Functional characterization of the *Ustilago maydis* protein Acb1 and its derived peptide SDF-2

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Marburg, den 28.09.2016

Joachim Jungmann

“If you live long enough, you'll make mistakes. But if you learn from them, you'll be a better person. It's how you handle adversity, not how it affects you. The main thing is never quit, never quit, never quit.”
-William J. Clinton

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Summary

The plant pathogenic fungus *Ustilago maydis* secretes effector proteins during the biotrophic interaction with its host plant *Zea mays*, with the aim being the suppression of the plant immune response virulence. All effector proteins virulence characterized to date were shown to be conventionally secreted proteins. However, over the last decade it could be established that proteins without a signal peptide can also be targeted to the outside of the cell in an ER/Golgi independent manner. A proteomic analysis on the apoplastic fluid of *U. maydis* infected maize plants identified 65 fungal proteins which did not contain a signal peptide. One of these proteins is the Acyl-CoA binding protein (ACBP) of *U. maydis*, Acb1, which will be investigated in this thesis.

Over the years many studies have been dedicated to the functional analysis of Acyl-CoA binding proteins, in which ACBPs were shown to have many distinct intracellular functions, such as control over the intracellular Acyl-CoA pool size, roles in vesicular trafficking and their relevance for fatty acid biosynthesis. Further, in addition to their metabolic roles, ACBPs were shown to be involved in gene regulation, and also signaling by receptor binding, as was shown for the ACBP of the amoeba *Dictyostelium discoideum* (AcbA). In this organism it was shown that AcbA is secreted under nutrient limitation and extracellularly processed into the Spore Differentiation Factor 2 (SDF-2), which induces terminal spore differentiation.

In this thesis it could be shown that the Acb1 protein of *U. maydis* is unconventionally secreted, subsequently processed into a SDF-2 like peptide and that this peptide is capable of inducing the sporulation of *D. discoideum* cells. The study of the protein's extracellular function in *U. maydis*, as a full length secreted protein or in its processed form as the SDF-2 like peptide was hampered by the important intracellular functions of Acb1. Deletion of the *acb1* gene resulted in strong growth defects and an accumulation of suppressor mutations, which were able to restore growth in a mutation dependent manner. Ultimately it could be shown that a virulence related function of the SDF-2 like peptide in both the solopathogenic strain SG200, as well as the compatible wild type strains FB1 and FB2, has to be considered unlikely. A potential extracellular role of the full length secreted protein has to date been neither confirmed nor ruled out.

Zusammenfassung

Der Pflanzen-pathogene Pilz *Ustilago maydis* sekretiert während seiner biotrophen Interaction mit seiner Wirtspflanze *Zea mays* Effektor-proteine, mit dem Ziel die pflanzliche Immunantwort zu unterdrücken. Für alle bisher charakterisierten Effektoren wurde gezeigt, dass sie konventionell sekretiert werden. Über die letzten Jahrzehnte konnte jedoch gezeigt werden, dass Proteine, die nicht mit einem Signalpeptid transkribiert werden, auf einem ER/Golgi unabhängigen Weg aus der pilzlichen Zelle transportiert werden können. In einer proteomischen Analyse der apoplastischen Flüssigkeit von *U. maydis*-infizierten Mais Pflanzen konnten 65 pilzliche Proteine identifiziert werden die über kein Signalpeptid verfügen. Eines dieser Proteine ist das Acyl-CoA Binde Protein (ACBP), Acb1, welches in dieser Studie untersucht wurde.

Über die vielen Jahre in denen Acyl-CoA Binde Proteine bereits untersucht wurden, konnten viele verschiedene Funktionen der Proteine entdeckt werden. Diese schließen die Kontrolle über intrazelluläre Acyl-CoA Vorräte, eine Funktion bei dem Transport intrazellulärer Vesikel und Beteiligung an der Fettsäuresynthese ein. Des Weiteren wurde gezeigt, dass ACBPs in der Genregulation eine wichtige Rolle spielen können und, wie für das homologe ACBP von *Dictyostelium discoideum* (AcbA) gezeigt wurde, als Signalstoffe für Rezeptorbindung agieren könne. Für AcbA konnte gezeigt werden, dass es spezifisch unter Nährstoffmangel sekretiert wird und extrazellulär in den Sporen Differenzierungs Faktor 2 (SDF-2) prozessiert wird, der endgültige Sporendifferenzierung einleitet.

In dieser Arbeit konnte gezeigt werden, dass das Acb1 Protein von *U. maydis* auch unkonventionell sekretiert wird und nach der Sekretion in ein SDF-2 ähnliches Peptid prozessiert wird. Für das SDF-2 ähnliche Peptid konnte gezeigt werden, dass es Sporendifferenzierung in *D. discoideum* in einleitet. Das Untersuchen der extrazellulären Funktionen des in voller Länge sekretierten Proteins, oder des daraus entstehenden Peptids, wurde durch die wichtigen intrazellulären Aufgaben von Acb1 erschwert. Die Deletion des *acb1* Gens war durch erhebliche Wachstumsdefekte charakterisiert, welche durch Suppressormutationen ganz oder teilweise unterdrückt werden konnten. Letztendlich konnte eine für die Virulenz relevante Rolle des SDF-2 ähnlichen Peptids in dem solopathogenen Stamm SG200, also auch den kompatiblen Wildtyp Stämmen FB1 und FB2, für unwahrscheinlich erklärt werden. Eine mögliche Rolle des unprozessierten Acb1 Proteins konnte bisher weder bestätigt, noch ausgeschlossen werden.

Abbreviations

ACBP	Acyl-CoA bindin protein
ACC	Acetyl-CoA carboxylase
AM	Ammonium Medium
APF	apoplastic fluid
bp	base pair
cAMP	cyclic adenosine monophosphate
CUPS	compartments of unconventional secretion
DAPI	4',6-diamidino-2-phenylindole
Δ	deletion
dH_20	distilled water
ddH_2O	double distilled water
ER	endoplasmatic reticulum
FAS	Fatty acid synthase
Indel	insertion or deletion
kDA	kilodalton
MNP	multiple nucleotide polymorphism
MVB	Multi-vesicular body
NM	Nitrate Medium
OD_{600}	optical density at 600 nm
SDF-2	Spore differentiation factor 2
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-Polyacrylamid-Gelelectrophoresis
SNP	single nucleotide polymorphism
TCA	Trichloroacetic acid
TPCK	Tosyl phenylalanyl chloromethyl ketone
Tris	Trisaminomethane

1 Introduction

1.1 The corn smut *Ustilago maydis*

The heterobasidiomycetic fungus *Ustilago maydis* is ranked amongst the smut fungi and is the causative agent of the so called corn smut disease. This plant disease is characterized by plant tumors, filled with pigmented teliospores, which are induced in all above ground parts of the maize plant (Fig. 1) [Ruiz-Herrera *et al., 1999*]. When these tumors crack open, large amounts of spores are released providing a burnt appearance to the plant. Hence, it is these visual cues from which the species derives its name (latin: *ustilare* = partially burnt/charred). The host range of *U. maydis* includes corn (*Zea mays*) and its progenitor teosinte.

Figure 1: *Ustilago maydis* tumor in the field. Picture provided by R. Rösser.

As a crop plant maize is cultivated extensively, not only as a food source for humans, but also as a fodder plant and a source of renewable energy (e.g. biodiesel). Hence, the losses generated by *U. maydis* infections are of economic relevance [Christensen, 1963]. This is contrasted in part by the Mexican cuisine which considers on the tumors induced by *U. maydis* as a delicacy [Juarez-Montiel *et al.*, 2011; Ruiz-Herrera and Martinez-Espinoza, 1998]. Due to its cultivability in axenic culture, genetic accessibility and its relatively short life cycle of only two to three weeks under optimal conditions, *U. maydis* presents itself as an excellent model organism for the study of biotrophic phytophatogenic fungi [Brefort *et al.*, 2009; Kahmann and Kämper, 2004]. Further, the *U. maydis* genome has been fully decrypted [Kämper *et al.*, 2006] allowing not only for easy bioinformatic analysis, but also enables comparisons to other sequenced genomes.

1.1.1 The *Ustilago maydis* life cycle

Ustilago maydis is a facultative biotrophic fungus whose pathogenic development is closely coupled to its sexual development. Its life cycle begins with haploid, saprophytically growing sporidia that proliferate asexually by yeast-like budding (Fig. 2A). Following the fusion of two sporidia of different mating type loci, pathogenic development of *U. maydis* is initiated [Rowell, 1955; Rowell and Devay, 1954]. Fusion is controlled by the bi-allelic *a* locus encoding a pheromone/pheromone-receptor system, which enables mate detection and cell fusion [Bölker *et al.*, 1992]. The perception of the pheromone results in the formation of non-septated conjugation hyphae (Fig. 2B), which grow towards each other directed by the pheromone gradient and fuse at their tips [Snetselaar and Mims, 1992]. The resulting so called b-filament represents the infectious form of *U. maydis* and requires the multi-allelic *b*-mating type loci, encoding the bE and bW transcription factors, for its establishment.

bE and bW only form an active b-protein complex when expressed from different alleles [Banuett and Herskowitz, 1996; Gillissen *et al.*, 1992; Holliday, 1961; Rowell, 1955; Yee and Kronstad, 1993]. The dikaryotic filament displays tip growth, where distal hyphal segments are separated from the cytoplasm-filled tip cell by septation (Fig. 2C) [Christensen, 1963; Freitag *et al.*, 2011].

Contact with the plant surface plays an essential role for the early differentiation processes in *U. maydis* [Apoga *et al.*, 2004]. *In vitro*, the formation of septated filaments and appressoria is induced by physical stimuli such as hydrophobicity and can be further stimulated by chemical signals, i.e. cutin monomers (Fig. 2D) [Mendoza-Mendoza *et al.*, 2009]. The addition of hydroxy fatty acids (16-hydroxy-palmitic acid) to cells encoding an active bE/bW heterodimer leads to the induction of filaments in axenic cultures, which are filled with cytoplasm. In liquid culture these filaments do not form appressoria. However, spraying of such cultures on hydrophobic surfaces such as parafilm in the presence of HPS will induce the formation of septated filaments, which will readily form appressoria [Mendoza-Mendoza *et al.*, 2009]. Appressoria in *U. maydis* are characterized by a swelling of the hyphal tip. In comparison to many other pytophatogenic fungi, *U. maydis* appressoria are not melanized [Bell and Wheeler, 1986; Tucker and Talbot, 2001], which means that the penetration of the plant surface is not relying on mechanical pressure, but based on the local secretion of lytic enzymes [Heiler *et al.*, 1993; Kämper *et al.*, 2006]. The penetrating hypha becomes encased by the cytoplasmic membrane of the host cell (Fig. 2E). The result is a so-called biotrophic interaction zone which facilitates "communication" between fungus and plant and provides *U. maydis*'s supply with nutrients. By means of effector secretion into the apoplastic space, *U. maydis* can suppress the plant associated molecular pattern triggered plant immune responses and produce a biotrophic interaction [Doehlemann *et al.*, 2008]. After the initial successful penetration, *U. maydis* grows intracellularly in epidermal cells.

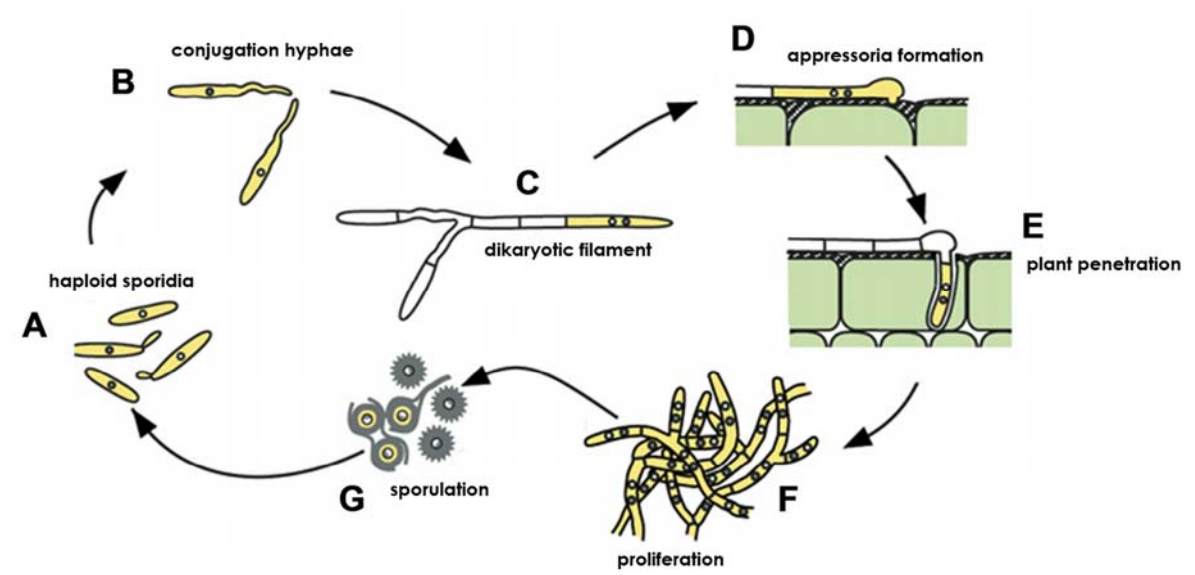

Figure 2: Schematic representation of the *Ustilago maydis* life cycle. The life cycle of *U. maydis* can be divided into a saprophytic (A-C) and a biotrophic stage (D-F). Modified from Kämper *et al.* (2006).

In the later stages of infection the hyphae penetrate into the deeper cell layers of the mesophyll, where massive proliferation takes place (Fig. 2F). During the entire infection cycle the plant tissue remains intact and an immune response of the plant is not observed. Initial tumor development can be observed approximately 4 days after infection under greenhouse conditions. During this stage tumors are induced, which contain enlarged plant cells that have resumed mitotic divisions [Callow, 1975]. In the tumor tissue karyogamy takes place, the hyphae fragment and mature into diploid teliospores, which are embedded in a mucilaginous matrix (Fig. 2G) [Banuett and Herskowitz, 1996]. Following rupture of the tumors, spores are released and are spread by wind, rain or animals. Under suitable conditions they germinate to form a probasidium in which meiosis occurs and from which haploid cells pinch off [Christensen, 1963]. The formation of the haploid sporidia completes the life cycle.

1.1.2 The importance of effectors for the *U. maydis* / *Zea mays* pathosystem

Resulting from its biotrophic lifestyle, *U. maydis* is dependent on the survival of its host plant, *Z. mays,* for a constant supply with nutrients. Hence, damage to the plant tissue, which would cause an immediate immune response of the plant, must be avoided. The successful suppression of the plant immune response and the reprogramming of the plant metabolism are requirements for the establishment of a compatible biotrophic interaction. To achieve this, *U. maydis* is equipped with a large arsenal of different secreted effectors, who can either be active in the apoplastic interaction zone or, following translocation, within the plant cell [Djamei et al, 2011; Doehlemann *et al.*, 2009; Hemetsberger *et al.*, 2012].

Compared to other phytopathogenic fungi, the genome of *U. maydis* is with 20.5 Mb, encoding for 6900 proteins, relatively small [Kämper *et al.*, 2006]. The genome codes for 466 proteins predicted to be secreted conventionally as well as

an unknown number of unconventionally secreted proteins. More than 50% of the secretome are putative effectors, which lack domains of known function and are expressed only during plant colonization. Many of the genes coding for these proteins are located in clusters; and many of the clusters contribute to virulence, either positively or negatively [Kämper *et al.*, 2006]. For some of the *U. maydis* effectors it could already be shown that they modulate the plant defenses directly. For example, the apoplastic effector Pep1 is essential for the establishment of a biotrophic interaction. While *pep1* deletion mutants are capable of forming appressoria and of plant penetration, they induce a strong plant defense reaction [Doehlemann *et al.*, 2009]. Subsequent studies revealed that Pep1 directly influences the virulence of *U. maydis* by inhibition of *Zea mays* peroxidases, thereby suppressing the early immune response of the plant [Hemetsberger *et al.*, 2012]. Another example for an apoplastic effector is Pit2, which is required for the successful suppression of the plant defenses by inhibiting a set of plant cysteine proteases [Doehlemann et al, 2011; Mueller et al, 2013]. Further, there are several translocated effectors whose biological importance has already been studied to completion. Following translocation, Cmu1 functions as a chorismate mutase in the shikimate pathway, converting chorismate to prephenate. The reduced pool of chorismate available to the maize plant results in a decreased production of salicylic acid [Djamei *et al.*, 2011]. Similarly, the effector Tin2 also weakens the plant defenses through its interaction with the maize protein kinase ZmTKK1, preventing its degradation. The result of increased ZmTKK1 activity is a rechanneling of metabolites into the anthocyanin pathway and thereby reduction of the amount of resources available for plant defense reactions [Tanaka *et al.*, 2014].

To understand the manipulative potential of the *U. maydis* secretome the localization and mechanism of action of all secreted proteins needs to be studied.

1.2 Unconventional pathways of protein secretion

Conventional secretion of proteins depends on an N-terminal hydrophobic signal peptide, which translocates the protein in question into the lumen of the endoplasmic reticulum from where the Golgi apparatus mediates the transport out of the cell via exocytosis. However, more recently, more and more proteins are shown to be targeted to the extracellular space although they do not possess a signal peptide.

Initial evidence for unconventionally secreted proteins was provided by the observation that the secretion of these proteins is not inhibited by Brefeldin A [Flieger *et al.*, 2003; Nickel, 2003; Rubartelli *et al.*, 1990]. Brefeldin A is an anti-fungal drug that interferes with the anterograde transport of proteins from the ER to the Golgi and causes a rapid fragmentation of the Golgi [Lippincott-Schwartz *et al.*, 1989; Orci *et al.*, 1991]. By now it is known that several discrete mechanisms exist for the unconventional secretion of proteins. These include vesicular transport via lysosomes and exosomes, vesicle independent transfer via plasma membrane shedding, as well as transporter mediated translocation (Fig. 3) [Nickel, 2005].

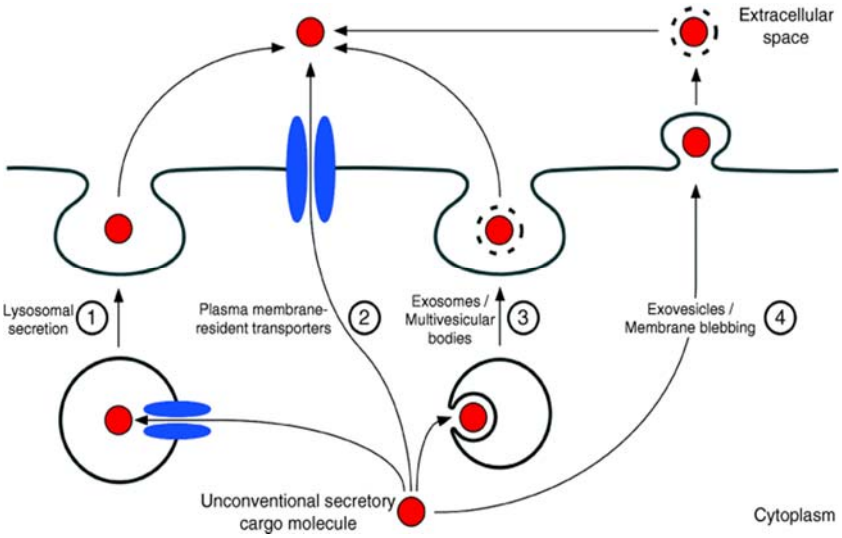

Figure 3: The four proposed pathways for unconventional secretion. Unconventional secretion of proteins can occur via secretory lysosomes (1), transporter mediated secretion (2), secretion by means of multi-vesicular body (MVB) derived exosome release (3) and secretion mediated by the shedding of plasma membrane derived microvesicles (4) [Figure taken from Nickel, 2005].

In the extracellular space, unconventionally secreted proteins usually serve distinct functions that can be completely different from their cytoplasmic function. An MS/MS/MS analysis of the apoplastic fluid (APF) of infected *Z. mays* plants (K. Schipper, T. Brefort, B. Macek, M. Mann and R. Kahmann, unpublished) uncovered a number of potentially unconventionally secreted *U. maydis* proteins, one of which was the *U. maydis* Acyl-CoA binding protein Acb1 (*um02959*).

1.3 Acyl-CoA binding proteins

1.3.1 The intracellular roles played by Acyl-CoA binding proteins

Acyl-CoA binding proteins (ACBPs) are highly conserved proteins, averaging in size around 10kDa, that are found in all four eukaryotic kingdoms, as well as in some eubacterial species [Burton *et al.*, 2005]. Named for their capability to bind to their ligand, the binding affinity of ACBPs is highly specific for long saturated Acyl-CoA esters, with the highest binding affinity being towards molecules of 14 to 22 carbons and highly saturated chains [Faergeman *et al.*, 1996; Frolov and Schroeder, 1998; Huang *et al.*, 2005; Knudsen *et al.*, 1989; Mikkelsen and Knudsen, 1987; Mogensen *et al.*, 1987; Schroeder *et al.*, 2008]. No affinity towards non-esterified fatty acids and only low affinity towards free CoAs could be shown [Rosendal *et al.*, 1993].

Studies in yeast, rat hepatoma cell lines and mouse liver, both *in vitro* and *in vivo*, have determined that ACBPs participate in a large number of cellular functions such as the regulation of the fatty acid metabolism (β-oxidation and complex lipid synthesis), the modulation of the intracellular Acyl-CoA pool size and vesicular trafficking. Additionally, ACBPs are known for their ability to bind to type A gamma-

aminobutyrate ($GABA_A$) receptors and to temper the function of peripheral-type benzodiazepine receptors [Besman *et al.*, 1989; Guidotti *et al.*, 1983].

More concretely, the regulation of the Acyl-CoA pool was extensively studied by overexpression of the native *acbp* gene in *Saccharomyces carlsbergensis*, resulting in a significantly larger intracellular Acyl-CoA level [Knudsen *et al.*, 1994; Yang *et al.*, 2001]. From mammalian models, it was determined that this occurs thanks to ability of the ACBPs to extract Acyl-CoAs from the membrane [Rasmussen *et al.*, 1994]. Further, it was found that ACBPs can also release the Acyl-CoA synthetase, an enzyme that converts fatty acids to Acyl-CoAs, from its end-product inhibition and thereby heighten its turnover rate [Rasmussen *et al.*, 1993].

ACBPs participate in the fatty acid metabolism in different ways depending on the concentration of ACBP bound Acyl-CoA. Given their ability to bind to Acyl-CoA molecules, ACBPs can transport the bound Acyl-CoAs to the mitochondria or the endoplasmic reticulum (ER), where they can be used by the cell for metabolic processes like β-oxidation or glycerolipid synthesis, depending on requirement [Abo-Hashema *et al.*, 2001; Rasmussen *et al.*, 1994].

Interestingly, *acbp* mutations have extensively been studied in *S. cerevisiae*. Initial studies concerning the disruption of the *acb1,* encoding the yeast ACBP, showed a growth defect in comparison to the wild type when the cells were grown on a poor carbon media as ethanol, but no differences were observed when cells were grown on a rich media containing glucose [Schjerling *et al.*, 1996]. However, when grown together on rich media, the mutant could not compete with wild type cells. Deeper examination showed that lack of ACBP result in the synthesis of long Acyl-CoA chains by the fatty acid synthase (FAS) and consequently in a ~2 fold increase in overall intracellular Acyl-CoA levels. Thus it was suggested that, in *S. cerevisiae,* ACBPs are essential for the proper synthesis of Acyl-CoA molecules and therefore for processes where fatty acids are needed [Schjerling *et al.*, 1996].

In order to further determine the effect of *acb1* on growth rate, a depletion strain was created, which initially showed a much stronger growth defect. This was subsequently suppressed, leading to a faster growing phenotype [Gaigg *et al.*, 2001]. No changes were observed in relation to general glycerolipid, phospholipid or the total Acyl-CoA pool, but a significantly increased Stearoyl-CoA (C18:0) and reduced Cerotic acid (C26:0) levels were detected [Gaigg *et al.*, 2001]. As a consequence, the strain accumulated autophagocytotic vesicles in the cytosol and exhibited perturbed plasma membrane structures. From these results, it was deduced that Acb1 holds an important function during yeast membrane assembly and organization, by providing a specific pool of Acyl-CoAs required in membrane trafficking [Gaigg *et al.*, 2001]. Studies of the native ACBPs of numerous mammal derived organs or cell lines corroborate the discoveries made in *S. cerevisiae* [Chao *et al.*, 2003; Faergeman and Knudsen, 2002; Fyrst *et al.*, 1995].

