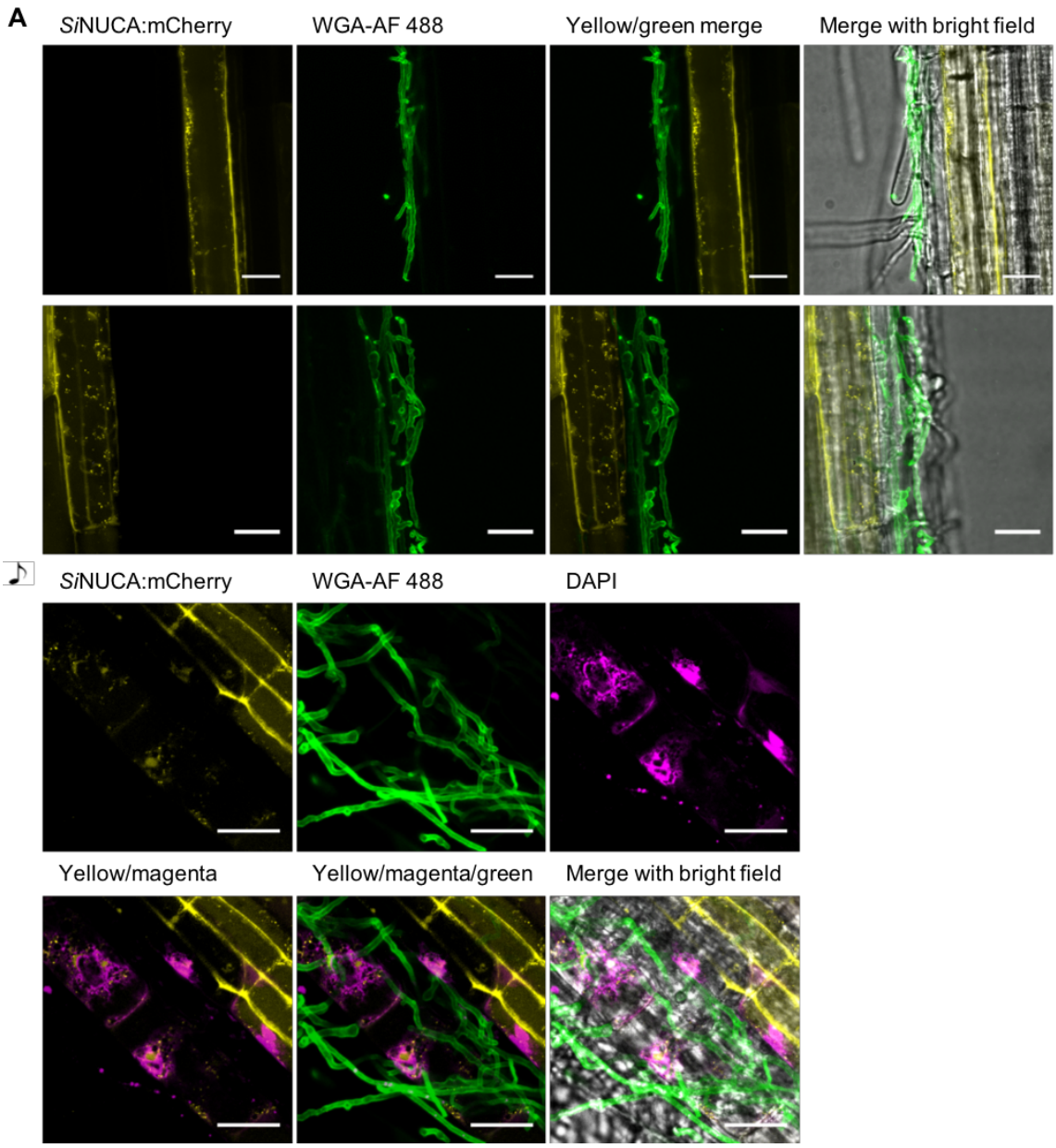

Fig. 2.20: Localisation of *Si*NUCA in colonised Arabidopsis heterologously expressing *Si*NUCA:mCherry.

CLSM live cell images of Arabidopsis root expressing *Si*NUCA:mCherry inoculated with *S. indica* wt. Fungal cell wall of hyphae stained with WGA AF-488 and nuclei with DAPI. **A)** *Si*NUCA:mCherry localises to the cell wall and accumulates around penetrating hyphae (intracellular hyphae in living cells not stainable with WGA AF-488 in the biotrophic interaction phase) **B)** In a later phase, *Si*NUCA:mCherry is mainly present at bursting nuclei in colonised cells.

2.2.11 Antibacterial properties of *Si*NUCA

SiNUCA was hypothesised to be a bacterial-derived gene. Therefore, its antibacterial properties were tested. 20 µM purified *Si*NUCA protein was added to liquid *E. coli* cultures, incubated for 5 h and dilution series were dropped on plates (Fig. 2.21A). No differences in growth were observed between the *Si*NUCA treatment and control treatments of *E. coli* liquid cultures. Next, *Si*NUCA protein was pipetted on a filter platelet on plates inoculated with *E. coli* (Fig. 2.21B). No hallows around the platelets with *Si*NUCA were observed. Furthermore, it was possible to express *SiNUCA* in *E. coli*. In summary, no antibacterial activity of *Si*NUCA against *E. coli* was noticed in the different experiments.

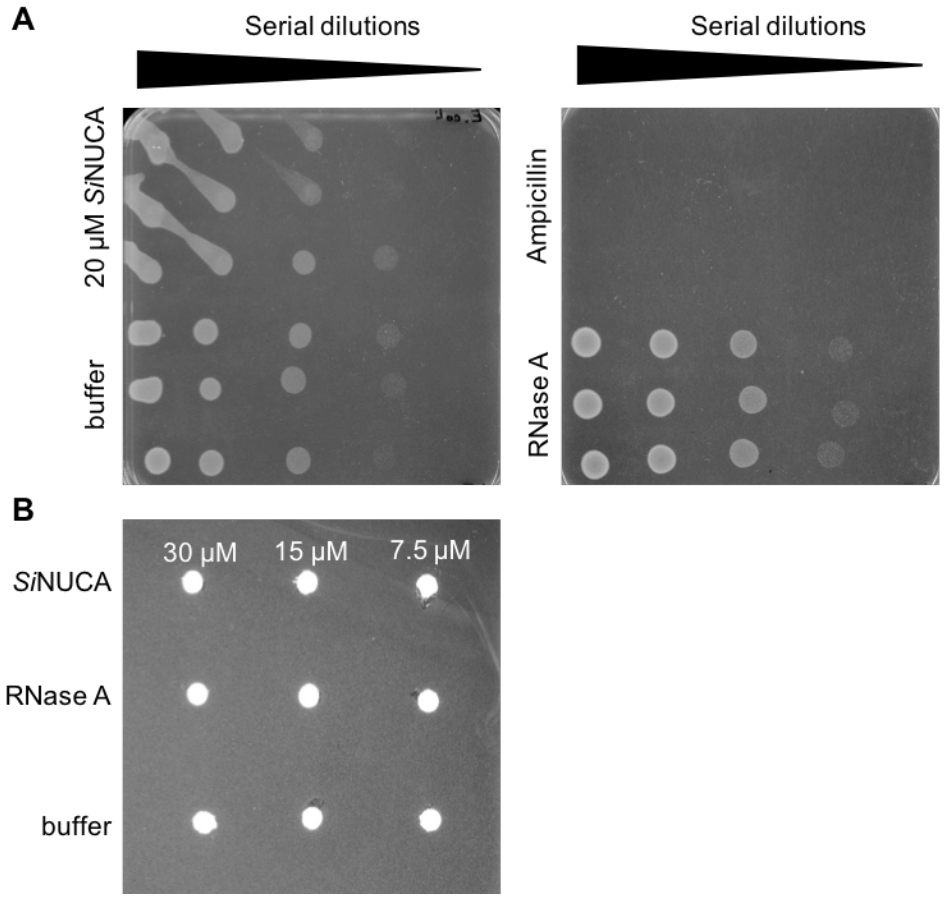

Fig. 2.21: Antibacterial activity of *Si*NUCA.

A) *E. Coli* was growing in the presence of 20 µM *Si*NUCA, 130 µg/ml ampicillin, 3 µg/ml RNase A or buffer (20 mM Tris pH 8) for five hours before plating of 1:10 dilution series on plates. **B)** *E. Coli* was sprayed on plates and filter rondels with different *Si*NUCA concentrations, 3 µg/ml RNase A or buffer (20 mM Tris pH 8) were put on the plates.

Pictures were taken after overnight growth.

3 Discussion

3.1 Application of *S. indica* as a protein production system

A protein production system was established in *S. indica* in order to express and secrete homologous and heterologous proteins for further protein purification from culture filtrate. For high protein production the choice of the promoter for gene expression is a key factor (Ward, 2012). As the use of established promoters from other species failed the endogenous constitutive promoters TEF and GPD were applied for gene expression in *S. indica* so far (Hilbert et al., 2012; Zuccaro et al., 2009). However, problems with gene silencing of endogenous genes emerged using these promoters (Wawra et al., 2016). In order to strongly express genes in *S. indica* other promoters were screened for their suitability.

First of all, the TEF promoter of the closely related fungus *S. vermifera* was cloned in front of GFP to test its performance in *S. indica*. Strong GFP signals were detected under the microscope confirming its functionality in *S. indica* (Fig. 2.1). Yet it remains to be tested whether constitutive expression of endogenous genes under this promoter can be achieved without gene silencing. FGB1 is one of the most abundant secreted proteins in CM but not in YNB medium (Wawra et al., 2016). The repressibility and strong activity of the FGB1 promoter was confirmed by analysing *S. indica* strains harbouring FGB1 with a C-terminal GFP fusion driven by the FGB1 promoter (Fig. 2.2). Therefore, the FGB1 promoter was picked as first choice for protein production.

Next, media conditions were improved for optimal protein expression and secretion. Using the FGB1:GFP expressing strains large amounts of protein secretion into the culture medium was achieved by specifically growing *S. indica* in CM to gain biomass and then switching to MYP medium for protein secretion (Fig. 2.3). However, mainly free GFP was detected urging the improvement of fusion protein stability. Therefore, different tags and linkers between the gene of interest and tags were tested.

The modular vector pFGB1 was generated to facilitate cloning and testing of different constructs (Fig. 2.4). In the FGB1:GFP strains only a small linker consisting of the restriction site was applied. Linkers are not only important for protein flexibility and stability but can also improve their activity and yield (Chen et al., 2013; Waugh, 2011). Therefore, a flexible linker and the PreScission site for cleavage of the tags after protein

purification were tested. Moreover, mRFP instead of GFP was used as its sequence differs at the beginning and end of the protein, which might lead to less protein cleavage. However, no significant improvements were achieved. The advantage of flexible linkers is the mobility of the different protein domains to allow correct protein folding and activity (Chen et al., 2013). On the other hand, testing of a rigid linker, which separates and reduces the interference between the protein domains, can lead to higher stability and activity in some fusion proteins (Chen et al., 2013). Linker design is not trivial and databases or bioinformatic tools should be considered for assistance in linker optimisation (Crasto & Feng, 2000; C. Liu et al., 2015).

Protease activity in the culture medium is one of the biggest draw backs of fungal expression systems (Ward, 2012). Edman degradation might be performed to identify putative cleavage sites between the protein of interest and tags or between the tags. Consequently, these sequences could be avoided to prevent fusion protein cleavage by proteases. Proteomic analysis revealed that *S. indica* secretes metalloproteinases, serine proteases, aspartic proteases and aminopeptidases into the culture filtrate. Therefore, deletion of the major secreted proteases or protease regulators might be a big step forward and is frequently applied in optimised protein production systems (Sarkari et al., 2014; Ward, 2012). Establishment of CRISPR/Cas9 in *S. indica* could make it feasible to delete more than one protease (Schuster et al., 2018). Alternatively, a proteinase inhibitor can be expressed in parallel with the protein of interest to protect its cleavage (Erjavec et al., 2012). FGB1:GFP production was low but stable in YNB medium (Fig. 2.2). In order to overcome this observed trade-off of protein production versus stability the YNB medium might be adjusted for higher protein yields or the CM medium for less protease activity. It was shown that the pH and the metabolites in the culture medium have influence on protease activity (Ward, 2012).

FGB1:GFP moved in vesicles towards the hyphal tip (Fig. 2.2). This and the predicted SP of FGB1 were hints for conventional secretion of FGB1 via the ER and Golgi apparatus (Nevalainen & Peterson, 2014; Rodrigues et al., 2011). Interestingly, higher expressing FGB1:GFP(:HA:His) strains tended to secrete less fusion protein compared to lower expressing strains. Therefore, conventional secretion of fusion proteins might reach a limit. The major bottlenecks of high protein production in filamentous fungi are not only at the transcriptional and translational level but also protein secretion (Ward, 2012).

Proteins can get trapped or lost during protein translocation, folding, processing and transport in the ER respectively the Golgi apparatus (Nevalainen & Peterson, 2014; Ward, 2012). Therefore, the overexpression of foldases and chaperones, which help correct protein folding, or fusion to highly secreted carrier proteins might improve protein secretion (Nevalainen & Peterson, 2014; Ward, 2012).

As shown by addition of FITC-FGB1 to the hyphae FGB1 localised at the cell wall and a matrix surrounding the hyphae (Fig. 2.5). Therefore, production of a large amount of cell wall-binding lectins in the culture filtrate is an additional task to solve. The capacity of *S. indica* to produce this matrix around its hyphae seemed to be culture condition dependent. Hence, the culture medium might also be optimised regarding matrix production to increase the amount of proteins like FGB1 in the culture filtrate. Alternatively, a method for matrix collection and protein purification thereof could be developed.

Despite the drawbacks of the present protein production system homologous and heterologous expression of several proteins was successful. The tested heterologous proteins were an E3 ubiquitin ligase form *S. vermifera* and the FGB1 homolog in *B. sorokiniana*. Proteins overexpressed in *S. indica* besides FGB1 were the SSPs DLD1, WSC3 and *Si*NUCA. Some of these proteins were not possible to express in the commonly applied systems *P. pastoris* or *E. coli*.

High amount of secreted protein in the culture filtrate was achieved for *Si*NUCA:HA:His. Since cleavage of the His tag in a large fraction of the secreted protein protein purification relied on ammonium sulphate precipitation and subsequent size exclusion chromatography (Fig. 2.6). Applying this method a protein purity of ca. 70 % and a protein yield of 13 mg/L was achieved. If higher purity is needed for specific biochemical assays the protein might be further purified using alternative methods like HA affinity beads since this tag was still intact. To increase fusion protein stability the mentioned methods above for FGB1 might be implemented. In *U. maydis* expression of mCherry:*Si*NUCA instead of *Si*NUCA:mCherry was non-functional. Therefore, C-terminal protein tags were also used in *S. indica* for *Si*NUCA expression. However, it is still possible that small N-terminal tags like the His-tag might be functionally tolerated by *Si*NUCA and lead to higher fusion protein stability. Moreover, the reversed tag His:HA might be constructed with the idea that only HA would be cleaved and therefore,

protecting the His tag for protein purification. On top, other tags commonly used for protein purification like the Glutathione S-transferase tag could be tested (Kimple et al., 2013).

In conclusion, a protein production system was established in *S. indica* by implementing the FGB1 promoter, optimising culture conditions, linkers and tags for fusion protein production. Not only homologous but also heterologous proteins were successfully expressed in this system. *Si*NUCA was produced in *S. indica*, purified from the culture filtrate with a yield of 13 mg/L and its activity was confirmed. With further improvement of fusion protein stability this system has the potential for upscaling, cheap protein production and easy purification from culture filtrate complementing the few established systems in Basidiomycetes. Protein production in *S. indica* can bring an added value as especially proteins of Basidiomycete are difficult to express in prokaryotic, yeast and even filamentous fungal systems (Corchero et al., 2013; Erjavec et al., 2012).

