Optimizing the expression of antibody formats in protease-deficient *Ustilago maydis* strains

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To Amit and Parents

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Summary

The global market for protein biopharmaceuticals is increasing at a tremendous rate. In order to provide the full repertoire of biopharmaceutical proteins, an alternative expression platform based on unconventional secretion of the endochitinase Cts1 was developed in *Ustilago maydis*. Although *U. maydis* displays several features that indicate a great potential for its utilization in biotechnology, some shortcomings are needed to be solved. During initial expression studies using an α-myc scFv as a proof-of-principle, one such major limitation observed was low yields of secreted full-length protein. This was mainly due to the proteolytic degradation by host proteases. Hence, the main goal in this study was to tackle this proteolysis problem by elimination of harmful proteases.

Initially, the key protease Kex2 known to activate several secreted proteases was targeted. Deletion of the *kex2* gene in expression strains led to reduced heterologous protein degradation. However, this deletion mutant also exhibited a peculiar phenotype and lowered growth rates, which can lead to problems in bioreactor studies. Hence, in the next step homologs of some crucial proteases know to be responsible for proteolysis in other fungi were targeted. However, the resulting single deletion mutants did not show any significant improvement in the stability or yields of secreted proteins. Therefore, the most promising approach to tackle the protease problem was to generate a multiple-protease deficient expression strain. An elegant technique of Golden Gate cloning coupled with FLP-FRT marker recycling system was thus employed to generate a quintuple protease deletion strain. This engineered strain displayed much reduced proteolytic activity. Coupled with a strong promoter and an optimized expression cassette, this multiple-protease deficient strain led to much improved stability and enhanced yields of the active secreted scFv-Cts1 fusion protein.

To prove the versatility of this expression host, different other antibody formats were attempted to produce. Besides the α-myc scFv, successful production of an active α-Gfp nanobody was shown while expression of a diabody against the myc epitope could not be achieved. Nevertheless, in this study, the *U. maydis* expression system was optimized significantly in terms of reducing its proteolytic potential. Various antibody formats could thus be produced in this simple eukaryotic organism. With this optimized strain the system is now on a promising way to be exploited for different biotechnological applications.

Zusammenfassung

Der globale Markt für Biopharmazeutika steigt immens. Um die volle Bandbreite biopharmazeutischer Proteine herstellen zu können, wurde in *Ustilago maydis* eine alternative Expressionsplattform basierend auf der unkonventionellen Sekretion der Endochitinase Cts1 etabliert. Auch wenn *U. maydis* diverse Eigenschaften mit sich bringt, die ein großes biotechnologisches Potential versprechen, müssen jedoch zunächst einige Defizite behoben werden. In initialen Expressionsstudien mit einem α-myc scFv Antikörperfragment stellte sich heraus, dass nur geringe Mengen Volllängen-Protein sekretiert wurden. Dies konnte hauptsächlich auf einen proteolytischen Abbau durch Wirtsproteasen zurückgeführt werden. Daher war das Hauptziel dieser Arbeit, das Degradierungsproblem durch die Eliminierung von Proteasen zu beheben.

Zunächst fokussierten die Untersuchungen auf der Schlüsselprotease Kex2, die als Aktivator zahlreicher sekretierter Proteasen bekannt ist. Die Deletion des entsprechenden Gens führte zu einem stark reduzierten proteolytischen Abbau. Zudem zeigte die Mutante jedoch einen auffälligen Phänotyp sowie verlangsamtes Wachstum, was in Bioreaktorstudien zu Problemen führen könnte. Daher wurden in einem nächsten Schritt *U. maydis* Homologe bekannter schädlicher Proteasen aus filamentösen Pilzen entfernt. Die entsprechenden Einzelmutanten zeigten jedoch keine signifikant verbesserte Stabilität oder höhere Ausbeuten der sekretierten Proteine. Daher war der vielversprechendste Ansatz, einen Stamm mit multiplen Proteasedefizienzen herzustellen. Die Herstellung eines entsprechenden Fünffach-Deletionsstammes wurde mit einer eleganten Golden Gate Klonierungsmethode in Kombination mit einem FLP-FRT Marker-Recyclingsystem bewerkstelligt. Dieser gentechnisch veränderte Stamm zeigte eine stark verringerte proteolytische Aktivität. Im Zusammenspiel mit einem starken Promotor und einer optimierten Expressionskassette konnten so eine deutlich höhere Stabilität und größere Ausbeuten aktiven sekretierten scFv-Cts1 Fusionsproteins erzielt werden.

Um die Flexibilität des Expressionswirts zu zeigen, sollten zudem weitere alternative Antikörperformate hergestellt werden. So konnte neben dem α-myc scFv ein aktiver α-Gfp Nanobody hergestellt werden. Dahingegen war die Herstellung eines gegen das myc-Epitop gerichteten aktiven Diabodys nicht möglich. Zusammenfassend konnte jedoch in dieser Arbeit das *U. maydis* Expressionssystem besonders in Bezug auf die proteolyische Aktivität stark

optimiert werden. Verschiedene Antikörperformate konnten erfolgreich in diesem einfachen Eukaroyoten produziert werden. Mit dem hier optimierten Stamm zeigt das Expressionssystem somit großes Potential für diverse biotechnologische Anwendungen.

List of abbreviations

Acb	Acyl-CoA binding protein
Acyl-CoA	Acyl coenzyme A
AM	Ammonium minimal
AmpR	Ampicillin resistance
Asn	Asparagine
Bsa	Bovine serum albumin
C_H	Constant heavy chain
ChrR	Chloramphenicol resistance
C_L	Constant light chain
CM	Complete medium
DNA	Deoxyribonucleic acid
dsFv	Disulfide-stabilized scFv
Fab	Fragment antigen binding
Fc	Fragment crystallizable
FLP	Flippase-recombinase
Fw	Forward
Gfp	Green fluorescent protein
Glc	Glucose
Gst	Glutathione S-transferase
h	Hours
HCAb	Heavy chain antibody
HCl	Hydrochloric acid
HIV	Human Immunodeficiency Virus
HygR	Hygromycin resistance
Ig	Immunoglobulin
IMAC	Immobilized metal ion affinity chromatography
Ip	Iron-sulfur protein subunit
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kb	Kilobases
kDa	Kilodalton
l	Liter
mAb	Monoclonal antibody
min	Minutes
mRNA	Messenger ribonucleic acid
µl	Microliter
µm	Micrometer
NatR	Nourseothricin resistance
Ni^+-NTA	Nickel-nitrilotriacetic acid
NM	Nitrate minimal
OD	Optical density
Rev	Reverse
UV	Ultraviolet light
v/v	Volume by volume
V_H	Variable heavy chain
w/v	Weight by volume

1. Introduction

1.1. Overview of protein expression systems

Currently, there are more than 200 marketed biopharmaceutical products. The global sales of recombinant proteins itself crossed the 100 billion dollar mark in 2011 and it is expected to reach 170 billion dollars by 2014 [Berlec and Štrukelj 2013, Spadiut et al., 2014]. This clearly emphasizes the significant impact of protein production on the overall pharmaceutical market. A repertoire of excellent platforms exists to produce recombinant proteins for applications in therapeutics, research, and industry. Living cells are harnessed as factories to synthesize proteins based on molecular engineering. The bacterium *Escherichia coli* and mammalian systems are currently the main workhorses of biopharmaceutical production [Andersen and Krummen 2002, Walsh 2010], although new and emerging systems like fungi [Mattanovich et al., 2012, Punt et al., 2002], plants [M Twyman et al., 2013, Ma et al., 2003], transgenic animals [Houdebine 2009] and cell-free synthesis [He et al., 2011] also account for some of these products. The selection of the expression system depends on the type of protein to be expressed, its requirements for functional activity and the desired yield [Berlec and Štrukelj 2013, Demain and Vaishnav 2009]. Moreover, it is worthwhile to mention that the downstream processing can amount up to 80% of the total manufacturing costs [Roque et al., 2004]. Thus, the systems in which a protein is secreted into the medium are favorable to use because of the simplicity in downstream processing and thus reduced overall expenses [Moir and Mao 1990].

Among the prokaryotic expression systems, *E. coli* is most widely employed due to its excellent properties such as the huge variety of genetic tools available for cloning and expression, quick growth in inexpensive media, and high product yields [Huang et al., 2012, Swartz 2001, Waegeman and Soetaert 2011]. Hence, 31% of the biopharmaceuticals approved till now are produced in this system [Berlec and Štrukelj 2013]. However, it is apt for production of small and structurally simple proteins but mostly not suitable for expressing huge proteins with complex tertiary structures or for proteins requiring disulfide bridges or other post-translational modifications [Gellissen 2006]. The proteins produced either accumulate intracellular or are secreted into the periplasmic space. Some examples of protein secretion in the medium have also been reported [Mergulhão et al., 2005, Ni and Chen 2009, Parente et al., 1998, Schwarz et al., 2012, Zhang et al., 2006]. The tendency for protein

aggregation in the form of inclusion bodies, however, necessitates employing cumbersome protein purification and refolding methods to obtain active protein [Lilie et al., 1998, Sørensen and Mortensen 2005].

Mammalian systems are preferred expression hosts due to their ability to carry out correct post-translational modifications required for many eukaryotic proteins. These include important human therapeutic proteins like e.g. erythropoietin, interferons or monoclonal antibodies [Wurm 2004, Zhu 2012]. Accordingly, 43% of the approved biopharmaceuticals are produced in this system. Mainly rodent cells such as Chinese hamster ovary (CHO), baby hamster kidney (BHK) and mouse myeloma (NS0) cells are employed. Less frequently, cells of human descent like human embryonic kidney (HEK-293) and human-retina-derived cells (PER-C6) are also applied [Berlec and Štrukelj 2013]. However, high cultivation expenses, lengthy generation of stable expression clones and the risk of viral contamination still remain bottlenecks, which can account for very high costs of protein drugs produced in these systems [Berlec and Štrukelj 2013, Spadiut et al., 2014, Wurm 2004].

Fungal expression systems not only offer the advantage of high density culture capacity in inexpensive media as for bacterial systems, but also possess the ability to perform post-translational modifications as for mammalian systems [Mattanovich et al., 2012]. Furthermore, these systems offer a simple and cheap downstream processing because proteins are secreted into the medium [Berlec and Štrukelj 2013]. The most commonly used fungi are the yeasts *Saccharomyces cerevisiae* (15% of approved biopharmaceuticals) and *Pichia pastoris* (1% of approved biopharmaceuticals) while several other yeasts such as *Hansenula polymorpha, Kluyveromyces lactis, Schizosaccharomyces pombe, Yarrowia lipolytica* and *Arxula adeninivorans* are reported only in rare cases [Berlec and Štrukelj 2013, Çelik and Çalık 2012]. Besides the yeasts, some species of filamentous fungi originating for example from the genera *Trichoderma*, *Aspergillus* or *Penicillium* have been exploited for their efficient secretion capacity and hence, their excellent ability of extracellular enzyme synthesis [Conesa et al., 2001, Ward 2012]. Despite this promising potential, there are some downsides to fungal expression platforms that often turn out difficult to eliminate. Especially in the case of filamentous fungi, proteolytic degradation by host proteases has been one of the major problems limiting the yields of heterologous proteins [Ward 2012]. Furthermore, although post-translational modifications are performed in these systems, inappropriate glycosylation

patterns like hyper-mannosylation can result in an interference with protein activity or impart harmful immunogenicity in humans [Gerngross 2004]. To solve this issue, humanization of the glycosylation machinery has been successfully performed in yeast cells to produce various glycoproteins like recombinant erythropoietin in *P. pastoris* [Hamilton et al., 2006, Hamilton and Gerngross 2007] or IgGs [Li et al., 2006]. Strategies to engineer the glycosylation machinery also exist for other fungi [De Pourcq et al., 2010].

Many alternate platforms are being explored but their commercialization has been slow till now [Corchero et al., 2013, Demain and Vaishnav 2009]. In general, every system has some drawbacks and none of them is suitable for production of all heterologous proteins. Hence, there is a strong demand for exploring alternative, novel cell factories to fill the existing gaps and to expand the repertoire of expression systems [Corchero et al., 2013, Feldbrügge et al., 2013, Gellissen 2006]. In the future, this will broaden the selection and bring down the costs of clinical relevant biopharmaceuticals, making them more affordable.

1.2. Exploiting novel routes of secretion for biotechnological applications

Downstream processing can be simplified if heterologous proteins are secreted into the culture medium. In a eukaryotic cell, proteins are usually secreted by the conventional secretory pathway which starts from recognition and hence translocation of signal peptide-containing proteins across the membrane of the endoplasmic reticulum (ER). After folding and quality control, secretory proteins are packaged into transport vesicles in the ER lumen, followed by delivery of the cargo to the Golgi apparatus. After further modification of the cargo, post-Golgi transport carriers fuse with the plasma membrane eventually resulting in the release of secretory proteins into the extracellular milieu [Nickel 2005].

Additionally, in some eukaryotes, protein secretion via non-conventional pathways is observed. Unlike the conventional pathway, this route does not require a canonical N-terminal secretion signal [Cleves 1997, Nickel 2005]. Non-classical secretion of proteins has been described to occur either via vesicular or non-vesicular transport (Fig. 1) [Rabouille et al., 2012].

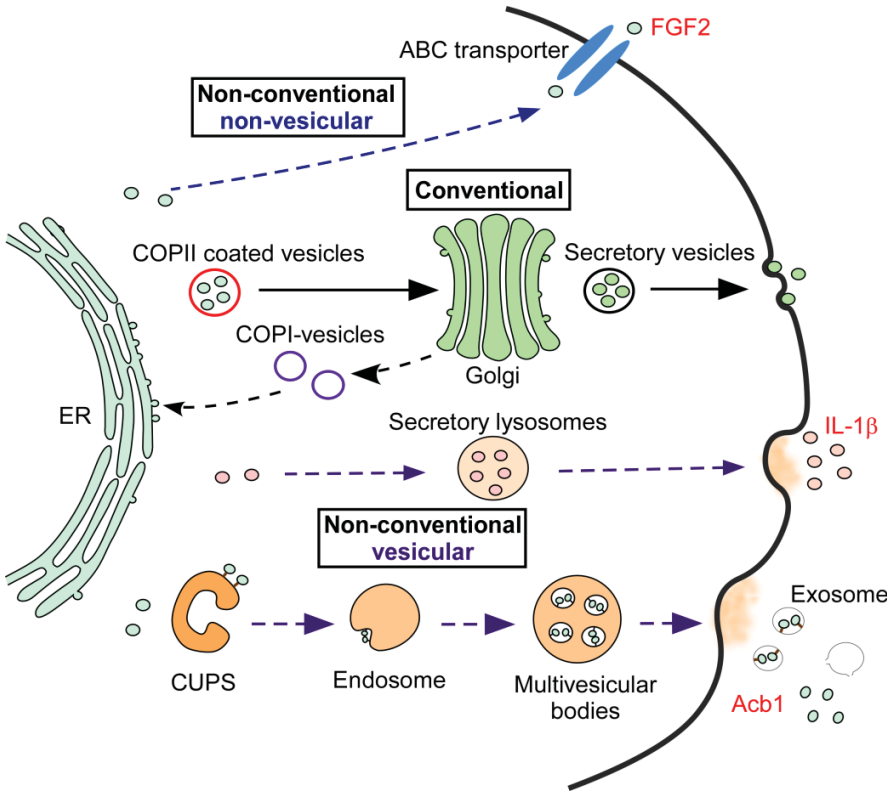

Figure 1. Scheme depicting the modes of protein secretion in eukaryotes

This model schematically shows conventional and unconventional pathways of protein secretion in eukaryotes. During conventional secretion, the proteins pass the ER and Golgi apparatus (black arrows). For unconventional secretion different pathways have been proposed that can be discriminated into vesicular and non-vesicular. In one example of non-vesicular pathway (dark blue arrow), the protein is directly translocated across the plasma membrane via ABC transporters (like shown for FGF2). In one example of the vesicular pathway (purple arrows), autophagosome-like vesicles containing the Acb1 protein forms at CUPS, (compartment for unconventional protein) which subsequently fuses with endosomes to form multivesicular bodies (MVB). These MVBs then fuse to the plasma membrane to release the exosomes filled with protein. In another example of vesicular pathway (purple arrows), protein secretion is mediated through secretory lysosomes (as in the case of IL-1β) Model adapted from [Malhotra 2013, Rabouille et al., 2012].

Representatives of both types have already been shown for higher eukaryotes like mammalian cells. For example, the vesicle-independent secretion of fibroblast growth factor 2 (FGF2) by direct translocation across the plasma membrane in a range of mammalian cells [Nickel 2011] or in the case of vesicular transport, the export of Interleukin 1β via secretory lysosomes from monocytes during inflammation [Malhotra 2013, Nickel and Rabouille 2009]. Different examples of vesicular transport routes have been discovered in lower eukaryotes too. One well-described example is the secretion of the acyl-CoA binding protein in *S. cerevisiae* (Acb1) and *Dictyostelium* (AcbA) via autophagosome-like vesicles [Rabouille et al., 2012]. Various routes observed till now with representative examples are briefly

summarized in Figure 1 [Malhotra 2013, Rabouille et al., 2012]. The biotechnological exploitation of these alternate secretion pathways by either presenting heterologous fusion proteins on the cell surface or secreting them into the extracellular medium would be a novel step towards broadening the repertoire of expression platforms [Nickel 2010].

1.3. *U. maydis* - a model microorganism on the rise

U. maydis is a dimorphic basidiomycete that causes smut disease on corn. This fungus is well known for extensive research conducted since several years in various fields of molecular biology such as DNA recombination and repair [Holliday 2004, Kojic et al., 2013, Yu et al., 2013], plant-pathogen interaction [Bölker 2001, Brefort et al., 2009, Djamei and Kahmann 2012], post-transcriptional regulation [Vollmeister and Feldbrügge 2010] or molecular transport [Göhre et al., 2012, Steinberg 2014, Vollmeister et al., 2012]. Its genome is sequenced and manually annotated with very high quality [Kämper et al., 2006]. As a consequence, this dimorphic fungus develops more and more towards a popular fungal model system [Dean et al., 2012, Steinberg and Perez-Martin 2008, Vollmeister et al., 2012].

U. maydis cells can grow in a yeast-like form dividing by budding (Fig. 2a) while for pathogenic development cells switch to filamentous growth. This filamentous form infects corn plants turning the cobs into tumors filled with fungal spores (Fig. 2b) [Vollmeister et al., 2012]. Interestingly, these infected corn cobs have been served as a delicacy since centuries in Central America suggesting that this fungus is harmless for human consumption (Fig. 2c). Hence, *U. maydis* could be safely used as an expression host for production of protein biopharmaceuticals [Feldbrügge et al., 2013].

With respect to its use in biotechnology, *U. maydis* is known as a promising host for production of secondary metabolites like glycolipids or itaconic acid [Hewald et al., 2006, Klement et al., 2012, Teichmann et al., 2007]. Furthermore, the fungus harbors hydrolytic enzymes that could be of use [Feldbrügge et al., 2013]. For example, a novel lipase (Uml2) was recently described that has been discovered by genome mining and displays homology to the industrially important lipase CalB from *Candida antarctica*. Uml2 not only exhibits esterase activity comparable to CalB but it also harnesses an additional ability to hydrolyse phospholipids making it a potential alternate biocatalyst for biotechnological purposes [Buerth et al., 2014].

Figure 2. The corn smut fungus *U. maydis*

a) Microscopic image of yeast-like cells growing in liquid medium. **b)** Infected maize plant showing tumour development on corn cobs (picture taken near Regensburg, Germany). **c)** Corn smut galls conserved in tins and sold as the delicacy "cuitlacoche" (picture taken by Steffen Köhler, HHU Düsseldorf). **d)** Yeast-like cells in shake flask cultures. **e)** 3 L bioreactor cultivation of yeast-like cells. **f)** Transformants on a regeneration agar plate after five days of transformation of the protoplasts.

The prime prerequisite for a ubiquitous protein expression host is easy handling along with low demands with respect to growth conditions. *U. maydis* meets these criterions quite well: in its yeast form, the fungus grows by budding in standard complete as well as minimal medium [Holliday 1974] with a growth rate of about two hours in shake flask cultures (Fig. 2d). Albeit in nature filamentous growth is restricted to the plant stage, engineered strains can also grow in the hyphal form in axenic culture. The coordinated induction of filamentous growth in these strains can be achieved by a simple medium switch [Brachmann et al., 2001].

Furthermore, cells are robust and well suited for bioreactors which can be grown to high densities in short time, without loss of strain fitness [Maassen et al., 2014] (Fig. 2e). Several groups are employing this fungus to produce bio-based platform chemicals. Aerobic fermentation of cellulosic biomass has been successfully employed for production of itaconic acid, a platform chemical for the synthesis of potential biofuels [Klement et al., 2012, Maassen et al., 2014]. A metabolic model for optimized itaconic acid production has been

developed which can eventually be helpful in reactor studies and downstream processing of broth for protein expression purposes [Voll et al., 2012].

Also, as an important basis for strain generation, molecular cloning techniques are well established in *U. maydis* [Brachmann et al., 2001, Brachmann et al., 2004, Kämper 2004, Kojic and Holloman 2000, Stock et al., 2012, Terfrüchte et al., 2014]. Deletion and insertion mutants can be generated easily. A versatile set of genetic tools is available that includes not only integrative plasmids allowing targeted insertion at defined genomic loci but also self-replicating plasmids as well as constitutive and inducible promoters [Brachmann et al., 2001, Kojic and Holloman 2000, Stock et al., 2012, Terfrüchte et al., 2014]. In general, genetically modified *U. maydis* strains can be generated quickly in around two to three weeks (Fig. 2f) with verification using simple PCR reactions and Southern blot analysis. This results in genetically stable transgenic strains, without the need to keep selective conditions. Its fast growth rate coupled with excellent molecular handling thus makes this fungus a good starting point to develop it as a valuable protein production factory.

1.4. Microtubule-dependent transport and unconventional secretion

Besides the omnipresent eukaryotic secretion apparatus, a second, alternative secretion process was uncovered in *U. maydis* a few years ago [Koepke et al., 2011, Stock et al., 2012]. In particular, unconventional secretion of a bacterial-type endochitinase (Cts1) was found to be dependent on long distance transport of mRNAs along microtubules in fungal filaments. This cytoskeletal trafficking of mRNAs is mediated by the RNA binding protein Rrm4 [Becht et al., 2005, Becht et al., 2006]. Deletion of *rrm4* led to impaired filamentous growth and reduced virulence, indicating the significance of this microtubule-dependent mRNA transport for polar growth of filaments and thus successful infection of corn plants [Becht et al., 2006, Vollmeister and Feldbrügge 2010, Zarnack and Feldbrügge 2007].

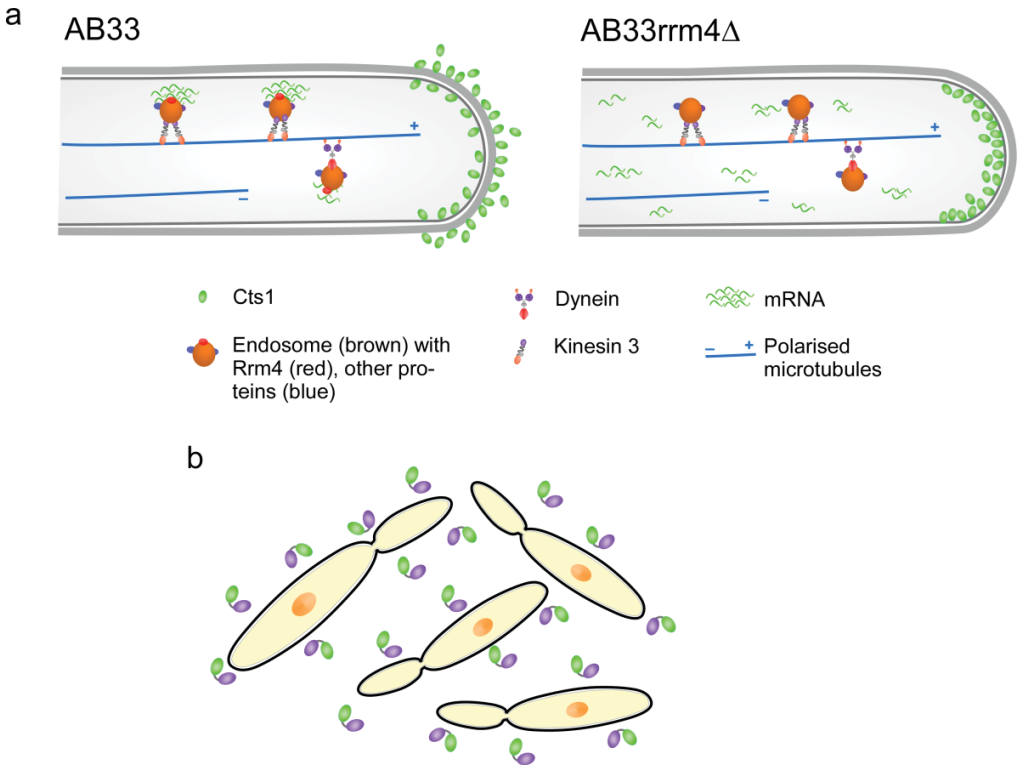

Figure 3. Unconventional Cts1 secretion in yeast-like cells and filaments
a) Scheme showing Cts1 secretion in *U. maydis* hyphae in AB33 and *rrm4* deletion strain. A specific set of mRNAs bound to Rrm4 is transported in mRNPs (ribonucleoparticles) and with endosomes along the microtubules across the filament. Cts1 is localized at the hyphal tip and after secretion it is most likely associated with the cell wall. In *rrm4* deletion filaments, microtubule-dependent shuttling of mRNA is disrupted. Therefore, secretion of Cts1 is impaired leading to accumulation of Cts1 at the hyphal poles [Koepke et al., 2011, Vollmeister et al., 2012]. Picture adapted from (Janpeter Stock Dissertation 2014). **b)** Schematic representation of Cts1 fusion proteins secreted by yeast-like cells. Unconventional secretion of Cts1 was also demonstrated in yeast-like cells, showing that the role of Rrm4 mediated mRNA shuttling is not essential for Cts1 secretion [Stock et al., 2012], lilac: heterologous protein, green: Cts1.