1.3.2 Unconventional secretion of Acb1 in *S. cerevisiae*

Unconventional secretion of Acb1 was also studied in *S. cerevisiae*. It was shown that starvation induces ACBP secretion and triggers the generation of a so called 'compartment of unconventional secretion' (CUPS), a process which requires, amongst others, the Grh1 protein. A model was proposed in which acetylated Acb1 proteins, binding an Acyl-CoA ligand, associate with the CUPS. The CUPS snare off vesicular intermediates containing Acb1 on their cytoplasmic face, which then fuse with the endosomes. The Acb1 protein, transported on the surface of the vesicular intermediates, is deposited on the external face of the endosome and then internalized into intraluminal vesicles. This is followed by the release of the Acb1 containing exosome-like vesicle into the extracellular space after fusion of the endosome to the plasma membrane. Subsequent lysis of the exosome-like vesicles release Acb1 to the extracellular space (Fig. 4) [Bruns, 2011; Duran *et al.*, 2010; Kinseth *et al.*, 2007; Manjithaya *et al.*, 2010; Manjithaya & Subramani, 2010; Manjithaya & Subramani, 2011].

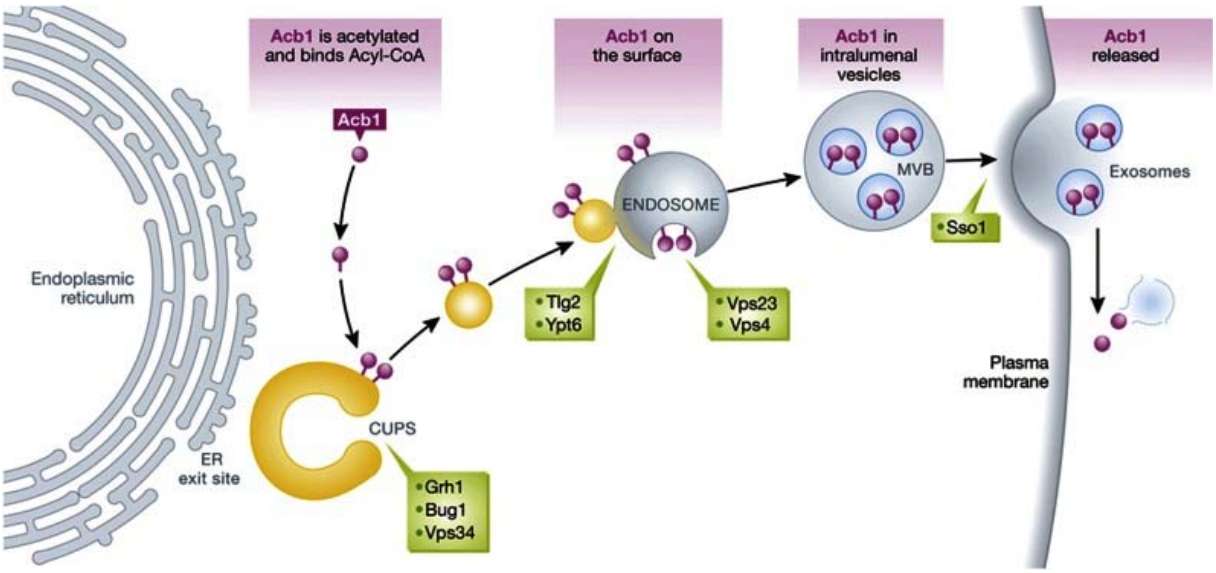

Figure 4: Model for the unconventional secretion of Acb1 based on *S. cerevisiae*. [Figure taken from Malhotra, 2013]

1.3.3 The amoeba *Dictyostelium discoideum* and the life cycle essential role of its ACBP

In the social amoeba Dictyostelium *discoideum*, the native ACBP was also studied for its relevance in the spatio-temporal regulation of the later stages of development. In the presence of nutrients, the unicellular amoeba *D. discoideum* moves towards nutrient sources, such as bacteria found on decaying vegetation, and divides via binary fission. Upon starvation, cyclic AMP (cAMP) is emitted by pulses, to which other *D. discoideum* cells are attracted [Manahan *et al.*, 2004]. In response to the perceived cAMP signal, cells move towards its source and emit their own pulse of cAMP, thus amplifying and propagating the signal. As a result, large numbers of cells congregate forming streams of migrating cells, which ultimately merge into an aggregate containing up to 100,000 cells. This aggregate initially forms the shape of

a mound that develops into a so called 'slug', which migrates to a favorable environment for the formation of a fruiting body. Upon release of the spores from the fruiting body, in the presence of nutrients, germination can occur to complete the life cycle [Christenholm and Firtel, 2004].

For the formation of a functional fruiting body, differentiated cell types, such as the basal disc cells, spore cells and stalk cells, are essential. Originating from a homogeneous population in the early slug, the precursors of these cell types, which can be identified by specific markers already prior to their differentiation, are pre-allocated in the slug. Following reception of external stimuli indicating a suitable location, transition of the slug into the mature fruiting body is initiated [Esch and Firtel, 1991; Fosnaugh and Loomis, 1993; Ozaki *et al.*, 1993; Williams *et al.*, 1989; Yoder and Blumberg, 1994]. It is at this point that apically localized pre-stalk cells invaginate into the pre-spore mass, differentiate into stalk cells and form the stalk tube, which elongates through the pre-spore mass, raising it off the substratum [Jermyn *et al.*, 1996]. Simultaneously, the anterior slug cells migrate to form part of the basal disc. As the stalk tube is formed, pre-spore cells enter the terminal differentiation pathway and generate spores [Aubry and Firtel, 1999]. In order for this process to function properly, temporal and spatial organization is essential [Loomis, 1998].

The spatio-temporal regulation of sporulation is controlled by a complex signaling pathway, dependent on the unconvential secretion of the *D. discoideum* Acyl-CoA binding protein AcbA by the pre-store cells [Duran *et al.*, 2010; Abrahamsen und Stenmark, 2010]. Resulting from the proteolytic cleavage of the AcbA protein by the TagC protease expressed by the pre-stalk cells, an SDF-2 peptide is generated by removal of the two termini, which binds to the histidine kinase receptor DhkA found on the surface of both the pre-spore and pre-stalk cells. The binding to the DhkA prevents a phosphorelay, via the phosphodonor RdeA, to the cAMP phosphodiesterase RegA thereby reducing its activity. Resulting from the reduced activity of RegA, the intracellular cAMP levels generated by the late adenylyl cyclase ACR are increased. As a result the regulatory subunit of the PKA is disassociated from the catalytic subunit, increasing the activity of the PKA, which results in spore encapsulation. Similarly the increased PKA activity has an effect on the pre-stalk cells, resulting in the increased expression of the TagC protease (Fig. 5) [Anjard and Loomis, 2005].

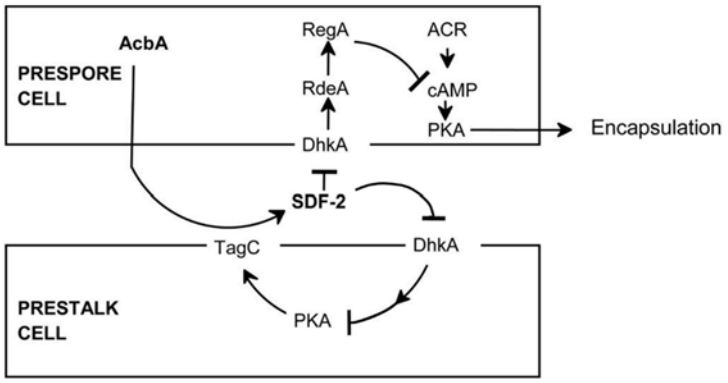

Figure 5: Schematic representation of the *D. discoideum* AcbA dependent signaling pathway leading to sporulation. Modified from Anjard and Loomis, 2005.

1.3.4 The Acyl-CoA binding protein Acb1 of *U. maydis*

The gene *um02959*, providing the transcript for the *Ustilago maydis* Acyl-CoA binding protein Acb1, is located on chromosome 7 and encodes a protein of 106 amino acids in length [Pedant3 database, June 2015], translating to approximately 12 kDa. The protein is transcribed without a signal peptide [SignalP version 4.1, May 2016], and contains a confidently predicted Pfam ACBP domain [http://smart.embl-heidelberg.de, June 2016], characterizing Acb1 as an Acyl-CoA binding protein. No further functional domains could be identified. In an RNAseq experiment it was shown that *acb1* is strongly expressed in all stages of the *U. maydis* life cycle, with upregulation in planta. The regulation pattern resembles the regulation of other fatty acid synthesis pathway related genes (D. Lanver, personal communication).

U. maydis Acb1 has been shown capable of functionally replacing the intracellular function of the *S. cerevisiae* Acb1 protein [J. Jungmann, unpublished]. In *U. maydis*, a protein Um11226 (536 amino acids) exists, which contains a region with high Acb1 homology. Controls throughout the experiments exclude an effect of this protein.

1.7 Aim of the PhD

Over the course of this thesis, the *U. maydis* Acb1 protein was to be characterized. Based on the gathered information from the ACBPs of *S. cerevisiae* and *D. discoideum*, as well as the apoplastic fluid proteomic dataset (K. Schipper, T. Brefort, B. Macek, M. Mann and R. Kahmann, unpublished), a model was proposed that stated an unconventional secretion of the Acb1 protein to the biotrophic interaction zone. Further, on account of the processing of *D. discoideum* AcbA, extracellular processing of Acb1 was postulated and a signaling function of the resulting peptide was also suggested (Fig. 6).

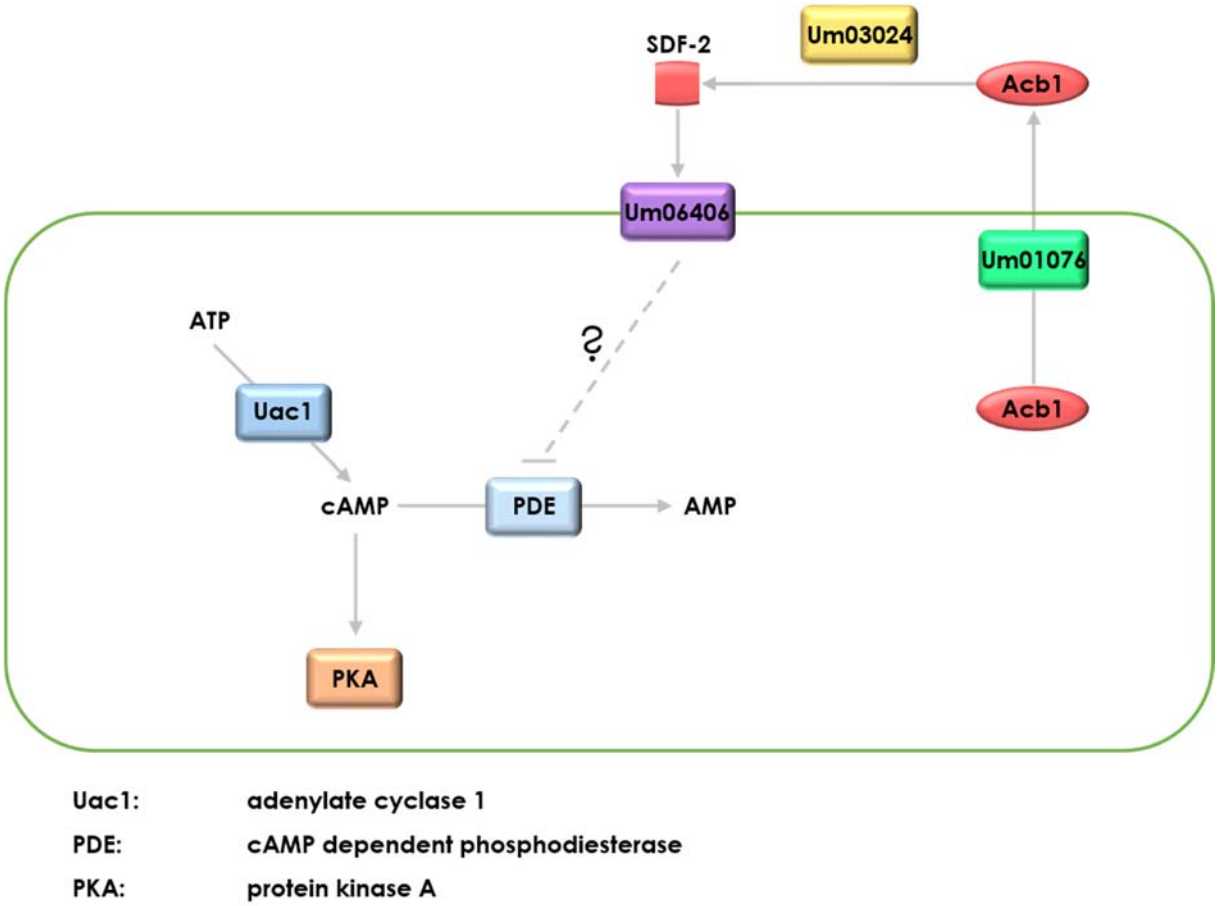

Figure 6: Model for the secretion of the *U. maydis* Acb1 protein. As depicted in this scheme the model proposes a secretion pathway for the Acb1 protein to be dependent on the compartments of unconventional secretion (CUPS) as published for the *S. cerevisiae* ACBP. Um01076 a homologue of the pathway essential Grh1 protein was suggested to secrete Acb1 in *U. maydis*. The Acb1 protein would then be processed by the secreted protease Um03024, analogous to the processing of the *D. discoideum* ACBP protein, producing an SDF-2 like peptide through the removal of the two termini. A receptor for the SDF-2 like peptide was proposed to be Um06406. Finally it was suggested that through a, further not described, signaling cascade downstream of the putative receptor, an inhibition of the cAMP dependent phosphodiesterase (PDE) would occur, raising the intracellular cAMP levels.

Scrutiny of the *U. maydis* genome determined Um01076 to be a distant homologue to Grh1 protein, shown to be essential for the assembly of the CUPS in *S. cerevisiae*. Concerning the extracellular processing of the secreted Acb1 protein analogous to the ACBP of *D. discoideum* by the TagC protease, no homologue of the TagC protease could be identified. However, a secreted *U. maydis* protease Um03024 was found, which was described to perform a trypsin like cleavage, similar to the one performed by the TagC protease.

Also with respect to the candidate receptor of the SDF-2 like peptide, no homologue of the *D. discoideum* DhkA protein could be found. However, *U. maydis* does have a membrane bound peripheral-type benzodiazepine receptor Um06406 and since it was known that ACBPs are capable of interacting with this type of

protein [Besman *et al*., 1989; Guidotti, 1983], it was proposed that this protein functionally replaced the DhkA in the proposed pathway.

Finally, from the proposed receptor Um06406, an indistinct signaling cascade would inhibit the activity of the cAMP dependent phosphodiesterase thereby raising the intracellular level of cAMP. Preceding studies had already established that, also in *U. maydis,* the intracellular cAMP level do regulate the activity of the PKA and thereby all the life cycle relevant downstream processes.

Hence, the aim of this thesis was to provide evidence for the unconventional secretion of the *Ustilago maydis* Acb1 protein and its subsequent processing into an SDF-2 like peptide. Additionally, the involvement of the Grh1 homologue Um01076 and the secreted protease Um03024 in the secretion of Acb1 and its processing were investigated. Advancing, a putative effector function of the secreted population was to be investigated, with the focus of the research being on purpose the SDF-2 like peptide.

2 Results

2.1 The secretion of the unconventionally secreted protein Acb1 in the axenic culture of *U. maydis*

In the aforementioned apoplastic fluid proteomic dataset (K. Schipper, T. Brefort, B. Macek, M. Mann and R. Kahmann, unpublished) the Acb1 protein was identified by a single C-terminal peptide, as one of 65 proteins lacking a signal peptide. To rule out that this was due to cell lysis, proof of unconventional secretion needed to be provided. An axenic culture assay was designed based on the AB33 strain. The AB33 strain was chosen due to the possibility to simulate both, the budding and the filamentous stages of the life cycle, by using Ammonium Medium (AM) or Nitrate Medium (NM) respectively [Brachmann *et al.*, 2001].

In this background a Strep-HA tagged *acb1* allele, as well as a GFP-myc allele, was integrated as single copy into the *ip*-locus. The *acb1* allele was under control of its native promoter and the GFP expression was controlled by the strong constitutive P_{otef} promoter (SR1116). In culture supernatants of this strain only the unconventionally secreted Acb1 would be detected and the cytoplasmically expressed GFP would act as a lysis control.

Given the potential processing of Acb1 into an SDF-2 like peptide, the protease inhibitor Tosyl phenylalanyl chloromethyl ketone (TPCK) was added, as TPCK was known to inhibit the *D. discoideum* TagC protease [Cabral *et al.*, 2006]. SR1116 was grown in AM medium to an OD of 1.0, then shifted into either fresh AM or NM and harvested after 8 hours. It was found that the Strep-HA-Acb1 could be enriched from the culture supernatant and visualized by a slot blot. However, the reproducible detection of the protein in the supernatants, in the absence of the lysis control, was enigmatic. The protein could only be detected in a fraction of the repetitions of the assay (~40%); hence, the experimental conditions were put under scrutiny. Various means of protein enrichment, such as lyophilization and Trichloroacetic acid (TCA) precipitation were assayed and it was found that with TCA precipitation, in combination with Western, rather than Slot blots, the reproducibility was improved (50%-60%).

It had been published that secretion of the AcbA protein of *D. discoideum* and the Acb1 protein of *S. cerevisiae* depend on the autophagy machinery which involves the *atg* genes [Duran *et al.*, 2010]. When the expression of the exophagy pathway essential protein Atg8 was tested in AB33, following the shift into NM, by means of a real-time polymerase chain reaction (RT-PCR) experiment, it was found that the exophagy pathway was activated very quickly following the shift into NM (F. Hartwig, personal communication).

Based on these results, the AB33 supernatant was analyzed over a time course in the presence of TPCK. Supernatants of filamentous cells were harvested every 2 hours following the shift into nitrate containing medium and TCA precipitated. Two identical Western Blots were run with the resulting samples, one was developed with anti-HA antibodies, the other using anti-GFP antibodies (Fig. 7).

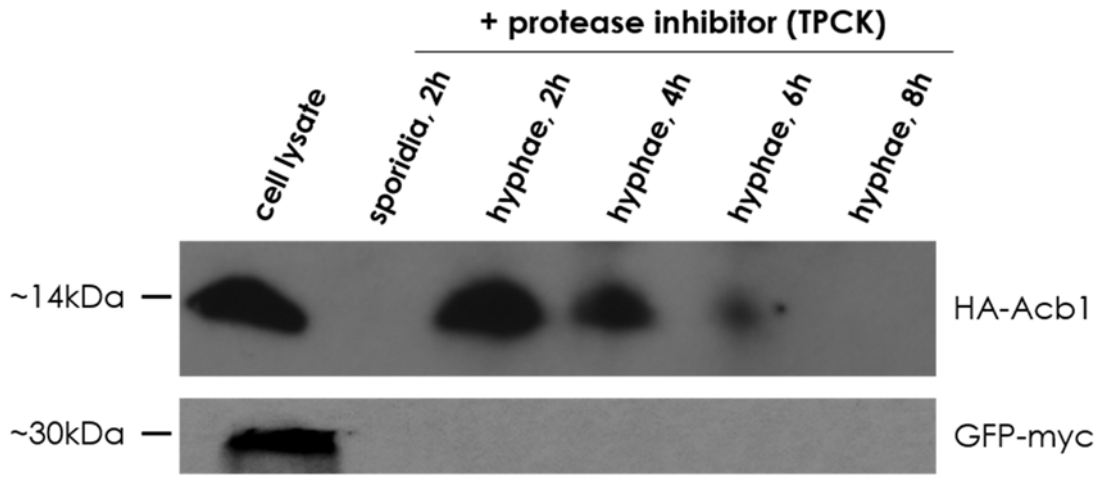

Figure 7: Secretion time course of the Acb1-HA protein from SR1116. SR1116 was grown in AM medium to an OD of 1.0. Cells were then shifted into fresh AM for the sporidia samples, or NM to produce hyphae. The supernatants of the AM medium as well as one of the NM samples were harvested after 2 hours; the remaining NM cultures were harvested subsequently every 2 hours. The entire experiment was carried out in the presence of the protease inhibitor TPCK. The supernatants were TCA precipitated and protein quantities present in each sample were visualized via Western Blot. For each sample 2 blots were done, one with anti-HA and one with anti-GFP antibodies. A total cell lysate is shown to confirm that the proteins are expressed in the experimental strain.

The anti-HA Western blot shows a strong signal for the Acb1 protein in the anti-HA blot at the 2 hour time point. Over the course of eight replicates it could be shown that the amount of detectable Acb1 in the supernatant decreases over time. Detection of the Acb1 protein is no longer possible after 6 to 8 hours, depending on variations within the replicates (Fig. 7). In budding cells grown in AM, Acb1 cannot be detected at the 2 hour time point. The anti-GFP blot shows not signal in any of the samples.

2.1.1 The secreted *U. maydis* Acb1 protein is processed

Having established the reproducible detection of Acb1 in the supernatants of SR1116 in the presence of TPCK, the next step was to address the question of the processing of the protein. For this purpose the secretion assay was carried out by growing SR1116 in NM in the presence and absence of the TPCK. Supernatants were harvested 2 hours after the shift to NM and following TCA precipitation the presence of Acb1 in the samples was assayed by Western Blot. The Acb1 protein could be detected in the control sample grown in the presence of TPCK. However, in the absence of TPCK no signal was seen. The anti-GFP blot providing the cell lysis control gives no indication that cell lysis occurred (Fig. 8).

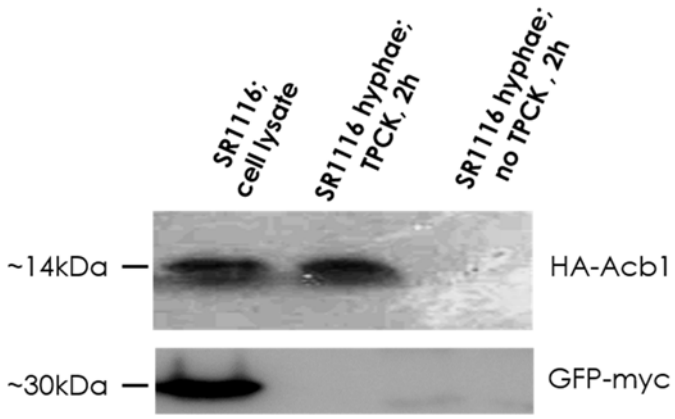

Figure 8: Processing of the Acb1-HA protein in the absence of TPCK. SR1116 was grown in AM to an OD of 1.0, at which point the cells were shifted into NM and grown for a further 2 hours, once in the presence and once in the absence of the protease inhibitor TPCK. The supernatants were harvested, TCA precipitated and tagged proteins were visualized via Western Blot. For each sample 2 blots were done, one was developed with anti-HA antibodies (top panel) and one with anti-GFP antibodies (lower panel).

Given the observation that Acb1 is processed in the absence of TPCK, it was decided to determine whether Um03024 is responsible of this process. By means of homologous recombination of the *um03024* gene with a hygromycin (hyg) cassette, a deletion of *um03024* was created in the SR1116 strain. The resulting SR820 strain showed no significant difference in growth rate when compared to its progenitor (data not shown). Secretion of Acb1 was assayed in the SR820 strain. Following the growth in AM, the SR820 was grown into NM in the presence and comparatively in the absence of the TPCK. Supernatants were harvested after 2 hour time point and TCA precipitation was used for the enrichment of the proteins (Fig. 9).

