Role of Striatin Complexes in the Control of Development and Secondary Metabolism of the Filamentous Fungus *Aspergillus nidulans*

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Thesis submitted to Maynooth University

for the degree of Doctor of Philosophy

October 2017

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Table of contents

Declaration of Authorship ……... **V**
Acknowledgements ……. **VI**
Abbreviations …….. **VII**
List of tables ……..... **XI**
List of figures …….. **XII**
Summary ……. **XIV**

Chapter 1: Introduction……..... **1**
Signalling pathways co-ordinate characteristic responses to stimuli Striatin…… **2**
The Structure of Striatin …… **2**
The Localization of Striatin ……... **5**
The Functions of Striatin ……... **6**
The Striatin Family Complex …… **6**
The STRIPAK complex …… **7**
The STRIPAK accessory protein SLMAP ……... **9**
Evolutionary conservation of the STRIPAK complex ……..... **9**
Fungi ……..... **11**
The Fungal Kingdom ……... **11**
Aspergillus nidulans, a model organism for fungal development and secondary metabolism ……..**12**
Vegetative growth in *A. nidulans*……..... **13**
Asexual development in *A. nidulans*…… **14**
Sexual development in *A. nidulans*…….. **16**
The role of the striatin family complex in fungi …….. **21**
The STRIPAK complex in the model fungus *Sordaria macrospora* ….. **21**
The *Aspergillus nidulans* striatin homolog StrA ……. **23**

Aim of this study……..... **24**

Chapter 2: Materials and Methods …….. **26**

Strains, media and growth conditions ……..... **27**

Nucleic acid methods ……. **32**

Transformations ……. **32**

Plasmids constructions details ……... **32**

Generation of *strA* deletion cassette, complementation and tagging protein-encoding gene with *sgfp*, *mrfp* and *ctap* ……..... **32**

Generation of striatin interacting protein encoding gene, *sipA*, *sipB*, *sipC*, *sipD* and *sipE* deletion, complementation, *sgfp* and *ctap* tagging plasmids…….. **43**

Generation of *histone 2A mRFP* fusion strains ……. **47**

Hybridization techniques ……... **47**

Fungal physiology…….. **52**

Phenotypic assays …….. **52**

Stress tests ……. **52**

Protein methods ……. **53**

Immunoblotting ……. **53**

Protein extraction …….. **53**

For Western Blot ……... **53**

Tandem Affinity Purification (TAP) and GFP-TRAP protocol and LC-MS/MS Protein identification …… **54**

Confocal microscopy…….. **54**

Secondary metabolite analysis by Shimadzu Reverse Phase - High Performance Liquid Chromatography (RP-HPLC) ……... **54**

Quantitative real time PCR …… **55**

Statistical Analysis ……. **56**

Chapter 3: Results …….. **57**
Striatin is required for proper fungal development and light response…….. **58**
StrA is highly expressed during early stage of asexual development ….. **60**
The StrA is localized to nuclear envelope and possibly to endoplasmic reticulum …… **60**
Determination of the striatin StrA-interacting proteins (SIPs) …….. **61**
SipA and SipB are abundantly expressed whereas SipC, SipD and SipE are poorly expressed during late fungal development ……. **64**
Reciprocal TAP-LC-MS's of SipA to SipE confirm the presence of STIPAK complex made of StrA-SipA-SipB-SipC-SipD-SipE ……. **65**
All members of STRIPAK complex are required for proper development except for SipA ……... **66**
STRIPAK complex controls expression of master regulators of asexual and sexual development …… **71**
STIPAK complex is involved in response to various environmental stress factors ……. **73**
STRIPAK complex is required for proper expression of reactive oxygen species (ROS) scavenging enzyme-encoding genes ……..… **80**
STRIPAK complex is essential for secondary metabolite production ….. **82**
StrA and SipA to SipE proteins are co-localized to nuclear envelope and endoplasmic reticulum …… **85**
StrA is required for proper subcellular localization of SipA, SipB, SipC, SipD and SipE on nuclear envelope …….. **88**
Lack of Striatin disrupts the interactions of the STRIPAK complex…..... **91**

Chapter 4: Discussion …….. **93**
STRIPAK complex role in fungal light responses and development …... **96**
STRIPAK complex regulates secondary metabolism …….. **97**
Stress responses are governed by STRIPAK complex ……. **99**
SipA has an epistatic role in the control of development and stress responses in STRIPAK complex ……...**100**
References …… **102**
Supplemented Materials ……. **121**

Declaration of Authorship

This thesis has not been previously submitted in whole or part to this or any other University for any other degree. This thesis is the sole work of the author.

Signature:……

Nadia Ali Elramli.

Acknowledgements

I would like to express my sincere appreciation and gratitude to Dr. Özgür Bayram for suggesting the research project, supervision, guidance and encouragement. The invaluable consultation and assistance of Professor Dr. Kevin Kavanagh and Professor Dr. Christine Griffin should be acknowledged with appreciation. I must point out, specifically, to the outstanding efforts of Dr. Özlem Sarikaya Bayram.

I would highly appreciate to the Libyan higher ministry of education and embays Libyan staff for granting all the requirements and facilities that are needed for successful study. Sincere appreciation and best regards ought to go the Benghazi University, to the Faculty of the Science for support to carry on my study.

I want to thank very much my brothers at lab: Mevlut Ulas, Betim Karahoda and Dean Frawley for the assistance and encouragement me in hard periods are appreciated. I would like also to thank everyone in Maynooth Biology Department.

My family, my husband, brothers, sisters and close relatives have been supporting and encouraging me throughout the course of study. I must say to all of them: thanks for everything including your patience….

Abbreviations

aa……. Amino acid

AspGD…… *Aspergillus* Genome Database

AREs……. AbaA Response Elements

bZIP…… Basic leucine zipper transcription factor.

Ca++……..... Calcium ion

C-terminus…….. Carboxy-terminus

CCM3……. Cerebral Cavernous Malformation 3 Protein

CDP…… Central Developmental Pathway

C2H2…….. Cys2-His2 zinc finger

CBP…… Calmodulin binding peptide

DNA …….. Deoxyribonucleic Acid

DEL……..... Deletion

DIC……..Differential interference contrast

DIG……. Digoxigenin

ddH2O……..... double-distilled water or deionized water

DTT……..... DL-dithiothreitol

EDTA……..... Ethylenediamine teraacetic acid

ER…… Endoplasmic Reticulum

ESID…… Extracellular Sporulation-Inducing Factor

EMS……. Ethyl methanesulfonate

GCKIII kinases…… Germinal Center Kinases III

GPCRs……. G-protein coupled receptors

GFP……. Green Fluorescent Protein

GMM…….Glucose Minimal Medium

gDNA……Genomic Deoxyribonucleic Acid

g……....Gram

H2O2…… Hydrogen peroxide

HCl……Hydrochloric Acid

h……Hours

H. sapiens……Homo sapiens

HMG……... High-mobility box genes

HU…….. Hydroxyurea

IgG……...Immunoglobulin G

KDa …… Kilodaltons

kbp…….. Kilobase pairs

L……...Liters

LB……Luria Bertani Broth

LC-MS…….Liquid chromatography, tandem mass spectrometry

MAPK …… Mitogen-activated protein kinase

Min…….. Minute

Mob3…….. .Monopolar Spindle One-binding Proteins

MAT…… Mating type genes

MS……Mass Spectrometry

MMS……Methyl methanesulfonate

mRFP……. Monomeric red fluorescent protein

mM…….. millimolar

mg…… milligram

ml……. milliliter

N. crassa ……. *Neurospora crassa*

NLS……. Nuclear localization signal

N-terminus…….. (NH2) Amino-terminus

natR…… nourseothricin resistance gene

nm…….. nanometer

ORF…… Open Reading Frame

NaCl…… Sodium Chloride

NaOH……. Sodium Hydroxide

P……. Probability value

PP2A…….. Protein phosphatase 2A

pH…….. Potential of hydrogen

PRO……... PRO genes

PCR…… Polymerase chain reaction

PMSF……. Phenylmethylsulfonyl fluoride

PN……. ß-lactam Antibiotic Penicillin (Penicillin)

ptrA…… Pyrithiamine resistance gene A

qRT-PCR……....Quantitative real-time polymerase chain reaction

qPCR…….....Quantitative polymerase chain reaction

RT…….. Room Temperature

RP-HPLC……Reversed phase-high performance liquid chromatography

ROS……..……. Reactive Oxygen Species

RNA…….. Ribonucleic acid

SG2NA…….... Striatin G2 nuclear autoantigen

STRIPAK……... Striatin-Interacting Phosphatases and Kinases complex

STRIP1/STRIP2…….. Striatin-Interacting Proteins

STK24…….. Striatin Kinase24

STK25……. Striatin Kinase25

SLMAP…….Sarcolemmal-Membrane-Associated Protein

SIKE……..Suppressor of IkB kinase ε

SR……..Sarcoplasmic Reticulum

SIP complex …….Septation initiation complex 2 Inhibitory PP2A

SMs……Secondary metabolites

ST……..Sterigmatocystin

Sips……Striatin A interacting proteins

strA……... Striatin

SDS…….. Sodium dodecylsulfate

TFA……..Tri Fluoroacetic Acid

TAP……..Tandem Affinity Purification.

TQ……Antitumour Polyketide Terrequinone (Terrequinone)

TORC2……... Target Of Rapamycin Complex 2

TBS…….. Tris buffered saline

TBST…… Tris buffered saline tween

TAP-MS…….. Tandem Affinity Purification- Mass Spectrometry

UV…….Ultraviolet radiation

UDAs……... Upstream Developmental Activators

UTR……..... Untranslated region.

v/v……..... Volume per volume.

WT…….. Wild-type.

µm…….. micrometer

µg …….. microgram

Δ……. deletion

N	D e s c r i p t i o n	Page
2.1	Fugal strains used in this study.	28
2.2	Oligonucleotides for Plasmids employed in this study.	34
2.3	Plasmids employed in this study.	41
3.1	Confirmation of STRIPAK complex by reciprocal TAP-LC-MS	66
3.2	Confirmation of the interaction between StrA and Sip-complex in (A) presence or (B) absence StrA.	92

N	D e s c r i p t i o n	Page
1.1	The molecular structure of the striatin family proteins	3
1.2	The mammalian STRIPAK complex including a range of accessory proteins.	8
1.3	Subunits of STRIPAK complexes in other organisms including fungi.	10
1.4	Regulation of asexual and sexual development in A. nidulans	18
1.5	(A) General organization of the STRIPAK complex in eukaryotes, (B) Model of the STRIPAK complex in S. macrospora.	22
2.1	Generation of single and double deletion combinations of sip genes.	49
2.2	Generation of endogenously expressed striatin interacting protein (sip) sgfp and ctap fusions.	51
3.1	Growth, expression pattern and subcellular localization of StrA.	59
3.2	Discovery and expression levels of StrA interacting proteins (Sips) during fungal development.	63
3.3	Role of STRIPAK complex in Light dependent fungal development.	69
3.4	Complementation of SIP deletion strains.	70
3.5	Expressional analysis of asexual and sexual development regulatory genes by qRT-PCR.	72
3.6	Growth responses of the STRIPAK complex mutants in the presence of reactive oxygen species (ROS) and cell wall stress.	75
3.7	Growth responses of the STRIPAK complex mutants in the presence of DNA damaging stressors.	77
3.8	Comparison of the radial growth rates of STRIPAK mutants that were exposed to amino acid starvation and osmotic stress agents.	79
3.9	Levels of reactive oxygen scavenging (ROS) enzyme- encoding genes in STRIPAK mutants by q-RNA-PCR.	81

3.10	Function of STRIPAK complex in secondary metabolite production.	84
3.11	Co-localizations of Sip proteins and Striatin in nuclear envelope and endoplasmic reticulum.	87
3.12	Expression and subcellular localization of Sip-GFP fusions in the absence of functional striatin.	90
4.1	Current model of STRIPAK complex and its functions in *A. nidulans*.	95

Summary

Striatin is a highly conserved eukaryotic protein first discovered in neuron cells of mammals. It is involved in many molecular processes, including cellular signaling, cell division and development. It has been shown in several fungi that striating complexes are important for development. Fungal development and secondary metabolism (SM) requires regulatory complexes which are under control of the environmental signals such as light, CO_2 and pH . *Aspergillus nidulans* is a model for eukaryotic systems as well as fungal development and SM. It produces more than forty identified compounds with potent biological activities. Function of the striatin gene, *strA* was previously shown to influence development in *A. nidulans*. However, detailed molecular function of StrA in fungal development as well as SM production is still unknown. This study focuses on the interactome of striatin protein by using tandem affinity purification (TAP) and green fluorescent protein (GFP) tags in *A. nidulans*. Five proteins interacting or associated with StrA protein were identified TAP and GFP-TRAP by using mass spectrometry (MS). They were named as StrA interacting proteins (Sips), SipA, SipB, SipC, SipD and SipE. The single and double deletion combinations of all *sipA*, *sipB*, *sipC*, *sipD* and *sipE* genes led to serious developmental as well as SM defects in *A. nidulans*. Localization and pull-down experiments suggest that StrA is required for the full assembly of the functional STRIPAK complex at the nuclear envelope. Furthermore, assembly dynamics of the STRIPAK complex was also determined. StrA interacts with SipA constantly during all developmental stages. However, SipB-SipD and SipC-SipE are found as heterodimers, which later assemble to StrA-SipA core heterodimer, which constitute the hexameric STRIPAK complex. This study determines the presence of STRIPAK complex by biochemical, genetic and cell biological methods, which is required for light responses, fungal fruit body formation, asexual conidiation and secondary metabolite SM production.

1.Introduction

1.1. Signalling pathways co-ordinate characteristic responses to stimuli:

All organisms, including fungi, have a strong relationship with their environment (Kuck et al., 2016) and possess the capability to sense and adapt to a myriad of intra- and extracellular stimuli (Irniger et al., 2016). In fungal species, upon detection of a stimulus, many underlying regulatory complexes can be activated that, in turn, contribute to the coordination of fungal development and secondary metabolite production (Bayram et al., 2016). Regulatory factors present within fungal hyphae are responsible for promoting stage-specific growth and differentiation via distinct protein-protein interactions (Etxebeste et al., 2010). Signalling pathways that regulate morphological and physiological processes in response to stimuli are often highly conserved throughout eukaryotes, signifying their importance (Kuck et al., 2016). Striatin is one of the regulatory proteins that is proposed to act as a signalling hub in response to environmental stimuli and for control of many cellular processes including development, cellular transport and signal transduction, which is a research topic of this study.

1.2. Striatin

1.2.1. The Structure of Striatin

Striatin is a protein of approximately 100-kDa that consists of 780-amino acids (Castets et al., 1996). Each of the 3 members of the striatin family has identical protein interaction domains (Gaillard et al., 2006). As can be seen in **Figure 1.1**, The C-terminus of striatin contains 6 tryptophan-aspartic acid dipeptide (WD) repeats which are characteristic of proteins involved in signal transduction (Neer et al., 1994). WD-repeat proteins have a characteristic series of 4-8 conserved repeated units. These units are stretches of 23-41 amino acids, which usually start with the amino acids

glycine and histidine (GH) and terminate with tryptophan and aspartic acid (WD). In between these amino acid pairs are variable short sequences (Neer et al., 1994). As a result, striatin is classified as a WD-repeat family member. These WD repeats occur at the following amino acid positions: 461-500, 514-553, 567-606, 662-701, 704-743 and 750-779.

Striatin contains several domains, other than the WD-repeat domain, that allow for protein-protein interactions (**Figure 1.1**). These domains span across the N and C-termini and are as follows: a caveolin-binding motif (amino acid position 55-63), a coiled-coil domain (amino acid position 53-120) and a calmodulin-binding site (amino acid position 149-166) (Castets et al., 1996; Bartoli et al., 1998). The caveolin-binding domain is essential for binding the internal membrane protein known as caveolin. In mammals, this protein is found to be enriched in caveolae (Rothberg et al., 1992), which are vesicles that enable the transport of various molecules across endothelial cells (Anderson, 1998). Caveolae are classified as lipid rafts. Proteins and lipids can aggregate in these rafts and are then capable of transmitting amplified signalling cascades (Boscher and Nabi, 2012). It is also evident that caveolae can function in the exocytic and endocytic pathways as carriers and their activity may be linked to changes in the plasma membrane (Parton and Simons 2007). Each caveola is surrounded by a striated coat (Rothberg et al., 1992) and caveolin acts as the basic building blocks for the filaments that compose the coat structure (Fernandez et al., 2002). Aside from this, caveolins also act as scaffold proteins and are capable of interacting not only with lipid rafts but with a wide range of signaling proteins like G-protein coupled receptors (GPCRs) and serine-threonine kinases, to name a few (Gaillard et al., 2001).

Figure 1.1. The molecular structure of the striatin family proteins. From the N-terminus to the C-terminus, the following protein domains are present in striatin family members: Brown square represent Caveolin-binding motif (55-63aa), Green square; Coiled-coil domain (53-120aa), Yellow square, Calmodulin binding site (149-166aa) and Red squares represent 6 WD-40 repeats (461-500, 514-553, 567-606, 662-701,704-743 and 750-779aa). Modified and redrawn from (AspGD.org, String-db.org).

The coiled-coil domain exists in between the caveolin-binding motif and the calmodulin-binding site and consists of 7 repeats of 7 amino acid residues (Gaillard et al., 2006). This domain is a highly conserved motif (94% identity), which suggests that it serves a crucial function(s) (Castets et al., 2000). A study by (Gaillard et al., 2006) showed that the coiled-coil domain of striatin proteins in mammals is critical for the localisation of striatin to dendritic spines. Striatin proteins that do not possess a coiled-coil domain accumulate at the peri-nuclear region. Many scaffold proteins, including striatin family members, homo/hetero-multimerise and utilise the coiled-coil domain for this purpose. Coiled-coils are the most frequent oligomerisation domains known in proteins (Lupas, 1996; Yun et al., 2002; Kammerer, 1997).

The calmodulin binding site allows for the binding of the calcium sensor protein calmodulin (Gaillard et al., 2001). Calmodulin acts as a sensor for intracellular calcium ion (Ca^{2+}) levels and also functions as a secondary messenger, interacting with a myriad of proteins (Sorensen et al.,

2013). In humans, calmodulin is capable of binding to at least 300 targets (Yap, 2000; Halling et al., 2016). The functions of calmodulin are diverse and are crucial for survival, highlighted by its evolutionary conservation. Roles of this protein include influencing cardiac excitation-contraction coupling via the modulation of specific proteins (Tang et al., 2002) and regulating cardiovascular ion channel activity (Pitt, 2007), to name a few.

1.2.2. The Localization of Striatin

In mammals, striatin can be found in a few subsets of neurons and is equally distributed in the cytosol and membrane fraction (Castets et al., 1996; Salin et al., 1998; Bartoli et al., 1998; Gaillard et al., 2001). It is present in high abundance in brain regions like the spinal cord, the striatum and in dendritic spines, which are compartments at the postsynaptic regions of excitatory synapses (Neer et al., 1994). Striatin was first isolated, in 1996, from a rat brain synaptosomal fraction (Castets et al., 1996). Apart from localising to the brain tissue, striatin is also expressed in cardiac tissue; specifically localising to the cardiac desmosomes which are adhesion molecules that function in the heart, skin and the cornea of the eyes (Stern et al., 2015; Moqrich et al., 1998; Gaillard et al., 2006; Meurs et al., 2010).

In fungi, striatin is present and functions in a homologous STRIPAK complex, which will be discussed later. Depending on the species of fungus and the specific functions it performs, striatin will have different localisations. Striatin in the fungus *Neurospora crassa* has been shown, via biochemical, genetic and cell imaging techniques, to localise to the nuclear envelope when associated with the STRIPAK complex. This allows for the complex to be involved in the regulation of nuclear import of the MAP kinase MAK-1 (Dettmann et al., 2013).

1.2.3. The Functions of Striatin

Striatins are proteins that contain an array of protein interaction domains and as a result, act as scaffolds. Because of these particular motifs, striatins are capable of participating in numerous protein-protein interactions, assembling proteins in distinct signalling complexes and regulating signal transduction downstream of these complexes (Hwang and Pallas 2014). Early studies showed that striatin proteins are capable of acting as scaffolds in a range of animal and fungal species (Wang et al., 2010). The protein-protein interactions that striatin are involved in may be mediated via calcium ion fluxes as striatin binds calmodulin when Ca^{2+} ions are present (Salin et al., 1998). It has also been shown that calcium ions influence the subcellular distribution of striatin, likely via calmodulin interactions (Bartoli et al., 1998). The presence of WD repeats in striatin; coupled with its interactions with calmodulin, provide evidence that striatin may be involved in calcium-dependent signalling (Bartoli et al., 1998).

1.2.4. The Striatin Family Complex

The striatin family complex is a small group of eukaryotic proteins that exists in fungi and mammals but is not present in plants and budding yeast (Benoist et al., 2006). In mammals, 3 scaffold proteins make up the striatin family. These proteins are striatin (STRN), S/G2 nuclear autoantigen (SG2NA/STRN3) and zinedin (STRN4) (Benoist et al., 2006). All 3 share the same overall structure and possess identical protein interaction domains. By performing phylogenetic analysis of the striatin family, it was found that they make up a multigenic family that is derived from a single ancestral gene (Castets et al., 2000). The SG2NA sequence is 80% homologous to and 66% identical to striatin sequences (Castets et al., 1996). A study by Tanti (2014) analysed the conserved features of striatin throughout evolutionary history. It was found that a bacterial motif known as pCM-I is

present in SG2NA. This led to the hypothesis that the striatin family members may have evolved from prokaryotic organisms and that SG2NA was the first member of the striatin family to arise (Tanti et al., 2014). The expression of the striatin family members occurs during the S to G2 phase of the cell cycle (Muro et al., 1995; Soni et al., 2014). In mammals, the striatin family is thought to be important for brain function due to their expression in both the central and peripheral branches of the nervous system. However, they are also highly expressed in other tissues and so, they may possess an array of biological functions (Hwang and Pallas 2014, Benoist et al., 2008).

1.3. The STRIPAK complex

The striatin-interacting phosphatases and kinases complex (STRIPAK) is a large multi-protein complex that is highly conserved in eukaryotes. It was originally identified in mammals via mass spectrometric analysis (Goudreault et al., 2009), and has been found to have an influence on mammalian cell size, morphology and migration (Hwang and Pallas 2013). It also plays a role in the polarisation of the golgi apparatus and is implicated in the process of mitosis, through tethering vesicles of the golgi to the nuclear membrane and centrosomes (Kean et al., 2011). The mammalian STRIPAK complex consists of a multitude of core members and a variety of further associated proteins, which can be seen in **Figure 1.2**.

Core proteins include (i) Striatins (ii) Striatin-interacting proteins (STRIP1/STRIP2), (iii) Mob3/phocein (monopolar spindle one-binder (Mob) protein) which plays a role in vesicular trafficking (Baillat et al., 2001), (iv) CCM3 (cerebral cavernous malformation 3 protein), (v) Protein phosphatase 2A (PP2A) and the PP2A subunits, termed PP2AA and PP2Ac,

which have structural and catalytic functions respectively. The roles of PP2A will be discussed in later sections. Further associated proteins include multiple GCKIII kinases, such as STK24, STK25 and MST4. These are germinal centre kinases that belong to the STE20 kinase family (Goudreault et al., 2009; Kuck et al., 2016). The germinal centre kinases have recently been discovered to be involved in the control of the cell cycle, polarity and migration (Preisinger et al., 2004; Cornils et al., 2011) and their functionality is reliant on CCM3 which is involved in stabilising the kinases (Fidalgo et al., 2010).

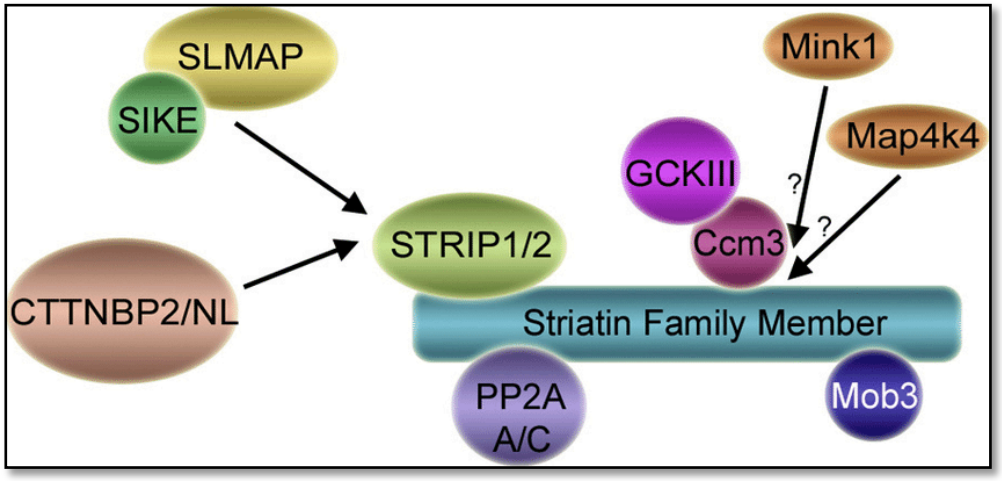


Figure 1.2. The mammalian STRIPAK complex including a range of accessory proteins (Goudreault et al., 2009)**.** The core members of this complex have been discussed (see text). Interacting partners of the STRIPAK complex include SLMAP, SIKE, CTTNBP2/NL and kinases. These associated proteins can vary, generating a multitude of potential combinations for this complex. Kinases for the STRIPAK complex include those listed in the text above as well as Map4k4, Mink1 and Tnik.

1.3.1. The STRIPAK accessory protein SLMAP

The STRIPAK complex is capable of interacting with accessory proteins, such as SLMAP (Sarcolemmal-Membrane-Associated Protein) and SIKE (suppressor of IkB kinase ε) (Hwang and Pallas 2014). SLMAPs are a family of tail-anchored membrane proteins that are expressed at specific developmental stages and in specific tissues (Wigle et al., 1997; Wielowieyski et al., 2000). Studies have shown that SLMAP expression occurs early during skeletal myogenesis and that this protein localises within specialised membrane systems that are implicated in calcium signalling. As a result, it is proposed that SLMAP may be involved in membrane function and myoblast fusion (Guzzo et al., 2004). Other locations for SLMAP include compartments within the cardiomyocytes like the sarcolemma, the sarcoplasmic reticulum/endoplasmic reticulum (SR/ER) and the transverse tubules (Guzzo et al., 2004; Guzzo et al., 2004(b)). Other studies have shown that SLMAP plays important roles in excitation-contraction coupling and centrosomal organisation (Guzzo et al., 2004(b); Byers et al., 2009). While all of the members and interaction partners listed above are well defined for the mammalian STRIPAK complex, it is likely that other proteins interact with this complex either directly or indirectly.

1.3.2. Evolutionary conservation of the STRIPAK complex

It has been shown that STRIPAK complexes exist in a wide variety of eukaryotes from fungi to humans, as is evident in **Figure 1.3.** The dSTRIPAK complex in fruit fly *Drosophila melanogaster* acts as a negative regulator of the Hippo signalling pathway (Ribeiro et al., 2010). In *Saccharomyces cerevisiae*, the homologous complex is termed the Far complex and this is involved in the mating process. It is implicated in cell cycle arrest and acts as an antagonist towards TORC2 (Target Of

Rapamycin Complex 2) signalling (Kemp and Sprague, 2003; Pracheil et al., 2012). In the fungus *Schizosaccharomyces pombe,* the STRIPAK complex is known as the SIP complex [SIN (septation initiation complex 2) Inhibitory PP2A]. This complex is necessary for coordinating mitosis and cytokinesis (Singh et al., 2011). In *Neurospora crassa,* the STRIPAK complex is involved in the nuclear accumulation of MAK-1, which is a mitogen-activated protein kinase (MAPK), and in turn, this complex is implicated in regulating chemotropic interactions between conidia (Dettmann et al., 2013).

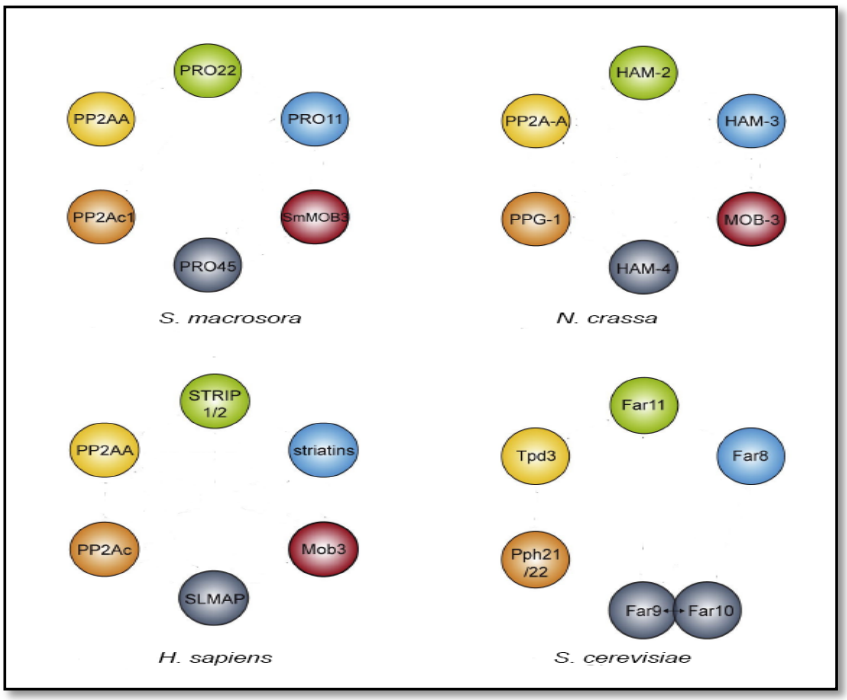


Figure 1.3. Subunits of STRIPAK complexes in other organisms including fungi. The core components for the *H. sapiens* STRIPAK complex have been discussed in **Section 1.3.** The homologous proteins are colour-coded in *N. crassa, S. macrospora* and *S. cerevisiae* (Kuck et al., 2016).