Meanwhile, the molecular process of mRNA shuttling is well described. mRNA binding by Rrm4 is accomplished by three N-terminal RNA recognition motifs (RRMs) present in the Rrm4 protein while a C-terminal MademoiseLLE domain (MLLE) is required for the formation of shuttling mRNA particles [Becht et al., 2006]. mRNA trafficking along microtubules mediated by Rrm4 requires a molecular motor-based movement of endosomes using Dyn1/2 for minus-end directed transport and Kin3 for plus-end delivery [Schuster et al., 2011b, Steinberg 2007] (Fig. 3a). Intriguingly, co-localisation studies demonstrated that Rrm4 and bound mRNAs hitchhike on endosomes that were known to shuttle along microtubules [Baumann et al., 2012, Schuster et al., 2011a]. Recently, live-cell imaging studies on polarized fungal filaments further showed co-localization of septin mRNA and encoded septin

protein as well as the presence of ribosomes on shuttling endosomes supporting the hypothesis of local translation on moving endosomes for delivery of proteins to distinct subcellular sites [Baumann et al., 2014, Higuchi et al., 2014].

To further characterize the role of Rrm4 during pathogenic development, differential proteomic studies were performed on parental and *rrm4* deletion filaments. Several proteins were differentially abundant in *rrm4Δ* filaments such as three mitochondrial proteins, one ribosomal protein and the potential bacterial-type endochitinase Cts1. In particular, Cts1 was shown in increased protein amounts in *rrm4Δ* filaments while losing its usual extracellular activity. Although its localization at the growth pole was not affected, secretion of Cts1 was drastically reduced in the absence of Rrm4 (Fig. 3a). These results illustrate the importance of a functional microtubule-dependent mRNP transport for efficient secretion of Cts1 which is probably achieved by mRNA transport of the respective interacting export factors [Koepke et al., 2011]. Interestingly, according to bioinformatics predictions Cts1 does not harbor a conventional N-terminal secretion signal. It thus constituted an interesting candidate for a novel carrier that can be tested for its use in applied research.

1.5. Cts1 mediated export of heterologous proteins

Since Cts1 lacks a predictable N-terminal secretion signal needed for conventional secretion, the possibility of secretion by an alternate pathway was investigated. This assumption was proved using β-glucuronidase (Gus) from *E. coli* as a reporter enzyme [Stock et al., 2012]. This bacterial enzyme by chance contains a eukaryotic N-glycosylation site at Asn_{345}, which renders the enzyme inactive upon glycosylation. Therefore, after secretion via the conventional pathway involving passage through ER and Golgi apparatus, the enzyme is not active anymore. If in contrast it takes an unconventional route bypassing the ER, it would circumvent glycosylation and the active enzyme would be secreted into the medium.

Based on this principle, Gus activity in strains expressing fusion proteins of Cts1 and Gus was assayed. The enzyme was indeed active in the supernatant when fused to the N-terminus of Cts1, suggesting that the fusion protein was secreted by an unconventional route [Stock et al., 2012]. Importantly, the observation that about 100 amino acids of the N-terminus are dispensable for Cts1 secretion further strengthened this hypothesis, since the

signal sequence for classical secretion is usually present at the immediate N-terminus [Stock et al., 2012].

The discovery of unconventional Cts1 secretion prompted the idea to test the feasibility of *U. maydis* as a protein expression system. As an alternative to the commonly employed signal recognition particle (SRP)-dependent export of glycosylated proteins, unglycosylated heterologous proteins with therapeutic or industrial use can be exported to the medium as Cts1 fusion proteins in this system (Fig. 3b) [Stock et al., 2012]. Besides the original finding in fungal filaments, unconventional secretion was later also demonstrated in yeast-like cells, permitting usage of both types of cells for protein expression [Feldbrügge et al., 2013, Stock et al., 2012].

1.6. Antibody formats as an interesting target for protein expression

Antibodies are high affinity immunoglobulins (Ig), a class of glycoprotein molecules that provide defense against pathogenic organisms and toxins [Abbas et al., 1994]. The high market turnover (see 1.1) is mainly due to the marketing of monoclonal antibodies (mAbs) and antibody fragments which currently represent the fastest growing class of approved biopharmaceutical products [Spadiut et al., 2014].

Among several formats of antibody molecules, IgGs constitute the main serum antibodies and the format used as therapeutic antibodies [Holliger and Hudson 2005]. IgGs are bivalent in nature so that each molecule can bind two antigens. This property increases its functional affinity and confers high retention times [Woof and Burton 2004]. They are Y-shaped molecules made up of two identical heavy chains and two identical light chains, linked by di-sulfide bridges [Huber et al., 1976]. If the molecule is cleaved at the hinge region, it separates into three pieces, two pieces of which are identical and consist of the complete light chain (V_L and C_L) associated with the V_H-C_H1 fragment of the heavy chain (Fig. 4a). These fragments retain the ability to bind the antigen due to paired V_H and V_L domains and hence, they are called Fabs (fragment, antigen binging). The third piece consists of two identical di-sulfide linked peptides consisting of C_H2 and C_H3 domains, which have the ability to self-associate and to crystallize into a lattice, and hence is called Fc (fragment, crystallizable) [Abbas et al., 1994]. This Fc portion mediates effector functions through the human complement system and/or interactions with gamma Fc receptors.

There is a range of therapeutic and medical diagnostic applications in which the Fc-mediated effects of IgGs are not required or even detrimental [Weisser and Hall 2009]. For example, a long serum half-life results in poor contrast in imaging applications, and inappropriate activation of Fc receptor-expressing cells can lead to massive cytokine release and associated toxic effects [Holliger and Hudson 2005]. Other shortcomings of full-length antibody molecules include batch to batch variability of polyclonal antibodies, high costs and long production times of monoclonal antibodies or size limitations for certain medical diagnostic and therapeutic purposes such as tumor penetration and passage through the blood-brain barrier [Cuesta et al., 2010, de Marco 2011]. These bottlenecks motivated many groups to develop strategies for the production of alternative scaffolds and recombinant antibodies of smaller sizes, the respective genes of which can be manipulated and optimized by standard molecular biology techniques [Gebauer and Skerra 2009]. By exploiting the molecular domain architecture of antibodies, a repertoire of alternative formats was created. Initially, the Fc domain was removed through proteolysis using enzymes like papain and pepsin. However, proteolysis was unspecific and did not yield molecules smaller than Fab fragments [Hudson 1998]. Therefore, later, genetically engineered molecules were synthesized such as monovalent variants like the scFv (single-chain variable fragment), the dsFv (disulfide-stabilized scFv) or bivalent fragments (Fab_2, diabodies, triabodies, minibodies, etc.) [Carter 2006].

Single-chain variable fragments (scFvs), one of the more commonly employed formats consists of only the V_H and V_L domains joined with a flexible polypeptide linker preventing its dissociation (Fig. 4a) [Bird et al., 1988]. scFvs (≈ 25 kDa) are smaller than intact immunoglobulin molecules (150 kDa) but retain the specific, monovalent, antigen-binding affinity of the parent IgG, while showing improved pharmacokinetics for tissue penetration [Hudson 1999]. They have reduced plasma half-life resulting in less whole body exposure to the drug, faster body clearance and improved target to non-target ratios [Carter 2006]. Due to their excellent pharmacokinetic properties, scFvs have varied applications such as in medical diagnostics i.e. imaging or HIV diagnosis or as therapeutics to treat cancer, infections or inflammatory diseases [Holliger and Hudson 2005]. They are also applied in immunodetection, purification and bioseparation applications [de Marco 2011]. Furthermore, scFvs have been forged into multivalent and multispecific reagents which are also known as

immunoconjugates or 'magic bullets' [Wu and Senter 2005]. For *in vivo* application, they are linked to therapeutic cargo [Monnier et al., 2013] such as radionuclides for imaging or immunotherapy [Mayer et al., 2000], toxins for cancer treatment [Carter 2006, Schrama et al., 2006], enzymes for prodrug therapy [Bagshawe 2004], liposomes for improved drug delivery [Park et al., 1997], viruses for gene therapy or biosensors for real-time detection of target molecules [Jespers et al., 2004].

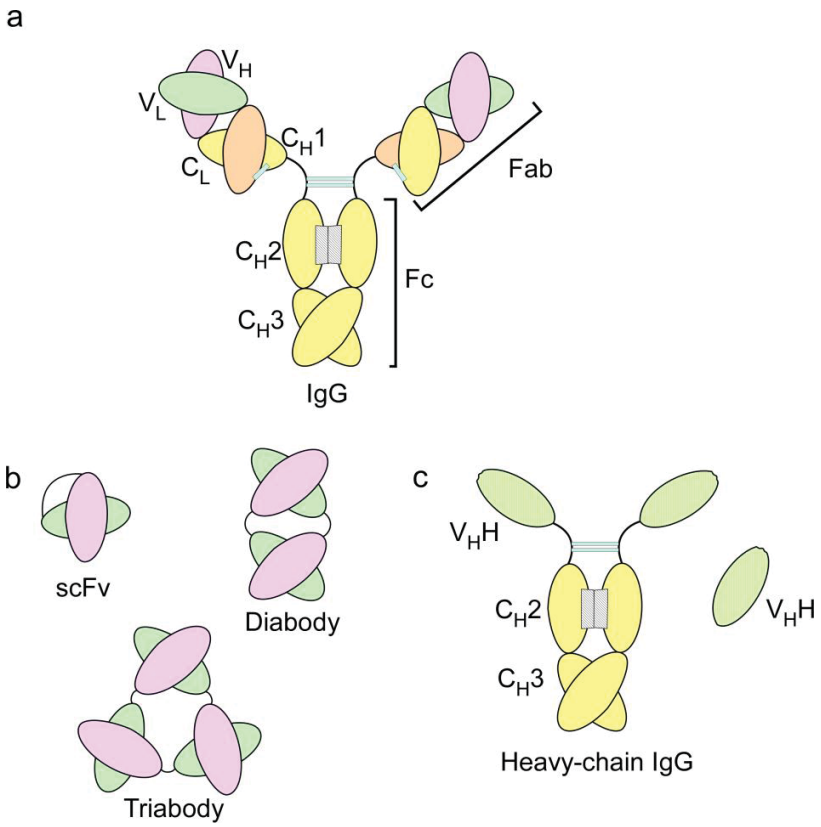

Figure 4. Schematic representation of different antibody formats

a) Scheme of an intact Ig antibody. Each structural unit consists of two identical heavy (H) and light (L) chains linked by di-sulfide bridges (shown as light blue bands). Each light chain consists of a variable domain (V_L) and a constant domain (C_L) while each heavy chain consists of a variable domain (V_H) and three constant domains (C_{H1}, C_{H2}, C_{H3}). The variable domains of the heavy and light chain associate to form the antigen-binding site. Hydrolysis by the enzyme papain leads to the cleavage of the molecule into 3 parts, 2 Fab pieces (Fragment antigen binding) and an Fc (Fragment crystallizable). A glycan portion, which influences the effector function, is present in C_{H2} domain, marked with the hashed box. **b)** Schematic view of different antibody fragments. One of the simplest formats is scFv (single-chain variable fragment) which consists of V domains of heavy and light chain (V_H and V_L) joined by a peptide linker of about 15 amino acids residues. These single-chain formats can be associated into multimeric forms resulting in a dimer (diabody), or a trimer (triabody), etc. [Carter 2006]. **c)**

Representation of a camelid heavy chain antibody consisting of two identical heavy chains but lacking light chains. Each heavy chain has one variable and two constant domains. The single-domain antigen binding entity which is derived from a camelid heavy-chain antibody is called V_HH or nanobody (depicted in light green) [Muyldermans 2001].

To improve the functional affinity and reduce the dissociation rates for target antigens, multivalent antibody formats can also be generated by reducing the scFv linker length yielding diabodies (55 kDa), triabodies (80 kDa) or tetrabodies (110 kDa) (Fig. 4a) [Hudson and Kortt 1999]. Bivalent formats can either be produced as a single chain molecule called bis-scFv, or they can be stabilized by non-covalent bonds like di-sulfide bridges in form of a diabody. These bivalent molecules can be designed in a way to confer multiple specificities i.e. bind to two different epitopes on a single antigen to increase avidity and specificity, or bind to two different antigens for various applications [FitzGerald et al., 1997, Holliger and Winter 1997]. One interesting example is the BiTE (Bispecific T-cell Engager). BiTEs are bispecific diabodies designed to bind to the CD3 T-cell co-receptor and thereby recruiting cytotoxic T-cells to the tumor site. Preclinical tests of BiTEs in animal models of human tumors have successfully demonstrated their effectiveness [Wolf et al., 2005].

Moreover, different type of antibody molecules from other species like heavy-chain antibodies from *Camelidae* (camels, dromedaries and llamas) [Hamers-Casterman et al., 1993] and Ig-NAR from cartilaginous fish (sharks, skates and rays) [Streltsov and Nuttall 2005] exist. The former type of antibodies is devoid of light chains and hence is called heavy-chain IgG (HCAb) [Muyldermans et al., 1994]. Its antigen binding domains consist only of the heavy-chain variable domains, called as V_HH or a nanobody and it represents the smallest intact antigen-binding fragment [Muyldermans 2001]. These molecules of only 15 kDa have high antigen affinity, high solubility and are more stable at higher temperatures than their corresponding derivatives of scFv and Fab fragments [Harmsen and De Haard 2007, Joosten et al., 2003]. These single-domain antibodies have expanded the vast repertoire of antibody-based reagents with wide applications such as in different therapeutics for their property to detect target sites inaccessible for monoclonal antibodies or antibody fragments [Stijlemans et al., 2004], in different consumer products [Joosten et al., 2003], or industrial or research purposes like immunopurification [Pichler et al., 2012].

In sum, engineered antibody formats are coming-of-age biopharmaceuticals with around 54 targets already in clinical development and 38 in preclinical trials [Nelson 2010].

1.7. Aim of the doctoral thesis

Based on the future goal to apply the fungus *U. maydis* as an alternate expression system to expand the repertoire and produce better quality protein biopharmaceuticals at affordable prices, the following aims and objectives were laid down in this work:

A. Production of different antibody formats to demonstrate the versatility of *U. maydis* as an expression host

To this end, a scFv against the c-myc epitope was chosen as a first proof-of-principle. The corresponding commercially available monoclonal antibody Myc1-9E10 has a broad range of diagnostic and research based applications for detection of nuclear proto-oncogene c-myc [Evan et al., 1985]. Besides the full-length mAb (150 kDa), the respective smaller (25 kDa) scFv format, which has the same selectivity, can be employed [Fuchs et al., 1997, Fujiwara et al., 2002]. Firstly, expression and secretion of active α-myc scFvs using Cts1-mediated unconventional secretion in *U. maydis* should be investigated. To further characterize the binding properties of this antibody fragment, the active protein should be enriched from the culture broth and analyzed by antigen-binding assays. To finally prove the versatility of the system, other antibody formats should also be expressed. In particular, a camelid-derived nanobody against Gfp as well as a diabody against c-myc were selected for this study.

B. Tackling the proteolytic degradation problem of the *U. maydis* expression system

Initial studies pointed towards protein degradation as one of the major problems in this system, leading to very low yields of the secreted heterologous proteins [Stock et al., 2012]. It is known that in case of fungal expression systems the main causes of protein degradation are host proteases [Idiris et al., 2010]. Hence, a parallel goal of the present study was to tackle the proteolytic degradation problem and thereby improve the secreted heterologous protein yields in the *U. maydis* expression system. To achieve this goal, different strategies were laid down:

i) Targeting a key protease involved in activation of several secreted proteases. This can prove the impact of secreted proteases on the stability of secreted heterologous proteins.

ii) In the next step, proteases known to reduce protein yields in other expression systems should be eliminated to generate a multiple-protease deficient strain with overall reduced proteolytic potential and hence achieve improved heterologous protein yields in *U. maydis* expression system.

2. Results

2.1. Expression of an α-myc scFv

To demonstrate the applicability of Cts1-mediated unconventional secretion for the export of pharmacological relevant proteins in *U. maydis*, a single chain antibody (scFv) [Bird et al., 1988] directed against the cMyc epitope EQKLISEEDL of the human oncogene product cMyc was expressed as a proof-of-principle. To this end, a modified version of the gene encoding the α-myc scFv described by Fujiwara et al. [Fujiwara et al., 2002] was codon-optimized for *U. maydis* to avoid premature polyadenylation [Zarnack et al., 2006] (Fig. 5a) and inserted into the expression vector pRabX1 (Fig. 5b). In this vector, the *scFv-cts1* fusion gene is under the control of constitutively active promoter P_{otef} [Spellig et al., 1996]. Furthermore, this expression plasmid harbors an SHH linker between the gene of interest and *cts1* (Fig. 5b). The SHH linker consists of a One-STReP tag (IBA, Göttingen), a triple HA tag and a 10xHis tag. These small protein extensions provide the flexibility for purification and detection of Cts1 fusion proteins. Also, the 3′UTR of *ubi1*, a target transcript of Rrm4, was inserted downstream of *cts1* to test, if the protein secretion is increased by enhanced mRNA transport. Earlier results demonstrated that this sequence contains a functional RNA element that promotes frequency and processivity of microtubule-dependent mRNA transport [Koepke et al., 2011, König et al., 2009]. However, no influence of *ubi1* 3′UTR could be detected using Gus as a reporter [Janpeter Stock, Dissertation 2014]. The novel expression vector was designed in a way that the α-myc scFv gene or the SHH linker can be replaced with other genes of choice or linkers containing i.e. protease cleavage sites by simple one-step cloning method (Fig. 5b). This allows using this vector for the expression of various high valued proteins [Janpeter Stock, Dissertation 2014].

The integrative pRabX1scFv-SHH-Cts1 plasmid was used to transform *U. maydis* strain AB33 that not only can grow yeast-like in complete medium but also allows efficient induction of filamentous growth in nitrate minimal medium [Brachmann et al., 2001]. To this end, the plasmid was linearized within the ip^r allele (e.g., using AgeI; Fig. 5b) and integrated at the genomic ip^s locus by homologous recombination (Fig. 5c). The ip^s locus codes for the iron-sulphur subunit of the succinyl dehydrogenase (Um00844/Sdh2; here designated ip^s). An amino acid exchange (H253L) encoded by the ip^r allele mediates carboxin resistance of the

enzyme [Broomfield and Hargreaves 1992, Keon et al., 1991]. Thus, carboxin selection of the transformants leads to single- or multi-copy plasmid integration at the *ip* locus. In addition, unwanted ectopic integrations as well as gene conversions occur occasionally. To verify correct insertion and copy number, carboxin resistant transformants were verified by Southern blot analysis and the resulting positive strains were used for expression analysis.

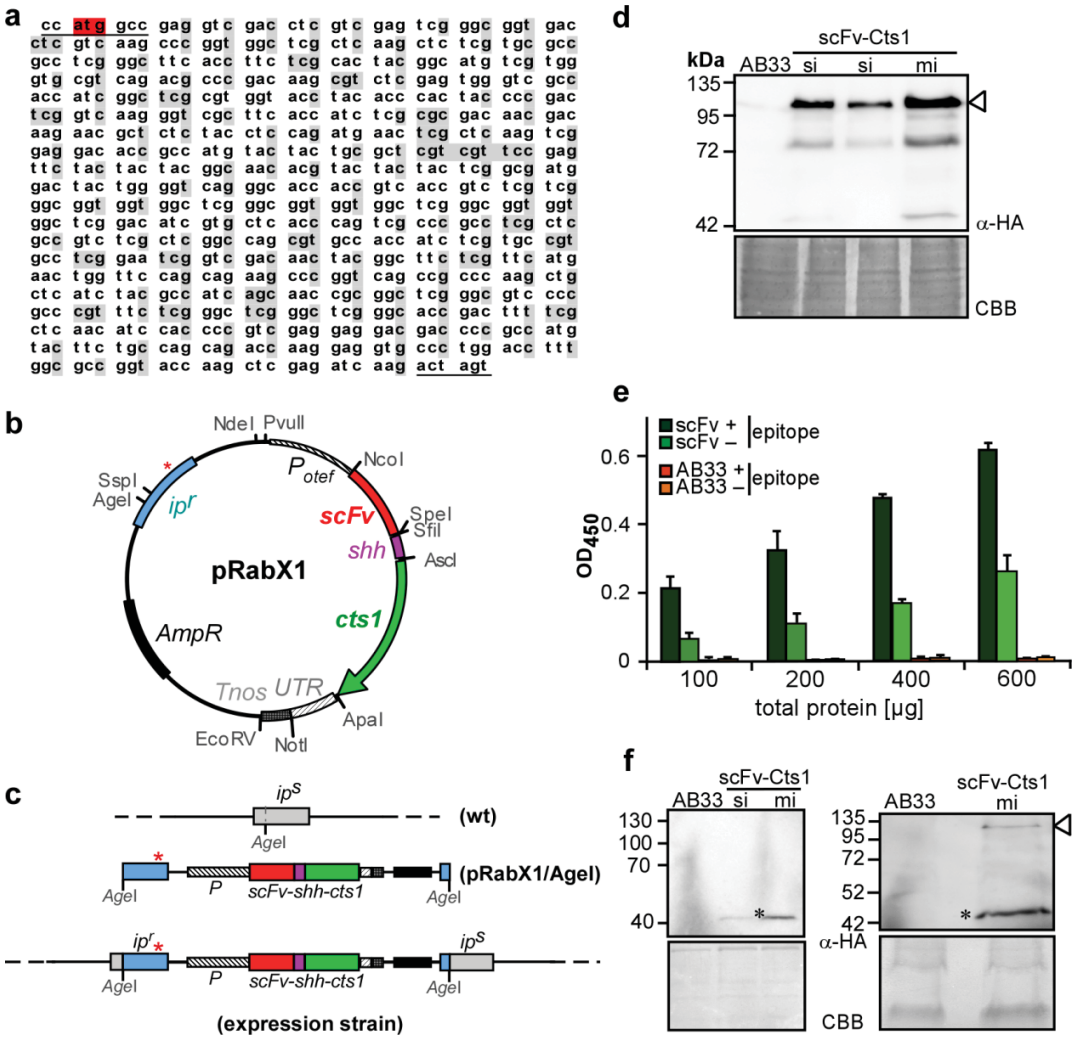

Figure 5. Expression of single-chain antibodies in *U. maydis*

a) DNA sequence of a synthetic scFv α-myc which was adapted to the context-dependent codon usage of *U. maydis*. Bases that were changed are shaded and mostly locate to the wobble position. Restriction sites (NcoI, SpeI) that were introduced for cloning purposes are underlined. The translational start codon ATG is marked in red. **b)** Schematic representation of the integrative plasmid pRabX1 which contains an expression cassette that allows the expression of N-terminal protein fusions with Cts1 (here: scFv-Cts1). The gene encoding the protein of interest can be inserted in one-step cloning via NcoI and SpeI. An internal linker encoding different tags for purification and detection of the corresponding fusion protein was inserted. In the depicted version, this linker (SHH, purple) consists of a One-STReP tag (IBA, Göttingen), a triple HA tag and a 10x His-tag (SHH). This

linker can easily be exchanged *e.g.,* by a sequence comprising a protease cleavage site, using SpeI and AscI restriction. In addition, the cassette harbors a sequence corresponding to the *ubi1* 3´UTR. It also contains an ip^r allele (blue) for heterologous recombination at the *ip* locus (ip^s; grey). The position of the mutation leading to the H253L exchange in the ip^r protein is indicated by an asterisk [Broomfield and Hargreaves 1992, Keon et al., 1991]. The *scFv-cts1* construct is under control of the constitutive promoter P_{otef} and the transcription termination sequence T_{nos} [Brachmann et al., 2004]. *AmpR*, gene mediating ampicillin resistance. **c)** Schematic view of the genomic region of the *ip* locus that can be used to integrate plasmids containing the ip^r allele (blue region) that confers carboxin resistance [Broomfield and Hargreaves 1992, Keon et al., 1991]. The organization of the wild type *ip* locus (wt) containing the ip^s allele (grey region; carboxin sensitive) as well as the architecture of the same region after single integration of the expression plasmid pRabX1 encoding scFv-Cts1 connected by an SHH linker (expression strain) are depicted (not to scale). The restriction endonuclease AgeI was used for linearization of the plasmid. **d)** Western blot depicting the expression of the scFv-Cts1 fusion protein. 10 µg of whole cell protein extracts were subjected to SDS PAGE and analyzed with α-HA antibodies by Western blotting. The Coomassie Brilliant Blue (CBB) stained membrane shows equal loading of protein. The migration of the fusion protein was slightly above the expected size of about 93 kDa (arrowhead). **e)** Protein extracts of *U. maydis* yeast cells expressing α-myc scFv-Cts1 (scFv) and of the progenitor strain AB33 were tested by indirect ELISA against Gst-MH and Gst-H with and without the cMyc epitope (+ and – epitope), respectively. The assay was performed in biological triplicates (error bars represent standard deviation). A proportional increase in activity was observed with increased protein amounts. **f)** Western blot depicting the detection of the scFv-Cts1 fusion protein in cell-free culture supernatants of yeast-like (50 ml) (left panel) and filamentous (200 ml) (right panel) cultures. The fusion protein was enriched by TCA precipitation and subjected to SDS PAGE and Western blotting. Degradation of protein is indicated by asterisks in both blots. The full-length fusion protein was observed only in filamentous cultures (arrowhead). AB33 was used as negative control.