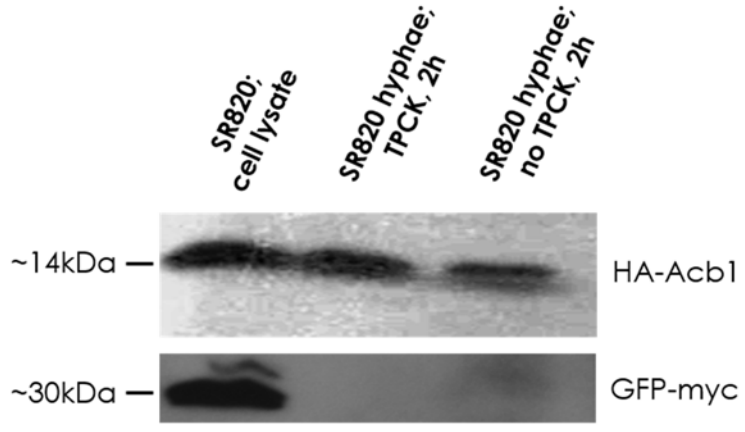

Figure 9: Abolishment of processing of Acb1-HA in the Δ*um03024* strain (SR820). SR820, a deletion strain of *um03024* in the SR1116 background, was grown in AM to an OD of 1.0. The cells were shifted into NM and grown for 2 hours, in the presence or absence of TPCK. Following harvesting of the supernatants and their TCA precipitation, the tagged proteins were visualized via Western Blot. For each sample, 2 blots were prepared, one was developed with anti-HA antibodies (top panel) and one with anti-GFP antibodies (lower panel).

It was shown that the Acb1 protein of strain SR1116 can be detected in full length both in the presence and the absence of the protease inhibitor. This shows that the Acb1 protein is no longer processed in SR820 and hence, by extension, that Um03024 is the protease responsible for the processing of the Acb1 protein.

2.1.2 The role of the Grh1 like protein Um01076 in the secretion of Acb1

An amino acid sequence comparison of the Grh1 protein of *S. cerevisiae* and the Um01076 protein showed the existing homology to be low; hence, a functional conservation could not be assumed [G. Mannhaupt, personal communication]. However, on account of Um01076 being the only homologue of Grh1 that could be identified in *U. maydis*, it was decided to study its role in the secretion of Acb1.

In order to investigate whether the *U. maydis* Acb1 protein follows the same unconventional secretion pathway as the ACBPs of *D. discoideum* and *S. cerevisiae,* the AB33 secretion assay was carried out using a deletion mutant of the *um01076* gene in the experimental strain background (SR564). The supernatant was harvested from filamentous cells at the 2 hour time point in the presence of TPCK. Via Western Blot it was shown that in the *um01076* deletion strain, the Acb1 protein is still secreted (Fig. 10).

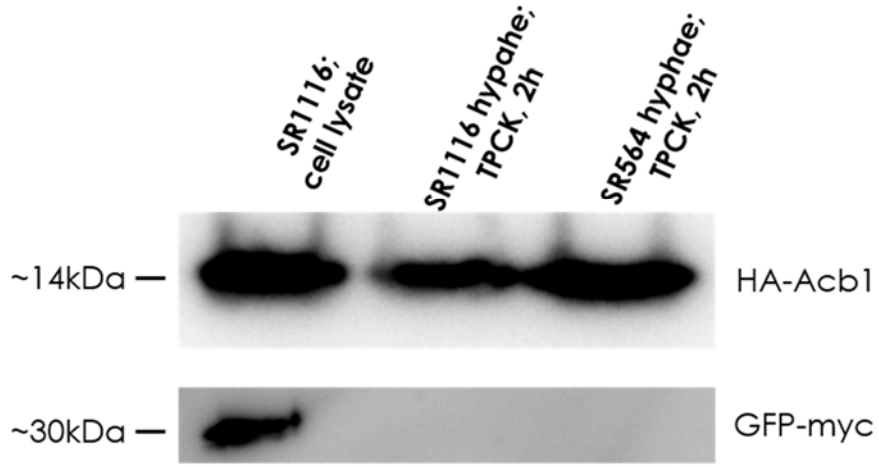

Figure 10: Secretion of the Acb1-HA protein SR564, carrying a deletion of *um01076*. SR1116 and SR564 were grown in AM to an OD of 1.0; the cells were shifted into NM and then grown for 2 more hours in the presence of TPCK. Following the harvesting of the supernatant, and their TCA precipitation, the contained proteins were visualized via Western Blot. For each sample, 2 blots were prepared, one was developed with anti-HA antibodies (top panel) and one with anti-GFP antibodies (lower panel).

2.2 The biological activity of the *Ustilago maydis* SDF-2-like peptide in the *D. discoideum* bioassay

Given that the Acb1 protein can be processed by a tryspin like protease, the next objective of the study was to determine whether this processing resulted in the proposed *U. maydis* SDF-2 like peptide, and subsequently whether it had biological activity in the *D. discoideum* based bioassay, analogous to the activity of the *D. discoideum* SDF-2 peptide as published by Anjard and Loomis [2005]. Preliminary data obtained by T. Ringel in collaboration with C. Anjard could show that the trypsination of Acb1 released a peptide with SDF-2 like activity and that such a peptide could be found in AB33 supernatants, as well as in the apoplastic fluid of SG200 infections (T. Ringel, personal communication). Lack of replicates, as well as inconsistencies in the results, required that the *D. discoideum* bioassay was established. To this end the KP4++ strain, a recreation of the published KP4 strain kindly provided by W. Nellen, was used. All the bioassay data shown in this thesis are based on the KP4++ strain.

2.2.1 The biological activity of the synthetic SDF-2 peptide of *D. discoideum* in the KP4++ strain-based bioassay

To establish the bioassay with the KP4++ strain, the SDF-2 peptide of *D. discoideum* was synthesized and its activity was determined. For this purpose, KP4++ cells were cultivated in HL5+ medium and set to a cell density of 10^3 in a cAMP containing priming buffer. Following 24 hours of growth in cell tissue plates, the cultures were supplemented with the synthetic SDF-2 peptide and following 2 hours of incubation time the relative amounts of spores and amoeba were determined by microscopy. Addition of the SDF-2 peptide increased the amount of cells undergoing sporulation. The highest possible concentration of spores that could be achieved in the KP4++ strain is about 40%. The buffer control was not supplemented with the SDF-2 peptide, showing that a basal level of sporulation activity exists under these conditions, which results in about 10% of the cells becoming (Fig.11).

To determine the range in which the SDF-2 peptide is active, serial 1:2 dilutions were assayed. It was establish that 1 unit of activity (U) of the synthetic *D. discoideum* peptide equates to approximately 1pM, where U is defined as the minimal concentration of SDF-2 required to achieve maximum sporulation under the experimental conditions (Fig. 11).

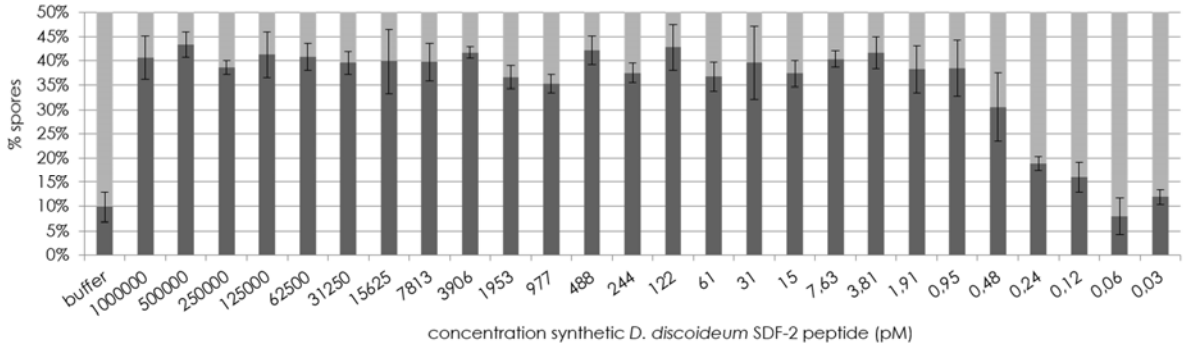

Figure 11: Determination of the detection limit of the SDF-2 peptide of *D. discoideum*. *D. discoideum* KP4++ cells were set to a cell density of 10^3 and grown in a cAMP containing priming buffer for 24 hours in cell culture plates. The culture wells were then supplemented with the respective amounts of peptide to reach the indicated concentrations and spores were counted 2 hours later. The basal level of sporulation was determined in Kp4++ cells that were not treated with any peptide. The average of 3 experiments is presented; error bars depict the standard deviation.

An identical assay was carried out making use of a synthetic negative control peptide, which contained the same amino acids as the native *D. discoideum* peptide, but in a random order. This peptide exhibited no biological activity at any of the tested concentrations (Fig. 12).

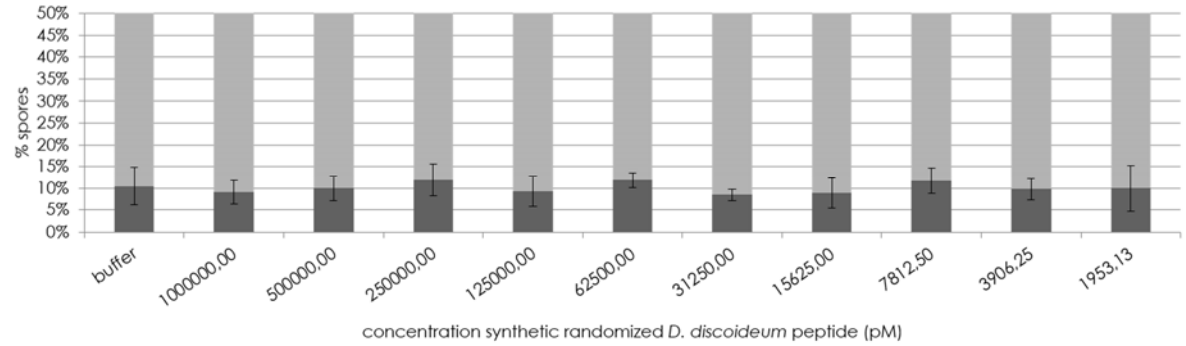

Figure 12: Determination of the detection limit of a randomized SDF-2 peptide of *D. discoideum*. *D. discoideum* KP4++ cells were set to a cell density of 10^3 and grown in a cAMP containing priming buffer for 24 hours in cell culture plates. The culture wells were then supplemented with the amounts of respective peptide to reach the indicated concentrations and spores were counted 2 hours later. The basal level of sporulation was determined in Kp4++ cells that were not treated with any peptide. The average of 3 experiments is presented; error bars depict the standard deviation.

2.2.2 Biological activity of the synthetic SDF-2 like peptide of *U. maydis*

An amino acid comparison between the Acb1 protein of *U. maydis* and the AcbA protein of *D. discoideum* was done and based on the known processing sites, a SDF-2 like peptide was proposed (Fig. 13).

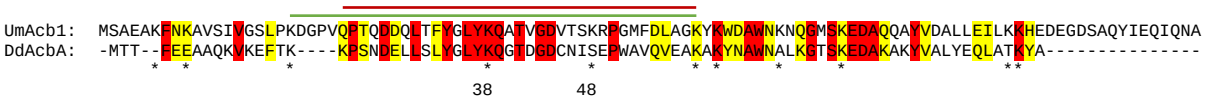

Figure 13: Amino acid sequence comparison between Acyl-CoA binding protein of *U. maydis* and *D. discoideum*. The *U. maydis* protein shares an amino acid sequence identity (indicated red) of 45% and sequence similarity of 62% (indicated yellow) with *D. discoideum* [NCBI blastp, 07.06.2016]. Indicated are also the SDF-2 peptide of *D. discoideum* (red bar), the proposed SDF-2 like peptide of *U. maydis* (green bar), as well as potential trypsin cleavage sites (*, as predicted by ExPASy (June, 2013)). The trypsination sites at amino acid 38 and 48 are located within the proposed SDF-2like peptide.

Within the proposed SDF-2 like peptide, two trypsin cleavage sites exist at amino acid position 38 and 48, the first of which can also be found in the sequence of the *D. discoideum* peptide. On account of the site not being cleaved in the SDF-2 peptide of *D. discoideum*, it was discounted in the synthetic SDF-2 like peptide of *U. maydis* as well. It was proposed that, potentially through the binding of an Acyl-CoA ligand, the cleavage sites within the peptide were not accessible. Similarly, it was assumed that a second cleavage site, exclusive to the proposed SDF-2 like peptide, was not digested for the same reason. The proposed SDF-2 like peptide of *U. maydis* is shown in figure 13.

Next, the bioassay was carried out making use of the synthetic SDF-2 like peptide of *U. maydis* (Fig. 14). This peptide of 39 amino acids differs from the *D. discoideum* peptide at 27 positions.

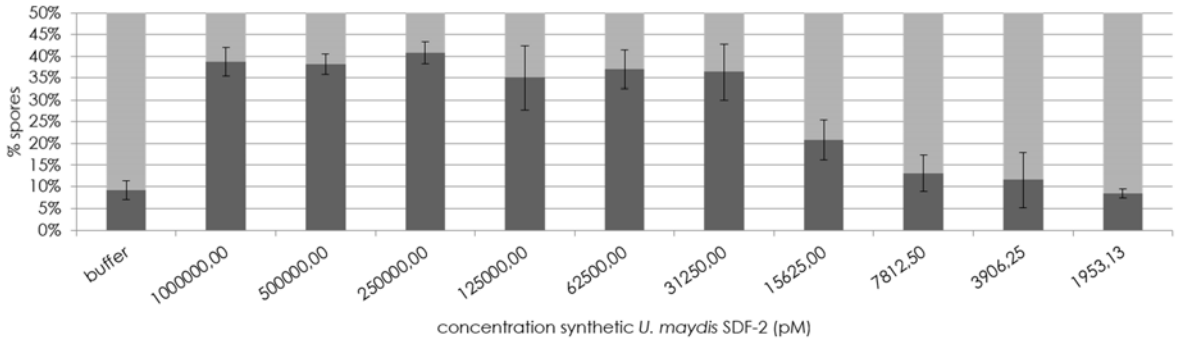

Figure 14: Determination of the detection limit of the synthetic SDF-2 like peptide of *U. maydis*. *D. discoideum* KP4++ cells were set to a cell density of 10^3 and grown in a cAMP containing priming buffer for 24 hours in cell culture plates. The culture wells were then supplemented with the respective amounts of peptide to reach the indicated concentrations and spores were counted 2 hours later. The basal level of sporulation was determined in Kp4++ cells that were not treated with any peptide. The average of 3 experiments is presented; error bars depict the standard deviation.

The synthetic SDF-2 like peptide of *U. maydis* was able to induce sporulation in the *D. discoideum* bioassay. However, its biological activity was considerably lower than that of the *D. discoideum* peptide and as a result, between 15 and 30nM of peptide were required to achieve maximum sporulation.

To show that *U. maydis* Acb1 does contain the SDF-2 like peptide and that it can be released by *in vitro* trypsin digestion of the purified Acb1 protein, *U. maydis* Acb1 protein was purified from the cytosolic fraction of SR1116. For this purpose, the full length UmAcb1-Strep-HA protein was purified from the cytoplasm via Strep-

affinity-column-chromatograph and via a Bradford assay the purified protein amount was determined to be 60 µg/ml. A grand total of 120 µg were isolated. A fraction of 100 µl of the purified protein was hydrolyzed via trypsin treatment overnight. As a control the same amount of protein was submitted to the same treatment procedure, without the addition of trypsin. The trypsin treated sample was enriched for the SDF-2 like peptide via an A25-matrix anion-exchange-chromatography, as it was published for the *D. discoideum* SDF-2 peptide [Anjard *et al.*, 1998a; Anjard *et al.*, 1998b]. The two samples were equalized in terms of volume and then assessed for biological activity in the *D. discoideum* bioassay (Fig. 15).

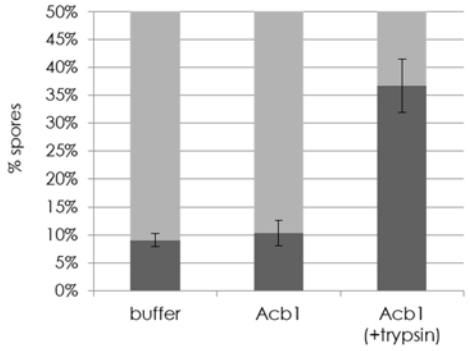

Figure 15: SDF-2 like activity of the full length *U. maydis* Acb1 protein prior and subsequent to trypsin treatment. KP4++ cells were grown to a cell density of 10^3 and primed in a cAMP containing buffer for 24 hours in cell culture plates. The cells were then subjected to either the full length Acb1 [~1 µg] protein or to the peptide, following its trypsin digestion [amount of peptide released from 1µg of full length Acb1, ~59nM]. The average of 3 experiments is presented; error bars depict the standard deviation.

From the results, it can be concluded that the full length Acb1 protein does not have SDF-2 activity in the bioassay. However, after trypsin treatment of Acb1, a peptide with biological activity can be enriched.

In order to determine if the activity of the purified SDF-2 like peptide and the synthetic peptide are comparable, the activity of the trypsinated Acb1 was assayed in a bioassay (Fig. 16).

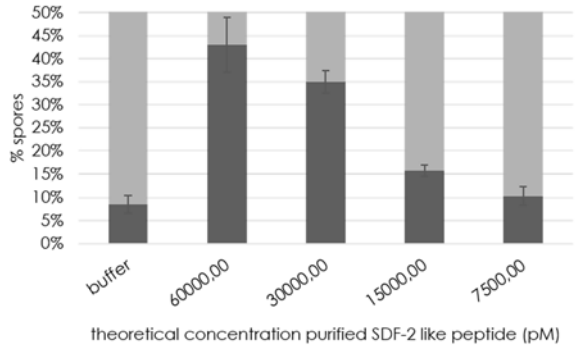

Figure 16: Determination of the detection limit of the SDF-2 like peptide purified from trypsinated Acb1. *D. discoideum* KP4++ cells were set to a cell density of 10^3 and grown in a cAMP containing priming buffer for 24 hours in cell culture plates. The culture wells were then supplemented with the SDF-2 like peptide purified from the trypsinated Acb1 protein to reach the indicated concentrations and spores were counted 2 hours later. The indicated concentrations are based on the assumption that all Acb1 was successfully trypsinated and no peptide was lost during the purification. The basal level of sporulation was determined in Kp4++ cells that were not treated with any peptide. The average of 3 technical replicates is presented; error bars depict the standard deviation.

The SDF-2 like peptide purified from trypsinated Acb1 was able to induce sporulation in the *D. discoideum* bioassay. Its U was determined to be in the range of 30 – 60nM.

2.2.3 The biological activity of the SDF-2 like peptide contained in *U. maydis* axenic culture supernatants

To address the next question, whether processing of Acb1 in *U. maydis* supernatants also resulted in a peptide that has SDF-2 like activity, supernatants were collected from the hyphae of SR1116, an AB33Δacb1 strain (SR616), an AB33Δ01076 strain (SR564) as well as an AB33Δ*um03024* strain (SR820), at the 2 hour time point. Supernatants were also collected from SR1116 sporidia, at the same time point. Samples were enriched for SDF-2 like peptides using the A25-matrix. All experiments were carried out in the presence and absence of TPCK (Fig. 17).

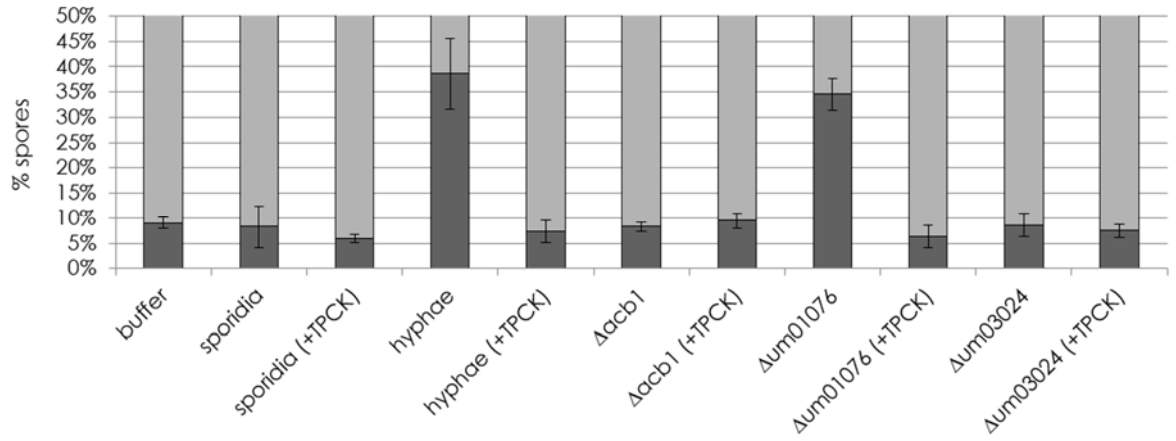

Figure 17: Activity of the SDF-2 like peptide in AB33 secretion assay supernatants. KP4++ cells were grown to a cell density of 10^3 and primed in a cAMP containing buffer for 24 hours in cell culture plates. The cells were then subjected to the samples, which were enriched for the SDF-2 like peptide derived from the supernatants of SR1116 grown as sporidia or hyphae, as well as SR616, SR564 and SR820, grown as hyphae. For each strain, samples were taken in the presence and absence of TPCK.

The results of the assay showed that in sporidia the SDF-2 like peptide is not produced, which is consistent with the previously described lack of secretion of Acb1 in sporidia. In hyphae, SDF-2 like activity could be detected confirming that Acb1 is secreted and that it is also processed into an SDF-2 like peptide *in vivo*. Additionally, SDF-2 like activity could be detected in the supernatants of the SR564 strain, carrying the *um01076* deletion, but not in SR820, carrying the *um03024* deletion. This supports the conclusion that Um01076 is not important for the secretion of Acb1, whereas Um03024 is important for the processing of Acb1. These results prove that no other protease secreted by *U. maydis* is capable of processing Acb1 into an active SDF-2 like peptide. In strains where an SDF-2 like activity was detected in the supernatant, this activity was gone when TPCK was added. This supports that the SDF-2 peptide is released by the activity of a TPCK inhibited protease.

2.3 The effect of Acb1 depletion on *Ustilago maydis*

To study the function of Acb1 in *U. maydis* and assign a function to the extracellular and intracellular forms, deletion mutants of *acb1* were constructed. From the literature it is known that the deletion of the AcbA protein of *D. discoideum* and the Acb1 protein of *S. cerevisiae* resulted in a prominent growth defect, which was spontaneously suppressed by suppressor mutations, which restored wild type-like growth [Gaigg *et al.*, 2001]. Similarly, when deleting the *acb1* gene of *U. maydis* in the FB1 wild type strain, an initial strong reduction in growth rate was suppressed by spontaneous selection for naturally occurring mutations providing a higher growth rate. It was observed that these mutations were accumulated immediately following transformation on the recovery plates, and had to be selected by singling out. Since this accumulation of mutations occurred very rapidly, a characterization of the *acb1* deletion strain, prior to accumulation of the suppressor mutants, proved impossible. To generate a strain in which the Acb1 protein could be transiently downregulated,

an *acb1* allele, under control of the arabinose inducible *crg1* promoter, was introduced into the *ip*-locus of FB1. Next, the native *acb1* gene of this strain was deleted and replaced by a hygromycin cassette to yield the SR966 strain. All of these steps were done in media containing arabinose to assure expression of the *acb1* gene in the *ip*-locus. Growth of SR966 was then to be evaluated comparatively to the FB1 wild type strain. In a first growth assay, it was investigated whether the amount of Acb1 produced under control of the *crg1* promoter resulted in wild type like growth. For this purpose, growth curves of SR966 as well as FB1 were conducted in CM-arabinose (Fig. 18A) and it could be shown that, under these conditions, growth of the two strains was comparable.

