Calcium signal generation and Transient Receptor Potential channel homologues in the fungal pathogen *Colletotrichum graminicola*

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1 General Introdution

1.1 Biology of *Colletotrichum graminicola*

Colletotrichum graminicola is an ascomycete fungus belonging to the pezizomycotina within the class of sordariomycetes [Lumbsch and Huhndorf, 2007]. The genus *Colletotrichum* harbours a large range of plant pathogens infecting a broad spectrum of hosts [Perfect et al., 1999]. *C. graminicola* is a hemibiotrophic pathogen of maize that can lead to drastic yield losses [Bergstrom and Nicholson, 1999]. In its native environment it survives the winter season on post-harvest residues of maize where it grows saprophytically and forms large numbers of spore-containing acervuli [Bergstrom and Nicholson, 1999]. Within these acervuli, the spores are embedded in protective mucilage. This gel contains glycoproteins preserving the spores from desiccation, as well as proline-rich proteins that can bind and thereby inactivate toxic polyphenols [Hagerman and Butler, 1981; Nicholson and Moraes, 1980; Ramados et al., 1985]. Moreover, in the mucilage there is a germination inhibitor, mycosporine-alanine, which prevents spore germination within the acervulus [Leite and Nicholson, 1992]. In spring, splashing rain drops can transmit spores from the acervuli to young maize plants [Bergstrom and Nicholson, 1999]. The rain also dilutes mycosporine-alanine below its effective concentration [Leite and Nicholson, 1992]. Germination of spores is initiated when they sense a contact with the hydrophobic maize leaf. A first sign of germination is the secretion of heavily mannosylated glycoproteins that fix the spores to the maize leaf [Mercure et al., 1994]. After a few hours a short germ tube emerges, and at its apex a specialized cell named appressorium is formed [Howard, 1997]. The appressorial cell wall is strongly melanized and therefore very rigid [Horbach et al., 2009]. The appressorium also secretes adhesive compounds to get fixed to the maize leaf [Sugui et al., 1998]. Next, this cell generates, in an orchestrated way, a high turgor pressure of over 5 MPa in concert with the secretion of hydrolytic enzymes to penetrate the maize leaf epidermis by an infection hypha [Bechinger et al., 1999; Nicholson et al., 1976; Pascholati et al., 1993]. Thereafter, an infection vesicle is formed between cell wall and plasma membrane of the infected epidermal

maize cell. After a short period of time, primary hyphae, that still do not destroy the host's cell membrane, emerge. This developmental stage is named biotrophic as no maize cell is killed so far [Bergstrom and Nicholson, 1999]. During this stage the fungal cell wall does not contain β-1,3-glucan and there are no siderophores secreted, both in order to avoid recognition of the fungus by the host [Albarouki et al., 2014; Oliveira-Garcia and Deising, 2013]. This specific regulation therefore prevents defence reactions of the host. During the later necrotrophic phase, the fungus forms secondary hyphae that actively kill maize cells. In this stage the fungus grows very aggressively, secretes siderophores to acquire iron, and has a strong, β-1,3-glucan-containing cell wall [Albarouki et al., 2014; Oliveira-Garcia and Deising, 2013]. After some days of necrotrophic growth new acervuli containing many spores are formed. The next rain will start the cycle again. *C. graminicola* thereby spreads from maize plant to maize plant as well as within the same plant. The fungus can infect the stems in a similar way as the plant's leaves. In addition, insect vectors, like the larva of the European corn borer, can transmit *C. graminicola* deep into the stem of maize plants [Bergstrom and Nicholson, 1999]. The severity of infection pressure within the field depends on weather, genotype, and mineral nutrition [Bergstrom and Nicholson, 1999; Ye et al., 2014]. In autumn the cycle closes: *C. graminicola* grows saprophytically on post-harvest residues and sporulates to allow infection in the next year [Bergstrom and Nicholson, 1999].

In the course of the infection cycle and the year, *C. graminicola* grows in the lifestyles of biotrophy, necrotrophy, and saprophytism. These adaptations are associated with differential gene expression, changes in cell wall composition, and different iron acquisition strategies [Albarouki and Deising, 2013; O'Connell et al., 2012; Oliveira-Garcia and Deising, 2013]. To allow these responses, the fungus needs to perceive its environment, and the resulting signals have to be transmitted within the cell. In this signalling process, Ca^{2+} is a pivotal second messenger, the importance of which has been described for many fungi [Nguyen et al., 2008; Wang et al., 2012].

1.2 Calcium signals in fungi - the peak and its physiological relevance

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1.2.1 Abstract

The key players of calcium signal generation, i.e. Ca^{2+} channels, Ca^{2+}/H^{+} antiporters, and Ca^{2+} ATPases, are present in all fungi. They keep a low Ca^{2+} baseline, allow a fast increase in Ca^{2+} concentration upon a stimulus, and terminate the elevation by an exponential decrease - hence forming the typical Ca^{2+} peak. In this respect, there are only nuances between different fungi. However, does the similarity of the genetic inventory that shapes the Ca^{2+} peak imply that "I've seen one, I've seen 'em all" in terms of physiological relevance? This review focuses, as the original literature does, on this aspect, highlighting common and divergent functions of homologous proteins. In conclusion, for the physiological role of these key players, "seen one", in many cases, does not mean: "seen 'em all".