Western blot analysis using whole cell extracts of the resulting expression strains confirmed that the scFv-Cts1 fusion protein is produced (Fig. 5d), migrating slightly above the expected size of 93 kDa. Importantly, strains with more than one copy of the inserted plasmid (Fig. 5d) show higher expression, which could be advantageous in biotechnological applications. Next, the biological activity of the scFvs-Cts1 in cell extracts was demonstrated by indirect enzyme-linked immunosorbant assays (ELISA) using a Gst-cMyc- 6xHis (Gst-MH) fusion protein as antigen containing the cMyc epitope (Fig. 5e). Gst-6xHis (Gst-H) was used as an antigen control in the assay. Cell extracts of AB33 were employed as negative controls. A proportional increase in binding activity was observed with increasing protein concentrations for scFv-Cts1 containing cell extracts. However, some nonspecific activity for Gst-H was also observed reaching about one third of the activity detected against Gst-MH (Fig. 5e). Furthermore, Western blot analysis of supernatant proteins of yeast-like and filamentous cultures revealed that full length fusion protein could be detected in cell-free culture supernatants of filamentous growing cells (Fig. 5f, right panel) while no specific signal was observed in supernatants of yeast-like cells (Fig. 5f, left panel). This demonstrates that the fusion protein is secreted into the culture medium of filamentous cells. In contrast

only degradation products were detected for cell-free supernatants of yeast-like growing cells (Fig. 5f, left panel; marked by an asterisk). Also in the supernatants of filaments, the detected amounts of full length protein were very low and much of the protein was detected as smaller degradation products (Fig. 5f, right panel; marked by asterisk). This observation indicated the presence of protease activity that leads to the degradation of secreted proteins.

The degradation of the recombinant proteins in the supernatant was also likely the reason for the failure to enrich active protein from supernatants (see above). Hence, tackling the problem of protein degradation became the next imperative step to achieve active full-length proteins in the supernatant and thereby improving the protein yields of this expression host.

2.2. Elimination of Kexin2 to reduce the proteolytic potential of *U. maydis*

Extracellular host proteases constitute a major factor limiting the yield of secreted proteins in the culture medium in filamentous fungi [Idiris et al., 2010, Ward 2012]. To test if proteases are indeed the cause of degradation of heterologous protein in *U. maydis*, the gene *kex2 (um02843;*http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Db=p3_t237631_Ust_maydi_v2&Method=ReportGene&GeneticelemlD=3300) encoding the putative subtilisin type serine protease Kexin2 (Kex2) was targeted. This protein resembles the well-described KEX2 (YNL238W) pro-protein convertase from *S. cerevisiae* by 31% amino acid identity (Fig. 6a). KEX2 is known to localize in the late Golgi where it plays a central role in activating secreted proteins for example, several secreted proteases by removing their N-terminal pro-sequences [Brenner and Fuller 1992, Fuller et al., 1988] (Fig. 6b).

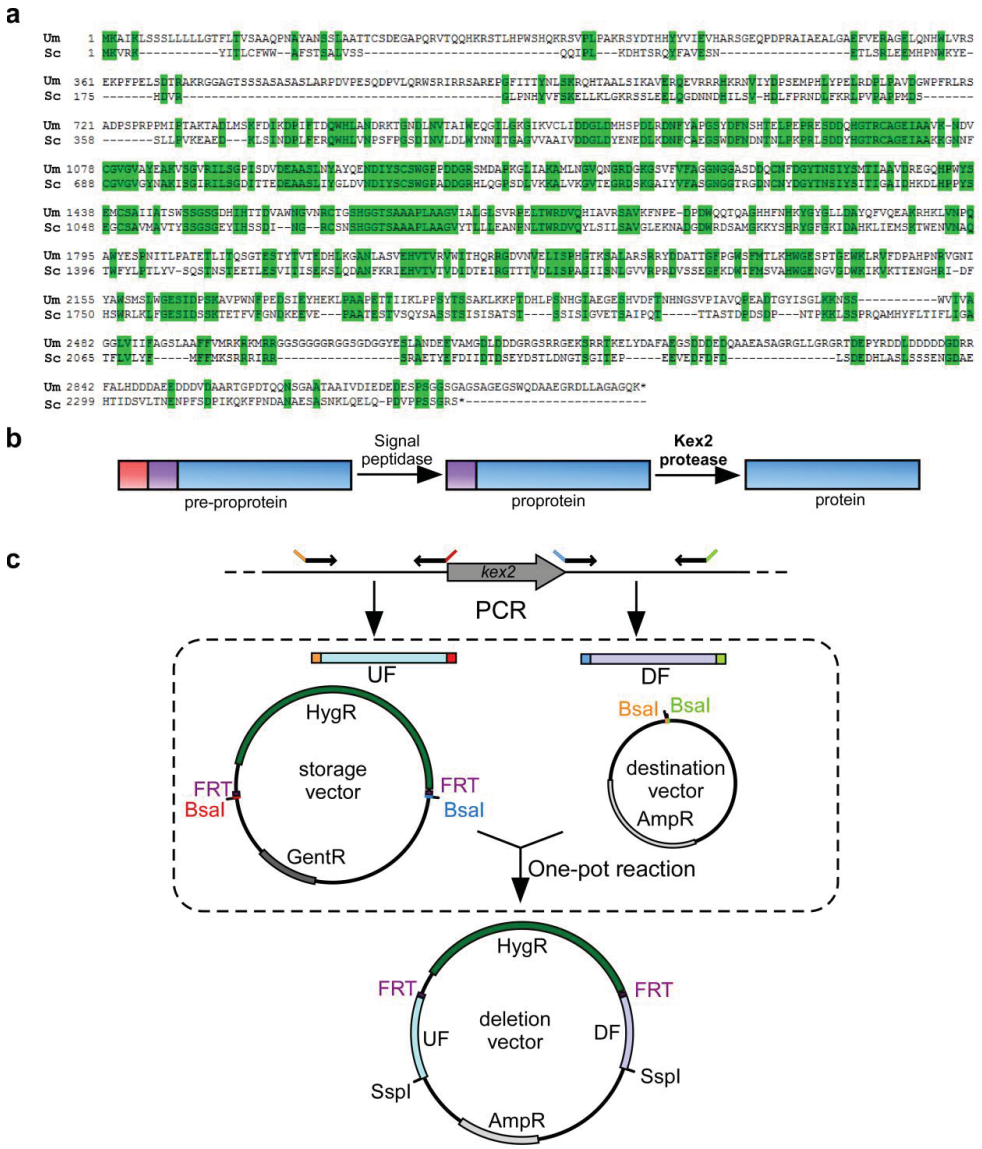

Figure 6. Strategy for reducing the proteolytic activity by elimination of the key protease Kex2

a) Amino acid sequence comparison of Kexin2 proteins from *S. cerevisiae* (ScKEX2) and *U. maydis* (UmKex2). Identical amino acids are highlighted in green. The overall sequence identity is 31%. **b)** Scheme showing the putative function of Kex2 as a pre-proprotein convertase: Upon entering of a preproprotein into the ER, a signal peptidase cleaves off the signal peptide sequence (pre-sequence). The proprotein then enters the Golgi where it encounters the Kex2 protease which cleaves off the N-terminal pro-sequence yielding active protein which is then secreted [Redding et al., 1991]. **c)** Gene deletion using a BsaI-mediated Golden Gate cloning strategy. Deletion constructs for a gene-of-interest (in the displayed case *kex2*) are generated by amplifying flanking sequences, concomitantly inserting the appropriate BsaI sites (colored primer appendages). Subsequently, flanks are combined with an appropriate resistance cassette in a one-pot reaction. At the same time, the deletion construct is inserted into a vector backbone for amplification. Specific restriction sites (i.e., SspI) can be used to remove the linear transformation construct from the resulting deletion plasmid generating perfect homologous ends [Terfrüchte et al., 2014] (HygR, hygromycin resistance cassette; GentR, gentamycin resistance; AmpR, ampicillin resistance; UF, upstream flank; DF, downstream flank).

To investigate the role of Kex2 in activating the secreted proteases of *U. maydis*, the corresponding gene was deleted in AB33 using established gene deletion techniques [Brachmann et al., 2001, Brachmann et al., 2004, Terfrüchte et al., 2014]: Firstly, the deletion plasmid was generated using a straight-forward Golden Gate cloning method [Terfrüchte et al., 2014, Marius Terfrüchte, Bachelorarbeit 2011] (Fig. 6c). The principle of this method is based on the special property of type II restriction enzyme BsaI that it cleaves few base pairs downstream of its recognition sequence allowing seamless joining of DNA fragments without introducing foreign nucleotides [Engler et al., 2008]. The flanking regions surrounding the gene-of-interest (here *kex2*, shown in Fig. 6c) are amplified by PCR introducing distinct BsaI sites at the ends. The flanks are then ligated to a resistance cassette of choice and inserted into a vector backbone. The storage vector contains a resistance cassette which mediates hygromycin resistance for selection in the fungal host, surrounded by distinct BsaI sites. The destination vector contains compatible BsaI sites for insertion of the deletion construct. To assemble the final deletion vector, a one-pot reaction is carried out in which the amplified flanks along with storage and destination vectors are added in a reaction mix containing the enzymes BsaI and DNA ligase (Fig. 6c). This enables restriction by BsaI and ligation of the resulting appropriate fragments in a single step, while the respective recognition sites are lost. Then, the linear deletion construct to be used for transformation is designed to contain blunt end restriction sites so that after excision of the linear construct, perfect homologous ends are generated, which increases the rate of homologous recombination [Terfrüchte et al., 2014] (Fig. 6c).

After generating the deletion mutant AB33kex2Δ, casein plate assays were performed to determine the proteolytic activity in the culture broth. In this assay the general proteolytic potential of tested strains is indicated by the formation of a turbid halo [Cowan and Daniel 1982] in casein-containing medium plates. Yeast cells of the progenitor strain AB33 induced such a turbid halo (Fig. 7a), indicating that homologous proteases could indeed be responsible for the extracellular protein degradation. Interestingly, strains in which the *kex2* gene was deleted showed a diminished proteolytic potential (Fig. 7a). Silver staining of proteins isolated from the culture supernatant revealed strong differences in the pattern of AB33kex2Δ compared to AB33 (Fig. 7b). This result is in line with the assumption that in the *kex2*Δ background many secreted proteases are not activated.

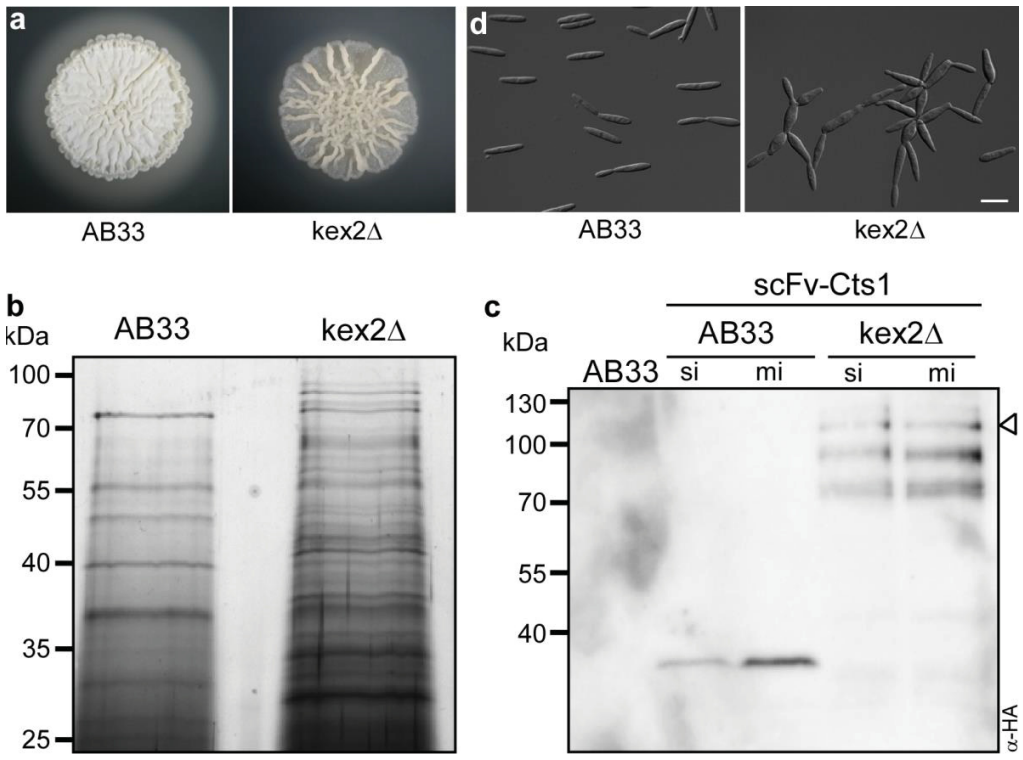

Figure 7. Effects of *kex2* deletion on the proteolytic activity

a) Casein plate assay visualizing proteolytic activity of the strains AB33 and AB33kex2Δ lacking the pre-proprotein convertase Kex2. AB33 cells produce a turbid halo that is generated by the degradation of casein by extracellular proteases. In contrast, AB33kex2Δ cells show a strongly reduced halo size indicating that major proteases are inactive. **b)** Secretome comparison of AB33 and AB33kex2Δ cultures. Secreted proteins were concentrated and analyzed by SDS PAGE and subsequent silver staining. Severe differences are evident throughout the range of detected proteins. **c)** Detection of scFv-Cts1 in culture supernatants. Culture supernatants of AB33 and AB33kex2Δ expressing the α-myc scFv-Cts1 fusion protein were harvested, TCA precipitated and subjected to SDS PAGE and Western blot analysis. Proteins were detected using antibodies directed against an internal HA-tag. Full-length fusion proteins (92.6 kDa; protein runs higher as mentioned in section 2.1) could only be observed in AB33kex2Δ (marked by arrowhead). To judge the effect of the number of gene copies, strains harboring single (si) and multiple insertions (mi) of the corresponding *scFv-cts1* gene in the *ip* locus were used. **d)** Morphology of AB33 and AB33kex2Δ cells during yeast-like growth. Cells of the progenitor strain AB33 multiply by budding. By contrast, AB33kex2Δ cells display a cytokinesis defect leading to the formation of aggregates (scale bar, 10 µm).

To further investigate the role of Kex2-activated proteases in degradation of heterologous proteins, the gene for the scFv-Cts1 fusion protein was inserted in the defined *ip* locus in single or multiple copies resulting in the expression strain AB33kex2Δ/scFv-Cts1. Western blot analyses of precipitated supernatant proteins showed that in the kex2Δ background full-length protein could be detected, whereas only low amounts of smaller degradation products were visible in the control strain (Fig. 7c). Thus, the degradation of heterologous proteins in the culture broth was strongly reduced in the absence of Kex2.

However, it was also evident that deletion strains recovered very slowly after transformation and had an abnormal cell morphology showing a cytokinesis defect (Fig. 7d) with the formation of visible aggregates in the culture broth (data not shown). This reduced strain fitness could constitute a limiting factor in biotechnological applications.

2.3. Deletion of key proteases

The reduced proteolysis in AB33kex2Δ is likely due to the inactivation of several host proteases that attack heterologous proteins in parenteral AB33 strains and thereby reducing their yields. To obtain an expression strain with reduced proteolytic potential but lacking the side-effects observed upon *kex2* deletion, an alternative approach was used that specifically targeted potentially interfering proteases. Deletion of key proteases to reduce such type of proteolytic degradation and hence improve secreted heterologous protein yields has been shown as one of the promising optimizing steps for strain improvement in other systems [Idiris et al., 2006, Yoon et al., 2011]. Unlike many filamentous fungi, *U. maydis* has a relatively low number of predicted secreted proteases which makes it is easier to tackle the proteolytic problem in this organism. Table 1 shows a list of proteases and peptidases predicted to be secreted according to the *Ustilago maydis* genome database (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t237631_Ust_maydi_v2) and the SignalP 4.1 program (http://www.cbs.dtu.dk/services/SignalP/). Along with several proteases, there are many predicted secreted peptidases in *U. maydis* like aminopeptidases, carboxypeptidases and di/tri-peptidylpeptidases (Table 1) which could also play a role in protein degradation [Monod et al., 2002].

Table 1. List of predicted secreted proteases and peptidases in *U. maydis*

No.	*um* number	Chromosome	Bioinformatic annotation	Predicted subcellular location[1] (Reliability class[2])
1	*um00064*	chr 01	related to pepsin precursor (aspartate protease)	secreted (RC 1)
2	*um00411*	chr 01	related to metalloprotease	secreted (RC 1)

3	*um00688*	chr 01	related to bacterial leucyl aminopeptidase precursor	secreted (predicted by homology) (RC 1)
4	*um01886*	chr 03	related to carboxypeptidase	lysosome-like vacuoles (RC 1)
5	*um01888*	chr 03	probable serine-type carboxypeptidase f precursor	secretory pathway (RC 2)
6	*um02178*	chr 05	related to aspartic protease	secreted (RC 2)
7	*um02597*	chr 06	related to extracellular aspartic proteinase	cell membrane (RC 1)
8	*um02711*	chr 06	related to ADAM protease ADM-B	membrane-bound; single-pass type I membrane protein (RC 2)
9	*um03024*	chr 07	related to subtilisin-like serine protease	secretory pathway (RC 1)
10	*um03630*	chr 09	related to metalloprotease MEP1	secreted (predicted by homology) (RC 1)
11	*um03860*	chr 10	related to pepsinogen precursor	secretory pathway (RC 2)
12	*um03947*	chr 11	probable carboxypeptidase 2	cell membrane; lipid-anchor, GPI-anchor (Potential) (RC1)
13	*um03975*	chr 11	related to carboxypeptidase Y precursor	vacuolar (predicted by homology) (RC 3)
14	*um04400*	chr 14	probable PRB1 protease B, vacuolar	secreted (predicted by homology) (RC 1)
15	*um04641*	chr 13	related to PRC1 - carboxypeptidase y, serine-type protease	vacuolar (predicted by homology) (RC 2)
16	*um04926*	chr 15	probable PEP4 - aspartyl protease	lysosome-like vacuoles (Potential) (RC 2)
17	*um05097*	chr 04	related to secreted	secretory pathway (RC 1)

			aspartic protease 2	
18	*um05558*	chr 18	related to ATP-dependent protease La	peroxisomal matrix (predicted by homology) (RC 3)
19	*um05774*	chr 16	related to metalloprotease MEP1	secreted (predicted by homology) (RC 1)
20	*um06098*	chr 21	related to extracellular elastinolytic metalloproteinase precursor	secreted (predicted by homology) (RC 2)
21	*um06118*	chr 21	related to tripeptidyl-peptidase I precursor	secreted, extracellular space (predicted by homology) (RC 2)
22	*um06456*	chr 23	related to aminopeptidase Y precursor, vacuolar	secreted (predicted by homology) (RC 2)
23	*um10493*	chr 10	related to aminopeptidase	secretory pathway (RC 1)
24	*um11398*	chr 05	related to UBP1 - ubiquitin-specific protease	secretory pathway (RC 3)
25	*um11908*	chr 05	related to cathepsin d - lysosomal aspartyl protease	secreted (RC 1)
26	*um15029*	chr 04	related to SPC3 - signal peptidase subunit	secretory pathway (RC 1)

[1]Candidate proteases and peptidases were selected by a PEDANT search and based on TargetP program, their subcellular location was predicted (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t237631_Ust_maydi_v2; date accessed 06/30/2014).

[2] RC (reliability class) values indicate the strongest prediction. This value is a measure of the size of the difference (diff) between the highest and the second highest output scores in TargetP program. There are five reliability classes as defined as follows:

1: diff > 0.800; 2: 0.800 > diff > 0.600; 3: 0.600 > diff > 0.400; 4: 0.400 > diff > 0.200; 5: 0.200 > diff

Thus, lower the RC value, safer the prediction.

Subcellular location of the candidates was verified by SignalP4.1 (http://www.cbs.dtu.dk/services/SignalP/, accessed on 06/30/2014) analyses (candidates with RC values 1 or 2 were shown to be secreted while ones with RC value 3 or above were shown as non-secreted).

To investigate the effect of single key protease deletions, firstly, homologs of the proteases known to be responsible for proteolytic degradation in other established fungal systems like *Aspergillus niger* [van den Hombergh et al., 1997a], *Schizosaccharomyces pombe* [Idiris et al., 2006], *Aspergillus oryzae* [Jin et al., 2007] were selected , namely Pep4 (Um04926), Prb1 (Um04400), and TppA (Um06118) (Table 2).

Table 2. List of three key proteases that are homologs to major proteases known from other fungal expression systems. Proteases are named according to the homologs and the respective homology results are given.

No.	*um* number	Predicted protein classification	Amino acid homology[1]
1	*um04926*	Pep4, aspartic protease	55% to PepE (*A. niger*) 54% to PepE (*A. oryzae*)
2	*um04400*	Prb1, serine protease	43% to PRB1 (*S. cerevisiae*) 38% to Isp6 (*S. pombe*) 31% to AlpA (*A. oryzae*)
3	*um06118*	TppA, tripeptidyl-peptidase A	36% TppA (*A. oryzae*)

[1] Amino acid sequence homology was determined over the full protein length using the global protein alignment function (Scoring Matrix: BLOSUM 62) in the Clone Manager 9.1 program.

Secondly, the respective genes were deleted in AB33 and the AB33 Gus-Cts1 expression strain. Deletion mutants were subsequently analyzed for their proteolytic activity and extracellular Gus activity. A slight reduction in the size of the turbid halo was observed for *pep4* and *prb1* deletion strains, while no effect was observed in strains carrying a *tppA* deletion (Fig. 8a).

A fluorimetric assay to monitor Gus activity of the secreted Gus-Cts1 fusion protein was performed using supernatants of yeast-like and filamentous cultures. While no activity differences were observed for samples from yeast-like cells (Fig. 8b, upper panel), an increase of about 26 % in Gus activity was noted for supernatant samples of filamentous growing cells lacking *pep4* (Fig. 8b, lower panel). However, no full-length Gus-Cts1 fusion protein could be detected in yeast-like supernatant samples of either of the protease deletion strains, indicating a strong protease activity still present in the expression strains. Hence, single deletions neither lead to a drastic reduction in proteolytic activity nor to any improvement of protein stability.

Thus, to mimic the effect obtained by *kex2* deletion, it seemed likely necessary to delete multiple protease genes in a single expression strain.