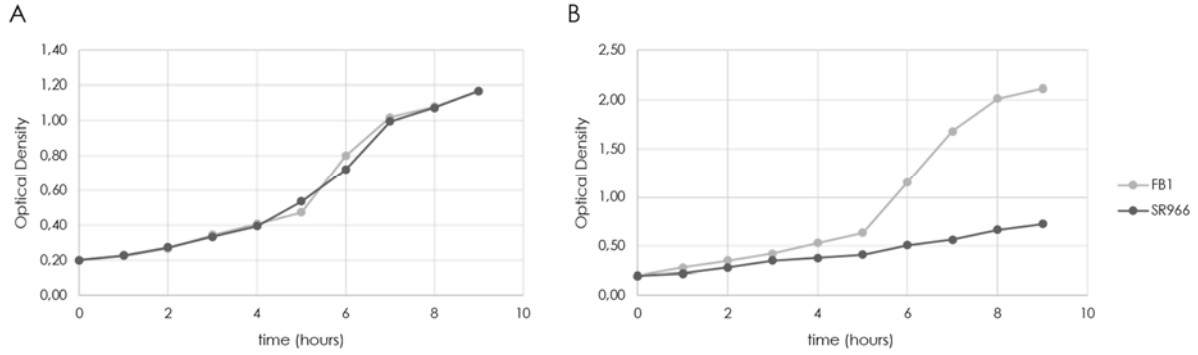

Figure 18: Growth curves of SR966 and FB1. (A) The strains were grown in CM-arabinose over a time course of 9 hours. **(B)** SR966 was depleted in CM-glucose for three days until the intracellular Acb1 amount was reduced sufficiently to observe a diminished growth rate. The two strains were then grown comparatively for 9 hours in CM-glucose.

For a conditional deletion strain of the ACBP in the parasitic protozoan *Trypanosoma brucei,* it had been shown that it took only approximately 9 generations of depletion in the exponential growth phase to reduce the intracellular amount of the ACBP to a low enough level to observe a detrimental effect on growth rate [Milne *et al.*, 2001]. Hence, the SR966 strain was depleted in CM+glucose over the course of several days, continuously being diluted to remain in the exponential phase. It was found that after ~48 hours (~24 generations) the strain started growing slower. After 72 hours (~36 generations) the intracellular Acb1 concentration was sufficiently diminished to not reduce the growth rate any further. From this time point on, the growth rate of the Acb1 depleted SR966 was compared to FB1 over the course of 9 hours. It can easily be discerned that the growth of the depletion strain is markedly reduced (Fig. 18B).

2.4 *acb1* deletion strains, suppressors and virulence in SG200

In order to analyze the function of the extracellular Acb1 and the SDF-2 like peptide, the *acb1* gene was deleted in SG200. SG200 is a haploid solopathogenic strain which was engineered to express an active bE1/bW2 hetrodimer and display autocrine pheromone stimulation [Kämper *et al.*, 2006]. SG200Δ*acb1* showed poor growth, however within the previously described short timeframe, the observed growth rate was increased, presumably due to an accumulation of suppressor

mutations. Three independent suppressor strains were isolated (SR444, SR465 & SR467), in which the suppressor mutations lead to a differential recovery of the growth defect. The suppressor strains were complemented by the integration of a single copy of the *acb1* allele under control of the native promoter into the *ip*-locus (SR456, SR478 & SR477 respectively). A growth comparison between the suppressor strains and complementation strains was carried out in $YEPS_{light}$ medium (Fig. 19).

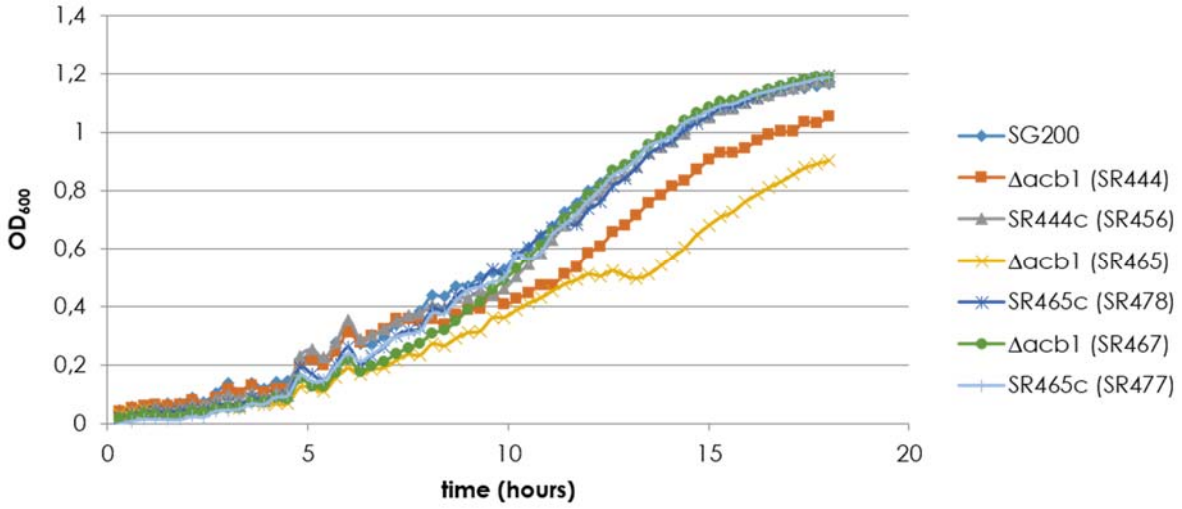

Figure 19: Growth rates of the SG200Δ*acb1* suppressor strains and their complementation strains. The SG200 strain, the SG200Δ*acb1* suppressor strains (SR444, SR465 & SR467) and the respective complementation strains (SR456, SR478 & SR477) were cultivated in $YEPS_{light}$ medium and their cell density was determined photometrically over time.

The assay revealed that all complementation strains show SG200 like growth. In addition, some of the spontaneous suppressor mutations were capable of fully storing the growth defect of an *acb1* deletion strain, as exemplified by the SR465 strain. Further, it could be shown that not all suppressor strains fully restore wild type like growth.

By whole genome resequencing it was found that all three strains have a single point mutation in the fatty acid synthase 1 (FAS 1, *um10339*) and that the SR465 strain has an additional mutation in the *um01335* gene, a mitochondrial Acyl-CoA dehydrogenase. It was concluded that the suppressor mutations are likely restoring processes related to the intracellular function of Acb1 (J. Jungmann, not published).

In order to investigate whether the suppressor strains were differentially affected under stress conditions, the three suppressor strains and their respective complementation strains were subjected to various stressors. To this end, the strains were spotted on CM agar plates supplemented to induce osmotic stress (NaCl, Sorbitol), oxidative stress (H_2O_2) and cell wall stress (Calcofluor, Congored). The ability to form b-filaments was tested on charcoal supplemented medium (Fig. 20).

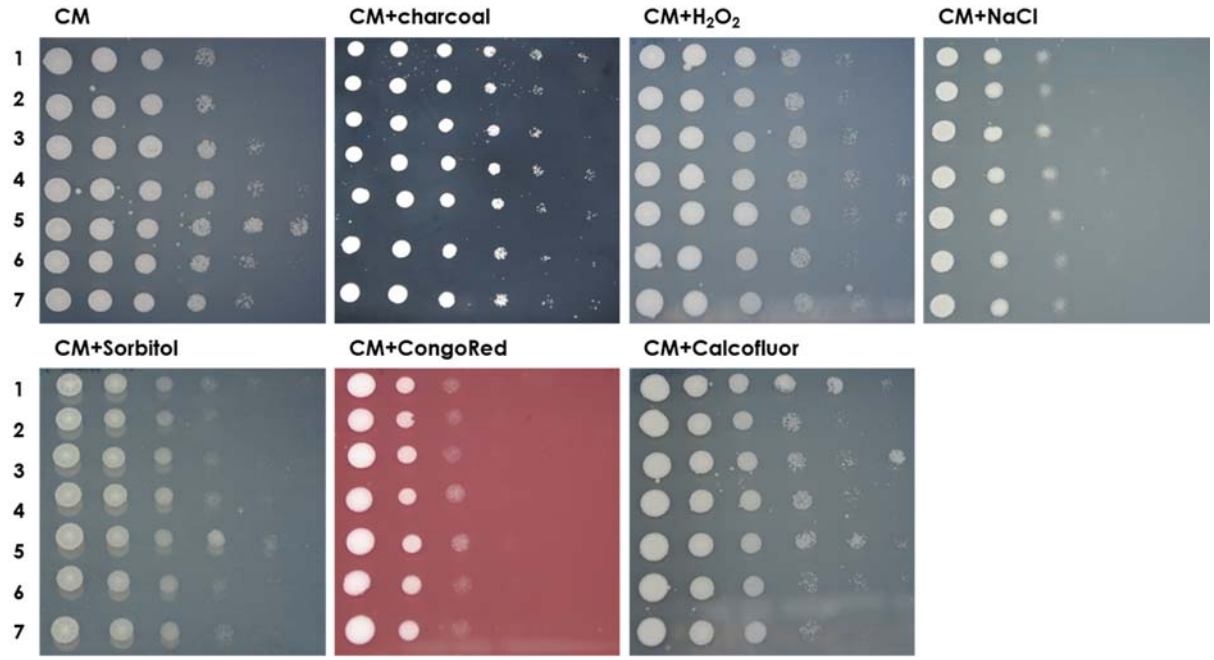

Figure 20: Stress sensitivity assays of the Δ*acb1* suppressor. Serial dilutions of the strains SG200 (1), SG200Δ*acb1* (SR444; 2), SR444 complemented (SR456; 3), SG200Δ*acb1* (SR465; 4), SR465 complemented (SR478; 5), SG200Δ*acb1* (SR467; 6), SR467 complemented (SR477; 7) strains were spotted on stress and charcoal media, and incubated for two days at 28°C.

The results for the stress assays confirmed a minor growth defect for the SR444 strain as well as the SR467 strain, though on the growth plates it was less distinct than in the growth curves and could not be seen on all the plates. It can be concluded that the *acb1* suppressor strains do not show an enhanced sensitivity to the tested stressors.

To study the extracellular role of Acb1 and the SDF-2 like peptide, the SR465 strain was chosen, since it showed a complete suppression of the initial growth defect. Furthermore, because of the deletion of the *acb1* gene in SR465, this strain should not produce the SDF-2 like peptide. To prove this, seven day old *Z. mays* plants were infected with SG200 as well as the SR465 strain and apoplastic fluid (APF) was extracted 6 days later. The APF was enriched for the SDF-2 like peptide via the A25-matrix and the samples were then tested in the *D. discoideum* bioassay (Fig. 21).

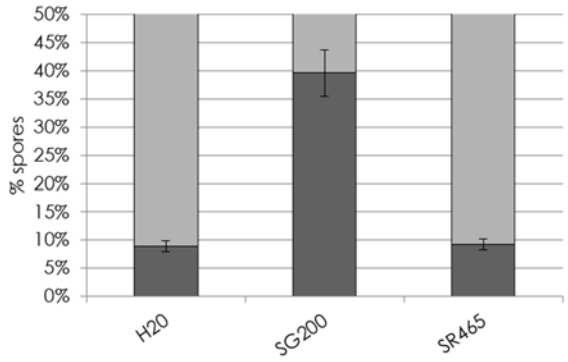

Figure 21: The SDF-2 like peptide acting in apoplastic fluid of SG200 and SR465 infected plants. Apoplastic fluid was extracted from *Z. mays* plants infected with the indicated strains. Following anion-exchange-chromatography enrichment for the SDF-2 like peptide, the samples were tested for the presence of the SDF-2 like peptide by means of the *D. discoideum* bioassay. The average of 3 independent biological experiments is presented; error bars depict the standard deviation.

The bioassay established that an SDF-2 like peptide is detectable in the apoplastic fluid of SG200 infected *Z. mays* plants 6 days post infection, but not in the apoplastic fluid of infections with the *SR465* strain. This indicates that under these conditions, Acb1 is the only *U. maydis* protein capable of producing such a peptide, and similarly that *Z. mays* does not produced an SDF-2 like peptide.

In a follow-up to the *D. discoideum* bioassay, maize plants were infected with SG200, the SG200Δ*acb1* suppressor strains SR444, SR465 & SR467 and the respective complementation strains. The virulence symptoms were evaluated 12 days post infection and sorted into categories as published by Kämper *et al.* [2006] (Fig. 22).

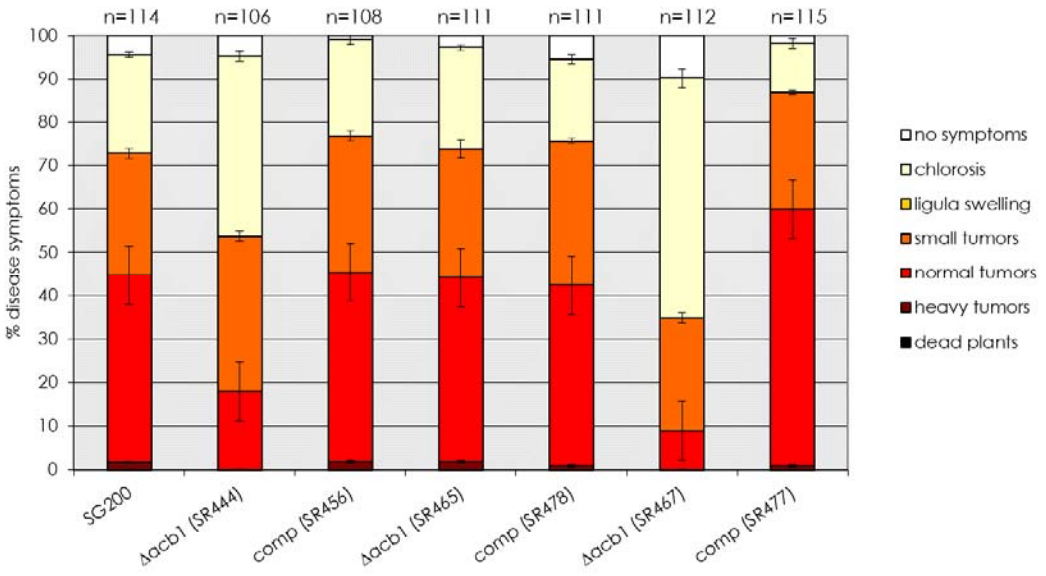

Figure 22: Virulence of the SG200Δ*acb1* suppressor strains and their complementation strains. Cell suspensions of the described strains were injected into 7 day old maize seedlings via syringe infection. Plant symptoms were quantified 6 days post infection and differentiated into the published disease scoring categories shown on the right [Kämper *et al*, 2006]. For this data, three independent infections were carried out, the average of which is displayed here as a percentage of the total number of infected plants (n). The error bars depict the standard deviation.

It could be shown that the SR465 strain, which was fully suppressed for the growth defect, exhibited comparable virulence as to the SG200 reference strain. Further, the SR444 strain characterized in the growth assay by a minor growth defect showed a minor virulence reduction. Finally, the virulence of the SR467 strain was reduced to a larger extent, corresponding to the respective larger reduction in growth rate. All complementation strains showed virulence comparable to the reference strain SG200.

Additionally, 30 days post infection, all suppressor strains and the complementation strains displayed spores in stem tumors. Comparative quantification of spores in planta is not feasible. Macroscopically however, neither spore distribution nor quantity of the spores in the suppressor or the complementations seemed to differ from the wild type. To ensure that the observed spores are capable of germination, a spore germination assay was conducted with representative stem tumors being harvested from SG200 or SR465 infected plants. Spores were isolated from the tumors via mechanical separation (see materials & methods). The spore concentration was determined by means of a Neubauer improved cell counting chamber and adjusted to a concentration of 500 spores/ml for both samples. Spore suspensions were plated on PD agar plates and the percentage of germination spores was determined following an incubation of two days at 28°C (Fig. 23). Spores resulting from the infection of plants with SR465 germinate, as well as those from the SG200 infection.

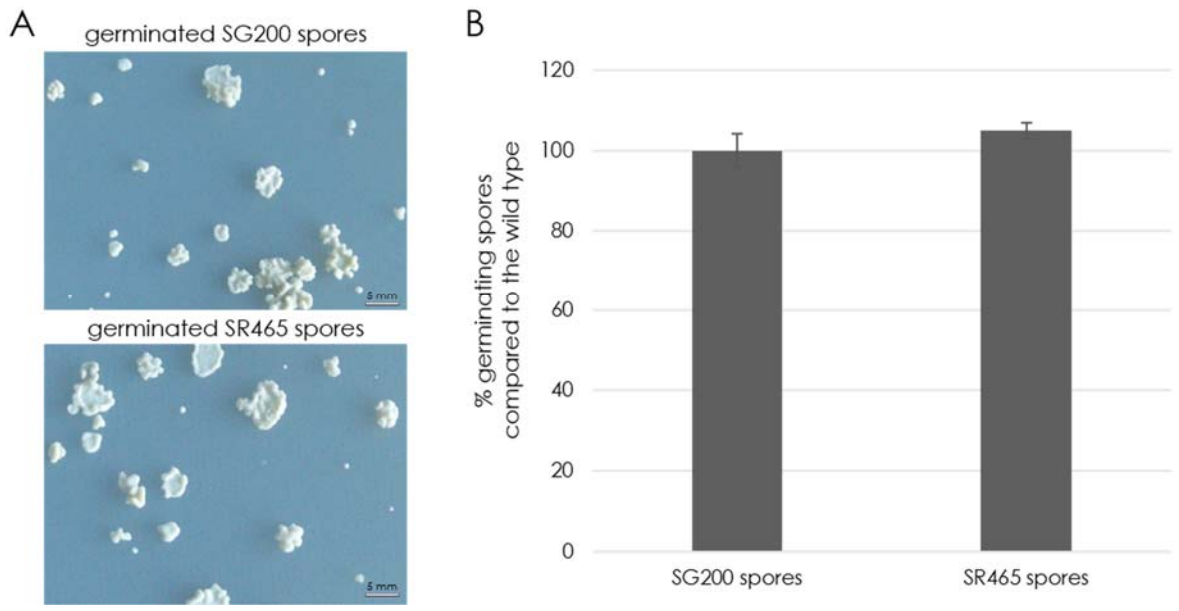

Figure 23: Spore germination of SG200 spores and SR465 spores in comparison to the wild type (FB1/FB2). (A) Representative images of the growth plates are shown. **(B)** Quantification of spore germination. The amount of germinating spores of the wild type was set to 100%, the number of germinating spores of the mutant is given as a percentage of the wild type. The assay was performed for tumors harvested from three independent infections in three technical replicates each. The error bars represent the standard deviation.

Given the absence of any observable virulence related defects in the SR465 strain, a virulence related function of the extracellular Acb1 or the SDF-2 like peptide in the solopathogenic strain SG200 is considered unlikely.

The deletion of *um01076,* encoding the *grp1* gene, as well as the double deletion of *um03023* and *um03024*, encoding a putatively secreted uncharacterized protein and the trypsin like protease respectively, did also not result in attenuated virulence symptoms (S. Thiele and N. Rössel, personal communication). This reinforces that a contribution of the extracellular Acb1 to virulence in SG200 is unlikely.

2.5 Compatible haploid *acb1* deletion strains

2.5.1 Characterization of suppressor mutants for the initial growth defect

Given that an extracellular function of secreted Acb1 in SG200 was considered unlikely, it was decided to study the importance of extracellular Acb1 in the haploid wild type strains. Deletions of *acb1* were produced in FB1 and FB2, by replacing their *acb1* genes by hygromycin cassettes. Each of the mating partners was grown in several tubes containing $YEPS_{light}$, to accumulate different suppressor mutations, and subsequently plated on hygromycin containing medium such that individual colonies were produced. Over 20 different suppressor mutations were screened for each mating partner and a pair of suppressor mutants, with comparable growth rates, was selected (SR526/SR529). None of the other suppressor strains showed a higher growth rate than that of the selected strains (data not shown). The growth assays comparing SR526 and SR529 to their progenitors is shown in figure 24.

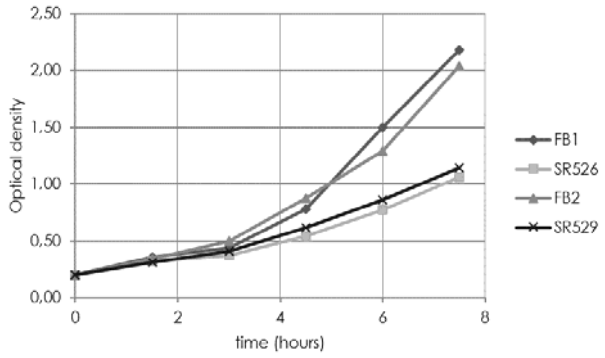

Figure 24: Growth rate determination of the SR526/SR529 suppressor strains in comparison to the respective wild type strains. The FB1 and FB2 strains and their derived Δ*acb1* strains suppressed for the prevalent growth defect, were cultivated in $YEPS_{light}$ medium and their cell density was determined photometrically over time.

The growth assay revealed that the growth rate of the suppressor strains was still significantly reduced compared to that of the progenitor strains.

Despite the incomplete suppression, mating assays were carried out between SR526 and SR529, as well as with their respective wild type mating partners FB1 and FB2 (Fig. 25).

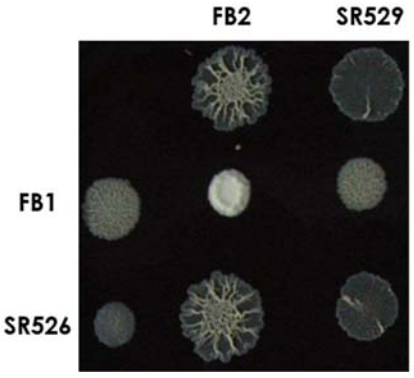

Figure 25: Mating competence of the suppressor strains SR526 and SR529. The indicated strains were grown to an OD of 1.0 and either spotted alone or in the indicated mixtures, on CM charcoal agar plates. The image was taken after 48 hours of incubation at room temperature.

The suppressor strains SR526 and SR529 were not able to produce the characteristic white dikaryotic filaments when mated with each other. They were also not able to produce these filaments when mated with the compatible wild type mating partner. The morphology of the FB1 SR529 mating colony resembles that of the FB1 colony without a mating partner. The same holds true for the SR526 FB2 mating colony which looks akin to the FB2 colony. Since the formation of the dikaryon is a requirement for plant colonization, infections with mixtures of these suppressor mutants displayed no disease symptoms (data not shown).

2.5.2 Identification of SR526 and SR529 derivatives suppressed for the mating defect

In a repetition of the original mating assay it was found that the suppressor strains, previously characterized by their inability to mate on charcoal, now produced small amounts of filaments. Following screening for this new phenotype, two strains could be isolated which are able develop filaments when co-spotted on CM-charcoal plates. These new strains (SR1202/SR1204) were further characterized.

To determine whether the additional suppressor mutations had an effect on the growth rate, growth of SR1202 and SR1204 was compared to the FB1 and FB2 wild type strains (Fig. 26).

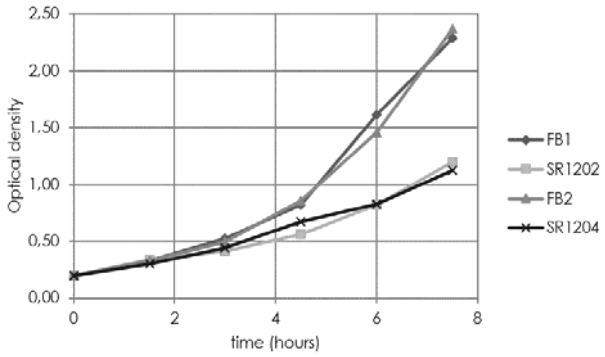

Figure 26: Growth rate of SR1202 and SR1204 in comparison to the respective wild type strains. The FB1 and FB2 strains and the suppressor strains SR1202 and SR1204, partially suppressed for the growth defect as well as the mating defect, were grown in $YEPS_{light}$ medium and their cell density was determined photometrically over time.

The assay showed that the restoration of filamentation had not suppressed the growth defect observed in SR526 and SR529 any further (Fig. 24).

Analogous to the characterization of SR526 and SR529, mating assays were carried out for SR1202 and SR1204 in comparison to the wild type mating partners FB1 and FB2 (Fig. 27).

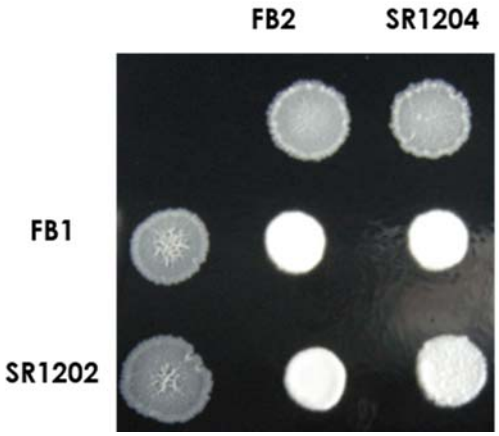

Figure 27: Mating competence of the suppressor strains SR1202 and SR1204. The indicated strains were grown to an OD of 1.0 and either spotted alone or in the indicated mixtures, on CM charcoal. After 48 hours at room temperature the plate was photographed.