In the ascomycetous model fungus *Sordaria macrospora*, a functional homologous STRIPAK complex has been recently discovered and characterized via Tandem Affinity Purification (TAP) coupled with Mass Spectrometry (MS). The subunits of this complex are involved in the regulation of sexual development, hyphal fusion and vegetative growth (Bloemendal et al., 2012; Dettmann et al., 2013; Poggeler and Kuck 2004). This complex will be discussed in more detail in later sections.

1.4. Fungi

1.4.1 The Fungal Kingdom

Fungi are eukaryotic organisms that share a common ancestor with animals. Their divergence from that ancestor is estimated to have occurred roughly 1 billion years ago, plus or minus 500 million years (Berbee and Taylor, 2010). The fungal kingdom is diverse and various estimations have been proposed to calculate the number of species that belong to this group. These numbers have reached as high as 5.1 million species (O'Brien et al., 2005). Members of the fungal group include yeasts, mushrooms and moulds along with a range of commonly used model organisms like *N. crassa*, *S. cerevisiae* and *Aspergillus nidulans,* to name a few (Blackwell, 2011).

Many known fungal species are significant for industrial, health and research purposes. In industry, specific species of fungi are used to produce beers, wines and bread. With regards to health, certain fungi have the capability to produce a wide range of metabolites, which can be beneficial or harmful to humans. Examples of metabolites include penicillin, cyclosporine and fusidic acid (Brakhage 2004, Brakhage 2006). While many fungi are not harmful to humans, the production of certain metabolites enables specific species to cause disease. *Aspergillus fumigatus,* which

produces the secondary metabolite gliotoxin, is an example of a fungal species that is capable of causing systemic infections in immunocompromised individuals (Jarvis et al., 1996, Annamaria et al., 2000). Fungi can also cause significant disease in plants, resulting in dramatic declines in crop yields annually (Knogge 1996). *Aspergillus flavus* is an example of a fungus that infects crops like maize, rice and peanuts (Fakruddin et al., 2015) and produces secondary metabolites known as aflatoxins. With respect to research, there are multiple fungal species that are commonly used as model organisms to gain insight on areas like genetics, pathogenesis and cell biology. In the next section, the model organism *A. nidulans* will be discussed in detail.

Fungi have the ability to adapt to many different environments, such as wet and dry soils, and also fresh and saline water. Fungi exhibit a range of morphologies, ranging in shape and size, from microscopic yeast to visible mushrooms. Fungi are heterotrophic, and depend on other organisms and their environment to obtain their carbon sources necessary for growth. Fungi possess cell walls to protect themselves from the environment and to serve as a signalling centre. These cell walls are not homologous to plant cell walls and consist of an array of polysaccharides like chitin and glucan, along with various glycoproteins (Bowman 2006, Latgé 2007).

1.4.2 *Aspergillus nidulans*, a model organism for fungal development and secondary metabolism

The filamentous fungus *A. nidulans* belongs to the Phylum *Ascomycota*. It is a commonly used model organism in research and has helped provide extensive knowledge of fungal morphology, cell biology and genetics. *A. nidulans* has a number of characteristics that enable it to be efficiently used as a model. It possesses a haploid genome, and so, contains only one copy

of each of its genes. As a result, gene deletion studies can be easily performed, unlike in diploid organisms (Galagan et al., 2005, Bayram & Braus, 2012). Another feature of *A. nidulans* is that it can be cultured with ease in laboratory conditions and is non-pathogenic, minimizing health risk to researcher. This fungus is capable of reproducing by asexual and sexual means. This has allowed for detailed studies to be performed on the methods of reproduction in fungi (Casselton and Zolan, 2002) and has led to the characterization of a wide range of genes and proteins involved in each response.

A. nidulans is capable of undergoing three different methods of reproduction, depending on it's surrounding environmental conditions. Different stimuli from the environment trigger genetically determined developmental states which lead to the creation of specialized structures for vegetative, sexual or asexual growth (Bloemendal et al., 2012).

1.4.2.1. Vegetative growth in *A. nidulans*

Vegetative growth begins when a spore of *A. nidulans* receives sufficient nutrients and begins to establish polarity, leading to it's germination in a fixed direction (Oiartzabal-Arano et al., 2016; Garzia et al., 2009). Germination can be affected by a variety of factors, with light being a major influence (Rohrig et al., 2013). Continued polarized growth leads to the formation of a germ tube which extends via the continuous transport of materials for cell wall formation to the hyphal apex (Riquelme, 2013; Takeshita et al., 2014). As the hyphae extend, branches can occur where a new point of polarity is defined and hyphae extend in different directions from the original hypha. This ultimately leads to the development of a network of cells that are interconnected and are collectively termed the mycelium (Oiartzabal-Arano et al., 2016; Garzia et al., 2009).

1.4.2.2. Asexual development in *A. nidulans*

When vegetative growth is completed, *A. nidulans* becomes competent for the detection of an array of environmental signals, which can influence whether this fungus undergoes asexual or sexual reproduction. These modes of growth are summarised in **Figure 1.4**. Asexual development can be triggered by a combination of external and internal stimuli like light, stress and metabolites (Etxebeste et al., 2010; Rodriguez-Urra et al., 2012). The transition from vegetative to asexual growth is characterized by the emergence of a specialized thick-walled cell, known as a foot cell, from the vegetative hypha. This foot cell begins to elongate upwards to form a structure known as the stalk. As this stalk grows, the tip begins to swell, producing the multinucleate vesicle. This vesicle then generates structures on its surface termed the metulae, which are finger-like in appearance and emerge due to repeated nuclear divisions. These metulae continue to grow and eventually bud to produce phialides, which are the uttermost structures, giving rise to long chains of mitotic asexual spores (conidia), by means of repeated asymmetric mitotic divisions. These conidia are haploid cells, capable of disseminating via the air and germinating to produce new colonies (Adams et al., 1998; Park and Yu, 2012; Etxebeste et al., 2010; Oiartzabal-Arano et al., 2016; Garzia et al., 2009).

The regulation of conidiophore development is governed by genes belonging to the central developmental pathway (CDP) (Adams et al., 1998; Park and Yu, 2012). The first CDP gene is *brlA,* which encodes a C_2H_2 transcription factor responsible for the control of several early developmental genes like *yA, abaA, wetA and rodA* (Boylan et al., 1987; Timberlake and Clutterbuck, 1994). *brlA* is required for formation of the foot cell and stalk structures and when *brlA* is deleted, strains exhibit aerial hyphae with bristle-like appearances, rather than conidiophores (Park and

Yu, 2012). Transduction of environmental signals that promote asexual reproduction to the *brlA* promoter is governed by upstream developmental activators (UDAs), which encode various transcription factors and signalling elements. These UDA genes are collectively known as *fluffy* genes, as the UDA mutants exhibit cotton-like colony phenotypes due to the accumulation of undifferentiated masses of vegetative hyphae (Etxebeste et al., 2010; Cortese et al., 2011; Wieser et al., 1994; Weiser et al., 1997).

The UDA FluG is essential for commencing conidiation. It is necessary for the production of an extracellular sporulation-inducing factor (ESID) (Lee and Adams, 1994). FluG inhibits vegetative growth by activating the UDA FlbA. This protein regulates G-protein signalling and inhibits the G-protein complex required for vegetative growth (Yu et al., 1996). FluG also activates two pathways that mediate asexual development. These involve the UDAs FlbB, FlbC, FlbD and FlbE. FlbC is a C_2H_2 transcription factor that binds to the regulatory elements of *brlA* and, together with the FlbB/FlbD/FlbE pathway, activates *brlA* expression (Kwon et al., 2010; Garzia et al., 2010).

FlbB, a basic leucine zipper (bZIP)-type transcription factor, localizes to polarized germling tips and nuclei in mature hyphae (Etxebeste et al., 2008). Localisation and stability of FlbB at the hyphal tip is influenced by a second UDA, FlbE. FlbB contains a dimerization domain, which allows it to interact with FlbE (Wieser et al., 1994; Garzia et al., 2009; Herrero-Garcia et al., 2015). FlbB migrates to the nucleus with FlbE and accumulates due to the presence of a bipartite nuclear localization signal (NLS) which exists in close proximity to it's bZIP domain (Herrero-Garcia et al., 2015). When FlbB enters the nucleus, it induces expression of FlbD, which is also a UDA transcription factor. The activation of FlbD is critical

for inducing *brlA* expression and both FlbB and FlbD bind to target sites of the *brlA* promoter (Garzia et al., 2010).

brlA is required for the activation of the *abaA* gene during the middle stages of asexual development. *abaA* is critical for the differentiation of phialides and mutants are defective in conidia formation (Sewall et al., 1990; Andrianopoulos and Timberlake, 1994). *abaA* possesses a DNA binding motif known as an ATTS/TEA motif, as well as a leucine zipper. *abaA* binds to cis-acting AbaA Response Elements (AREs), which commonly reside in the promoters of a variety of genes involved in development (Andrianopoulos and Timberlake, 1994; Boylan et al., 1987; Aramayo and Timberlake, 1993). *brlA* also induces expression of the *wetA* gene during the late stage of asexual development. *wetA* is essential for the production of the cell walls of conidia, which allow conidia to mature and to become impermeable and is also required for activating genes that have spore specific functions (Sewall et al., 1990; Marshall and Timberlake, 1991). The *brlA, abaA* and *wetA* genes are considered to function in co-operation to coordinate gene expression, ultimately leading to conidiophore production and spore maturation (Boylan et al., 1987; Mirabito et al., 1989).

1.4.2.3. Sexual development in *A. nidulans*

The minority of *Aspergillus* species (36%) undergo a sexual cycle of reproduction (Dyer and O'Gorman, 2011). 2 mating type (*MAT*) genes are required for sexual reproduction to proceed. These genes can belong to either the MAT1-1 family, which encode an α-domain protein, or the MAT1-2 family, which encode a high-mobility (HMG) box protein (Turgeon and Yoder, 2000). These transcription factors determine sexual identity and regulate expression of the pheromone signaling system (Czaja *et al*., 2011; Kronstad and Staben, 1997). Fungi can be either heterothallic

or homothallic, possessing one or both mating type genes respectively (Yun *et al.*, 2000). Both mating types will produce a particular pheromone that is detected by the opposite mating type, allowing for signal transduction via MAPK cascade activation, ultimately resulting in the induction of mating (Lengeler *et al.,* 2000; Banuett, 1998). *A. nidulans* is a homothallic fungus and so is capable of, but not restricted to, sexual reproduction by means of self-fertilisation. This process is termed 'selfing' and entails that both nuclei are derived from the same fungus (Paoletti *et al.,* 2007; Turgeon, 1998; Yun *et al.*, 2000). It is proposed that this is a method that has evolved to ensure reproduction when mating partners are scarce and to maintain particular genotypes (Nasrallah *et al*., 2004; Shimizu *et al*., 2004).

Aspergilli reproduce sexually by producing sexual spores (ascospores), which are contained within fruit bodies known as cleistothecia. Cleistothecia formation is preceded by the aggregation of hyphae, forming a structure termed a 'nest'. Two hyphae fuse in the nest to generate dikaryotic heterokaryons, which lead to the construction of the 'crozier' structures. Two nuclei are trapped in the topmost crozier cell and fuse to produce a zygote. This zygote then undergoes meiotic and mitotic divisions, ultimately resulting in a sac-like structure, known as an ascus, containing 8 ascospores. (Braus et al., 2002; Champe et al., 1994; Sohn & Yoon, 2002). Mature cleistothecia can harbour up to 100,000 asci and in *A. nidulans,* a single cleistothecium can contain 80,000 ascospores on average (Pontecorvo, 1953; Braus et al., 2002). Cleistothecia are surrounded by several hundred Hulle cells which are large, thick-walled cells that exhibit laccase and chitin synthase activity. It is proposed that Hulle cells assist the cleistothecia during development (Sarikaya Bayram et al., 2010; Bayram & Braus, 2012; Wei et al., 2001)

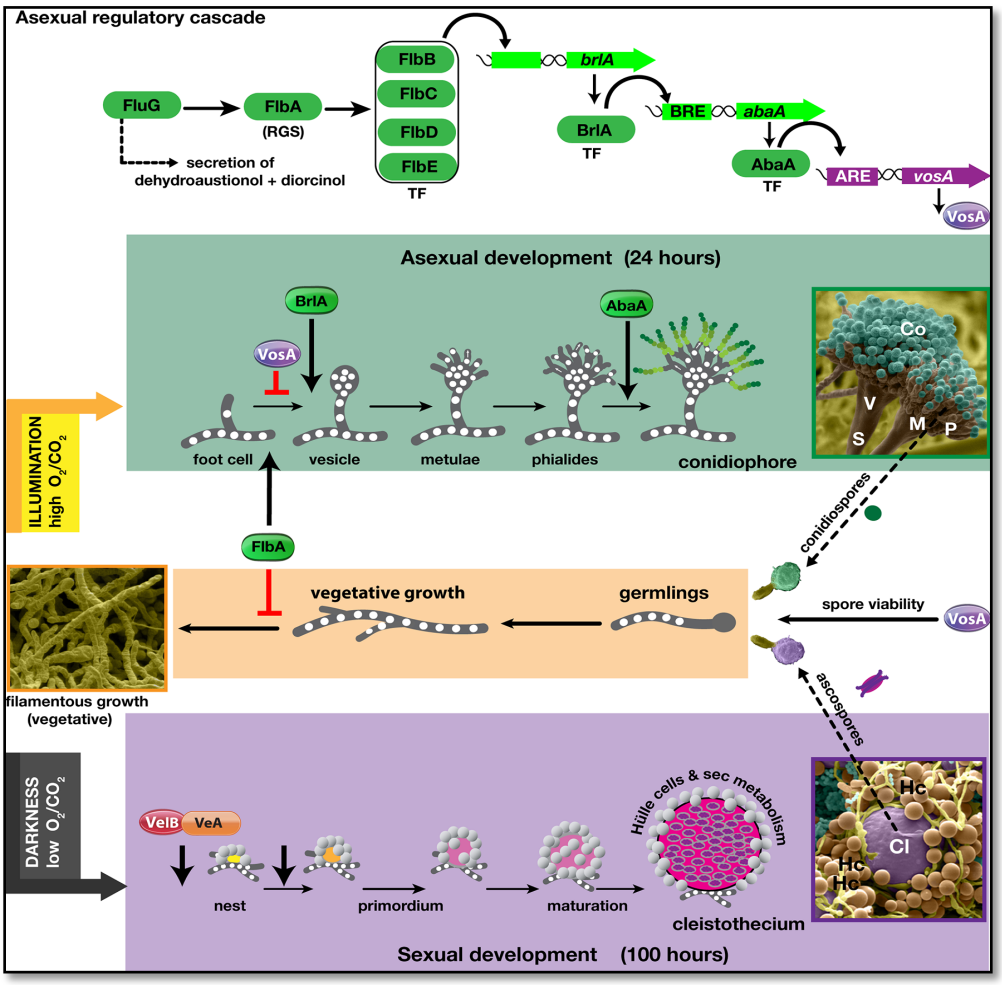


Figure 1.4. Regulation of asexual and sexual development in *Aspergillus nidulans*. In the presence of light, different types of light receptors and a transcription factor cascade triggers asexual reproduction, producing haploid spores that are clones of the original fungus. These spores can be dispersed and undergo vegetative growth to colonise new areas. In the absence of light, this fungus undergoes sexual reproduction and secondary metabolism, transducing signals via the velvet complex. Sexual structures known as cleistothecia are produced, which contain meiotically formed sexual spores, termed ascospores. These spores have undergone genetic mixing and are capable of growing vegetatively to colonise new areas (Sarikaya-Bayram O, 2015).

Factors that induce/repress sexual development are diverse. Examples include growth medium compositions (pH, nutrient content, carbon/nitrogen ratio, amino acid sources), various atmospheric gases, temperature and light (Dyer and O'Gorman, 2011). The presence/absence of light is often considered a major factor for sexual development regulation. In *A. nidulans,* the sexual cycle can precede in both light and dark conditions, however, the presence of light delays the cycle by roughly 15 hours (Yager, 1992). Signal transduction of the presence/absence of light is conducted via the velvet complex, which is critical for the coordination of fungal development and secondary metabolism (Bayram and Braus, 2012).

The *velvet* protein VeA is the founding member of the *velvet* complex. This protein exhibits increased expression during sexual development and it's transport to the nucleus is inhibited in the presence of light (Kim et al., 2002; Stinnett et al., 2007). It is also known that VeA negatively regulates asexual development (Mooney and Yager, 1990). VeA deletion strains are unable to produce any fruit bodies even in favourable conditions, while overexpression of VeA results in constitutive formation of cleistothecia in both the presence and absence of light (Kim et al., 2002). Other members in the *velvet* family include VelB, VosA and VelC. VelB (velvet like B) interacts with VeA to regulate development and secondary metabolism in the absence of light (Bayram et al., 2008). VosA (viability of spores A) interacts with VelB and negatively regulates asexual development (Ni and Yu, 2007; Sarikaya Bayram et al., 2010). This protein is involved in trehalose accumulation in asexual and sexual spores and is proposed to influence mycotoxin production (Ni et al., 2010). VelC interacts with VosA and positively regulates sexual development (Park et al., 2014).

VelB and VeA interact with the methyltransferase LaeA in a trimeric complex that coordinates secondary metabolism and sexual development in

the absence of light (Bayram et al., 2008). Secondary metabolites (SMs) are small bioactive compounds that are non-essential for fungal development but can have medical and industrial importance. Fungi produce SMs to protect themselves from other organisms like bacteria. VeA initially interacts with VelB in the cytoplasm and the dimer migrates into the nucleus via the aid of the KapA α-importin. In the nucleus, VeA acts as a bridge, connecting VelB to LaeA. VelB and VeA then mediate fruit body formation while LaeA influences Hulle cell production, sexual spore formation and regulation of SM genes (Bayram et al., 2008; Sarikaya Bayram et al., 2010).

LaeA is required for expression of AflR, which is a binuclear zinc finger transcriptional activator that regulates the secondary metabolite gene cluster for the aflatoxin precursor sterigmatocystin (ST) in *A. nidulans*. ST is carcinogenic, capable of causing hepatocellular carcinomas and severe damage to the human liver (Brown et al., 1996; Fernandes et al., 1998). Aside from regulating ST production, LaeA is also critical for the production of the antibiotic penicillin (PN) and the cholesterol-lowering drug lovastatin, to name a few (Bok and Keller, 2004; Bayram and Braus, 2012). In *A. fumigatus,* LaeA has been proposed to be essential for expression of 13 of 22 currently characterized gene clusters (Perrin et al., 2007). Consequently, LaeA has been defined as a global regulator of secondary metabolism in *Aspergilli* (Bok and Keller, 2004).

1.5. The role of the striatin family complex in fungi

1.5.1. The STRIPAK complex in the model fungus *Sordaria macrospora*

As discussed in **Section 1.3.2.**, the STRIPAK complex is highly conserved throughout eukaryotes including fungi. In the model fungus *S. macrospora,*

several proteins have been characterised as being crucial for fruit body formation and have been termed PRO proteins (Poggeler and Kuck 2004; Bloemendal et al., 2010; Engh et al., 2010). One of these PRO proteins, PRO11 is homologous to striatin in humans (Castets et al., 1996; Moqrich et al., 1998). A study by (Bloemendal et al., 2012) identified and characterised a homologous STRIPAK complex in *S. macrospora*, using yeast-two-hybrid analysis, tandem-affinity purification (TAP) and multi-dimensional protein identification technology. This work found that PRO11 interacts with PRO22 (a STRIP homolog that is highly conserved and is necessary for sexual development), SmMOB3 (MOB3 homolog that functions as a kinase activator and is essential for fruit body formation) and SmPP2AA (homolog of the structural subunit of human PP2A) (**See Figure 1.5**). It is shown that this complex is critical for regulating sexual development and cell fusion in *S. macrospora* and it is hypothesised that this complex influences the developmental switch from vegetative to sexual growth.

As mentioned earlier (**see Section 1.3.**), striatin is present in the multi-protein STRIPAK complex in eukaryotic organisms (Bloemendal et al., 2012). Striatin, alongside SG2NA/STRN3, binds PP2A, the serine/threonine protein phosphatase in the STRIPAK complex and acts as a platform to recruit and assemble the C (catalytic) and A (structural) subunits of PP2A (Dettmann et al., 2013).

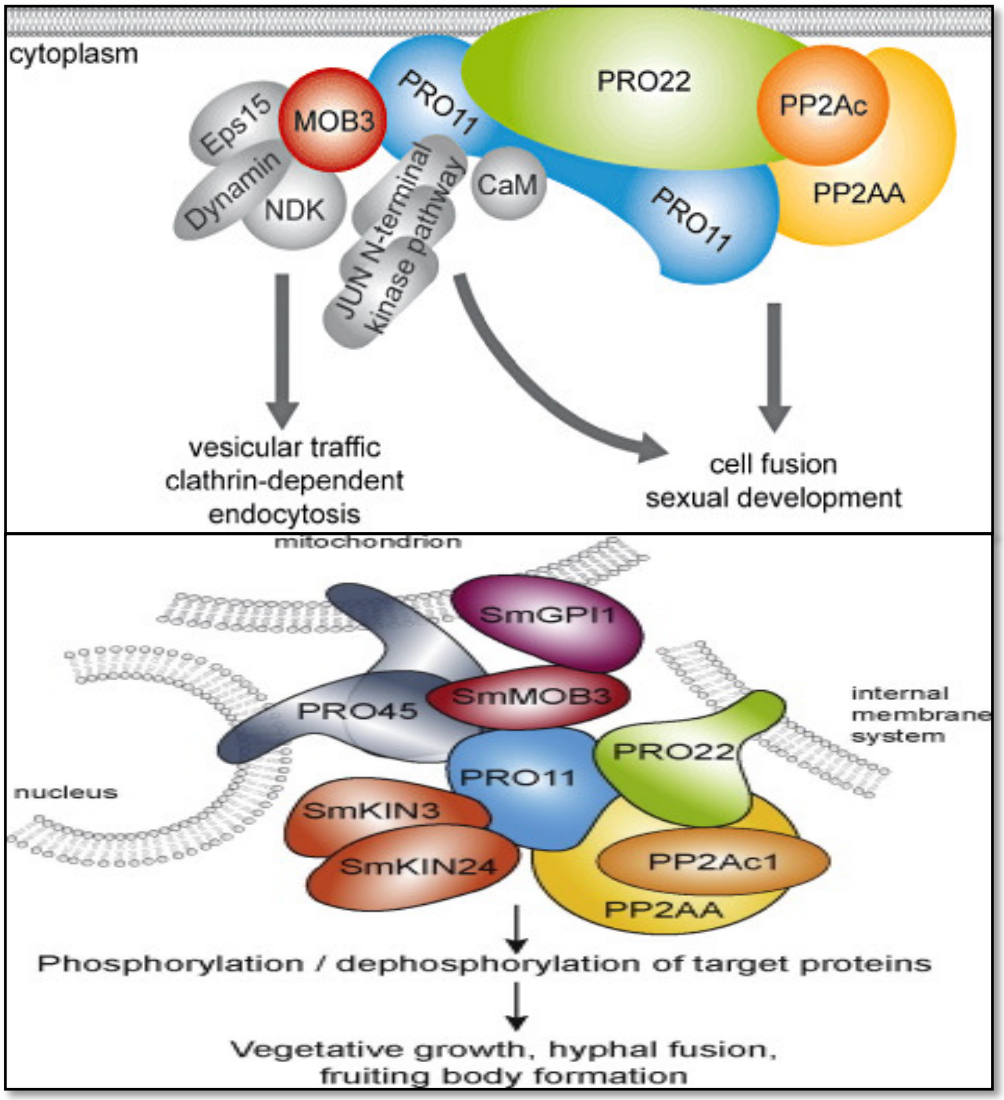


Figure 1.5. General organization of the STRIPAK complex in eukaryotes (upper panel). Anchoring of the striatin scaffold (PRO11) to the membrane via PRO22 (STRIP1/2) interactions allows for the assembly of the STRIPAK complex and mediation of downstream signaling (Bloemendal et al., 2012). **Model of the STRIPAK complex in *S. macrospora* (lower panel)** The STRIPAK complex becomes anchored to internal membrane systems via interactions with membrane proteins like PRO45 (SLMAP) and PRO22. Binding of the striatin scaffold (PRO11) to the phosphatase subunits mediates phosphorylation/dephosphorylation of proteins, influencing processes like vegetative growth and sexual development (Kuck et al., 2016).

Striatin also acts as a regulatory (B-type) subunit, directing PP2A to specific locations and signalling complexes, while also regulating the activity of the catalytic subunit of PP2A with various substrates (Moreno, Park et al. 2000, Kuck, Beier et al. 2016). PP2A is crucial for an array of processes in mammalian cells like development, cell cycle regulation and neuronal signalling (Moreno, Lane et al. 2001). It has also been found that PP2A is involved in the development of certain cancers like lung and colon cancers (Wang et al., 1998).

1.6. The *Aspergillus nidulans* striatin homolog StrA

Orthologs of striatin in filamentous fungi like *S. macrospora* and *A. nidulans* have been shown to be required for perithecium development (Poggeler and Kuck 2004). Perithecia are a type of fruit body that are produced by the Ascomycetes. A study by (Wang et al., 2010) characterised the influence of the striatin ortholog StrA in sexual development in *A. nidulans*. StrA is an 816-amino acid protein that contains each of the 4 domains that are present in human striatin, suggesting that it may also function as a scaffold protein. This work showed that striatin over-expression results in enhanced sexual development. Striatin mutants are still capable of producing cleistothecia containing ascospores, however, they are significantly smaller. It was also found that *strA* mutants exhibit reduced conidiation and delayed germination. Using GFP-tagged strains, it was observed that this protein is found to localise at the endoplasmic reticulum and the nuclear envelope. In *A. nidulans*, a homolog of human PP2Ac (*Anppg1*) has been characterised and mutants were found to display reduced rates of growth and germination, similar to the effects of the *strA* deletion (Son and Osmani, 2009; Wang et al., 2010).

1.7. Aim of this study

Fungal development occurs in response to environmental cues such as light, nutrient levels and stressors. In filamentous fungi, light triggers the induction of asexual conidiation and repression of sexual development. Light does not only control development but also influences production of SMs in *A. nidulans*. Several regulatory complexes have been identified to coordinate fungal development with SM production. Intensive studies of striatin showed that it is one of the signalling platforms that eukaryotes use to respond to environmental signals. Striatin was shown to make a STRIPAK complex in two other filamentous fungi. These species are *N. crassa* and it's closely related species *S. macrospora*, where striatin controls the production of fruit bodies. In *A. nidulans,* it was shown that the *strA* gene influences radial growth of the fungus, maturation of the fruit bodies (cleistothecia), production of sexual spores (ascospores) and pigment secretion. It localizes to the nuclear envelope or nuclear endoplasmic reticulum. It is currently unknown how StrA elicits its effect on fungal development at the molecular level.

This raises the following questions:

(I) Does StrA in *A. nidulans* form a functional STRIPAK complex?

(II) Are the STRIPAK members similar to those found in other eukaryotes and fungi?

(III) Where and how do they interact within the fungal cell?

(IV) Is the STRIPAK complex involved in the production of secondary metabolites and how does it regulate production?

Interatomic studies coupled with mass spectrometry, using functional synthetic green fluorescent protein (sGFP) and tandem affinity purification (TAP) epitope tag versions of StrA, led to the identification of a STRIPAK complex in *A. nidulans*. This complex contains striatin and five proteins termed striatin interacting proteins (SipA to SipE), one of which was not found in other fungi. Single and double mutant combinations of the *strA* gene with *sipA* to *sipE* resulted in developmental defects characterized by the lack of sexually formed ascospores, reduced conidiation, reduced colony diameter and increased sensitivity to various stress conditions.

Furthermore, the STRIPAK complex is involved in the control of fungal secondary metabolism. A single and double deletion combination of *strA* with *sipA* to *sipE* reveals dramatic alterations in the production of secondary metabolites. These results propose that there is a hexameric STRIPAK complex in *A. nidulans* which is made of at least six regulatory subunits, that are all equally important for fungal development and secondary metabolism.

2. Materials and Methods

2.1. Strains, media and growth conditions

Fungal strains used in this study are listed in **Table 2.1**. *A. nidulans* AGB551, which has wild-type *veA* allele, was used as recipient strain for transformation of all deletion and epitope tagging events. Stellar (Clontech) and MACH-1 competent *Escherichia coli* cells (Invitrogen) were used for recombinant plasmid DNA preparations. The wild type and transformed *A. nidulans* strains were grown in Glucose Minimal Media (GMM), supplemented with appropriate amounts of Uridine (0.25g/l), Uracil (1g/l) or Pyridoxine (1mg/l). For vegetative growth, Tandem Affinity Purification (TAP) and Green Fluorescent Protein (GFP) trap experiments, strains were grown in liquid minimal media, with appropriate supplements at 37°C for 6, 12, 24 hours.