3.2 Homokaryotic versus dikaryotic *S. indica* strains

To increase gene deletion efficiency, a homokaryotic *S. indica* strain was used. Additionally, the transformed strains derived from wt were often homokaryotic including the *SiNUCA* overexpression strains in this thesis. Thus, homokaryotic and dikaryotic *S. indica* strains were characterised in more detail. Griebel showed in her Bachelor thesis that homokaryotic strains are affected in saprotrophic growth compared to dikaryotic strains (Griebel, 2016). Impairment of growth on plates was shown in many wood decay Basidiomycetes but exceptions are known too (Fryar et al., 2002; Hiscox et al., 2010; Meng et al., 2013; Nazrul & YinBing, 2011). Moreover, homokaryons show differences in morphology and gene expression (Meng et al., 2013; Nazrul & YinBing, 2011) but not in competitivity and enzyme activity (Fryar et al., 2002; Hiscox et al., 2010). Barley colonisation by the *S. indica* homokaryotic GenR C2 strain was impaired at 5 and 14 dpi but not at 7 dpi compared to the dikaryotic wt strain (Fig. 2.16). At these time points, *SiNUCA* expression was higher in the GenR C2 strain and barley responded with a second peak of *PR10* expression at 7 dpi. As the GenR C2 strain proved to have a deletion in the hypothetical SSP *PIIN_08972* we speculated that *PIIN_08972* might be involved in plant immunity suppression at 7 dpi. With impaired *PIIN_08972* expression

the plant defence response was increased at 7 dpi and consequently, less colonisation was observed at later stages in the GenR C2 strain. At the same time, *SiNUCA* was induced at later stages probably attempting to increase colonisation or to allocate nutrients. However, these differences could not be confirmed by comparing other homokaryotic and dikaryotic *S. indica* strains. Therefore, *Si*NUCA gene deletion might be repeated in another homokaryotic strain. However, it must be further confirmed if *S. indica* homokaryotic strains are generally suitable for plant colonisation analysis before using them as substitution for the wt strain.

3.3 Disappearance of plant nuclei upon *S. indica* colonisation

S. indica has a biphasic lifestyle with an early biotrophic and a cell death-associated phase (Deshmukh et al., 2006; Jacobs et al., 2011; Zuccaro et al., 2011). The disappearance of plant nuclei in colonised Arabidopsis roots was observed by cytological studies (Fig. 2.7). *S. indica* was detected to grow through the nucleus suggesting that the fungus was actively feeding on the nucleus.

S. indica was extracellularly growing around the whole Arabidopsis root and interacting with BLC but colonisation of the root tip or BLC was not observed (Fig. 2.8 and Jacobs et al., 2011). Protection of root tip infection by border cells was shown for the root pathogens *Aphanomyces euteiches* and *Nectria haematococca* in pea, where border cell production is increasing upon pathogen infection (Cannesan et al., 2011; Gunawardena & Hawes, 2002). Hyphae in contact with border cells are fragmented and unable to reach the root cap itself (M. C. Hawes et al., 2011). Moreover, microbes are attracted by border cells but not able to colonise them (M. Hawes et al., 2016a). A connection between border cell production and mycorrhizae was suggested but its functional interaction is unknown (Driouich et al., 2013). Arabidopsis BLC are released in sheets and not as single cells like the border cells of other plant species (Vicre et al., 2005). However, it was suggested that BLC function in a similar way to border cells and are involved in pathogen response too (Driouich et al., 2010; Plancot et al., 2013). Therefore, BLCs could protect Arabidopsis root tips from colonisation by *S. indica* as well.

Nuclei fading was not only observed in colonised epidermal and cortex cells but also in BLC, which just have come in contact with *S. indica* (Fig. 2.8). Border cells were described as living cells, which are metabolically active (M. C. Hawes et al., 2011; Wen et al., 2007).

Therefore, nuclei fading, probably followed by cell death, in BLC in contact with *S. indica* might be due to the normal turn-over of BLC. It was shown that Arabidopsis BLC stay only alive for few days (Plancot et al., 2013; Vicre et al., 2005).

3.4 The features of the *S. indica* endonuclease *Si*NUCA

The *S. indica* endonuclease *Si*NUCA was found in the APF of *S. indica*-colonised barley roots at 5 dpi, where the transition from the biotrophic to the cell death-associated phase happens. *SiNUCA* expression was induced in barley with highest expression levels at 7 dpi (Fig. 2.10). In Arabidopsis, *SiNUCA* expression was highest at 14 dpi. However, *SiNUCA* expression of *S. indica* in Arabidopsis was not significantly higher than on ½ MS medium but significantly increasing over time. *Si*NUCA is 211 amino acids long, has a predicted SP and Pfam DUF1524 domain, which belongs to the His-Me finger endonuclease superfamily (Fig. 2.9). Therefore, *Si*NUCA shows typical features of effector proteins as it is small, has a SP, four cysteines and is induced *in planta*. In contrast, it is highly conserved in the Agaricomycetes, Ascomycetes and Actinobacteria. High conservation of effectors is uncommon but has been shown before (Lo Presti et al., 2015). Jablonska et al. suggested horizontal gene transfer as highly conserved genes with the DUF 1524 domain were found in the Actinomyces bacteria (belonging to the Actinobacteria) and Agaricomycetes fungi (Jablonska et al., 2017). Consequently, *SiNUCA* might be a bacterial-derived gene.

The homologous gene in *S. vermifera* was expressed in Arabidopsis and barley too but at earlier time points compared to *SiNUCA*. In accordance, Lahrmann et al. suggested that Arabidopsis colonisation by *S. vermifera* is faster than by *S. indica* (Lahrmann et al., 2015). As the homologous protein in *S. vermifera* has a SP and DUF1524 domain as well a nuclease effector function was suggested. Therefore, *Si*NUCA as a putative conserved effector was chosen for further molecular characterisation and its involvement in plant cell death was investigated.

First, secretion and nuclease activity were confirmed by expression of *SiNUCA* in *U. maydis* (Fig. 2.11). *Si*NUCA emerged to be an unspecific endonuclease as in *U. maydis* culture supernatant containing *Si*NUCA fungal and plant genomic DNA and RNA was digested. Moreover, EDTA inhibited digestion confirming the metal ion- dependent catalytic activity of the protein.

Second, *SiNUCA* driven by the FGB1 promoter was overexpressed in *S. indica*. Here, secretion and nuclease activity were observed in the culture filtrate of *Si*NUCA overexpressing *S. indica* strains compared to empty vector strains (Fig. 2.12). Further characterisation of *Si*NUCA was performed with the purified protein. Unspecific endonuclease activity was confirmed with the purified *Si*NUCA:HA:His as the protein was able to digest fungal and plant gDNA and RNA and linearised plasmids. Furthermore, low pH and high salt conditions reduced *Si*NUCA activity. It is unknown which metal ion *Si*NUCA is binding and also the HNN motif as catalytic side is not confirmed. Mg^{2+} is a common cofactor of His-Me finger endonucleases (Jablonska et al., 2017; M. J. Mate & Kleanthous, 2004; Rangarajan & Shankar, 2001). As small amounts of $MgCl_2$ and $CaCl_2$ increased *Si*NUCA activity, Mg^{2+} might bind *Si*NUCA. On the other hand, the protein showed a brown colour, which was lost upon addition of the reducing agent glutathione (data not shown). Therefore, an iron-sulphur complex might be responsible for this colour as demonstrated for other proteins (Huang et al., 2008; Yeeles, 2009). However, it was shown for nucleases that they are active with interchangeable metal ions (Garforth et al., 2001; Okafor et al., 2017). The DNase Ehe A from the bacteria *Exiguobacterium* sp. yc3 is the only biochemically characterised homologue of *Si*NUCA to our knowledge. This extracellular 20 kDa DNase with 48 % sequence identity to *Si*NUCA on protein level exhibits high activity with Mg^{2+}, Mn^{2+} and Co^{2+} (Zhou et al., 2015). Ehe A shows activity from pH 5- 10 and thermostability (Zhou et al., 2015). Moreover, mutation of the conserved HNN motif disrupts DNase activity (Zhou et al., 2015).

3.5 *SiNUCA* overexpression and deletion has no effect on plant colonisation

Gene deletion was established in *S. indica* to delete *SiNUCA* and analyse its effect on plant colonisation. By using 1.8 kb up- and downstream of the gene, splitting the fragment and adding more than 10 µg of each fragment for the transformation a homologous recombination frequency of 3-10 % was reached (Fig. 2.13). A higher homologous recombination frequency was shown in different fungal systems with longer flanking regions and when the flanking regions show 100 % homology at their ends (Bird & Bradshaw, 1997; Kamper, 2004; Krappmann et al., 2006; Ninomiya et al., 2004). A major increase in homologous recombination frequency was achieved by

deleting *KU* genes involved in non-homologous end-joining for example in *A. fumigatus* and *Neurospora crassa* (Krappmann et al., 2006; Ninomiya et al., 2004). Therefore, the rate of homologous recombination in *S. indica* might be further increased by preventing non-homologous end-joining. As the GenR C2 strain already harbours the Geneticin resistance cassette and on top only the hygromycin B resistance cassette is routinely used more antibiotics must be screened for selection purposes in *S. indica*. The attempt applied here to prevent non-homologous end-joining was to split the fragment as integration of the two fragments without homologous recombination led to a non-functional split resistance cassette. The established gene deletion system could be applied to delete other genes of interest in *S. indica* or could be adapted for *S. vermifera*.

The obtained *ΔsinucA* strain was compared to the background GenR C2 strain in its ability to colonise barley and Arabidopsis roots. No significant differences were observed at all analysed time points. Moreover, there were no differences detected in plant immunity response (analysed by *PR10* expression) or root cell death (analysed by VPE activity) in barley (Fig. 2.14). *PIIN_05889* expression, a marker for saprotrophic growth, was higher in the *ΔsinucA* strain in barley at 14 dpi probably compensating for *SiNUCA*. Moreover, no differences were observed in the timing of nuclei disappearance of stained nuclei with SYTOX orange or H2B:mCherry expressing Arabidopsis plants inoculated with the *SiNUCA* overexpression or *ΔsinucA* strain compared to the control strains (data not shown).

The *S. indica* secretome was analysed to find other secreted nucleases induced *in planta*, which could compensate for *SiNUCA* deletion. *PIIN_00073* (an endonuclease exonuclease phosphatase family protein) and *PIIN_09504* (a s1p1 nuclease) were confirmed to be induced in barley and Arabidopsis although their expression was not significantly elevated in the *ΔsinucA* strain *in planta* (Fig. 2.14). The ability of *ΔsinucA* to digest DNA in plates was not completely abolished (Fig. 2.15). These results suggested additional secreted nucleases playing a role and maybe compensating for *SiNUCA* deletion. There is the possibility of predicted nucleases without SP to be unconventially secreted or secreted proteins with unknown function exhibiting nuclease activity in *S. indica,* which should also be taken into consideration.

Despite the large variety and low sequence conservation of effectors, functional redundancy is recognised as a major problem in studying effector proteins (Plett &

Martin, 2015; Rafiqi et al., 2012; Win et al., 2012). Functional redundancy does not only include targeting of the same host protein by more than one effector but also the same host pathway by several effectors (Win et al., 2012). Saitoh et al. disrupted 78 putative effectors in the rice pathogen *Magnaporthe oryzae* but they only see a phenotype with one of the deleted effectors (Saitoh et al., 2012). The deletion of all six *mig2* genes in *U. maydis*, which show effector characteristics, does not alter tumour formation in maize (Farfsing et al., 2005). Furthermore, Kettles et al. showed that the fungal secreted ribonuclease Zt6 is phytotoxic but does not influence *Zymoseptoria tritici* virulence on wheat (Kettles et al., 2018). In addition, no differences in colonisation of the different strains might be due to not high enough sensitivity of the assays to see subtle effects (Lo Presti et al., 2015). Indeed, a tendency for lower colonisation by *ΔsinucA* correlated with tendentially higher expression of the two other tested nucleases in *ΔsinucA* compared to GenR C2 in barley at 3 dpi (Fig. 2.13& 2.14).

The effect of *SiNUCA* overexpression on plant colonisation was analysed as well. Arabidopsis colonisation at 7 dpi did not reveal any differences between *SiNUCA* overexpressor and empty vector strains (Fig. 2.17). Therefore, *SiNUCA* was heterologously expressed in Arabidopsis to analyse its effect on colonisation.

3.6 *SiNUCA* expression in Arabidopsis enhances colonisation and cell death

SiNUCA driven by the strong and constitutive 35S promoter was expressed in Arabidopsis. Arabidopsis lines harbouring *SiNUCA* showed induced colonisation of *S. indica* wt at 7 dpi (Fig. 2.18). This is in contrast to *SiNUCA* overexpression on fungal side, which had no effect on Arabidopsis colonisation (Fig. 2.17). A 3930- 6820-fold increase in *SiNUCA* levels were reached in the two highest expressing lines compared to a 7- 19-fold increase in *SiNUCA* overexpressing *S. indica* strains at 7 dpi. However, the *SiNUCA* expressing seedlings also showed shorter roots and less shoot weight suggesting a delay or impairment in seedling development (Fig. 2.18). Therefore, an effect of the seedling developmental stage on root colonisation could not be ruled out.

VPE activity was measured in the different Arabidopsis lines inoculated with *S. indica* to unravel the influence of *SiNUCA* on RCD. In general, colonisation by *S. indica* elevated VPE activity in roots as shown before (Fig. 2.18 and Lahrmann et al., 2013; Qiang et al., 2012b). VPE activity was higher in the colonised *SiNUCA* expressing plant line relative to

mock compared to the control line relative to mock. The calculation relative to mock inoculated plants revealed a normalisation for developmental effects of the different plant lines. This might be necessary as increased root cell death with root age was described in different plant species (Bagniewska-Zadworna & Arasimowicz-Jelonek, 2016; Liljeroth & Bryngelsson, 2001). Whether *Si*NUCA was directly involved in RCD and hence, a higher colonisation level resulted or *Si*NUCA was indirectly increasing colonisation that led to more RCD is unknown.

3.7 *Si*NUCA localisation

Since *in planta* localisation of *Si*NUCA with *Si*NUCA:GFP:HA:His and *Si*NUCA:HA:His expressing strains was not successful *Si*NUCA localisation was analysed via heterologous expression in Arabidopsis using a C-terminal mCherry fusion. mCherry signals were detected at the cell wall but after plasmolysis also in the whole protoplast (Fig. 2.19). In general, a cell wall localisation and a high background in vacuoles was detected. In order to verify secretion, plant lines expressing *Si*NUCA(w/o SP):mCherry were analysed as control. In this plant lines a clear cytoplasmic localisation was revealed and no specific accumulation in the nucleus. Accordingly, *Si*NUCA has no nuclear localisation signal in its protein sequence. These results suggested an extracellular localisation of the protein.

Upon inoculation of the *Si*NUCA:mCherry expressing Arabidopsis seedlings with *S. indica* mCherry signal translocated from the cell wall to focal accumulation around penetrating hyphae (Fig. 2.20). At later colonisation stages, *Si*NUCA:mCherry was observed at bursting nuclei of colonised host cells suggesting digestion of nuclear DNA.

However, the accuracy of *Si*NUCA localisation must be questioned as *Si*NUCA:mCherry was heterologously expressed under the strong and constitutive 35S promoter. The mCherry fusion might lead to artefacts in localisation and it is currently unknown where secreted mCherry alone localises in Arabidopsis roots. Wang et al. showed that localisation differed in their case if the mRFP fused effector was expressed in the host plant or in the fungus (Wang et al., 2017). Reitz et al. faced problems with different fusion protein localisation in Arabidopsis depending on where in the protein the GFP sequence was added (Reitz et al., 2013).