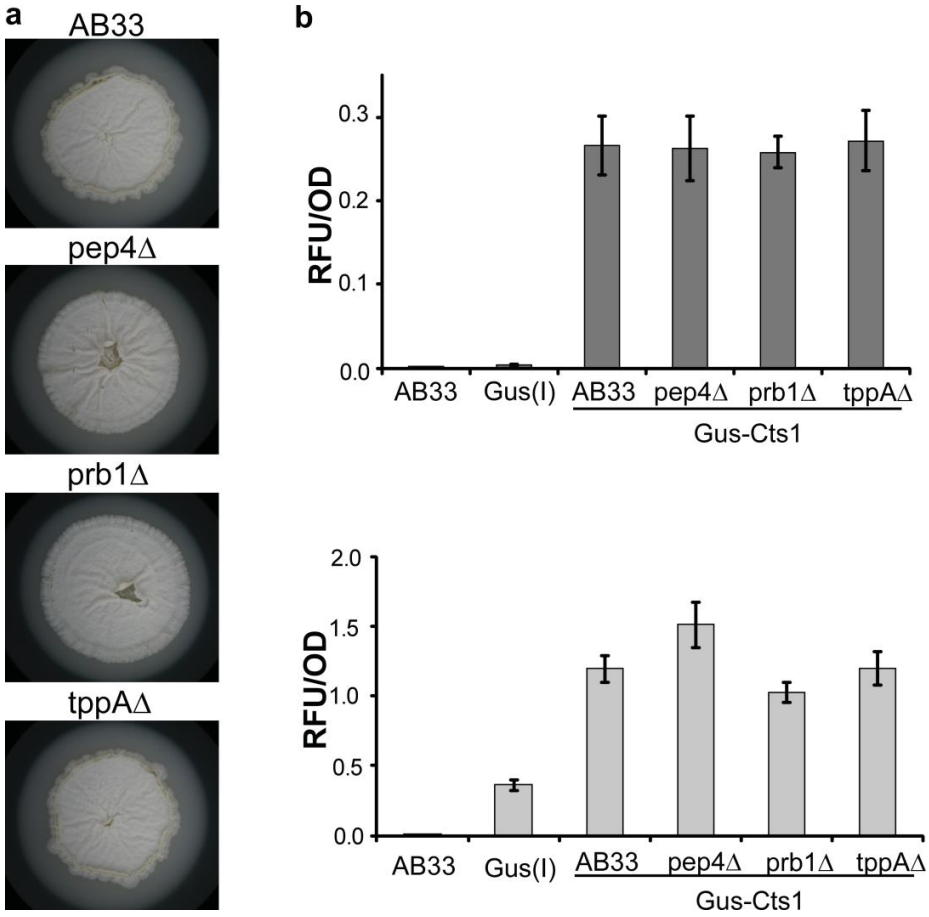

Figure 8. Effect of single protease deletions on the proteolytic activity

a) Casein plate assay visualizing proteolytic activity of the protease deletions (AB33, AB33pep4Δ, AB33prb1Δ, AB33tppAΔ). Cells of the progenitor strain AB33 produce a turbid halo. This halo is reduced to some extent in pep4Δ and prb1Δ strains as compared to the control strain AB33, while no effect was seen in the tppAΔ strain. **b)** Fluorimetric Gus activity determined in cell-free culture supernatants of the indicated AB33 and protease deletion strains grown in the yeast (upper panel) and filamentous form (lower panel). 4-methylumbelliferyl β-D-galactopyranoside (MUG) was used as a substrate. The graphs show mean values of three biological replicates (error bars represent standard deviation). While no significant difference was seen for yeast culture supernatants, an increase in activity of around 26% was observed for the pep4Δ strain in filamentous culture supernatants.

2.4. Sequential elimination of proteases to generate a multiple-protease deficient strain

To investigate the proteolytic potential of a multiple-protease deficient strain, five different proteases belonging to aspartic and serine protease families were sequentially deleted in the AB33 background (AB33P1Δ to AB33P5Δ; Fig. 9a). This time, the candidates were chosen based on two criteria, firstly by a detailed literature survey, identifying *U. maydis* homologs to

proteases known from other fungal expression systems and secondly, by the presence of putative Kex2 cleavage sites in the candidate proteases (Table 3).

Table 3. List of putative *U. maydis* proteases disrupted in the quintuple deletion strain (Fig. 9a). Proteases were named according to their homologs in other fungi. For those displaying low amino acid identities (< 40%) to described enzymes the term Upp (Ustilago predicted protease) was used.

No.	Protein	*um* number	Description according to bioinformatic predictions[1]	Predictied putative Kex2 sites[2]
1	Pep4	*um04926*	aspartic protease, related to PepE from *A. niger* [55% identity] (vacuolar), related to PepE from *A. oryzae* [54% identity]	-
2	Upp1	*um02178*	aspartic protease, related to *Saccharomyces cerevisiae* YPS3 [26% identity] (plasma membrane via GPI anchor)	2
3	Upp2	*um00064*	aspartic protease, related to *S. cerevisiae* PEP4 (proteinase A) [24% identity] (vacuolar), YPS3 and YAP3 [both 26% identity] (vacuolar or plasma membrane via GPI anchor)	3
4	Upp3	*um11908*	aspartic protease, related to *S. cerevisiae* PEP4 (proteinase A) [31% identity] (vacuolar) and YAP3 [27% identity] (vacuolar or plasma membrane via GPI anchor)	3
5	Prb1	*um04400*	serine protease, related to *S. cerevisiae* PRB1 [43% identity] (vacuolar), related to Isp6 from *S. pombe* [38%], related to AlpA from *A. oryzae* [31%]	-

[1]Amino acid sequence homology was determined over the full protein length using the global protein alignment function (Scoring Matrix: BLOSUM 62) in the Clone Manager 9.1 program.
[2] Predictions of putative Kex2 sites was based on the known property of this enzyme to perform endoproteolytic cleavage at the carboxyl side of a pair of basic residues, especially Lys-Arg [Bader et al., 2008, Fuller et al., 1991].

Selecting candidates with Kex2 sites was based on the idea that deletion of these candidates could potentially result in a similar reduction of proteolytic activity as observed upon *kex2* deletion. By contrast, any harmful phenotypic changes observed with *kex2* deletion

might be absent in such a protease deletion strain. *pep4* and *prb1* were both included in these sequential deletions based on their results of the single deletions (see 2.3).

Gene deletion vectors were again generated using the Golden Gate cloning strategy and gene deletion was carried out as previously described [Terfrüchte et al., 2014]. Since only a limited set of resistance markers exists in *U. maydis*, sequential deletions were carried out in an expression strain using a resistance-marker recycling method adapted to Golden Gate cloning [Khrunyk et al., 2010, Sarkari et al., 2014]. This marker recycling strategy is based on a property of the yeast flippase recombinase (FLP) to recognize and catalyze recombination between specific 34 bp sequences called FRT sites resulting in cleaving off the intervening DNA sequence and leaving one recombination site behind [Andrews et al., 1985]. Storage vectors are designed in a way that the resistance cassettes are surrounded by specific FRT sequences which are then introduced in the deletion construct (Fig. 9b). In this sequential protease deletion strategy, a hygromycin-resistance cassette (HygR) was employed. The resulting gene deletion strain is transformed with a free replicating FLP expression plasmid. Induction of FLP expression leads to removal of the HygR between the FRT sites leaving the strain marker-free (Fig. 9b). Hence, the same selectable marker can now be used for subsequent rounds of gene manipulation. Subsequently, the marker-free strain is grown in complete medium without antibiotics to eliminate the FLP expression plasmid and making the strain available for the next gene deletion round. Importantly, variations in the FRT core region prevent recombination between FRT scars that remain in the genome after each excision event [Khrunyk et al., 2010]. Excision events were detected using diagnostic PCR (Fig. 9c). In the future, the steps of gene deletion and FLP induction can also be combined by generating a plasmid that contains both inducible FLP and the resistance cassette flanked by FRT sites.

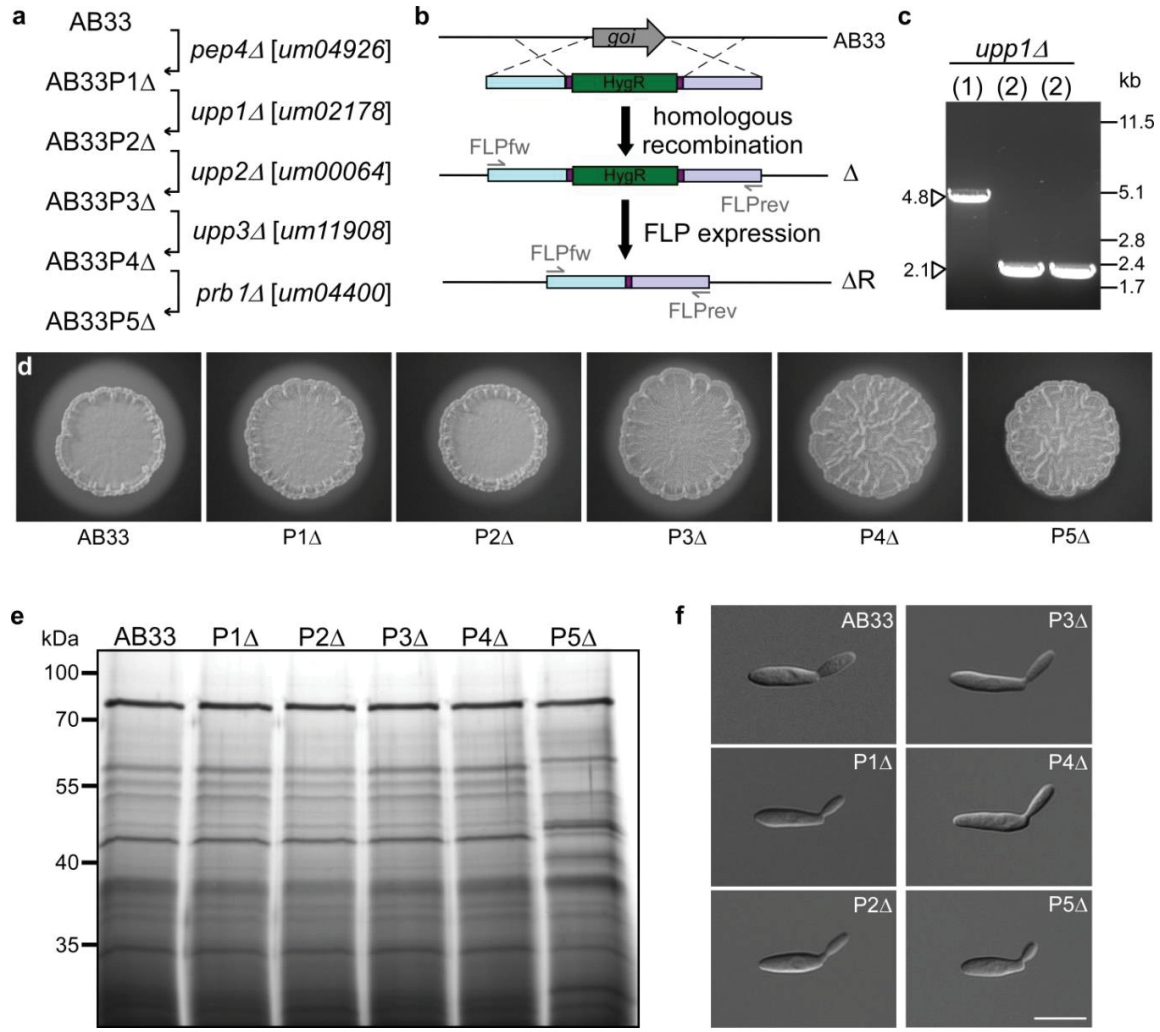

Figure 9. Effect of quintuple protease deletion on the proteolytic activity

a) Scheme depicting the flowchart of sequential protease deletions and the corresponding strain names. If applicable, proteases were named after homologous enzymes (Table 3). Proteases with low homologies to known proteins were termed Upp (Ustilago predicted protease). Provided *um*-gene numbers correspond to the PEDANT database nomenclature (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t237631_Ust_maydi_v2; accession on 06/30/2014). **b)** Scheme of FLP-mediated resistance-marker recycling. A protease-encoding gene (hatched arrow) is deleted using homologous recombination (flanking regions, blue and purple boxes); thereby replacing the gene with a HygR flanked by FRT recombination sites (maroon boxes). In the next step, the HygR cassette is excised using the recombinase FLP expressed from a free-replicating plasmid. Excision events were analyzed using primers binding in the flanking regions (FRTfw/FRThyg) with a large product after initial gene replacement (Δ) and a small product after subsequent cassette removal (ΔR). **c)** Agarose gel depicting PCR products obtained prior to and after HygR-cassette removal with the primer pair FLPfw and FLPrev for the flanking regions of gene *upp1*. A 4.8 kb product is obtained after replacing the original gene by the HygR-FRT cassette (lane 1). Subsequent excision of the cassette by FLP leads to detection of 2.1 kb products (lane 2). **d)** Casein plate assay depicting the proteolytic activity of the sequential protease deletions (AB33P1Δ-P5Δ). Cells of the progenitor strain AB33 produce a turbid halo which is diminished in AB33P5Δ lacking five proteases (see Table 2). **e)** Secretome comparisons of AB33 and multiple protease deletion strains. Secreted proteins were

concentrated using Amicon 30k tubes and analyzed by SDS PAGE and subsequent silver staining. Differences are evident after the deletion of five proteases. **f)** Representative microscopic images visualizing the morphology of AB33 and the mutants AB33P1Δ to AB33P5Δ in which up to five protease genes have been sequentially deleted. All mutants show normal cell morphology and yeast-like budding comparable to the progenitor strain AB33. Scale bar, 10 μm.

To judge the proteolytic potential of the different protease deletion strains (AB33P1Δ to AB33P5Δ), casein plate assays were performed (Fig. 9d). A slight reduction in halo size was obtained after deletion of the first protease gene, *pep4* (AB33P1Δ) as expected from previous results given in section 2.3. No significant change in the halo was observed after the deletion of the next three aspartic proteases. However, in the quintuple mutant (AB33P5Δ) in which a serine protease was eliminated the halo could no longer be observed (Fig. 9d). This suggests that the protease activity in the culture broth of the quintuple deletion strain is strongly reduced as comparable to AB33kex2Δ. In line with this observation, silver staining of secreted proteins in supernatants of yeast-like culture confirmed a distinctive secretome change along with the fifth protease deletion (Fig. 9e). Importantly, the morphology of yeast-like growing cells of all the mutants was not altered (Fig. 9f). These results indicate that the quintuple protease deletion strain resembles the *kex2* deletion strain with respect to the drastic reduction in the proteolytic potential while lacking the negative side-effects observed before in *kex2Δ* mutants.

2.5. Optimizing the system at the transcriptional level

Choosing the right promoter for the transcription of a target gene is crucial for successful recombinant expression [Berlec and Štrukelj 2013]. Thus, enhancing the promoter strength also became one of the steps to optimize this expression system. This should lead to an increase in the amount of transcripts and thus, result in enhanced protein synthesis. Until now, heterologous genes were expressed using the synthetic promoter P_{otef}. This promoter is made up of two direct repeats of a synthetic fragment (with seven copies of tetracycline-responsive elements each) fused to the transcription elongation factor 2 promoter [Spellig et al., 1996]. Promoter studies using Gus as a reporter were carried out and one promising candidate which led to strongly enhanced protein secretion was found i.e. the synthetic promoter P_{oma} [Michèle Reindl, Bachelorarbeit 2013]. This promoter is known to show high activity in axenic culture [Flor-Parra et al., 2006, Hartmann et al., 1999]. It had been artificially assembled from a minimal promoter of the *mfa1* gene encoding the small lipopeptide pheromone Mfa1, and

eight repetitive 40 bp upstream activating elements (UAS) taken from the promoter of the pheromone response factor Prf1, a central pathogenicity regulator (Fig. 10a) [Flor-Parra et al., 2006, Hartmann et al., 1999].

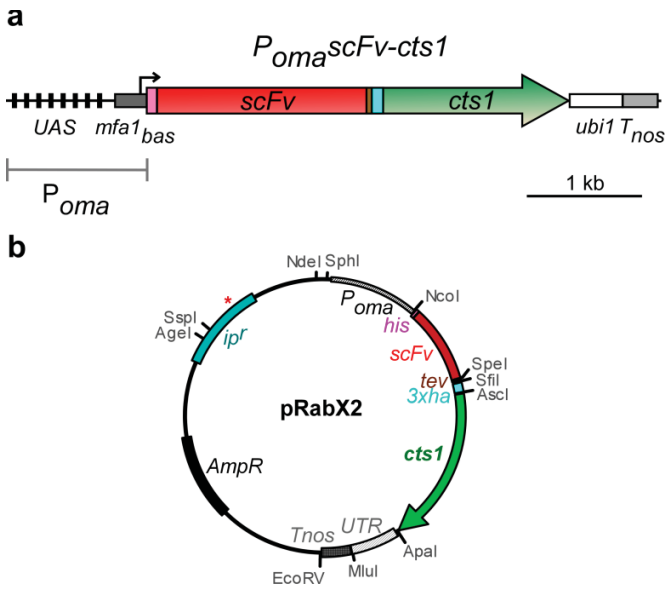

Figure 10. Architecture of the synthetic P_{oma} promoter and the corresponding optimized expression vector

a) Scheme of the fusion gene *scFv-cts1* controlled by P_{oma} [Hartmann et al., 1999]. P_{oma} consists of eight UAS fused to the minimal *mfa1* promoter ($mfa1_{bas}$). The bent arrow indicates the transcription initiation site. Repetitive UAS elements are indicated as filled boxes. Sequences encoding purification tags (Strep/10xHis, shown in pink) are placed on the 5´-terminal end of the gene of interest (here depicted is *scFv)* while the HA tag to be used for detection of protein is retained between the *goi* and *cts1* (shown in blue). A sequence for a TEV protease cleavage site is shown in brown. The 3´ UTR of *ubi1* and the terminator T_{nos} are located downstream of the fusion gene. **b)** Scheme of the optimized expression vector pRabX2 encoding scFv-Cts1. The position of purification tags (Strep/10xHis/3xHA; as described in **a**) were modified in the vector pRabX2 to facilitate purification and detection of the fusion protein. A sequence for a TEV protease cleavage site was introduced to facilitate the removal of the HA-Cts1 part from the purified scFv protein using the TEV protease.

Using P_{oma}, enhanced activity of the Gus enzyme was observed in both plate assay and fluorimetric liquid assay using yeast-like culture supernatants, with about 30-fold increase in the Gus activity. Quantification of the protein yields indicated a titer of about 35-40 µg/l of Gus-Cst1 in the culture supernatant of AB33 P_{oma}Gus-Cts1 (OD_{600} about 0.7) [Michèle Reindl, Bachelorarbeit 2013].

To exploit this result for optimizing scFv-Cts1 expression, the *scFv-cts1* fusion gene was inserted into a novel expression vector, pRabX2 (Fig. 10b) [Marius Terfrüchte, Masterarbeit 2013]. In this vector, the expression cassette was modified such that sequences encoding purification tags (Strep, 10xHis) were placed on the 5´-terminal end of the gene of

interest while the HA tag to be used for detection of protein was retained between the two genes. This strategy was adopted to facilitate purification of the secreted fusion proteins. Furthermore, a sequence for a TEV protease cleavage site was introduced upstream of the HA-tag, which would facilitate the removal of the HA-Cts1 part from the purified scFv protein using the TEV protease (Fig. 10b) [Marius Terfrüchte, Masterarbeit 2013]. The corresponding scFv-Cts1 expression construct was then introduced at the *ip*^{s} locus of the strains AB33 and AB33kex2Δ.

2.6. Combining the optimizing steps to generate a novel expression strain

To analyze the putative combinatorial effects of the reduced proteolytic potential and enhanced transcription strength, the strains AB33, AB33kex2Δ and AB33P5Δ harboring scFv-Cts1 expression constructs under the control of P_{oma} were compared. Firstly, scFv-Cts1 expression was analyzed in cell extracts of the different strains (Fig. 11a). Western blot analysis shows that the use of the promoter P_{oma} strongly increases the amounts of intracellular scFv-Cts1 while protease-deficiency did not influence the amount of detected protein. By contrast, cell-free supernatants of identical strains grown as yeast-like cells indicated a strong influence of the proteolytic potential on the amount of full-length fusion protein, as a clear full-length band could be detected only in the strains with reduced proteolytic activity. In line with the earlier results, expression with P_{oma} led to a further increase in protein yields (Fig. 11b).

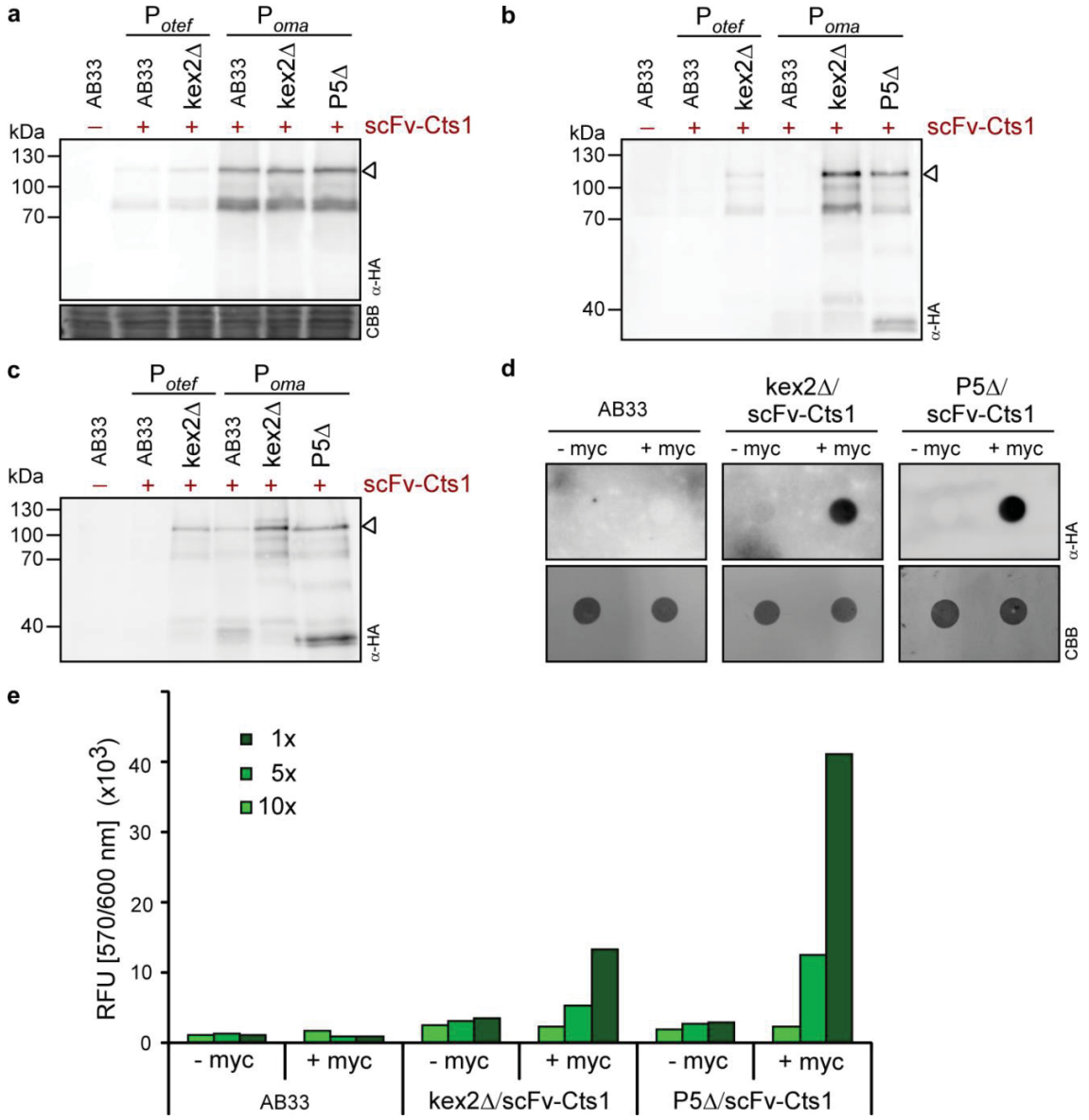

Figure 11. Purification of scFv-Cts1 from optimized expression strains

a) Comparative Western blot analysis of scFv-Cts1 expression in cell extracts of the indicated AB33 derivatives. scFv-Cts1 was detected using antibodies directed against an internal HA-tag. Arrowhead depicts the full-length fusion protein scFv-Cts1 which runs slightly higher than the expected size of 90.4 kDa. Use of P_{oma} for gene expression leads to an increase in protein amounts for all tested strains. **b)** Comparative Western blot analysis of scFv-Cts1 (arrowhead) secretion in the culture supernatant. 2 ml each of cell-free supernatant were used for the analysis. Cells lacking proteases show higher amounts of stable scFv-Cts1 in the supernatant while the parallel use of P_{oma} increases the protein amounts. **c)** Affinity purification of scFv-Cts1 (arrowhead) from cell-free culture supernatants of the indicated AB33 derivatives. IMAC was used to enrich the proteins via an N-terminal His tag. Strains lacking proteases and harboring P_{oma} for expression of scFv-Cts1 display best yields. **d)** Dot blot assay to detect the binding of α-myc scFv-Cts1 fusion proteins to the myc epitope. The assay visualize that the α-myc scFv-Cts1 fusion protein secreted by the protease-deficient strains AB33kex2Δ and AB33P5Δ are able to bind the myc-epitope. To visualize binding, glutathione S-transferase (Gst) fusion proteins Gst-MH and Gst-H

with and without the cMyc epitope (+ and – myc), respectively, were spotted onto a PVDF membrane (lower panels; CBB, Commassie Brilliant Blue staining). The membranes were then incubated with IMAC-purified proteins of the indicated strains and developed using primary mouse antibodies directed against an internal HA-tag and a secondary α-mouse HRP conjugate. scFv-Cts1 purified from the protease-deficient strains AB33kex2Δ/scFv-Cts1 and AB33P5Δ/scFv-Cts1was able to specifically bind the myc-tag. **e)** IMAC purified proteins of strains growing in the yeast form were tested in varying concentrations (1x/5x/10x) by indirect ELISA against Gst-MH and Gst-H with and without the cMyc epitope (+ and – myc), respectively. Active α-myc scFv-Cts1 fusion protein could be enriched from the protease deficient strains. Purified protein from AB33 lacking scFv-Cts1 was used as negative control. The assay has been performed in triplicates with similar results and a representative diagram of one assay is shown.