The suppressor strains SR1202 and SR1204, in contrast to SR526 and SR529, were able to produce dikaryotic filaments when mated with each other on charcoal. Mating with the compatible wild type mating partner was equally successful.

It was observed that during the mating assay, at 24 hours, the overall density of the filaments on the surface of the colony appears reduced in the suppressor strains as compared to the wild type strains, which may be connected to the reduced growth of the suppressor mutant (Fig. 28A). Next, the filaments were removed from the mating plate and stained with 4,6-diamidino-2-phenylindole (DAPI) to characterize them with respect to being dikaryotic (Fig. 28B).

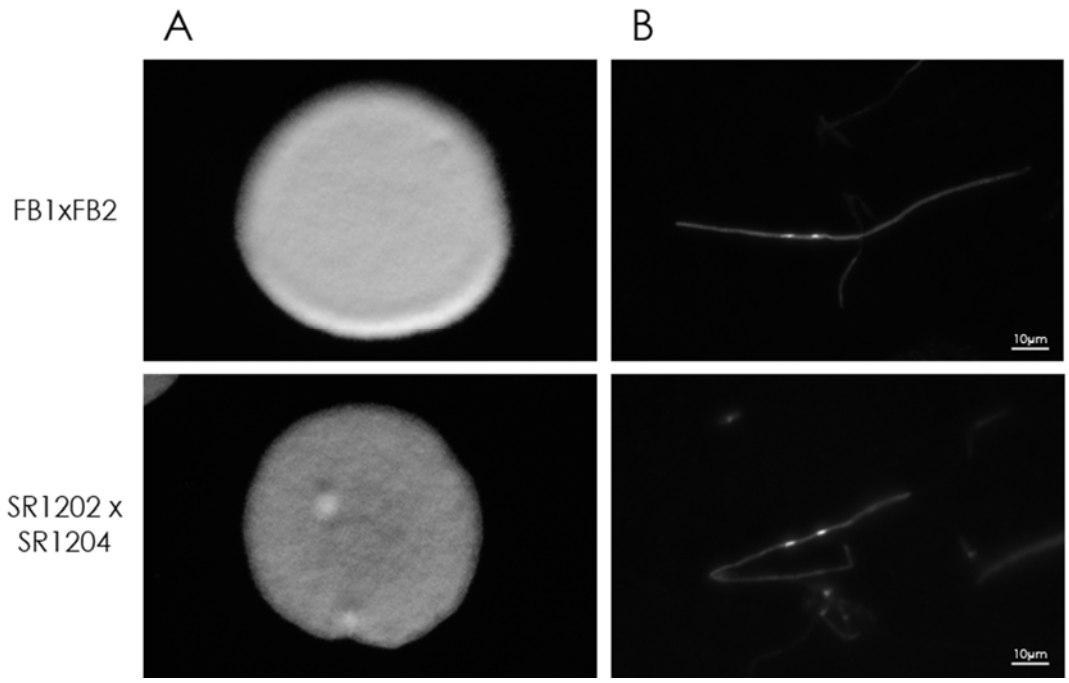

Figure 28: SR1202 x SR1204 mating on charcoal. (A) The indicated mixtures of strains were co-spotted on PD-charcoal. Following 24 hours of growth at room temperature the spots were photographed **(B)** The white filaments were pulled from the colony and resuspended in H_2O. Following fixation on object slides, the filaments were stained with DAPI.

Following DAPI staining, the microscopic analysis of mutant filaments revealed that they contain 2 nuclei like the wild type mixtures.

Given that the suppressor mutants SR1202 and SR1204 are capable of forming dikaryotic filaments, the strains were further investigated with respect to a putative function of the Acb1 protein and the SDF-2 like peptide in the infection of *Z. mays*. Prior to plant infections, it was decided to characterize the fitness of the SR1202 and SR1204 strains. The growth behavior of the mutants was assayed under different stress conditions and compared to that of the wild type strains (FB1 & FB2 respectively). The growth on full medium (CM) was assayed as a baseline, followed by growth on CM charcoal medium. The stress conditions comprised of the induction of oxidative stress (H_2O_2), Osmotic Stress (Sorbitol, NaCl) and cell wall stress (Congo red, Calcofluor) (Fig. 29).

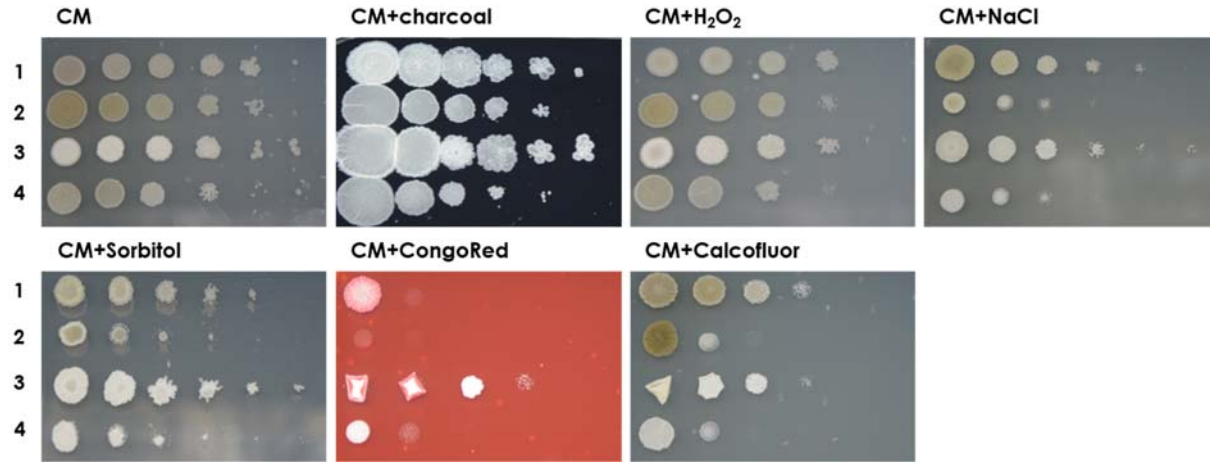

Figure 29: Stress sensitivity assays of the Δ*acb1* suppressor strains in comparison to the FB1 and FB2 background strains. Serial dilutions of the strains FB1 (1), FB1Δacb1 (2), FB2 (3) and FB2Δacb1 (4) were spotted on stress and charcoal media, and incubated for two days at 28°C.

On full medium (CM), as well as on the charcoal plates, a distinct defect in growth rate was observed. This defect confirms the data obtained for the growth assay. Under oxidative stress conditions, the growth pattern of the mutants is comparable to that in the respective CM control, indicating that deletion of *acb1* does not result in any disadvantage under oxidative stress conditions. For both, the osmotic and the cell wall stressor plates, a significant reduction in growth rate respective to the relevant controls can be attributed. It can be summarized that the SR1202 and SR1204 suppressor strains are detrimentally affected under stress conditions compared to the wild type.

Given that the FB1Δacb1 and FB2Δacb1 were capable of forming b-filaments on charcoal, it was decided to perform plant infection with the SR1202/SR1204 suppressor strains. Seven day old maize plants were infected with cell suspensions of the suppressor mutant mating, in comparison to the wild type mating (Fig. 30).

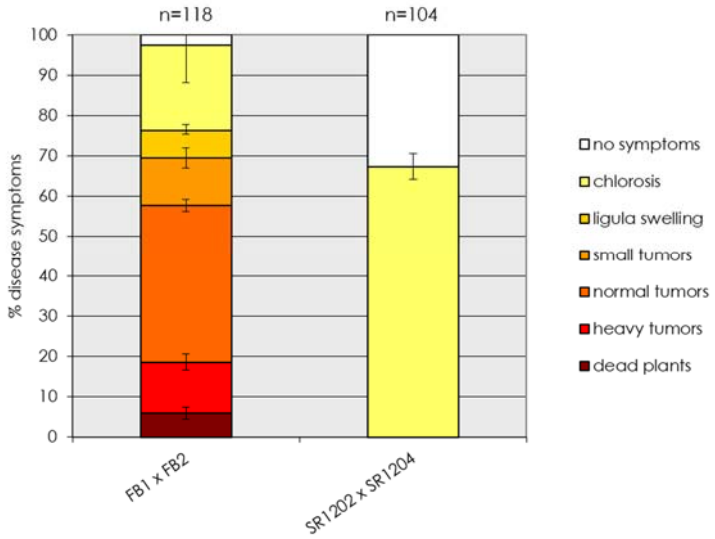

Figure 30: Virulence in the suppressor strains (SR1202/SR1204). Cell suspensions of the described strains were injected into 7 day old maize seedlings via syringe infection. Plant symptoms were quantified 6 days post infection and differentiated into the published disease scoring categories shown on the right [Kämper *et al*, 2006]. For this data three independent infections were carried out, the average of which is displayed here as a percentage of the total number of infected plants (n). The error bars depict the standard deviation.

The *acb1* deletion strains SR1202 and SR1204, partially suppressed for both the growth defect as well as the mating defect, were unable to cause disease symptoms such as tumors. Other indicators of successful plant colonization, e.g. the accumulation of anthocyanin, were also not observed.

To identify the accumulated mutations in SR1202 and SR1204 genomes which enabled partial growth recovery, as well as the restoration of mating, the genomes of the suppressor strains and their wild type progenitors were resequenced making use of the Illumina 454 platform. The resulting reads were in all cases mapped against the published *U. maydis* 521 genome [Kämper *et al.*, 2006] by the CLC genomic workbench (version 8.5.1). A mutation accumulation analysis was done in which single nucleotide polymorphisms (SNPs), multiple nucleotide polymorphisms (MNPs), and deletions and insertions (indels) were identified. The mutation list of the respective FB1 and FB2 wild type and mutant were then compared to detect mutations exclusive to the suppressor mutant (Table 1).

Table1: Suppressor mutations detected in SR1202 and SR104

strain	localization[1]	Type of mutation[2]	mutation	Gene/ position related to nearest gene	Coding region exchange	function of protein
SR1202	chr11; 690607-690608	MNP	TG->AC	*um04629*	Ala721Ser	Acetyl-CoA carboxylase
	chr13; 423762	SNP	C->A	end of chromosome; telomeric region		
	chr19; 1-2	insertion	TGCCTTTG CACTGAG CTCGGCA	beginning of chromosome; telomeric region		
SR1204	Chr01; 2148530	SNP	C->T	1107bp upstream of *um00727*		hypothetical protein
	Chr06; 987582-987583	insertion	TCCGTCTC CGTC	680bp upstream of *um02807*		hypothetical protein
				209bp upstream of *um02808*		related to nitrogen assimilation transcription factor nit-4
	Chr09; 506653-506776	deletion	124bp	818 bp upstream of *um03557*		related to nitrogen assimilation transcription factor
	Chr09; 200925-200942	deletion	18bp	*um03463*	Gly1570Glu 1575 deletion	putative protein: lipid methabolism
	Chr14; 4-5	insertion	TGGTCGAT T	telomeric region first bps of the chromosome		
	Chr15; 178658-178659	insertion	GTGAATG GTGAATG	280 bp upstream of *um11770*		putative lysine methyltransferase

Further study of the strains was intended in order to characterize the incidence of defect on planta. First and foremost, it was intended complement the deletion strains with a signal peptide equipped *acb1 allele*, to investigate whether the virulence defect could be rescued by an extracellular population of Acb1, in the absence of intracellular Acb1. Similarly, complementation with a signal peptide coupled SDF-2 like peptide was planned. Further, in order to determine if the SR1202 and SR1204 strains were capable of mating on planta, it was deliberated to integrate a

[1] localization: The chromosome on which the mutation is located and the base pair position on said chromosome
[2] SNP: single nucleotide polymorphisms
MNP: multi nucleotide polymorphisms

constitutively expressed, cytoplasmic GFP into the *ip*-locus of one of SR1202, and an analogous mCherry into SR1204. If membrane fusion were to happen on the plant surface, filaments would contain both fluorescent proteins. Advancing from there, integration of both an appressorial marker and a plant penetration marker were planned.

Conduction of these experiments was prohibited by an inability to transform the strains. Following transformation, no transformants could be observed on transformation plates. Regeneration of protoplasts on non-selection growth plates was also unsuccessful.

2.6 Contribution of the *Ustilago maydis* SDF-2-like peptide to virulence

To investigate a putative virulence related function of Acb1 or the SDF-2 like peptide in crosses of haploid mating partners, the suppressor problem had to be circumvented. Hence, the *acb1* gene would have to remain untouched and either the secretion of Acb1 or its processing into the SDF-2 like peptide would have to be prevented. Therefore, deletion mutants lacking *um01076* or *um03024* were generated in the FB1 and FB2 strains. The growth rate of FB1Δ*um01076* (SR775), FB2Δ*um01076* (SR689), FB1Δ*um03024* (SR784) and FB2Δ*um03024* (SR787) was not attenuated (data not shown).

In a CM-charcoal mating assay, the mating capability of the *um01076* deletion strains, as well as the *um03024* deletion strains, was investigated and proved that neither the deletion of *um01076*, nor that of *um03024*, affects mating on charcoal plates significantly (Fig. 31A and B respectively).

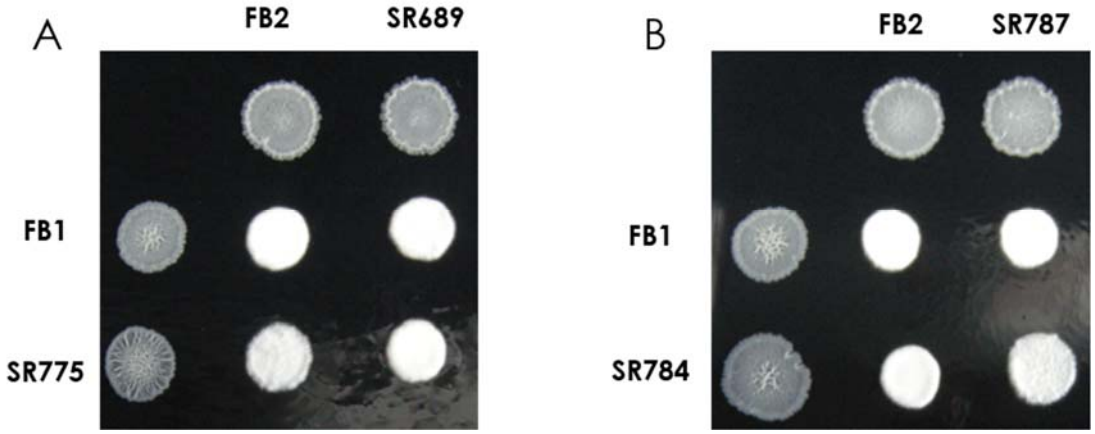

Figure 31: Mating competence of the *um01076* and *um03024* deletion strains. SR775 and SR689 **(A)**, as well as SR784 and SR787 **(B)** were grown to an OD of 1.0 and either spotted alone or in the indicated mixtures, on CM charcoal agar plates. After 48 hours at room temperature the plate was photographed.

Based on this result, SR775 and SR689, as well as SR784 and SR787, were also used for plant infection. Seven day old maize plants were infected with cell suspensions of the suppressor mutant mating partners, in comparison to the wild type mating (Fig. 32).

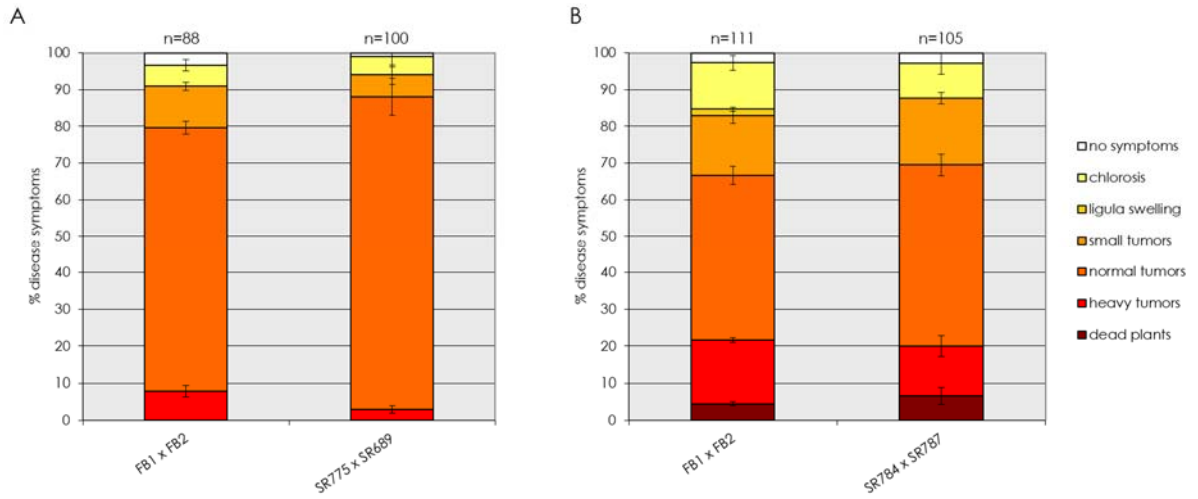

Figure 32: (A) Virulence in *um01076* deletion strains (SR775/SR689), (B) as well as *um03024* deletion strains (SR784/SR787). Cell suspensions of the described strains were injected into 7 day old maize seedlings via syringe infection. Plant symptoms were quantified 6 days post infection and differentiated into the published disease scoring categories shown on the right [Kämper *et al*, 2006]. For this data three independent infections were carried out, the average of which is displayed here as a percentage of the total number of infected plants (n). The error bars depict the standard deviation.

The plant infection experiment demonstrated that the deletion of *um01076* or *um03024* in haploid mating partners does not result in reduced virulence.

Next, a *D. discoideum* assay was carried out, in which apoplastic fluids of maize plants infected with the FB1Δ*um01076*/FB2Δ*um01076* and the FB1Δ*um03024*/FB2Δ*um03024* strains were biologically tested for the presence of the SDF-2 like peptide (Fig. 33).

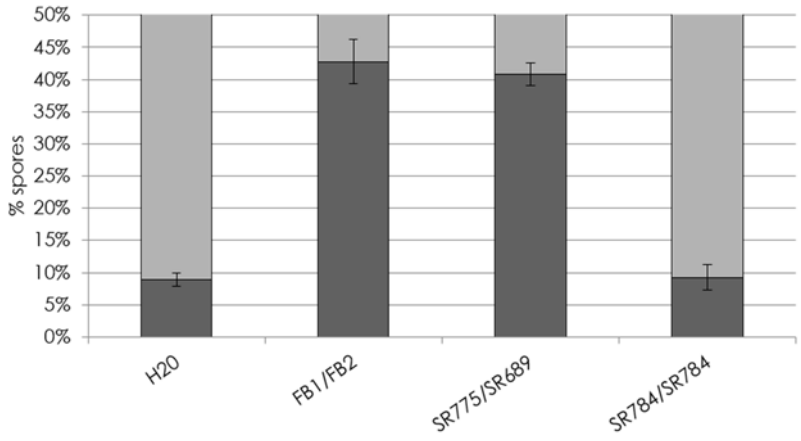

Figure 33: Detection of the SDF-2 like peptide in the apoplastic fluid samples of SR775 x SR689 and SR784 x SR787 infected plants. Apoplastic fluid was extracted from *Z. mays* plants infected with the indicated strains. Following anion-exchange-chromatography enrichment for the SDF-2 like peptide, the samples were tested for the presence of the SDF-2 like peptide by means of the *D. discoideum* bioassay. The average of 3 independent biological experiments is presented; error bars depict the standard deviation.

The assay illustrated that in the SR775 and SR689 strains, the SDF-2 like peptide can still be detected. Hence, this data supports the preceding observations. In apoplastic fluid of plants infected with SR784 and SR787 lacking the trypsin-like protease, it could be shown that the SDF-2 like peptide is no longer detectable.

Since it had been shown that the *um01076* deletion does not influence the secretion of the Acb1 protein nor does it result in other Acb1 unrelated virulence phenotype, the characterization of *um01076* was considered completed with respect to the plant infections. In contrast, the *um03024* deletion strains were further characterized, since it was possible that lack of theSDF-2 like peptide might result in a later virulence defect.

Subsequently, the ability of SR784 and SR787 to produce functional spores was investigated. Since spore quantification was not feasible, it was macroscopically concluded that no significant differences in spore quantity were observable. The ability of the observed spores to germinate was also assayed. For this purpose tumors induced by FB1Δ*um03024* x FB2Δ*um03024* were harvested 30 days post infection and the contained spores were liberated from the surrounding matrix. The spore concentration was determined by means of a Neubauer improved cell counting chamber and adjusted to a concentration of 500 spores/ml for both samples. Spore suspensions were plated on PD agar plates and the percentage of germination spores was determined following an incubation of two days at at 28°C (Fig. 34).

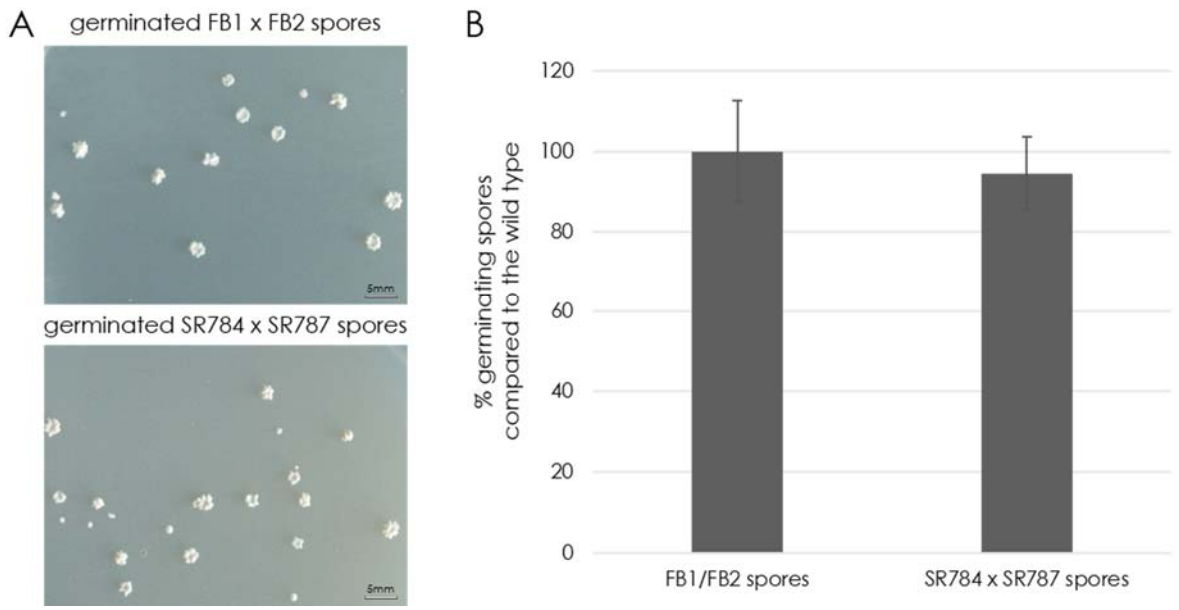

Figure 34: Spore germination FB1Δ*um03024* x FB2Δ*um03024* spores in comparison to the wild type (FB1/FB2). (A) Representative images of the growth plates are shown. **(B)** Quantification of spore germination. The amount of germinating spores of the wild type was set to 100%, the number of germinating of spores of the mutant is given as a percentage of the wild type. The assay was done in for tumors harvested from three independent infections, in three technical replicates each. The error bars represent the standard deviation.

Spores resulting from the infection of FB1Δ*um03024* and FB2Δ*um03024* germinated as well as those resulting from the infection with FB1 and FB2. It can therefore be concluded that any contribution by the SDF-2 like peptide in late stages of *U. maydis* development in the plant is unlikely.

3 Discussion

In this thesis, the function of the secreted Acb1 protein, and similarly the SDF-2 like peptide resulting from the extracellular processing of Acb1, were investigated with respect to a putative effector function.