3.8 Function of *Si*NUCA *in planta*

Secreted nucleases are not only abundant in bacterial and fungal pathogens but also in soil microbes (Hadwiger et al., 2013; M. Hawes et al., 2016a; M. C. Hawes et al., 2011). Searching the literature, these secreted nucleases are connected to host immunity, microbial competition, protection from foreign DNA, nutrition and biofilm modulation with some proteins having dual function. Therefore, the experimental data of *Si*NUCA was compared to other characterised secreted nucleases to hypothesise on its function.

3.8.1 Nucleases with cell death executor function

Nucleases were shown to localise in the nucleus and degrade chromosomal DNA upon apoptosis in animal systems (Hsia et al., 2005). CAD/FDD40 is activated by caspase-3, enters the nucleus and digests chromosomal DNA, which finally leads to cell death (Enari et al., 1998; X. Liu et al., 1997). Moreover, endoG in the mitochondria is translocated to the nucleus to degrade chromosomal DNA upon induction of apoptosis (Li et al., 2001). In plants, the nuclease ZEN1 degrades nuclear DNA in tracheary elements following vacuolar collapse (Ito & Fukuda, 2002). Host nuclear localisation and induction of the DNA damage response was shown for the pathogenic oomycete effector nucleases CRN13 of the legume root pathogen *Aphanomyces euteiches* and amphibian pathogen *Batrachochytrium dendrobatidis* (Ramirez-Garces et al., 2015). Therefore, *Si*NUCA might be involved in host cell death as shown for the above-mentioned nucleases.

In accordance, VPE activity was induced in *SiNUCA*-expressing Arabidopsis lines upon inoculation with *S. indica,* a mCherry fusion localised at the degrading nucleus at late colonisation stages, *Si*NUCA activity was higher at pH 8 and *SiNUCA* transcript levels were highest in Arabidopsis at later colonisation stages. In contrast, *Si*NUCA was present in the barley APF already at 5 dpi and its expression was highest at 7 dpi. Since *Si*NUCA was successfully expressed in Arabidopsis and these plants did not show extensive cell death *Si*NUCA might be a cell death executor and not a cell death trigger. However, cytosolic *Si*NUCA:mCherry signal was much weaker than extracellular *Si*NUCA:mCherry signal and a high signal background in the vacuole was detected, which speaks for a certain phytotoxicity of *Si*NUCA.

Many nucleases inducing cell death have an immunity protein to inhibit enzyme activity before arrival at the place of action (Meiss et al., 1998; Woo et al., 2004). However, no clear protein interaction with *Si*NUCA was found in yeast two hybrid assays (Bachelor thesis Charura, 2016). In contrast, a protein of ca. 50 kDa was co-eluted with *Si*NUCA by size exclusion chromatography in all prepared samples. This protein could interact with *Si*NUCA and needs to be sequenced. The type of cell death related to *Si*NUCA or even the function of *Si*NUCA might be different in barley and Arabidopsis. The involvement of *Si*NUCA in cell death execution and the type of cell death induced by *S. indica in planta* might be further investigated using Arabidopsis cell death mutants and specific stainings for cytological analysis.

3.8.2 Nucleases in immunity and cooperation with nucleotidases

The release of nucleic acid extracellular traps (NETs) from root border cells was described as response to environmental stimuli (Cannesan et al., 2011; Driouich et al., 2013; M. Hawes et al., 2016a; M. C. Hawes et al., 2016b; Tran et al., 2016). NETs are composed of DNA, proteins, polysaccharides and antimicrobial compounds (Cannesan et al., 2012; Driouich et al., 2013; Wen et al., 2007; Wen et al., 2009). The soil-borne bacterial plant pathogen *Ralstonia solanacearum* was shown to get trapped by pea root border cell NETs but can escape by digestion of the NET with the help of two secreted DNases (Tran et al., 2016). In contrast, the double DNase mutant of *R. solanacearum* is not able to escape NETs and besides has reduced virulence on tomato plants (Tran et al., 2016).

Nucleases responsible for bacterial release from neutrophil extracellular traps (the pendant to plant border cell NETs in animal systems) was originally shown for group A *Streptococcus* and later for many other bacterial animal pathogens (Buchanan et al., 2006; M. Hawes et al., 2016a; Sumby et al., 2005). Trapping by NETs was demonstrated for the fungal human pathogen *Candida albicans* and the fungal plant root pathogen *Nectria haematococca* (Urban et al., 2006; Wen et al., 2009). Therefore, the question arose if *Si*NUCA is involved in NETs escape. *SiNUCA* was not induced *in planta* at early colonisation time points and induction of NETs was not observed in Arabidopsis upon *S. indica* colonisation. However, in the APF of *S. indica*-colonised barley histone H4 was detected, which is a component of NETs (Wen et al., 2007). The model plant for NETs,

pea, might be utilised for further studies of NETs in connection with *S. indica* and its *Si*NUCA.

Thammavongsa et al. revealed that not only the nuclease Nuc is involved in NETs counter defence by *Staphylococcus aureus* but also the nucleotidase AdsA is required to induce apoptosis of macrophages (Thammavongsa et al., 2013). Nuc digests DNA of NETs and produces 3'-phosphomononucleotides and -dinucleotides (Berends et al., 2010). AdsA can not only process the substrates adenosine tri-, di- and monophosphate (ATP, ADP and AMP) to the immunosuppressive adenosine but also 2'-deoxyadenosine monophosphate (dAMP) to 2'-deoxyadenosine (dAdo) (Thammavongsa et al., 2013; Thammavongsa et al., 2011). dAdo is toxic and leads to apoptosis in macrophages (Thammavongsa et al., 2013). A similar mechanism was shown for the *Streptococcus pyogenes* nucleotidase A (S5nA) working together with the nuclease SpnA to produce toxic dAdo (Zheng et al., 2015).

Interestingly, a *S. indica* ecto-5'-nucleotidase (*Si*E5'NT) is homologous to AdsA. The effector *Si*E5'NT was shown to hydrolyse ATP, ADP and AMP and to be involved in suppression of the DAMP extracellular ATP (eATP) upon *S. indica* plant colonisation (Nizam et al., 2018). Analogous to AdsA and S5nA, it would be interesting to see if *Si*E5'NT can produce dAdo from substrates digested by *Si*NUCA. dAdo but not adenosine was shown to be toxic to Arabidopsis seedlings too (personal communication Dr. H. Rövenich). Therefore, *Si*NUCA and *Si*E5'NT might work together to trigger cell death. As *S. indica* only induced cell death in colonised cells after the biotrophic phase (Jacobs et al., 2011), dAdo production would need to be tightly controlled regarding time and location. *Si*E5'NT was found in the APF of barley at all analysed colonisation time points and Arabidopsis seedlings heterologously expressing *Si*E5'NT leads to increased colonisation at all time points. This is in contrast to *Si*NUCA, which was only found at 5 dpi in the APF and colonisation was only enhanced at 7 dpi in Arabidopsis seedlings expressing *Si*NUCA. Therefore, *Si*E5'NT might have a dual function involved in eATP-triggered immunity and production of phytotoxic dAdo. In accordance, eATP levels in the apoplast decrease after the early colonisation phase (Nizam et al., 2018). The open question is which DNA would be the substrate for *Si*NUCA- NETs, plant or fungal extracellular DNA?

In soil, DNA is abundant and it was shown that many organisms can distinguish between self and non-self extracellular DNA (exDNA) as only self exDNA is inhibiting growth (Duran-Flores & Heil, 2015; Mazzoleni et al., 2015a; Mazzoleni et al., 2015b). Duran-Flores and Heil suggested that self exDNA acts as DAMP inducing ROS and mitogen-activated protein kinase signalling (Duran-Flores & Heil, 2017). Only self exDNA fragments with a size of less than 700 bp are inhibiting growth and digestion by DNase I abolishs the effect (Duran-Flores & Heil, 2017). However, it is unknown if this self exDNA is released from living cells (NETs?) or upon RCD, which tissue and cells are involved and if exDNA is recognised by receptors or toxic per se (Bhat & Ryu, 2016; Duran-Flores & Heil, 2015). If self exDNA acts as DAMP *Si*NUCA might digest plant exDNA to avoid DAMP-triggered immunity response.

3.8.3 Nucleases and nutritional aspects

Involvement of secreted nucleases in nutrition was shown for different organisms. A 3' nucleotidase/nuclease of *Leishmania* sp. is not only acting in digestion of NETs but also in nutrient acquisition (Guimaraes-Costa et al., 2014; Sacci et al., 1990). A secreted DNase of *P. aeruginosa* is induced under phosphate limiting conditions and allocates phosphate, nitrogen and carbon (Mulcahy et al., 2010). Nucleases and nucleotidases are also induced under phosphate starvation in the marine bacteria *Shewanella* ssp., which are able to grow on DNA as phosphate, nitrogen and carbon source (Pinchuk et al., 2008). However, it was also shown that extracellular DNA digestion by secreted enzymes is not needed as DNA fragments itself can be taken up by *E. coli* and root hairs for nutrition (Finkel & Kolter, 2001; Paungfoo-Lonhienne et al., 2010).

As *S. indica* was growing through plant nuclei, which disappeared with progression of colonisation and the sugar non-specific nuclease *Si*NUCA was secreted the involvement of *Si*NUCA in plant or fungal nutrition must be considered. Lahrmann et al. showed that the cell death-associated phase in barley is triggered by nitrogen starvation sensed by the fungal transporter Amt1 (Lahrmann et al., 2013). Interestingly, transcriptomic analysis of *S. indica* wt compared to downregulated *Amt1* strains exhibited downregulation of *SiNUCA* at the same time (Ding, 2014). Therefore, growth of *S. indica* strains with different *SiNUCA* levels were analysed growing on DNA as nitrogen source. A *S. indica* strain overexpressing *Si*NUCA was growing better on these plates compared

to empty vector strains or wt and these were growing with denser mycelium compared to the *ΔsinucA* strain (Fig. 2.15). These results suggest a nutritional aspect of *Si*NUCA. The nitrogen and amino acid status of colonised plants expressing *SiNUCA* compared to control plants could be measured by inductively coupled plasma-mass spectrometry. However, *SiNUCA* was not induced under nitrogen starvation in contrast to *Amt1*. Next, the influence of *ΔsinucA* was tested on phosphate and sulphate uptake in colonised Arabidopsis seedlings in collaboration with Prof. Dr. S. Kopriva. No differences in uptake of radioactively labelled phosphate or sulphate into Arabidopsis roots or shoots in 30 min was observed between colonisation with *ΔsinucA,* control or wt strains (data not shown). However, compartmentalised experiments over days might reveal differences. As starvation and RCD are connected through autophagy (Üstün et al., 2017) disentangling *Si*NUCA function in nutrition and RCD might be difficult.

3.8.4 Antimicrobial nucleases

Nucleases with the role of antimicrobial toxicity for competition are well known for colicins. Colicins are stress induced, secreted proteins from *E. coli* with different functions like membrane pore formation, RNase or HNH endonuclease activity (James et al., 2002). The colicins E2, 7, 8, and 9 exhibit DNase activity and enter competing bacterial cells through a pore forming complex (Cascales et al., 2007). They enter the hostile nucleus to unspecifically degrade chromosomal DNA, which leads to cell death (M. J. Mate & Kleanthous, 2004; Pommer et al., 2001). Kettles et al. proposed for the secreted ribonuclease Zt6 a second role in antimicrobial competition and niche protection as the purified protein shows antimicrobial activity against *E. coli* and yeasts *in vitro* (Kettles et al., 2018). Nucleases as antimicrobial effectors in the microbiome of plants might have an important role in nutritional and niche competition and against invading pathogens (Hacquard et al., 2017; Rövenich et al., 2014). Therefore, the toxicity of *Si*NUCA was tested in different assays against *E. coli*. No antibacterial activity of *Si*NUCA was observed but activity against other microbes cannot be ruled out (Fig. 2.21). However, Ma et al. showed that antimicrobial activity can be environment dependent as the *Agrobacterium tumefaciens* secreted DNases Tde are only effective against *P. aeruginosa in planta* (Ma et al., 2014).

3.8.5 Conclusion

The cell death-associated colonisation phase of *S. indica* was further depicted by cytological studies and by using the *S. indica* effector nuclease *Si*NUCA. *Si*NUCA emerged as a conserved small secreted protein with sugar non-specific nuclease activity. Expression in Arabidopsis revealed a higher colonisation rate, more cell death and a final localisation in host nuclei upon *S. indica* colonisation. A function of *Si*NUCA in digestion of the DAMP self exDNA in the apoplast to avoid plant immunity was proposed (Fig. 3.1A). *Si*NUCA might work together with the nucleotidase *Si*E5'NT to produce toxic dAdo, which triggers host cell death. In addition, *Si*NUCA could be a cell death executor by degradation of chromosomal DNA in the plant nucleus (Fig. 3.1.B). Both could also have a benefit in the generation of fungal or plant nutrients.

These data provide a deeper insight into effector biology and how cell death is involved in symbiotic plant-microbe interactions. Understanding the function of highly abundant secreted nucleases in microbes in interaction with plants is important to develop new strategies against plant diseases.

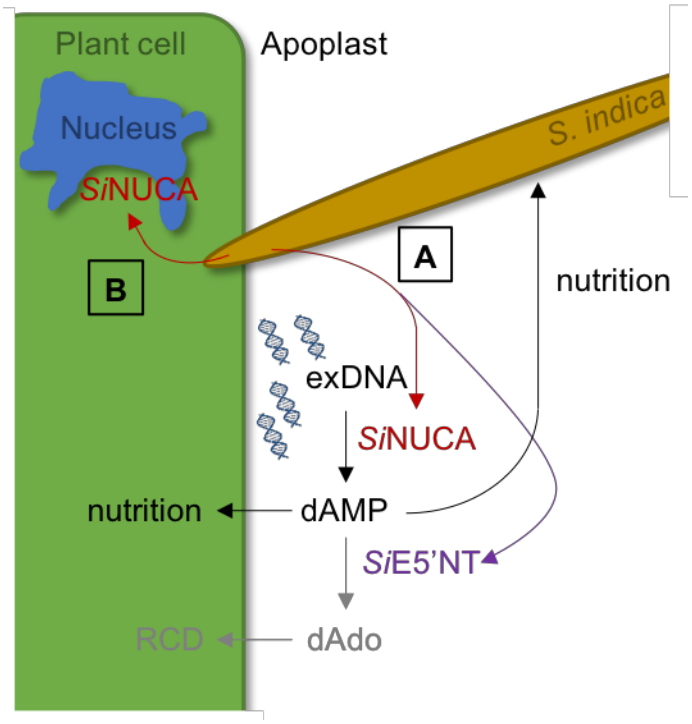

Fig. 3.1: Model for the *Si*NUCA function *in planta*.

A) As plant immune response to *S. indica* colonisation the plant could release the DAMP self exDNA. *S. indica* secretes the endonuclease *Si*NUCA into the apoplast to digest this exDNA and therefore, avoiding DAMP-triggered immunity. Digested exDNA could serve as nutritional building blocks for the plant and/or the fungus. Moreover, digested exDNA in form of dAMP could be further processed by the *S. indica* nucleotidase *Si*E5´NT producing toxic dAdo. dAdo might trigger host regulated cell death (RCD). **B)** *Si*NUCA could also degrade nuclear plant DNA and therefore, being a cell death executor. Digested nuclear DNA could serve as nutrition for the plant or fungus too.