Next, the scFv-Cts1 fusion proteins were purified from culture supernatants using affinity chromatography via the N-terminal 10xHis tag. Full length protein was obtained from all protease-deficient strains. However, it was evident that in combination with the P_{oma} promoter a better yield was obtained (Fig. 11c). Thus, the secreted full-length scFv-Cts1 protein could be purified for the first time. To test whether purified scFv-Cts1 is active, dot blot assays were performed (Fig. 11d). In these assays, purified Glutathione-S-transferase tagged with a 6xHis-Myc tag (Gst-MH) was used as an antigen. Gst-H harboring only 6xHis tag served as negative control. Defined amounts of both proteins were spotted onto PVDF membranes and incubated with IMAC-purified proteins from scFv-Cts1 expression strains in kex2Δ and P5Δ background. Purified proteins from the progenitor strain AB33 were employed as a negative control. The assay confirmed that α-myc scFv-Cts1 purified from these protease-deficient strains AB33kex2Δ and AB33P5Δ is able to bind to Gst-MH. In contrast, no binding could be observed for proteins purified from AB33.

The activity of the scFvs-Cts1 fusion proteins obtained from protease-deficient strains was corroborated by indirect ELISA, again using Gst-MH as an antigen (Fig. 11e). A concentration-dependent binding of the antibody fragments to the epitope was detected, confirming the secretion of active protein. Interestingly, scFv-Cts1 purified from the quintuple protease deletion strain displayed enhanced activity compared to protein obtained from the *kex2* deletion strain. This indicates that the quintuple protease deletion strain is a promising candidate for the expression of antibody formats and other protein biopharmaceuticals. Therefore, the combination of optimization steps at the transcriptional and post-translational level led to a strong improvement of unconventional scFv secretion in *U. maydis*.

2.7. Expression of other antibody formats

2.7.1. Generation of a camelid-derived nanobody against Gfp

Another interesting type of antibody fragment called as nanobody comprises of only a variable heavy chain (V_HH) domain derived from camelid antibodies [Muyldermans 2001] (Fig. 4c). These molecules of about 15 kDa represent the smallest intact antigen-binding fragments [Joosten et al., 2003]. In this work, a nanobody against the green fluorescent protein Gfp (α-Gfp Nb) derived from a llama antibody [Rothbauer et al., 2006] was chosen for expression in *U. maydis*. To this end, a codon-optimized synthetic α-Gfp Nb gene (Fig. 12a, Section 2.1) was introduced into the expression vector pRabX2 harbouring P_{oma} and the corresponding expression strains were generated in the AB33 and AB33kex2Δ background.

Western blot analysis using whole cell extracts of yeast-like growing cells confirmed the expression of similar amounts of the α-GfpNb-Cts1 fusion protein in both types of strains (Fig. 12b, left panel), with the fusion proteins migrating slightly above the expected size of 75.8 kDa. The full-length fusion protein was furthermore detected in cell-free culture supernatants of yeast-like growing cells with higher yields obtained in the *kex2*Δ background, confirming the reduced proteolytic degradation observed for scFv expression strains before (Fig. 12b, right panel). However, bands of lower molecular weights were also detected, still indicating residual proteolytic activity (Fig. 12b; marked by asterisks).

Next, the α-Gfp Nb-Cts1 fusion protein was purified from yeast-like cell extracts of the strain AB33kex2Δ_P_{oma}α-GfpNb-Cts1 by affinity chromatography via an N-terminal His tag. The fusion protein could be obtained in full-length in the eluate fractions (Fig. 12c) and was thus further used for antigen-binding assays. To this end, purified proteins were tested for Gfp binding activity using a modified Western blot protocol and ELISA. In Western blotting, cell extracts of *U. maydis* strain expressing a triple Gfp-fusion (3xGfp) were used, in which the present Gfp acts as an antigen while the cell extracts of AB33 (C) served as an antigen control (Fig. 12c). Defined protein amounts of cell extracts were separated on SDS gels and transferred onto a PVDF membrane. After blotting, IMAC-purified α-GfpNb-Cts1 protein was used as primary antibody to bind to the Gfp antigen. The Gfp:α-GfpNb-Cts1 complex was then detected using mouse α-HA antibody followed by an α-mouse antibody targeting an internal HA tag of the fusion protein. Bands were detected at three different molecular

weights of around 27 kDa, 54 kDa, and 81 kDa, indicating not only the presence of 3xGfp, but also of degradation products consisting of 1xGfp and 2xGfp (Fig. 12d). In contrast, no specific signal was observed for protein purified from AB33 cell extracts which were used as a negative control. Hence, the assay confirmed the binding of purified α-Gfp Nb-Cts1 to Gfp demonstrating the expression of functional nanobodies in *U. maydis*.

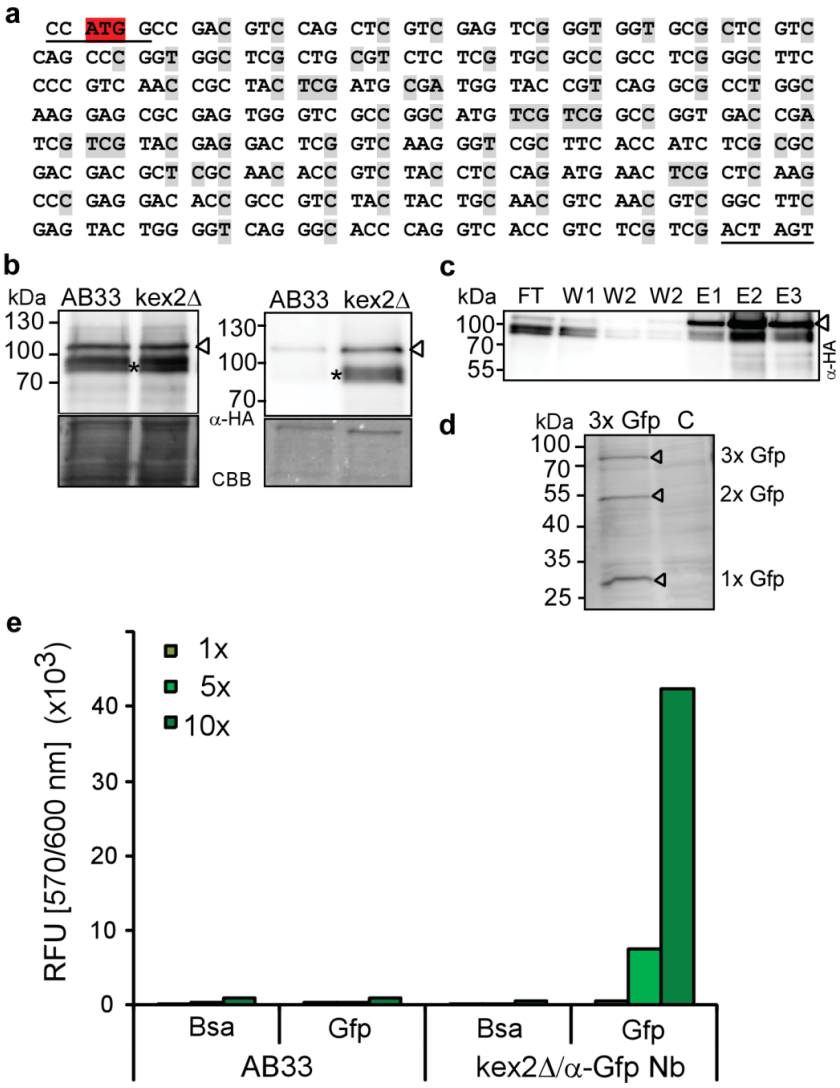

Figure 12. Expression of a camelid derived nanobody

a) DNA sequence of a synthetic V_HH or nanobody directed against Gfp which was adapted to the context-dependent codon usage of *U. maydis*. Bases that were changed are shaded and mostly locate to the wobble position. Restriction sites (NcoI, SpeI) that were introduced for cloning purposes are underlined. The translational start codon ATG is shown in red. **b)** Western blots depicting the expression of the α-Gfp Nb-Cts1 fusion protein. 10 µg of whole cell protein extracts of the AB33/α-Gfp Nb-Cts1 and AB33kex2Δ/α-Gfp Nb-Cts1 expression strains (left panel) and 2 ml cell-free culture supernatants of yeast-cell cultures that were enriched by TCA precipitation to detect the secreted proteins (right panel) were used. Proteins were subjected to SDS PAGE

and analyzed by Western blotting using α-HA antibodies. Coomassie Brilliant Blue staining shows equal loading of proteins. The fusion protein migrates slightly above the expected size of 75.8 kDa. Protein degradation is indicated by asterisks. **c)** Affinity purification of α-Gfp Nb-Cts1 (arrowhead) from whole cell extracts of AB33kex2Δ/α-Gfp Nb-Cts1 expression strain. IMAC was used to enrich the proteins via an N-terminal His tag. Different fractions obtained during purification were analyzed by Western blotting using α-HA antibodies. Eluate fractions (E1-E3) showed an enrichment of the fusion protein and were therefore employed for activity analysis. **d)** Western blotting to analyze the activity of α-Gfp Nb. Whole protein extracts (10 µg) of yeast-like *U. maydis* cells expressing 3xGfp (AB33/3xGfp) was used as an antigen sample (3xGfp) and similar amounts of the progenitor strain AB33 were used as antigen control (C). IMAC purified proteins as explained in **c** were used as primary antibody to detect 3xGfp. Along with the detection of 3xGfp at 81 kDa, degraded proteins most likely corresponding to 2xGfp at 54 kDa and 1xGfp at 27 kDa (marked with arrowheads) were also detected, indicating successful binding of the α-Gfp Nb-Cts1 to all Gfp variants. **e)** IMAC purified proteins from cell-free supernatants of the AB33kex2Δ/α-Gfp Nb-Cts1 strain growing in the yeast form were tested in varying concentrations (1x/5x/10x) by indirect ELISA against His-1xGfp and Bsa (+ and – antigen, respectively). The α-Gfp Nb-Cts1 fusion protein showed activity while purified protein from the progenitor strain AB33 only led to very low background signals (negative control).

Finally, α-Gfp Nb-Cts1 fusion protein was purified from yeast-like culture supernatants of the AB33kex2Δ expression strain using affinity chromatography via the N-terminal 10xHis tag. The activity of the α-Gfp Nb-Cts1 fusion proteins obtained from this IMAC purification was tested by indirect ELISA (Fig. 12e). Here, purified 1xGfp tagged with a 6xHis tag (Gfp-H) was used as an antigen and bovine serum albumin (Bsa) was employed as a negative control. A concentration-dependent binding of the antibody fragments to the Gfp-H epitope was detected which confirmed the secretion of active α-Gfp Nb.

In summary, the successful production of a camelid-derived nanobody was demonstrated using unconventional Cts1-mediated secretion in optimized *U. maydis* expression strains.

2.7.2. Generation of an α-myc diabody

Another antibody fragment which was tested for its Cts1-mediated secretion is an α-myc diabody (section 2.1) derived from the α-myc scFv. Diabodies are dimers of single-chain variable fragments showing much improved affinity for the target antigen owing to the presence of two antigen binding sites [Holliger et al., 1993]. In this type of antibody format, the heavy chain variable domain (V_H) of one polypeptide chain is non-covalently associated to a light-chain variable domain (V_L) of another polypeptide chain (V_H-V_L), leading to formation of a dimeric molecule. Dimerization occurs if the linker between the V_H and V_L domain is shortened in comparison to the linker used in scFvs to prevent intrachain domain

pairing. The domains are then forced to pair with the complementary domains of another chain resulting in a dimer with two antigen-binding sites [Holliger et al., 1993].

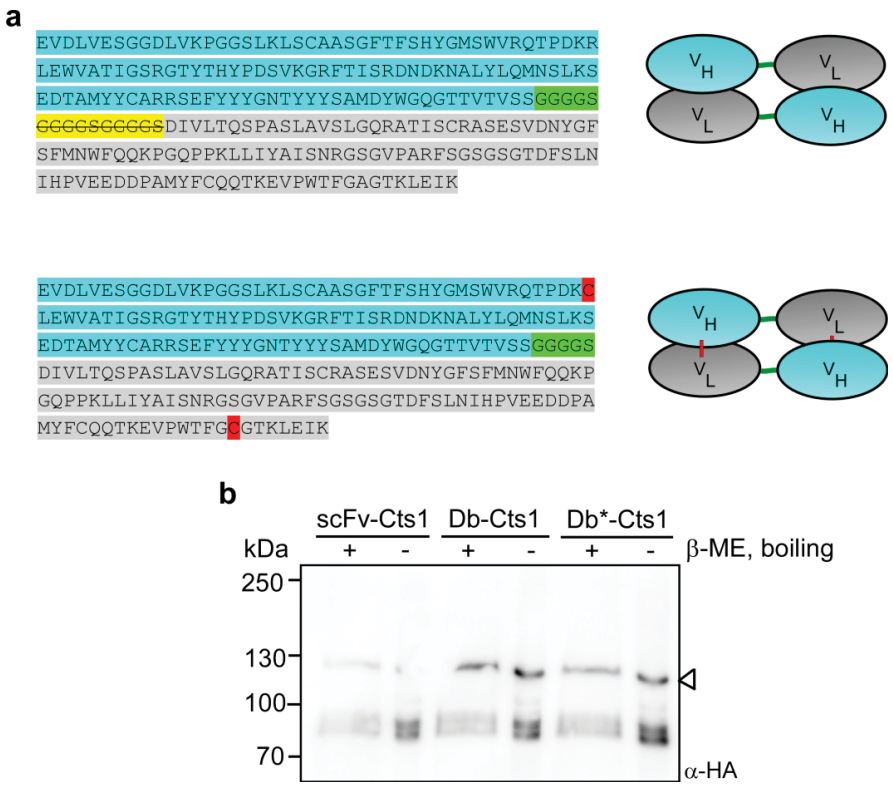

Figure 13. Expression of an α-myc diabody

a) Amino acid sequence of expressed α-myc diabody (Db) formats. In this variable fragment (Fv) molecule (upper panel, right scheme) the peptide linker is reduced from 15 to 5 amino acids (marked in green, amino acids in yellow are removed) compared to the corresponding scFv. This results in the association of the variable domains (V_H marked in blue; V_L marked in grey) of one molecule with the respective variable domains of another molecule yielding a protein dimer (diabody). In the di-sulfide stabilized diabody (lower planel), two amino acid residues, one in each variable domain, were replaced by cysteines (red bars; right scheme). This should lead to the formation of di-sulfide bonds between two scFv molecules resulting in a strongly associated and more stable diabody molecule. **b)** Western blot depicting expression of the α-myc diabody and its di-sulfide bridged format as Cts1 fusions (Db-Cts1 and Db*-Cts1, respectively) in the AB33 background in comparison to the α-myc scFv (scFv-Cts1) format. Proteins were subjected to reducing (+ β-ME, boiling) and non-reducing conditions (− β-ME, no boiling) to visualize dimer formation. The monomeric scFv-Cts1 fusion protein of 91 kDa migrates at a higher molecular weight level (as observed earlier). The dimeric form of this molecule was expected to migrate at a size of at least 182 kDa. However, only the monomeric version along with some degradation bands was detected using α-HA antibodies.

To this end, the linker sequence present in the α-myc scFv molecule (section 2.1) was reduced from 15 to 5 amino acid residues which should in principle result in formation of a dimer and thus, yield a diabody instead of an scFv (Fig. 13a, upper planel). To improve the association between the two chains, cysteine mutations were introduced at specific amino acid

positions in V_H and V_L domains in a second protein variant, theoretically leading to the formation of a stable covalent association between the domains by di-sulfide bridges and hence preventing the dissociation of the dimer (Fig. 13a, lower panel) [FitzGerald et al., 1997]. For generation of the two corresponding α-myc diabody expression strains, the respective genes were introduced into the pRabX1 expression vector and expression strains were generated in the AB33 and AB33kex2Δ background as described above (Section 2.1).

To investigate the expression and formation of diabody molecules Western blot analysis using whole cell extracts of yeast-like growing cells was performed. Firstly, to check the expression of the monomeric scFv-Cts1 fusion protein, denatured samples were subjected to SDS PAGE and subsequently, Western blot analysis was performed using α-HA antibodies. For investigating the successful formation of dimer between two fusion protein molecules, samples were treated in a different way so that the proteins are non-denatured. In this case, neither the cell extract samples were treated with β-mercaptoethanol nor were the samples boiled so as to retain the association between two monomers. These non-reduced samples were subjected to SDS PAGE and analyzed in a similar way as above to detect the dimer form. Successful expression of the monomeric forms was observed in the reduced samples, although the protein runs little higher than the expected size of 91 kDa (Fig. 13b). However, in the non-reducing samples no dimeric form of the protein at the expected size of 182 kDa could be detected (Fig. 13b). This result indicates that although the monomeric form of the fusion protein is successfully expressed, dimers were not formed. In line with that, due to the absence of the dimeric form, the activity of the diabody could not be detected. Therefore, the production of active diabodies could not be demonstrated in this study.

3. Discussion

This doctoral work deals with the optimization and application of a novel protein expression system in the corn smut fungus *U. maydis* which is based on the unconventional secretion of the endochitinase Cts1. This simple eukaryotic expression host has various advantages to offer which include easy and inexpensive culture conditions and the export of proteins directly into the culture medium allowing simple downstream processing. The unconventional pathway furthermore enables the production of high molecular weight proteins without N-glycosylation as well as proteins requiring disulfide bridges [Feldbrügge et al., 2013].

However, some shortcomings were observed with this expression host and therefore it became imperative to solve these problems so that this fungal system can achieve protein yields comparable to other established expression systems. In particular, the problem of protein degradation was tackled in this work which led to a significant improvement in the yields of secreted heterologous proteins. Furthermore, different active antibody formats were successfully produced in this system. In the end, an optimized expression strain was generated which shows the potential for the production of various protein biopharmaceuticals and also proteins for industrial or research purposes.

3.1. Tackling the problem of proteolytic degradation in *U. maydis*

One key problem observed during the initial studies was the degradation of heterologous proteins which led to low yields and failure to purify proteins from the supernatant [Stock et al., 2012]. A major factor known to contribute to this problem is fungal proteases which are responsible for protein degradation and thereby reduce the heterologous protein yields [Idiris et al., 2010, van den Hombergh et al., 1997b]. In nature, the large number of proteolytic enzymes present in fungi plays an important role in their nutrition and virulence [Monod et al., 2002]. However, in axenic culture there is often no need for such a broad repertoire of proteases as these nutritional bottlenecks can be solved by growth of cells in nutrient-rich medium. Hence, elimination of proteases can be fruitful for improving an expression host. Therefore, the reduction of proteolytic degradation was one of the key optimizing steps undertaken in this work to optimize the expression system.

Several strategies have been described to solve the problem of proteolysis. Traditionally, one of the methods to reduce the proteolytic degradation was to carry out the downstream

processing at lower temperature aided by the use of protease inhibitors. This led to a reduction in the rate of proteolysis and hence prevented further degradation at later stages [Enfors 1992]. At the bioprocess level, altering the culture conditions can also partially reduce the activity of proteases. During growth of fungal cells, the pH of the medium becomes acidic and it is known that aspartic or acid proteases are expressed at these low pH levels [Denison 2000]. Thus, maintaining the pH of the culture medium at 6 to 7 can help in reducing the activity of extracellular proteases which in turn, can improve the yields of secreted protein [O'Donnell et al., 2001]. Optimizing the medium components for example by using competitive substrates for proteases like peptone or casamino acids can also help in reducing the proteolysis in shake-flask cultures [Jahic et al., 2003]. However, as these strategies are effective only to some level, the need for more permanent solutions is imperative in the long run.

Gene silencing approaches using antisense constructs have been tested in some filamentous fungi and this has led to reduced protease activities [Moralejo et al., 2002, Zheng et al., 1998]. Although this is a powerful approach in cases where deletion of the respective gene may be lethal for the host, gene expression is not reduced to zero levels indicating the limited efficiency of this method [Meyer 2008]. Complete abolishment of the expression of harmful proteases by deletion of the respective genes thus seems to be the best strategy to tackle this problem. Several groups have reported that the deletion of key proteases in different fungi led to an improvement in heterologous protein expression. Some examples of such fungal systems are *A. niger* [van den Hombergh et al., 1997a], *S. pombe* [Idiris et al., 2010], and *A. oryzae* [Jin et al., 2007]. Along with secreted proteases, proteases found in different organelles of the endomembrane system like in vacuoles and organelles involved in the secretory pathway can also play a role in proteolytic degradation and hence, elimination of these intracellular proteins can also be useful [van den Hombergh et al., 1997a].

The protease Kexin2 (Kex2) is a membrane bound serine protease of the subtilisin superfamily known to reside in the late Golgi compartment [Mizuno et al., 1988, Mizuno et al., 1989]. It is responsible for the activation of several secreted proteins by cleaving off N-terminal extensions (pro-peptides) to generate active proteins [Fuller et al., 1991]. This enzyme was first identified during studies of processing of the yeast pheromone α-factor

required for mating, and the killer toxin needed for infection [Julius et al., 1984]. Because of the localization of the Kex2 protein in the late trans Golgi network and an endocytic, pre-vacuolar compartment [Abazeed et al., 2005, Redding et al., 1991], it can be assumed that the target spectrum of this protease is limited to proteins attached to the cell surface, those proteins which are secreted into the environment or to the luminal domains of integral membrane proteins passing through these compartments [Bader et al., 2008]. Therefore, it can also be speculated that this protein could be involved in the activation of several secreted proteases by removing their pro-peptides. For example, the disruption of a *kex2* homolog in *P. pastoris* prevented proteolysis of mammalian gelatin protein at a basic amino acid (Arg) residue and this led to an improvement in protein production [Werten and de Wolf 2005]. In line with these observations, eliminating a homologous protein (Um02843/Kex2) also resulted in a diminished proteolytic activity in *U. maydis* as observed in casein plate assays. Furthermore, the stability of secreted scFv-Cts1 fusion proteins was improved. These results indicated that secreted proteases activated by Kex2 indeed seem to be responsible for proteolytic damage of heterologous proteins. Albeit these positive effects, severe pleiotropic phenotypic effects were observed in the *kex2* deletion mutant, such as a reduced growth rate and cell clumping in liquid cultures. These observations correlate with a cytokinesis defect detected by microscopy and with the comparatively slow recovery of transformants. Similar types of morphological changes have been observed before in the respective *kex2* deletion mutants of several fungi like *S. cerevisiae*, *Candida albicans, Candida glabrata, P. pastoris, S. pombe, and Yarrowia lipolytica* and moulds such as *A. niger, A. oryzae* or *Trichoderma reesei* [Bader et al., 2008]. The observed phenotypic spectrum in *kex2* deletion mutants is likely due to the lack of processing events in substrate proteins rendering them non-functional. For example, in the case of the α-pheromone, the lack of processing renders the *kex2* mutant of *S. cerevisiae* deficient in mating [Julius et al., 1984]. Also, in the case of *C. albicans*, reduced virulence is observed in the *kex2* deletion mutant probably due to the lack of activity from cell-wall modifying enzymes [Newport et al., 2003]. Similarly, mutants of *C. glabrata* showed hypersensitivity to anti-mycotic drugs that target integrity of cell wall or plasma membrane [Bader et al., 2001]. Disruption of the XPR6 gene, a *kex2* homolog in *Y. lipolytica*, led to reduced secretion of mature alkaline protease due to which the strains grew poorly at higher pH. Cells remained physically attached after budding and continued to

bud, forming large balloon-like structures which aggregated forming visible clumps in liquid culture. Although these growth aberrations were largely eliminated by growing cells in medium at pH 4, no mycelial forms were observed regardless of the pH [Enderlin and Ogrydziak 1994]. Similarly, pleiotrophic effects have also been observed in *kex2* mutants in *A. oryzae* [Mizutani et al., 2004]. Therefore, these results indicate the importance of this protein for proper integrity of the cell which can also be observed in the *U. maydis kex2* mutant.