For induction of development, vegetative mycelia was then filtered through miracloth and shifted on solid GMM containing 2% Agar. To induce the cultures asexually, the plates containing the mycelia were incubated in the presence of light. For sexual induction, plates were covered by aluminium foil and incubated in the dark. Developmentally induced cultures were grown for 12, 24, 48 hours. *E. coli* strains were grown in LB Broth agar and liquid LB medium supplemented with Ampicillin (100μg/ml) at 37°C overnight.

Table 2.1. Fugal strains used in this study.

Strain	Genotype	Plasmid used	Reference
AGB551	*nkuAΔ::argB,pyrG89,pyroA4,veA+*	not applied	(Bayram et al., 2012)
StrA-GFP-RFP	*gpdA::mrfp::h2A::pyroA;strA::sgfp::natR;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB340 in StrA-GFP	This study
StrA-GFP	*strA::sgfp::natR;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB480 in AGB551	This study
StrA-TAP	*strA::ctap::natR;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB481 in AGB551	This study
StrA-DEL1	*strAΔ::prtA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB525 in AGB551	This study
StrA-DEL2	*strAΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB526 in AGB551	This study
StrA-mRFP	*strA::mrfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB527 in AGB551	This study
ANNE1.1	*sipAΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE1 in AGB551	This study
ANNE1.2	*sipAΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE2 in AGB551	This study
ANNE2.1	*sipBΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE3 in AGB551	This study
ANNE2.2	*sipBΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE4 in AGB551	This study
ANNE3.1	*sipCΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE5 in AGB551	This study
ANNE3.2	*sipCΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE6 in AGB551	This study
ANNE4.1	*sipDΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE9 in AGB551	This study
ANNE4.2	*sipDΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE10 in AGB551	This study
ANNE5.1	*sipEΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE11 in AGB551	This study
ANNE5.2	*sipEΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE12 in AGB551	This study
ANNE1.3	*sipAΔ::AfpyrG;strAΔ::ptrA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE1 in StrA-DEL1	This study
ANNE2.3	*sipBΔ::AfpyrG;strAΔ::ptrA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE3 in StrA-DEL1	This study

ANNE3.3	*sipCΔ::AfpyrG;strAΔ::ptrA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE5 in StrA-DEL1	This study
ANNE4.3	*sipDΔ::AfpyroA;strAΔ::ptrA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE9 in StrA-DEL1	This study
ANNE5.3	*sipEΔ::AfpyroA;strAΔ::ptrA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE11 in StrA-DEL1	This study
ANNE6	*sipAΔ::AfpyrG;sipBΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE4 in ANNE1.1	This study
ANNE7	*sipAΔ::AfpyrG;sipCΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE6 inANNE1.1	This study
ANNE8	*sipAΔ::AfpyrG;sipDΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE10 in ANNE1.1	This study
ANNE9	*sipAΔ::AfpyrG;sipEΔ::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE12 in ANNE1.1	This study
ANNE10	*sipBΔ::AfpyroA;sipCΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE4 in ANNE3.1	This study
ANNE11	*sipBΔ::AfpyroA;sipDΔ::AfpyG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE4 in ANNE4.1	This study
ANNE12	*sipBΔ::AfpyroA;sipEΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE4 in ANNE5.1	This study
ANNE13	*sipCΔ::AfpyroA;sipDΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE6 in ANNE4.1	This study
ANNE14	*sipCΔ::AfpyroA;sipEΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE6 in ANNE5.1	This study
ANNE15	*sipDΔ::AfpyroA;sipEΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE10 in ANNE5.1	This study
ANNE16	*sipA::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE13 in AGB551	This study
ANNE17	*sipB::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE15 in AGB551	This study
ANNE18	*sipC::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE17 in AGB551	This study
ANNE19	*sipD::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE19 in AGB551	This study
ANNE20	*sipE::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE21 in AGB551	This study

ANNE21	*sipA::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE14 in AGB551	This study
ANNE22	*sipB::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE16 in AGB551	This study
ANNE23	*sipC::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE18 in AGB551	This study
ANNE24	*sipD::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE20 in AGB551	This study
ANNE25	*sipE::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE22 in AGB551	This study
ANNE26	*sipA::sgfp::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE23 in AGB551	This study
ANNE27	*sipB::sgfp::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE24 in AGB551	This study
ANNE28	*sipC::sgfp::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE25 in AGB551	This study
ANNE29	*sipD::sgfp::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE26 in AGB551	This study
ANNE30	*sipE::sgfp::AfpyroA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE27 in AGB551	This study
ANNE31	*strAΔ::ptrA;sipA::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB525 in ANNE21	This study
ANNE32	*strAΔ::ptrA;sipB::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB525 in ANNE22	This study
ANNE33	*strAΔ::ptrA;sipC::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB525 in ANNE23	This study
ANNE34	*strAΔ::ptrA;sipD::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB525 in ANNE24	This study
ANNE35	*strAΔ::ptrA;sipE::ctap::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB525 in ANNE25	This study
ANNE36	*sipA::sgfp::AfpyroA;strA::mrfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB527 in ANNE26	This study
ANNE37	*sipB::sgfp::AfpyroA;strA::mrfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB527 in ANNE27	This study
ANNE38	*sipC::sgfp::AfpyroA;strA::mrfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB527 in ANNE28	This study

ANNE39	*sipD::sgfp::AfpyroA;strA::mrfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB527 in ANNE29	This study
ANNE40	*sipE::sgfp::AfpyroA;strA::mrfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB527 in ANNE30	This study
ANNE41	*^{p}sipA::sipA::sipA^{t}-AfpyroA;sipAΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE28 in ANNE1.1	This study
ANNE42	*^{p}sipB::sipB::sipB^{t}-AfpyroA;sipBΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE29 in ANNE2.1	This study
ANNE43	*^{p}sipC::sipC::sipC^{t}-AfpyroA;sipCΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE30 in ANNE3.1	This study
ANNE44	*^{p}sipD::sipD::sipD^{t}-AfpyroA;sipDΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE31 in ANNE4.1	This study
ANNE45	*^{p}sipE::sipE::sipE^{t}-AfpyroA;sipEΔ::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE32 in ANNE5.1	This study
ANNE46	*^{p}strA::strA::strA^{t}-AfpyroA;strAΔ::ptrA;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pNE33 in StrA-DEL1	This study
ANNE47	*^{p}gpdA::mrfp::h2A::AfpyroA;sipA::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB340 in ANNE16	This study
ANNE48	*^{p}gpdA::mrfp::h2A::AfpyroA;sipB::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB340 in ANNE17	This study
ANNE49	*^{p}gpdA::mrfp::h2A::AfpyroA;sipC::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB340 in ANNE18	This study
ANNE50	*^{p}gpdA::mrfp::h2A::AfpyroA;sipD::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB340 in ANNE19	This study
ANNE51	*^{p}gpdA::mrfp::h2A::AfpyroA;sipE::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB340 in ANNE20	This study
ANNE57	*^{p}gpdA::mrfp::h2A::phlR;sipA::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB111 in ANNE16	This study
ANNE58	*^{p}gpdA::mrfp::h2A::phlR;sipB::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB111 in ANNE17	This study
ANNE59	*^{p}gpdA::mrfp::h2A::phlR;sipC::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB111 in ANNE18	This study
ANNE60	*^{p}gpdA::mrfp::h2A::phlR;sipD::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB111 in ANNE19	This study
ANNE61	*^{p}gpdA::mrfp::h2A::phlR;sipE::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB111 in ANNE20	This study
ANNE62	*strAΔ::AfpyroA;pgpdA::mrfp::h2A::phlR;sipA::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB526 in ANNE57	This study
ANNE63	*strAΔ::AfpyroA;pgpdA::mrfp::h2A::phlR;sipB::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB526 in ANNE58	This study
ANNE64	*strAΔ::AfpyroA;pgpdA::mrfp::h2A::phlR;sipC::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB526 in ANNE59	This study

ANNE65	*strAΔ::AfpyroA;pgpdA::mrfp::h2A::phlR;sipD::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB526 in ANNE60	This study
ANNE66	*strAΔ::AfpyroA;pgpdA::mrfp::h2A::phlR;sipE::sgfp::AfpyrG;nkuAΔ::argB,pyrG89,pyroA4,veA+*	pOB526 in ANNE61	This study

2.2. Nucleic acid methods

2.2.1. Transformations

Transformation of *E. coli* and *A. nidulans* were performed as explained in detail (Hanahan et al., 1991; Punt et al., 1992; Punekar et al., 2004).

2.2.2. Plasmids constructions details

The oligonucleotide sequences and the plasmids used and created in this study are listed in **Table 2.2 and 2.3**, respectively.

2.2.2.1. Generation of *strA* deletion cassette, complementation and tagging protein-encoding gene with *sgfp*, *mrfp* and *ctap*

In order to create the *strA* deletion construct, the 5' UTR region of *strA* was amplified from wild-type genomic DNA (AGB551) using primers OZG1025/1110 and 3' UTR with OZG1112/1028 for *ptrA*. 5' UTR and 3 UTR were amplified with (OZG1025/1111, OZG1113/1028) for *AfpyroA*. The two fragments were fused to the *ptrA* and *AfpyroA* markers and amplified by a fusion PCR, using oligos OZG1025/1028 (4 -4.2 kbp). Both deletions cassettes were cloned in *Sma*I site of pUC19 leading to the plasmids pOB525 (*strA*Δ::*ptrA*) and pOB526 (*strA*Δ::*AfpyroA*). Both plasmids were digested with *Pme*I (*Mss*I) and linear deletion fragments were transformed into AGB551 generating *strA* deletions STRA-DEL1 and STRA-DEL2, respectively. pOB525 was transformed to ANNE21, ANNE22, ANNE23, ANNE24, ANNE25, resulting in ANNE31, ANNE32, ANNE33, ANNE34 and ANE35 strains.

For complementation of the *strA* deletion, the *strA* genomic locus (6.6 kbp), containing 2 kbp promoter and terminator regions, were amplified from gDNA (NE94/NE95) and cloned into the *Pme*I site of pOSB114, which yielded pNE33. Then, pNE33 was introduced into *strA* deletion strains (STRA-DEL1), generating the ANNE46 strain.

For the creation of *strA::sgfp* and *strA::ctap* fusions, the promoter and ORF (4.4 kbp) (OZG1025/1026), and terminator sequences (OZG1025/1028) of *strA* were amplified. These two fragments were fused to *sgfp::natR*, *ctap::natR* cassettes in *Sma*I site of pUC19 by using in-fusion HD cloning kit leading to plasmids pOB480 (*strA::sgfp*) and pOB481 (*strA::ctap*). In order to create *strA::mrfp* fusion, the promoter as well as ORF of *strA* (OZG1025/1114) and terminator sequences (OZG1115/1028) were amplified and fused to *mrfp::AfpyrG* by cloning in *Sma*I site of pUC19 leading to pOB527. These cassettes were released by *Pme*I digestion and introduced into AGB551 strain and ANNE26, ANNE27, ANNE28, ANNE29 and ANNE30. All deletions and epitope tags were confirmed by the Southern blots.

Table 2.2. Oligonucleotides and Plasmids employed in this study.

Designation	Sequence in 5' > 3' direction	Size
NE1 (5UTR AN6190 pUC tail)	TTC GAG CTC GGT ACC CGA CAG GAC TGG TCC AGA GTG	36 mer
NE2 (5UTR AN6190 nest)	GTG GTC GTG ATC AGA ACC GTC	21 mer
NE3 (5UTR AN6190 pyrG tail)	GAG CAT TGT TTG AGG CAG TCA GGA AGG TGA ATT TGG GAG	39 mer
NE4 (3UTR AN6190 pyrG/pyroA tail)	GCC TCC TCT CAG ACA GTG TGA CGA ACG GCG CTC AAA C	37 mer
NE5 (3UTR AN6190 pUC tail)	ACT CTA GAG GAT CCC CGT ACTG TCG ACG TTG AAT AGA TAC	40 mer
NE6 (3UTR AN6190 nest)	GCT ATA TCC TCC ACG CAC CTA G	22 mer
NE7 (5UTR AN6190 pyroA tail)	CCA GCA TCT GAT GTC CAG TCA GGA AGG TGA ATT TGG GAG	39 mer
NE8 (5UTR AN1010 pUC tail)	TTC GAG CTC GGT ACC CGT AAG TTC AAT GCA CTC GCC TTG	39 mer
NE9 (5UTR AN1010 nest)	CAA CTA GCG TTG TCG CAT TCA C	22 mer
NE10 (5UTR AN1010 pyrG tail)	GAG CAT TGT TTG AGG CGG TGT TCT ACA GGA ATG TGA GAT G	40 mer
NE11 (3UTR AN1010 pyrG/pyroA tail)	GCC TCC TCT CAG ACA GTG TAG CGC ACG CTG CTG ATA C	37 mer
NE12 (3UTR AN1010 pUC19 tail)	ACT CTA GAG GAT CCC CGT CAG ATG AGG GAG TAG TTG AC	38 mer
NE13 (3UTR AN1010 nest)	GTG TGA TTG GGT TCT CAA GAG G	22 mer
NE14 (5UTR AN1010 pyroA tail)	CCA GCA TCT GAT GTC CGG TGT TCT ACA GGA ATG TGA GAT G	40 mer
NE15 (5UTR AN6611 pUC tail)	TTC GAG CTC GGT ACC CGT AGG CCG CAG AAG GTT ACG	36 mer
NE16 (5UTR AN6611 nest)	GTG CCA CTC GGT CTA GTG TC	20 mer
NE17 (5UTR AN6611 pyrG tail)	GAG CAT TGT TTG AGG CGA TCG GGT ATA GTA ATT AAG AAG GTG	42 mer
NE18 (3UTR AN6611 pyrG/pyroA tail)	GCC TCC TCT CAG ACA GCT ACT TCA TAG TAC AGT TAT GTA CCT C	43 mer
NE19 (3UTR AN6611 pUC tail)	ACT CTA GAG GAT CCC CCT CAT CAC TAT CCT CCT CAT CC	38 mer
NE20 (3UTR AN6611 nest)	TCG TCG GAA TAA TCA TCG CTA GAG	24 mer

NE21 (5UTR AN6611 pyroA tail)	CCA GCA TCT GAT GTC CGA TCG GGT ATA GTA ATT AAG AAG GTG	42 mer
NE22 (5UTR AN4085 pUC tail)	TTC GAG CTC GGT ACC CGT TTC AGA ATA TGG TGC GTA CCG	39 mer
NE23 (5UTR AN4085 nest)	TGA GTT GGG GTG GTT CCT GC	20 mer
NE24 (5UTR AN4085 pyrG tail)	GAG CAT TGT TTG AGG CAC CAG AGG GCA AGG ATG TGA C	37 mer
NE25 (3UTR AN4085 pyrG/pyroA tail)	GCC TCC TCT CAG ACA GGT TGA CAC ACT AGG GCT TGA AG	38 mer
NE26 (3UTR AN4085 pUC tail)	ACT CTA GAG GAT CCC CGC AAC ACA CAG CAA GAC CAC C	37 mer
NE27 (3UTR AN4085 nest)	GAA AGA TGC ACA GCG TAG AAA GC	23 mer
NE28 (5UTR AN4085 pyroA tail)	CCA GCA TCT GAT GTC CAC CAG AGG GCA AGG ATG TGA C	37 mer
NE29 (AN1010 5UTR OUT)	CAG CAG CTT AGA CCA ACT GCC	21 mer
NE30 (AN1010 ORF FWD)	GTG GCT CCT TTG AGA ATG GTG	21 mer
NE31 (AN1010 ORF RVS)	TCC CAG CCG TAC TCT TCC TC	20 mer
NE32 (AN6190 5UTR OUT)	CAG CCA GAC ATC TAC GAG AGG	21 mer
NE33 (AN6190 ORF FWD)	GTT CAC AGA GGT GCA GAT TGG	21 mer
NE34 (AN6190 ORF RVS)	GCT CCT CTT CCT TCT GTC TTG	21 mer
NE35 (AN6611 5UTR OUT)	CCT ACT TCC CCT TAC CTG AGC	21 mer
NE36 (AN6611 ORF FWD)	CAT CCG CTG ATG CTT CCT TGG	21 mer
NE37 (AN6611 ORF RVS)	CTG TAG CAT GCC ATC CCA TTC	21 mer
NE38 (AN4085 5UTR OUT)	GTC ATG AGT ACC AAT GGA GAC G	22 mer
NE39 (AN4085 ORF FWD)	GCT CTA TCC TAT CGC TGT TCT C	22 mer
NE40 (AN4085 ORF RVS)	CCG AAC ATC AAC ATC ATC ATC GC	23 mer
NE41 (AN4632 5UTR pUC tail)	TTC GAG CTC GGT ACC CCG CAC CCA TCT TCA CTG TAG C	37 mer
NE42 (AN4632 5UTR pyrG tail)	GAG CAT TGT TTG AGG CGG TGT TAT GGA TGC TCG ACG C	37 mer
NE43 (AN4632 3UTR pyrG/pyroA tail)	GCC TCC TCT CAG ACA GCT GAT CAT TCA TGG CTC TGT TAT C	40 mer

NE44 (AN4632 3UTR pUC tail)	ACT CTA GAG GAT CCC CGA ATT ACG GTC CGT CTA CGT TG	38 mer
NE45 (AN4632 5UTR nest)	CAC TAG AGC GGA TAC TGA CAT TG	23 mer
NE46 (AN4632 3UTR nest)	GTG TAT CAG TGC TAC GGG ATC	21 mer
NE47 (AN4632 5UTR OUT)	CAA GCG CAA GCG TAT CGA GG	20 mer
NE48 (AN4632 ORF nest FWD)	TCG CTT GGG ATG GTA CGA TTC	21 mer
NE49 (AN4632 ORF nest RVS)	CTT GCT CAA CAG CGT CAA GAC	21 mer
NE50 (AN0164 5UTR pUC tail)	TTC GAG CTC GGT ACC CCG ACT ACT GCA GGT GAG CAG	36 mer
NE51 (AN0164 5UTR pyrG tail)	GAG CAT TGT TTG AGG CGC CGA GAG AAT GGT GGT ACA G	37 mer
NE52 (AN0164 3UTR pyrG/pyroA tail)	GCC TCC TCT CAG ACA GCG CCG CTT TAT GTT TCC TTT GAC	39 mer
NE53 (AN0164 3UTR pUC tail)	ACT CTA GAG GAT CCC CCC ATT AAC TAA CGC CAC TCT CAC	39 mer
NE54 (AN0164 5UTR nest)	TGA AGC GAA CAT AGC TGC CTC	21 mer
NE55 (AN0164 3UTR nest)	GAC GAA TCC CTC AAC CGT TGG	21 mer
NE56 (AN0164 5UTR OUT)	CAA CAG ATC CTG CTC TTA GGA G	22 mer
NE57 (AN0164 ORF nest FWD)	CAT CGA CCT GGA CGA GTG C	19 mer
NE58 (AN0164 ORF nest RVS)	GTA GTC GAT CAC AGG ACC CTG	21 mer
NE59 (AN4632 5UTR pyroA tail)	CCA GCA TCT GAT GTC CGG TGT TAT GGA TGC TCG ACG C	37 mer
NE60 (AN0164 5UTR pyroA tail)	CCA GCA TCT GAT GTC CGC CGA GAG AAT GGT GGT ACA G	37 mer
NE61 (AN6190 ORF linker tail)	CAC CGC TAC CAC CTC CGT CTT TTT TGG CAG GCT CCT CTT	39 mer
NE62 (AN6190 3UTR pyrG/pyroA tail)	GCC TCC TCT CAG ACA GTT GCT GAC ATG TGG AAA CCA AAT TAT C	43 mer
NE63 (AN1010 ORF linker tail)	CAC CGC TAC CAC CTC CCT CAG AGC CTC CCG GAA CAT	36 mer
NE64 (AN1010 3UTR pyrG/pyroA tail)	GCC TCC TCT CAG ACA GTG TAG CGC ACG CTG CTG ATA C	37 mer
NE65 (AN6611 ORF linker tail)	CAC CGC TAC CAC CTC CTG CCC CCT CCA CCT GTA GC	35 mer

NE66 (AN6611 3UTR pyrG/pyroA tail)	GCC TCC TCT CAG ACA GTA GAT CTG AAA TAT CTC GCA TAT GAT G	43 mer
NE67 (AN4632 ORF linker tail)	CAC CGC TAC CAC CTC CCT TAT CCA TTT TCT GCC ACC CGT	39 mer
NE68 (AN4632 3UTR pyrG/pyroA tail)	GCC TCC TCT CAG ACA GCT GAT CAT TCA TGG CTC TGT TAT C	40 mer
NE69 (AN0164 ORF linker tail)	CAC CGC TAC CAC CTC CCA GAA AGT AGT CGA TCA CAG GAC	39 mer
NE70 (AN0164 3UTR pyrG/pyroA tail)	GCC TCC TCT CAG ACA GTA CCC TTT TTC ATT CGT ATT TCG CC	41 mer
NE71 (AN0164 3UTR pUC tail)	ACT CTA GAG GAT CCC CTA CCT GCT GAA TCC AGA CAG TAC	39 mer
NE84 (5UTR AN6190 pUC tail)	AGC TCG GTA CCC ATT TGA ATT CTC TCT TGG GTG ATC TAG A	24 mer
NE85 (3UTR AN6190 pUC tai)	CAT CTG ATG TCC ATT TAG TCC TAT CCA CAC TCC CTA AC	22 mer
NE87 (3UTR AN1010 pUC tail)	ATC TGA TGT CCA TTT TCG ACC TTA GCT CTG GTG CAC	20 mer
NE86 (5UTR AN1010 pUC tail)	AGC TCG GTA CCC ATT TGC CAA CTA CCG TGA GGG GA	19 mer
NE88 (5UTR AN6611 pUC tail)	CTC GGT ACC CAT TTA ACC TCT TTC TCA CAA CCG ACA ATG	23 mer
NE89 (3UTR AN6611 pUC tail)	GCA TCT GAT GTC CAT TTA CGT TGT TAG AGG TTG CGA TCA C	22 mer
NE90 (AN4632 5UTR pUC tail)	GAG CTC GGT ACC CAT TTG TGG AGA TGC GGG GAT GGC	19 mer
NE91 (AN4632 3UTR pUC tail)	CAT CTG ATG TCC ATT TGT GAA GAC CGT GAG TTG CTT GAT	23 mer
NE92 (AN0164 5UTR pUC tail)	AGC TCG GTA CCC ATT TCT CAC ATC TGG CGT CTG AAG C	21 mer
NE93 (AN0164 3UTR pUC tail)	CAT CTG ATG TCC ATT TTC CAT AAT GGT TCT CTT GCA CAG C	24 mer
NE94 strA(5UTR AN8071 pUC tail)	AGC TCG GTA CCC ATT TGT TCT AGA AGA GTG GCC ACA AG	22 mer
NE95 strA(3UTR AN8071 pUC tail)	CGC AGT AGC CGT AAC AGC AG	20 mer
OZG1110 (*strA* ptrA 5)	ACA ATG CGA AGC TGA AGC GAC	21 mer
OZG1111 (*strA* pyroA 5)	ACA ATG CGA AGC TGA AGC GAC	21 mer
OZG1112 (*strA* ptrA 3)	TAA CGC AGT GGA TGT GGC CT G	21 mer
OZG1113 (*strA* pyroA 3)	CAT CAG TGC CTC CTC TCA GAC	21 mer

OZG1114 (*strA* 3 for mRFP)	CAC CGC TAC CAC CTC CTC TCG AG	23 mer
OZG1115 (*strA* 3 UTR pyrG)	GCC TCC TCT CAG ACA GCG C	19 mer
OZG1025 (*strA* 5 UTR PmeI)	CCC AGG TTC CTT TGC GAT ATA G T	23 mer
OZG1027 (*strA* 3 natR)	CAT GCC CTG CCC CTG ACG C	19 mer
OZG1028 (*strA* 3 UTR PmeI)	ACT CTA GAG GAT CCC CGT TTA AA	23 mer
OZG1026 (*strA* GFP & TAP fuser)	TAC CAC CGC TAC CAC CTC TCG AG	23 mer
OSBRT1 (*laeA* 5UTR)	CAC AAC CAC TAC AGC TAC CAC	21 mer
OSBRT2 (*laeA* 3UTR)	GCA ACC GCG TAT CTG GTC G	19 mer
OSBRT7 (*ipnA* 5UTR)	GAG AGT AGC CCA GCA AAT CG	20 mer
OSBRT8 (*ipnA* 3UTR)	GGC ACG AAT CGC AAG GTC C	19 mer
OSBRT9 (*acvA* 5UTR)	GAC AAG GAC AAC CGT GAT G	19 mer
OSBRT10 (*acvA* 3UTR)	GCA CAC CAT TAC TGC TAG AGG	21 mer
OSBRT11 (*aatA* 5UTR)	CCA TTG ACT TCG CAA CTG GC	20 mer
OSBRT12 (*aatA* 3UTR)	CGT ACG AGT GTT GAG CAT GAC	21 mer
OSBRT13 (*tdiA* 5UTR)	CGA TGC CTG GAG TGC GAA TG	20 mer
OSBRT14 (*tdiA* 3UTR)	GCC GTT GCT GTC AAT GAA CG	20 mer
OSBRT19 (*brlA* 5UTR)	CTC GAA GAC ATG CGA AAT CAG	21 mer
OSBRT20 (*brlA* 3UTR)	CAG GAG TTC GTT CGT ACG TG	20 mer
OSBRT57 (*aflR* 5UTR)	CCT TCG CTT CTT GAG GGT ATG G	22 mer
OSBRT58 (*aflR* 3UTR)	GCA GTA GGA GTG GCT TGT GGT G	22 mer
OSBRT68 (*stcE* 5UTR)	GCA TCT CGA TGT AGT GAT CG	20 mer
OSBRT69 (*stcE* 3UTR)	CTA GTC GCC TGG AAC AGT AG	20 mer
OSBRT70 (*stcQ* 5UTR)	GGT TGT AGC GTC TTT GCA ACG	21 mer

OSBRT71 (*stcQ* 3UTR)	GAA CAT CGT TGC AGA ACG TGG	21 mer
OSBRT76 (*veA* 5UTR)	CGA TCC AGA GCC TCT CAG AG	20 mer
OSBRT77 (*veA* 3UTR)	GGT CAT CAT GAC CGA ACG AC	20 mer
OSBRT78 (*velB* 5UTR)	CCT CCC ACA ATC GGA TAT TGC	21 mer
OSBRT79 (*velB* 3UTR)	GGG ATC TTG ATT CCT TGG TTC	21 mer
OSBRT88 (*abaA* 5UTR)	GCA ACC CGA GTG TAT GGT ATC	21 mer
OSBRT89 (*abaA* 3UTR)	CAC GTT CCC AGA CGT ATT CTG	21 mer
OSBRT102 (*flbA* 5UTR)	CTA CAA CAA CTA CCT TCT CAA TG	23 mer
OSBRT103 (*flbA* 3UTR)	GTT TCT CTG ACA GAA CCT CTG	21 mer
OSBRT106 (*flbC* 5UTR)	TGA CAG TGT TTA CCA GCA TCC	21 mer
OSBRT107 (*flbC* 3UTR)	CTA GTC TCG TCC TGC TTG ATG	21 mer
OSBRT112 (*39oda* 5UTR)	GCT TCC ACA TCC ACC AGT TC	20 mer
OSBRT113 (*39oda* 3UTR)	GTC TTG AAG TTA CCA AGG TCA C	22 mer
OSBRT114 (*catB* 5UTR)	CAA GAG CTC CAC CTT CGA TGA TG	23 mer
OSBRT115 (*catB* 3UTR)	CTG AAC GAT GAC GTT GTT CTT C	22 mer
OSBRT116 (*catC* 5UTR)	GCC ACC ATG TTC TGG GAC TAC	21 mer
OSBRT117 (*catC* 3UTR)	GTA GGT GTG ACC GGA GTA GC	20 mer
OSBRT118 (*sodB* 5UTR)	CTA CGT CAA CAG CTA CAA CAC	21 mer
OSBRT119 (*sodB* 3UTR)	GAG AGA GCA CCA GAA GGA G	19 mer
OSBRT120 (*nsdD* 5UTR)	GGC ATT ATG CAA ACA GAG CAC	21 mer
OSBRT121 (*nsdD* 3UTR)	GCA TCA GAG CGT CGT GGT TC	20 mer
OSBRT122 (*nsdC* 5UTR)	CAC CTC CTC ACA CAT AGT CA	20 mer
OSBRT123 (*nsdC* 3UTR)	GGA CAT ATA TGG GGT CGC TC	20 mer

BK280 (*benA* 5UTR)	GAT GGC TGC CTC TGA CTT C	19 mer
BK281 (*benA* 3UTR)	GCA TCT GGT CCT CAA CCT C	19 mer
BK293 (*matB* 5UTR)	GAC CGA AGC TAC AGA ACT CC	20 mer
BK294 (*matB* 3UTR)	GAG GTG CGA GAT TGT GGA C	19 mer
BK295 (*setA* 5UTR)	CAC TCA GGA GCG ACC ATA TC	20 mer
BK296 (*setA* 3UTR)	CTT GCT GAG CCT CGT GAA TG	20 mer

Table 2.3. Plasmids employed in this study.