4 Material and Methods

4.1 Chemicals, media and buffers

Chemicals and media components were mainly ordered from Carl Roth (Karlsruhe, Germany), Sigma Aldrich (Taufkirchen, Germany) and VWR (Darmstadt, Germany). Arabidopsis growth media was ordered from Duchefa (Haarlem, The Netherlands). In general, media and buffer were autoclaved at 121°C for 20 min 1.5 bar. Heat sensitive components were sterile filtrated through 0.22 µm Rotilabo syringe filters (Carl Roth, Karlsruhe, Germany) or for bigger volumes through 0.22 µm bottle top vacuum filtration systems (VWR, Darmstadt, Germany).

4.2 Bacterial, fungal and plant material

4.2.1 *E. coli* strains

The *Escherichia coli* strains Top10 or Mach1 (Invitrogen, Thermo Fisher Scientific, Schwerte, Germany) were used for experiments in this thesis.

4.2.2 *A. tumefaciens* strain

The *Agrobacteria tumefaciens* strain GV3101 was used for Arabidopsis transformation.

4.2.3 *U. maydis* strains

All transformed *Ustilago maydis* strains in this thesis derived from the haploid solopathogenic strain SG200 (Kämper et al., 2006).

Table 4.1 *U. maydis* strains used in this thesis

Name	Description
mCherry	Cytosolic mCherry expression driven by the otef promoter (provided by Dr. S. Nizam)
*Si*NUCA	Expressing full length *SiNUCA* driven by the otef promoter
*Si*NUCA:mCherry	Expressing full length *SiNUCA* with C- terminal mCherry fusion driven by the otef promoter
SP^{SiNUCA}:mCherry	Secreting mCherry by using the SP of *SiNUCA* driven by the otef promoter

4.2.4 *B. sorokiniana* strain

The *Bipolaris sorokiniana* strain ND90Pr (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany) was cultivated for microscopy.

4.2.5 *S. indica* strains

The tranformed *Serendipita indica* strains used in this thesis all derived from the dikaryotic *S. indica* strain DSM11827 (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany).

Table 4.2 *S. indica* strains mentioned in this thesis

Name	Karyotype	Description
GFP	Dikaryotic	Cytosolic *GFP* expression driven by the GPD promoter (Hilbert et al., 2012)
*Sv*TEF::GFP	n.d.	Cytosolic *GFP* expression driven by the TEF promoter from *S. vermifera*
FGB1:GFP	Homokaryotic	Expressing full length *FGB1* (PIIN_03211) with C-terminal *GFP* driven by the FGB1 promoter (This thesis and Wawra et al., 2016)
FGB1:GFP:HA:His	n.d.	Expressing full length *FGB1* with C- terminal GFP:HA:His driven by the FGB1 promoter
FGB1:mRFP:HA:His	n.d.	Expressing full length *FGB1* with C- terminal mRFP:HA:His driven by the FGB1 promoter
SP^{FGB1}: Sebve1_14646:GFP	n.d.	Expressing *Sebve1_14646* without SP with SP of *FGB1* and C- terminal *GFP* driven by the FGB1 promoter

Name	Karyotype	Description
*Bs*FGB1:GFP:HA:His	n.d.	Expressing the full length *FGB1* homologue of *B. sorokiniana* (19137836) with C- terminal GFP:HA:His driven by the FGB1 promoter
*Bs*FGB1:mRFP:HA:His	n.d.	Expressing the full length *FGB1* homologue of *B. sorokiniana* (19137836) with C- terminal mRFP:HA:His driven by the FGB1 promoter
*Bs*FGB1:HA:His	n.d.	Expressing the full length *FGB1* homologue of *B. sorokiniana* (19137836) with C- terminal HA:His driven by the FGB1 promoter
*Si*NUCA:HA:His	Homokaryotic	Expressing full length *SiNUCA (PIIN_02121)* with C-terminal HA:His tag driven by the FGB1 promoter
HA:His	Homokaryotic	Control strains for *Si*NUCA:HA:His
GenR C2 16/3	Homokaryotic	Sequenced, homokaryotic reference strain harbouring LB^{FGB1}:GPD::GenR: RB^{FGB1} (Wawra et al., 2016)
ΔsinucA	Homokaryotic	*SiNUCA* gene deletion in GenR C2 16/3 strain, *SiNUCA* replaced by Hygromycin B resistance cassette
RNAi-*Si*NUCA	Dikaryotic	Fragment of *SiNUCA* between the GPD and TEF promoter for gene silencing
GPD::GenR	Dikaryotic/ Homokaryotic	Dikaryotic and homokaryotic strains harbouring the Geneticin resistance cassette (Griebel, 2016)

4.2.6 *S. vermifera* strain

The *S. vermifera* strain MAFF305830 (National Institute of Agrobiological Sciences, Tsukuba, Japan) was used for microscopy.

4.2.7 Arabidopsis lines

The *Arabidopsis thaliana* ecotype Columbia 0 (Col-0) was used for most of the experiments. The other used lines had the background Col-0.

Table 4.3 *A. thaliana* lines used in this thesis

Name	Description	Reference
atg5	*atg5* mutant line (N674040, SALK_020601C)	Dr. C. Masclaux-Daubresse (INRA, Versaille, France)
atg10	*atg10* mutant line (N39994, SALK_084434)	Dr. C. Masclaux-Daubresse (INRA, Versaille, France)
H2B:2xmCherry	Constitutive expression of Histone H2B fused to mCherry in root cells under the UBQ10 promoter	(Marquès-Bueno et al., 2016)
*Si*NUCA	Constitutive expression of *Si*NUCA driven by the 35S promoter	This thesis
*Si*NUCA:mCherry	Constitutive expression of *Si*NUCA C-terminally fused to mCherry driven by the 35S promoter	This thesis
*Si*NUCA(w/o SP):mCherry	Constitutive expression of *Si*NUCA without SP C- terminally fused to mCherry driven by the 35S promoter	This thesis

4.2.8 Barley line

The line *Hordeum vulgare* L. cv. Golden Promise was used (Prof. R. Hückelhoven, Technical University of Munich, Germany).

4.3 Microbiological and plant biological methods

4.3.1 *E. coli*

4.3.1.1 Cultivation

E. coli was growing in low salt lysogeny broth (LB) medium (0.5 % (w/v) yeast extract, 1 % (w/v) tryptone, 0.5 % (w/v) NaCl) with 1.5 % (w/v) agar for pouring of plates. Depending on the required selection 100 µg/ml ampicillin or 50 µg/ml kanamycin were added. *E. coli* was growing at 37 °C on plates or in Erlenmeyer flasks at 37 °C 220 rpm.

4.3.1.2 Heat shock transformation

For the preparation of chemo competent *E. coli* the RbCl/$CaCl_2$ method was applied. A 1 ml of a 5 ml *E. coli* overnight liquid culture was added to 600 ml medium with 10 mM $MgSO_4$ and 10 mM $MgCl_2$ and this culture grown to OD_{600}= 0.4- 0.6. Afterwards, the

culture was put on ice for 20 min and all the following steps were performed with pre-cooled equipment and solutions. The cells were centrifugated at 3000 rpm for 15 min and resuspended in 100 ml RF I solution:

100 mM RbCl

50 mM $MnCl_2$ x $4H_2O$

30 mM CH_3CO_2K

10 mM $CaCl_2$ x $2H_2O$

15 % (w/v) glycerol

The pH was set to 5.8 with acetic acid and the solution was sterile filtered.

The resuspended cells were left on ice for 15- 30 min, centrifugated at 3000 rpm for 15 min and resuspended in 6 ml RF II solution:

10 mM MOPS

10 mM RbCl

75 mM $CaCl_2$ x $2H_2O$

15 % (w/v) glycerol

The pH was set to 6.8 with NaOH and the solution was sterile filtered.

The suspension was put on ice for 15 min, divided into 50 µl aliquots and flash-frozen in liquid nitrogen before stored at -80 °C.

For heat shock transformation, an aliquot was thawed on ice and 1 µl plasmid or 5 µl Gibson assembly mix were added. The tube was left on ice for 5 min followed by 45 s at 42 °C and another 5 min on ice. 250 µl low salt LB was added, the transformed cells were incubated for 30-45 min at 37 °C and plated on low salt LB with appropriate antibiotics.

4.3.2 *U. maydis*

4.3.2.1 Cultivation

U. maydis was cultivated on potato dextrose agar (PDA) plates or in liquid YEPS light medium (0.4 % (w/v) yeast extract, 0.4 % (w/v) peptone, 2 % (w/v) sucrose) at 28 °C with 220 rpm shaking for liquid cultures. For selection, 2 µg/ml carboxin was added to the medium.

4.3.2.2 Transformation

A 2 ml YEPS light pre-culture was diluted 1:1000 into 55 ml YEPS light and grown until an OD_{600} of 0.6-0.8 was reached. The culture was centrifugated at 3000 rpm for 10 min and the pellet resuspended in 25 ml SMC (1.33 M sorbitol, 50 mM $CaCl_2$, 20 mM MES pH 5.8). The suspension was centrifugated again and resuspended in 40 mg lysing enzymes from *Trichoderma harzianum* (Sigma Aldrich, Taufkirchen, Germany) dissolved in 2 ml SMC and sterile filtrated. The process was checked every 10 min until 30- 40 % of the cells were protoplasted. Subsequently, 10 ml cold SMC was added and the protoplasts centrifugated at 2400 rpm at 4 °C for 10 min. The pellet was resuspended in 10 ml cold SMC. This washing step was repeated twice but at last resuspended in 10 ml cold STC (100 mM $CaCl_2$, 10 mM Tris-HCl pH= 7.5, 1M sorbitol). The suspension was centrifugated again and the pellet was resuspended in 500 µl cold STC. The protoplasts were divided into 50 µl aliquots on ice and directly used for transformation or frozen at - 80 °C.

A protoplast aliquot was thawed on ice and 1 µl heparin (15 mg / ml) and 5 µg plasmid (in 10 µl, linearised with SalI) added. 500 µl 40 % polyethylene glycol PEG 3350 in STC (0.45 µm sterile filtered) were add and the protoplasts were gently resuspended. This was incubated for 15 min on ice and plated by pipetting and gently spreading. The plates consisted of a first layer of 10 ml regeneration agar with antibiotics (4 µg/ml carboxin) and a second layer of 10 ml regeneration agar without antibiotics. The regeneration agar contained:

1 % (w/v) yeast extract
2 % (w/v) bacto peptone
2 % (w/v) sucrose
18.22 % (w/v) sorbitol
1.5% (w/v) agar

The plates were incubated at 28 °C until emerging colonies appeared to transfer on PDA plates with antibiotics. Transformation of single colonies was confirmed by PCR on the inserted plasmid.

4.3.3 *B. sorokiniana*

4.3.3.1 Cultivation

B. sorokiniana was cultivated on CM plates for 10- 12 days at 25 °C to get spores.

CM medium for *B. sorokiniana*:	1 % (v/v) Solution A
	1 % (v/v) Solution B
	0.05 % (v/v) Srb's micronutrients
	0.05 % (v/v) iron solution
	0.1% (w/v) yeast extract
	0.05% (w/v) peptone
	0.05% (w/v) Casamine acids
	1 % /w/v) glucose
	1.5% (w/v) Agar
Solution A:	10% (w/v) Ca(NO3)2 x 4H2O
Solution B:	2 % (w/v) KH2PO4
	2.5 % (w/v) MgSO4 x 7H2O
	1.5 % (w/v) NaCl
Srb's micronutrients:	0.006 % (w/v) MnSO4 x H2O
	0.0057 % (w/v) H3BO3
	0.049% (w/v) ZnSO4 x 7H2O
	0.0013% (w/v) KI
	0.0037 (w/v) (NH4)6M07O24 x 4H2O
	0.039% (w/v) CuSO4 x 5H2O
Iron solution:	94.8 % (w/v) FeCl3 x 6H2O

B. sorokiniana was cultivated in MYP medium (0.7 % (w/v) malt extract, 0.1 % (w/v) peptone, 0.05 % (w/v) yeast extract) at 28°C 120 rpm. The fungus was incubated for 3 days, the culture filtered with miracloth (Millipore Merck, Darmstadt, Germany), crushed in new medium (MicrotronR MB550 homogenizer (Kinematica, Luzern, Switzerland) and regenerated for 1 day. This young mycelium was used for microscopy.

4.3.3.2 Spore collection

Spores from 10-12-day old plates were scratched from the plate with a spreader and 5 ml 0.002 % Tween20 was added. The spore solution was filtered through miracloth and centrifugated for 10 min at 4000 rpm. The spores were washed twice and were finally resuspended in 10 ml ddH_2O. The concentration was adjusted to 5000 spores / ml using a Neubauer improved counting chamber.

4.3.4 *S. vermifera* cultivation

S. vermifera was cultivated in MYP medium (see 4.3.3.1) at 28°C with 120 rpm agitation. The fungus was incubated for 5 days, the culture filtered with miracloth, crushed in new medium (MicrotronR MB550 homogenizer (Kinematica, Luzern, Switzerland) and regenerated for 1 day. This young mycelium was used for microscopy.

4.3.5 *S. indica*

4.3.5.1 Cultivation

S. indica was growing in complete medium (CM): 5 % (v/v) 20x Salt solution
0.1 % (v/v) Microelements
0.2 % (w/v) Peptone
0.1 % (w/v) Yeast extract
0.1 % (w/v) Caseinhydrolysat
1.5 % (w/v) Agar (for solid medium only)

After autoclaving 2 % (w/v) Glucose (filter sterilised) was added.

20x salt solution: 12 % (w/v) $NaNO_3$
1.04 % (w/v) KCl
1.04 % (w/v) $MgSO_4$ x $7H_2O$
3.04 % (w/v) KH_2PO_4

Microelements: 0.6 % (w/v) $MnCl_2$ x $4H_2O$
0.15 % (w/v) H_3BO_3

Microelements (continued):	0.265 % (w/v) $ZnSO_4$ x $7H_2O$
	0.075 % (w/v) KI
	0.24 ‰ (w/v) Na_2MO_4 x $2H_2O$
	0.013 % (w/v) $CuSO_4$ x $5H_2O$

S. indica was growing at 28 °C and 120 rpm for liquid cultures. 80 µg/ml Hygromycin B or 100 µg/ml Geneticin (G418) were added to solid medium for selection purpose (Carl Roth, Karlsruhe, Germany). To set up liquid cultures, spores or small plugs punched from plates were added to CM in an Erlenmeyer flask.

The following protocol was used for medium tests and microscopy if not otherwise described: A seven-day old 50 ml liquid culture was filtered with miracloth and the mycelium was washed with 50 ml 0.9 % NaCl. The mycelium was crushed in new medium in a blender (MicrotronR MB550 homogenizer (Kinematica, Luzern, Switzerland) and the culture was grown for two days to regenerate. The mycelium was filtered, washed and cultured again in different media. The tested media besides CM were MYP (see 4.3.3.1), YEPS light (see 4.3.2.1), MEP (2 % (w/v) malt extract and 0.2 % (w/v) peptone), YNB without amino acids (Sigma Aldrich, Taufkirchen, Germany) supplemented with 2 % (w/v) glucose and YNB without amino acids and ammonium sulphate (BD Difco, Fisher Scientific, Schwerte, Germany) supplemented with 5 mM asparagine and 2 % (w/v) glucose. YNB and asparagine were sterile filtrated.

4.3.5.2 Collection of chlamydospores

Chlamydospores were harvested from three- to four-week old plates by adding few ml of 0.002 % (v/v) Tween20 and scratching with a scalpel. The spore suspension was filtered through miracloth to get rid of mycelium and medium. The spores were centrifugated at 3500 rpm for 7 min. The pellet was washed with 20 ml 0.002 % (v/v) Tween20 twice and after the last washing step the pellet was resuspended in 1 ml ddH_2O. The spore concentration was counted with a Neubauer Improved counting chamber and the concentration was adjusted to 500000 spores per ml.