Since the pleiotropic growth effects observed for *kex2* deletion mutants are undesirable in protein production strains, instead of targeting this key protease required for normal morphogenesis and cellular growth, selective crucial proteases were targeted. According to predictions obtained from the *U. maydis* genome database (http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t237631_Ust_maydi_v2) at least 72 putative proteases and peptidases exist, of which 26 are likely to be secreted (Section 2.3, Table 1). Apart from the essential mitochondrial proteases or signal peptidases which likely have no role in the proteolytic degradation of heterologous proteins, many of these candidate proteins could be relevant and hence can be good targets for deletion.

The major vacuolar proteases PrA and PrB of the budding yeast (encoded by PEP4 and PRB1, respectively) are required for the maturation and activation of several vacuolar proteases. Hence, the double deletion of these genes resulted in a strain with reduced proteolytic activity [Jones 1991]. Similar results were obtained when homologous genes were eliminated in *P. pastoris* [Gleeson et al., 1998] and *C. boidinii* [Komeda et al., 2002]. Furthermore, deletions of the genes for the *S. pombe* proteases Isp6 and Psp3, which are similar to the *S. cerevisiae* vacuolar protease Prb1, showed a significant reduction in extra- and intracellular protease activity [Idiris et al., 2006]. Also in filamentous fungi such as *A. niger* or *A. oryzae* homologs of these proteases were eliminated and a significant improvement in protein production was reported [Jin et al., 2007, van den Hombergh et al., 1997a]. The group of Kitamoto carried out a deletion of the exopeptidase *tppA* gene in *A. oryzae* which resulted in an increase of 32% in human lysozyme production [Jin et al., 2007]. This protease is known to be responsible for cleaving off tripeptides from proteins and

therefore destabilizing the protein conformation triggering degradation by other proteases [Jin et al., 2007].

Knowing about the important role of Pep4, Prb1, and TppA in proteolysis, homologs of these proteins were initially targeted in *U. maydis*. With the presence of a haploid yeast-like growth phase where cells carry only one copy of each gene, gene deletion is straightforward in *U. maydis* and gene deletion techniques are well-established [Brachmann et al., 2001, Terfrüchte et al., 2014]. In accordance with the published results in other fungi, *pep4* as well as *prb1* deletion mutants showed a lower general proteolytic activity which became evident in casein plate assays. However, no positive effect was observed for the stability of heterologous proteins like Gus although a minor improvement was detected in the activity of this reporter enzyme.

It is rational to suggest that each heterologous protein is targeted by a specific set of proteases and hence it is essential to target multiple proteases to reduce the overall proteolytic potential of the expression host. In line with this assumption, the strategy which seemed to be the most effective way to achieve reduced proteolysis and secretion of stable heterologous proteins was generating a multiple protease-deficient strain. Similarly, Idiris et al. (2006) analyzed a set of 52 single protease deletion strains in *S. pombe* for reduced degradation of human growth hormone (hGH) and then constructed a multi protease-deficient strain, which lacked 13 crucial proteases selected from the screening [Idiris et al., 2006]. Indeed, this strain demonstrated a 30-fold increase in secretion of hGH [Idiris et al., 2006]. Also the group of Kitamoto reported a 30% increase in bovine chymosin production by generating a decuple protease deletion stain in *A. oryzae* [Yoon et al., 2011]. In the present study, 5 proteases of the aspartic and serine type were sequentially deleted in *U. maydis* to generate a quintuple protease deficient background. Remarkably, with the fifth protease deletion, the general proteolytic activity was almost diminished as observed in casein plate assays. This result demonstrates that yields of heterologous proteins can be improved by deleting several harmful proteases in a single expression strain. Interestingly, the preliminary characterization of the secretome in cooperation with Prof. B. Macek and Dr. M. Franz (Proteome Center, University of Tübingen) confirmed that 4 of the targeted proteases could be detected and furthermore revealed the presence of at least 15 more potentially harmful proteases in the culture

supernatant of the progenitor strain AB33. Deletion of several of these candidates should be undertaken to achieve even better yields and less degradation of heterologous proteins. Among these protease candidates, some might have redundant function in axenic cultures and therefore, their elimination may not induce any harmful phenotypic effects. For example, several carboxypeptidases detected in the secretome analysis could be targeted, thereby reducing the secretome of this host.

It is to be noted that no harmful phenotypic effects were observed in the quintuple protease deficient strain, indicating that the respective proteases might not have an indispensable role for the yeast-like growth of the cells. However, there could be one or more proteases or peptidases that would have an important role for the cellular growth and deletion of such candidates can be lethal for the cell. Such an observation was even made with the attempt of deleting the subtilisin type serine protease gene *um03024* during the generation of the multiple-protease deficient strain (data not shown).

3.2. Strain optimization at transcriptional level

Along with the protease deletion strategy, improvement at the transcriptional level was accomplished by selecting a more suitable promoter exhibiting stronger activity than the previously used one (Michèle Reindl, Bachelorarbeit 2013). Choosing the right promoter for the transcription of a target gene is crucial for successful recombinant expression [Berlec and Štrukelj 2013]. In general, using a stronger promoter should lead to increased transcript amounts and hence result in enhanced protein synthesis. Some examples of strong fungal promoters commonly employed for heterologous protein production are the AOX1 (alcohol oxidase) promoter of *P. pastoris* [Porro et al., 2005], the c*bh*I (cellobiohydrolase I) promoter of *T. reesei* or the *gla*A (glucoamylase A) promoter of *A. niger* [Nevalainen et al., 2005]. Eukaryotic promoters often show quite complex structures, harboring enhancing and regulatory elements like, for example, upstream activating sequences (UAS) [Gagniuc and Ionescu-Tirgoviste 2012]. Taking advantage of this observation, a synthetic promoter that had been assembled from different genetic elements was employed for gene expression. This highly active synthetic promoter P_{oma}, build up by artificial multimerization of UAS (upstream activating sequences) *cis*-active elements in front of a core promoter [Flor-Parra et al., 2006, Hartmann et al., 1999] was employed for recombinant expression of scFv-Cts1

fusion protein. In comparison to the previously used P_{otef} promoter, this resulted in a strong increase in unconventionally secreted protein. Moreover, the P_{oma} promoter activity could be fine-tuned to the specific needs of the individual target by altering the number of UAS [Hartmann et al., 1999]. In addition, to prevent the saturation of the system for example, with respect to insufficient supply of factors required for promoter regulation, overexpression of such regulatory proteins could result in more enhanced promoter activity [Nevalainen et al., 2005]. Therefore, overexpression of transcription factor Ncp1 that regulates the UAS elements [Hartmann et al., 1999] can be tested to check for further increase in protein amounts.

Coupling both the optimization steps of improved transcriptional strength and reduced proteolytic potential, active full-length scFv-Cts1 fusion protein could be purified from culture supernatants for the first time. The use of the multiple protease-deficient strains led to an improved stability of the secreted scFv-Cts1 fusion protein. The combination with the strong promoter finally resulted in strongly improved protein yields.

3.3. Expression of different antibody formats as a proof-of-principle

Successful expression of proteins of different classes forms the basis for the establishment of a novel expression system. Initially, the expression of the bacterial derived enzyme Gus was demonstrated in *U. maydis*, unraveling its potential to deal as an expression system by exploiting unconventional secretion [Janpeter Stock, Dissertation 2014]. In the present study, different antibody formats were selected as proof-of-principle to demonstrate the expression of biopharmaceutical proteins. Antibody-based drugs are one of the fastest growing classes of drugs. One of the major problems is that they are expensive, which limit their use for serious medical conditions [Carter 2011]. Factors which contribute to their high costs are the large expenses in drug development and manufacturing along with the large doses that are often required to treat the disease [Kelley 2009]. Most often these costly antibody therapeutics are produced in mammalian cells, commonly Chinese hamster ovary cells (CHO), NS0 mouse myeloma cells or hybridoma cells. However, by using alternative hosts for production, the costs can be reduced to some extent [Carter 2006, Chadd and Chamow 2001]. For example, various bacteria (most commonly *E. coli*), fungi (yeasts, filamentous fungi), insect cells and transgenic plants have been explored for production of antibody fragments [Joosten et al.,

2003, Verma et al., 1998]. Expression of antibody fragments in *E. coli* has been successful in many cases, even reaching g/l titer in fermenter cultures [Harrison and Keshavarz-Moore 1996]. However, some bottlenecks such as costly and laborious downstream processing of protein aggregates (inclusion bodies) [Somerville Jr et al., 1994, Wülfing and Plückthun 1994] or problems associated with cell lysis during periplasmic or secretory protein production [Skerra 1993] reduce the attractiveness of *E. coli* as a host for large-scale production of antibody fragments [Joosten et al., 2003]. Many groups have been working on these shortcomings to improve the production of soluble and functional scFvs in this system, for example, by improving disulfide bond formation [Jurado et al., 2006] or using the efficient twin-arginine translocation system for secretion of antibody fragments [Choi and Lee 2004]. Alternative bacterial systems have also been used to express single chain antibodies. Recently, a psychrophilic bacterium, *Pseudoalteromonas haloplanktis,* was successfully utilized for scFv production, showing a better solubility of proteins as compared to *E. coli*. This Antarctic gram-negative bacterium can hence deal as a useful unconventional expression system for such proteins [Giuliani et al., 2014].

Since scFvs contain intramolecular di-sulfide bridges and require proper folding for activity, it is not always easy to produce these antibody formats in prokaryotic systems. Hence, different fungi have been used as alternate hosts for production of antibody fragments. Some examples include yeasts such as *S. cerevisiae* [Evans et al., 2010, Shusta et al., 1998], *S. pombe* [Davis et al., 1991] and *P. pastoris* [Fischer et al., 1999, Gasser et al., 2006]. Even filamentous fungi have been used for antibody fragment production like different *Aspergillus* species [Sotiriadis et al., 2001]. Another alternate approach reported for scFv production is based on cell-free systems based on cultured insect cells using the eukaryotic translation machinery [Stech et al., 2014].

Here, employing the Cts1-based *U. maydis* expression system, successful production of an α-myc scFv was shown. After optimizing the strain, the purification of a scFv-Cts1 fusion protein from the culture medium was successful and the protein was shown to be active by ELISA. Of note, in the future, the relevant scFv fragment can be cleaved off from the fusion protein using the TEV protease to get untagged active scFv protein.

Another antibody format called as nanobody (V_HH) which is derived from the heavy chain camelid antibodies (Section 1.6) was tested for successful expression in *U. maydis*. In particular, a V_HH directed against the fluorescent protein Gfp was selected. The α-Gfp nanobody can for example be fused to fluorescent proteins which can be utilized for various studies like protein localization, protein dynamics, protein-protein interactions in cells [Rothbauer et al., 2006, Rothbauer et al., 2008]. Although these antibody formats have been previously shown to be expressed in different microbial systems like *E. coli* [Arbabi Ghahroudi et al., 1997], *S. cerevisiae* [Frenken et al., 2000] and *P. pastoris* [Rahbarizadeh et al., 2006], some bottlenecks have been observed in each of the systems. For example, yeast produced V_HHs can sometimes be glycosylated which can affect antigen binding and also led to nonspecific immunogenicity [Harmsen and De Haard 2007]. In this study, successful expression and purification of the α-Gfp nanobody from the supernatant of *U. maydis* cultures was shown. Binding of the nanobody to Gfp was also demonstrated by Western blotting and ELISA. Therefore, *U. maydis* also displays the potential to deal as an alternate expression system for the production of such type of antibody formats.

One more antibody format, the diabody, which constitutes an *in vivo* association of two monomeric scFvs, was selected for this study. Successful diabody formation has been reported previously [FitzGerald et al., 1997], where the expression of a bispecific diabody against a carcinoembryonic antigen (CEA) and the T-cell co-receptor CD3, was shown successfully in *P. pastoris* while only the monomeric form was detected in *E. coli* [FitzGerald et al., 1997]. In this study, the expression of an α-myc diabody and its di-sulfide bridged variant with improved dimer stability was tested. Although the monomeric form could be detected, the dimeric form could not be observed in non-reducing SDS PAGE. The most probable reason for the failure to achieve dimer formation could be a steric hindrance by the Cts1 moiety present in the fusion protein. Like mentioned above, in the future, the fusion proteins can be processed to cleave off the Cts1 fragments and hence achieve dimerization of the scFv fragments.

Hence, it was successfully demonstrated in this study that the *U. maydis* expression system can be employed as an alternative platform to produce different antibody formats like scFvs and nanobodies. However, there is still a lot of room for improvement in this system.

Further careful studies will reveal if it is also suited for producing antibody formats like diabodies and other higher valency formats which require covalent association between monomeric forms to obtain the functional antibody formats.

3.4. Conclusion and future prospects

Although the production of various antibody fragments has been reported in different expression systems, each of them also includes some pitfalls. In antibody fragments, the *V* domains itself differ widely in their biophysical properties such as stability, solubility and folding kinetics, all of which can affect the recombinant expression [Carter 2006]. Therefore, each protein has specific requirements which cannot be met by any single expression system. This means that no universal expression system exists which can assure expression and high yields for every recombinant protein [Corchero et al., 2013, Feldbrügge et al., 2013]. Therefore, an alternate expression system like *U. maydis* can eventually be applied for the production of 'hard to express' or other niche proteins and help in reducing the overall costs for these antibody molecules [Stock et al., 2012]. Optimization of this system already led to an increase in protein yields from about 1 µg/l to 40 µg/l. Although these values are quite low compared to other established fungal systems, the expression studies were carried out only at shake flask levels till now, using culture densities of no more than 1.0. In the near future, at the shake-flask level an improvement in protein expression can be achieved by monitoring several complex factors like oxygen limitations or nutritional limitations. For example, a state of the art system called as Cultivation in Respiration Activity MOnitoring System (RAMOS) could be employed, which allows the online monitoring of important culture parameters such as the oxygen transfer rate (OTR), the carbon dioxide transfer rate (CTR), and the respiratory quotient (RQ) in shake-flasks [Klement et al., 2012]. In this way, limiting factors can be identified and eliminated by altering the growth medium and the cultivation conditions. In the long run, the production can be scaled up to the bioreactor level where most likely much higher protein yields can be achieved. Preliminary studies support this assumption [Marius Terfrüchte, Masterarbeit 2013]. In this respect, a metabolic model has been developed for optimized itaconic acid production in *U. maydis* which can eventually be helpful in bioreactor studies and downstream processing of broth for protein expression purposes [Voll et al.,

2012]. The optimized strain generated in this study will build the foundation for these experiments.

Moreover, besides going ahead with culture optimization and scale up studies, it is important to tackle all the limiting steps and engineer the strain at various molecular levels. In this study, the expression system was already improved in terms of reducing the proteolytic degradation of heterologous proteins and thereby improving their stability. Coupled with an efficient promoter, active antibody formats were produced and purified from the culture medium. However, the molecular pathway of unconventionally Cts1 secretion is not yet deciphered. It can furthermore be hypothesized that an interesting connection exists between endosome-coupled mRNA shuttling and unconventional secretion in filaments [Baumann et al., 2014, Koepke et al., 2011]. Through genetic screening studies one can identify key players involved in the unconventional secretion process. Subsequent ingenious genetic engineering will lead to an improved system with enhanced yields of secreted proteins in the future.

The expression platform used in this study enables the production of non N-glycosylated proteins which may be essential for certain therapeutic or industrial proteins [Stock et al., 2012]. However, one can harness the full potential of this eukaryotic host by also exploiting the conventional secretory pathway, allowing the production of glycosylated proteins. Interestingly, the glycosylation machinery of *U. maydis* could likely be humanized with fewer efforts as compared to other fungal species [Fernández-Álvarez et al., 2010].

In summary, in this study the *U. maydis* expression platform based on unconventional secretion was optimized to obtain improved yields of secreted proteins and applied for the production of antibody formats. However, the black box of this fungal expression system could be opened even more such that in the end it may constitute a biotechnological advancement to provide affordable biopharmaceuticals.

4. Materials and Methods

4.1. Materials

4.1.1. Chemicals, enzymes and kits

Chemicals:

Chemicals used in this work were purchased from the following companies unless otherwise specified: Sigma-Aldrich, Merck, Thermo Scientific, Fluka, Roche, Difco, Serva, BioRad, GE Healthcare, Pharmacia, Invitrogen and Carl Roth.

Table 3: Enzymes used in the work

Name	Application	Company
Alkaline phosphatase	De-phosphorylation of DNA fragments	Roche
Lysozyme	Plasmid isolation	Merck
Novozyme	Protoplast preparing of *U. maydis*	Novo Nordisc
Phusion DNA polymerase	DNA amplification	Finnzymes or prepared in laboratory
Restriction enzymes	DNA restriction	New England Biolabs
RNase A	Plasmid isolation, gDNA isolation	Boehringer Ingelheim
T4-DNA ligase	DNA ligation	Roche
Quick-DNA ligase	DNA ligation	New England Biolabs

Table 4: Kits used for molecular cloning and protein biochemical work

Name	Application	Company
CDP-Star® (chemiluminescent substrate for alkaline phosphatase)	Southern blot analysis	Invitrogen
Amersham™ ECL™ Prime Western blotting detection kit (chemiluminescent substrate for horseradish peroxidase, HRP)	Western blot analysis	GE Healthcare Life Sciences
Aceglow™ Ultrasensitive chemiluminescence substrate (for	Western blot analysis	Peqlab

horseradish peroxidase, HRP)		
QuantaRed Enhanced Chemifluorescent HRP substrate	ELISA	Thermo Scientific
High Pure plasmid isolation kit	Preparation of plasmid DNA	GE Healthcare Life Sciences
High Pure PCR product purification kit	PCR product clean-up	GE Healthcare Life Sciences
JetSorb® gel extraction kit	DNA fragment isolation from agarose gels	Genomed
JetQuick® DNA purification kit	Clean-up of plasmid	Genomed
JetQuick® Plasmid Miniprep Spin Kit	Preparation of plasmid DNA	Genomed
PCR DIG Labeling Mix	Digoxigenin-labelling of PCR products (for DNA probes used in Southern blots)	Roche
Plasmid Midi Kit	Preparation of plasmid DNA	Qiagen
SureClean	Clean-up of PCR products	Bioline

4.1.2. Solutions and media

Solutions

Standard buffers and solutions were prepared according to [Ausubel et al., 1987] and [Sambrook et al., 1989] protocols. Special buffers and solutions are listed under the respective methods.

Media

For the cultivation of *E. coli*, dYT, YT liquid and solid medium were used [Ausubel et al., 1987, Sambrook et al., 1989]. Media were autoclaved for 5 min at 121 °C. Following antibiotics were employed: Ampicillin (100 µg/ml), Gentamycin (50 µg/ml), Chloramphenicol (32 µg/ml) and Kanamycin (50 µg/ml).

For the cultivation of *U. maydis*, following media were used. Unless otherwise stated, all were autoclaved for 5 min at 121 °C. For agar-containing plates, 2.0 % (w/v) agar (Difco)

was added to medium before autoclaving. The sterile medium was stirred, and then antibiotics were added when medium was cooled to 60 °C and then poured in plates. Following antibiotics were used in liquid medium- Carboxin (2 µg/ml), Hygromycin (200 µg/ml) and Nourseothricin (150 µg/ml)

CM Complete medium [Banuett and Herskowitz 1989, Holliday 1974] 0.25% (w/v) casaminoacids (Difco) 0.1% (w/v) yeast extract (Difco) 1.0% (v/v) vitamin solution 6.25% (v/v) salt solution 0.05% (w/v) DNA degr. free acid (Sigma, D-3159) 0.15% (w/v) NH_4NO_3 (Sigma, A9642) Dissolve in MilliQ water, adjust pH to 7.0 with NaOH, autoclave after autoclaving: Add 2.0% (v/v) 50% glucose solution	**NM minimal medium** [Holliday 1974] 0.3% KNO_3 6.25% salt solution Dissolve in MilliQ water, adjust pH to 7.0 with NaOH, autoclave after autoclaving: Add 2.0% (v/v) 50% glucose solution **AM minimal medium** 0.3 % (w/v) $(NH_4)_2SO_4$ 6.25% (v/v) salt solution Dissolve in MilliQ water, adjust pH to 7.0 with NaOH, autoclave after autoclaving: Add 2.0% (v/v) 50% glucose solution
YEPS light medium modified from [Tsukuda et al., 1988] 1% (w/v) yeast extract (Difco) 0.4% (w/v) BactoTM peptone (Difco) 0.4% (w/v) Sucrose Dissolve in MilliQ water, autoclave	**NSY-Glycerin (for glycerol stocks)** modified from [Tsukuda et al., 1988] 0.8% (w/v) nutrient broth 0.1% (w/v) yeast extract 0.5% (w/v) sucrose 69.6% (v/v) glycerin
Salt Solution [Holliday 1974] 16.0% (w/v) KH_2PO4 4.0% (w/v) Na_2SO4 8.0% (w/v) KCl 1.32% (w/v) $CaCl_2x2H_2O$	**Trace-Elements** [Holliday 1974] 60 mg H_3BO_3 140 mg $MnCl_2$ x 4 H_2O 400 mg $ZnCl_2$ 40 mg $NaMoO_4$ x 2 H_2O

8.0% (v/v) trace elements	100 mg $FeCl_3$ x 6 H_2O
2.0% (w/v) $MgSO_4$ (water free)	40 mg $CuSO_4$ x 5 H_2O
Dissolve in MilliQ water, filter sterilize	Dissolve in MilliQ water, filter sterilize
Vitamin solution [Holliday 1974]	**Regeneration agar** [Schulz et al., 1990]
100 mg thiamine	**top agar:**
50 mg riboflavin	1.5% (w/v) Bacto agar
50 mg pyridoxine	1 M Sorbitol
200 mg calcium pantothenate	in $YEPS_L$-Medium
500 mg p-aminobenzoic acid	**bottom agar:**
200 mg nicotinic acid	Top-Agar with 4 µg/ml carboxin or 400 µg/ml hygromycin or 300 µg/ml nourseothricin
200 mg choline chloride	
1000 mg myo-inositol	
Dissolve in MilliQ water in 1 l and filter sterilize	

4.1.3. Oligonucleotides

All the oligonucleotides were synthesized by Metabion GmbH (Table 5).