Plasmid	Description	Reference
pUC19	*E. coli* cloning plasmid with *bla* (ampicillin resistance gene) gene	Thermo Fisher
pOB111	*pgpdA::mrfp::h2A::trpCt* (histone 2A) with phleomycin (*phlR*) marker	(Bayram et al., 2012)
pOSB114	*Pme*I*::AfpyroA::Swa*I inserted in *Sma*I site of pUC19 (for complementation experiments)	This study
pOB340	*bioA5ORF::AfpyroA::pgpdA::mrfp::bioA3ORF* (histone 2A) with *bla* (*E.coli*) and *AfpyroA* (*A. nidulans*)	(Sarikaya Bayram et al., 2014)
pOB480	*strA::sgfp::natR* cassette with *Pme*I in *Sma*I site of pUC19	This study
pOB481	*strA::ctap::natR* cassette with *Pme*I in *Sma*I site of pUC19	This study
pOB525	*strA* deletion (1025/1110) +(1112/1028) with *ptrA* in *Sma*I site of pUC19	This study
pOB526	*strA* deletion with *Afpyro*A (1025/1111) +(1113/1028) in *Sma*I site of pUC19	This study
pOB527	*strA::mrfp* with *AfpyrG* (1025/1114)+(1115/1028) in *Sma*I site of pUC19	This study
pNE1	*sipA* (AN6190) deletion (NE1/3)+(NE4/5)with *AfpyrG* in *Sma*I site of pUC19	This study
pNE2	*sipA* (AN6190) deletion (NE1/7)+(NE4/5) with *AfpyroA* in *Sma*I site of pUC19	This study
pNE3	*sipB* (AN1010) deletion (NE8/10)+(NE11/12) with *AfpyrG* in *Sma*I site of pUC19	This study
pNE4	*sipB (AN1010)* deletion (NE8/14)+(NE11/12) with *AfpyroA in Sma*I site of pUC19	This study
pNE5	*sipC* (AN6611) deletion (NE15/17)+(NE18/19) with *AfpyrG* in *Sma*I site of pUC19	This study
pNE6	*sipC* (AN6611) deletion (NE15/21)+(NE18/19) with *AfpyroA* in *Sma*I site of pUC19	This study
pNE9	*sipD* (AN4632) deletion (NE41/42)+(NE43/44)with *AfpyrG* in *Sma*I site of pUC19	This study

pNE10	*sipD* (AN4632) deletion (NE59/42)+(NE43/44)with *AfpyroA* in *Sma*I site of pUC19	This study
pNE11	*sipE* (AN0164) deletion (NE50/51)+(NE52/53) with *AfpyrG* in *Sma*I site of pUC19	This study
pNE12	*sipE* (AN0164) deletion (NE51/60)+(NE4/5) with *AfpyroA* in *Sma*I site of pUC19	This study
pNE13	*sipA* (AN6190) *sgfp::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE1/61)+(NE62/5)	This study
pNE14	*sipA* (AN6190) *ctap::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE1/61)+(NE62/5)	This study
pNE15	*sipB* (AN1010) *sgfp::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE8/63)+(NE64/12)	This study
pNE16	*sipB* (AN1010) *ctap::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE8/63)+(NE64/12)	This study
pNE17	*sipC* (AN6611) *sgfp::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE15/65)+(NE66/19)	This study
pNE18	*sipC* (AN6611) *ctap::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE15/65)+(NE66/19)	This study
pNE19	*sipD* (AN4632) *sgfp::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE41/67)+(NE68/44)	This study
pNE20	*sipD* (AN4632) *ctap::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE41/67)+(NE68/44)	This study
pNE21	*sipE* (AN0164) *sgfp::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE50/69)+(NE70/71)	This study
pNE22	*sipE* (AN0164) *ctap::AfpyrG* cassette with *Pme*I in *Sma*I sit of pUC19 (NE50/69)+(NE70/71)	This study
pNE23	*sipA* (AN6190) *sgfp::AfpyroA* cassette with *Pme*I in *Sma*I sit of pUC19 (NE1/33)+(NE62/5)	This study
pNE24	*sipB* (AN61010) *sgfp::AfpyroA* cassette with *Pme*I in *Sma*I sit of pUC19 (NE1/33)+(NE62/5)	This study
pNE25	*sipC* (AN6611) *sgfp::AfpyroA* cassette with *Pme*I in *Sma*I sit of pUC19 (NE1/33)+(NE62/5)	This study
pNE26	*sipD* (AN4632) *sgfp::AfpyroA* cassette with *Pme*I in *Sma*I sit of pUC19 (NE1/33)+(NE62/5)	This study
pNE27	*sipE* (AN0164) *sgfp::AfpyroA* cassette with *Pme*I in *Sma*I sit of pUC19 (NE1/33)+(NE62/5)	This study
pNE28	*sipA* (AN6190) genomic locus in *Pme*I site of the pOSB114	This study
pNE29	*sipB* (AN1010) genomic locus in *Pme*I site of the pOSB114	This study
pNE30	*sipC* (AN6611) genomic locus in *Pme*I site of the pOSB114	This study

pNE31	*sipD* (AN4632) genomic locus in *Pme*I site of the pOSB114	This study
pNE32	*sipE* (AN0164) genomic locus in *Pme*I site of the pOSB114	This study
pNE33	*strA* (AN8071) genomic locus in *Pme*I site of the pOSB114	This study

2.2.2.2 Generation of striatin interacting protein encoding gene, *sipA*, *sipB*, *sipC*, *sipD* and *sipE* deletion, complementation, *sgfp* and *ctap* tagging plasmids

For the creation of the *sipA* deletion plasmid construct, the 5' UTR region of *sipA* was amplified from AGB551 (WT) genomic DNA using primers (NE1/3 and NE1/7) resulting in approximately 1.5 kbp fragments. The 3' UTR region was amplified with (NE4/N5). The two fragments were fused to the *AfpyrG* and *AfpyroA* markers and inserted into *Sma*I site of pUC19 leading to the plasmids pNE1 (*sipA*Δ::*AfpyrG*) and pNE2 (*sipA*Δ::*AfpyroA*) then were transformed to MACH-1 competent *Escherichia coli* cells as described previously. Plasmid constructs were isolated using Qiagen Mini-Prep Plasmid Purification (Cat no: 27104) kit. To amplify the deletion cassette, NE2/6 primers were used and 4.2kbp linear fragment was extracted from the gel. These linear deletion fragments were used to transform AGB551, generating the *sipA* deletion strains, termed (ANNE1.1 and ANNE1.2), and StrA-DEL1 for double deletion combinations with *strA* (ANNE1.3). The *sipA* complementation plasmid was generated by amplifying *sipA* genomic locus including, 1.9 kbp promoter and 1.9 kbp terminator regions using primers (NE84/85) and cloned into the *Pme*I site of pOB114 that yielded pNE28, which was introduced into *sipA* deletion strain (ANNE1.1), generating the ANNE41 complementation strain. All deletion strains were verified by the Southern hybridization (**Figure 2.1**).

For the creation of *sipA::sgfp* and *sipA::ctap* fusions, the promoter (NE1/61) and ORF (5.5kbp), along with the terminator sequences (NE5/62) of *sipA* were amplified from gDNA. These two fragments were fused to *sgfp::AfpyrG , sgfp::AfpyroA* and *ctap::AfpyrG* cassettes in *Sma*I site of pUC19 by in-fusion HD cloning kit creating pNE13 (*sipA::sgfp::AfpyrG*), pNE23 (*sipA::sgfp::AfpyroA*), pNE14 (*sipA::ctap::AfpyrG*) plasmids respectively. The nest oligos (NE2/6) amplified these cassettes from pNE13, pNE23, and pNE14, which were introduced into the wild-type (ABG551) yielding strains ANNE16, ANNE26, ANNE21. These plasmids were also introduced into *strA* deletion strain STRA-DEL1 as explained in Table 1.

The construction of *sipB* deletion strains (ANNE2.1 and NANE2.2) was performed as explained above, using the primers NE8/10 and NE8/14 for amplifying the 5' UTR and NE11/12 for amplifying the 3' UTR. These fragments were fused to *AfpyrG* and *AfpyroA* markers and inserted into *Sma*I site of pUC19 leading to pNE3 (*sipB*Δ::*AfpyrG*) and pNE4 (*sipB*Δ::*AfpyroA*). The nest oligos NE9/13 were used to amplify deletion cassettes (4.2 kbp), which were ultimately transformed into ABG551 to generate *sipA* deletion (ANNE2.1 and ANNE2.2) and double deletion combinations with *strA* (ANNE2.3), *sipA* (ANNE6), *sipC* (ANNE10), *sipD* (ANNE11), and *sipE* (ANNE12).

The *sipB* genomic locus (5.4kbp), containing the 2kb promoter region and 2 kbp terminator regions, was amplified from genomic DNA (NE86/87) and used for the complementation of *sipB* deletion strains. This fragment was cloned into the *Pme*I site of pOSB114 that yielded pNE29 that was introduced into *sipB* deletion strains to generate ANNE42 strain. The *sipB::sgfp* and *sipB::ctap* cassettes were generated by using *AfpyrG* or *pyroA* markers in the same way that was explained previously, using

primers NE8/63 and NE12/64 and pNE15 /pNE16. The resulting strains were named ANNE17 (*sipB::sgfp::AfpyrG*), ANNE27(*sipB::sgfp::AfpyroA*) and ANNE22 (*sipB::ctap::AfpyrG*).

In order to create ANNE3.1 and ANNE3.2, the 5' UTR region of *sipC* was amplified from WT gDNA using primers NE15/17 or NE15/21. The 3' UTR regions were amplified with NE18/19. The two fragments were fused to the *AfpyrG* and *pyroA* markers and inserted into *Sma*I site of pUC19 leading to pNE5 (*sipC*Δ::*AfpyrG*) and pNE6 (*sipC*Δ::*AfpyroA*). Linear deletion cassettes (4.2kbp), which were amplified by PCR, using nest oligos (NE16/20), were transformed to AGB551 generating *sipC* single deletion (ANNE3.1 and ANNE3.2) and double mutant combinations of *sipC* with *strA* (ANNE3.3), *sipA* (ANNE37), *sipD* (ANNE13) and *sipE* (ANNE14). For construction of complementation plasmid, pNE30, a 7kbp *sipC* genomic fragment (NE88/89) was cloned into the *Pme*I site of pOSB114. The pNE30 was then transformed into *sipC* deletion ANNE3.1 Yielding ANNE43 as described previously.

To create pNE17, pNE18 and pNE25, the *sipC* promoter and ORF was amplified with NE15/65 and the terminator region with NE19/66, using AGB551 genomic DNA as a template. These fragments were fused to *sgfp::AfpyroA, sgfp::AfpyrG* or *ctap:: AfpyrG* cassettes and cloned in pUC19 as described for the *sipA* and *sipB*. The primers NE16/20 amplified 10 kbp linear fragments from (pNE17, pNE18 and pNE25). These fragments were transformed into the wild type to generate ANNE18, NANE23, NANE28, respectively. Gene replacement events were verified by the Southern hybridization.

For the creation of the *sipD* deletion construct, the 5' UTR (NE41/42 or NE59/42) and the 3' UTR (NE43/44) were amplified and fused to the

AfpyrG and *AfpyroA* markers and inserted into *Sma*I site of pUC19 leading to the plasmids pNE9 (*sipD*Δ::*AfpyrG*) and pNE10 (*sipD*Δ::*AfpyroA*), respectively. Deletion cassettes were amplified with NE45/46 and used to transform AGB551, generating the *sipD* single deletion ANNE4.1 and ANNE4.2 and double deletion combinations with *strA/sipD* (ANNE4.3), *sipA/sipD* (ANNE8), and *sipE*/*sipD* (ANNE15). For complementation of the *sipD* deletion, *sipD* genomic DNA including 1.97 kbp of the promoter region and 2 kbp of the terminator, was amplified (NE90/91) and cloned into pOSB114 at the *Pme*I site, generating pNE31, which was then transformed into ANNE4.1. This yielded the ANNE44 strain.

To generate the *sipD::sgfp* and *sipD::ctap* strains, the primers NE41/67 were used to amplify the *sipD* promoter and ORF for *sgfp* and *ctap* cassettes, while the primers NE44/68 were used to replicate the *sipD* terminator. These fragments were fused to *sgfp::AfpyrG, sgfp::AfpyroA* and *ctap::AfpyrG* with NE45/NE46. Created endogenous fragments (pNE19, pNE26, pNE20) were transformed into AGB551, yielding ANNE19, ANNE29 and ANNE24 respectively.

To generate the *sipE* deletion construct, *sipE* 5' UTR (NE50/51 and NE50/60) and the 3' UTR (NE52/53) were amplified. The two fragments were fused to the *AfpyrG* and *AfpyroA* markers and inserted into *Sma*I site of pUC19 yielding pNE11 (*sipE*Δ::*AfpyrG*) and pNE12 (*sipE*Δ::*AfpyroA*). The liner deletion fragments, which were amplified with nest oligos NE54/55, then used to transform AGB551, generating *sipE* deletion strains (ANNE5.1 and ANNE5.2, respectively) and double deletions *sipE*/*strA* (ANNE5.3), *sipE*/*sipA* (ANNE9).

For complementation of the *sipE* deletion, the *sipE* genomic locus (6.8 kbp), containing 2 kbp of the promoter region and 2 kbp of the

terminator regions, was amplified (NE92/93) cloned into the pOSB114 (pNE32). Then pNE32 was introduced into *sipE* deletion strain (ANNE5.1), generating the ANNE45 strain. To generate the *sipE::sgfp* and *sipE::ctap* strains, primers NE50/69 were used to amplify the *sipE* promoter and ORF for *sgfp* and *ctap*, while the primers NE71/70 were used to amplify the *sipE* terminator. These fragments were fused to *sgfp::AfpyrG, sgfp::AfpyroA* and *ctap::AfpyrG* cassettes with NE54/55. Endogenous fragments (pNE21, pNE27, pNE22) were transformed into AGB551, yielding ANNE20, ANNE30 and ANNE25, respectively. All deletions and epitope tags were confirmed by the Southern blots (**Figure 2.1 and 2.2**).

2.2.2.3. Generation of histone 2A mRFP fusion strains

The pOB340 plasmid was digested with *Pme*I at 37°C overnight, then transformed to ANNE16, ANNE17, ANNE18, ANNE19 and ANNE20, resulting ANNE47, ANNE48, ANNE49, ANNE50 and ANNE51. The same strains were transformed with pOB111, which led to strains ANNE57, ANNE58, ANNE59, ANNE60 and ANNE61. These strains were introduced with pOB526 (*strA*Δ*::AfpyroA*) in order to delete *strA* gene generating the strains ANNE62, ANNE63, ANNE64, ANNE65 and ANNE66.

2.3. Hybridization techniques

Southern hybridization experiments were performed as given in detail (Bayram et al., 2008). Fungal genomic DNA was prepared from plates using the ZR Fungal/Bacterial DNA MiniPrep TM (Zymo Research) kit. 700ng of isolated genomic DNA was used for restriction enzyme digestion. The southern hybridization was performed with non-radioactive probes by using DIG labeling (Roche) as described in the user protocol. DIG labeled DNA probes of the corresponding ORFs, 5' UTR or 3' UTR were amplified by

the PCR DIG Probe Synthesis kit (Roche). Digested gDNA was run on a 0.7% Agarose gel at 100 Volts for 99 min. The gel was then washed with 0.25M HCl, 0.5M NaCl and 1.5M NaOH at room temperature for 10, 25 and 30 minutes respectively. For dry blotting, the Amersham Hybond TM-N+(GE Healthcare) membrane was used and UV cross-linking was performed (UV Stratalinker 1800). The membrane was then washed with 2x SSC (Saline Sodium Citrate) twice for 2min and dried at 70°C for 7min. Preparation of the membrane for Immunodetection was achieved by incubating the membrane in a rotating tube at 42°C for 1 hour with 15ml of pre-hybridization buffer, followed by addition of probe and incubation at 42°C for over night in rotator. Next day, the membrane was washed by 2x SSC with 0.1% SDS for 5min at RT two times and more washing by pre-warmed of 0.1x SSC with 0.1% SDS at 68°C for 20min two times, followed washing by 1x DIG buffer1 for 5mins. Then, 1:10.000 Alkaline phosphatase conjugated anti-DIG fab fragment (Roche 11093274910) was used with DIG buffer1, which was named DIG buffer2, followed by two more washing steps. For the Alkaline Phosphatase reaction, the membrane was incubated with 10 ml of DIG buffer 3 for 5 minutes. For chemiluminescent detection, the membrane was placed on a nylon bag and CSPD (Roche) was added and left to incubate at RT for 5 minutes. The membrane was then exposed, using the G: BOX Chemi XRQ (Syngene) by using specific settings for CSPD reagent.

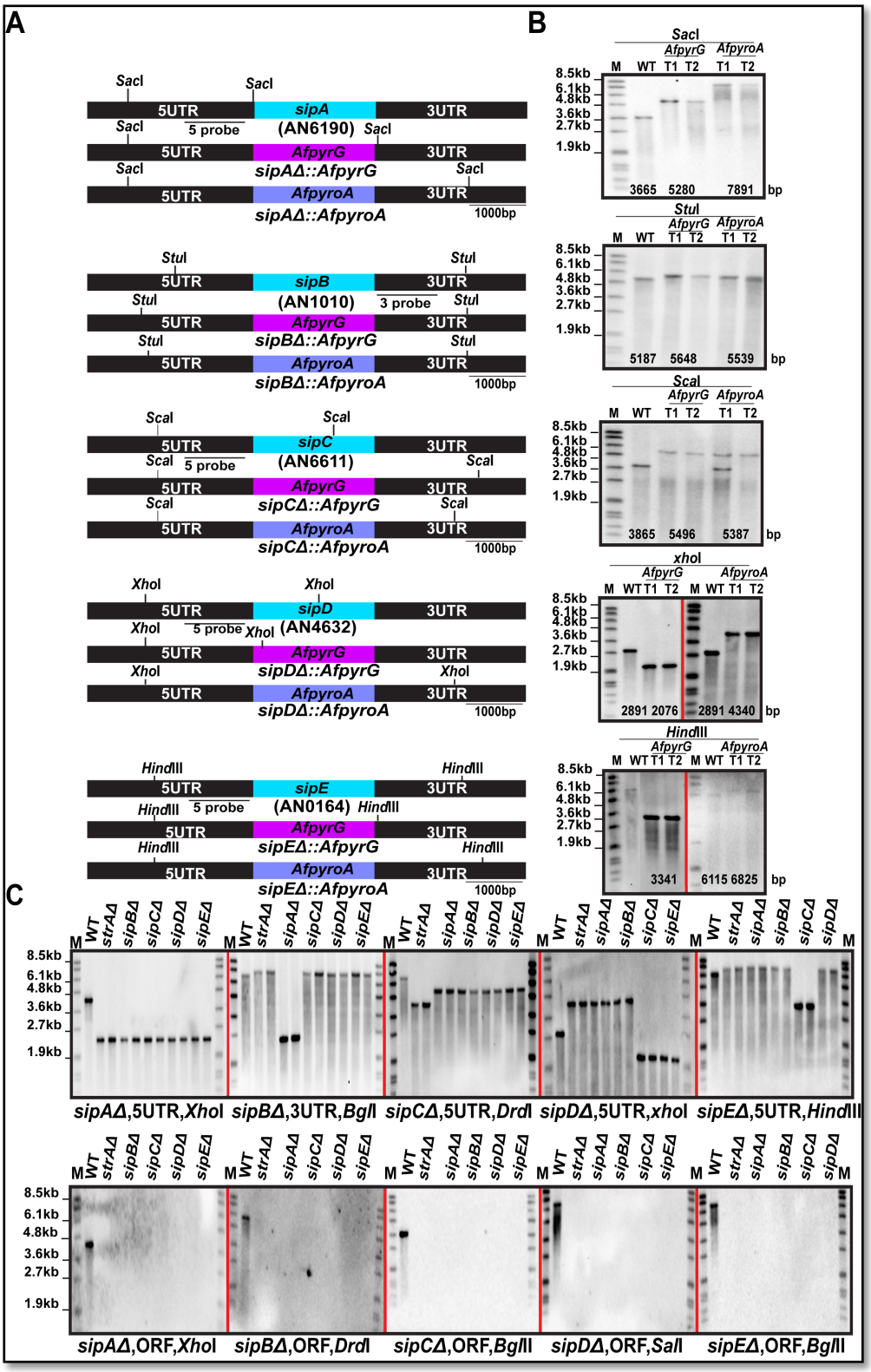

A
SacI
5UTR
sipA
(AN6190)
3UTR
5 probe
AfpyrG
sipAΔ::AfpyrG
AfpyroA
sipAΔ::AfpyroA
1000bp
StuI
sipB
3 probe
sipBΔ::AfpyrG
sipBΔ::AfpyroA
ScaI
sipC
sipCΔ::AfpyrG
sipCΔ::AfpyroA
XhoI
sipD
(AN4632)
sipDΔ::AfpyrG
sipDΔ::AfpyroA
HindIII
sipE
(AN0164)
sipEΔ::AfpyrG
sipEΔ::AfpyroA
B
SacI
AfpyrG AfpyroA
M WT T1 T2 T1 T2
8.5kb
6.1kb
4.8kb
3.6kb
2.7kb
1.9kb
3665 5280 7891 bp
StuI
5187 5648 5539 bp
ScaI
3865 5496 5387 bp
xhoI
2891 2076 2891 4340 bp
HindIII
3341 6115 6825 bp
C
M WT strAΔ sipBΔ sipCΔ sipDΔ sipEΔ
sipAΔ,5UTR,XhoI
sipBΔ,3UTR,BglI
sipCΔ,5UTR,DrdI
sipDΔ,5UTR,xhoI
sipEΔ,5UTR,HindIII
sipAΔ,ORF,XhoI
sipBΔ,ORF,DrdI
sipCΔ,ORF,BglII
sipDΔ,ORF,SalI
sipEΔ,ORF,BglII

Figure 2.1. Generation of single and double deletion combinations of *sip* genes. (**A)** General depiction of the wild-type *sipA* to *sipE* deletion loci. Scale bar (1000 bps), restriction enzymes, probes used for Southern hybridizations are shown in the graph. A small proportion of 5 un-translated region (5 UTR) or 3 UTR were used as Southern probes. Deletions were created with both *AfpyrG* and *AfpyroA* markers. (**B)** Southern hybridizations of *sip* single deletions with both marker combinations. Southern hybridizations confirm the replacement of endogenous loci by either *AfpyrG* or *AfpyroA* markers. M: Molecular marker in kbp, T1&T2; Transformant 1&2, respectively. Sizes of the bands are in agreement with theoretical maps shown in A. **(C)** Southern hybridizations of *strA/sip* and *sip/sip* double deletions. The Southerns in upper panel show all double deletion combinations by use of either 5 UTR or 3 UTR probes. The Southerns in lower panel display the lack of respective ORFs of *sipA* to *sipE* in double deletion combinations. Respective ORFs of *sipA* to *sipE* were used as the Southern probe in lower Southern.

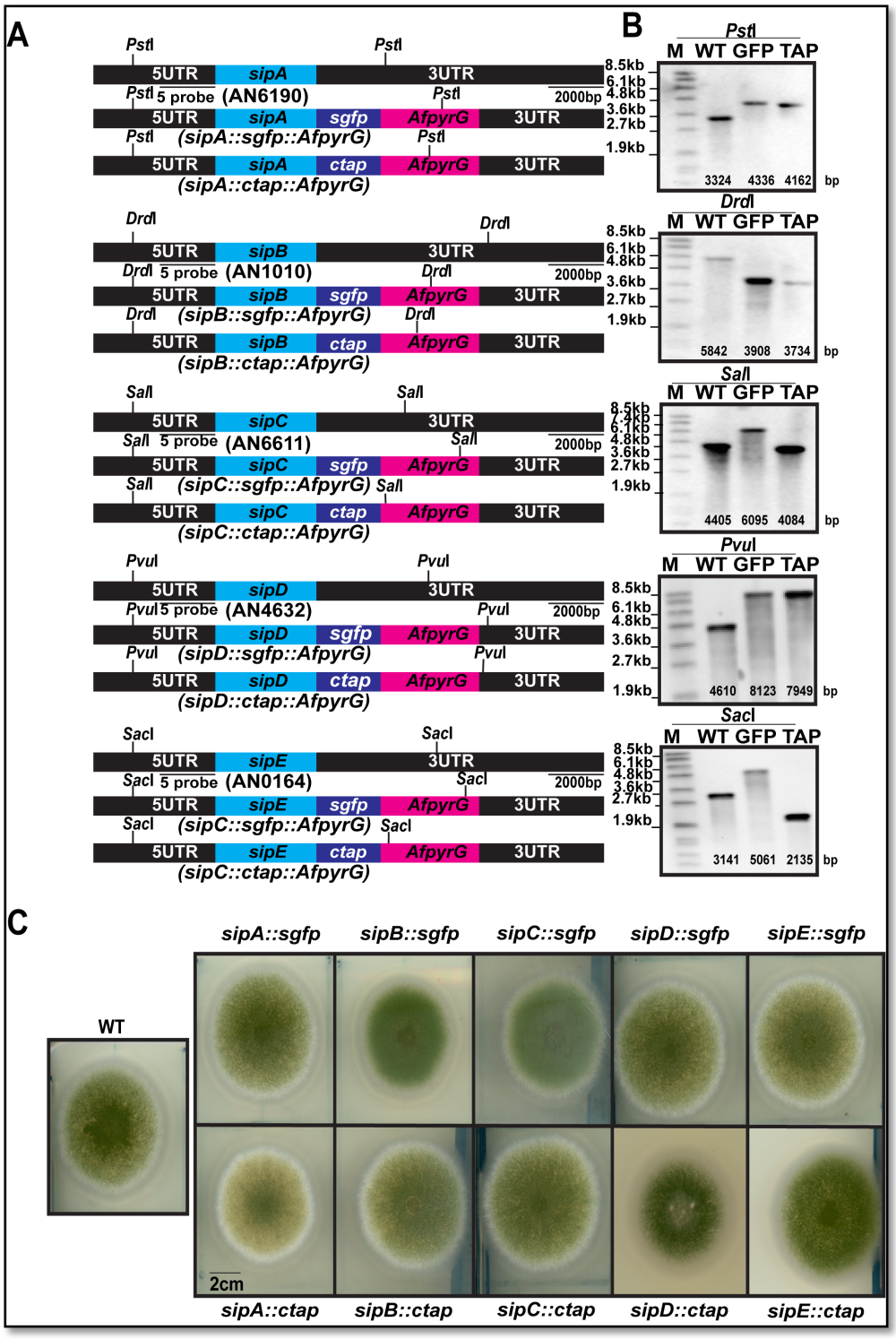

A
PstI
5UTR
sipA
3UTR
5 probe (AN6190)
2000bp
sgfp
AfpyrG
(sipA::sgfp::AfpyrG)
ctap
(sipA::ctap::AfpyrG)
DrdI
sipB
(AN1010)
(sipB::sgfp::AfpyrG)
(sipB::ctap::AfpyrG)
SalI
sipC
(AN6611)
(sipC::sgfp::AfpyrG)
(sipC::ctap::AfpyrG)
PvuI
sipD
(AN4632)
(sipD::sgfp::AfpyrG)
(sipD::ctap::AfpyrG)
SacI
sipE
(AN0164)
(sipC::sgfp::AfpyrG)
(sipC::ctap::AfpyrG)
B
M WT GFP TAP
8.5kb
6.1kb
4.8kb
3.6kb
2.7kb
1.9kb
3324 4336 4162 bp
5842 3908 3734 bp
4405 6095 4084 bp
4610 8123 7949 bp
3141 5061 2135 bp
C
sipA::sgfp
sipB::sgfp
sipC::sgfp
sipD::sgfp
sipE::sgfp
WT
2cm
sipA::ctap
sipB::ctap
sipC::ctap
sipD::ctap
sipE::ctap

Figure 2.2. Generation of endogenously expressed striatin interacting protein (*sip*) *sgfp* and *ctap* fusions. (A) General depiction of the wild type as well as *sgfp* and *ctap* fused *sip* loci. Scale bar (2000 bps), restriction enzymes, probes used for Southern hybridizations are shown in the graph. A small proportion of 5 un-translated region (5UTR) were used as Southern probes. **(B)** Southern hybridizations of *sip::sgfp* and *ctap* fused loci. M: Molecular marker in kbp. Sizes of the bands are in line with theoretical maps shown in A. **(C)** Growth tests of the *sip::sgfp* and *ctap* fusions in comparison to the wild type. Tagged strains behave similar to untagged strain (wild type), indicating functionality of the fusions. $5x10^3$ fungal spores were point inoculated on solid GMM plates and incubated for 5 days at 37°C under constant light.

2.4. Fungal physiology

2.4.1. Phenotypic assays

The phenotypes and quantification of sporulation for wild-type and deletion strains were examined by performing the following procedure. Fungal spores were counted using a haemocytometer. Approximately $5x10^3$ spores (5µl) were used to point inoculate solid GMM, containing appropriate supplements. Plates were incubated in the light (for asexual development) and in the dark (for sexual development) for 4-5 days at 37°C. Colonies were observed using the Olympus szx16 microscope with olympussc30 camera. Digital pictures were taken and processed with the Cell Sens Standard software (Olympus). Quantifications were performed in triplicate.