3.1 The suppression of growth and mating defects in *acb1* deletions

The first step in order to investigate Acb1 activity was to construct a deletion mutant. The deletion of *acb1* in SG200 resulted in a slow growth phenotype which was suppressed by spontaneous mutations, as it had been published for *S. cerevisiae* [Gaigg *et al.*, 2001]. In addition, a depletion assay in FB1 had determined the full extent of the growth defect resulting in the absence of Acb1 prior to suppression. It was noted that while all the SG200Δ*acb1* suppressor strains improved upon the growth defect, the extent by which this was done varied. While the SR465 strain showed SG200 like growth in liquid culture, the SR444 and SR467 were characterized by reduced growth rates (Fig. 19). Whole genome sequencing of the suppressor strains had discovered non-identical mutations, with each strain having a distinct mutation in the Fatty Acid Synthase 1 (FAS 1) and the SR465 strain having and additional mutation in Um01335, a protein annotated as a Mitochondrial Acyl-CoA dehydrogenase (J. Jungmann, unpublished). Interestingly, previous connections between ACBPs, the FAS 1 and the mitochondrial Acyl-CoA dehydrogenase were described in the literature [Franch *et al.*, 2002; Vock *et al.*, 2010]. In two of the strains, the FAS 1 mutations are located in the active ketoacyl synthase (KS) domain, whereas the mutation in the third strain is found in the dehydratase (DH) domain (Fig. 35) [Rangan *et al.*, 1998; Smith, 2005].

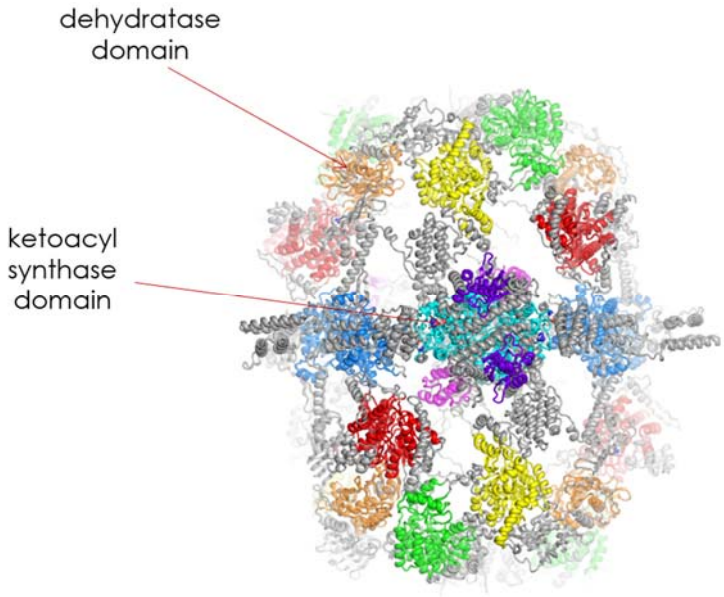

Figure 35: Structural model of the *S. cerevisiae* FAS with arrows indicating the ketoacyl synthase domain and dehydratase domain [M. Leibundgut, personal communication]**.** The KS domain is responsible for the condensation of Acetyl with Malonyl to form β-Ketobutyryl, in the first step of the fatty acid synthesis. The DH domain performs a dehydration reaction required for fatty acid chain elongation [Anselmi *et al.,* 2010].

While the mutations in the KS domain do not interfere with the catalytic center of the FAS directly, their vicinity to it suggests that they might induce structural changes, resulting in reduced stability of the protein (M. Leibundgut, personal communication). An effect of the DH domain mutation on the FAS cannot be predicted, as a single amino acid exchange outside of the catalytic center is not predicted to induce structural changes. Additionally, FAS proteins without DH domain have been described in Mycobacteria (M. Leibundgut, personal communication). A direct interaction of the FAS with the Acb1 protein of *S. cerevisiae* is shown, in which the Acb1 protein shuttles *de novo* synthesized Acyl-CoA molecules from the FAS 1 to the Endoplasmatic Reticulum (ER) for glycerolipid synthesis [Kajiwara *et al.*, 2001; Rasmussen *et al.*, 1994]. However, an explanation for the relevance of these mutations in circumventing the absence of Acb1 can at this point not be given, on account of there being insufficient information concerning how the mutations change the mechanism of action of the FAS 1 (M. Leibundgut, personal communication). In order to understand the impact of the mutations, biochemical and structural analysis in the presence of Acyl-CoA molecules and ACBPs are needed.

Concerning the Um01335 mutation identified in SR465, it is known that in *U. maydis* β-oxidation happens in the mitochondrial, as well as in the peroxisomes [Kretschmer *et al.*, 2012], and that an Acyl-CoA dehydrogenase is required for the initial step in each cycle of fatty acid β-oxidation [Thorphe and Kim, 1995]. This fits with the data showing an indirect regulatory effect of the Acb1 concentrations on the regulation of β-oxidation [Harris *et al.*, 2014]. It can be proposed that the mutation identified in SR465 absolves Um01335 of its Acb1 regulation.

In conclusion, in the presence of the suppressor mutations, the SG200Δacb1 strains were fully capable of filamenting on charcoal; plant infections with these strains were able to produce disease symptoms according to their growth defect.

To study if *acb1* deletion resulted in any defects in the wild type, which would have been overlooked in SG200, deletions of *acb1* were constructed in FB1 and FB2. In these strains, the initial growth defect resulting from *acb1* deletion was also rescued by spontaneous suppressors, however the extent of recovery was significantly lower than in the SG200Δacb1 strains. Screening for fast growing suppressor strains was unable to identify suppressed strains with a growth rate comparable to the respective type strains. The fastest growing suppressor strains found in FB1 and FB2 had a growth rate approximately 50% as fast as the wild type.

Being a derivate of the FB1 strain created by the integration of compatible mating type loci, SG200 was not modified with respect to any metabolic processes [Bölker et al., 1995][3]. While it is known that the creation of SG200 involved an UV mutagenesis step which likely resulted in genome wide variations, the inability of FB1, and by extension FB2, to completely suppress the observed growth defect was not expected. The variations resulting from the UV mutagenesis are not characterized,

[3] The published strain is SG100. SG200 differs from SG100 exclusively through its Phleomycin resistance.

hence, no explanation can be given as to which mutations present in SG200, and lacking in the wild type strains, could provide the ability to fully suppress the growth defect.

On account of the FB1 and FB2 wild type strains having near identical growth rates, a mating pair with a near identical growth defect was chosen in SR526 and SR529 for further study. Mating assays with these strains were characterized by the inability of the suppressor strains to mate with each other or even with the respective wild type mating partner. It was proposed that the inability of the SR526 and SR529 strains to establish a dikaryotic filament was a result of a defect in the production, or sensing, of the *U. maydis* mating pheromones [Spellig *et al.*, 1994]. Confrontation assays of the mutants were conducted and it could be shown that pheromone recognition of the SR523 and SR529 was fully functional (in collaboration with C. Trippel) [Fuchs *et al.*, 2004]. Hence, as an alternative justification for the mating defect it was proposed that due to the absence of Acb1 the cell membranes of the deletion strains would be unable to fuse.

This defect was however not further characterized, as a repetition of the mating assay revealed that small amounts of white filaments could be observed. It was concluded that a subpopulation of the suppressor strains had mutated further and it could be shown that these strain had reacquired the ability to mate. Through screening for the filamentation phenotype two strains were isolated, SR1202 and SR1204, whose growth defect did not improve over that off their progenitors, but which were capable of forming wild type like dikaryotic filaments.

In order to study the function of Acb1 on virulence in FB1 and FB2, the ability to form filaments is essential. Therefore in order to distinguish the defects caused by lack of secreted Acb1 or the SDF-2 like peptide from the suppressor mutations, characterization of the genome was needed. By resequencing of the genomes of SR1202 and SR1204, an attempt was made at understanding how the mutations accumulated by the suppressor strains could restore the defects resulting from the deletion of *acb1*. It was expected that in each strain two or more distinct mutations would be identified, which would be separated into two distinct groups, the first of which would be related to the metabolic function of Acb1 and its suppression, i.e. be analogous to the FAS 1 mutations identified in the SG200 suppressor mutants. For the second category, mutations in genes suppressing the mating defect were expected.

In SR1202, the expectation for the first category was fulfilled by the identification of a mutation in the gene *um04629*, coding for the Acetyl-CoA carboxylase (ACC). It has been described that the activity of the ACC is downregulated by Acyl-CoA inhibition and that through binding to the Acyl-CoA, ACBPs have the ability to release the inhibition [Faergeman and Knudsen, 1997; Rasmussen *et al.*, 1993]. The identified mutation was determined to result in an exchange of the 17th amino acid of the ACC-central domain, a 701 amino acid region described as being 'not relevant' for the catalytic activity of the ACC. It is proposed however, that this central domain has relevance for the regulation of the ACC though this process is poorly understood [Hunkeler *et al.*, 2016]. It is proposed

that a mutation, for example in an Acyl-CoA binding site, could prevent the downregulation of the ACC, by preventing the binding of an Acyl-CoA to the regulatory unit. On account of the catalytic activity of the ACC being the conversion of Acetyl-CoA to Malonyl-CoA, the upregulation of the protein would result in an enlargement of the resource pool available for all subsequent fatty acid synthesis processes [Schjerling *et al.*, 1996]. It is proposed that by means of this mutation increasing the Malonyl-CoA pool, the defects resulting from acb1 deletion can be compensated.

The two other identified mutations in SR1202 are located in the telomeric regions of the chromosomes 13 and 19. Given the difficulties of sequencing telomeric regions, it is very likely that these differences to the reference stem from sequencing errors in either of the sequences. Hence, a second category mutation explaining the ability to mate could not be identified.

In SR1204, linking of the identified mutations to either, the metabolic function of Acb1 or the restored mating, was more difficult, because none of them have a clearly annotated function with respect to either process. Discarding the mutation in the telomeric region of chromosome 14 as a sequencing aberration as was done for those of SR1202, a total of five mutations remained, only one of them being in a coding region and resulting in an amino acid exchange. This Um03463 protein, annotated as being putatively lipid metabolism related is most likely the strongest candidate for the partial restoration of the growth defect, as Acb1 is highly relevant for the lipid metabolism [Knudsen *et al.*, 1993]. On account of no exact function within the lipid metabolism being annotated to the Um03463 protein, no conclusions as to how the protein is related to Acb1 can be drawn. All the other identified mutations were located in intergenic regions, in the close upstream regions of *um00727*, *um02807*, *um02808*, *um03557* and *um11770*. Since essential features of promoters in *U. maydis* are unknown, it can be postulated only that mutations in these upstream regions could potentially influence the regulation of these gene.

One of the genes whose regulation might in this way be influenced is *um11770*, coding for a probable lysine methyltransferase. Two theories were proposed for how this mutation could assist in the restoration of the mating defect. Firstly, it had been shown that *U. maydis* wild type cells exposed to methyltransferase inhibitors are impaired in mating [Fischer *et al.*, 2011] and thus it could be proposed that the observed mating defect is derived from reduced methyltransferase activity, resulting from a putative Acb1 related regulation defect. The mutation in the promoter region of *um11770* would then upregulate the expression of the gene, thereby restoring mating. Secondly, it had been shown that histone methyltransferases hold a function in epigenetic gene regulation [Zhang *et al.*, 2012]; as the methylation of histones can both activate and repress gene transcription [Chen *et al.*, 2010]. This might be connected to the mating defect, as it was shown that in *U. maydis* histone deaceylation is responsible for direct regulation of the mating type genes [Elías-Villalobos *et al.*, 2015].

Two further mutations are of note since both are in the putative promoter regions of two independent transcription factors, annotated as being related to the nitrogen assimilation pathway of *U. maydis*. Neither of these transcription factors has been studied sufficiently to be connected to any of these processes directly; it could be shown however, that the nitrogen starvation-induced filamentation of *U. maydis* is under the regulation of the related transcription factor nit2 [Horst *et al.,* 2012]. It is hence possible that these mutations are related to the restoration of the mating defect, though on charcoal plates nitrogen starvation is not an issue.

The remaining two genes, in whose upstream regions mutations were found, code for hypothetical proteins. It is not known if these proteins are truly produced and, if that were the case, how they could be connected with the deletion of *acb1*.

For the aim of identifying the suppressor mutations responsible for the partial recovery of the growth defect, and the subsequent restoration of mating, the analysis of the suppressor mutants proved rather unsatisfying. It was only possible to identify one mutation and suggest a mechanism of compensation resulting from *acb1* deletion inSR1202. For the rest of the mutations, no clear picture presents itself.

3.2 The unconventional secretion pathway of Acb1 in *U. maydis*

The next question to address was to characterize the secretion of Acb1 in *U. maydis* and its processing in the supernatant. It could be shown that the full length Strep-HA tagged Acb1 protein can be found to be secreted into the supernatants of AB33 hyphae. However, detection of the secreted protein was only possible in the presence of TPCK. The secretion of Acb1 was induced following the shift into NM and hence, occurred in parallel with initiation of hyphae formation. In supernatants of sporidia, Acb1 could not be detected. The highest amount of secreted Acb1 was detected following the induction of filamentation. Even in the presence of the protease inhibitor, the detectable amount of Acb1 went down over time, indicating degradation by a secreted protease which is not inhibited by TPCK. The fact that the amount of detectable Acb1 decreases over time, rather than increasing or staying constant, indicates that secretion of Acb1 happens only within a short time frame.

As the ACBPs of *S. cerevisiae* and *D. discoideum* were shown to be secreted unconventionally via a Grh1 dependant secretion pathway (Fig. 4), which relies on components of the exosomes [Malhotra, 2013], it was considered likely that Acb1 would be secreted by a similar route in *U. maydis*. In collaboration with F. Hartwig it was shown that in the AB33 strain, the initiation of hyphae formation coincides with the upregulation of *atg8*, a marker gene for the exophagy pathway, providing support to the hypothesis that the Acb1 protein is secreted via exophagy. This notion gains further support from preliminary data showing an association of Acb1 with the membranes of exosomes isolated from AB33 hyphae supernatants [L. LoPresti, personal communication]. However, when the secretion of Acb1 was examined in an AB33Δum01076 strain, Acb1 was still detected in the supernatant suggesting that Um01076 is not involved in the secretion of Acb1. Um01076 is the only orthologue of

Grh1 detected in the *U. maydis* genome and attempts to find a second orthologue by lowering the threshold below 20% identity failed to yield a related protein which could have a related function. Assuming that the secretion of the Acb1 protein is mediated by the same pathway as established for *S. cerevisiae* and *D. discoideum*, it could be consider that the Acb1 protein enters these secretion vesicles in a CUPS independent manner. Preliminary data by L. LoPresti suggests that Acb1 is attached to the outside of *U. maydis* exosomes. Hence, it might be proposed that rather than associating with the surface of the CUPS and then being internalized into the MVBs, as was proposed for *S. cerevisiae*, the acetylated Acyl-CoA bound Acb1 of *U. maydis* would associate directly with the surface of the exosomes. In line with these observations, no evidence for the existence of *U. maydis* CUPS could be found to date (K. Schipper, personal communication).

After providing biochemical proof for the unconventional secretion of Acb1 in the AB33 based secretion assay, attention was directed at determining whether the Acb1 protein was processed by a trypsin like protease analogous to the published AcbA protein of *D. discoideum*. First indications that such processing did take place were already provided by the fact that the full length Acb1 protein could only be detected in hyphal supernatants if a protease inhibitor was added. The only secreted *U. maydis* protease with trypsin like activity is encoded by *um03024*. When the secretion assay was carried out with AB33Δum03024, full length Acb1 was detected in the supernatants also in the absence of TPCK. Hence, it was proven that Um03024 is the protease that processes Acb1 in culture supernatants.

3.3 Secreted Acb1 produces a peptide which has biological activity in the *D. discoideum* bioassay

A *D. discoideum* based bioassay, capable of detecting *D. discoideum* SDF-2 activity, was employed to determine whether the peptide released from Acb1 by proteolytic cleavage also contained activity analogous to *D. discoideum* SDF-2. Following the establishment of the assay, using the KP4++ strain and making use of a synthetic *D. discoideum* SDF-2 peptide, an SDF-2 like peptide was defined from the Acb1 amino acid sequence (Fig. 12). Using the two cleavage sites producing the SDF-2 peptide as a reference, the length of the SDF-2 like peptide was defined. Within the proposed SDF-2 like peptide, two putative trypsin cleavage sites exist. One of these is conserved in the SDF-2 peptide and is known to not be processed in *D. discoideum*. The second cleavage site within the proposed *U. maydis* peptide does not exist in *D. discoideum*. It was proposed that since the common cleavage site is not utilized in *D. discoideum*, the same would most likely apply to *U. maydis*. A possible explanation as to why the cleavage site is not hydrolyzed could be that it is not accessible, e.g. through a bound ligand. It is known that the central part of the AcbA destined to become the SDF-2 peptide coincides with the predicted Pfam ACBP domain of the protein and hence, the binding site to its ligand [http://smart.embl-heidelberg.de, June 2016]. Based on this information it was assumed that neither of the two cleavage sites in the proposed SDF-2 like peptide would be cut.

The synthetic version of the so defined SDF-2 like peptide did have biological activity in the bioassay, with 1 unit of activity being achieved by adding between 15 – 30nM of peptide. Comparatively, the activity contained in the *D. discoideum* peptide was 30'000 times higher. It was proposed that the *U. maydis* peptide was incapable of activating the positive feedback loop of *D. discoideum*, in which the detection of SDF-2 stimulates the additional secretion of AcbA [C. Anjard, personal communication]. Alternatively, it could be suggested the affinity of the synthetic SDF-2 like peptide for the receptor is not as high as for the native one.

Advancing from the knowledge that SDF-2 like activity was contained in the Acb1 protein, the question whether such a peptide was released from the full length protein by trypsin digestion was addressed. Implementation of the *D. discoideum* bioassay with trypsinated Acb1 revealed that this was indeed the case. The activity of this peptide was however lower than that of the synthetic peptide, as 1 unit of activity was determined to be in the range of 60nM. Possible explanations for the reduced activity of the trypsinated Acb1 peptide are that one of the trypsin cleavage sites within the peptide, which were assumed to not be used, is processed during the *in vitro* digestion of Acb1. Preliminary data with a synthetic SDF-2 like peptide truncated at the non-conserved cleavage site (amino acid 48) showed that the contained activity is reduced; determination of the unit of activity is still outstanding.

To determine whether a peptide, like the one shown to be released by *in vitro* Acb1 trypsination, is detectable in the supernatants of axenically grown *U. maydis* cultures, supernatants of the AB33 strain were harvested at the same time points as when secretion of the full length Acb1 protein was highest. Following enrichment for the putative presence of the SDF-2 like peptide using the A25 anion exchange resin, the bioassay was able to detect the peptide in the supernatants of hyphae, and not in those of sporidia. This provided evidence that, also *in vivo,* an SDF-2 like peptide was produced by *U. maydis* cells and that the SDF-2 like peptide was only detected in samples were the Acb1 protein had previously been detected. Additionally, Um11226 was found to share 48% of identity with Acb1 when comparing the ACBP domain, which later becomes the SDF-2 like peptide. A SDF-2 like peptide resulting from Um11226 could be ruled out since no SDF-2 like activity was found in *acb1* deletions. Likewise, the absence of the peptide in an *um03024* deletion was able to prove that Um03024 is the protease responsible for the processing of Acb1 into the SDF-2 like peptide, in accordance with the model.

3.4 The function of the secreted Acb1 protein and the derived SDF-2 like peptide in *U. maydis*

In a thesis preceding experiment, it had been tested whether the synthetic SDF-2 like peptide would be able to influence the intracellular cAMP levels as had been proposed in the model (Fig. 6). A FB1 strain was used, in which deletion of the *uac1* gene resulted in lowered cAMP levels and a pseudofilamentation phenotype. It had

previously been shown that raising the intracellular cAMP levels of this strain, by cAMP supplementation of the medium, restored wild type like cell morphology. It was expected that treating the cells with the synthetic SDF-2 like peptide would raise the intracellular cAMP though the proposed inhibition of the cAMP dependent phosphodiesterase and hence, the SDF-2 like peptide would put an end to the pseudofilamentation phenotype. However, while the synthetic peptide was shown to have significant activity in the *D. discoideum* bioassay at concentrations as low as 30 nM, no effect on the filamentation phenotype could be observed at peptide concentration up to 2µM. This indicates that either the receptor to which the SDF-2 like peptide binds is not expressed in FB1 in axenic culture, or that the SDF-2 like peptide does not have an influence on the intracellular cAMP levels (J. Jungmann, unpublished data).

Despite these results, the detection of a SDF-2 like peptide in axenic culture supernatants of filamentous cells, as well as the apoplastic fluid of SG200 and, FB1 and FB2 infected maize plants was strongly suggestive for the existence of a signaling function of the SDF-2 like peptide in planta. Hence, a virulence related function of the secreted Acb1 protein, or the SDF-2 like peptide, was sought in infected maize plants.

In order to study if the lack of secreted Acb1 or its derived peptide would result in any virulence defect plant, infections with the suppressor strains were carried out. It was observed that the SR465 strain showed wild type like disease symptoms, whereas the SR444 and SR467 strains were reduced in virulence according to their growth defect. It was concluded that suppressor mutations, as exemplified by those found in the SR465 strain, are capable of completely suppressing the intracellular functions of the Acb1 protein and that in a strain suppressed to this extent wild type like disease symptoms can be observed at the tumor stage. To exclude a late defect resulting from the absent Acb1, or the peptide, the spores of the SR465 infection were examined. Since no feasible method for the quantification of the spores produced by an infection exists, a macroscopic observation had to suffice. Qualitatively, no difference in the amount of spores produced by the SR465 infection, as compared to the wild type, could be observed. Following spore isolation, it could also be shown that the spores resulting from SR465 infection germinate as well as those of the SG200 strain. Since it was shown, by means of a *D. discoideum* bioassay on the SDF-2 peptide enriched apoplastic fluid from SR465 infected maize plants, that no SDF-2 like peptide is produced in SG200Δ*acb1* plant infections, a contribution of the extracellular Acb1 or the derived SDF-2 like peptide on virulence can be considered unlikely in SG200.

In *D. discoideum*, the function of the SDF-2 like peptide had been shown to be essential for the organization of the pre-stalk and pre-spore cells, spatio-temporally organizing the fruiting body formation [Anjard and Loomis, 2005]. Since the SG200 strain is an artificial, solopathogenic strain, those parts of the *U. maydis* life cycle which involved the mating process could not be studied in this system. It is for example known that for SG200, certain differences in the manifestation of disease

symptoms exist apart from the not requiring mating to be capable of infecting plants. For example, it had been shown that organ specificity exists in the infection of maize with *U. maydis* [Schilling *et al.*, 2014]. Compared to the wild type, SG200 does not produce spores in leaf tumors and hence, the fact that SG200 can produce spores in cobs, does not allow for the conclusion that sporogenesis in leaves would also be functional. Having established that in SG200 neither the secreted Acb1, nor the SDF-2 like peptide, had an observable virulence related function, attention was turned to the wild type strains FB1 and FB2.

In order to investigate a virulence effect of the secreted Acb1 or the SDF-2 like peptide on virulence in the FB1 and FB2, *acb1* deletion strains were created. These strains were characterized by growth defects, partially suppressed by suppressor mutations, as well as mating defects, suppressed by suppressor mutations at a later time point. Plant infections with the suppressor strains were devoid of disease symptoms.

Given the absence of disease symptoms and since no indications could be found that these strains ever penetrated the plant, the extraction of apoplastic fluid from infected maize plants with the purpose of proving the absence of the SDF-2 like peptide in these infections was considered futile. However, from the SG200 results, it could be inferred that an SDF-2 like peptide is also not produced in FB1 and FB2 *acb1* deletion strains.

Based on this conjecture it could be stated that neither, the secreted full length Acb1 protein nor the SDF-2 like peptide, are required for functional mating of the FB1 and FB2 strains on charcoal. On planta however, FB1 and FB2 *acb1* deletion strains were incapable of producing virulence symptoms. While the absence of disease symptoms might be an indicator for a virulence related function of the extracellular Acb1, or the SDF-2 like peptide, the multitude of defects exhibited by the suppressor strains, such as the sensitivity of the strains to osmotic and cell wall stresses, the reduced growth rate and the observed delay in mating on charcoal, does not allow for that conclusion to become validated. Further study of the suppressor strains was prohibited by the inability to complement the mutant due to the failure of the cells to survive protoplastation. Hence, it could not be studied if the lack of virulence was due to the lack of Acb1 or the SDF-2like peptide or due to the suppressor mutations.