Table 5: List of oligonucleotides used in the study

Designation	**Nucleotide sequence (5´- 3´)**
Primers used for protease deletions	
RL172 U2um02843	GGTCTCGCCTGCAATATTTGCTGTTCCTCTTGCTCGC
RL173 U3um02843	GGTCTCCAGGCCCGAGAGTCTCAGTTTGCCACATGT
RL174 D1um02843	GGTCTCGCTGCAATATTGTGTGTGATGAACGATCGAGAG
RL175 D2um02843	GGTCTCCGGCCAAGGAGCCTGTAGAGCAGAAGCG
RL434 D2um04926	GGTCTCGCTGCAATATTCAAGTGGGCAAGCTGATCC
RL435 U2um04400	GGTCTCGCCTGCAATATTGTGAGCCACGGACGAAAAAAAGG
RL436 U3um04400	GGTCTCCAGGCCATTGCGGATGGGGTGATGATCAGG
RL437 D1um04400	GGTCTCCGGCCAAGTGCCACCCTACCAGAGC
RL438 D2um04400	GGTCTCGCTGCAATATTGGCGTCGACATTGAAAACTCG

RL439 BsaIfw um04400	TTTGGTCTCATGTCGGGATGGTAAGAAGGGGAAGAG
RL440 BsaIrev um04400	TTTGGTCTCTGACACCATCTTGCGTGCACTCTGATCC
RL441 U2um06118	GGTCTCGCCTGCAATATTCGTCTGCGGTGAGGTCTTCAGC
RL442 U3um06118	GGTCTCCAGGCCGCGTCAATAGCCTAGTCTGG
RL443 D1um06118	GGTCTCCGGCCAGTGGTGGTAGTAGTCAATCAGAACG
RL444 D2um06118	GGTCTCGCTGCAATATTCACGATTGTTAGTCTCACTCACC
RL445 BsaIfw um06118	TTT GGTCTCATGTCGGCTGCATATGGTGTCTTCG
RL446 BsaIrev um06118	TTTGGTCTCTGACACCAAATCGTGAATGTTGGTGC
RL569 P1um04926	TACGCTGAAGCCAAGAAGG
RL570 P2um04926	CCTCGCCGCCATCCTCTTCG
RL571 U1um04926	GCCAAGACCTGTGACTGC
RL572 D3um04926	GCGCGTGATCGCATCAACC
RL573 P1um04400	GACCATGCCCCAGAACGACG
RL574 P2um04400	TGCCCGTGTTCCAAGTGG
RL575 U1um04400	TCGATCGAGATGACTGAGC
RL576 D3um04400	GCCGGTCTGGACTCGACCG
RL577 P1um06118	TTCTTATCCGGGATGAAGG
RL578 P2um06118	CTTGCTCTACGTTCACCCG
RL579 U1um06118	TTCACAGCAGGCCCCGACG
RL580 D3um06118	CTTGACTCAACTCTCTTGC
RL946 U2um02178	GGTCTCGCCTGCAATATTTGAATCGCACTACGGTTG
RL947 U3um02178	GGTCTCCAGGCCTTCTGTAATAAAGCAGCAGTAAAG
RL948 D1um02178	GGTCTCCGGCCTCGCGCCAGCACCTACAAC
RL949 D2um02178	GGTCTCGCTGCAATATTCAGCGCTGAGCACGAG
RL950 BsaIUfw um02178	CACGGTCTCGTGTCTCTCTCTTTCTCTTTTGCGCCGAC

RL951 BsaIUrev um02178	GACGGTCTCTGACACCCTGAATTCTTCTCAGTCTC
RL985 U1um02178	CAGCACAAACTCGACACTAG
RL986 D3um02178	CGTTGCATGCTGCAACAGTGT
RL1095 U2um00064	GGTCTCGCCTGCATTTAAATCCAATTGCCAATTGCCAATTG
RL1096 U3um00064	GGTCTCCAGGCC CTTGACTGTGTGTAGAGGCTTG
RL1097 D1um00064	GGTCTCCGGCC ACATTCACGATTGTGACTCG
RL1098 D2um00064	GGTCTCGCTGCATTTAAATGGACCAAGGAGAGCC
RL1099 P1um00064	GCTGTGGACCGGTACTTTGG
RL1100 P2um00064	CTCGATGCTCTGGCCGTTG
RL1101 U1um00064	GAGCCGGTTATCAGTCTC
RL1102 D3um00064	CACGATCGCTCTTGACGC
RL1176 U2um11908	GGTCTCGCCTGCATTTAAATGTGTCAGGGTCGCAG
RL1177 U3um11908	GGTCTCCAGGCCTGCTATGGTGAGACGCGTGTC
RL1178 D1um11908	GGTCTCCGGCCTAGGCTCGCTTGTTCTTGA
RL1179 D2um11908	GGTCTCGCTGCATTTAAATGCTGATCCGCACATTG
RL1248 P1um11908	GCGTCAAACGATCGGATTG
RL1249 P2um11908	GCGGGTTCATGGACCAGCTC
RL1250 U1um11908	CCTCGAACATTCGTCAGAG
RL1251 D3um11908	CGACGATCGCTACCGTAGA
Primers for *E. coli* plasmids	
RL176	CATGGGTGAGGAGCAGAAGCTGATCTCAGAGGAGGACCTGCACCACC ACCACCACCACTG
RL177	AATTCAGTGGTGGTGGTGGTGGTGCAGGTCCTCCTCTGAGATCAGCTT CTGCTCCTCACC
RL188	TACATGGACCCAATGTGC
RL189	CTATCGCTACGTGACTGG
RL253	CATGGGTCACCACCACCACCACCACTG
RL278	AATTCAGTGGTGGTGGTGGTGGTGACC

Primers used to generate scFv, diabody and llama antibody plasmids	
RL565	CACCACCCATGGCCGAGGTCGACCTCGTCGAGTCGG
RL566	GTGGTGGGATCCCTATTAGCGCCGGTCCGGAGCGGC
RL880	GGTCTCGCTCGGACATCGTGCTCACCCAGTCGCCC
RL881	GGTCTCGCGAGCCACCACCGCCCGACGAGACGGTGACGGTGGTGCC
RL893	GGCGACCCACTCGAGGCACTTGTCGGGCGTCTG
RL894	CTCGAGCTTGGTACCGCAGCCAAAGGTCCAGGGC
RL908	TCAGACGCCCGACAAGTGCCTCGAATGGGTCGCCACCATC
RL909	GTGCCCTGGACCTTTGGCTGCGGCACCAAGCTCGAGATC
RL1331	CCATGGCGGCCCATCACCACCATCACCACCATCACCACCATGCGATGG CCGAGGTCGACCTCGTCG
RL1577	GCCATGGCGGCCCATCACCACCATCACCACCATCACCACCATCATATG GCCGACGTCCAGCT
RL1578	GACTAGTCGACGAGACGGTGA

4.1.4. Plasmids

For generation of plasmid vectors, standard molecular cloning techniques were followed [Sambrook et al., 1989]. All oligonucleotides used in this study are listed in Table 5. All the constructs were confirmed by sequencing at the Institute for Genetics in Ludwig-Maximilians-Universität München.

4.1.4.1. Plasmids for use in *E. coli*

pGEX-GST-1xcMyc-6xHis (pUMa1531): An insert coding for a combined cMyc-6xHis tag was generated by annealing primers RL176 and RL177 and was ligated to the pGEX-2T derivative pUMa429 [König et al., 2007] after restriction with NcoI and EcoRI [Stock et al., 2012].

pGEX-GST-6xHis (pUMa1562): An insert coding for a 6xHis tag was generated by annealing of the primers RL253 and RL278 and was ligated to the pGEX-2T derivative pUMa429 (König *et al*., 2007) after restriction with NcoI and EcoRI [Stock et al., 2012].

pET15b-6xHis-Gfp (pUMa2156): Gfp insert was amplified from pUMa828 using primers RL1385 and RL1384. A 722bp NdeI-BamHI Gfp fragment was then ligated to NdeI-BamHI fragment of vector backbone pUM1951 (This study).

4.1.4.2. Plasmids for *U. maydis*

Expression plasmids

For gene expression in *U. maydis*, integrative vectors were used. All integrative vectors were inserted via homologous recombination at the *ip* locus of *U. maydis* and were derived from p123 [Aichinger et al., 2003].

pRabX1 Potef scFv-SHH-Cts1 (pUMa1570): A 767 bp NcoI-SpeI fragment of the codon-optimized α-Myc scFv gene obtained from pMK-RQ Um-anti-cMyc-scFv (Geneart; Invitrogen-Life Technologies; pUMa1465) was inserted between the respective NcoI and SpeI sites of pRabX1Gus-SHH-Cts1, pUMa1521 [Stock et al., 2012].

pRabX2 Poma His-scFv-Tev-HA-Cts1 (pUMa2137): A 809 bp NcoI-10xHis-scFv-SpeI fragment was amplified from pUMa1465 using RL1331 and RL1289. It was ligated to 1.27 kb NcoI-SacI fragment having Poma promoter sequence from pUMa2113 and then introduced into SacI-SpeI vector backbone (6.92 kb) of pUMa2120 [Sarkari et al., 2014].

pRabX2 Potef His-scFv-Tev-HA-Cts1 (pUMa2273): A 1 kb NdeI-NcoI Potef fragment was introduced in NdeI-NcoI vector backbone of pUMa2137 [Sarkari et al., 2014].

pRabX2 Poma His-α-Gfp Nb-Tev-HA-Cts1 (pUMa2240): A 403 bp NcoI-SpeI fragment having codon-optimised anti-Gfp llama nanobody gene was amplified from pMA-T Um-anti-gfp-Nb (Geneart; pUMa2234) using RL1577 and RL1578 and it was inserted into the respective NcoI and SpeI sites of pRABX2 Poma His-scFv-Tev-HA-Cts1 (pUMa2137; this study).

pRabX1 Potef α-Myc Db-SHH-Cts1 (pUMa1809): A 399 bp NcoI-BsaI V_H fragment and 338 bp BsaI-SpeI V_L fragment is introduced into NcoI-SpeI vector backbone of pUMa1570 to generate pUMa1809 (This study).

pRabX1 Potef α-Myc S_2 Db-SHH-Cts1 (pUMa1834): Mutagenic PCR was carried out using RL908 and RL909 on pUMa1809, to introduce point mutations in V_H and V_L segments. Point

mutations in segments V_H and V_L checked by XhoI and KpnI digestion respectively and confirmed by sequencing (This study).

Gene deletion plasmids

Vectors for protease gene deletions were generated by a Golden Gate cloning strategy [Terfrüchte et al., 2014]. To this end, flanking regions of about 1 kb directly up- and downstream of the gene to be deleted were generated by PCR. Genomic DNA of UM521 was used as a template (DSM14603). The PCR products were purified by standard procedures using kits mentioned in Table 4. To generate the destination vectors containing the deletion constructs, Golden Gate reactions containing the two PCR products (flanks), a storage vector (pStorI derivative) with an appropriate HygR cassette which can be recycled by FRT/FLP and the destination vector (pDestI/pUMa1467) was conducted (Table 5) [Terfrüchte et al., 2014]. Primers used for flank generation are also described in Table 5. General components of the Golden Gate reaction and reaction conditions are as follows:

Components	**Thermocycler reaction**		
40 ng flank 1	37 °C	2 min	
40 ng flank 2	16 °C	5 min	50 cycles
75 ng storage vector	37 °C	5 min	
75 ng destination vector	50 °C	5 min	
0.5 µl BsaI.HF	80 °C	5 min	
0.75 µl T4 DNA ligase	16 °C	∞	
1.5 µl 10x ligase buffer			
Water q.s. 15 µl			

Table 6. Vectors generated by Golden Gate cloning: constituents of Golden Gate reactions and resulting deletion vectors

Final destination vector (pUMa)	**Linearization enzyme**	**Description / flanking FRT site**	**Upstream flank (U2, U3, BsaI primers)**	**Downstream flank (D1, D2, BsaI primers)**	**Storage vector (pUMa)**
pDest-Kex2Δ (pUMa1530)	SspI	Deletion of *kex2* (*um02843*) / FRT wt	RL172 x RL173 (1033 bp)	RL174 x RL175 (994 bp)	pStorI-1rh (pUMa1522)
pDest-Upp1Δ (pUMa1538)	SspI	Deletion of *um02178* / FRTwt	2 pieces: RL946 x RL951	RL948 x RL949 (946 bp)	pStorI-1rh/FRTwt (pUMa1522)

			(751 bp); RL947 x RL950 (347 bp)		
pDest-Upp2Δ (pUMa1549)	SwaI	Deletion of *um00064* / FRTm2	RL1095 x RL1096 (1144 bp)	RL1097 x RL1098 (1751 bp)	pStorI - 1rh/FRTm2 (pUMa1524)
pDest-Upp3Δ (pUMa1556)	SwaI	Deletion of *um11908* / FRTm3	RL1176 x RL1177 (1517 bp)	RL1178 x RL1179 (1888 bp)	pStorI-1rh/FRTm3 (pUMa1525)
pDest-Pep4Δ (pUMa1646)	SspI	Deletion of *pep4* (*um04926*) / FRTm1	RL431 x RL432 (844 bp)	RL433 x RL434 (749 bp)	pStorI-1rh/FRTm1 (pUMa1523)
pDest-Prb1Δ (pUMa1647)	SspI	Deletion of *prb1* (*um04400*) / FRTm2	RL435 x RL436 (889 bp)	2 pieces: RL437 x RL440 (483 bp); RL438 x RL439 (333 bp)	pStorI-1rh/FRTm2 (pUMa1524)
pDest-TppAΔ (pUMa1648)	SspI	Deletion of *tppA* (*um06118*) / FRTm3	2 pieces RL442 x RL445 (367 bp); RL441 x RL446 (573 bp)	RL443 x RL444 (866 bp)	pStorI-1rh/FRTm3 (pUMa1525)
pDest-Prb1Δ (pUMa2181)	SspI	Deletion of *prb1 / FRT*m5 (*um04400*)	RL435 x RL436 (889 bp)	2 pieces: RL437 x RL440 (483 bp); RL438 x RL439 (333 bp)	pStorI-1rh/FRTm5 (pUMa2045)

The generation of the pStorI-1rh derivatives (pUMa1522, 1523, 1524 and 1525) containing HygR cassettes flanked by different FRT sites (wt, m1-m3, m5) has been described before

[Khrunyk et al., 2010, Terfrüchte et al., 2014]. The pStorI-1rh version pHyg-FRTm5 (pUMa2045) was derived from pUMa1441 [Khrunyk et al., 2010] by introducing a point mutation in the FRT core region. To this end, the region containing the HygR cassette framed by FRTwt sites was amplified using primers RL1151 and RL1152, thereby introducing the respective point mutation termed m5. The PCR product was hydrolyzed with SfiI and introduced to the pStorI (pUMa1466) backbone [Terfrüchte et al., 2014].

Resistance marker recycling was conducted using the plasmid pFLPexpN (pUMa1889). This vector was derived from pFLPexpC [Khrunyk et al., 2010] by exchanging the CbxR cassette by a NatR cassette. A 881 bp partial P_{crg} promoter fragment of pFLPexpC (pUMa1446) [Khrunyk et al., 2010] was amplified using primers RL913 and RL914. A 4.4 kb BspEI/HindIII fragment of pFLPexpC and the BspEI/BamHI hydrolyzed PCR product were ligated to the 4.44 kb BamHI/HindIII fragment of pUMa419 (pNEBUN) [Brachmann et al., 2004].

4.1.5. Strains

4.1.5.1. *E. coli*

The *E. coli* K-12 derivate Top10 (Invitrogen/Life Technologies) was used for cloning purposes. *E. coli* Rosetta 2 (DE3) pLysS (Table 7) was employed for protein expression. Bacterial cells were grown at 37 °C with 110 rpm shaking.

Table 7. *E. coli* strains used in this study

Strains	Relevant genotype/ Resistances	UMa number	Plasmid transformed	Progenitor	Reference
E. coli Rosetta2 (DE3) pLysS	*F⁻ ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm* (DE3) pLysSRARE2 (ChrR)	791			Novagen [Merck-Millipore]
E. coli Rosetta2 (DE3) pLysS pGst-MH	*F⁻ ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm* pLysSRARE2 (CamR) pGst-MH (AmpR)	793	pGst-MH (pUMa1531) [expression of a GST-cMyc epitope-His fusion	UMa791	[Sarkari et al., 2014, Stock et al., 2012]

			protein]		
E. coli Rosetta2 (DE3) pLysS pGst-H	*F⁻ ompT hsdS$_B$* (r_B^- m_B^-) *gal dcm* pLysSRARE2 (CamR) pGst-H (AmpR)	806	pGst-H (pUMa1562) [expression of a GST-His fusion protein]	UMa791	[Sarkari et al., 2014, Stock et al., 2012]
E.coli Rosetta2 (DE3) pLysS pH-Gfp	*F⁻ ompT hsdS$_B$ (r_B^- m_B^-) gal dcm pLysSRARE2 (CamR) pH-Gfp (AmpR)*	1464	pGfp-H (pUMa2156) [expression of a His-Gfp fusion protein]	UMa791	This study

4.1.5.2. *U. maydis*

U. maydis strains used in this study are displayed in Table 8. Cells were incubated at 28 °C with 200 rpm shaking. Cultures were grown either in complete medium [Holliday 1974] or in ammonium minimal medium [Brachmann et al., 2004], both supplemented with 1% (w/v) glucose (CM-Glc/AM-Glc; see 4.1.2).

Deletion strains

U. maydis mutants (Table 8) were obtained by transformation of the progenitor strains with linearized plasmids. Gene deletion mutants were generated by the homologous recombination following established protocols [Brachmann et al., 2004, Kämper 2004, Terfrüchte et al., 2014]. In all deletion mutants generated in this study, the gene of interest was replaced by a hygromycin-resistance cassette (HygR) flanked by unique FRT sites for subsequent FLP-mediated excision [Khrunyk et al., 2010, Terfrüchte et al., 2014]. Homologous recombination was verified either by Southern blot analysis (gene deletion) or by diagnostic PCR (resistance-marker recycling).

For the recycling of resistance cassettes, an earlier described procedure [Khrunyk et al., 2010] was followed with slight modifications: First, gene deletion mutants were generated with HygR-cassettes framed by distinct FRT sites (termed wt or m1-m5; this study) [Khrunyk et al., 2010]. After confirmation of target gene deletion by Southern blot analysis, the respective mutants were transformed with a free-replicating plasmid containing the gene for the flippase (FLP) under control of the arabinose-inducible promoter P_{crg} (pFLP-Nat) using

nourseothricin as a selection marker (NatR). Transformants were singled out on CM-Nat plates. Single colonies were subsequently grown in CM liquid medium containing 1% (w/v) arabinose and 150 µg/ml nourseothricin (CM-Ara/Nat) to an optical density of about 1.0 for induction of FLP expression. Then, culture dilutions were spread on CM-Glc plates to obtain single colonies. These were screened for hygromycin sensitivity (HygS) by counter-selection on CM-Glc/Hyg and CM-Glc plates. Colonies which failed to grow on CM-Glc/Hyg indicate excision of the HygR cassette by FLP and were selected for diagnostic PCR using primer pairs (respective U1 and D3, given in Table 5) encasing the manipulated locus [Khrunyk et al., 2010]. Strains in which the PCR product confirmed HygR cassette removal were grown in CM-Glc medium in the absence of nourseothricin until the plasmid pFLP-Nat was lost, indicated by the inability of these strains to grow on CM-Glc/Nat plates.

Expression strains

For gene insertions, integrative expression plasmids derived from pRabX1 [Stock et al., 2012] or pRABX2 (adopted from Marius Terfrüchte, Masterarbeit 2013) [Sarkari et al., 2014] were used. These plasmids contain a region encoding an *ip* allele that confers resistance to the antibiotic carboxin (*ip*^{r}; [Broomfield and Hargreaves 1992, Keon et al., 1994]. For integration into the *ip*^{s} locus by homologous recombination, respective plasmids were linearized within the *ip*^{r} gene using either AgeI or SspI. Subsequently, protoplasts were transformed with the linearized plasmids following methods described below [Brachmann et al., 2004]. Homologous single or multiple integrations at the *ip* locus were verified by Southern blot analysis as described below, using a 2.1 kb probe obtained with the primer combination MF502/MF503 and the template pUMa260 [Brachmann et al., 2004, Loubradou et al., 2001].

Table 8. *U. maydis* strains used in this study

Strains	Relevant genotype	UMa number	Plasmid transformed	Locus	Progenitor	Reference
AB33	*a2 P*$_{nar}$*bW2bE1 PhleoR*	133		*b*	FB2	[Brachmann et al., 2001]

AB33P$_{otef}$ scfv-Cts1	*ip^r [P$_{otef}$scFv:shh:cts1 ubi1 3´UTR] ip^s PhleoR, CbxR*	829	pRabX1scFv-SHH-Cts1 ubi1 3´UTR (pUMa1570)	*cbx*	AB33	[Stock et al., 2012]
AB33P$_{otef}$ Gus-Cts1 ubi1 3´UTR	*ip^r [P$_{otef}$gus:shh:cts1 ubi1* 3´UTR*] ip^s PhleoR, CbxR*	843	pRabX1Gus-SHH-Cts1 ubi1 3´UTR (pUMa1521) /CbxR	*cbx*	AB33	[Stock et al., 2012]
AB33Gus$_{cyt}$	*ip^r [P$_{otef}$gus:gth] ip^s PhleoR, CbxR*	718	pGus-GTH (pUMa1403) /CbxR	*cbx*	AB33	[Stock et al., 2012]
AB33kex 2Δ	*FRTwt[um02843Δ]FRTwt PhleoR, HygR*	803	pKex2Δ (pUMa1530) /HygR	*um02843*	AB33	[Sarkari et al., 2014]
AB33kex 2Δ/P$_{otef}$scfv-Cts1	*FRTwt[um02843Δ::hyg]FRTwt ip^r [P$_{otef}$scFv:shh:cts1 ubi1 3´UTR] ip^s PhleoR, CbxR, HygR*	962	pRabX1scFv-SHH-Cts1 ubi1 3´UTR (pUMa1570) /CbxR	*cbx*	UMa803	[Sarkari et al., 2014]
AB33Pep 4Δ/P$_{otef}$ Gus-Cts1 ubi1 3´UTR	*ip^r [P$_{otef}$gus:shh:cts1 ubi1* 3´UTR*] ip^s FRT1[um04926Δ::hyg]FRT1 PhleoR,CbxR, HygR*	922	pPep4Δ (pUMa1646) /HygR	*um04926*	UMa843	this study
AB33Prb 14Δ/P$_{otef}$ Gus-Cts1 ubi1 3´UTR	*ip^r [P$_{otef}$gus:shh:cts1 ubi1* 3´UTR*] ip^s FRT2[um04400Δ::hyg]FRT2 PhleoR, HygR*	923	pPrb1Δ (pUMa1647) /HygR	*um04400*	UMa843	this study

AB33TppAΔ/P_{otef} Gus-Cts1 ubi1 3´UTR	*ip^r [P_{otef}gus:shh:cts1 ubi1* 3´UTR*] ip^s FRT3[um06118Δ::hyg]FRT3 PhleoR, HygR*	924	pTppAΔ (pUMa1648) /HygR	*um06118*	UMa843	this study
AB33Prb1Δ	*FRT2[um04400Δ::hyg]FRT2 PhleoR, HygR*	899	pPrb1Δ (pUMa1647) /HygR	*um04400*	AB33	this study
AB33TppAΔ	*FRT3[um06118Δ::hyg]FRT3 PhleoR, HygR*	900	pTppAΔ (pUMa1648) /HygR	*um06118*	AB33	this study
AB33P1Δ	*FRT1[um04926Δ::hyg]FRT1 PhleoR, HygR*	898	pPep4Δ (pUMa1646) /HygR	*um04926*	AB33	[Sarkari et al., 2014]
AB33P1ΔR	*FRT1[um04926Δ] PhleoR*	1118	pFLPexpC (pUMa1889) / NatR/free replicating [temporarily transformed, cured]		UMa898	[Sarkari et al., 2014]
AB33P2Δ	*FRTwt[um02178Δ::hyg]FRTwt FRT1[um04926Δ] PhleoR, HygR*	1119	pum02178Δ (pUMa1538) /HygR	*um02178*	UMa1118	[Sarkari et al., 2014]
AB33P2ΔR	*FRTwt[um02178Δ] FRT1[um04926Δ] PhleoR*	1154	pFLPexpC (pUMa1889) / NatR/free replicating [temporarily transformed, cured]		UMa1119	[Sarkari et al., 2014]
AB33P3Δ	*FRT2[um00064Δ::hyg]FRT2 FRTwt[um02178Δ] FRT1[um04926*	1156	pum00064Δ(pUMa1549)/HygR	*um00064*	UMa1154	[Sarkari et al., 2014]

	Δ] *PhleoR, HygR*					
AB33P3ΔR	*FRT2[um00064Δ]FRTwt[um02178Δ]* *FRT1[um04926Δ]* *PhleoR*	1205	pFLPexpC (pUMa1889) / NatR/free replicating [temporarily transformed, cured]		UMa1156	[Sarkari et al., 2014]
AB33P4Δ	*FRT3[um11908Δ::hyg]FRT3* *FRT2[um00064Δ]FRTwt[um02178Δ]* *FRT1[um04926Δ]* *PhleoR, HygR*	1231	pum11908Δ(pUMa1556)/ HygR	*um11908*	UMa1205	[Sarkari et al., 2014]
AB33P4ΔR	*FRT3[um11908Δ]* *FRT2[um00064Δ]* *FRTwt[um02178Δ]* *FRT1[um04926Δ]* *PhleoR*	1264	pFLPexpC (pUMa1889) / NatR/free replicating[temporarily transformed, cured]		UMa1231	[Sarkari et al., 2014]
AB33P5Δ	*FRT5[um04400Δ::hyg]FRT5* *FRT3[um11908Δ]* *FRT2[um00064Δ]* *FRTwt[um02178Δ]* *FRT1[um04926Δ]* *PhleoR, HygR*	1341	pum04400Δ (pUMa2181) /HygR	*um04400*	UMa1264	[Sarkari et al., 2014]
AB33P5ΔR	*FRT5[um04400Δ]* *FRT3[um11908*	1391	pFLPexpC (pUMa1889) / NatR/free		UMa1341	[Sarkari et al., 2014]

	Δ] FRT2[um00064 Δ] FRTwt[um02178 Δ] FRT1[um04926 Δ] PhleoR		replicating [temporarily transformed, cured]			
AB33P5Δ /P_{oma}scFv-Cts1	*FRT5[um04400 Δ::hyg]FRT5 FRT3[um11908 Δ] FRT2[um00064 Δ] FRTwt[um02178 Δ] FRT1[um04926 Δ] ip^r [P_{oma} his:scFv:tev:ha: cts1 ubi1 3´UTR] ip^s PhleoR, CbxR, HygR*	1392	pRabX2_P_{oma} His-scFv-tev-ha-Cts1 ubi1 3´UTR (pUMa2137) /CbxR	*cbx*	UMa1341	[Sarkari et al., 2014]
AB33-P_{oma}scfv-Cts1	*ip^r [P_{oma} his:scFv:tev:ha: cts1 ubi1 3´UTR] ip^s PhleoR, CbxR*	1315	pRabX2_P_{oma} His-scFv-tev-ha-Cts1 ubi1 3´UTR (pUMa2137) /CbxR	*cbx*	AB33	[Sarkari et al., 2014]
AB33kex 2Δ/P_{oma} scFv-Cts1	*FRTwt[um02843 Δ::hyg]FRTwt ip^r [P_{oma} his:scFv:tev:ha: cts1 ubi1 3´UTR] ip^s PhleoR, CbxR, HygR*	1267	pRabX2_P_{oma} His-scFv-tev-ha-Cts1 ubi1 3´UTR (pUMa2137) /CbxR	*cbx*	UMa803	[Sarkari et al., 2014]
AB33P_{otef} scfv-Cts1	*ip^r [P_{otef} his:scFv:tev:ha:*	1415	pRabX2_P_{otef} His-scFv-	*cbx*	AB33	[Sarkari et al.,

	cts1 ubi1 3´UTR] ip^s PhleoR, CbxR		tev-ha-Cts1 ubi1 3´UTR (pUMa2273) /CbxR			2014]
AB33kex 2Δ /scfv-Cts1	*FRTwt[um02843 Δ::hyg]FRTwt ip^r [P$_{otef}$ his:scFv:tev:ha: cts1 ubi1 3´UTR] ip^s PhleoR, CbxR, HygR*	1416	pRabX2_P$_{otef}$ His-scFv-tev-ha-Cts1 ubi1 3´UTR (pUMa2273) /CbxR	*cbx*	UMa803	[Sarkari et al., 2014]
AB33P$_{oma}$ His-α-gfp Nb-cts1	*ip^r [P$_{oma}$ his:Nb:tev:ha:cts 1 ubi1 3´UTR] ip^s PhleoR, CbxR*	1396	pRabX2_P$_{oma}$ His-α-Gfp Nb-tev-ha-Cts1 ubi1 3´UTR (pUMa2240) /CbxR	*cbx*	AB33	this study
AB33kex 2 Δ /P$_{oma}$His-α-Gfp Nb-cts1	*FRTwt[um02843 Δ::hyg]FRTwt ip^r [P$_{oma}$ his:Nb:tev:ha:cts 1 ubi1 3´UTR] ip^s PhleoR, CbxR, HygR*	1397	pRabX2_P$_{oma}$ His-α-Gfp Nb-tev-ha-Cts1 ubi1 3´UTR (pUMa2240) /CbxR	*cbx*	UMa803	this study
AB33α-myc diabody-SHH-Cts1	*ip^r [P$_{otef}$Db:shh:cts1 ubi1 3´UTR] ip^s PhleoR, CbxR*	1040	pRabX1Db-SHH-Cts1 ubi1 3´UTR (pUMa1809) /CbxR	*cbx*	AB33	this study
AB33Di-S$_2$ α-myc diabody-SHH-Cts1	*ip^r [P$_{otef}$Db:shh:cts1 ubi1 3´UTR] ip^s PhleoR, CbxR*	1067	pRabX1Di-S$_2$Db-SHH-Cts1 ubi1 3´UTR (pUMa1834) /CbxR	*cbx*	AB33	this study

4.1.6. Bioinformatic and computer programs

Clone Manager 9; Microsoft Office 2010; Canvas 12; NCBI; Genome database *U. maydis* 'mumdb' (http://mips.helmholtz-muenchen.de/genre/proj/ustilago);

PEDANT(http://pedant.helmholtz-muenchen.de/pedant3htmlview/pedant3view?Method=analysis&Db=p3_t237631_Ust_maydi_v2); Signal peptide prediction 'SignalP 4.1 (http://www.cbs.dtu.dk/services/SignalP/); SecretomeP 2.0 (http://www.cbs.dtu.dk/services/SecretomeP/).