2.4.3. Stress tests

5µl of spore suspension, containing $5x10^3$ spores of the wild type, complementation and the mutant strains, were spotted on solid GMM, containing appropriate supplements and stress agents. The plates were

incubated at 37°C for 72 hours as shown in results section. Each experiment was performed in triplicate.

2.5. Protein methods

2.5.1. Immunoblotting

Primary and secondary antibodies used during this study were applied in the following conditions and dilutions: For TAP tag, the primary antibody (polyclonal rabbit α-calmodulin binding peptide (CBP), 05-932, EMD Millipore Corporation) was used as a 1:1000 dilution in TBS with 5% milk. The secondary antibody (goat α-rabbit, G-21234, Life technologies) was also used as a 1:1000 dilution in TBS and 5% milk. For GFP detection, the primary antibody (α-GFP "mouse monoclonal IgG", SC-9996, Santa Cruz Biotechnology) was used as a 1:500 dilution in TBST with 5% milk. The secondary antibody (goat anti mouse, 170-6516, Biorad) was used as a 1:1000 dilution in TBST and 5% milk. For detection of loading control SkpA protein, custom made rabbit polyclonal α-SkpA 1:2000 dilutions in TBS and 5% milk with 0.2% Tween-20 (Genescript) was used. Goat α-rabbit was used as secondary antibody for SkpA.

2.5.2. Protein extraction

2.5.2.1. For Western Blot

Fungal mycelia were obtained from liquid cultures and broken using liquid nitrogen. Protein extracts were prepared by re-suspending the smooth mycelia in protein extraction buffer (B300 buffer) which contains 300mM NaCl, 100mM Tris-Cl pH: 7.5, 10% Glycerol, 1mM EDTA, 0.1% NP-40 and added with 1mM DTT, Protease inhibitor mix (Roche), 1.5mM Benzamidine, 1x Phosphatase inhibitors mix (4-(2-Aminoethyl benzenesulfony Fluoride Hydrochloride"CAS30827-99-7" ROCHE) and

1mM PMSF. Protein concentrations were calculated by performing Bradford assays. 100 µg of total protein extract was run on various percentages SDS gels as required (10% or 12%) and transferred to protean membrane with 0.45µm pore size (GE Healthcare).

2.5.3. Tandem Affinity Purification (TAP) and GFP-Trap protocol and LC-MS/MS Protein identification

Tap tag and GFP-Trap experiments and preparation of the protein crude extracts and analysis of the proteins were performed as explained in detail (Bayram et al., 2008 and Bayram et al., 2012b).

2.6. Confocal microscopy

Green and monomeric red fluorescent protein (GFP and mRFP) expressing strains were grown in 500µl liquid GMM media, with appropriate supplements in Lab-Tek Chambered Coverglass W/CVT (Thermo Scientific) 16 hours at 25°C. Localizations of the proteins were captured and recorded as published previously (Bayram et al., 2012a).

2.7. Secondary metabolite analysis by Shimadzu Reverse Phase - High Performance Liquid Chromatography (RP-HPLC)

The wild type and all deletion strains were grown as following: $5x10^3$ spores were inoculated on agar minimal medium supplemented with vitamins and 1% Oatmeal at 37°C for 5 days. A sample was cut from the center of the culture with the back of a 15 ml falcon tube and diced into pieces. 3 ml of ddH_2O was added to these pieces and vortexed for 5 min on

ice. 3 ml chloroform was then added, samples were vortexed for a further 5 min and left to incubate on a shaker at 4°C for 30 minutes. Chloroform extracted samples were centrifuged at 5000 rpm at 4 °C for 15 min. Lower phase was removed then dried in a speed vac at medium heat for 20-30 minutes. Samples were suspended in 200 µl Methanol. Sterigmatocystin (Sigma) 1mg/ml was used as the standard (About 2.5 µl of standard added in 47.5 µl of 100% Methanol). RP-HPLC analysis was carried out on a Shimadzu RP-HPLC with a photodiode array detector (PDA). 20µl of standard or sample was injected onto a Luna®omega 5µm polar C18 (LC column 150 x 4.6m.m) and separated across at water: acetonitrile gradient with 0.1% (v/v) TriFluoroacetic Acid (TFA). Gradient conditions of 5-100% acetonitrile over 30 min with a flow rate of 1 ml/min were used with PDA detection at 254 nm.

2.8. Quantitative real time PCR

The strains were grown in GMM, containing appropriate supplements at 37°C in three different developmental stages (48h Vegetative, 24h Asexual and 48h Sexual) as three biological replicates. Approximately 100 mg of mycelia was used for RNA isolation, by using DNAse I (Cat.NO.79254, Qiagen). Following quantity estimate of RNA by using Qubit RNA BR Assay Kit Protocol (Q10210). About 1 µg RNA was used for each cDNA synthesis, Transcriptor First Strand cDNA Synthesis Kit (Roche) was used. Different primers of target genes were used for qPCR reaction as described in the user protocol, by using LightCycler®480Sypr Green I Master (Roche). All qRT-PCR experiments were performed in triplicate. House-keeping gene, *benA* was used as standard. Relative Expression Analysis was performed by Light Cycler® 480 Software.

2.9. Statistical Analysis

All experiments were proceeding on three independent occasions and the mean ± SD and standard error were used to express numerical data. The corresponding means were compared for significant differences via employment of the student t-test and One-way ANOVA methods, by using the software Graphpad Prism Version 6. The calculated "p" value of this statistical test is presented in the figures to have an exact idea about the significant level means differences. P value of less than 0.05 was set to denote significance of the difference between the compared means that referred to as: * means there is slight difference $p < 0.05$, ** means more significant difference $p < 0.005$ and *** means strong significant difference $p < 0.0005$.

3. Results

3.1.1. Striatin is required for proper fungal development and light response

Previously *strA* gene was deleted and phenotypes caused by the lack of *strA* gene were reported as: lack of fully matured fruit bodies and reduced conidiation as well as vegetative growth (Wang et al., 2010). Therefore, in order to compare and assess the phenotypes of *strA* gene with the previous study, a deletion strains of *strA* was generated in a *veA*+ background and confirmed by a Southern hybridization as described in materials and methods (**Figure 2.1**). Growth of *strA* deletion strain was similar to what was described previously. Mutant strains were characterized by reduced hyphal growth (small radial growth, $P < 0.0001$), tiny fruit bodies (cleistothecia), reduced conidiation and brownish pigment secreted into the media in comparison to wild type strain (**Figure 3.1A, B**). While wild type showed a normal light response, *strA* mutant was blind to light and produced the same level of conidia both under light and dark conditions (**Figure 3.1B**). Furthermore, conidiation level of the *strA* mutant decreased by more than 90 % under light conditions in comparison to wild type, suggesting a special role of *strA* in asexual development programme. Both *strA::sgfp* (synthetic green fluorescent fusion) and *strA::ctap* (c terminal tandem affinity purification tag) cassettes under endogenous *strA* promoter were introduced into the *strA*Δ strain to restore the mutant phenotypes (**Figure 3.1A, B**). Both cassettes successfully complemented the *strA* mutant phenotype suggesting that both fusion genes of *strA* with *sgfp* and *ctap* fulfill the functions of untagged *strA* gene in both asexual, sexual development as well as fungal growth.

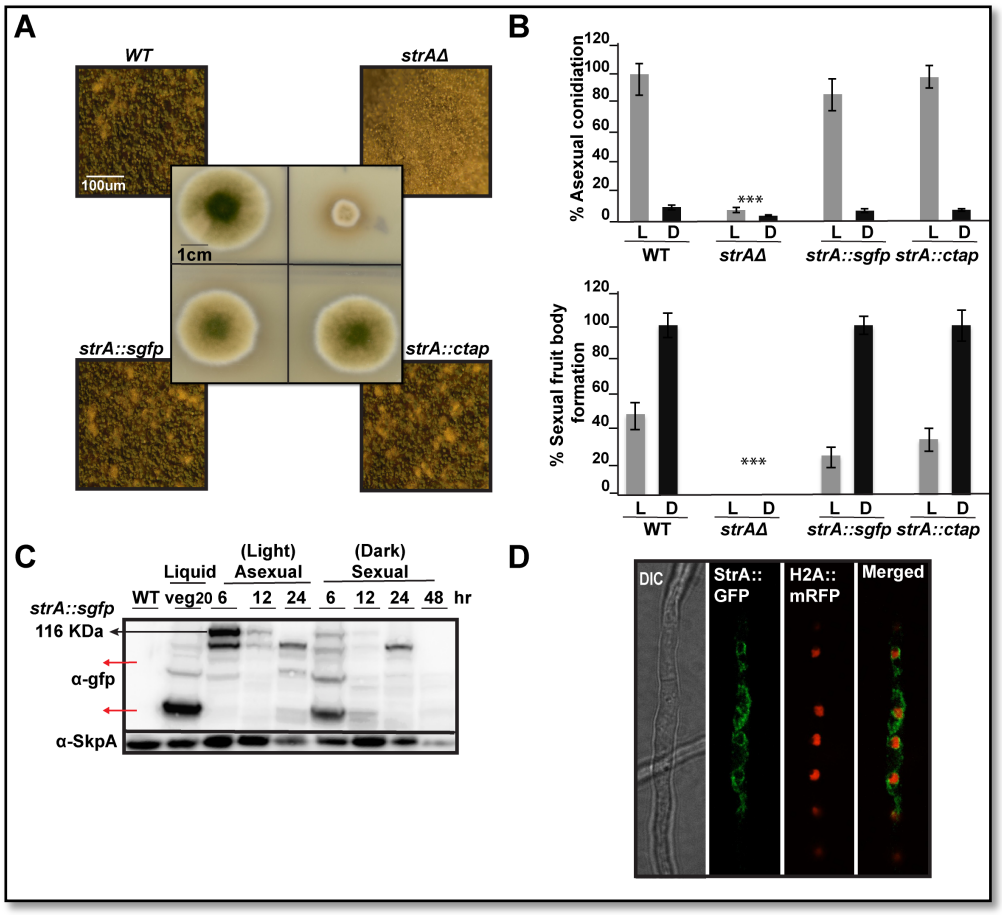


Figure 3.1. Growth, expression pattern and subcellular localization of StrA. **(A)** A comparative development of *strA*Δ, complementation (StrA::sGFP, StrA::cTAP) and wild type fungal strains. $5x10^3$ fungal spores were point inoculated on glucose minimal medium (GMM) plates for 5 days at 37°C under constant light and dark conditions (only dark is shown here). Central Square shows radial growth of colonies and small squares show the stereomicroscopic close-up pictures of the colonies. **(B)** Quantification of asexual spores and sexual fruit bodies from A. Values are the means of three replicates, and vertical bars represent standard errors. ***Represent values for significant when compared with wild type ($P < 0.0001$). **(C)** Protein expression levels of StrA fused to synthetic green fluorescent protein (sGFP) during different stages of fungal development. StrA::sGFP fusion was monitored in submerged vegetative (20h), on plates in light (asexual for 6, 12, 24 h) and in the dark (sexual 6, 12, 24, 48h). 100µg total protein

was applied on each lane. α-gfp detects StrA::sGFP fusion, α-SkpA detects SkpA levels used as loading control. Black arrow shows the full length StrA::sGFP (116 kDA). Red arrows indicate the degradation products of StrA::sGFP. **D.** Localization of StrA in living cells. StrA::sGFP localized at nuclear envelope. Histone 2A fused to monomeric red fluorescent protein (mRFP) marks the position of nucleus. Scale bar approximate 11μm.

3.1.2. StrA is highly expressed during early stage of asexual development

In order to gain insights into the expression pattern of *strA* gene, immunoblotting using the strain expressing fully functional *strA::sgfp* (c terminal fusion) was performed from different stages of development (**Figure 3.1C**). The strain was vegetatively grown in liquid submerged media for 20h, on plates under illumination (asexual development) for 6h, 12h and 24h, and under darkness (sexual development) for 6h, 12h, 24h and 48h. StrA::sGFP fusion (116 kDA) was slightly expressed during vegetative growth (20h), but showed an abundant expression pattern during early asexual development (6h) and reduced expression pattern towards the end of asexual development (24h). During early sexual development StrA::sGFP fusion was expressed at lower level and completely disappeared during late sexual development (48h). These results show that StrA protein is expressed during almost all developmental stages except for 48h sexual development.

3.1.3. The StrA is localized to nuclear envelope and possibly to endoplasmic reticulum

To understand localization pattern of StrA, the functional StrA::sGFP was used for subcellular localization experiments. StrA was previously reported

to be localized around the nucleus and endoplasmic reticulum (Wang et al., 2010). However, this study had used a constitutive promoter which expressed StrA::GFP at much higher than endogenous levels. Sometimes, overexpression of regulatory proteins might cause the mislocalization of excessive protein. Subcellular localization studies performed with a confocal laser microscope showed that StrA::sGFP was absent from the nuclei which was marked by a histone 2A fused to monomeric red fluorescent protein (H2A::mRFP) (**Figure 3.1D**). Furthermore, StrA::sGFP was associated with the periphery of the nucleus (nuclear envelope) as well as helical mesh-like endoplasmic reticulum along the hyphae. The localization study confirms the results of the previous report and show that endogenously expressed StrA is localized to nuclear envelope and endoplasmic reticulum (Wang et al., 2010).

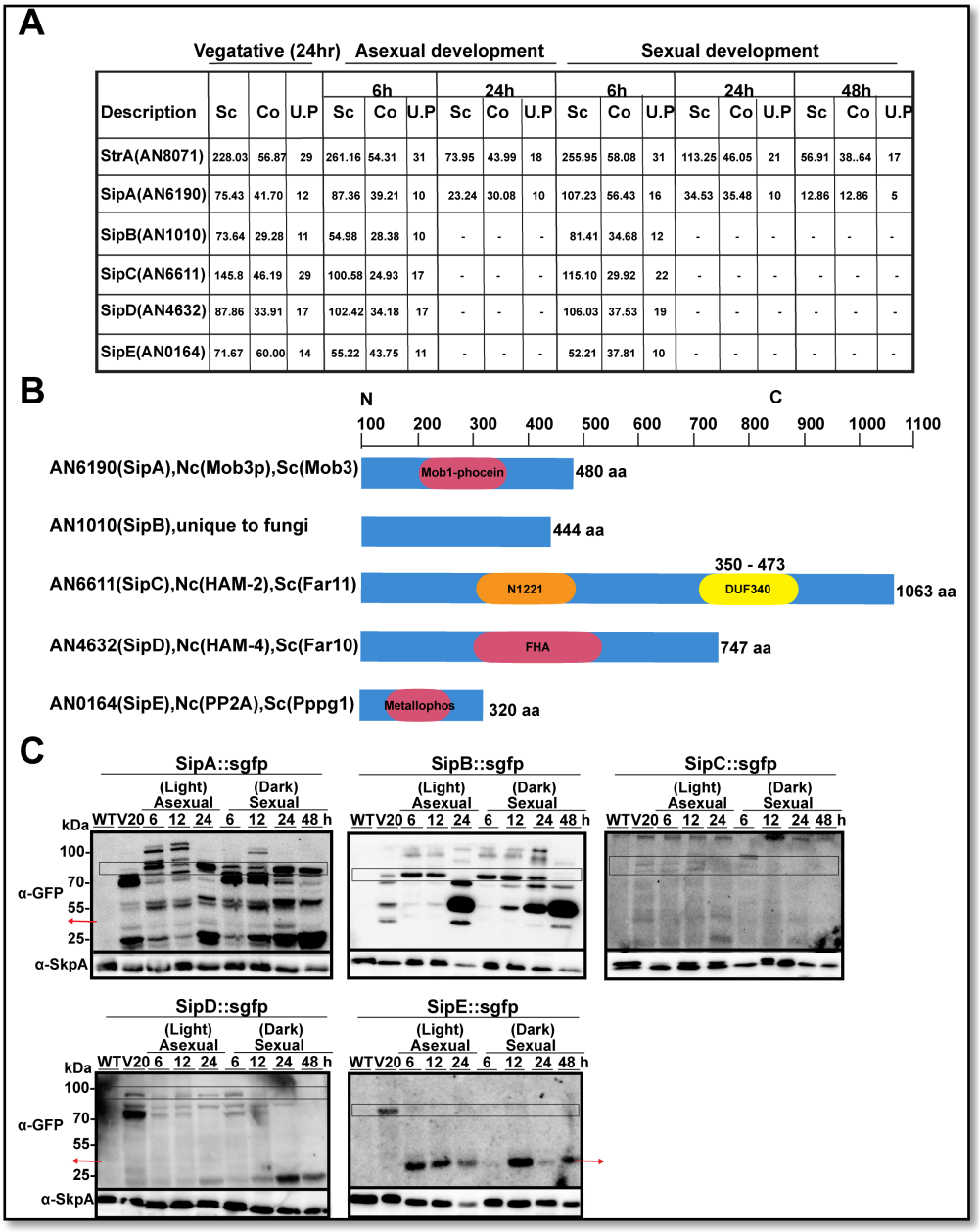
3.2.1. Determination of the striatin StrA-interacting proteins (Sips)

StrA plays an important role in eukaryotic and fungal development (Wang et al., 2010). Previous studies identified that striatin interacts with several proteins, which acts as a regulatory protein complex. To reveal the molecular interaction network of StrA and understand its molecular functions in fungal development and secondary metabolism in detail, a strain expressing fully functional StrA::cTAP fusion was used for tandem affinity purification (TAP) and mass spectrometry (MS) studies (**Figure 3.2A**). Initially, StrA-interacting proteins (Sips) were identified from vegetative cultures grown for 24h. From these TAP-MS studies five proteins associated with (AN8071) StrA were identified: (AN6190) SipA, (AN1010) SipB, (AN6611) SipC, (AN4632) SipD and (AN0164) SipE, respectively. StrA was expressed at higher levels during early stages of

asexual and sexual development (**6h, Figure 3.2C**). Therefore, in order to see if there were other interacting proteins during differentiation, TAP-MS performed from cultures grown under light and dark (6h). Interestingly, StrA interacted with the same set of proteins SipA to SipD. We further looked at late developmental stages, 24h asexual, 24 and 48h sexual stages (**Figure 3.2A**). StrA only recruited SipA during late developmental stages. In all purifications, StrA gave more peptides than any other Sip proteins.

SipA is homologous to yeast Mob3p (Phoecin) homolog in *A. nidulans* and is 480 amino acid long (**Figure 3.2B**). Mob3p, which is a kinsase co-activator, is a part of STRIPAK complex in *N. crassa* (Dettmann et al., 2013). SipB is a 444 amino acid protein with no known motif or domain. This protein is an uncharacterized protein and unique to fungi.

The third member of the complex SipC is the largest protein among the other Sips and is 1063 amino acid in length. SipC has two putative domains one N1221 and one domain of unknown function (DUF340). SipC is a homolog of *N. crassa* HAM-2, yeast Far11 and human adaptor protein group STRIP1/2. *N. crassa ham-2* is involved in ascospore formation, meiosis and vacuole organization (Xiang, 2002). *S. cerevisiae* Far11 controls recovery from cell cycle arrest and regulates intra-S DNA damage checkpoint and autophagy. SipD is 747 amino acid long and bears an FHA domain. SipD is a homolog of *N. crassa* HAM-4, yeast Far10 and human adaptor proteins group II SLMAP. Both proteins HAM4 and Far10p control similar developmental events as HAM2 and Far11p (Read, 2012). SipE is the smallest of the Sip proteins and is 320 amino acids in length. SipE bears a serine threonine phosphatase domain. SipE is a homolog of *N. crassa* PP2A, yeast Pppg1 and human PP2A triple complex. PP2A controls syncytium formation by plasma membrane fusion in *N. crassa* and glycogen accumulation in *S. cerevisiae* (Ci, 2011).



Description	Vegatative (24hr) Sc	Co	U.P	Asexual development 6h Sc	Co	U.P	24h Sc	Co	U.P	Sexual development 6h Sc	Co	U.P	24h Sc	Co	U.P	48h Sc	Co	U.P
StrA(AN8071)	228.03	56.87	29	261.16	54.31	31	73.95	43.99	18	255.95	58.08	31	113.25	46.05	21	56.91	38..64	17
SipA(AN6190)	75.43	41.70	12	87.36	39.21	10	23.24	30.08	10	107.23	56.43	16	34.53	35.48	10	12.86	12.86	5
SipB(AN1010)	73.64	29.28	11	54.98	28.38	10	-	-	-	81.41	34.68	12	-	-	-	-	-	-
SipC(AN6611)	145.8	46.19	29	100.58	24.93	17	-	-	-	115.10	29.92	22	-	-	-	-	-	-
SipD(AN4632)	87.86	33.91	17	102.42	34.18	17	-	-	-	106.03	37.53	19	-	-	-	-	-	-
SipE(AN0164)	71.67	60.00	14	55.22	43.75	11	-	-	-	52.21	37.81	10	-	-	-	-	-	-

Figure 3.2. Discovery and expression levels of StrA interacting proteins (Sips) during fungal development. (A) Identification of the StrA interacting proteins by tandem affinity purification (TAP). Strain expressing functional StrA::cTAP fusion was grown in GMM vegetatively (24 h) and induced for both light (6 and 24 h) and dark (6, 24 and 48h) development at 37°C. TAP-MS was performed as described in MM. Sc: Score, Co: Coverage and UP: unique peptides numbers. **(B)** Domain

structure of the striatin (StrA) and striatin interacting proteins (Sips) SipA , SipB, SipC, SipD and SipE. N; N-terminus, C; C-terminus, (aa) Number of amino acids. Mob1: Monopolar spindle one-binder protein; N1221: Acidic domain with possible transmembrane domains; DUF340: Unknown Function protein; FHA: Forkhead-associated domain. **(C)** Expression patterns of SipA to SipE proteins during fungal development. Similar experimental set up was used as in Figure 1 for developmental induction. sGFP fusions of Sip proteins (100µg total protein) were used for time course immunoblotting. α-sgfp antibody detects fusion proteins and α-SkpA shows equal loading on each lane.

3.2.2. SipA and SipB are abundantly expressed whereas SipC, SipD and SipE are poorly expressed during late fungal development

Since SipA was the only interaction partner of StrA during late developmental stages, we wondered whether this was due to the expression patterns of the Sip proteins. Therefore, c terminally sGFP tagged versions of SipA to SipE were constructed and used to replace the original loci of these genes, which were confirmed by the Southern hybridization (**Figure 2.2A-B**). Functionality of the fusions were verified by growing the strains expressing SipA to SipE::sGFP and comparing them with the wild type (**Figure 2.2C**). Expressions of Sip proteins were monitored during 20h vegetative submerged culture, 6h, 12h and 24h asexual development under light and 6h, 12h, 24h and 48h sexual development in the dark (**Figure 3.2C**). A 79kDa SipA-GFP protein was present during all developmental stages, a 76kDa SipB-GFP fusion was also present almost all developmental time points except for late asexual and sexual stages (24 and 48h respectively). SipC-GFP fusion was poorly expressed during all developmental time points with an approximately 145kDa molecular weight. A 107kDa SipD-GFP fusion was constantly expressed during all stages except for 48h sexual development. Interestingly, 63kDa SipE-GFP

fusion was only present during vegetative growth and degraded and disappeared during both asexual and sexual developmental stages (**Figure 3.2C, lower panel**). These results show that both SipA and SipB are abundant proteins whereas SipD is expressed at lower relative levels. Interestingly, both SipC and SipE show a weak expression pattern, suggesting that they might have important developmental roles in *A. nidulans*.

3.3. Reciprocal TAP-LC-MS's of SipA to SipE confirm the presence of STIPAK complex made of StrA-SipA-SipB-SipC-SipD-SipE

Tandem Affinity Purification (TAP) of StrA coupled with LC-MS identified five proteins SipA to SipE as interaction partners. In order to further confirm the existence of this complex and to gain insights into the more complex physical interactions of the Sip proteins, a reciprocal TAP-LC-MS was performed. To achieve this all five members of the complex *sipA* to *sipE* were fused to *ctap* and replaced the native loci of these genes, which were all similar to the wild type, suggesting fully functional nature of these fusions (**Figure 2.2C**). TAP of SipA (16 peptides) recruited StrA (34 peptides) along with SipB (12 peptides), SipC (22 peptides), SipD (17 peptides) and SipE (6 peptides) (Table 4). TAP of SipB (19 peptides) similarly pulled down StrA (31 peptides) and other Sips, SipA (18 peptides), SipC (30 peptides), SipD (24 peptides) and SipE (11 peptides). StrA (23 peptides), SipA (10 peptides), SipB (10 peptides), SipD (14 peptides), SipE (13 peptides) were found in TAP of SipC (44 peptides). Both SipD and SipE also recruited other members of the complex (**Table 3.1**). These TAP-MS results clearly underline that a hexameric STRIPAK complex made of a striatin StrA, a Mob1 kinase homolog SipA, a novel

protein SipB, a HAM2 homolog SipC, a HAM4 homolog SipD and a PP2A homolog SipE.

Table 3.1. Confirmation of STRIPAK complex by reciprocal TAP-LC-MS

Protein ID	SipA tag			SipB tag			SipC tag			SipD tag			SipE tag		
	Sc	Co	U.P	Sc	Co	U.P	Sc	Co	U.P	Sc	Co	U.P	Sc	Co	U.P
StrA (AN8071)	184.58	58.20	34	278.84	56.50	31	120.94	42.04	23	246.84	61.12	34	243.52	55.04	29
SipA (AN6190)	91.69	56.85	16	152.62	61.62	18	32.13	41.29	10	91.26	53.32	15	126.96	54.56	16
SipB (AN1010)	62.25	33.11	12	114.47	51.35	19	36.33	25.45	10	64.79	30.86	13	79.03	35.14	15
SipC (AN6611)	79.97	34.71	22	162.24	43.74	30	326.97	65.00	44	190.77	49.95	30	196.69	48.54	31
SipD (AN4632)	70.21	31.37	17	133.19	43.97	24	62.76	21.72	14	143.97	48.39	26	110.98	42.63	22
SipE (AN0164)	16.99	30.00	6	42.35	48.44	11	71.88	61.56	13	57.15	61.88	14	128.84	67.50	18

Sc marks Score, Co indicates to Coverage and U.P marks to unique peptides numbers.

3.4. All members of STRIPAK complex are required for proper development except for SipA

Striatin and STRIPAK complex play a major role in eukaryotic cellular processes. Striatin mutants show reduced hyphal growth, diminished asexual conidiation and tiny cleistothecia, which are devoid of ascospores. To understand the roles of STRIPAK complex members, *sipA* to *sipE* in fungal development, single deletions as well as double deletions of *sipA* to *sipE* were created and confirmed by the Southern hybridization (**Figure**

2.1). Furthermore, to understand the genetic interdependency between striatin (*strA*) and *sipA* to *sipE*, their double deletion combinations were created.

All single and double deletion strains were subjected to developmental tests by incubating them both in light and dark (**Figure 3.3A**). Growth rates, asexual conidiation and sexual development of the mutants were analyzed (**Figure 3.3B-D**). Ablation of *sipB*, *sipC*, *sipD* and *sipE* all resulted in similar phenotypes seen in *strA*Δ strain, characterized by slower growth rate, reduced conidiation and lack of sexual fruit bodies ($P < 0.0001$) (**Figure 3.3**). Mutant phenotypes were restored by introducing the genetic loci of the respective genes back into deletion strains (**Figure 3.4**). Furthermore, their double deletion combinations with each other and *strA* exhibited similar phenotypes to single deletion strains. Surprisingly, *sipA* deletion showed an opposite phenotype. Radial growth rate of *sipA* mutant slightly increased (**Figure 3.3B**) and produced two-fold more asexual conidia (* $P < 0.0288$) in comparison to the wild type (**Figure 3.3C**). In contrast to mutants of the other members, it did not show any defects in sexual fruit body formation (**Figure 3.3C**). Moreover, *strA*Δ/*sipA*Δ double mutant showed the phenotypes of *sipA* single mutant, suggesting epistatic effect of *sipA* over the *strA* gene. Even *strA*Δ/*sipA*Δ double mutant produced more asexual spores (*** $P < 0.0001$) than *sipA* single deletion. Interestingly, double mutant combinations of *sipA* with *sipB*, *sipC*, *sipD* and *sipE* all displayed similar defects of single deletions, showing that *sipA* is not epistatic to all other four members of the STRIPAK complex.

Detailed phenotypic dissection of the single and double deletion strains showed that all members of the STRIPAK complex are equally important for growth and light-dependent development except for SipA

which shows epistacy to StrA but not the other components of the STRIPAK complex.