In order to circumvent the suppressor problem to study the function of the secreted Acb1, a mutant in which the secretion of Acb1 would be blocked would be needed. Since the only candidate, Um01076, turned out not to be involved in secretion, a further characterization of secreted Acb1 was not possible. The integration of the suppressor mutations identified in SR465 into FB1 and FB2, prior to deletion of the *acb1* gene was planned to rectify this situation. Should the mutations, analogous to the situation observed in SR465, result in complete suppression of any growth rate and stress defects originating from *acb1* deletion, these strains could be used to determine whether a secreted Acb1 has a virulence related function.

Lack of the SDF-2 like peptide could however be studied in strains in which the trypsin like protease Um03024, responsible for the processing of Acb1, is deleted. However, it must be considered that in Δ*um03024* strains, any observed phenotype could also result from any SDF-2 like peptide unrelated function of the Um03024 protease, a distinction which would have to be made following phenotype identification. It was shown however, that in the *um03024* deletion strains mating on charcoal and virulence on planta appeared to be wild type like. Qualitative examination of the spores produced in an *um03024* deletion strain plant infection determined that the spore quantities did not significantly differ from the wild type infection. Spores also germinated wild type like. Hence, it could be concluded that the SDF-2 like peptide does not hold a virulence essential function in *U. maydis* under the studied conditions.

While a macroscopically observable life cycle essential function for the SDF-2 like peptide could be ruled out over the course of this thesis, a number of microscopic functions still remain possible. For example, it was proposed that the peptide fulfills a quorum sensing function in planta. As such, organizational differences, for example in the formation of the matrix, could be possible. From *D. discoideum* it is known that the native peptide is essential to the spatial organization of where the pre-spores become spores. In order to determine whether the arrangement of the matrix and spores in the *U. maydis* tumors remains the same in the absence of the peptide, FB1 and FB2 tumors were harvested comparatively to *um03024* deletion tumors and will be microscopically studied. Basic-fuchsin staining of the matrix is planned to distinguish any potential differences in spore distribution or matrix composition.

Further, it had been shown by GFP fusion to the promoter of the effector genes *stp1* and *pep1*, that gene regulation within the *U. maydis* tumors differs for cells located on the inside of a tumor or on its frontier with the plant tissue. It could be shown that these effectors are more strongly expressed on the tumor surface (D. Lanver, personal communication). The means by which the cells determine their position within a tumor is to date undetermined. It could be proposed that in *U. maydis,* the proposed Acb1 dependent signaling pathway is used, quorum sensing like, for spatial position determination. In order to investigate this claim, deletion of *um03024* in the relevant strains is planned.

Further, the possibility may be taken into account that the secreted Acb1 protein, or the SDF-2 like peptide, has functions involved in processes not assayed in this thesis. It is for example possible that the proposed signal pathway does exist, but is not related to virulence as was assumed. Relevance for a number of processes could be proposed, such as for example the defense against or the suppression of other microorganisms during either the saprophytic part of the *U. maydis* life cycle or the plant infections. Since competition with other organisms does not occur under laboratory conditions and effect in this regard would not have been observed. Competitive plant infections of, for example, the SG200 and SR465 strains with other maize pathogens could potentially shed light on this situation.

4 Materials and Methods

4.1 Materials and source of supplies

4.1.1 Chemicals

All chemicals used for the completion of this PhD thesis were of research grade and were acquired from Difco (Augsburg, Germany), Fluka (Buchs, Switzerland), Merck (Darmstadt, Germany), Riedel-de-Haën (Seelze, Germany), Roth (Karlsruhe, Germany), Formedium ltd (Hunstanton, England) and Sigma-Aldrich (Deisenhofen, Germany).

4.1.2 Buffers and solutions

Standard buffers and solutions were prepared as described in Ausubel, *et al.*, [1987] and Sambrook *et al.* [1989]. Solutions and buffers belonging to a certain method are listed in the corresponding method sections. Generally speaking all media, solutions and buffers were autoclaved for 5 min at 121 °C. Exceptions to this rule were heat-sensitive solutions, which were sterilized by filtration (pore size 0.2 µm; Merck, Darmstadt, Germany).

4.1.3 Enzymes and antibodies

All the restriction enzymes were purchased from New England Biolabs (NEB, Frankfurt am Main). DNA polymerases particularly the Phusion® Hot Start High-Fidelity DNA polymerase was procured from Thermo Scientific (Bonn) or the RedMix containing the Taq polymerase was obtained from Bioline, Luckenwalde. Ligation of DNA molecules was performed with T4 DNA ligase (Thermo Scientific, Bonn). For the enzymatic degradation of RNA RNase A (Serva, Heidelberg, Germany) was used. Enzymatic degradation of fungal cell walls was carried out by using Novozyme 234 (Novo Nordisk, Copenhagen, Denmark). Antibodies were purchased from Sigma-Aldrich (Deisenhofen).

4.1.4 Commercial kits

For purification of PCR products, plasmids as well as for the elution of DNA fragments from agarose gels, the Wizard® SV Gel and PCR Clean-Up System from Promega (Mannheim, Germany) was used. The isolation of plasmid DNA from bacterial cultures was carried out with QIAprep ® Mini Plasmid Kit from Qiagen (Hilden, Germany). For digoxigenin labeling of PCR products, the DIG High Prime kit (Roche, Mannheim, Germany) was used. The ECL Plus Western blot detection reagent (GE Healthcare, Munich) was used for chemiluminescence detection. Special kits used for specific experiments, are listed under the respective method.

4.2 Cell culture

4.2.1 Cultivation of Escherichia coli

E. coli cultures were grown in dYT liquid medium and plated on YT solid medium as described in Ausubel *et al.* (1987) and Sambrook *et al.* (1989). Where required, ampicillin (Amp) and kanamycin (Kan) were added to a final concentration of 100µg/ml and 40µg/ml, respectively. All cultures were incubated at 37°C; liquid cultures were shaken at 200 rpm.

dYT liquid medium (Sambrook *et al.*, 1989)
1.6% (w/v) Tryptone-Peptone
1.0% (w/v) Yeast-Extract
0.5% (w/v) NaCl
in dH_2O, autoclaved

YT - solid medium
0.8% (w/v) Tryptone-Peptone
0.5% (w/v) Yeast-Extract
0.5% (w/v) NaCl
1.3% (w/v) Agar
in dH_2O, autoclaved

4.2.2 Cultivation of *Ustilago maydis*

The U. maydis strains used in this study were incubated either in $YEPS_{light}$, CM (2% glucose) or CM (2% arabinose) at 28°C at 200rpm. For solid media Potato-Dextrose-Agar plates were used, selection of transformants was based on the supplementation with carboxin (Duchefa, Haarlem, Netherlands) and hygromycin B (Riedel de Haen, Seelze, Germany). Carboxin (cbx) was added to a concentration of 2 µg/ml, while hygromycin B (hyg) was dosed to 200 µg/ml. All cultures were incubated at 28°C; liquid cultures were shaken at 200 rpm. Cultures requiring permanent storage were produced by mixing exponentially growing cell cultures with NSY-glycerin at a 1:1 ratio and freezing in liquid nitrogen. The resulting stocks were stored at -80°C.

$YEPS_{light}$ (modified from Tsukuda *et al.*, 1988)
1.0% (w/v) Yeast-Extract
1.0% (w/v) Bacto-Peptone
1.0% (w/v) Saccharose
in dH_2O, autoclaved

Potato-Dextrose-Agar (PD-Agar)
2.4% (w/v) Potato Dextrose Broth
2.0% (w/v) Agar
in dH_2O, autoclaved

PD-Charcoal-Agar
Like PD, with an additional
1.0% (w/v) Charcoal

NSY-Glycerin
0.8% (w/v) Nutrient Broth
0.1% (w/v) Yeast-Extract
0.5% (w/v) Saccharose
69.6% (v/v) Glycerin
in dH_2O, autoclaved

CM (Holliday, 1974)
0.15% (w/v) NH_4NO_3
0.25% (w/v) Casaminoacids

0.05% (w/v) Heringsperm-DNA
0.1% (w/v) Yeast-Extract
1.0% (v/v) Vitamin solution
6.25% (v/v) Salt solution
2.0% Glucose (after autoclaving)
1.0% (w/w) 1M Tris-Cl pH 8.0 (f.c. 10mM)
in dH_2O, pH 7,0 (with NaOH)

Vitamin solution (Holliday, 1974)
0.1‰ (w/v) Thiamin
0.05‰ (w/v) Riboflavin
0.05‰ (w/v) Pyridoxine
0.2‰ (w/v) Calcium pantothenate
0.05‰ (w/v) p-Aminobenzoic-acid
0.2‰ (w/v) Nicotinic acid
0.2‰ (w/v) Choline chloride
1.0‰(w/v) myo-Inositol
in dH_2O, filter sterilized

Salt solution (Holliday, 1974)
1.6% (w/v) KH_2PO_4
0.8% (w/v) KCl
0.4% (w/v) $MgSO_4 \times 7H_2O$
0.1% (w/v) $CaCl_2 \times 2H_2O$
0.8% (v/v) Trace element solution
in dH_2O, autoclaved

Trace element solution (Holliday, 1974)
0.06‰ (w/v) H_3BO_3
0.14‰ (w/v) $MnCl \times 4H_2O$
0.4‰ (w/v) $ZnCl_2$
0.4‰ (w/v) $Na_2MoO_4 \times 2H_2O$
0.1‰ (w/v) FeCl3 x 6H2O
0.03‰ (w/v) CuSO4
in dH_2O, autoclaved

For stress assays CM was employed. Sorbitol (2M) and NaCL (1M) were added prior to autoclaving. Kongored (70 µg/ml), Calcofluor while (45 µg/ml) and H_2O_2 were supplemented after autoclaving. In order to test the sensitivity of strains to the stressors, strains were grown to an OD_{600} of 0.8 in YEPSL and set to an OD_{600} of 1.0 in H_20_{bid}. Serial dilutions (1:10) were produced and 7µl of each dilution were spotted on the respective stress plates. To test filamentous growth the cell suspensions were spotted on PD-Charcoal plates.

4.2.3 Cultivation of *Dictyostelium Discoideum*

For cultivation *D. discoideum* cells woken were grown in HL5 medium (Formedium ltd, Hunstanton, England), supplemented with Penicillin-Streptomycin [100µl/ml], Ampicillin [25µg/ml], Amphotericin B [0.25µg/ml] and Chloramphenicol [34µg/ml], all acquired from Sigma-Aldrich (Deisenhofen) (HL5+), at 22°C at 120rpm in a shaking incubator.

4.2.4 Determination of cell density

The optical cell density of liquid cultures was determined in a Novaspec II photometer (Pharmacia Biotech / GE Life Sciences, Munich, Germany) at 600 nm (OD_{600}). To guarantee a linear dependence, cultures were diluted by appropriate dilutions to values below 0.8. As a reference value, the OD_{600} of the corresponding culture medium was used. In *U. maydis* an OD_{600} corresponds to a cell count of 1.5 x 10^7 fungal cells. In *E. coli* this value corresponds to about 1 x 10^9 bacterial cells. *D. discoideum* cell densities were determined by optical counting in a Neubauer improved hemocytometer (Superior Marienfeld, Lauda-Königshofen, Germany).

4.3 Strains, oligonucleotides and plasmids

4.3.1 *E. coli* strains

For all cloning purposes *E. coli* TOPIO (Invitrogen, Karisruhe, Germany) was utilized, an E. coli K12 derivative with the genotype: F-, mcrA, Δ(mrr-hsd RMS-mcrBC), Φ80lacZΔM15 ΔlacO74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)*7697 gal*U *gal*K *rps*L (StrR) *end*A1*nup*G.

4.3.2 *U. maydis* strains

All strains used in this study are listed in Table 2. Deletion mutants were created by replacing the gene(s) of interest with a hygromycin B resistance cassette according to Kämper [2004] and Brachmann *et al.* [2004]. Integration of genes was done into the ip-locus (*sdh2*) by means of plasmids containing a carboxin-resistant ip-allele (Broomfield und Hargreaves, 1992). In both cases the integration of the relevant plasmids was performed via homologous gene recombination [Holloman *et al.*, 2008]. All strains were verified by Southern analysis.

Table 2: Strains of *U. maydis* used in this study

Strain name	Strain description[4]	Genotype	Resistance[5]	Reference
SG200	SG200	a1:mfa2 bE1 bW2 ble	P	Kämper *et al.*, 2006
SR444	SG200Δ*acb1* GSM	a1:mfa2 bE1 bW2 ble um02959::hyg GSM	H, P	T. Ringel
SR456	SR444 $P_{um02959}$-*acb1*	a1:mfa2 bE1 bW2 ble um02959::hyg GSM ipr[$P_{um02959}$-um02959]ip	C, H, P	S. Winterberg
SR465	SG200Δ*acb1* GSM	a1:mfa2 bE1 bW2 ble um02959::hyg GSM	H, P	F. Ahrens
SR478	SR465 $P_{um02959}$- *acb1*	a1:mfa2 bE1 bW2 ble um02959::hyg GSM ipr[$P_{um02959}$-um02959]ip	C, H, P	S. Thiele

[4] GSM, growth suppressor mutation; MSM, mating suppressor mutation

[5] C, Carboxin; H, Hygromycin; P, Phleomycin

SR467	SG200Δ*acb1* GSM	a1:mfa2 bE1 bW2 ble um02959::hyg GSM ipr[$P_{um02959}$-um02959]ip	C, H, P	F. Ahrens
SR477	SR467 $P_{um02959}$- *acb1*	a1:mfa2 bE1 bW2 ble um02959::hyg GSM	H, P	S. Thiele
FB1	FB1	a1 b1	-	Banuett and Herskowitz, 1989
FB2	FB2	a2 b2	-	Banuett and Herskowitz, 1989
SR526	FB1Δ*acb1* GSM	a1 b1 um02959::hyg GSM	H	J. Jungmann
SR529	FB2Δ*acb1* GSM	a2 b2 um02959::hyg GSM	H	J. Jungmann
SR1202	FB1Δ*acb1* GSM MSM	a1 b1 um02959::hyg GSM MSM	H	J. Jungmann
SR1204	FB2Δ*acb1* GSM MSM	a2 b2 um02959::hyg GSM MSM	H	J. Jungmann
SR775	FB1 Δ*um01076*	a1 b1 *um01076*::hyg	H	S. Reissmann
SR689	FB2 Δ*um01076*	a2 b2 um1076::hyg	H	F. Ahrens
SR784	FB1 Δ*um03024*	a1 b1 *um03024*::hyg	H	S. Reissmann
SR787	FB2 Δ*um03024*	a2 b2 *um03024*::hyg	H	J. Jungmann
SR616	AB33Δ*acb1*	a2 Pnar-bW2 Pnar-bE1, ble; um02959::hyg	H, P	J. Jungmann
SR966	FB1 P_{crg}-StrepHA-um*acb1*-Tnos Δum02959	a1b1 ble ipr[P_{crg}-Strep-HA-um02959]ips; um02959::hyg	C, H, P	F. Ahrens
SR1116	AB33 P_{acb1}-Strep-HA-*acb1* P_{otef}GFPmycstrep	a2 Pnar-bW2 Pnar-bE1 ble ipr[Potef-GFP-myc-Strep-Potef-Strep-HA-um02959)ips	C, P	L. Bender
SR820	AB33 P*acb1*-Strep-HA-*acb1* PotefGFPmycstrep Δ*um03024*	a2 Pnar-bW2 Pnar-bE1 ble ipr[Potef-GFP-myc-Strep-Potef-Strep-HA-um02959)ips *um03024*::hyg	C, P	J. Jungmann
SR564	AB33 P*acb1*-Strep-HA-*acb1* PotefGFPmycstrep Δ*um01076*	a2 Pnar-bW2 Pnar-bE1 ble ipr[Potef-GFP-myc-Strep-Potef-Strep-HA-um02959)ips *um01076*::hyg	C, P	S. Thiele

4.3.3 *D. discoideum* strains

The single *D. discoideum* strain used in this study was the KP4++ strain, a recreation of the K-P strain, created by the lab of W. Nellen [Fuhrmann, 2013]. The K-P strain, and by extension the KP4++ strain, is a derivative of the AX2 wild type strain that carries multiple copies of a construct in which the structural gene for protein kinase A catalytic subunit is regulated by its endogenous upstream region (pkaC:pkaC) [Anjard *et al.*, 1992].

4.3.4 Varieties of *Z. mays*

For all pathogenicity assays the maize variety Early Golden Bantam (Old Seed Company, Madison, Wisconsin, USA) was used.

4.3.5 Oligonucleotides

The oligonucleotides used in this study are listed in Table 3. They were procured in the quality 'salt free' from Eurofins MWG Operon (Ebersberg) and were used for plasmid construction and sequencing.

Table 3: List of oligonucleotides used in this study

Serial number	Nucleotide Sequence (5' > 3')	Application
SK105	CGAAGCGAGGAGGCGAGGAGAGG	amplifies upstream region of *um02959 (fw)*
SK106	GTGGGCCATCTAGGCCGGTTGACTTTGTATAACGATGAG	amplifies upstream region of *um02959 (rev)*
SK107	ATCGGGGCCTGAGTGGCCGCCTAAAGTTTGTCGATGGC TACC	amplifies downstream region of *um02959 (fw)*
SK108	GGTCAACGAGGTGATCGGCATCG	amplifies downstream region of *um02959 (rev)*
SR112	TAGATGTCGGGCCTGAGTGGCCCAATGTCTGCCGAAG CCAAATTC	amplifies *um02959 for use in complementation construct (fw)*
SR113	CTACGATCAGGCCGCGTTGGCCTTAGGCGTTCTGAATCT GCTCG	amplifies *um02959 for use in complementation construct (rev)*
SR313	AAGACTCAGGGCCATCTAGGCCACCTTCTATGGAGAGT TGTC	amplifies upstream region of *um03024 (fw)*
SR314	CTGAGTCTTAAGCTTGGGTTGCGACGAGATAAG	amplifies upstream region of *um03024 (rev)*
SR315	CCTGATGCTGGCCTGAGTGGCCATTCGCACCGAGACT CTTTGC	amplifies downstream region of *um03024 (fw)*
SR316	CTGCTATCGTGCATGAACTG	amplifies downstream region of *um03024 (rev)*
SR373	TGCAGTATCAAGCTTGTCATTGGTGGCCTTGATAG	amplifies upstream region of *um01076 (fw)*
SR374	GGCCATCTAGGCCGGTGGACAGCAGCCTCTAGC	amplifies upstream region of *um01076 (rev)*
SR375	GGCCTGAGTGGCCGTTTCAGTGCAGTTTCG	amplifies downstream region of *um01076 (fw)*
SR376	GACGGGAACTATCTGCTACC	amplifies downstream region of *um01076 (rev)*

4.3.6 Plasmids

All plasmids created in this work are described here. Plasmid sequences were verified by sequencing (Eurofins MWG Operon, Ebersberg).

pTR11 This plasmid contains a hygromycin resistance cassette flanked by the 1kb upstream and downstream regions of *um02959*. The deletion construct can be liberated from the plasmid by restriction with the enzyme SfiI.

pTR16 This construct contains the *um02959* gene under control of the P_{otef} promoter. For the transformation into *U. maydis*, the plasmid can be digested with the enzyme SspI and integrated into the cbx gene in the *ip*-locus

pSR128 This plasmid contains a hygromycin resistance cassette flanked by the 1kb upstream and downstream regions of *um03024*. The deletion construct can be liberated from the plasmid by restriction with the enzyme SfiI.

pSR169 This plasmid contains a hygromycin resistance cassette flanked by the 1kb upstream and downstream regions of *um01076*. The deletion construct can be liberated from the plasmid by restriction with the enzyme SfiI.

pSR260 This plasmid contains the Strep-HA-*acb1* allele under control of the *crg1* promoter. For the transformation into *U. maydis*, the plasmid can be digested with the enzyme SspI and integrated into the cbx gene in the *ip*-locus.

pSR352 This plasmid contains the Strep-HA-*acb1* allele under control of the *um02959* promoter as well as a GFP-myc allele under control of the *otef* promoter. For the transformation into *U. maydis*, the plasmid can be digested with the enzyme SspI and integrated into the cbx gene in the *ip*-locus.

4.4 Microbiological methods

4.4.1 Infections of Z. mays with *U. maydis*

In order to assess the virulence of *U. maydis* wild type and mutant strains, a pre-culture was grown in YEPSL over night at 28°C with shaking. A main culture was then inoculated with an OD_{600} of 0.1 and incubated at 28°C at 200 rpm until an OD_{600} of about 1.0 was reached (6-8 hours of incubation). The cell culture were harvested by centrifugation (3500 rpm, 10 min, room temperature) and set to an OD_{600} of 1.0 in ddH_2O. Compatible haploid type strains were mixed at a ratio of 1:1 (v/v) prior to infections. Four maize seeds were planted in one flowerpot containing 'Frühstorfer Pikiererde' of type 'P' or 'H'. Watering was done on a daily basis. Infections were done in seven days old maize seedlings by injecting cell suspensions in the leave whorl making use of a syringe. The infection site was about 1cm above the stem base. Plants were grown in a greenhouse with daylight temperature of 28 °C, for the night the temperature was lowered to 20°C. During the day phase, light intensity was a least 28,000 lux (with additional sun shine up to 90,000 lux). Relative humidity was kept between 40% and 60%. Symptom were scored 6 days post infection (dpi). The symptom scoring was done according to the categories described by Kämper *et al.*

[2006]. Each infection was done in three independent biological replicates with 30 - 40 plants (10 flower pots). Data is presented as the mean in relation to the total number of plants, with error bars depicting the standard deviation.

4.4.2 Rubidium-chloride mediated transformation of *E. coli*

The Rubidium-chloride mediated transformation of *E. coli* protocol was modified after Cohen *et al.* (1972). In order to obtain chemo-competent *E. coli* cells, 100 mL dYT medium was supplemented with 10 mM MgCl2 and 10 mM MgSO4 and inoculated with 1 mL of an overnight culture. The culture was grown at 37°C at 200 rpm to an OD_{600} of 0.5 and harvested by centrifugation (15 min, 3000 rpm, 4°C). The cell pellet was resuspended in 33 mL ice cold RF-1 solution and incubated for 30 min to 60 min on ice. This was followed by another centrifugation step (15 min, 3000 rmp, 4°C), cells were resuspended in 5 mL ice cold RF-2 solution and incubated on ice for 15 minutes. Aliquots of this cell suspension (50 µL) were shock frozen in liquid nitrogen and stored at -80◦C.

RF-1 solution
100mM RbCl
50mM $MnCl_2$ x 2 H_2O
30mM Potassium acetate
10mM $CaCl_2$ x 2 H_2O
15% (v/v) Glycerin
in dH_2O, pH 5,8 (with acetic acid), filter sterilized

RF-2 solution
10mM 3-(*N* -morpholino) propanesulfonic acid (MOPS)
10mM RbCl
75mM $CaCl_2$ x 2 H_2O
15% (v/v) Glycerin
in dH_2O, pH 5,8 (with NaOH), filter sterilized

For transformations, one aliquot of cells was thawed on ice and mixed with up to 20 µL plasmid solution and incubated on ice for 20 minutes. After a heat shock (1 min, 42◦C), the cells were chilled on ice of 1 minute, after which 150 µL of dYT were added. The cell suspension was incubated at 37°C at 950 rpm for 45 min in a Thermo mixer (Eppendorf). After that, the cell suspension was spread on plates containing the relevant antibiotic and incubated over night at 37°C.