4.3.5.3 PEG-mediated transformation

S. indica transformation was adapted from Hilbert et al., 2012. A seven-day old *S. indica* liquid culture was filtered with miracloth and the mycelium was washed with 50 ml 0.9 % NaCl. The mycelium was crushed in 20 ml medium in a blender and added to 130 ml fresh medium. The culture was grown for three days to regenerate. The mycelium was filtered with miracloth, washed with 50 ml 0.9 % NaCl and added to 20 ml lysing enzymes from *Trichoderma harzianum* solution (Sigma Aldrich, Taufkirchen, Germany, 0.4 g solved in 20 ml SMC (1.33 M sorbitol, 50 mM $CaCl_2$, 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) pH 5.8) and sterile filtered). This mix was shaken for 2 h at 32 °C for 100 rpm for protoplastation. Protoplast formation was verified under the microscope and the protoplasts were filtered through miracloth. To stop enzyme activity 20 ml cold STC (50 mM $CaCl_2$, 10 mM Tris-HCl pH= 7.5, 1M sorbitol) was added. The protoplasts were centrifugated at 4 °C 4000 rpm for 10 min and gently resuspended in 1 ml cold STC. The protoplasts were centrifugated at 4 °C 4000 rpm for 5 min and resuspended in 1 ml cold STC again. This washing step was repeated twice and the protoplast resuspended in the required volume for transformation.

In advance, the plasmid to be transformed was linearised by restriction digest overnight. The linearised plasmid was precipitated with 0.5 volumes 7.5 M ammonium acetate and 2 volumes isopropanol for 15 min and centrifugated for 30 min. The pellet was washed twice with 70 % ethanol and then air dried. The pellet was resuspended in 12 µl ddH_2O. 5- 10 µg linearised plasmid in a volume of 10 µl were added to 70 µl protoplasts on ice. 1 µl (15 mg/ml) heparin and 1 µl restriction enzyme used to linearise the plasmid was added and the tube left on ice for 10 min. 500 µl 40 % (w/v) PEG 3350 in STC (0.45 µm filter sterilised) were added and mixed by inversion of the tube for a few times. The tube was left on ice for another 15 min and the mix transferred to a 15 ml falcon tube. 5 ml 42 °C top medium (MYP medium supplemented with 0.3 M sucrose and 0.6 % (w/v) agar) was added, gently mixed and poured on plates with 20 ml bottom medium (MYP medium with 0.3 M sucrose and 1.2 % (w/v) agar and appropriate antibiotics). First transformants emerged around two weeks later and were transferred to CM plates with antibiotics using toothpicks.

Strains were confirmed by PCR on the inserted plasmid (see 4.4.2). The mating type was determined by PCR on the four mating type genes HD 1.1, HD 1.2, HD 2.1 and HD 2.2 (Wawra et al. 2016).

4.3.5.4 Growth on DNA as nitrogen source

For growth assays with DNA as nitrogen source, a medium composed of YNB without amino acids and ammonium sulphate, 100 µg /ml salmon testis DNA, 2 % (w/v) glucose and 1.5 % (w/v) agar was used. The agar was solved in H_2O, buffered with MES pH 5.6 and autoclaved. The other components were added prior to plate pouring. As a control, plates with YNB without amino acids (instead of YNB without amino acids and ammonium sulphate and DNA) were used. Plugs without remaining agar from old plate were put on plates and the plates incubated for 5- 7 days. The plates were stained with Ethidium bromide (500 ng/ml) to visualise DNA under UV light.

4.3.5.5 Germination of spores on glass slide

Collected *S. indica* spores (see 4.3.5.2) were pipetted on sterile cover slides on wet filter paper in petri dish. Cover slides were directly put on microscopy slide after overnight incubation to observe germinated spores by CLSM.

4.3.6 Arabidopsis

4.3.6.1 Agrobacteria-mediated transformation

A. tumefaciens was transformed by electroporation and the transformants checked by colony PCR. For Arabidopsis transformation 200 ml LB supplemented with 50 µg/ ml Kanamycin, 25 µg/ ml Gentamycin and 25 µg/ ml Rifampicin was inoculated with the *A. tumefaciens* strains overnight at 28 °C 220 rpm. At an OD_{600} of 1.5- 2 the cultures were centrifugated for 10 min at 5500 rpm and the pellet resuspended in 2 ml 5 % (w/v) sucrose. The suspension was diluted to 400 ml with 5 % (w/v) sucrose and 0.02 % (v/v) Silwet L-77 (Obermeier, Bad Berleburg, Germany) was added.

A. thaliana Col-0 was grown on soil in the greenhouse and the inflorescence was cut. When the inflorescence was regrown, the flowers were dipped in the *A. tumefaciens* suspension for 1 min and the plants left overnight in the dark at RT with high humidity. Plants were returned to the green house for seed building.

4.3.6.2 Selection of Arabidopsis lines

Arabidopsis was grown on soil in the greenhouse and dried seeds were collected. An aliquot of the seeds was sterilised and single seeds put in lines on ½ MS plates with sucrose (and 15 µg/ ml hygromycin B (Invitrogen, Thermo Fisher Scientific, Schwerte, Germany) (see 4.3.6.3). Seeds were stratified on plates at 4 °C in the dark and then put upright into the phytochamber. T1 seeds germinating and seedlings growing on plates with and without selection were transferred to soil. gDNA was extracted from leaves to confirm insertion of the plasmid by PCR (see 4.4.3). For T2 seeds the germination and seedling growth rate was calculated on plates with and without selection to calculate the segregation rate. Plant lines with a 3:1 segregation were selected for propagation and non-segregating T3 lines were used for the experiments. RNA was extracted from T3 plant lines and synthesised cDNA used for qPCR to compare expression levels of the inserted gene between the plant lines (see 4.4.4/6).

4.3.6.3 Growth under sterile conditions

Arabidopsis seeds were surface sterilized for 10 min with 70 % ethanol followed by 7 min with 100 % ethanol. Seeds were tried under the sterile bench and distributed on half-strength Murashige and Skoog medium (including modified vitamins, pH was adjusted with KOH to pH 5.7) supplemented with 1 % (w/v) sucrose and 0.4 % (w/v) Gelrite. Seeds on plates were stratified for 2- 3 days at 4°C in the dark. Afterwards, the plates were put into the phytochamber for 7 days and the seedlings were grown under short day conditions (8 h light, 16 h dark) with 130 µmol m^{-2} s^{-1} of light and 22 °C/18 °C.

4.3.6.4 *S. indica* – Arabidopsis interaction studies

Seven-day old seedlings were transferred to ½ MS plates without sucrose. Twenty seedlings of the same size were put in one row per plate. *S. indica* spore solution (500000 spores/ml) (see 4.3.5.2) was equally pipetted on, beside and below the roots. Mock-inoculated plants were treated with ddH_2O. The plates were dried under the sterile bench and then put upright into the phytochamber. Root parts were covered from light. 2- 3 plates were pooled for one biological replicate. Colonised roots were

washed with water and a paper towel several times, a root part of 4 cm starting below 0.5 cm of the shoot was cut and immediately frozen in liquid nitrogen.

4.3.6.5 *S. vermifera* – Arabidopsis interaction study

Seven-day old seedlings were transferred to ½ MS plates without sucrose. Twenty seedlings of the same size were put in one row per plate. *S. vermifera* plugs were set in-between the seedlings. Plates were put up right into the phytochamber. Root parts were covered from light. Root parts were covered from light. 2- 3 plates were pooled for one biological replicate. Colonised roots were washed with water and a paper towel several times, a root part of 4 cm starting below 0.5 cm of the shoot was cut and immediately frozen in liquid nitrogen.

4.3.6.6 *B. sorokiniana* – Arabidopsis interaction study

The protocol for Arabidopsis inoculation with *B. sorokiniana* spores was analogous to inoculation with *S. indica* spores (see 4.3.6.4). The spore concentration was set to 5000/ml (see 4.3.3.2).

4.3.7 Barley

4.3.7.1 *S. indica*- barley interaction studies

Barley seeds were surface sterilised with 70 % ethanol for 1 min, washed with ddH_2O and sterilized with 12 % sodium hypochlorite for 1.5 h while shaking. Afterwards, seeds were washed for 5- 10 times for 20 min with ddH_2O. Seeds without seed coat were put on wet filter paper in square petri dishes and germinated at room temperature for three days in the dark. Four germinated seeds of the same size were put into one 1062 ml Weck jar (Weck, Wehr- Öflingen, Germany) filled with 100 ml 1/10 PNM medium:

0.5 mM KNO_3

3.7 µM KH_2PO_4

1.4 µM K_2HPO_4

2 mM $MgSO_4$ x H_2O

0.2 mM $Ca(NO_3)_2$

0.25% (v/v) Fe-EDTA

4.3 µM NaCl

0.4% (w/v) Gelrite

Fe-EDTA: 0.55 % (w/v) $FeSO_4$ x $7H_2O$ and 0.75% (w/v) Na_2EDTA x $2H_20$

boiled and stirred for 30 min.

The pH was adjusted to 5.6 with HCl. 10 mM MES pH 5.6 (filter sterilised) was added after autoclaving.

3 ml *S. indica* spore solution (see 4.3.5.2) was pipetted along the roots on the medium and the open jars dried under the sterile bench. Mock-inoculated seedlings were treated with ddH_2O. Barley seedlings in jars were growing in the phytochamber under long day conditions (16 h light, 8 h dark) with 60 % humidity and 130- 165 µmol m^{-2} s^{-1} at 22 °C/16 °C. Colonised roots were thoroughly washed with water, the first 4 cm of the root cut and flash-frozen in liquid nitrogen. One jar was pooled for one biological replicate.

4.3.7.2 *S. vermifera*- barley interaction study

This study was performed like in 4.2.7.1. Instead of spores, mycelium was crushed, washed and pipetted on the barley roots.

4.4 Molecular biological methods

4.4.1 DNA isolation

Plant or fungal material was ground with a mortar in liquid nitrogen, up to ¼ of a 2 ml Eppendorf tube filled with ground material and 1 ml extraction buffer was added:

100 mM TrisHCl pH 7.5

50 mM EDTA pH 8

1.5 M NaCl

2 % (w/v) Cetyl trimethylammonium bromide

0.05 % (v/v) ß- mercaptoethanol

Tubes were mixed on a Stuart SB3 rotator (Cole-Parmer, Staffordshire, UK) at RT for 10 min and afterwards, 1 ml of a chloroform:isoamylalcohol mixture (24:1) was added and the tubes mixed for another 5 min. The tubes were centrifugated for 20 min at 10000 g. 800 µl of the upper phase were transferred to a new tube and 160 µl ethanol were added. Before and after the addition of 960 µl chloroform:isoamylalcohol mixture

(24:1), the tubes were mixed for 5 min. The tubes were centrifugated at 10000 g for 20 min and the upper phase was transferred to a new tube. 1 volume isopropanol was added and the DNA precipitated for one hour at 4 °C. The tube was centrifugated at 5000 g for 20 min and the pellet washed with 70 % cold ethanol. The pellets were air-dried and dissolved in 50 µl TE buffer (10 mM TrisHCl, 1mM EDTA pH 8). 1 µl RNAse A (10 mg/ml pH 7.4, Sigma Aldrich, Taufkirchen, Germany) was added and incubated for 30 min at 37 °C.

4.4.2 Quick and dirty DNA isolation *S. indica*

A small piece of mycelium was scratched from a plate and ground with a pestle in liquid nitrogen. 100 µl extraction buffer (10 mM Tris pH 8, 1mM EDTA and 0.1 % (w/v) SDS) was added and boiled for 10 min at 100 °C immediately after grinding. After centrifugation at 13300 rpm for 20 min, 0.5 volumes 7.5M ammonium acetate and 2 volumes isopropanol were added to the supernatant. The DNA was precipitated for one hour or overnight at 4 °C and subsequently, centrifugated for 30 min at 13300 rpm. The pellet was washed with cold 70 % ethanol, air-dried and dissolved in 30 µl ddH_2O. 1 µl was used as template for the PCR (see 4.4.5).

4.4.3 Quick and dirty DNA isolation Arabidopsis

A piece of a leave was ground with a pestle in a 1.5 ml Eppendorf tube. 400 µl extraction buffer were added:

250 mM TrisHCl pH 7.5

250 mM NaCl

25 mM EDTA

0.5 % (w/v) SDS

The leave was ground again and the tube vortexed for 5 s. 150 µl 3 M potassium acetate (pH5.5, adjusted with formic acid) was added, the tube inverted and centrifugated at 13300 rpm for 3 min. 500 µl supernatant was transferred to a new Eppendorf tube and 1 volume isopropanol was added. The tube was inverted and centrifugated at 13300 rpm for 10 min. The pellet was washed with 70 % ethanol, air-dried and 50 µl TE buffer (pH 7.5) added. 1 µl was used as template for PCR.

4.4.4 RNA isolation and cDNA synthesis

Plant or fungal material was ground with a mortar in liquid nitrogen, up to ¼ of a 2 ml Eppendorf tube was filled with ground material and 1 ml TRIzol (Invitrogen, Thermo Fisher Scientific, Schwerte, Germany) was added. The tube was vortexed at 1 500 rpm with a vibrax shaker (IKA, Staufe, Germany) until all material was solved. 200 µl chloroform were added and the tubes vortexed for 20 s. The tubes were centrifugated at 13300 rpm for 30 min at 4 °C and 500 µl supernatant were transferred to a new tube. The RNA was precipitated with 1 volume isopropanol for 1 h at 4 °C. The samples were centrifugated again and the pellet was washed with 75 % ethanol (water supplemented with 0.1 (v/v) % Diethyl pyrocarbonate (DEPC)) two times. The pellet was air-dried, 30 µl RNAse- free water were added and the pellet was solved by incubation at 65 °C for 5 min. The concentration was measured with a nanodrop 2000c (Thermo Fisher Scientific, Schwerte, Germany).

2 µg RNA were digested with 2 U DNase I in 10 x DNase I buffer (Thermo Fisher Scientific, Schwerte, Germany) for 30 min at 37 °C. 5 mM EDTA were added and the tubes incubated at 65 °C for 10 min to stop the reaction. The RNA was precipitated with 0.5 volumes of 7.5 M ammonium acetate (solved in DEPC water) and 2 volumes isopropanol for 1 h at 4 °C. The samples were centrifugated for 30 min at 13300 rpm and 4 °C and the pellets were washed with 75 % ethanol (diluted with DEPC water). The pellets were air-dried and 13.5 µl RNAse-free water were added. The pellet was suspended at 65 °C 700 rpm for 5 min. The concentration and purity of the RNA were verified at the nanodrop. The integrity was checked by loading of 1 µl RNA on a 2 % agarose gel (see 4.4.5).

cDNA was synthesised with the Fermentas First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Schwerte, Germany). 1 µg RNA in 9 µl were added to 1 µl Oligo dT_{18} primer and 1 µl random hexamer primers. This mix was incubated at 65 °C for 5 min. Then, 4 µl 5 x reaction buffer, 2 µl 10 mM dNTP mix, 1 µl RiboLock RNase inhibitor and 2 µl M-MuLV reverse transcriptase were added on ice. The tubes were incubated at 25 °C for 5 min followed by 1 h at 37 °C (42 °C for RNA from *S. indica*) and 5 min at 70 °C. The RNA was diluted to 5 ng/ µl for qPCR.

4.4.5 PCR and gel electrophoresis

PCRs were performed with the GoTaq G2 Flexi DNA Polymerase (Promega, Mannheim, Germany) or the Taq DNA Polymerase (New England BioLabs, Frankfurt, Germany) according to the manufacturer's manuals. PCR on quick and dirty purified DNA from *S. indica* was performed with Tag DNA Polymerase from New England BioLabs using self-made Pfu buffer (see 4.7.1), 2.5 mM $MgCl_2$ and 2 % dimethyl sulfoxide (DMSO) instead of the ThermoPol reaction buffer. The PCR reaction proceeded in a C1000 Touch Thermal Cycler (Biorad, Munich, Germany). 6x loading dye was added to the samples for loading on an agarose gel.