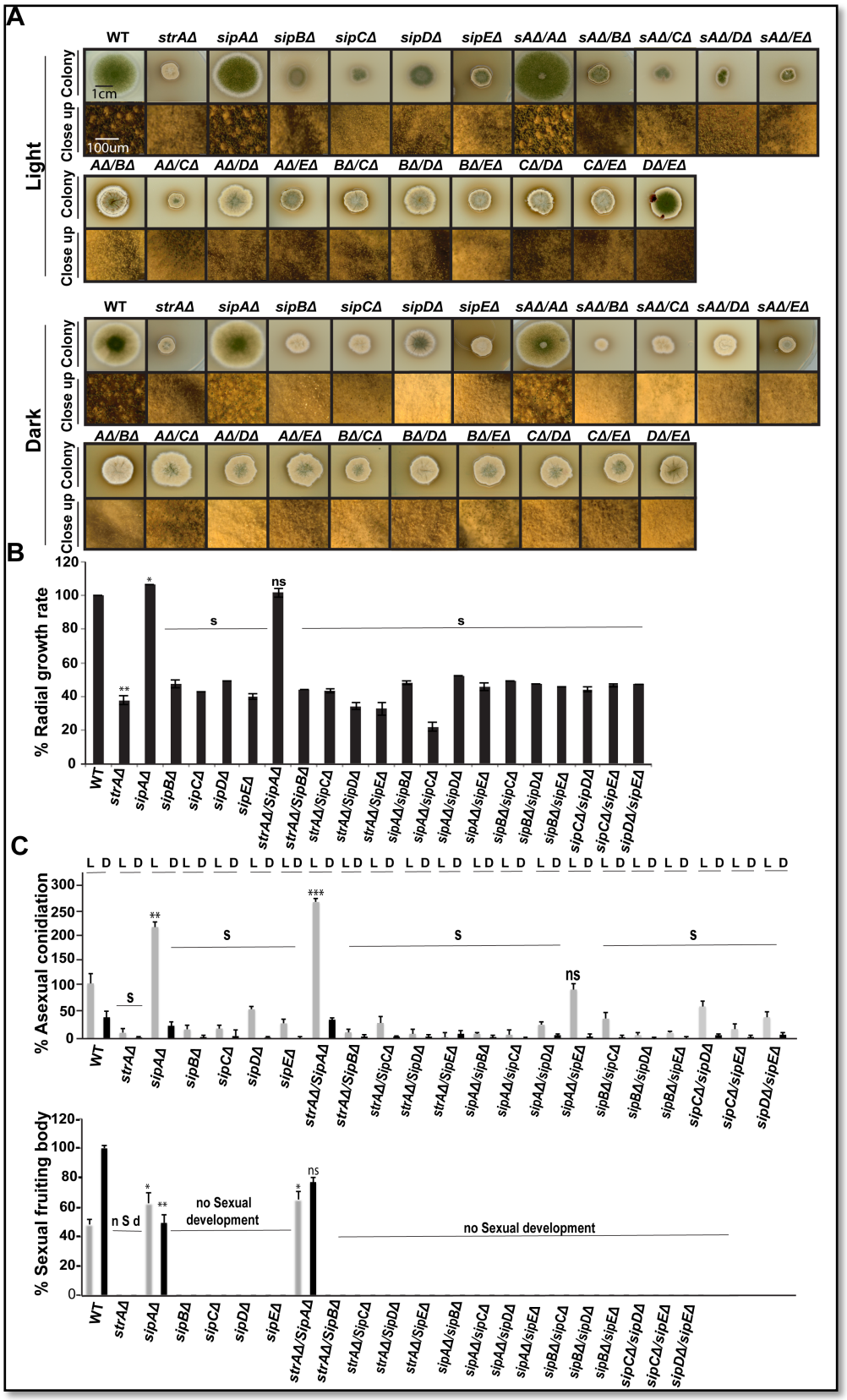

A
Light
Dark
Colony
Close up
WT
strAΔ
sipAΔ
sipBΔ
sipCΔ
sipDΔ
sipEΔ
sAΔ/AΔ
sAΔ/BΔ
sAΔ/CΔ
sAΔ/DΔ
sAΔ/EΔ
AΔ/BΔ
AΔ/CΔ
AΔ/DΔ
AΔ/EΔ
BΔ/CΔ
BΔ/DΔ
BΔ/EΔ
CΔ/DΔ
CΔ/EΔ
DΔ/EΔ
1cm
100um
B
% Radial growth rate
C
% Asexual conidiation
% Sexual fruiting body
L D
n S d
no Sexual development
ns
s

Figure 3.3. Role of STRIPAK complex in light dependent fungal development. (A) Light and dark induced development of single and double mutants of STRIPAK complex. $5x10^3$ fungal spores were point inoculated on GMM plates for 5 days at 37°C under constant light and dark conditions. Upper squares show colony development (scale 1 cm) from plates and lower squares are close up images of the colonies (200 µm). Whitish round structures represent fruit bodies. *sA*Δ represents *strA*Δ, *A*Δ represents *sipA*Δ, *B*Δ represents *sipB*Δ, CΔ; *sipC*Δ, *D*Δ; *sipD*Δ, *E*Δ; *sipE*Δ. **(B)** Quantification of comparative radial growth rate of the single and double mutants from light induced plates. NS; not significant, S; significant. Wild type growth rate serves as 100% standard. **(C)** Quantification of asexual and sexual development from STIPAK single and double mutants. L; light, D; Dark, S; significant. Asexual sporulation of WT in light represent 100% sporulation. Sexual fruit body formation of WT in dark used as 100%. Values are the means of three replicates, and error bars represent standard errors.

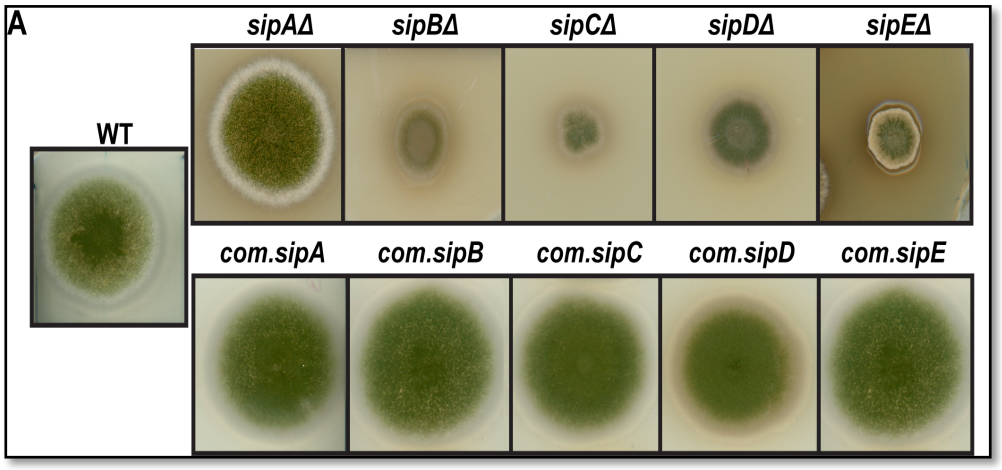


Figure 3.4. Complementation of *sip* deletion strains. $5x10^3$ spores of complemented and mutant strains were point inoculated and grown for 5 days at 37°C and pictures were taken from only asexual plates.

3.5. STRIPAK complex controls expression of master regulators of asexual and sexual development

All mutants of STRIPAK complex except for *sipA*, trigger a drastic reduction in asexual sporulation and loss of sexual development. Asexual and sexual development were regulated by a cascade of transcription factors. Therefore, expressions of several important asexual and sexual regulators were examined in STRIPAK single deletion strains. Expression of four major transcription factors *abaA*, *brlA*, *flbA* and *flbC* important for conidiophore development and conidiogenesis were analyzed during 24h asexual development (**Figure 3.5A**). Expression of three out of four, *abaA*, *brlA*, *flbA* showed significant reduction in the absence of *strA* ($P < 0.0003$, $P < 0.0003$ and $P < 0.0004$, respectively). *brlA* and seminal transcription factor *abaA* responsible for spore separation in conidiophores was reduced in all tested deletants except for *sipA*. *sipA* presented 2-3 fold higher *brlA* and *abaA* expression. Likewise, *brlA* and *flbA* were also reduced in most of the mutants excluding *sipA* and *sipD*. Only *flbC* showed a mild decrease in its expression in mutants excluding *strA* deletion.

Similar to asexual regulators, four important transcription factors essential for sexual development, *nsdD*, *nsdC*, *matB* and *steA* were tested in cultures of STRIPAK mutants induced for sexual development in the dark for 48 h (**Figure 3.5B**). Expression levels of *nsdD* and *steA* were diminished in almost all mutants of the STIPAK complex excluding *sipA* and *sipE*, which showed higher expression of these genes. *nsdC* was only lessened in *sipB* and *sipC* mutants. Interestingly, only *matB* expression had a general tendency to rise in the mutant strains.

These expression data propose that reduced asexual sporulation and lack of sexual development seen in STRIPAK mutants are as a result of

either reduced or imbalanced expression of developmental transcription factors.

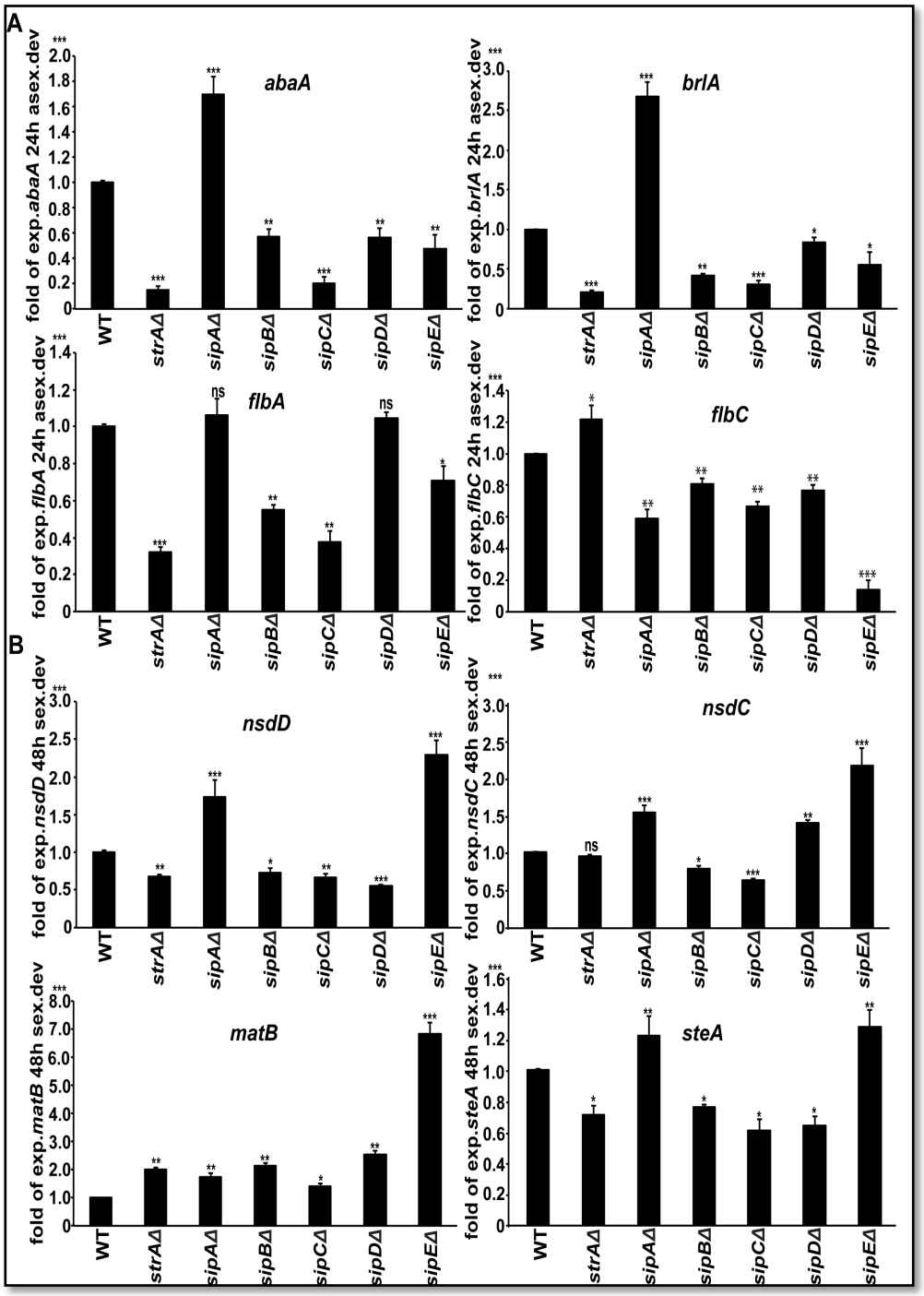


Figure 3.5. Expressional analysis of asexual and sexual development regulatory genes by qRT-PCR. (A) Expression of asexual regulatory transcription factor genes *abaA, brlA, flbA* and *flbC* in STRIPAK single mutants and wild type. **(B)** Expression levels of sexual regulatory transcription factors *nsdD, nsdC, matB* and *steA* in mutants and WT. All tested strains were grown on appropriate GMM solid media in light for 24 hours asexually or in dark for 48 hours sexually. Experiments were carried out in biological triplicates. The statistical significance is indicated as (*) $p < 0.05$, (**) $p < 0.001$, (***) $p < 0.0001$ and non-significant (ns) $p > 0.05$ compared with wild type.

3.6. STIPAK complex is involved in response to various environmental stress factors

The striatin complex plays important roles in growth and developmental programmes. In order to gain further insights into the function of STRIPAK complex in stress response pathways, it was investigated which stress responses were influenced in the absence of StrA and its associated proteins SipA to SipE. Single and double mutant strains were subjected to various stress conditions in solid minimal media for 5 days at 37°C (**Figure 3.6**).

To confer oxidative stress, Hydrogen peroxide (H_2O_2) and Menadione sodium bisulfate were applied to mutant strains. The radial growth of the all single and double deletion strains were significantly reduced in 1 mM H_2O_2 and 0.08 mM Menadione sodium bisulfate, when compared with the wild type and complementation strains. Only *sipA* deletion had increased radial diameter and was less sensitive to oxidative stress. Furthermore, *strA*Δ and its double deletion combinations with *sip* genes were much more sensitive to H_2O_2 and Menadione sodium bisulfate, which did not allow any colony formation (**Figure 3.6A&B**).

Cell wall stress responses of the all double and single deletions were measured by growing them on MM containing several cell wall stressors such as Congo Red, Calcoflour and Sodium Dodecyl Sulfate (SDS). Growth on Congo Red had most drastic effects causing total loss of growth for *strA* deletion and its double deletion combinations with sips (**Figure 3.6C**). *sipA*Δ was resistant to cell wall stressors more than wild type.

Strains were also monitored to see how they cope with DNA damage stress. To achieve this, 5.2 mM Hydroxyurea (HU), alkylating agents 0.03% Methyl methanesulfonate (MMS), 0.01% Ethyl methanesulfonate (EMS) were used as DNA damaging agents. *strA* double deletions with *sip* genes, interestingly including the *strA*/*sipA* double mutant were extremely sensitive to all three kind of stressors HU, MMS and EMS (**Figure 3.7**). Again *sipA* single deletion displayed more vegetative growth than the wild type strain under all tested conditions.

Mutant strains of STRIPAK genes were further tested to assess their responses against various stresses such as amino acid starvation using 3-amino 1,2,4 triazole (3-AT), Caffeine and osmotic stress 1M NaCl (**Figure 3.8**). Amino acid starvation mainly influenced *strA/sip* double deletions similar to previous stress treatments. Caffeine treatment did not influence the *strA* double mutants as much as the other stress conditions. All of the deletion strains were more sensitive to osmotic stress induced by NaCl than the wild type and complementation strains. Furthermore, *strA/sipB* double mutant was highly sensitive to osmotic stress (*** $p < 0.0001$).

These data clearly show that the lack of striatin together with one of the *sip* genes results in drastic problems combating various stress conditions. Furthermore, *strA*/*sipA* double deletion, which grows as well as

the wild type strain under non-stress conditions, exhibits the most dramatic sensitivity against stress conditions, suggesting an essential role of *sipA* regulating stress responses in the absence of striatin.

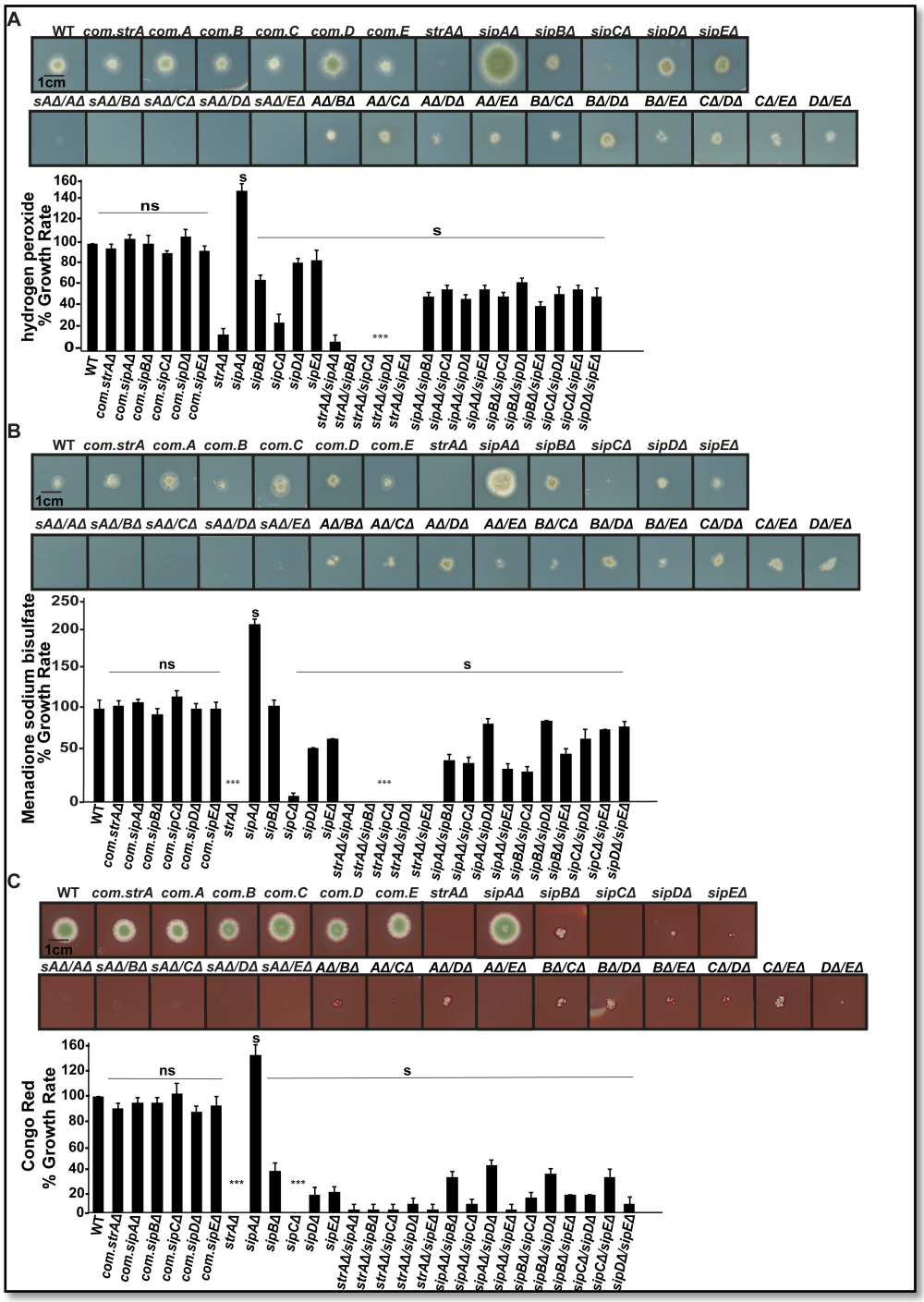


Figure 3.6. Growth responses of the STRIPAK complex mutants in the presence of reactive oxygen species (ROS) and cell wall stress. (A) Sensitivity tests of STRIPAK mutants and their complementation in the presence of hydrogen peroxide (H_2O_2, 1 mM) measured as radial growth rate. **(B)** Oxidative stress test in Menadione (0.08 mM). **(C)** Growth of the mutants in the presence of cell wall stress agent Congo red, 20ug/ml). The cultures ($5x10^3$ spores) were grown for 5 days at 37°C in light. Scale bar 1 cm. These experiments were repeated at least three times with the same results. Chart graphs show radial growth diameter compared with wild type, which was used as standard (100%). Data are indicated as average ± SD of three independent biological repetitions. Columns with (ns) denote non-significant but (n) denote significant difference and also (***) represent values for strong significant difference compared with wild type.

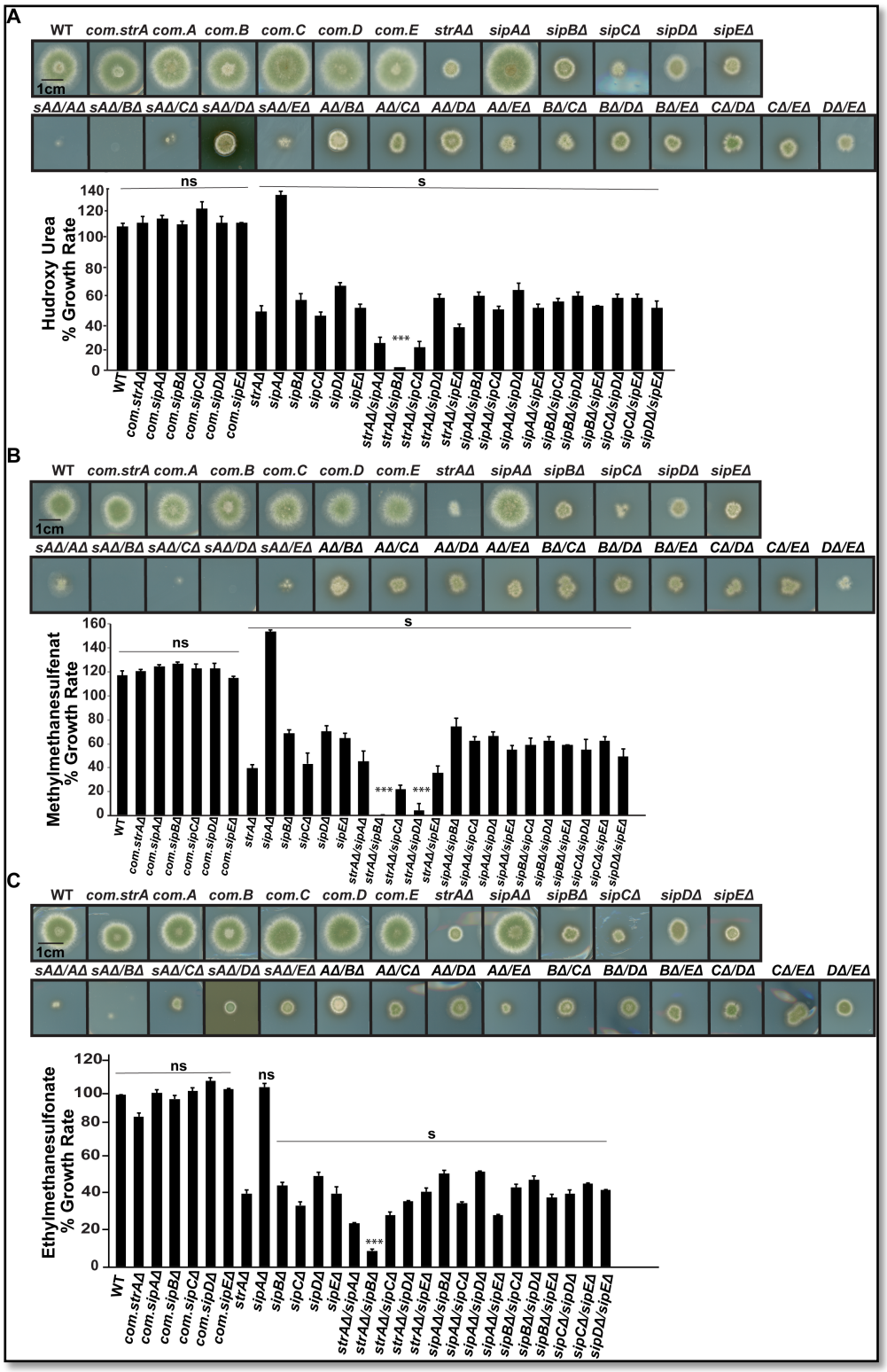

A
WT com.strA com.A com.B com.C com.D com.E strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ
1cm
sAΔ/AΔ sAΔ/BΔ sAΔ/CΔ sAΔ/DΔ sAΔ/EΔ AΔ/BΔ AΔ/CΔ AΔ/DΔ AΔ/EΔ BΔ/CΔ BΔ/DΔ BΔ/EΔ CΔ/DΔ CΔ/EΔ DΔ/EΔ
Hudroxy Urea % Growth Rate
ns
s
***
WT com.strAΔ com.sipAΔ com.sipBΔ com.sipCΔ com.sipDΔ com.sipEΔ strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ strAΔ/sipAΔ strAΔ/sipBΔ strAΔ/sipCΔ strAΔ/sipDΔ strAΔ/sipEΔ sipAΔ/sipBΔ sipAΔ/sipCΔ sipAΔ/sipDΔ sipAΔ/sipEΔ sipBΔ/sipCΔ sipBΔ/sipDΔ sipBΔ/sipEΔ sipCΔ/sipDΔ sipCΔ/sipEΔ sipDΔ/sipEΔ
B
WT com.strA com.A com.B com.C com.D com.E strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ
1cm
sAΔ/AΔ sAΔ/BΔ sAΔ/CΔ sAΔ/DΔ sAΔ/EΔ AΔ/BΔ AΔ/CΔ AΔ/DΔ AΔ/EΔ BΔ/CΔ BΔ/DΔ BΔ/EΔ CΔ/DΔ CΔ/EΔ DΔ/EΔ
Methylmethanesulfenat % Growth Rate
ns
s
***
WT com.strAΔ com.sipAΔ com.sipBΔ com.sipCΔ com.sipDΔ com.sipEΔ strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ strAΔ/sipAΔ strAΔ/sipBΔ strAΔ/sipCΔ strAΔ/sipDΔ strAΔ/sipEΔ sipAΔ/sipBΔ sipAΔ/sipCΔ sipAΔ/sipDΔ sipAΔ/sipEΔ sipBΔ/sipCΔ sipBΔ/sipDΔ sipBΔ/sipEΔ sipCΔ/sipDΔ sipCΔ/sipEΔ sipDΔ/sipEΔ
C
WT com.strA com.A com.B com.C com.D com.E strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ
1cm
sAΔ/AΔ sAΔ/BΔ sAΔ/CΔ sAΔ/DΔ sAΔ/EΔ AΔ/BΔ AΔ/CΔ AΔ/DΔ AΔ/EΔ BΔ/CΔ BΔ/DΔ BΔ/EΔ CΔ/DΔ CΔ/EΔ DΔ/EΔ
Ethylmethanesulfonate % Growth Rate
ns
ns
s
***
WT com.strAΔ com.sipAΔ com.sipBΔ com.sipCΔ com.sipDΔ com.sipEΔ strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ strAΔ/sipAΔ strAΔ/sipBΔ strAΔ/sipCΔ strAΔ/sipDΔ strAΔ/sipEΔ sipAΔ/sipBΔ sipAΔ/sipCΔ sipAΔ/sipDΔ sipAΔ/sipEΔ sipBΔ/sipCΔ sipBΔ/sipDΔ sipBΔ/sipEΔ sipCΔ/sipDΔ sipCΔ/sipEΔ sipDΔ/sipEΔ

Figure 3.7. Growth responses of the STRIPAK complex mutants in the presence of DNA damaging stressors. (A) Sensitivity tests of STRIPAK mutants and their complementation in Hydroxyurea (HU, 5.2 mM) **(B)** in Methyl methane sulfonate (MMS, 0.03% mM) and **(C)** Ethyl methanesulfonate (EMS0.01%). The cultures ($5x10^3$ spores) were grown for 5 days at 37°C in light. Scale bar 1 cm. These experiments were repeated at least three times. Chart graphs show radial growth diameter compared with wild type, which was used as standard (100%). Data are indicated as average ± SD of three independent biological repetitions. Columns with (ns) denote non-significant but (n) denote significant difference and also (***) represent values for strong significant difference compared with wild type.