4.2. Methods

4.2.1. Molecular biology methods

All the standard techniques of cloning such as DNA preparation, purification, restriction digestion, electrophoretic separation of DNA were followed as described in [Ausubel et al., 1987] and [Sambrook et al., 1989] or described in the instructions of the respective kits.

4.2.1.1. Diagnostic PCR (Colony PCR) method

Template DNA was prepared as follows: Some cell material was suspended in 20 µl 0.02 M NaOH and incubated at room temperature for 30 min. 1 µl supernatant of this mix was used for PCR. Primers P1 and P2 is a pair of sequence specific oligonucleotides which bind to gene of interest. PCR reaction was set as given below:

1 µl cell suspension (template DNA)
5 µl 5xPCR buffer (50 mM KCl, 1.5 mM $MgCl_2$, 10 mM Tris-HCl pH 8.3)
0.5 µl P1 primer (1 µM)
0.5 µl P2 primer (1 µM)
0.25 µl dNTPs (125 µM)
0.5 µl 1U Phu.HF
q.s. 25 µl H_2O

Program used for reactions in thermo cycler (PTC-200, MJ Research or Labcycler, SensoQuest) was denaturation at 96 °C for 1 min, 30 cycles each of 30 sec; denaturation at 96 °C, annealing at 65 °C and elongation at 72 °C (for 1 kb product) then a final elongation of 8

min at 72 °C. For products with expected different sizes, elongation time is adjusted accordingly.

4.2.1.2. Southern blot analysis

This method is modified by [Southern 1975]. *U. maydis* gDNA samples are prepared following the method described in [Hoffman and Winston 1987]. The samples are digested into smaller fragments using appropriate Restriction Type II enzymes. Digested DNA samples are applied on 0.8% agarose gels and electrophoresed at 75 V for 3 h. Then the agarose gel is incubated for 20 min each in 0.25 M HCl, DENAT and RENAT solutions to depurinate the DNA fragments breaking the DNA into smaller pieces, thus allowing efficient transfer from the gel to membrane. The transfer of the separated DNA fragments from an agarose gel to a nylon membrane is then performed by a capillary blot. Here, the transfer solution (20x SSC) from a buffer reservoir forces through the gel by capillary action and the DNA fragments are eluted and bind onto an overlying nylon membrane (Hybond-N^+, Amersham Pharmacia Biotech). After 4h to overnight capillary blot, DNA in membrane is fixed by UV irradiation.

DENAT solution:	**RENAT solution:**	**20x SSC:**
1.5 M NaCl	1.5 M NaCl	3.0 M NaCl
0.4 M NaOH	282 mM Tris-HCl	0.3 M sodium citrate x $2H_2O$
	218 mM Tris-Base	Set pH to 7 with HCl

Gene-specific probes were labeled by incorporation of digoxigenin-11-dUTP (DIG) during PCR. A PCR mixture used was 10 to 100 pg plasmid DNA or 100 ng of genomic DNA, 10 µl PCR buffer, 5 µl PCR DIG labeling mix (Roche), 20 pmol each of the two oligonucleotides and 0.5 µl Phusion polymerase. The reaction was performed in a thermocycler (PTC200, MJ Research or Labcycler, SensoQuest) similar to a standard PCR reaction. The blotted Hybond-N+ membranes were preincubated for saturation of nonspecific binding sites with Southern hybridization buffer for 20 min at 65 °C. The probes are denatured at 95 °C for 5 min and added in pre-warmed hybridization buffer.

Denhardt's solution:	**Southern hybridization buffer:**
2% (w/v) BSA fraction V	26% (v/v) SSPE (20x)
2% (w/v) Ficoll	5% (v/v) Denhardt's solution

2% (w/v) PVP (Polyvinyl Pyrrolidone, SIGMA PVP-360)	5% (v/v) SDS (10%)

This probe solution was added to the membranes and hybridized overnight at 65 °C. Subsequently, the membranes were washed for 15 min each at 65 °C with different wash buffers as given below:

20x SSPE buffer:	**Southern wash buffer I:**	**Southern wash buffer II:**	**Southern wash buffer III:**
3 mM NaCl, 227 mM NaH_2PO_4 x H_20 20 mM Na_2EDTA x $2H_2O$ Set pH to 7.4 with NaOH	2x SSPE (20x) 0.1% SDS (10%)	1x SSPE (20x) 0.1% SDS (10%)	0.1x SSPE (20x) 0.1% SDS (10%)

For detection, the membrane was incubated at room temperature in the following solutions: 5 min DIG Wash buffer, 30 min DIG2 solution, 30 min DIG2 antibody solution (1:20,000 anti-digoxigenin antibody Fab fragments (Roche) in DIG2 solution) and twice for 15 min in DIG Wash buffer. Thereafter, the membrane was equilibrated for 5 min in DIG3 solution and then for 5 min in chemiluminiscent substrate 1:100 CDP-Star (Roche) in DIG3 solution. The detection of chemiluminescence was performed in a LAS4000 ImageQuant (GE Healthcare Life Science).

DIG1:	**DIG2:**	**DIG3:**	**DIG Wash:**
0.1 M maleic acid 0.15 M NaCl Set pH to 7.5 using NaOH	1%(w/v) skimmed milk powder in DIG1	0.1 M Tris-HCl 0.1 M NaCl Set pH to 9.5	0.3% (v/v) Tween20 in DIG1 solution

4.2.2. Microbiological methods

4.2.2.1. Transformation of *U. maydis*

This protocol is modified according to [Schulz et al., 1990] and [Gillissen et al., 1992]. A starter culture was prepared by inoculation of some cell material from fresh CM plate into 3 ml YEPS light medium and incubated for 24 h at 28 °C on a rotary wheel. This starter culture was then diluted 1:2000 in 50 ml fresh YEPS light medium and allowed to grow at 28 °C, 200

rpm upto OD_{600} of 0.6 - 0.8. Then the cells were collected by centrifugation (3000 rpm, 5 min, 4 °C), washed once with 25 ml SCS. The washed cell pellet is then resuspended in 2 ml of Novozyme mix (3.5 mg /ml in SCS) and formation of protoplasts is monitored under a microscope. The rod shaped cells become spherical in shape after lysis of the cell wall. When around 40% cells became spherical or like protoplasts (3 - 10 min), 10 ml ice cold SCS was added and the protoplasts were pelleted by centrifugation at 2500 rpm, 4 °C, for 5 min. The wash cycle was repeated 2x with SCS to completely remove the novozyme. The protoplast pellet was then washed with 10 ml of STC in similar way. Then the pellet was resuspended in 1 ml of ice cold STC and aliquots were prepared and stored at -80 °C for several months till use.

100 µl aliquot of protoplasts was used for the transformation of protoplasts. After thawing, it was mixed with 1 to 5 µg of linearized plasmid DNA and 1 µl heparin solution for 10 min, on ice. For a transformation with free replicating plasmids, 250 - 500 ng of circular plasmid DNA was used. After addition of 0.5 ml of STC/PEG, mix was further incubated for 15 min on ice. Then, the entire transformation mixture was plated on a recently prepared two layered regeneration agar plate (Bottom layer with appropriate antibiotic, top layer with no antibiotic). After 4 - 10 days of incubation at 28 °C, the grown colonies were isolated on CM plates containing appropriate antibiotics to test the acquired resistance of strains due to transformed plasmid.

SCS:	**STC:**	**STC/PEG**
20 mM sodium citrate, pH 5.8	10 mM Tris-Cl, pH 7.5	15 ml of STC
1 M sorbitol (Sigma S-1876)	100 mM $CaCl_2$	10 g of PEG4000
in ddH_2O, Autoclave	1 M sorbitol in ddH_2O, Autoclave	

4.2.2.2. Induction of filamentous growth

AB33 derived strains were grown in CM liquid medium overnight at 28 °C, 200 rpm to an OD_{600} of 0.5. The cells were harvested by centrifugation 3000 rpm, 3 min, RT (Heraeus Biofuge) and cell pellet was washed in ddH_2O. Then the cells were resuspended in appropriate volume of NM-Glc liquid medium to OD of 0.2 to 0.5 and incubated for 6 - 16 h at 28 °C, 200 rpm for filamentous growth.

4.2.2.3. Microscopy of yeast-like cells and filaments of *U. maydis* strains

For microscopy of yeast-like cells, cell material from fresh plates were inoculated into 3ml CM liquid medium and shaken on rotary wheel overnight to obtain dense starter culture (OD_{600} 4 - 5). Then a 1:2000 dilution was prepared in fresh CM medium using this starter culture and allowed to grow at 28 °C, 200 rpm up to the OD_{600} of 0.5 - 0.6. 2 µl of this culture was mounted on agarose layered glass slide and the morphology of cells was observed under 63x EC-Plan Neofluar oil immersion objectives on Axio Imager M1 microscope (ZEISS) using DIC (differential interference contrast microscopy). Digital images of the cells were taken using a CCD camera Pursuit (SPOT) and using the program MetaMorph (version 7).

For microscopy of filaments, filamentous growth was induced for 6 h as described before. 2 µl of the culture was then used for analysis.

4.2.3. Protein biochemical methods

4.2.3.1. Protein extraction from yeast-like cells

Yeast-like cells were grown to an OD_{600} of 0.75 in CM-Glc medium. After harvest (5,000 g, 5 min, 4 °C) cells were resuspended in 2 ml ice-cold PBS (phosphate buffered saline, pH 7.4, 2x complete protease inhibitor cocktail, Roche). The cell suspension was subsequently frozen in liquid nitrogen and proteins were extracted using a Retsch pebble mill (10 min, 30 Hz, 4 °C). Cell debris was then pelleted at 13,000 g (30 min, 4 °C). Protein concentrations in the extracts were determined by Bradford assays (Bradford, 1976).

4.2.3.2. Protein enrichment from culture supernatants

For the enrichment of Cts1 fusion proteins from culture supernatants yeast-like cells were grown to an OD_{600} of 0.75. Supernatants were harvested by centrifugation (5,000 g, 5 min, 4 °C) and subsequently filtered (MN 615¼ filter paper, Macherey-Nagel). Proteins of the cell-free supernatants were precipitated using 10% TCA (2 h, on ice) followed by washing the pellets with ice-cold acetone. Centrifugation steps were carried out at 4 °C (30 min, 8,000 g). Finally, the pellets were dissolved in SDS sample buffer, boiled for 10 min and used for Western blot analysis.

For the enrichment proteins from filamentous cultures, filamentation was induced for six hours. The original protocol [Brachmann et al., 2001] for filament induction was modified

such that yeast cells were grown to an OD_{600} of 0.5 and subsequently shifted to an OD_{600} of 1 in nitrate-containing NM medium supplemented with 1.5% (w/v) glucose [Holliday 1974]. Similar procedure was then used for sample preparation and analysis as explained above.

4.2.3.3. Fluorimetric determination of Gus activity

Gus activity in culture supernatants of yeast-like cells and filaments was determined using the fluorogenic substrate 4-methylumbelliferyl β-D-glucuronide hydrate (MUG, Sigma-Aldrich; Stock et al., 2012). Cell-free culture supernatants of yeast-like cells grown for sixteen hours (final OD_{600} of about 2.0) were used for this assay. Cell-free culture supernatants of filament cells induced for sixteen hours (starting OD_{600} 0.2) were used for the assay. All activities were determined in technical triplicates. 4-methylumbelliferone (MU, Sigma Aldrich), the fluorescent product formed in the presence of Gus, was used to generate a calibration curve. Enzyme activities of mutants were compared to AB33 strain by plotting fluorescence/OD [Stock et al., 2012].

4.2.3.4. Protease-activity plate assay

Protease activity was assayed on minimal medium plates containing casein. Therefore, yeast-like cells were grown to an OD_{600} of around 0.75 in CM-Glc medium. The cells were washed once in sterile water and adjusted to an OD_{600} of 3. 3 µl of each sample were spotted on AM-Glc plates containing 1% (w/v) casein. Plates were incubated for 2 to 3 days at 28 °C until halo formation was observed.

For production of the indicator plates, a two-fold concentrated casein solution was prepared following published protocols [Berger et al., 1983, Cowan and Daniel 1982] with slight modifications: 2% (w/v) casein was dissolved in 0.1 N NaOH by gentle heating and stirring. After dissolving the pH was adjusted to 8.0 with HCl and the solution was sterilized in a 100 °C water bath for 30 min. The solution was mixed in a 1:1 ratio with warm two-fold concentrated sterile AM-Glc medium and used for pouring plates.

4.2.3.5. Western blot analysis

10 µg of denatured protein extracts or protein precipitated from defined volumes of culture supernatants were used for SDS-PAGE and transferred to a methanol-activated PVDF membrane. Cts1 fusion proteins were detected with primary α-HA antibodies (Roche;

1:3,000) and a secondary α-mouse IgG HRP conjugate (H+L; Promega; 1:5,000). HRP activity was detected using AceGlow (Peqlab) and a LAS4000 Mini chemiluminescence imager (Fuji).

4.2.3.6. Non-reducing SDS PAGE

The samples are treated in a way that di-sulfide bonds should not be destroyed in the protein if present. No β-mercaptoethanol is added in protein sample and the samples are not boiled for such non-reducing SDS PAGE.

4.2.3.7. Purification of scFv-Cts1

His-tagged scFv-Cts1 was purified by immobilized metal affinity chromatography (IMAC). To this end, yeast-like cells were grown to an OD_{600} of 0.6 to 0.75 in CM-Glc medium. Ni^{2+}-NTA slurry (Qiagen) was equilibrated with lysis buffer (50 mM sodium phosphate, pH 8.0, 300 mM NaCl) supplemented with 10 mM imidazole. Cell-free supernatant was adjusted to pH 8.0 using 10x lysis buffer. 2 ml slurry was added to 200 ml cell-free supernatant and stirred for one hour at 4 °C in a batch procedure. The suspension was then passed through a column and washed with three column volumes each of lysis buffer supplemented with 10 mM and 20 mM imidazole. Finally, bound protein was eluted stepwise in 500 ml fractions using lysis buffer supplemented with 250 mM and 500 mM imidazole. Fractions were checked by Western blot analysis for presence of full-length scFv-Cts1.

4.2.3.8. Expression and purification of proteins from *E.coli*

For production of purified Gst-MH and Gst-H, the *E. coli* strain Rosetta 2 (DE3) pLysS (Novagen) was transformed with the plasmids pUMa1531 and pUMa1562, respectively. Protein expression was induced with 0.5 mM IPTG for 2 hours at 37 °C and the purification was performed according to established protocols, using Ni^{2+}-NTA metal-affinity chromatography (The QIAexpressionist, Qiagen). Purity of the proteins was judged by SDS PAGE analysis and protein concentrations were determined by Bradford assays [Bradford 1976].

For production of purified Gfp-H, *E. coli* strain Rosetta 2 (DE3) pLysS (Novagen) was transformed with the plasmids pUMa2156. Later, similar steps were followed as described above.

4.2.3.9. Dot-Blot analysis

10 µg Gst-myc-his and Gst-his antigen proteins purified from *E. coli* [Stock et al., 2012] were spotted on a methanol-activated PVDF membrane. After the spots were dried, the membranes were incubated in PBS-T (PBS pH 7.4, 0.5% v/v Tween20) with 3% w/v skimmed milk for one hour at room temperature. Then the membranes were incubated with IMAC-purified scFv-Cts1 obtained from 200 ml culture supernatant overnight at 4 °C including 3% w/v skimmed milk. After washing with PBS-T bound scFv-Cts1 was detected using a primary mouse α-HA antibody (Roche, 1:2,000 in PBS-T/3% w/v skimmed milk, 2 h, room temperature) and a secondary α-mouse IgG-HRP conjugate (H+L, Promega; 1:5,000 dilution in PBS-T/3% w/v skimmed milk, 1 h, room temperature). HRP activity was detected using Ace Glow (Peqlab) and a LAS4000 Mini chemiluminescence imager (Fuji).

4.2.3.10. Indirect enzyme-linked immunosorbent assay (ELISA)

For ELISA, microtiter plates (Maxisorb, Nunc) were coated for two hours with 10 µg/well Gst-MH (+ epitope) in 100 mM bicarbonate coating buffer (pH 9.6) at room temperature. Similar amounts of purified Gst-H (– epitope) were used as negative control (Stock et al., 2012). After washing thrice with PBS-T, plates were blocked with 3% (w/v) skimmed milk dissolved in PBS (MPBS) for two hours at room temperature. The purified scFv-Cts1 obtained after IMAC was concentrated using Amicon 3k tubes and different concentrations of the sample were applied to the wells in the presence of 2% w/v MPBS overnight at 4 °C. After washing, a mouse α-HA antibody (Roche, 1:2,000 dilution in 3% MPBS) was added (2 h, room temperature), followed by an α-mouse IgG-HRP conjugate (H+L, Promega; 1:5,000 dilution in PBS-T/3% w/v skimmed milk) to detect binding of the scFv-Cts1 to the myc tag (1 h, room temperature). After washing, the reaction was developed with QuantaRed Enhanced Chemifluorescent HRP substrate and the fluorescence measured at excitation/emission wavelengths of 570 and 600 nm, respectively (Tecan Safire, Magellan Software).

To check the activity of α-Gfp Nb using ELISA, microtiter plates (Maxisorb, Nunc) were coated for two hours with 2 µg/well Gfp-H (+ epitope) in 100 mM bicarbonate coating buffer (pH 9.6) at room temperature. Similar amounts of Bsa (– epitope) was used as negative control. Further steps were followed same as described above.

4.2.3.11. Silver staining of supernatant proteins

50 ml cultures of yeast-like cells were grown overnight in CM-Glc to an OD_{600} of 0.75. The culture supernatant was harvested by centrifugation (5,000 g, 5 min, 4 °C). To remove residual cells, the supernatant was passed through paper filters (MN 615¼ filter paper, Macherey-Nagel) and subsequently concentrated 50-fold using Amicon tubes (30 kDa exclusion size; Millipore). 25 µl of the sample were subjected to SDS PAGE followed by silver staining [Blum et al., 1987].

4.2.3.12. Modified Western blot analysis for α-Gfp Nb activity

Cell extracts of *U. maydis* strain expressing 3xGfp (Uma587) was used as an antigen while the cell extracts of AB33 served as an antigen control. Defined amounts of cell extracts were run on SDS gel and later immuno-blotted on PVDF membrane. The membrane was then incubated with 3% blocking buffer (PBS-T/3% w/v skimmed milk). Followed by incubation with IMAC-purified protein from cell extracts of AB33 and AB33_kex2Δ α-Gfp Nb-Cts1 strains (Methods Section. 4.2.3.7). These purified elutes served as 1° antibody (overnight binding). After washing the membranes using 1xPBST, antigen-antibody complex was detected using Mouse α-HA and then secondary α-mouse IgG HRP conjugate (H+L; Promega; 1:5,000). HRP activity was detected using AceGlow (Peqlab) and a LAS4000 Mini chemiluminescence imager (Fuji).

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Author contributions

This dissertation is based on three publications as mentioned below with their respective status. This declaration is intended to clarify the contribution of Parveen Sarkari to these publications:

(1) Applying unconventional secretion of the endochitinase Cts1 to export heterologous proteins in *Ustilago maydis,* 2012, **Janpeter Stock, Parveen Sarkari, Saskia Kreibich, Thomas Brefort, Michael Feldbrügge, Kerstin Schipper**, *Journal of Biotechnology*, 161(2), 80–91 (Research on Industrial Biotechnology within the CLIB-Graduate Cluster - Part II).

(2) Improved expression of single-chain antibodies in *Ustilago maydis*, 2014, **Parveen Sarkari, Michèle Reindl, Janpeter Stock, Olaf Müller, Regine Kahmann, Michael Feldbrügge, Kerstin Schipper**, *Journal of Biotechnology*, doi: 10.1016/j.jbiotec.2014.06.028 (In Press, Corrected Proof).

(3) The corn smut fungus *Ustilago maydis* as an alternative expression system for biopharmaceuticals, **Parveen Sarkari, Michael Feldbrügge and Kerstin Schipper.** In: *Fungal Biology. Gene Expression Systems in Fungi: Advancements and Applications* (Schmoll and Dattenböck, eds), Springer Book Series. Invited review, submitted.

The following sections in this thesis are mainly based on the above publications as mentioned below:

Introduction:

Sections 1.1 to 1.5 excluding Figure 1, 3, and 4; and Figure 6c were taken from publication (3). The text was mainly written by Parveen Sarkari.

Results:

Section 2.1 was taken from (1) publication with few modifications (Figure 5b, c, and f). Work on scFv expression was carried out by Parveen Sarkari while work on establishment of expression system including the generation of expression plasmids and Gus expression strains were carried by Janpeter Stock. Work on establishing the Gus assay was carried out by Janpeter Stock, Saskia Kreibich and Thomas Brefort.

Sections 2.2, 2.4, 2.5 (Figure 10a modified), and 2.6 were taken from publication (2). In these sections, work described in Section 2.5 was partially carried out by Michèle Reindl during her Bachelor thesis. Work described in the remaining sections mentioned above was done by Parveen Sarkari. Olaf Müller and Regine Kahmann contributed instrumentally to the Kex2 part by providing information on a *kex2* deletion strain generated in another strain background.

Sections 2.3 and 2.7 describe the unpublished work carried out by Parveen Sarkari during her doctoral studies.

The summary was translated by Dr. Kerstin Schipper (Zusammenfassung).

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