6x loading dye:	10 mM TrisHCl pH 8
	60 mM EDTA pH 8
	60 % glycerol
	0.03 % bromophenol blue
	0.03 % xylene cyanol FF

0.8- 2 % agarose gels using the TAE buffer (40 mM Tris, 40 mM acetic acid, 1 mM EDTA pH 8) system were applied for gel electrophoresis at 110 V for 30- 40 min with a Power Pac Basic device (Biorad, Munich, Germany). The GeneRuler 1 kb DNA ladder was used to compare band sizes (Thermo Fisher Scientific, Schwerte, Germany). Ethidium bromide (500 ng/ ml) was added directly into the gels for subsequent band detection under UV light.

4.4.6 qPCR

For quantitative real-time PCR the 2x GoTaq qPCR master mix (Promega, Mannheim, Germany) was applied. 500 mM primer forward and reverse each and 10- 20 ng template cDNA or gDNA were added. The reaction was running in a CFX connect real time system (BioRad, Munich, Germany) with the following program:

1. 95 °C 3 min
2. 95 °C 15 s
3. 59 °C 20 s
4. 72 °C 30 s
5. Go back to step 2 39x
6. 95 °C 15s
7. Melt curve measurement: 65 °C – 95 °C with 0.5 °C per 0.05 s

Three technical replicates were pipetted per biological replicate. If possible, all replicates were analysed on one plate. Otherwise, the baseline was adjusted on the second plate according to the first plate. Relative expression was calculated using the $2^{-\Delta\Delta C_T}$ method (Livak & Schmittgen, 2001). Primer efficiencies were checked with 1:5 template dilution series and determined efficiencies used for calculation of relative expression levels.

4.4.7 RACE-PCR

The RACE-PCR was performed with the Ambion First Choice RLM-RACE kit (Thermo Fisher Scientific, Schwerte, Germany) according to the manual. RNA from an *S. indica* 5-day old liquid CM culture was used. The obtained fragments from 5´and 3´ RACE-PCR were cloned into the pJet 1.2 vector (Thermo Fisher Scientific, Schwerte, Germany) and sequenced.

4.4.8 Southern blot

100 µg extracted gDNA (see 4.4.1) was digested with EcoRI (for verification of gene deletion) or EcoRV (for verification of single integration of strains) restriction enzyme (New England BioLabs, Frankfurt, Germany) overnight. The digested DNA was precipitated with 0.5 volumes 7.5 M ammonium sulphate and 2 volumes isopropanol. The samples were incubated for 1 h and then centrifugated for 30 min at 13 300 rpm. The pellet was washed with 70 % ethanol, air-dried and dissolved in 25 µl ddH_2O. The samples were loaded on a 0.8 % agarose gel (see 4.4.5) and the gel run at 80 V for 3 h. The gel was depurinated in 0.25 N HCl for 15 min followed by denaturation in 0.4 M NaOH for 15 min. The transferring apparatus was built the following: A plate was laid on top of a tray filled with 0.4 M NaOH. A filter paper was soaked in 0.4 M NaOH and laid on the plate with the ends dipped in 0.4 M NaOH of the tray. The gel was laid on the filter paper upside down and the membrane (GE Healthcare Amersham Hybond-N^+, Sigma Aldrich, Taufkirchen, Germany) was put on top. The edges were sealed with parafilm, another wet filter paper and paper towels were laid on top and this tower fixed with a weight. The transfer of the DNA lasted for 3 h. The membrane was crosslinked in a UV crosslinker and transferred into a hybridisation tube with 30 ml hybridisation buffer

(0.5 M sodium phosphate pH 7, 7 % (w/v) SDS). The membrane was prehybridised at 65 °C for 1 h in a HB-1000 Hybridizer hybridisation oven (UVP, Analytik Jena, Jena, Germany).

The probe was produced with the PCR Dig labelling mix (Roche, Mannheim, Germany). A 50 µl PCR was performed with the Q5 High Fidelity DNA Polymerase (New England BioLabs, Frankfurt, Germany) according to the manual. Instead of dNTPs, labelled dNTPs from the PCR Dig labelling mix (Roche, Mannheim, Germany) were added. The probe was denatured for 10 min at 100 °C and added to 50 ml hybridisation buffer. Hybridisation took place overnight at 65 °C. The membrane was washed twice with 30 ml wash buffer (0.1 M sodium phosphate pH 7, 1 % (w/v) SDS) for 20 min at 65 °C and then with DIG wash buffer (0.3 % (v/v) Tween20, 0.1 M maleic acid and 0.15 M NaCl, pH adjusted to 7.5) for 5 min at RT. Afterwards, the membrane was incubated in 30 ml DIG II buffer (1 % blocking reagent (Roche, Mannheim, Germany) in 0.1 M maleic acid, 0.15 M NaCl, pH adjusted to 7.5) for 30 min. The membrane was incubated with 10 ml antibody solution (Anti-Digoxigenin AP Fab fragments (Roche, Mannheim, Germany) in DIG II buffer 1: 10000) for 30 min and then washed twice with 30 ml DIG wash buffer for 1 h and once for 15 min. The membrane was equilibrated with 30 ml DIG III buffer (0.1 M NaCl, 0.05 M $MgCl_2$ x 6 H_2O, pH adjusted to 9.5) for 5 min and subsequently, incubated for 5 min in CDP Star solution (1:100 in DIG III buffer, (Roche, Mannheim, Germany)). The membrane was transferred to a plastic foil and incubated for 15 min at 37 °C in the dark. The membrane was developed with a Fujifilm LAS4000.

4.4.9 VPE assay

The VPE assay was performed according to Lahrmann et al., 2013. 1 ml extraction buffer (10 mM sodium acetate pH 5.5, 100 mM NaCl, 1 mM EDTA, 2 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF)) was added to 100 mg in liquid nitrogen grinded material. For colonised Arabidopsis roots, 3- 6 plates were pooled per biological replicate. One jar with 4 barley plants was pooled per biological replicate. The tubes were centrifugated at 13 300 rpm for 10 min at 4 °C, the supernatant transferred to a new tube and centrifugated again. 100 µl supernatant was pipetted into a 96 well plate and 100 µM VPE substrate (Ac-ESEN-MCA, Peptide Institute, Osaka, Japan) added on ice. Fluorescence intensities were immediately measured in a Tecan infinite 200 plate reader

(Tecan, Männedorf, Switzerland) with 360 nm excitation wavelength and 465 nm emission wavelength. The increasing fluorescence intensities were measured over 1h for barley roots and 2h for Arabidopsis roots. Time point zero was subtracted from the end time point for each of the three technical replicates.

4.5 Biochemical methods

4.5.1 Protein sample preparation

For the preparation of samples from *U. maydis* for Western blot and nuclease activity, an overnight culture was diluted to OD_{600}= 0.2 and grown until an OD_{600}= 0.6 was reached. 1 ml of this culture was centrifugated at 8000 rpm for 2 min, the pellet resolved in 50 µl SDS sample buffer (0.1 M Tris pH 6.8, 2 M thiourea, 8 M urea, 8 % (w/v) SDS, bromophenol blue and 2 % (v/v) ß-mercaptoethanol) and cooked for 10 min at 95 °C. 20 ml culture was centrifugated at 3500 rpm for 5 min and the supernatant sterile filtered (0.45 µm). The proteins in the supernatant were precipitated with 10 % trichloroacetic acid overnight at 4 °C. The tubes were centrifugated at 10000 g for 30 min and the pellet was washed twice with acetone. The pellet was tried at 95 °C, 50 µl SDS sample buffer were added and the samples were cooked for 10 min at 95 °C.

S. indica liquid cultures were filtered with miracloth. An aliquot of the mycelium was cooked for 10 min at 95 °C in 100 µl SDS sample buffer. The proteins in the culture filtrate were precipitated with 10 % trichloroacetic acid overnight at 4 °C. The tubes were centrifugated at 10000 g for 30 min and the pellet was washed twice with acetone. The pellet was tried at 95 °C, SDS sample buffer was added and the samples were cooked for 10 min at 95 °C.

4.5.2 SDS-PAGE

SDS-PAGE was performed with self-made 10 % Bis-Tris gels:

323 mM Bisamino-trismethan (Bis Tris) pH 6.4

10 % (v/v) acrylamide/bisacrylamide (30 %, 37.5:1)

0.1 % (w/v) ammonium persulfate (APS)

0.001 % (v/v) Tetramethylethylenediamine (TEMED)

The stacking gel contained 5 % acrylamide/bisacrylamide. Besides the protein samples, the protein marker Page Ruler (Plus) Prestained (Thermo Fisher Scientific, Schwerte, Germany) was loaded on the gel. The gel was run in MES- SDS running buffer:

50 mM MES

50 mM Tris

1 mM EDTA

0.1 % (w/v) SDS

5 mM $Na_2S_2O_5$

The gel run at 180 V for 35 min in a Mini PROTEAN Tetra cell system (BioRad, Munich, Germany) with a Life technologies Powerease 90 W device (Thermo Fisher Scientific, Schwerte, Germany). Afterwards, the gel was stained overnight in Coomassie solution:

30 % (v/v) methanol

17.5 % (v/v) ethanol

10 % (v/v) acetic acid

2 % (w/v) Coomassie R-250

0.2 % (w/v) Coomassie G-250

The gel was destained in destaining solution (50 % (v/v) methanol and 10 % (v/v) acetic acid) and water.

4.5.3 Western blot

For Western blotting, an SDS-PAGE was run (see 4.5.2) and the gel blotted on GE Healthcare Amersham nitrocellulose blotting membrane Protran 45 µm NC (Sigma Aldrich, Taufkirchen, Germany) using the semidry Fastblot B44 device (Biometra, Göttingen, Germany). The following transfer buffer was used:

48 mM Tris

20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)

1 mM EDTA pH 8

1.3 mM $Na_2S_2O_5$

1.3 mM dimethylformamide

The proteins were blotted at 375 mA for 30 min. Subsequently, the membrane was washed in membrane wash buffer (0.3 % (v/v) Tween20, 0.1 mM maleic acid and 0.15 mM NaCl, pH adjusted to 7.5 for 5 min. The membrane was stained with Ponceau S

solution (0.1 % (w/v) Ponceau S, 1 % (v/v) acetic acid) to confirm equal loading and then destained in phosphate-buffered saline (PBS) buffer:

0.138 M NaCl

2.7 mM KCl

10.14 mM Na_2HPO_4 x $2H_2O$

1.76 mM KH_2PO_4

The membrane was incubated with the primary antibody in PBS buffer supplemented with 2.5 % (w/v) milk powder and 0.1 % (v/v) Tween20 for 1h. The membrane was washed three times for 5 min with PBS buffer and then, the secondary antibody in PBS buffer supplemented with 2.5 % (w/v) milk powder and 0.1 % (v/v) Tween20 was applied for 1h. The membrane was washed again, transferred to a plastic foil and Pierce ECL Western Blotting substrate (Thermo Fisher Scientific, Schwerte, Germany) was distributed on the membrane. The chemiluminescence was detected with a GelDoc XR+ device (BioRad, Munich, Germany) or Fujifilm LAS4000.

Table 4.4 Antibodies used for Western blot

Name	Description	Working concentration	Company
Anti-Penta His-HRP conjugate	Directly coupled	1:10000	Qiagen (Hilden, Germany)
Anti-HA	Primary antibody, mouse	1:5000	Sigma Aldrich (Taufkirchen, Germany)
Anti-GFP	Primary antibody, mouse	1:1000	Roche (Mannheim, Germany)
Anti-mCherry	Primary antibody, rabbit	1:1000	Biovision (Biozol Diagnostica, Eching, Germany)
Anti-mouse	Secondary antibody	1:1000	Sigma Aldrich (Taufkirchen, Germany)
Anti-rabbit	Secondary antibody	1:1000	Sigma Aldrich (Taufkirchen, Germany)

4.5.4 Purification of *Si*NUCA from *S. indica* culture filtrate

A 100 ml CM culture from spores was filtered with miracloth and the mycelium grown in 200 ml new CM and after another medium exchange in 500 ml CM. Subsequently, the mycelium was crushed and regenerated for two days. The culture filtrate was filtered with miracloth and through a 0.45 µm membrane filter (Hartenstein, Würzburg,

Germany). 1 mM Phenylmethanesulfonyl fluoride (PMSF) was added, the pH set to 7 with 1M Tris pH 8 and the culture filtrate precipitated with 80 % ammonium sulphate for 1h while stirring. The precipitate was centrifugated for 20 min at 43000 g and the resulting protein pellet was resuspended in 20 mM Tris pH 8. The soluble protein was concentrated with a 5 kDa Spin-X UF Concentrator (Corning, Flintshire, UK) to 1 ml volume. The soluble protein was further purified by size exclusion chromatography with a Sephadex G 200 column (Hiload 6/600) in collaboration with Prof. Dr. J. Riemer. The buffer 20 mM Tris pH8/150 mM NaCl was used and 5 ml elution fractions were collected. The fractions were verified by anti-HA Western blot to contain the target protein. The selected fractions were concentrated, desalted (dialysis tubing visking cellulose 12-14 kDA, Carl Roth, Karlsruhe, Germany) and loaded on an SDS-PAGE to estimate yield and purity. The protein was stored in 20 mM Tris pH 8 at – 20 °C. Trypsin-digested protein in 20 mM Tris pH 8 was analysed by LC-MS/MS.

4.5.5 Nuclease assays

To test nuclease activity in *U. maydis* supernatant, cultures of the same OD_{600} were prepared as described in 4.4.1. 50 µl supernatant was pipetted in 96 well plates, linearised plasmid, gDNA or RNA was added and the mixtures incubated for several time points. The reaction was stopped by adding loading dye and the samples were loaded on an agarose gel (see 4.4.5). As controls, 1 U DNase I, 10 µg RNase A, 1 mM EDTA or 20 U RiboLock RNase inhibitor were used (all purchased from Thermo Fisher Scientific, Schwerte, Germany).

S. indica cultures were grown in CM medium for 5 days, crushed and incubated for another 3 days (see 4.3.5.1). The culture was filtered with miracloth. 50 µl culture filtrate was pipetted on 96 well plates and 100 ng linearised plasmid was added. The mixture was incubated for several time points in parallel and the reaction stopped with loading dye. The samples were loaded on a 1 % agarose gel (see 4.4.5).

Generally, nuclease activity assays with 10 nM purified *Si*NUCA were performed in 5 mM Tris pH 8 supplemented with 0.1 % (v/v) microelement solution (see 4.3.5.1), 1 mM $MgCl_2$ and 1 mM $CaCl_2$. 100 ng linearised plasmid, 1 µg gDNA or 1 µg RNA were added, the mix incubated for several time points in parallel and the reaction stopped with loading dye. The samples were loaded on a 1- 2 % agarose gel. 1.25 mM EDTA or 20 U

RiboLock RNase inhibitor were used for inhibition (purchased from Thermo Fisher Scientific, Schwerte, Germany).

4.5.6 Antibacterial tests with *Si*NUCA

A *E. coli* overnight culture was set to OD_{600}=0.2 and grown for 1.5 h. The culture was diluted to an OD_{600}= 0.01 and 10 µl aliquots were pipetted in a 96 well plate. 20 µl 20 mM Tris pH8 supplemented with 30 µM *Si*NUCA, 200 µg/ml ampicillin or 5 mg/ml RNAse A were added. The plate was shaken at RT for 5h and 1:10 dilutions were dropped on plates. The plates were incubated overnight at RT. The diluted culture with an OD_{600}= 0.01 was also sprayed on plates, dried under the sterile bench and filter platelets distributed. 10 µl *Si*NUCA in 20 mM Tris pH 8, 10 µl RNAse A (10 mg/ml) in 20 mM Tris pH 8 or 10 µl 20 mM Tris pH 8 were pipetted on the filter platelets. The plates were incubated overnight at RT.