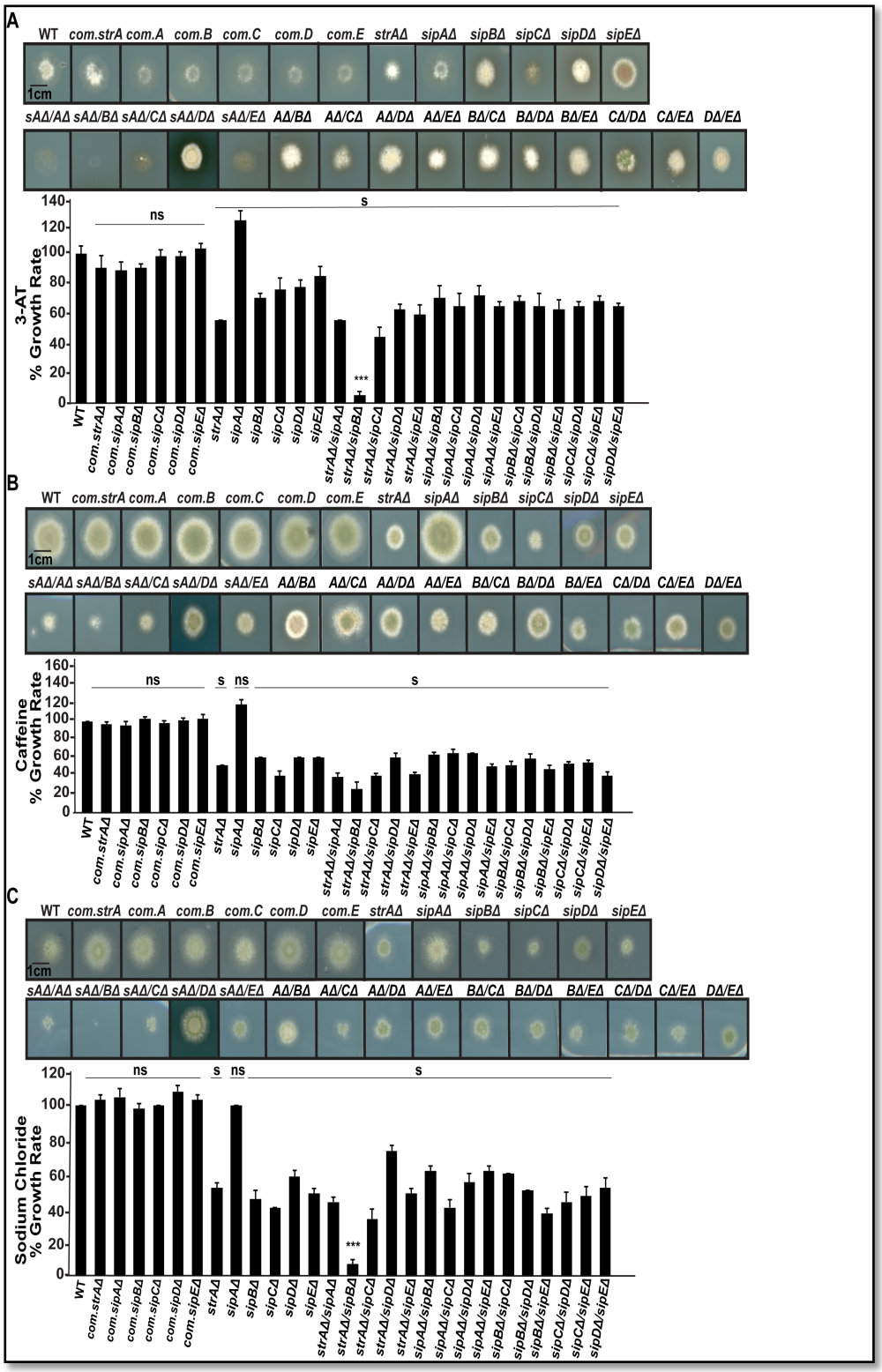

A
WT com.strA com.A com.B com.C com.D com.E strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ
1cm
sAΔ/AΔ sAΔ/BΔ sAΔ/CΔ sAΔ/DΔ sAΔ/EΔ AΔ/BΔ AΔ/CΔ AΔ/DΔ AΔ/EΔ BΔ/CΔ BΔ/DΔ BΔ/EΔ CΔ/DΔ CΔ/EΔ DΔ/EΔ
3-AT % Growth Rate
ns
s
***
WT com.strAΔ com.sipAΔ com.sipBΔ com.sipCΔ com.sipDΔ com.sipEΔ strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ strAΔ/sipAΔ strAΔ/sipBΔ strAΔ/sipCΔ strAΔ/sipDΔ strAΔ/sipEΔ sipAΔ/sipBΔ sipAΔ/sipCΔ sipAΔ/sipDΔ sipAΔ/sipEΔ sipBΔ/sipCΔ sipBΔ/sipDΔ sipBΔ/sipEΔ sipCΔ/sipDΔ sipCΔ/sipEΔ sipDΔ/sipEΔ
B
WT com.strA com.A com.B com.C com.D com.E strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ
1cm
sAΔ/AΔ sAΔ/BΔ sAΔ/CΔ sAΔ/DΔ sAΔ/EΔ AΔ/BΔ AΔ/CΔ AΔ/DΔ AΔ/EΔ BΔ/CΔ BΔ/DΔ BΔ/EΔ CΔ/DΔ CΔ/EΔ DΔ/EΔ
Caffeine % Growth Rate
ns s ns s
WT com.strAΔ com.sipAΔ com.sipBΔ com.sipCΔ com.sipDΔ com.sipEΔ strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ strAΔ/sipAΔ strAΔ/sipBΔ strAΔ/sipCΔ strAΔ/sipDΔ strAΔ/sipEΔ sipAΔ/sipBΔ sipAΔ/sipCΔ sipAΔ/sipDΔ sipAΔ/sipEΔ sipBΔ/sipCΔ sipBΔ/sipDΔ sipBΔ/sipEΔ sipCΔ/sipDΔ sipCΔ/sipEΔ sipDΔ/sipEΔ
C
WT com.strA com.A com.B com.C com.D com.E strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ
1cm
sAΔ/AΔ sAΔ/BΔ sAΔ/CΔ sAΔ/DΔ sAΔ/EΔ AΔ/BΔ AΔ/CΔ AΔ/DΔ AΔ/EΔ BΔ/CΔ BΔ/DΔ BΔ/EΔ CΔ/DΔ CΔ/EΔ DΔ/EΔ
Sodium Chloride % Growth Rate
ns s ns s
***
WT com.strAΔ com.sipAΔ com.sipBΔ com.sipCΔ com.sipDΔ com.sipEΔ strAΔ sipAΔ sipBΔ sipCΔ sipDΔ sipEΔ strAΔ/sipAΔ strAΔ/sipBΔ strAΔ/sipCΔ strAΔ/sipDΔ strAΔ/sipEΔ sipAΔ/sipBΔ sipAΔ/sipCΔ sipAΔ/sipDΔ sipAΔ/sipEΔ sipBΔ/sipCΔ sipBΔ/sipDΔ sipBΔ/sipEΔ sipCΔ/sipDΔ sipCΔ/sipEΔ sipDΔ/sipEΔ

Figure 3.8. Comparison of the radial growth rates of STRIPAK mutants which were exposed to amino acid starvation and osmotic stress agents. (A) Growth behavior of the STRIPAK mutants in 3-amino 1,2,4 triazole (3-AT, 1 mM) containing GGM media, **(B)** Caffeine containing media and **(C)** in osmotic stress NaCl (1 M) media. Strains were grown and statistically analyzed as in Figure 2.2 and 3.1.

3.7. STRIPAK complex is required for proper expression of reactive oxygen species (ROS) scavenging enzyme-encoding genes

Sensitivity of the STRIPAK complex mutants against various cellular stressors including the oxidative stress raised the question how the expression of reactive oxygen species (ROS) scavenging enzyme genes were regulated in these mutants. Therefore, expression of four ROS scavenging enzyme encoding genes, two catalases *catB*, *catC* and two superoxide dismutase *sodA* and *sodB* were quantified during 48 h vegetative growth (**Figure 3.9**). Expression of *catC* and *sodB* were significantly decreased in deletions of STRIPAK complex genes. In contrast, *catB* was up regulated in all mutants whereas *sodA* showed a variable pattern where it was down regulated in *sipB* as well as *sipC*. Interestingly, *strA* deletion strain expressed both *catB* and *sodA* at normal levels. *sipA* showed higher levels of expression for *catC*, *sodA* and *sodB* but not for *catB*.

These expression data demonstrate that STRIPAK complex is required for expression of at least two ROS scavenging enzyme-encoding genes indicating that many other ROS scavenging enzymes may be misregulated in the absence of STRIPAK complex.

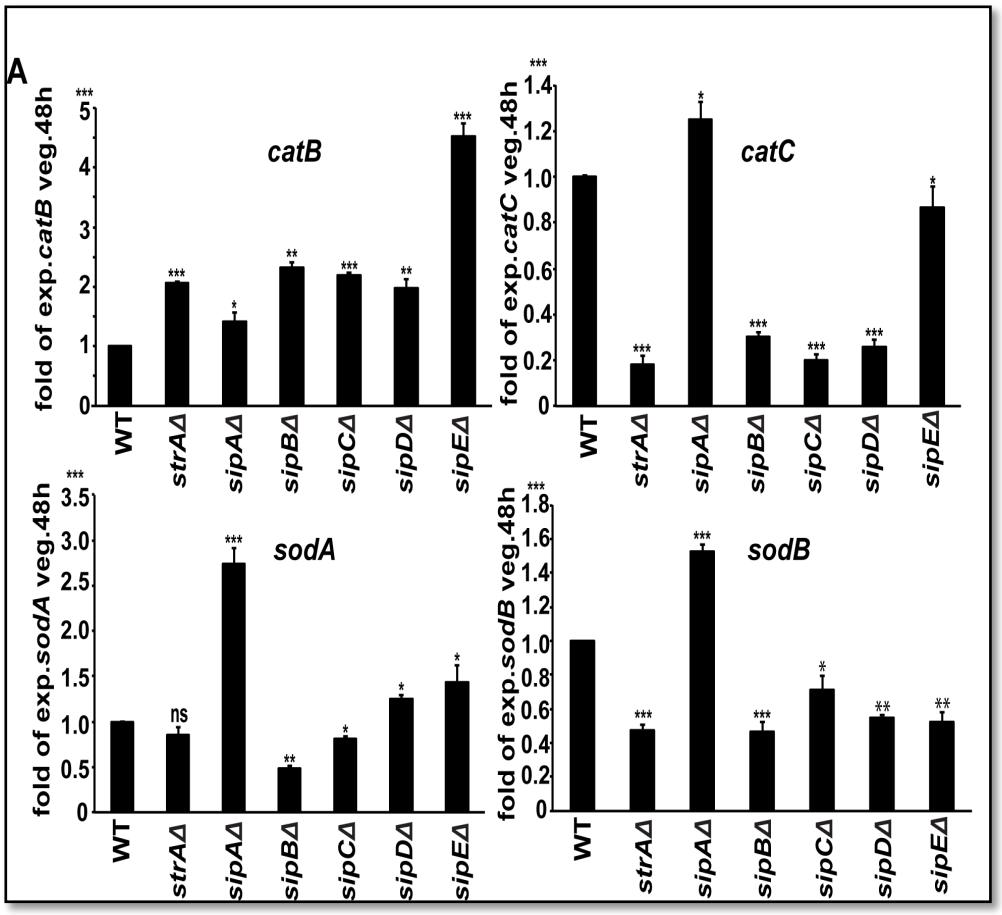


Figure 3.9. Levels of reactive oxygen scavenging (ROS) enzyme encoding genes in STRIPAK mutants by q-RNA-PCR. Expression levels of two catalase (cat) encoding genes, *catB* and *catC*, two superoxide dismutase (sod) encoding genes *sodA* and *sodB* in STRIPAK mutants. Mutant strains and wild type were grown in appropriate liquid media for 48 hours at 37°C vegetatively. Experiments were carried out in biological triplicates and the statistical significance of (*) $p < 0.05$, (**) $p < 0.001$, (***) $p < 0.0001$ and (ns) $p > 0.05$.

3.8. STRIPAK complex is essential for secondary metabolite production

Fungal development and SM production are coordinately regulated by the regulatory protein complexes. A defect in fungal development particularly in fruit body formation is often associated with changes in SM production. All members of STRIPAK complex are involved in growth, fruit body formation, asexual conidiation and stress responses excluding the SipA protein. To verify the previous hypothesis whether STRIPAK complex plays any role in production of SMs, fungal mycotoxin sterigmatocystin (ST) levels were measured by extracting metabolite in chloroform and running the extracts in HPLC (**Figure 3.10A**). Production of ST was significantly reduced ($p < 0.0001$) in comparison to the wild type.

strA and *sipA* mutants produced less ST than wild type but more than *sipB*, *sipC*, *sipD* and *sipE* mutants. ST production of *strA*/*sipA* double mutant was slightly less than their single mutants. However, double deletion of *strA* with *sipB*, *sipC*, *sipE* resulted in extremely reduced ST (less than 10 % of wild type). Interestingly, *sipA*Δ/*sipC*Δ strain recorded lowest ST production rate at about < 5 %.

The velvet complex is one of the key regulatory complex controlling both fungal development and SM production (Bayram and Braus 2012). A lack of each member, *veA*, *velB* and *laeA* results in loss of ST production caused by a reduced expression of transcription factor *aflR*, which induces expression of the ST gene cluster (Bayram et al, 2008). To see how velvet complex expression is regulated in the mutants of STRIPAK complex, *veA*, *laeA*, *velB* were quantified from 48 h vegetative cultures by qRT-PCR (**Figure 3.10B**). Expression of the velvet complex was generally diminished in the absence of STRIPAK complex. Most drastic down regulation was

observed in *velB* and *veA* expression. To see if the reduced expression of the velvet complex is translated into ST gene cluster, transcription factor *aflR* and two structural genes *stcQ* and *stcE* were quantified (**Figure 3.10C**). Indeed, *aflR*, *stcQ* and *stcE* expressions sharply dropped in STRIPAK complex mutants except for *sipA* deletion. ß-lactam antibiotic penicillin (PN) and antitumour polyketide terrequinone (TQ) are two other important SMs produced by *A. nidulans*. To understand if the STRIPAK complex is required for production of other SMs, expression of PN and TQ gene cluster were examined in STRIPAK complex mutants (**Figure 3.10D and E**). Surprisingly, expression of *acvA*, *ipnA* and *attA* required for PN production was reduced in STRIPAK mutants. *ipnA* expression in *sipB*Δ and *aatA* expression in *sipD*Δ did not change. Similar to PN, *tdiA* and *tdiB* genes of TQ cluster were highly down regulated in STRIPAK mutants except in the *sipA* mutant, which was higher (**Figure 3.10E**).

These metabolite and expression data demonstrate that STRIPAK complex is vital for production of ST and expression of the velvet complex components. Furthermore, expression of at least three different gene clusters ST, PN and TQ need an intact STRIPAK complex.

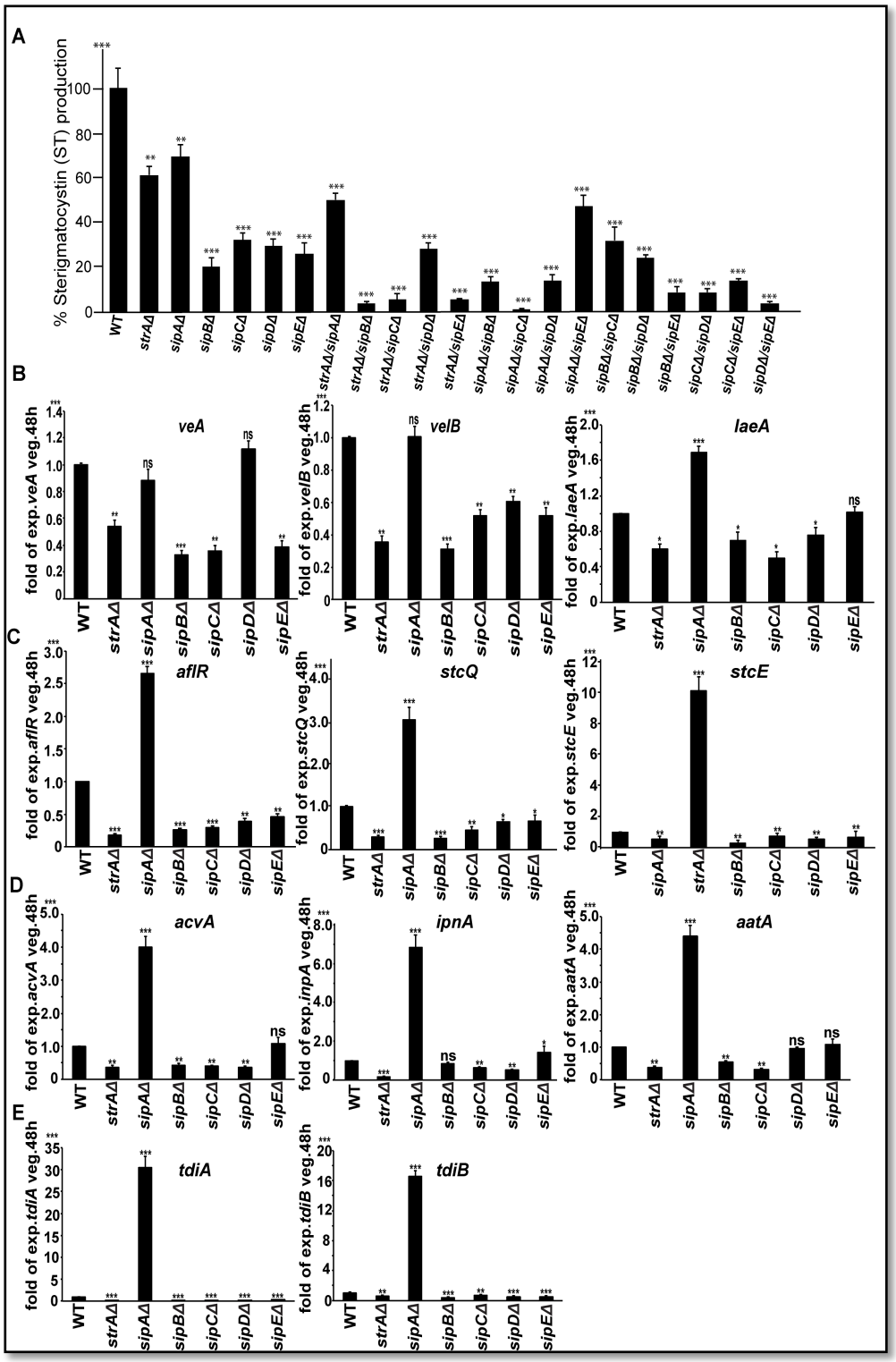

A
% Sterigmatocystin (ST) production
WT
strAΔ
sipAΔ
sipBΔ
sipCΔ
sipDΔ
sipEΔ
strAΔ/sipAΔ
strAΔ/sipBΔ
strAΔ/sipCΔ
strAΔ/sipDΔ
strAΔ/sipEΔ
sipAΔ/sipBΔ
sipAΔ/sipCΔ
sipAΔ/sipDΔ
sipAΔ/sipEΔ
sipBΔ/sipCΔ
sipBΔ/sipDΔ
sipBΔ/sipEΔ
sipCΔ/sipDΔ
sipCΔ/sipEΔ
sipDΔ/sipEΔ
B
veA
velB
laeA
fold of exp.veA veg.48h
fold of exp.velB veg.48h
fold of exp.laeA veg.48h
C
aflR
stcQ
stcE
fold of exp.aflR veg.48h
fold of exp.stcQ veg.48h
fold of exp.stcE veg.48h
D
acvA
inpA
aatA
fold of exp.acvA veg.48h
fold of exp.inpA veg.48h
fold of exp.aatA veg.48h
E
tdiA
tdiB
fold of exp.tdiA veg.48h
fold of exp.tdiB veg.48h

Figure 3.10. Function of STRIPAK complex in secondary metabolite production. (A) Levels of mycotoxin Sterigmatocystin (ST) production in STRIPAK mutants by using HPLC. $5x10^3$ spores were point-inoculated at the center of the GMM plates and incubated in light at 37°C asexually for 5 days. Values are the means of three replicates, and error bars represented standard errors. The statistical analyses were done by t.test and one way-ANOVA **(B)** Expression of the velvet complex encoding genes, *veA*, *velB* and *laeA* in STRIPAK complex mutants by a qRT-PCR. **(C)** Expression of ST gene cluster (regulatory *alfR,* structural *stcQ, stcE*) in the mutants. **(D)** Expression of Penicillin gene cluster genes (*acvA, ipnA, aatA*). **(E)** Expression of Terrequinone gene cluster genes, *tdiA* and *tdiB* in mutants. The wild type and mutant strains were grown for 48 hours at 37°C in appropriate liquid media vegetatively. The statistical significance is expressed as (*) $p < 0.05$, (**) $p < 0.001$, (***) $p < 0.0001$ and non-significant (ns) $p > 0.05$ compared with wild type.

3.9.1. StrA and SipA to SipE proteins are colocalized to nuclear envelope and endoplasmic reticulum

Striatin StrA is localized to nuclear periphery and endoplasmic reticulum where it possibly controls activity or interaction of regulatory proteins. SipA to SipE were found to be interacting with StrA constituting the hexameric STRIPAK complex. This interaction data raises two questions:

(I) Where the Sip proteins are localized within the cell?

(II) Are Sip proteins localized with StrA protein or not?

Therefore, a functional StrA::mRFP fusion expressed under native locus was co-expressed with SipA, SipB, SipC, SipD and SipE::sGFP fusions which were also expressed under their native promoter (**Figure 3.11**). StrA::mRFP fusion clearly decorated the perinuclear space where the nuclear envelope is found. However, it was not found inside the nucleus.

Furthermore, StrA was also present on long string- like extensions, representing the endoplasmic reticulum (**Figure 3.11**). Endogenously expressed SipA::sGFP fusion was also found to be accumulated around the nucleus and string like extensions but excluded from inside the nucleus (**Figure 3.11A**). Both GFP and RFP signals clearly overlapped on nuclear envelope and ER. Similarly, GFP fusions of SipB, SipC, SipD and SipE were also found to be colocalised with StrA::mRFP fusion protein (**Figure 3.11B to E**). Real time live images of StrA and Sip proteins also showed similar cellular movement patterns around the nuclear envelope (not shown). None of the Sip proteins accumulated inside the nucleus.

These localization data display that StrA and SipA to SipE colocalized to nuclear envelope and extensions or ER, forming a functional STRIPAK complex, acting as a molecular hub on the nuclear envelope where the complex possibly controls or modulates regulatory proteins entering the nucleus.

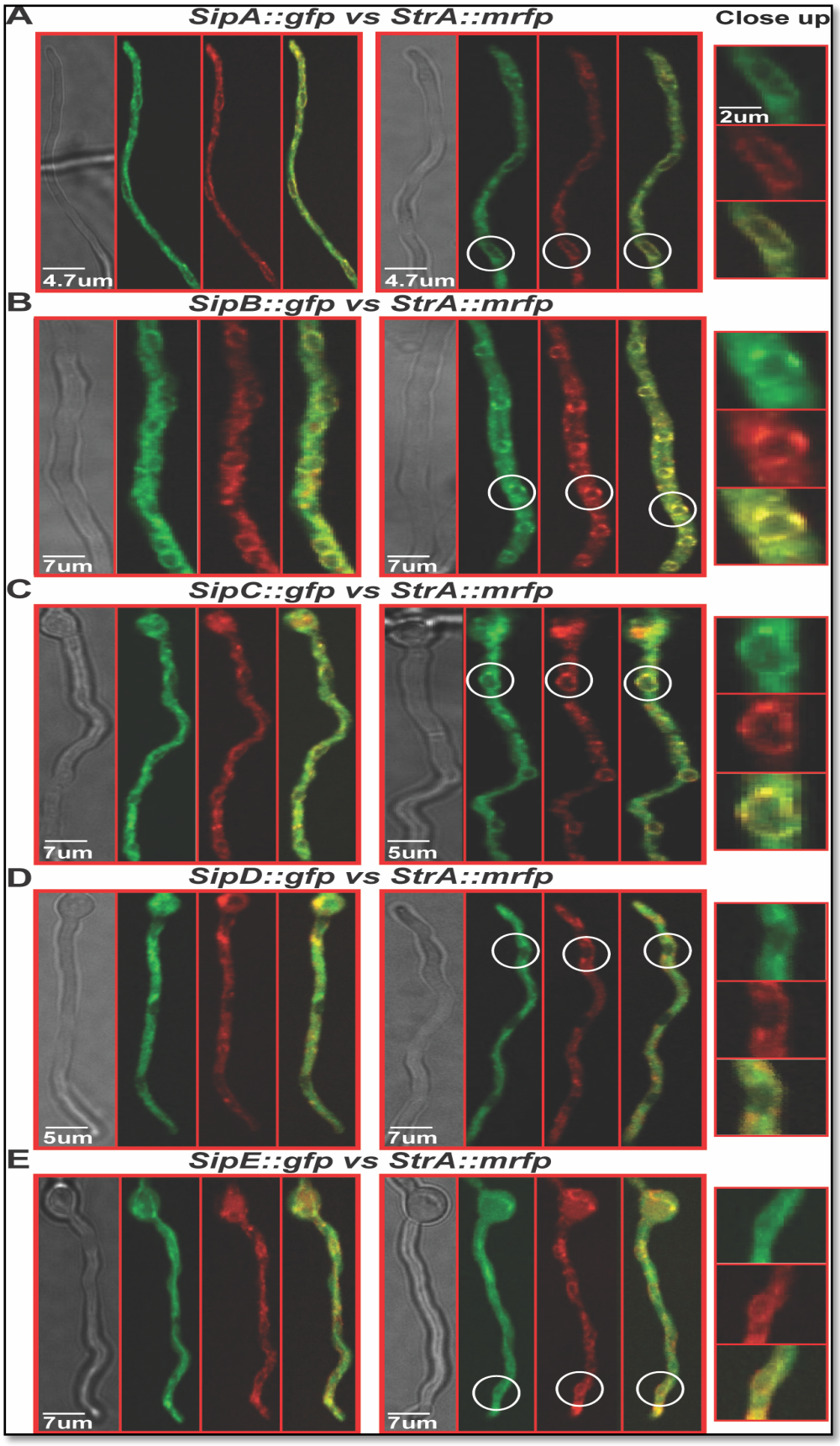

A
SipA::gfp vs StrA::mrfp
Close up
4.7um
4.7um
2um
B
SipB::gfp vs StrA::mrfp
7um
7um
C
SipC::gfp vs StrA::mrfp
7um
5um
D
SipD::gfp vs StrA::mrfp
5um
7um
E
SipE::gfp vs StrA::mrfp
7um
7um

Figure 3.11: Colocalizations of Sip proteins and Striatin in nuclear envelope and endoplasmic reticulum. Subcellular location of StrA::mRFP in combination with **(A)** SipA::sGFP, **(B)** SipB::sGFP, **(C)** SipC::sGFP, **(D)** SipD::sGFP, **(E)** SipE::sGFP in cells grown in liquid media for 20 h at 37°C vegetatively. White arrows indicate the position of nuclei around which, StrA::mRFP shows ring-like accumulations together with Sip::sGFP fusions. Scale bars are in the range of 4 to 7 µm.

3.9.2. StrA is required for proper subcellular localization of SipA, SipB, SipC, SipD and SipE on nuclear envelope

Striatin StrA, SipA to SipE are all colocalized around the nuclear envelope and partially in endoplasmic reticulum. Their double deletions with *strA* lead to more sensitive phenotypes suggesting the key role of StrA in molecular function of the STRIPAK complex. This raised the question how subcellular localizations and distributions of the STRIPAK complex members are influenced in the absence of StrA protein. SipA to SipE::sGFP fusions were expressed in a strain devoid of StrA. To exclude the possibility of improper expression of Sip proteins due to lack of StrA, expression levels of Sip proteins were determined both in the presence and absence of StrA (**Figure 3.12A**). Lack of StrA did not influence expression of the most of the Sip proteins except for SipD, which showed a higher molecular weight band. Seeing that all proteins show stable expression patterns in wild type and striatin deletion strain, subcellular localizations of them were determined. As expected SipA::sGFP fusion protein accumulated on the nuclear envelope, showing ring-like green fluorescent around the nucleus (**Figure 3.12B**). Interestingly, lack of StrA led to loss of nuclear envelope localization of SipA, which became more diffused to the cytoplasm (**Figure 3.12B**). SipB::sGFP showed similar pattern in the absence of StrA, indicating that both SipA and SipB need StrA for proper nuclear envelope

localization. Further, accumulations of SipC, SipD and SipE::sGFP fusions on the nuclear envelope was negatively influenced in the *strA* mutant. Both SipC and SipE diffused to the cytoplasm, SipD protein lost nuclear envelope accumulation but it also showed a punctate pattern, which was different than the other four proteins. This was attributed to high molecular weight version and partial degradation product of SipD the absent of StrA availability.

These confocal images and expression data show that all members of STRIPAK complex SipA to SipE needs the molecular scaffold StrA in order to accumulate or attach to the nuclear envelope membrane.