4.4.3 Transformation of *U. maydis*

For the transformation of *U. maydis* a protocol modified from Schulz *et al.* (1990) and Gillissen *et al.* (1992) was used. A cell culture of the required strain was grown in 50 mL YEPSL (28°C, 200 rpm) to an OD_{600} of 0.8 - 1.0, the cells were harvested (3500 rpm, 5 min, room temperature) and then resuspended in 25 mL SCS. Following an additional centrifugation (3500 rpm, 5 min, room temperature), the cells were resuspended in 2 mL Novozyme solution (2.5 mg/mL SCS; filter sterilized) and incubated at room temperature, until about 80% of the cells began to protoplast (about 2 - 10 min). The protoplast formation was monitored microscopically and was stopped by adding 20 mL of ice cold SCS. Subsequent to a further centrifugation step (10 min, 2300 rpm, 4°C), the cells were resuspended carefully in 20 mL ice cold

SCS and again centrifuged (10 min, 2300 rpm, 4°C). The cells were resuspended in 10 mL ice cold SCS and following centrifugation (10 min, 2300 rpm, 4°C) were resuspended in 10 mL ice cold STC. After a final centrifugation step (10 min, 2400 rpm, 4°C), the cells were resuspended in 0.5 mL ice cold STC. Aliquots of 70 µL were used immediately or stored at -80°C for up to several months. For integrative transformations, one aliquot was thawed on ice, mixed with up to 10 µL linearized DNA (in total up to 5 µg) and 1 µL Heparin solution (10 mg/mL) and incubated 10 min on ice. The addition of 0.5 mL STC/PEG was followed by incubation on ice, for 15 min. The complete transformation mix was spread on a Reg-Agar plate containing double concentrated antibiotics. Plates were grown at 28°C and colonies were singled out 3 - 7 days later. Singling out was done on PD plates containing the respective antibiotic. Potential transformants were verified by Southern analysis.

SCS solution
1M Sorbitol
20mM Sodium acetate
in ddH_2O (pH 5.8; autoclaved)

STC solution
1M Sorbitol
10mM Tris-HCl (pH 7.5)
100mM CaCl2
in ddH_2O (autoclaved)

STC/PEG
40% (w/v) PEG 3350 in STC (filter sterilized)

RegAgar
1.0% (w/v) Yeast Extract
2.0% (w/v) Bacto-Pepton
2.0% (w/v) Sucrose
1M Sorbitol
1.5% (w/v) Bacto-Agar
in ddH_2O (autoclaved)

4.4.4 Protein isolation from the cytoplasm of *U. maydis*

For the purification of Strep-HA tagged proteins the relevant *U. maydis* strain was grown over night in 50ml $YEPS_{light}$. The following morning this pre-culture was used to inoculate 3x 200ml of $YEPS_{light}$ to an OD_{600} of 0.2, in 1000 ml Erlenmeyer flasks, and the cells were grown to an OD_{600} of 0.8. The cells were then pooled, centrifuged (15 min, 15000rpm, 4°C) and the cell pellet was resuspended in 50ml ddH_2O. The resuspended cells were transferred to 50ml Falcon tubes, centrifuged (10 min, 3000rpm, 4°C) and the cell pellet was shock frozen in liquid nitrogen. In the next step the cells were resuspended in 20ml Buffer W, containing 100 µM PMSF, 1 mM DTT und 1x Protease Inhibitor Mix tablet (Roche). Three French Press cycles were used to lyse the cells by mechanical force, followed by centrifugation (15 min, 3000rpm, 4°C). The protein containing supernatants were then purified for the tagged proteins by StrepTactin column chromatography.

4.4.5 Protein isolation via StrepTactin column chromatography

10ml StrepTactin Columns (Bio-Rad, München) were prepared with 200µl of a StrepTactin Sepharose matrix (Bio-Rad, München) and washed with 2x 5ml Buffer W. The protein samples were then loaded onto the column and following complete passage of the sample, the column was washed 2x with 5ml Buffer W. Proteins were eluted in 6x 100µl of Buffer E into separate 1.5 ml Eppendorf tubes. 30 µl of each elution fraction were mixed with 10µl 4x SDS sample buffer, boiled for 15 min at 99°C and centrifuged (3 min, 13000rpm). The samples were run on a Western blot and the protein containing fractions were pooled. Quantitative protein concentrations were determined via Bradford assay.

Buffer W
100 mM Tris-Cl pH 8,0
150 mM NaCl
1 mM EDTA

Buffer E
100 mM Tris-Cl pH 8,0
150 mM NaCl
1 mM EDTA
2,5 mM Desthiobiotin

Buffer R
100 mM Tris-Cl pH 8,0
150 mM NaCl
1 mM EDTA
1 mM HABA (2-(4-Hydroxyphenylazo) benzoic acid)

4.4.6 Trypsin treatment of purified UmAcb1 protein

For the trypsination of Acb1 50µl of a purified cytoplasmic Acb1 sample were treated with 10µl of Trypsin (1mg/ml), 0,1 µl 1 M $CaCl_2$, 10 µl 1 M Tris HCl pH 8 and 30 µl ddH_20, overnight at 37°C.

4.4.7 AB33 secretion assay

For the axenic culture AB33 secretion assay the relevant strains were grown in a $YEPS_{light}$ pre-culture of 3ml over night at 28°C. From this pre-culture an overnight culture was grown in 100ml AM medium such that the following morning an OD_{600} of 1.0 was reached. The cells were harvested (5 min, 3500rpm, room temperature), washed 2 times in 50ml ddH_2O and then set to an OD_{600} of 0.8 in 100ml of AM or NM, depending on the assay. The cultures were grown at 28°C at 200rpm, for varying time periods, depending on the experimental setup. Supernatants were harvested by two centrifugation steps (5 min, 3500rpm, 4°C) and subsequently supplemented with ice cold Trichloroacetic acid (TCA) to a final TCA concentration of 20%. Samples were stored at 4°C for 12 hours and their protein content was then precipitated by centrifugation (2 hours, 8000rpm, 4°C). TCA/supernatant mixture was removed and the protein pellets were washed three times with aceton (2 hours, 8000rpm, 4°C). Subsequent to the last washing step the protein pellets were dried and then resuspended in 4x sample buffer. Detection of the relevant proteins was done by Western Blot (chapter 4.5.4).

AM
0,3% (w/v) $(NH_4)_2SO_4$
6,25% (v/v) Salt solution
2.0% Glucose (after autoclaving)

NM
0,3% (w/v) KNO_3
6,25% (v/v) Salt solution
2.0% Glucose (after autoclaving)

4.4.8 *D. discoideum* bioassay

For the *D. discoideum* bioassay KP4++ cells were grown in HL5 medium (chapter4.3.3) and then set to a cell density of 10^3 cells/ml in a cAMP containing priming buffer in cell culture plates. Cell priming took place over 24 hours and was followed by supplementation of the culture wells with the respective samples. Sporulation was allowed to take course over 2 hours, at the conclusion of which the spore count in the wells was determined by microscopy (Leica DM IRE 2) as a percentage of the total number of cells.

Priming buffer
10mM MES
10mM NaCl
1mM KCl
1mM $CaCl_2$
1mM $MgSO_4$
5mM cAMP
in ddH_2O (filter sterilized)

4.4.9 Spore germination assay

For the spore germination assay Zea mays plants were infected with the relevant strains as described in chapter 4.4.1. Comparable tumors were harvested 30 dpi and dried at 37°C over the course of two days. Of each strain one tumor was ground in mortar, in the presence of 1ml of ddH_20, until the black spores separated from the surrounding tissue. The spore containing water was removed and the grinding step was repeated 3 times with fresh water. The spore containing water was centrifuged (3 min, 2000rpm, room temperature) and the water was removed via pipetting. The spore pellet was resuspended in 2ml water and 2ml 3%$CuSO_4$ solution was added. Following an incubation of 15 min at room temperature the solution was centrifuged (3 min, 2000rpm, room temperature), the supernatant was removed and following 3 washing steps in 10ml water (3 min, 2000rpm, room temperature) the spores were again resuspended in 2ml of water (supplemented with ampicillin [100µg/ml] and tetracyclin [25µg/ml]). The spore count was then determined making use of a Neubauer improved hemocytometer (Superior Marienfeld, Lauda-Königshofen, Germany). For the spore germination assay the spore concentration was set to 500 spores/ml in H_2O and 200µl of this spore suspension were plated on PD agar plates. Colonies originating from the germinating spores were counted after 2 - 4 days.

4.5 Methods of molecular biology

4.5.1 Methods for the *in vitro* modifications of nucleic acids

4.5.1.1 Restriction of DNA

Restrictions of DNA were carried out via a type II endonuclease (NEB, Frankfurt) for 2 - 16 h at the enzyme-specific optimal temperature. A typical reaction mix was set up as follows:

XµL DNA (0.1 - 5 µg)
5µL Enzyme-specific 10x buffer (NEB)
5µL BSA (if required; NEB)
0.5-1U Restriction endonuclease
ad 50µL ddH_2O

4.5.1.2 Ligation of DNA fragments

Ligation of DNA fragments was done using the T4 ligase (Roche, Mannheim), where ligations of a linearized vector and a DNA fragment were prepared in such a way that the fragment was at least three times more abundant than the vector. Ligations with more than two fragments were carried out at an equal molar ratio of all fragments. Typically, ligations were done in a total volume of 10µL with 1U of T4 DNA ligase at 16°C over night.

4.5.1.3 Polymerase chain reaction

For the amplification of DNA fragments the polymerase chain reaction (PCR) was used. For all reactions the Phusion® High-Fidelity DNA Polymerase was employed. A PCR cycle consisted typically of the following steps: Initial denaturation (98°C, 1 min), denaturation (98°C, 10 sec), annealing (55°C, 30 sec), elongation (72°C, 30 sec), finale elongation (72°C, 10 min). In all cases, 35 cycles were run. The annealing temperature and elongation duration was adjusted to the primers used and to the length of the fragment (30 sec/1000 bp). PCR reactions were performed in a TProfessional Thermocycler (Biometra, Göttingen). A typical reaction mix was set up as follows:

10.0µL 5x HF-Buffer (Finnzymes)
1.5µL DMSO
0.4µL dNTPs (1:1:1:1 ratio)
1.0µL Oligonucleotide 1 (100pM/µL)
1.0µL Oligonucleotide 2 (100pM/µL)
1.0µL gDNA (as template)
0.5µL Phusion® High-Fidelity DNA polymerase
34.6µL ddH2O

4.5.2 Isolation of nucleic acids

4.5.2.1 Isolation of plasmids from E. coli

Plasmids were isolated from a densely grown cell culture using the QIAprep Mini Plasmid Kit (Quiagen, Hilden) with 1.5mL - 2.0mL of culture. Plasmids were eluted with 30µL - 50µL ddH2O. Yields were typically around 250 ng/µL.

4.5.2.2 Isolation of genomic DNA from *U. maydis*

The method for the extraction of genomic DNA from *U. maydis* cells was modified from Hoffman & Winston [1987]. 2 mL of a dense overnight culture, grown in $YEPS_{light}$, were centrifuged (5 min, 13.000rpm, room temperature). The pellet was resuspended in 500µL *Ustilago*-lysis buffer and 500µL TE-Phenol/Chloroform. Approximately 200µL (around 0.3g) of glass beads were added. Samples were shaken for 15 min on a Vibrax VXR shaker (IKA, Staufen) at 1500rpm. Following centrifugation (20 min, 13.000rpm, room temperature) to separates the phases, 400µL of the supernatant were transferred into 1mL 70% (v/v) ethanol. After centrifugation (15 min, 13.000rpm, room temperature), the pellet was washed once with 500µL 70% (v/v) ethanol (5 min, 13.000rpm, room temperature) and solved in 30µL TE/RNase A (50:1) at 55°C for 15 min in a Thermomixer (Eppendorf). DNA could be stored for several months at -20°C.

***Ustilago*-lysis-buffer**
50mM Na_2-EDTA
1% (w/v) SDS
in 50mM Tris-HCl (pH 7.5)

TE-Phenol/Chloroform
1:1 mixture of phenol (equilibrated with TE-buffer) and chloroform

TE-buffer
1mM Na2-EDTA
in 10mM Tris-HCl (pH 8.0)

4.5.3 Separation and detection of nucleic acids

4.5.3.1 Agarose-Gelelectrophoresis

DNA fragments were separated according to their size in an electric field, where, due to its negative charge, DNA migrates towards the anode. The agarose concentration varied between 0.8% and 1.0% (w/v) in TAE-buffer according to the fragment length, with shorter fragments being run at higher concentrations. Prior to use the agarose was supplemented with ethidium bromide (1mg/mL). TAE served as the buffer in the running chambers. DNA was mixed with a loading buffer and transferred to the gel. The electrophoresis was done at 80mA until the desired separation was achieved. As size standards, either the 1 kb ladder (0.5kb – 10kb; NEB, Frankfurt) or the 100 bp ladder (0.1kb – 1.5kb; NEB, Frankfurt) were used. DNA was detected under UV light (254 nm). Photographs for documentation were taken with the BioDoc-IT-system; UVP).

50x TAE-Buffer
2M Tris-Base
2M acetate
50mM Na2-EDTA
in ddH_2O

6x Loading buffer
50% (w/v) Saccharose
0.01% (w/v) Bromphenol blue
in TE-buffer

4.5.3.2 Southern Blot analysis

Genomic DNA was isolated from *U. maydis* transformants as described in chapter 4.5.2.2. Around 5 µg of DNA were used for restriction, with enzymes chosen in such a way that the transformed constructs alter the number and/or length of the resulting fragments as compared to the wild type locus. Restriction occurred over night at the enzyme-specific temperature. Restricted DNA was separated via agarose gel electrophoresis (see chapter 4.5.3.1) and transferred to a PVDF membrane according to ta a method modified from Southern [1975]. Prior to transfer, the gel was incubated in 0.25M HCl for 20 min – 30 min, leading to depurination of the DNA. After that, the gel was equilibrated for 15 min in 0.4 M NaOH. Transfer to the positively charged PVDF membrane (Roche, Mannheim) was carried out by using capillary forces created by a stack of paper towels and 0.4M NaOH as transfer buffer. Due to this flux, DNA fragments are eluted from the gel and bound to the membrane. Transfer was done at room temperature over night (typically around 16 h). To detect DNA fragments, probes were generated by PCR using the PCR DIG labeling mix (Roche, Mannheim). The recombination flanks (ca. 1 kb) served as templates. The PCR products were purified from an agarose gel, eluted in 50µL H_2O and mixed with 30mL Southern Hybridization Buffer. Prior to use, the probe was denatured at 99°C for 20 min. Membranes were pre-hybridized with Southern Hybridization Buffer at 65 °C for 30 min – 120 min, which was subsequently replaced by the denatured probe. Hybridization occurred for at least one day in a hybridization oven at 65°C under constant slow turning. The membrane was washed twice with Southern Wash Buffer each washing step talking 20 min at 65°C. After washing with DIG Wash Buffer (5 min, room temperature), the membrane was incubated in 20mL - 30mL DIG II buffer for 30 min – 60 min at room temperature for the purpose of masking non-hybridized areas of the membrane. The membrane was then incubated with 10mL Antibody Solution for 30 min at room temperature, the antibody being covalently coupled to an alkaline phosphatase. After washing twice with DIG Wash Buffer (15 min, room temperature), the membrane was equilibrated with 30mL DIG III buffer (5 min, room temperature). After incubation in 10mL CDP Star Solution (5 min, room temperature), which serves as substrate for the phosphatase, any excess CDP Star Solution was removed and the membrane was sealed in a plastic bag for further incubation (15 min, 37°C). For detection of the luminescence signal from the phosphatase the membrane was placed in a film cassette together with an X-ray film (Medical X-Ray Screen Film Blue Sensitive; CEA, Hamburg). A signal was typically monitored for 10 min – 12 hours and detected by developing the film in an x-ray film developer machine (QX-60; Konica or AGFA CP 1000; Mortsel, Belgium).

Na-Phosphate Buffer
Mix solution 1 and 2 (ratio ca. 4:1) (pH 7.0)

Solution 1
1M Na_2HPO_4 in ddH_2O

Solution 2
1M NaH_2PO_4 x H_2O in ddH_2O

Southern Hybridization Buffer
7.0% (w/v) SDS in 0.5M Na-Phosphate Buffer

Southern Wash Buffer
1.0% (w/v) SDS in 0.1M Na-Phosphate Buffer

DIG I Buffer
0.1M Maleic acid
0.15M NaCl
in ddH2O
adjust pH to 7.5 (with NaOH); autoclaved

DIG Wash Buffer
0.3% (v/v) Tween-20 in DIG I Buffer

DIG II Buffer
1.0% (w/v) Powdered Milk in DIG I Buffer

DIG III Buffer
0.1M NaCl
0.05M $MgCl_2$ x 6 H_2O
in ddH_2O, adjust pH to 9.5 (with 1M Tris-HCl)

Antibody Solution
1µL Anti-DIG antibody (Anti Digoxigenin Fab Fragment; Roche)
in 10mL DIG II Buffer

CDP Star Solution
100µL CDP Star (Roche)
in 10mL DIG II Buffer

4.5.4 Separation and detection of proteins

4.5.4.1 Protein quantification

The quantification of soluble proteins in protein extracts and enrichments was done with the aid of the Bradford method [1976], making use of the Roti®-Quant reagent (Carl Roth). For calibration purposes Bovine serum albumin (BSA) was used.

4.5.4.2 SDS-PolyacrylAmide Gel Electrophoresis

Separation of protein samples was performed using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) making use of a method modified from Laemmli (1970). In this method, all proteins receive a constant negative charge via the binding to SDS, which allows for separation in an electric field. For this purpose proteins were boiled in the presence of 1x SDS sample buffer at 99°C. Gel chambers (Mini Protean System; Bio-Rad, München) were filled with SDS-Running Buffer. Gels were composed of a stacking gel and a running gel. The stacking gel is used to concentrate the proteins in one layer prior to entering the separation gel. The running gel separates the proteins in a polyacrylamide matrix according to their molecular size. Separation was performed at 40 mA/gel. The approximate protein mass was assessed with the help of a stained mixture of standard proteins (15 kDa – 170 kDa; Prestained Page Ruler; Fermentas, St. Leon-Roth).

6x SDS sample buffer
1,5mM Tri-HCl, pH 6,8
30% (w/v) SDS
0,15% (w/v) Bromphenolblue
10% Glycerin

Stacking gel
5% (v/v) Acrylamid
0.1% (w/v) SDS
in 125mM Tris-HCl (pH 6.8)

to start polymerization:
0.1% (w/v) Ammonium persulfate (APS)
0.05% (v/v) Tetramethylethylenediamine (TEMED)

Running gel
12% (v/v) Acrylamid
0.1% (w/v) SDS
in 375mM Tris-HCl (pH 8.8)

to start polymerization:
0.1% (w/v) APS
0.05% (v/v) TEMED

SDS running buffer
25mM Tris-HCl, pH 8,3
192mM Glycin
4mM SDS

4.5.4.3 Immunological protein detection via chemiluminescence

The proteins were transferred from the gel to a PVDF-membrane via the Trans-Blot® Turbo™ Transfer System (Bio-Rad, München). The membrane was activated by submersion in methanol. The 'Mixed Protein Sizes' program (7 min) was used for blotting according to the manufacturer's instructions. The transferred proteins were detected by means of an immunological assay making use of chemiluminescence. The membrane was incubated for 1h at room temperature in Blocking Solution, followed by a short washing in TBS-T. The membrane was then incubated with an antibody solution containing the primary antibody (mouse anti-HA, diluted 1:10000 or mouse anti-GFP, diluted 1:4000; both obtained from Sigma-Aldrich, Deisenhofen) at 4°C for at least 4 hours, preferentially over night, with constant slow shaking. After three washing steps in TBS-T, each lasting 10 min, the membrane was incubated in an antibody solution containing the secondary antibody (anti-mouse horse radish peroxidase (HRP)-linked, diluted 1:10000; Cell Signaling Technology, Danvers, USA) for 1 h at room temperature with constant slow shaking. After a final three washing steps with TBS-T, each lasting 10 min, the membrane was incubated with an ECL solution (GE Healthcare, München), which serves as substrate for the HRP, for 10 min at room temperature. After removing any excess ECL solution, the membrane was sealed in a plastic bag and placed in a film cassette, together with an X-ray film (Medical X-Ray Screen Film Blue Sensitive; CEA, Hamburg). The signal was typically monitored for 1 min – 12 hours and detected by developing the film in an x-ray film developing machine (QX-60; Konica or AGFA CP 1000; Mortsel, Belgium).

TBS-T
150mM NaCl
0.1% (v/v) Tween20
in 50mM Tris-HCl (pH 7.5)

Blocking solution
10% (w/v) Powdery milk in TBS-T

Antibody solution
Antibodies diluted in 2,5% (w/v) Powdery milk in TBST

4.6 Staining, Microscopy and Image Processing

4.6.1 Fluorescence microscopy and image processing

The microscopic examination of *U. maydis* cell morphology was performed making use of a light microscope (Axiophot, Zeiss) using Nomarski optics. Cells were viewed with either a 40x or a 60 x Plan-Apochromat objective (Zeiss), with a 1.4 numerical aperture for DIC microscopy and fluorescence microscopy. Images were taken with a digital high-resolution CCD camera (Photometrics CoolSnap HQ, Visitron Systems, Puchheim, Germany) and were processed with the programs MetaMorph (version 6.2.6) and Photoshop 5.5 (Adobe).

4.6.2 Colony observation by stereomicroscopy

For the macroscopic documentation of charcoal colonies stereo microscopy was used (Leica stereomicroscope M165 FC) at a 12x magnification.

4.6.3 DAPI staining

The nuclei of *U. maydis* can be visualized by staining with 4,6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich, Deisenhofen). This staining method was used investigate charcoal filaments for cell fusion. For this purpose mating partners of the relevant strains were grown to an OD_{600} of 1.0, mixed in a ratio of 1:1 and then spotted (7µl) on PD-charcoal agar plates. Following 24 hours of growth filaments were plucked from the colonies using tweezers, and resuspended in H_2O and transferred to Poly-L-Lysine (Sigma-Aldrich, Deisenhofen) coated object slides. Following fixation in PBS with 3.5% Formaldehyde the cells were stained in a DAPI solution (0.5µg/ml DAPI in PBS) for 5 minutes. Following 3 washing steps of 30 seconds in PBS the cell staining was documented by fluorescence microscopy using the DAPI emission filter (>395nm).

4.7 Strain resequencing with Illumina 454

4.7.1 Isolation of genomic DNA from *U. maydis* for sequencing

For isolation of the genomic DNA of the suppressor strains, for subsequent resequencing of their genomes, the "Isolation of genomic DNA from *U. maydis*" (chapter 4.5.2.2) protocol was used. To ensure quality and purity of the DNA an additional cleaning step was carried out making use of the MasterPure Complete DNA & RNA purification kit (Epicenter Biotechnologies, Madison, Wisconsin, USA),

according to their protocol. Afterwards the gDNA concentration was determined making use of a Qubit Fluorometer (Thermo Scientific, Bonn).

4.7.2 Library production and sequencing

The high purity DNA was subsequently sent to the Max Planck Genome Center in Cologne for quality assessment and subsequent library production. The strains were sequenced using the Illumina HiSeq2000 platform. For each strain in excess of 25'000'000 reads of 100bps were generate, providing an average coverage of 80.

4.7.3 Data analysis

The reads generated by the Max Planck Genome Center in Cologne were loaded into the CLC Genomic Workbench (version 8.5.1) and mapped against the published *U. maydis* reference genome [Kämper *et al.*, 2006]. Following local realignment of the four mapped genomes (FB1, FB2, SR1202 & SR1204) a variant detection analysis was carried out determining the variations (SNPs, MNPS & indels) of each strain compared to the published reference. Subsequently, the variant lists of the suppressor strains were compared to those of their respective wild type progenitor, such that only mutations found exclusively in the mutant would remain and the original reads of the suppressor strains were mapped against the list of remaining mutations. For all analysis steps the standard settings of the CLC software were used. The mutation list provided by the CLC software was in the end further shortened by setting a cutoff of 95% mutation certainty. The final result of this analysis is shown in table 1.

4.8 Bioinformatic methods

The cloning strategies and amino acid or nucleotide sequence comparisons was carried out with the program Clone Manager 9.0 (SciEd Software; Denver, USA). Sequence data was obtained from the National Center for Biotechnology Information (NCBI; www.ncbi.nlm.nih.gov) and the Pedant *U. maydis* Database (http://pedant.helmholtz-muenchen.de). Both nucleotide and protein sequences were compared using web-based alignment tools such as BLAST (Basic Local Alignment Search Tool; Altschul *et al.*, 1990). Domain analyzes were performed using the SMART (Simple Modular Architecture Research Tool) program (http://smart.embl-heidelberg) and proteins were analyzed by the program SignalP 4.0 program (Nielsen *et al.*, 1997; Bendtsen *et al.*, 2004; www.cbs.dtu.dk/services/SignalP/) for prediction of an N-terminal signal sequence.

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"There is no real ending. It's just the place where you stop the story."
— **Frank Herbert**

Curriculum Vitae