4.6 Microscopy

4.6.1 Fluorescence microscopy

Light and fluorescence microscopy of living fungal and plant material was performed with a Leica DM2500 light microscope (Leica, Wetzlar, Germany).

4.6.2 Sample preparation

For microscopy of living fungal mycelium, young crushed cultures with 1- 2 days regeneration phase were used (see 4.3.3, 4.3.4, 4.3.5.1). Stainings were applied in Eppendorf tubes, samples were washed with ddH_2O and the mycelium was directly put on the microscopy slide. The cover slide was carefully added on top without pressure.
S. indica spores germinated on glass slides were directly subjected to the microscope.
Inoculated Arabidopsis plants were transferred to an Eppendorf tube with the staining solution. The plants were carefully washed with PBS pH 7.4 and put on the microscopy slide. The cover slide was carefully added on top of the root part only without pressure.
For microscopy of inoculated barley roots, side roots were cut and transferred to an

Eppendorf tube for staining. Otherwise, the procedure was equal to Arabidopsis plants. For plasmolysis, 1 M NaCl or 1 M sorbitol were directly added on the slide.

For visualisation of BLC, microscopy slides were poured into the ½ MS plate. Therefore, inoculated Arabidopsis roots were growing on top of the slide with a layer of ½ MS medium. The slide was carefully cut from the plate without moving the plant. Staining solution was directly added on the root, incubated for 15 min and a cover slide put on top.

Table 4.5 Stainings for microscopy

Dye	Description	Working concentration	Company
WGA-AF 594	Fungal cell wall staining (chitin), Wheat Germ Agglutinin Alexa Fluor 594 conjugate	5 µg/ml in PBS pH 7.4 (plant roots) or ddH_2O (mycelium)	Life technologies (Thermo Fisher Scientific, Schwerte, Germany
WGA-AF 488	Fungal cell wall staining (chitin), Wheat Germ Agglutinin Alexa Fluor 488 conjugate	5 µg/ml in PBS pH 7.4 (plant roots) or ddH_2O (mycelium)	Life technologies (Thermo Fisher Scientific, Schwerte, Germany
FGB1-FITC	Fluorescein isothiocyanate -labelled FGB1, labelling efficiency= 30 %	1 µM in ddH_2O, incubation for 15 min (0.2 µM for direct staining on slide)	SureLINK Fluorescein-X (FAM-X) Labeling Kit (SeraCare Life Sciences, Milford, USA)
DAPI	4',6-Diamidine-2'-phenylindole dihydrochloride, Nuclei staining	250 ng/ml, 2h incubation	Provided by Dr. S. Wawra
SYTOX Orange	Nuclei staining of dead cells	500 nM in ddH_2O	Life technologies (Thermo Fisher Scientific, Schwerte, Germany

4.6.3 Confocal laser scanning microscopy

A TCS SP8 confocal microscope (Leica, Wetzlar, Germany) was used for live cell CLSM. For excitation of AF488, GFP and FITC fluorescence an Argon laser at 488 nm was used and the signal detected with a hybrid detector at a wavelength of 500- 550 nm. AF594, mCherry and SYTOX orange fluorescence was excited at 561 nm with a DPSS laser and the emitted light was detected with a hybrid detector at a wavelength of 590-660 nm. DAPI was excited with a diode laser at 405 nm and the signal was detected with a hybrid

detector at a wavelength of 415-460 nm. The picture resolution was 1024 x 1024 pixels with a line average of 4. Z stacks were accumulated using the Fiji software version 2.0 (Schindelin et al., 2012). Background subtraction was done with the rolling ball method and brightness and contrast were adjusted.

4.7 Vectors

4.7.1 Cloning

Plasmids were purified form *E. coli* liquid cultures with the DNA QIAprep Spin Miniprep Kit (QIAGEN, Hiden, Germany), Nucleo Spin plasmid kit (Macherey-Nagel, Düren, Germany) or in bigger amounts with the PROMEGA PureYield Plasmid Midiprep (Mannheim, Germany) kit according to the manuals. Plasmid digests were performed with restriction enzymes from New England Biolabs (Frankfurt, Germany) according to the manual. For amplification of fragments by PCR, the proof reading self-made Pfu polymerase was used with the following protocol for a total reaction volume of 20 µl:

2 µl 10x Pfu buffer
0.4 µl dimethyl sulfoxide (DMSO)
0.4 µl dNTPs (10 mM)
0.4 µl primers each (10 µM)
2 µl $MgCl_2$ (25 mM)
0.6 µl Pfu (1:4 diluted)
1 µl template
12.8 µl ddH_2O

10x Pfu reaction buffer:
200 mM Tris pH 8.8
100 mM $(NH_4)_2SO_4$
100 mM KCl
1% (v/v) Triton X-100
1 mg/ml BSA (Bovine serum albumin)
20 mM $MgSO_4$

Buffer of Pfu polymerase:	20 mM Tris
	1mM DTT
	0.1 mM EDTA
	100 mM KCl
	0.1 % (v/v) Nonidet P40
	0.1 % (v/v) Tween20
	50 % (v/v) glycerol
	pH 8.2

The following PCR protocol was applied:

1. 98 °C 1 min
2. 98 °C 15 s
3. Annealing temperature 30 s
4. 72 °C elongation time (1 kb/ min)
5. Back to step 2. for 25-35 times
6. 72 °C 4 min

Plasmid or PCR fragments were cleaned up from agarose gels with the PROMEGA Wizard SV Gel and PCR Clean-Up System (Mannheim, Germany) or NucleoSpin Gel and PCR Clean-up (Macherey-Nagel, Düren, Germany). Fragments and plasmid backbones were ligated with the Gibson assembly method (Gibson, 2011). Fragments to be inserted into the restriction enzyme-digested backbone were designed with 20 bp overlaps. 100 ng backbone and 3 times more insert(s) were added to the 2x Gibson assembly mix:

40 % 5x Isothermal reaction mix

0.08 % T5 Exonuclease (10U/µl) (New England Biolabs (Frankfurt, Germany)

2.5 % µl Phusion DNA-Polymerase (2U/ µl) (Thermo Fisher Scientific, Schwerte, Germany)

20 % Taq DNA-Ligase (40U/ µl) (New England Biolabs (Frankfurt, Germany)

5x Isothermal reaction mix:

0.5 M TrisHCl pH 7.5

0.05 M $MgCl_2$

1 mM dNTPs each

0.05 M DTT

250 µg/ml PEG-8000

5 mM NAD^+ (Nicotinamide-Adenine-Dinucleotide)

The tube was incubated at 50 °C for 1 h and 5 µl mix were used for *E. coli* transformation (see 4.3.1.2). The obtained plasmids were confirmed by restriction digest and sequencing of the modified plasmid region (GATC, Eurofins Genomics, Ebersberg, Germany).

4.7.2 Vectors for *U. maydis*

p123-SiNUCA: p123-DLD1 (harbouring the otef promoter, *DLD1* gene and carboxin resistance, provided by Dr. S. Nizam) was digested with BamHI/NotI and *DLD1* replaced with PCR-amplified *SiNUCA* from cDNA.

p123-SiNUCA-mCherry: PCR- amplified *SiNUCA* and *mCherry* amplified from p123-E5NTSP-mCherry-E5NTwoSP (provided by Dr. S. Nizam) were inserted into the BamHI/NotI cut p123 backbone.

p123-SP^{SiNUCA}-mCherry: To obtain p123-SP^{SiNUCA}-mCherry-SiNUCA, PCR- amplified *mCherry* and *SiNUCA* (without SP) were inserted into the BamHI/NotI cut p123 backbone. This new vector was digested with BamHI/NcoI and the SP inserted by ligation with denatured 5´- phosphorylated primers using the T4 ligase (New England Biolabs, Frankfurt, Germany). The obtained vector was digested with XbaI/NotI and religated with a denatured 5´- phosphorylated primer mix to get rid of *SiNUCA*.

4.7.3 Vectors for *S. indica*

pFGB1-FGB1-oGFP: The vector pGoGFP harbours the GPD promoter, oGFP and the Hygromycin B resistance cassette (Hilbert et al., 2012). pGoGFP was modified by addition of a second SfiI site at the beginning of the Hygromycin B resistance cassette (provided by Dr. S. Wawra). Therefore, the resistance cassette can be exchanged using

the SfiI sites. This vector was digested with ApaI/ClaI to remove the GPD promoter and insert the FGB1 promoter. The first part of the FGB1 promoter was PCR- amplified from gDNA, the second part was synthesised including the SP of FGB1 and the ClaI site was added (also compare to supplementary in Wawra et al., 2016). These two fragments were inserted into the digested vector. Afterwards, the obtained vector was digested with ClaI/HindIII to insert PCR- amplified FGB1 without SP from cDNA.

pSvTEF-oGFP: pFGB1-FGB1-oGFP was digested with ApaI/HindIII to insert the TEF promoter of *S. vermifera*, which was PCR-amplified from gDNA.

pFGB1-SP^{FGB1}-Sebve1_14646-oGFP: The vector pFGB1-FGB1-oGFP was digested with ClaI/HindIII and *Sebve1_14646* without SP was inserted (PCR-amplified from cDNA).

pFGB1-SiNUCA-2HA-His$_6$: pFGB1-FGB1-oGFP was digested with ApaI/ClaI and the FGB1 promoter replaced by the FGB1 promoter with an additional NheI site before the ClaI site. Afterwards, this vector was digested with NheI/EcoRV and a synthesised piece containing SP^{DLD1}-2HA-His$_6$-oGFP-PreSc-FGB1 was inserted to obtain the vector pFGB1-SP^{DLD1}-2HA-His$_6$-oGFP-PreSc-FGB1. This is the pFGB1 version with N- terminal tags.

This vector was cut with SwaI/PmeI and religated to get read of the N- terminal tags. Subsequently, the vector was opened with SnaBI and *oGFP* was inserted with the flexible linker (YSSGSGSSAQ) in front of it. The vector was digested with SnaBI again and the 2HA-His$_6$ tag was inserted to obtain the vector pFGB1-SP^{DLD1}-FGB1-oGFP-2HA-His$_6$.

The vector pFGB1-SP^{DLD1}-FGB1-oGFP-2HA-His$_6$ was cut with NheI/PmeI and *SiNUCA* was inserted into the vector to obtain pFGB1-SiNUCA-2HA-His$_6$.

pFGB1-2HA-His$_6$: To obtain the empty vector pFGB1-SiNUCA-2HA-His$_6$ was digested with NheI/PmeI and ligated with a denatured 5´-phosphorylated primer mix using the T4 ligase.

pFGB1-Bs19137836-PreSc-oGFP-2HA-His$_6$: The vector pFGB1-SP^{DLD1}-FGB1-oGFP-2HA-His$_6$ was digested with NheI/PmeI and the *B. sorokiniana* gene 19137836 and PreSc-oGFP were inserted (in collaboration with Dr. S. Wawra).

pFGB1-FGB1-PreSc-oGFP-2HA-His$_6$: The pFGB1-Bs19137836-PreSc-oGFP-2HA-His$_6$ vector was digested with NheI/HindIII to replace *Bs19137836* with FGB1 (in collaboration with Dr. S. Wawra).

pFGB1-Bs19137836-PreSc-mRFP-2HA-His$_6$: The oGFP was exchanged with mRFP by digestion with PmeI of pFGB1-Bs19137836-PreSc-oGFP-2HA-His$_6$. mRFP was PCR-amplified from pET21b-nterHAHismRFP (provided by Dr. S. Wawra).

pFGB1-Bs19137836-PreSc-2HA-His$_6$: The PmeI digested vector pFGB1-Bs19137836-PreSc-oGFP-2HA-His$_6$ was religated.

pFGB1-FGB1-PreSc-mRFP-2HA-His$_6$: pFGB1-FGB1-PreSc-oGFP-2HA-His$_6$ was digested with PmeI and *oGFP* replaced by *mRFP*.

pPiRNAi-SiNUCA: The vector pPiRNAi contains the GPD and TEF promoters with an EcoRV restriction site in the middle to clone a gene fragment (Hilbert et al. 2012). The gene fragment is expressed from both sites with the promoters to obtain a double stranded RNA for RNAi. pPiRNAi was digested with EcoRV and a 550 bp fragment of SiNUCA was inserted.

pJet-LB^{SiNUCA}-HygB-RB^{SiNUCA}: For construction of the fragments used for transformation to obtain *ΔsinucA,* 1 kb upstream (LB) and 1 kb downstream (RB) of the *SiNUCA* open reading frame were amplified from gDNA by PCR. Moreover, the Hygromycin B resistance cassette including the TEF promoter and Nos terminator were amplified from pGoGFP (Hilbert et al., 2012). These three fragments were joined by Gibson assembly, the new big fragment amplified by PCR and cloned into pJet (linearised with EcoRV, Thermo Fisher Scientific, Schwerte, Germany) to obtain the vector pJet-LB^{SiNUCA}-HygB-RB^{SiNUCA}.

This vector was digested with BglII to obtain one fragment or ClaI/EcoRI+ XhoI to obtain two fragments for *S. indica* transformation. The fragments were cleaned up from an agarose gel, precipitated for an additional cleaning step and used for transformation.

pJet-LB$^{SiNUCA,1.8kb}$-HygB-RB$^{SiNUCA,1.8kb}$: The vector pJet-LB^{SiNUCA}-HygB-RB^{SiNUCA} was digested with SacI/ClaI and the LB replaced with a 1.8 kb fragment of the LB amplified from gDNA. This new vector was cut with BamHI in the RB region and the RB prolonged to 1.8 kb by insertion of a PCR-amplified RB piece from gDNA. The obtained vector pJet-LB$^{SiNUCA,1.8kb}$-HygB-RB$^{SiNUCA,1.8kb}$ was digested with ClaI/NaeI for transformation of one fragment or with ClaI/SacII + EcoRI/NaeI for transformation of two fragments.

4.7.4 Vectors for Arabidopsis

pCXSN-SiNUCA, pCXSN-SiNUCA-mCherry, pCXSN-SiNUCA(w/o SP)-mCherry: The vector pCXSN harbouring the CaMV 35S promoter and Hygromycin B resistance cassette (provided by Dr. S. Nizam) was digested with BamHI. *SiNUCA*, *SiNUCA:mCherry* or *SiNUCA*(w/o SP):*mCherry* were PCR-amplified from p123-SiNUCA or p123-SiNUCA:mCherry and inserted.

4.8 Oligonucleotides

Oligonucleotides were purchased at Sigma Aldrich (Taufkirchen, Germany).