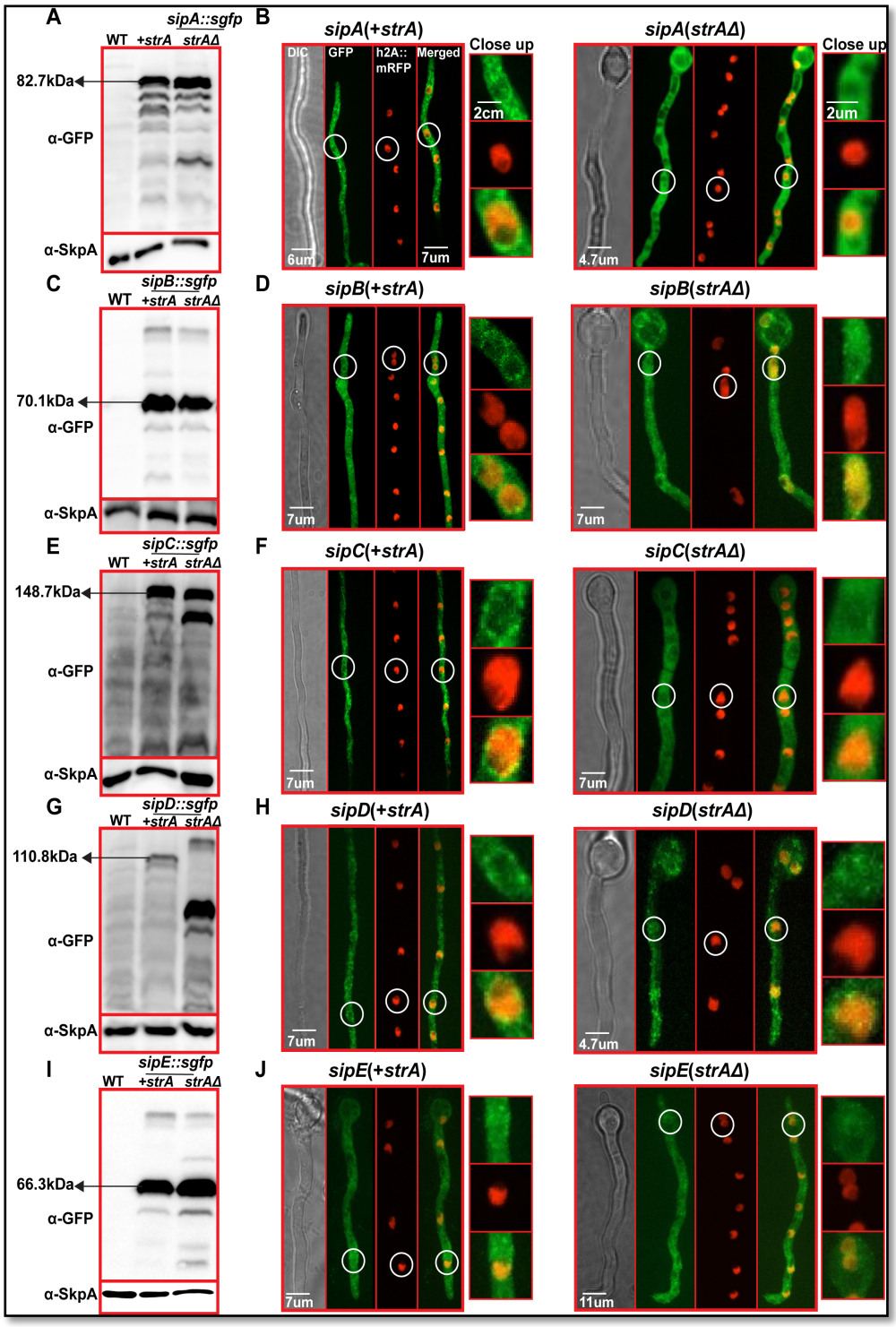


Figure 3.12. Expression and subcellular localization of Sip-GFP fusions in the absence of functional striatin. Protein expression levels **(A)** and subcellular

localization **(B)** of SipA::sGFP fusion in the presence (+*strA*) and absence of striatin (*strA*Δ). Protein expression levels **(C)** and subcellular localization **(D)** of SipB::sGFP fusion in the presence (+*strA*) and absence of striatin (*strA*Δ). Influence of StrA on SipC **(E-F)**, SipD **(G-H)** and SipE **(I-J)** expression and localization. For immunoblotting and microscopy, strains were grown in liquid media for 20 h at 30°C vegetatively. 100 µg total crude extract was applied on each lane. α-gfp detects Sip::sGFP fusions, α-SkpA detects SkpA levels used as loading control. Black arrow shows the full length of the Sip::sGFP fusion proteins. H2A::mRFP fusions indicate the position of nucleus. White circles show the close-up sections from each localization image. Scale bar about 7µm.

3.10. Lack of Striatin disrupts the interactions of the STRIPAK complex

Seeing that StrA is required for proper subcellular localization of the STRIPAK complex members, it became intriguing whether this mislocalizations influence the interdependent interactions of the complex members. To adders this question, fully functional c terminally tagged fusions of SipA to SipE were expressed both in wild type and *strA* deletion strains and interaction partners were identified through TAP-MS (**Table 3.2**). SipA to SipE all recruited each other with high peptide number in the absence of StrA (**Table 3.2B**). However, surprising interaction data was uncovered in the *strA* deletion strain. Firstly, SipA did not recruit any other members of the complex, indicating that SipA only interacts with StrA. Secondly, SipB was only able to bind to SipD but nothing else. Furthermore, SipD TAP also resulted in only SipB, which confirms the SipB TAP result, suggesting that SipB-SipD form a heterodimer independent of StrA and then attach to StrA. A similar heterodimer also existed for SipC-SipE, because their reciprocal TAP-MS only led to identification of each other but neither SipA, nor SipB-SipD heterodimer.

These TAP data comparing the physical interaction dynamics of STRIPAK members in presence and absence of StrA plainly display that

(I) SipA only interacts with StrA, therefore recruited to STRIPAK complex via StrA.

(II) SipB-SipD forms a heterodimer and then associates with StrA.

(III) A second heterodimer complex is formed by SipE-SipC, which is then attached to StrA to form the fully functional STRIPAK complex.

Table 3.2: Confirmation of the interaction between StrA and Sip-complex in (A) presence or (B) absence StrA.

A

SipA (+StrA)	SipB (+StrA)	SipC (+StrA)	SipD (+StrA)	SipE (+StrA)
StrA (AN8071)	StrA (AN8071)	StrA (AN8071)	StrA (AN8071)	StrA (AN8071)
SipB (AN1010)	SipA (AN6190)	SipA (AN6190)	SipA (AN6190)	SipA (AN6190)
SipC (AN6611)	SipC (AN6611)	SipB (AN1010)	SipB (AN1010)	SipB (AN1010)
SipD (AN4632)	SipD (AN4632)	SipD (AN4632)	SipC (AN6611)	SipC (AN6611)
SipE (AN0164)	SipE (AN0164)	SipE (AN0164)	SipE (AN0164)	SipD (AN4632)

B

SipA (-StrA)	SipB (-StrA)	SipC (-StrA)	SipD (-StrA)	SipE (-StrA)
	SipD (AN4632)	SipE (AN0164)	SipB (AN1010)	SipC (AN6611)

4. Discussion

STRIPAK complexes or STRIPAK-like complexes are highly conserved in eukaryotic organisms and STRIPAK signaling is involved in many cellular processes ranging from cell-cell fusion and fruit body formation in fungi, proper neuronal and cardiac function in mammals (Hwang and Pallas 2014, Teichert and Kuck 2016). This PhD study has revealed the molecular nature and functions of *A. nidulans* STRIPAK complex, which consist of at least six proteins **StrA**/STRIP/HAM-3, **SipA**/Phoecin/MOB-3, **SipB**/CTTNBP2, **SipC**/STRIP/HAM-2, **SipD**/SLMAP/HAM-4, **SipE**/PP2A/PPG-1. Detailed phenotypic, genetic, biochemical, live cell imaging and chemical biology approaches identified the StrA as the core scaffolding protein which assembles at least five other STRIPAK members at nuclear envelope to control intracellular signaling events (**Figure 4.1**). Striatin protein StrA is found in heterodimer state with Phoecin protein SipA, and further recruits two heterodimers SipB/SipD (CTTNBP2/SLMAP) and SipC/SipE (STRIP/PP2A) to establish fully functional hexameric STRIPAK complex. STRIPAK complex controls multicellular phase of the fungus defined as sexually formed fruit bodies and furthermore, it is also required for production of secondary metabolites.

Although the molecular composition of the STRIPAK complex is conserved, the described functions of the complex show highly diverse roles in fungi, flies and mammals. In baker´s yeast *S. cerevisiae*, FAR (STRIPAK) complex acts as an antagonist of target of rapamycin (TOR) pathway and counteracts recovery from pheromone arrest (Kemp and Sprauge 2003, Pracheil et al, 2012) whereas in fission yeast *S. pombe*, the STRIPAK complex acts as a negative regulator of septation initiation (Singh et al 2011). In closely related filamentous fungi *S. macrospora* and *N. crassa*, STIPAK complex regulates hyphal fusion and fruit body formation (Bloemendal et al, 2012, Dettmann et al, 2013, Teichert et al, 2016). In two *Fusarium* species, which are plant pathogenic fungi, striatin is required for

pathogenicity on host organisms (Islam et al., 2017 and Zhang et al., 2017). In the fruit fly *D. melanogaster*, STRIPAK complex controls epithelial cell movement and tissue size by modulating two different signaling pathways (Chen et al., 2002, Ribeiro et al., 2010, Horn et al., 2011), whereas in the nematode *C. elegans*, members of the complex control polarity establishment during embryogenesis (Pal et al., 2017). In humans, STRIPAK has a variety of roles, including embryonic stem cell differentiation, proper cardiac function, dendritic spine morphology and cancer (Nader et al., 2017; Lin et al., 2017, Shi et al., 2016).

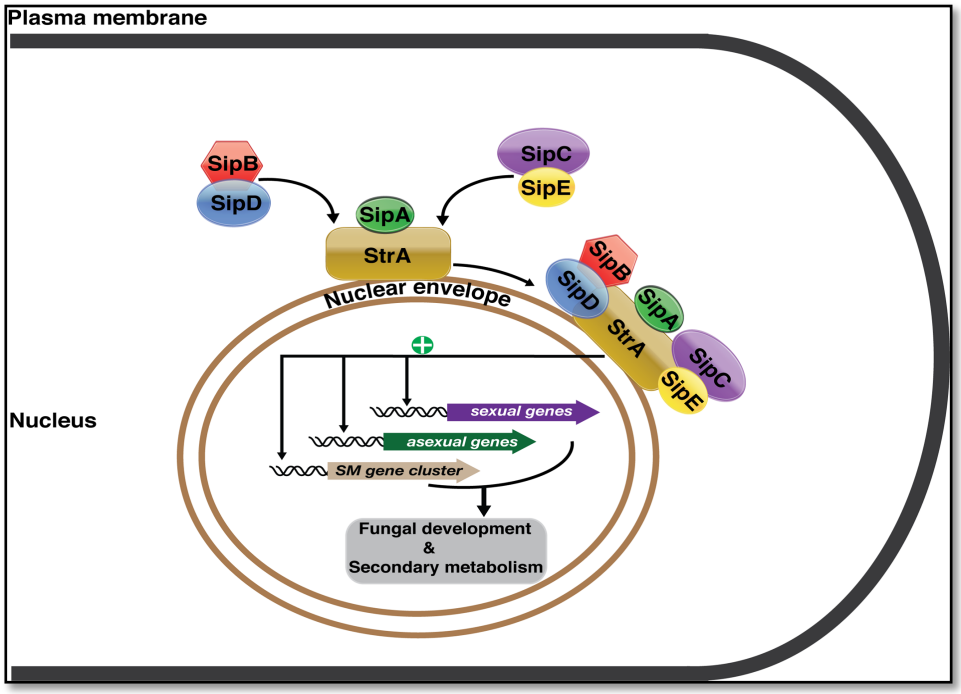


Figure 4.1: Current model of STRIPAK complex and its functions in *A. nidulans*. Hexameric STRIPAK complex assembles at the nuclear envelope. SipA-StrA heterodimer act as a seed to establish the complex by recruiting two heterodimer subpopulations SipB-SipD and SipC-SipE. Fully functional complex acts as a signaling hub for various singalling elements, which finally controls development and SM production by controlling gene dosage of major regulatory genes.

STRIPAK complex role in fungal light responses and development:

In the model organism *A. nidulans*, STRIPAK complex controls light-dependent fungal development. In this fungus, light controls mode of reproduction through the various light receptors including, red light receptor phytochrome FphA, blue light receptors LreA/LreB, UV-blue light receptor CryA and green light receptor NopA. Lack of fully assembled STRIPAK kinase-phosphatase complex results in loss of proper light response as a result of reduction of asexual spores and loss of sexual fruit bodies. Reduced asexual sporulation stems from down regulation of asexual regulatory genes. Particularly, two major asexual transcription factors *abaA* and *brlA* are downregulated in the absence of STRIPAK complex. The role of STRIPAK complex in fungal fruit body formation might be somewhat complicated. Several regulators of sexual development are misregulated in STRIPAK mutants. STRIPAK probably controls formation of fruit bodies by properly dosing expression of sexual transcription factors such as *nsdD*. It is known from *N. crassa* and *S. macrospora* that the STRIPAK complex is involved in cellular fusion, which finally leads to fruit body formation in these fungi. In *A. nidulans*, sexual fruit body formation is also controlled by formation of cell-cell fusions. Loss of fruit bodies also indicate that there are defects in cell-cell fusions in the absence of STRIPAK members. Furthermore, in *N. crassa*, STRIPAK complex controls nuclear accumulation of MAK-1 which controls cell wall stress. However, there is no reported functions of STRIPAK mutants in cell-wall pathway. NRC-1-MEK-2-MAK-2 kinase cascade are the central components of self singaling machinery in *N. crassa* (Fleissner et al., 2009; Dettmann et al., 2012). MOB-3 component of the *N. crassa* STRIPAK complex interact with MAK-2. However, nuclear localization of MAK-2 is not influenced by STRIPAK complex. In *A. nidulans*, sexual pathway is contolled by a pheromone response pathway module, consisting of MAPKKK (AnSte11, SteC), MAPKK (AnSte7, MkkB), adaptor protein AnSte50 (SteD), and

MAPK (AnFus3, MpkB). The SteC-SteD-MkkB-MpkB module moves from plasma membrane to nuclear envelope to deliver MpkB into the nucleus. Phenotypes of pheromone pathway mutants are very similar to STRIPAK complex mutants (Bayram et al., 2012) with reduced asexual sporulation and loss of fruit body formation along with no hyphal fusion. Furthermore, mutants of pheromone pathway and STRIPAK complex produce masses of huelle cells in submerged culture (unpublished data). Interestingly, in time course purifications during two sexual stages, StrA-TAP fusion copurified SteD, which is the adaptor domain of pheromone response pathway. Since STRIPAK complex sits at the nuclear envelope and SteC-SteD-MkkB-MpkB complex also transiently touches the nuclear envelope, it is highly likely that this module and STRIPAK complex might have mutual interaction and interdependency to control light-dependent development.

STRIPAK complex regulates secondary metabolism

One of the diverse functions of STRIPAK complex in eukaryotes has turned out to be regulation of secondary metabolism in fungi. This body of work revealed the functions of STRIPAK complex is in secondary metabolite production. Fungal secondary metabolism (SM) is controlled by upstream signaling elements such as receptors at plasma membrane. The receptors transduce the signals into downstream elements such as transcription factors or chromatin elements which control expression of SM genes. In *N. crassa* or *S. macrospora*, the involvement of STRIPAK complex in SM has not been reported yet. STRIPAK complex controls production of carcinogenic mycotoxin Sterigmatocystin. Production of the toxin is downregulated in single and double mutants of STRIPAK complex, which might stem from reduced expression of the components of the velvet complex VeA-VelB and

LaeA. Accordingly, the expression of three selected genes of the ST gene cluster are also drastically reduced in the absence of STRIPAK complex except for SipA. Expression of Penicillin and Terrequinone gene clusters are also drastically diminished in the STRIPAK mutants. *A. nidulans* produces forty to fifty metabolites (Inglis et al., 2013), although only three gene clusters were examined in this PhD thesis, the effect of loss of STRIPAK complex might be similar in other gene clusters. How does the complex control the SM production? Transcriptional downregulation of the velvet complex might be the first reason why SM production is drastically affected in STRIPAK mutants because this complex was shown to be required for production of ST in a light-dependent fashion (Bayram et al., 2008). Another scenario might be that STRIPAK is important for vital functions of pheromone response module SteC-SteD-MkkB-MpkB. Because it was shown that this module uses the nuclear envelope to interact with nucleus and deliver the MAPK MpkB into the nucleus. MpkB then interacts with the VeA member of the velvet complex, which is required for development and SM production. Since STRIPAK and pheromone response pathway mutants show similar defects both in development and ST production, loss of STRIPAK functions also presumably leads to loss of pheromone response pathway functions, which finally causes developmental and SM malfunctioning. STRIPAK might be required for nuclear delivery of MAPK MpkB from nuclear envelope into the nucleus. In future, it will be interesting to check to subcellular dynamics of the MAPK module and nuclear import of the MAPK MpkB in the absence of the STRIPAK members in order to reveal the exact molecular mechanism.

Stress responses are governed by STRIPAK complex

Previously it was not shown that STRIPAK complex is important for stress responses in any eukaryotes. Here it was shown that STRIPAK complex play an important role in various stress responses. Single mutants of STRIPAK complex are sensitive to oxidative and cell wall stress, however they are not extremely influenced by the osmotic stress. At least one catalase *catC* and one superoxide dismutase *sodB* are down regulated in the absence of the STRIPAK complex. In *A. nidulans*, stress responses are mediated by three MAPK proteins, MpkA, SakA and MpkC. MpkA regulates cell wall integrity (Irniger et al., 2016). *mpkA* mutants needs high osmolarity media to grow. STRIPAK double deletions which are sensitive to oxidative stress and cell wall stress show improved growth under high osmolarity media indicating the involvement of MpkA in STRIPAK mutants. In *N. crassa*, MAK-1 is transported into the nucleus in a MAK-2-dependent manners, which might be a likely scenario in *A. nidulans*. SakA-MpkC MAPKs control oxidative stress responses interdependently in *A. nidulans* (Hagiwara et al., 2008, 2014). In addition to oxidative stress, they are also involved in trehalose accumulation, thermotolerance and conidial viability (Sarikaya Bayram., 2017). However, STRIPAK mutants do not show a decreased viability in spores (not shown), indicating that SakA-MpkC-dependent functions might be partially controlled by the STRIPAK complex. It will be interesting to study the subcellular behaviours of these three MAPKs and their phosphorylation states in the absence of STRIPAK members in future.

SipA has an epistatic role in the control of development and stress responses in STRIPAK complex

The *sipA* mutant and its double mutant combinations showed an opposite phenotype to STRIPAK gene deletions, indicating an epistatic function of SipA over the StrA but not over SipB-SipC-SipD-SipE complex. This is not only specific to development but also stress responses. Secondary metabolite production was slightly influenced in the absence of *sipA* and expression of all other gene clusters were upregulated in the absence of *sipA*. SipA is the only member, which interacts with StrA constantly during all stages of development and does not depend on other members of the complex. Therefore, in the absence of SipA, absence of StrA does not influence development and stress responses. StrA requires SipA to show its influence on various processes. MOB-3 (SipA homolog) in *N. crassa* interacts with NRC-1-MEK-2-MAK-2 kinase cascade of self-signaling (Dettmann et al., 2012). These interactions are similar to CKA protein of *Drosophila*. However, although SipA does not interact with the components of MAPK pathway in the absence of StrA in TAP or GFP pull downs, it might transiently interact with MAPK pathway to elicit its effect on development. Further work will be required to dissect the molecular functions of SipA in the absence of STRIPAK members.

In conclusion, this study revealed the composition and partial role of STRIPAK complex in model fungus *A. nidulans*. It was surprising that STRIPAK complex also regulates light and stress responses, production of SMs in addition to developmental programmes. Since *A. nidulans* represents more than three hundred biotechnologically and medically relevant species, the information gained through this study will be useful to apply the knowledge to other important Aspergilli to understand their biology, to use their full potential in SM and enzyme production. A possible link between

STRIPAK complex and MAPK pathways will also reveal how fungi sense various signals and how they control signal flow on nuclear envelope and how they convert these signals into appropriate responses.

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Supplemented Materials

Glucose Minimal Medium (GMM):

50x AspA (20ml), 5% Glucose (11g), 1M MgSO4 (2ml) and 2% Agar (20g) were add to 1000ml distilled water and autoclaved at 121^{o}C for 20mins. The solution was allowed to cool to about 50^{o}C than add 1000X Trace elements (1ml) and add supplementary material that was needed for strains. The media was poured into petri dishes than the plates were allowed to set and stored at 4^{o}C.

Liquid minimal media:

50x AspA (16ml), 5% Glucose (8.8g), 1M MgSO4 (1.6ml) and 16g Agar were add to 800ml distilled water and autoclaved at 121^{o}C for 20mins. After the solution was cooled about 50^{o}C, 1000X Trace elements (800ul) was added and also supplementary vitamins deepened on strains were needed. The media was stored at room temperature.

LB Broth agar and liquid medium:

Luria Bertani Broth (SIGMA) (25g) was dissolved in distilled water (1L) for liquid media or LB Broth with agar (35g) was dissolved in distilled water (1L) and autoclaved prior to use. The media was allowed to cool and than Ampicillin (1ml) was added. The liquid media was stored at RT but solid media was stored at 4^{o}C.

Sorbitol Minimal Media:

218g of sorbitol were dissolved in 400ml of distilled water and all compound of GMM was added. The solution was made up to 1L with distilled water and than was autoclaved at 121^{o}C for 20mins. After the media was cooled at RT, the 1000X Trace elements (1ml) and supplementary vitamins was deepened on strains needed. The media was

poured into petri dishes than the plates were allowed to set and stored at 4°C. This media was used for transformation process.

50x AspA:

Sodium citrate (297.2g), potassium chloride (26g) and H2KPO4 (76g) were dissolved in deionized water 1L. The pH was adjusted to 5.5 using Potassium. The solution was stored in RT.

1M MgSO4:

Magnesium Sulfate hepta-hydrate (60.18g) was dissolved in 500ml ddH2O and autoclaved at 121°C for 20mins. The solution was stored at RT.

Supplementary to M.M:

Uridine 5% (0.25g/l):

5g of Uridine was dissolved in 100ml ddH2O. The solution was divided into 50ml tubes and stored at 4°C.

Uracil (1g/l):

240 mg of Uracil was dissolved in 100ml ddH2O and than filtered. The solution was stored in 4°C.

Pyridoxine 0.1% (1mg/l):

1g of Pyridoxine-HCL was dissolved in 1000ml of ddH2O. The solution was divided into 50ml tubes and stored at 4°C.

Oat meal media:

10g of grinded Oat meal was added to 1L of Agar M.M before autoclaved.

Antibiotice to be added to MM:

Nourseothricin (*natR*)

1g of Nourseothricin (Jena bioscience) were dissolved into 10ml ddH2O and than filtered. The solution was divided on 10 of 1ml e-cup. 120ul of *natR* solution was used for 800ml of MM.

Pyrithiamine (*ptrA*)

1g of pyrithiamine hydrobromide (Sigma) were dissolved into 10ml ddH2O and than filtered. The solution was divided on 10 of 1ml e-cup. 120ul of *natR* solution was used for 800ml of MM.

Ampicillin:

1g of Ampicillin Sodium Salt (Sigma) was dissolved in 10ml ddH_2O and filtered by using Filtropur S 0.2. The solution was divided on 1.5ml e-cup and stored at -20 ^oC.

0.7% Agarose gel:

About 800mg of Ultra pure^{TM} Agarose (Invitrogen) was dissolved in 1x TAE buffer (120ml) using microwaves for 2min and than 5ul of a SYBR$^{®}$Safe(Invitrogen) DNA gel stain (10.000x concentrate in DMSO) was added. The solution was allowed to cool and poured into a casting rig.

50x TAE:

Tris-Base (242g) and EDTA (18.6g) were dissolved in ddH2O 800ml and than added to Glacial acetic (57.1ml). The solution was completed to 1000ml by ddH2O and autoclaved at 121^oC for 20min.

0.25M Hydrochloric Acid (HCl):

Hydrochloric acid (7.6ml) was added slowly to deionized water (1L) and than autoclaved at 121^{o}C for 20mins. The solution was stored at RT.

0.5M Sodium Chloride (NaCl):

Sodium chloride (87.66g) was dissolved into deionized water (1L) using a magnetic stirrer and than autoclaved at 121^{o}C for 20mins. The solution was stored at RT.

1.5M Sodium Hydroxide (NaOH):

Sodium Hydroxide pellets (20g) were dissolved into deionized water (1L) using a magnetic stirrer and than autoclaved at 121^{o}C for 20mins. The solution was stored at RT.

20x SSC (Saline Sodium Citrate) Buffer:

Sodium Chloride (350g) and Na-Citrate (176g) were dissolved in distilled water (dH2O) (1800ml). The pH was adjusted to 7 by using 5M Hydrochloric Acid (HCl). The final volume was adjusted to 2l with deionized water and than autoclaved at 121^{0}C for 20min. The solution was stored at room temperature.

Pre-hybridization buffer:

75ml of 20x SSC, 60ml of 10%Block Solution, 3ml of 10%SDS, 6ml ddH2O and 150ml of Pure Formamide were mixed and dissolved at 68^{0} C on rotator. The solution was divided in 50ml tubes and stored at -20^{0} C.

10x DIG Buffer1:

Maleic Acid (116g) and Sodium Chloride (88g) were dissolved in 1L deionised water. The pH was adjusted to 7.5 using Sodium Hydroxide (NaOH). The solution was autoclaved and stored at RT.

DIG Buffer3:

100mM Trisa-Base (6.05g), 100mM Sodium Chloride (2.29g) and 50mM Magnesium Chloride $MgCl_2$ (5.08g) were dissolved in deionized water (500ml) and autoclaved at 121^0 C for 20min. The solution was divided on tubes 50ml and stored at RT.

10x TBS (Tris-Buffered Saline):

24g Tris base and 88g NaCL were dissolved in 900ml of distilled water. The pH was adjusted to 7.6 using 5M Hydraulic Acid. The solution was adjusted to 1 liter with distilled water. The solution was autoclaved and stored at RT. 1x TBS was used in experiments.

5% Milk:

10g Dried skimmed milk was dissolved into 1x TBS.

TBST (Tris-Buffer Saline, 0,1% Tween-20):

For 1liter solution, 100ml of TBS and 1ml of Tween-20 were added to 900ml of distilled water. The solution was autoclaved and stored at RT.

Protein extraction buffer (B300 buffer):

The 300mM NaCl, 100mM Tris-HCl (pH: 7.5), 10% Glycerol, 1mM EDTA and 0.1% NP-40 were dissolved in distilled water (1L). The solution was autoclaved at 121^oC for 20min and stored at 4^oC. Immediately prior to use, the 1mM DTT, Protease inhibitor mix (Roche), 1.5mM Benzamidine, 1x Phosphatase inhibitors mix (4-(2-Aminoethyl benzenesulfony Fluoride Hydrochloride"CAS30827-99-7" ROCHE) and 1mM PMSF were added to appropriate amounts of solution.

1M Tris-HCl (pH: 7.5):

157.6 g Trizma-hydrochloride was dissolved in 500 ml distilled water and the pH was adjusted to 7.5 by using 1M NaOH the volume was adjusted to 1L and stored at RT.

1mM EDTA:

4.0445mg of Ethylenediaminetetraacetic acid dipotassium salt dehydrate was dissolved into 8ml of distilled water. The pH adjusted to… in order to dissolved the salt. The solution stored at -20^{o}C.

Protease inhibitor mix (Roche):

Two Roche tablets EDTA free were dissolved in 1ml H2O. The solution can stroes at -20^{o}C until using.

100mM PMSF:

Phenylmethylsulfonyl fluoride (1g) was dissolved in 60ml Ethanol 100%. The solution was stored at 4^{o}C.

Sodium Dodecyl Sulfate (SDS) gels:

For a 5ml stacking gel:

500ul of 10% (w/v) SDS was added, 1.25ml of 0.5% of Tris-HCL (pH6.8), 0.67ml of Acrylamide/Bis-acrylamide 30%, 500ul of 10% (w/v) Ammonium Persulfate and 0.005ml of TEMED were added to 2.975ml and mixed gently by hand.

For a 10ml separating gel:

1. 10% separating gel was prepared as following: 3.8ml H2O, 3.4ml Acrylamide/Bis-acrylamide 30%, 2.6ml of 1.5M Tris (pH8.8), 0.1ml 10% (w/v) SDS, 100ul of 10% (w/v) ammonium persulfate and 10ul TEMED were mixed together by hand.

2. 12% separating gel was prepared as following: 3.2ml H2O, 4ml Acrylamide/Bis-acrylamide 30%, 2.6ml of 1.5M Tris (pH8.8), 0.1ml 10% (w/v) SDS, 100ul of 10% (w/v) ammonium persulfate and 10ul TEMED were mixed together gently by hand.

Stacking gel was poured on top of the separating gel after it was solidification.

10% (w/v) Sodium Dodecyl Sulfate (SDS):

A bout 10g of SDS was added to 100ml of distilled water and dissolved. The solution was stored at room temperature.

0.5M Tris-HCL pH6.8:

About 7.8g of Trizma-hydrochloride were dissolved in 80ml of dH2O. The pH was adjusted to 6.8 using 4M NaOH. The volume was adjusted to 100ml with dH2O and stored at RT.

10% (w/v) Ammonium Persulfate:

About 100mg of Ammonium persulfate was dissolved in 1ml dH2O. The solution was stored at -20^{o}C.

Reagents and concentrations used for test stress process:

Substance	Stock solution	Solvent	For 100ml media
3-AT (3-amino1,2,4triazole)	100mg/ml	H2O	83.3µl for 1mM final conc.
			250µl for 3mM final conc.
CPT (Camptothecin)	10mg/ml (store 2-8°C)	DMSO	261µl for 75 µM final conc.
MMS (Methylmethanesulfonate)	Clear-liquid (100%)		30µl for 0.03%

EMS (Ethyl methanesulfonate)	Liquid (100%)		50µl for 0.05%
4-NQO (4-Nitroquinoline *N*-oxide)	25mg/ml	Acetone	1µl for 0.25 µg/ml
Hydroxyurea (HU)	50mg/ml	H2O	197.6µl for 5.2mM
Benomyl	1mg/ml	DMSO	30µl for o.3g/ml
Nocodazole	5mg/ml	DMSO	2µl for o.1µg/ml
Latrunculin B	1mg/ml	DMSO	20µl for 0.2µg/ml
SDS	10%	H2O	50µl for 0.005%
1M NaCl			5.84g
1M KCl			7.45g
H2O2	30%	H2O	10µl for 1mM 20µl for 2mM
Menadione sodium bisulfite (water soluble form of Menadione)	50mg/ml	H2O	44.2µl for 0.08mM
Diamide (Tetramethylazodicarbox amide)	20mg/ml	H2O	430.45µl for 0.5mM
Calcoflour			1ml for 5mg/ml
Congo Red	10mg/ml	H2O	40µl for 20µm
Caffeine		DMSO	400µl for 2mM