Table 4.6 Oligonucleotides used in this thesis

Name	Sequence (5´-3´)	Purpose
AtUBI_F	CCAAGCCGAAGAAGATCAAG	qPCR *AtUBI*
AtUBI_R	ACTCCTTCCTCAAACGCTGA	qPCR *AtUBI*
HvUBI_F	CAGTAGTGGCGGTCGAAGTG	qPCR *HvUBI*
HvUBI_R	ACCCTCGCCGACTACAACAT	qPCR *HvUBI*
Hv_U35_15826_for	GGAGGGCGACAAGGTAAGTG	qPCR *HvPR10*
Hv_U35_15826_rev	CGTCCAGCCTCTCGTACTCT	qPCR *HvPR10*
TEF_Piri_QPCR_F	GCAAGTTCTCCGAGCTCATC	qPCR *SiTEF*
TEF_Piri_QPCR_R	CCAAGTGGTGGGTACTCGTT	qPCR *SiTEF*
PIIN02121_5inner	TCCAGATGCACCAGACCTCCA	qPCR *SiNUCA*, also RACE PCR
PIIN02121_3outer	GGTACTCAAGCGTGACGGAA	qPCR *SiNUCA*, also RACE PCR
05889_QPCR_F	CTTCTCAGCAGCAAACAAGC	qPCR *PIIN_05889*
05889_QPCR_R	AGAGTACCAGGCGTTCGAGT	qPCR *PIIN_05889*
PIIN_00073_fw2	ATCCATGGAGATGCTCGTCC	qPCR *PIIN_00073*
PIIN_00073_rv2	TCAAAGACAGCACCCGTTTG	qPCR *PIIN_00073*
PIIN_09504_fw	GGATCCAACCAAGTTTGCGT	qPCR *PIIN_09504*
PIIN_09504_rv	CCATCGTCAAAGCTACCGTG	qPCR *PIIN_09504*
PIIN_08972_fw	TTCATGACCACCAACACGAC	qPCR *PIIN_08972*
PIIN_08972_rv	AGCGGCAAGAATATCCCTGA	qPCR *PIIN_08972*
Sebve1_TEF_qPCR_new_FW	ATCCCAAGCAAGCCAATGTG	qPCR *Sv*TEF
Sebve1_TEF_qPCR_new_RV	TGCCGTCAGTCTTCTCAACA	qPCR *Sv*TEF
K284_Sebve12299_qPCR_fw	ACCCTGCTAAATGGATGCC	qPCR *Sebve1_12299*
K285_Sebve12299_qPCR_rv	CGTTGTACTCTGCTTGGTCG	qPCR *Sebve1_12299*
K174_NewHD1.2 left	AGATATCCGGAGGCGAGTTT	Mating type HD 1.2
K175_NewHD1.2 right	CCTGAATCTGCTGTTCGTCA	Mating type HD 1.2
K176_NewHD2.2 left	ACATCTGGCTCCCATTTACG	Mating type HD 2.2
K177_NewHD2.2 right	GTTGAGCTTTGGCTCGTTTC	Mating type HD 2.2
K178_NewHD2.1 left	ATGAGTACGATTGCCCAAGG	Mating type HD 2.1
K179_NewHD2.1 right	TCGTCTCGTAGGCGACTTTT	Mating type HD 2.1
K180_NewHD1.1 left	CGATACCTACCCGCCTACAA	Mating type HD 1.1
K181_NewHD1.1 right	CTTTTTAAGCGGTGCTGGAG	Mating type HD 1.1
K403_RNAi_PIIN02121_fw	CAACCTCATGGAGAGTCGTG	PCR *SiNUCA, also plant line screening, cloning for RNAi*
K404_RNAi_PIIN02121_rv	CTTGTACAATGCATTGTACTCG	PCR *SiNUCA, also plant line screening, cloning for RNAi*

Name	Sequence (5´-3´)	Purpose
PIIN02121_5outer	GCGATGAGTTGAGGACGAGT	RACE PCR *SiNUCA*
PIIN02121_3inner	CGACCATCTTGTTCCATTAGCC	RACE PCR *SiNUCA*
K262_PIIN02121_5UTR_fw	GCGTTTCTGGCTATAAGAACTGCG	*ΔsinucA* screening
K263_PIIN02121_3UTR_rv	TAAACAGACCAAGAACGATTGTATTGCG	*ΔsinucA* screening
Hyg-03211RBcPCR primer FW	AGCGCGCAAACTAGGATAAA	*ΔsinucA* verification
TEFprom_begR	CAGAGGAACCGATGCTGAAT	*ΔsinucA* verification
PIIN_02121_upstream_fw	TGAATAGCGCGTCAATTGTCTA	*ΔsinucA* verification
PIIN_02121_downstream_rv	CACAGGTGGACCCTGGAGGT	*ΔsinucA* verification
PIIN_02121_fw2	CCCCGAAAATAGCAGTGTCG	*ΔsinucA* Southern blot probe
PIIN_02121_rv2	GGGGTGCCAAACATGAAGG	*ΔsinucA* Southern blot probe
PIIN_02121_RB_rv	TAAGCATGCACGCTTCTTC	*ΔsinucA* Southern blot probe
PIIN_02121_RNAi_qPCR_fw2	GCTAGGAAGAGGCAGAATTTCG	*ΔsinucA* Southern blot probe
Kan_FWD	TGGAGAGGCTATTCGGCTAT	Southern blot probe GenR
Kan_REV	AACTCGTCAAGAAGGCGATA	Southern blot probe GenR
K417_SP02121_NcoI_fw	catggATGATACCTACTTTCTCTTTTGCCGCCCTCATACTCGGCATGCTTGCAACCGTGAGCGCTGC	Verification of *A. tumefaciens* transformants
mCherry_Rev_2	AGCCCATCGTCTTCTTCTGC	Verification of *A. tumefaciens* transformants
mCherry_Fwd_1	GGGCGAGGAGGATAACATGG	Verification of *A. tumefaciens* transformants
K409_Potef_02121_fw	ACAGACAACATCATCCACGGGATCCATGATACCTACTTTCTCTTTTGC	Cloning of *SiNUCA* into p123
K410_Potef_02121_rev	TGAACGATCTGCAGCCGGGCGGCCGCCTAGCATGCGTACAAATACTTG	Cloning of *SiNUCA* into p123
K411_mcherry_02121_rv	ATGGCGGTGGCGATCGAGCGGCATGCGTACAAATACTTGTAC	Cloning of *SiNUCA:mCherry* into p123
K412_02121_mcherry_fw	GCCGCTCGATCGCCACCGCCATGGTGAGCAAGGGCGAGG	Cloning of *SiNUCA:mCherry* into p123
K413_Potef_mcherry_rv	TGAACGATCTGCAGCCGGGCGGCCGCCTACTTGTACAGCTCGTCCATGC	Cloning of *SiNUCA:mCherry* into p123
K414_Potef_mcherry_fw	ACAGACAACATCATCCACGGGATCCGCCATGGTGAGCAAGGGCGAGG	Cloning of SP^{SiNUCA}:*mCherry:* into p123
K415_02121_mcherry_rv	AGCCGGAGCCGGAGGAtgctctagaTGCCTTGTACAGCTCGTCCATGCCGC	Cloning of SP^{SiNUCA}:*mCherry:* into p123
K416_mcherry_02121_fw	gagcaTCCTCCGGCTCCGGCTCCTCCGCGCAAGCTCCGCTCAACCTCATGG	Cloning of SP^{SiNUCA}:*mCherry:* into p123
SP02121_BamHI_fw	gatccATGATACCTACTTTCTCTTTTGCCGCCCTCATACTCGGCATGCTTGCAACCGTGAGCGCTGC	Cloning of SP^{SiNUCA}:*mCherry:* into p123
02121SP_NcoI_rv_2	CATGGCAGCGCTCACGGTTGCAAGCATGCCGAGTATGAGGGCGGCAAAAGAGAAAGTAGGTATCATg	Cloning of SP^{SiNUCA}:*mCherry:* into p123
XbaINotI_fw	ctagagctagttcactgGC	Cloning of SP^{SiNUCA}:*mCherry:* into p123
XbaINotI_rv	GGCCGCcagtgaactagct	Cloning of SP^{SiNUCA}:*mCherry:* into p123
K89>Prom03211_NotI RV	AACGTGCGGCCGCTGAAT	FGB1 promoter cloning
Altern Prom03211_ApaI FW	CGATAGAGTTGGAGGCAAATC	FGB1 promoter cloning
RV_03211 ORF+SP HindIII dStop	CAG TAA GCT TGC ACG AGA GCT TCC CGC AC	Cloning of pFGB1-FGB1-oGFP
K13703211_ORF ClaI FW	ACTA AT CGAT GATGACGAAACAGCGGTCATTG	Cloning of pFGB1-FGB1-oGFP

Name	Sequence (5´-3´)	Purpose
SebaTEFprom_ApaI_fw	GGGAACAAAAGCTGGTACCGGGCCC GTTTATAGATGCGCTGCTTTTG	Cloning of p*Sv*TEF-oGFP
SebaTEFprom_HindIII_rv	CCTTGGAGACCATGATATCGCTAGCT TTTGAGAGAAAAAAGTGTGTGAG	Cloning of p*Sv*TEF-oGFP
Sebve1_14646_RV_dStop_HindIII	gttgaagcttCTGATCGTACTGACCGCG C	Cloning of *Sebve1_14646:GFP* into pFGB1
Sebve1_14646_FW_dSP	AGCAAACTTGTTCCCGCCCTCCC	Cloning of *Sebve1_14646:GFP* into pFGB1
K405_FGB1promApaI_fw	GGGAACAAAAGCTGGTACCGGGCCC TATGTCCAAATGATCACATAAC	Cloning of pFGB1- SP^{DLD1}-2HA:His_6-oGFP-PreSc-FGB1
K406_FGB1promNheIClaI_rv	GACCGCTGTTTCGTCATCATCGATGC TAGCGACTGTTTTTGGAGTGATGC	Cloning of pFGB1- SP^{DLD1}-2HA:His_6-oGFP-PreSc-FGB1
K407_synthnterNheI_fw	CATCACTCCAAAAACAGTCGCTAGCA TGCGCGTCGGT	Cloning of pFGB1- SP^{DLD1}-2HA:His_6-oGFP-PreSc-FGB1
K408_synthnterStop_rv	CCGGGCTGCAGGAATTCGATCTATA CGTAAAGCTTGCACG	Cloning of pFGB1- SP^{DLD1}-2HA:His_6-oGFP-PreSc-FGB1
GFP_linkerPmeI_fw	AGCTCTCGTGCAAGCTTTACTCCTCC GGCTCCGGCTCCTCCGCGCAAAGTTT AAACATGGTCTCCAAGGG	Cloning of pFGB1-SP^{DLD1}-FGB1-oGFP-2HA-His_6
GFP_PmeISnaBI_rv	TGCAGGAATTCGATCTATACGTAGTT TAAACTCTTGTAGAGCTCGTCCATAC C	Cloning of pFGB1-SP^{DLD1}-FGB1-oGFP-2HA-His_6
HAHis_EcorV_fw	TCTACAAGAGTTTAAACTACGATATC TACCCATACGATGTTCC	Cloning of pFGB1-SP^{DLD1}-FGB1-oGFP-2HA-His_6
HAHis_EcoRV_rv	TGCAGGAATTCGATCTATACGATATC CTAgTGGTGGTGGTG	Cloning of pFGB1-SP^{DLD1}-FGB1-oGFP-2HA-His_6
PIIN2121_olK91_fw	CATCACTCCAAAAACAGTCGCTAGCA TGATACCTACTTTCTCTTTTGC	Cloning *SiNUCA* into pFGB1
PIIN2121_olK91_EcorV_rv	ATGGGTAGATATCGTAGTTTGAGCA TGCGTACAAATACTTGTAC	Cloning *SiNUCA* into pFGB1
P-pGoGFPHaHis_fw	CTAGCTTGATCGCTAGGTTT	Cloning of empty vector pFGB1
P-pGoGFPHaHis_rv	AAACCTAGCGATCAAG	Cloning of empty vector pFGB1
>K605_BiPo7697489.1_FW	GCATCACTCCAAAAACAGTCGCTAGC ATGCATTTCCAACACGCGCTATCTC	Cloning of *Bs19137836* into pFGB1
>K606_BiPo7697489.1_RV	TTGGCCCCTGGAACAGAACTTCCAG AAGCTTATTCGGTGAACCCTGTCGGC	Cloning of *Bs19137836* into pFGB1
>K607_GFP+Nterm_PreS	CTGGAAGTTCTGTTCCAGGGGCCAA GTTTAAACATGGTCTCCAAGGGCGA GG	Cloning of *Bs19137836* into pFGB1
>K608_RV_PmeI/EcoRV for K91	CGTATGGGTAGATATCGTAGTTT	Cloning of *Bs19137836* into pFGB1
K627_3211dSP_RV_Gibs_Hind_K106	GGAACAGAACTTCCAGAAGCTTGCA CGAGAGCTTCCCGCACTTC	Cloning of pFGB1-FGB1-PreSc-oGFP-2HA-His_6
K624_3211SP_FW_Gibson_NheI_K106	GCATCACTCCAAAAACAGTCGCTAGC ATGAAGTTCACTACCGTCTTCG	Cloning of pFGB1-FGB1-PreSc-oGFP-2HA-His_6
mRFP_PmeI_fw	CTGTTCCAGGGGCCAAGTTTAAACat ggcctcctccgagg	Cloning of *Bs19137836:mRFP* into pFGB1
mRFP_PmeI_rv	ATGGGTAGATATCGTAGTTTAAACTg gcgccggtggagtggc	Cloning of *Bs19137836:mRFP* into pFGB1
PIIN_02121_LB_fw	CACATGCGTTCCATAACCG	Cloning of LB SiNUCA for *ΔsinucA* vector
PIIN_02121_LB_Hyg_rv	AAAGTAGGTCCCTGGACAAACAAGG GGAC	Cloning of LB SiNUCA for *ΔsinucA* vector

Name	Sequence (5´-3´)	Purpose
PIIN_02121_RB_Hyg_fw	CAAACATGAGGAAGAGGCAGAATTTCGATAG	Cloning of RB SiNUCA for *ΔsinucA* vector
PIIN_02121_RB_rv	TAAGCATGCACGCTTCTTC	Cloning of RB SiNUCA for *ΔsinucA* vector
PIIN_02121_Hyg_LB_fw	TTTGTCCAGGGACCTACTTTTGATGAGATTATTC	Amplification of HygB cassette for *ΔsinucA* vector
PIIN_02121_Hyg_RB_rv	CTGCCTCTTCCTCATGTTTGACAGCTTATC	Amplification of HygB cassette for *ΔsinucA* vector
LB2121_SacI_fw	GACAAACAAGGGGACTGAAAG	Cloning of LB 1.8 kb SiNUCA for *ΔsinucA* vector
LB2121_ClaI_rv	agagaataaaagaagaacatcgatATGTACGAACGATCATCAAGTC	Cloning of LB 1.8 kb SiNUCA for *ΔsinucA* vector
RB2121_BamHI_rv	CGCCATCCACATGTCGAGAG	Cloning of RB 1.8 kb SiNUCA for *ΔsinucA* vector
PIIN2121_RBlong_NaeI_fw	GCGACCTTGATCACCCAATGCCGGCGTGTCCTCGCAAGGGTATCA	Cloning of RB 1.8 kb SiNUCA for *ΔsinucA* vector
pCXSN_2121_fw	TACGAACGATACTCGAGGGGGATCCATGATACCTACTTTC	Cloning of *SiNUCA* into pCXSN
pCXSN_2121_rev	ATCGGGGAAATTCGCTAGTGGATCCCTAGCATGCGTACAAATACTT	Cloning of *SiNUCA* into pCXSN
pCXSN_2121woSP_fw	TACGAACGATACTCGAGGGGGATCCGCTCCGCTCAACCTCATGG	Cloning of *SiNUCA* (w/o SP) into pCXSN
pCXSN_2121mcherry_rv	ATCGGGGAAATTCGCTAGTGGATCCCTACTTGTACAGCTCGTCCAT	Cloning of *SiNUCA:mCherry* into pCXSN

4.9 Programs

Homologous proteins were found with the NCBI blastp algorithm using the non-redundant protein sequences (nr) database.

SP were analysed using SignalP 4.1 with default settings for eukaryotes (Petersen et al., 2011). Protein domains were detected with the SMART tool (Letunic & Bork, 2018).

A protein logo was created with WebLogo using default settings (Crooks et al., 2004).

Statistical analysis was performed with R studio mainly using the multcomp and car packages (RCoreTeam, 2017).

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Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit − einschließlich Tabellen, Karten und Abbildungen −, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie − abgesehen von unten angegebenen Teilpublikationen − noch nicht veröffentlicht worden ist, sowie, dass ich eine solche Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen der Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Frau Prof. Dr. Alga Zuccaro betreut worden.

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