1.2.2 Introduction

Stimuli from outside and inside the fungus require the initiation of appropriate responses [Read, 2007]. Many of these stimuli, including touch, changes in osmolarity or carbon source, exposure to oxidizing agents or xenobiotics, as well as the initiation and continuation of hyphal growth, branching and spore germination, are transmitted by calcium (Ca^{2+}) signaling [Binder et al., 2010; Gonçalves et al., 2014; Harren and Tudzynski, 2013; Kim et al., 2012; Lange and Peiter, 2016; Nelson et al., 2004]. Ca^{2+} signals are usually characterized by a sharp rise in free cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) concentration that is followed by an exponential decrease. A low basal level of $[Ca^{2+}]_{cyt}$ is maintained by Ca^{2+} pumps and antiporters that export Ca^{2+} or sequester it into organelles [Dinamarco et al., 2012; Fan et al., 2007; Hu et al., 2014; Kmetzsch et al., 2010; Lustoza et al., 2011; Yang et al., 2001]. In response to a stimulus Ca^{2+} channels open and Ca^{2+} enters the cytosol from the extracellular space or intracellular stores [Harren and Tudzynski, 2013; Muller et al., 2003; Zhou et al., 2005]. Ca^{2+}-sensitive Ca^{2+} channels may further amplify the signal by Ca^{2+}-induced Ca^{2+} release (CICR) [Gonçalves et al., 2014]. Finally, Ca^{2+}/H^{+} antiporters and Ca^{2+} pumps decrease the $[Ca^{2+}]_{cyt}$ again to the basal level [Demaegd et al., 2013; Kmetzsch et al., 2013]. The amplitude, duration, shape,

and number of Ca^{2+} peaks vary with stimulus and organism [Gonçalves et al., 2014; Harren and Tudzynski, 2013; Kim et al., 2012; Lange and Peiter, 2016].

These signals are decoded and modulated by Ca^{2+}-sensitive fungal proteins, such as calmodulin (CaM) and calcineurin (CN). CaM binds to a variety of CaM-dependent proteins and modulates their activity by Ca^{2+}-induced conformational changes, as reviewed by [Brown et al., 2007]. The protein phosphatase calcineurin (CN) is activated by Ca^{2+} itself and by CaM. CN activates the transcription factor Crz1 by dephosphorylation [Choi et al., 2009].

In recent years, a considerable number of studies have elucidated the processes that contribute to the generation of Ca^{2+} signals in fungi (Fig. 1.1). Here, these mechanisms will be reviewed with a particular focus on functional similarities and differences between species.

1.2.3 Ca^{2+} channels: Starting the burst upon a stimulus

1.2.3.1 Acquiring extracellular Ca^{2+} for signals in low [Ca^{2+}] environments by the high affinity Ca^{2+} uptake system (HACS) in the plasma membrane

In *Saccharomyces cerevisiae*, a HACS for Ca^{2+} is formed by a homolog of mammalian voltage-gated Ca^{2+} channels, Cch1 [Fischer et al., 1997], and an interacting membrane protein, Mid1 [Locke et al., 2000]. Coherently, most phenotypes of deletions in either or both HACS genes are identical. Ca^{2+} entry via HACS is important for yeast to survive ionic, ER, cold, and iron stress, as well as for mating and survival in low [Ca^{2+}] media [Bonilla et al., 2002; Fischer et al., 1997; Matsumoto et al., 2002; Peiter et al., 2005a]. Ecm7 is another subunit of the HACS and needed for HACS-mediated Ca^{2+} influx in response to high pH and fungicides, but not during mating [Martin et al., 2011]. Ecm7 is protected from protein degradation by Mid1, while Mid1 is protected from protein degradation by Cch1 [Martin et al., 2011]. The cell integrity MAP kinase pathway activates Cch1 during ER stress and another yet not specified kinase during mating [Bonilla and Cunningham, 2003]. The Ca^{2+}-activated phosphatase CN negatively regulates Cch1 in *S. cerevisiae* and *Schizosaccharomyces pombe* [Bonilla and Cunningham, 2003; Ma et al., 2011]. Next to plasma membrane (PM) localization, Mid1 resides also in the ER and may form a stretch-activated Ca^{2+} channel without Cch1, but its physiological relevance of this is unclear [Kanzaki et al., 1999; Yoshimura et al., 2004].

In filamentous fungi, HACS is important for colony growth, especially in low- [Ca^{2+}] media. This was shown for *Cch1* in *Fusarium graminearum* [Hallen and Trail, 2008], for *Mid1* in

Aspergillus fumigatus [Jiang et al., 2014b], *F. graminearum* [Cavinder et al., 2011] and *Neurospora crassa* [Lew et al., 2008], and for both genes in *Aspergillus nidulans* citeWang2012 and *Botrytis cinerea* [Harren and Tudzynski, 2013]. In the dimorphic, animal pathogenic fungus *Candida albicans Cch1* is more important for colony growth in Ca^{2+}-limited environments than *Ecm7* [Ding et al., 2013]. *Mid1* deletion in *Claviceps purpurea* also results in poor growth, but low [Ca^{2+}] actually rescues this partially [Bormann and Tudzynski, 2009]. Reduced growth is associated with hyperbranching in *A. fumigatus* [Jiang et al., 2014b], *A. nidulans* [Wang et al., 2012] and *N. crassa* [Lew et al., 2008] but not in *B. cinerea* [Cavinder et al., 2011] and *C. purpurea* [Bormann and Tudzynski, 2009]. In *C. albicans* (but not in *N. crassa*), surface recognition also

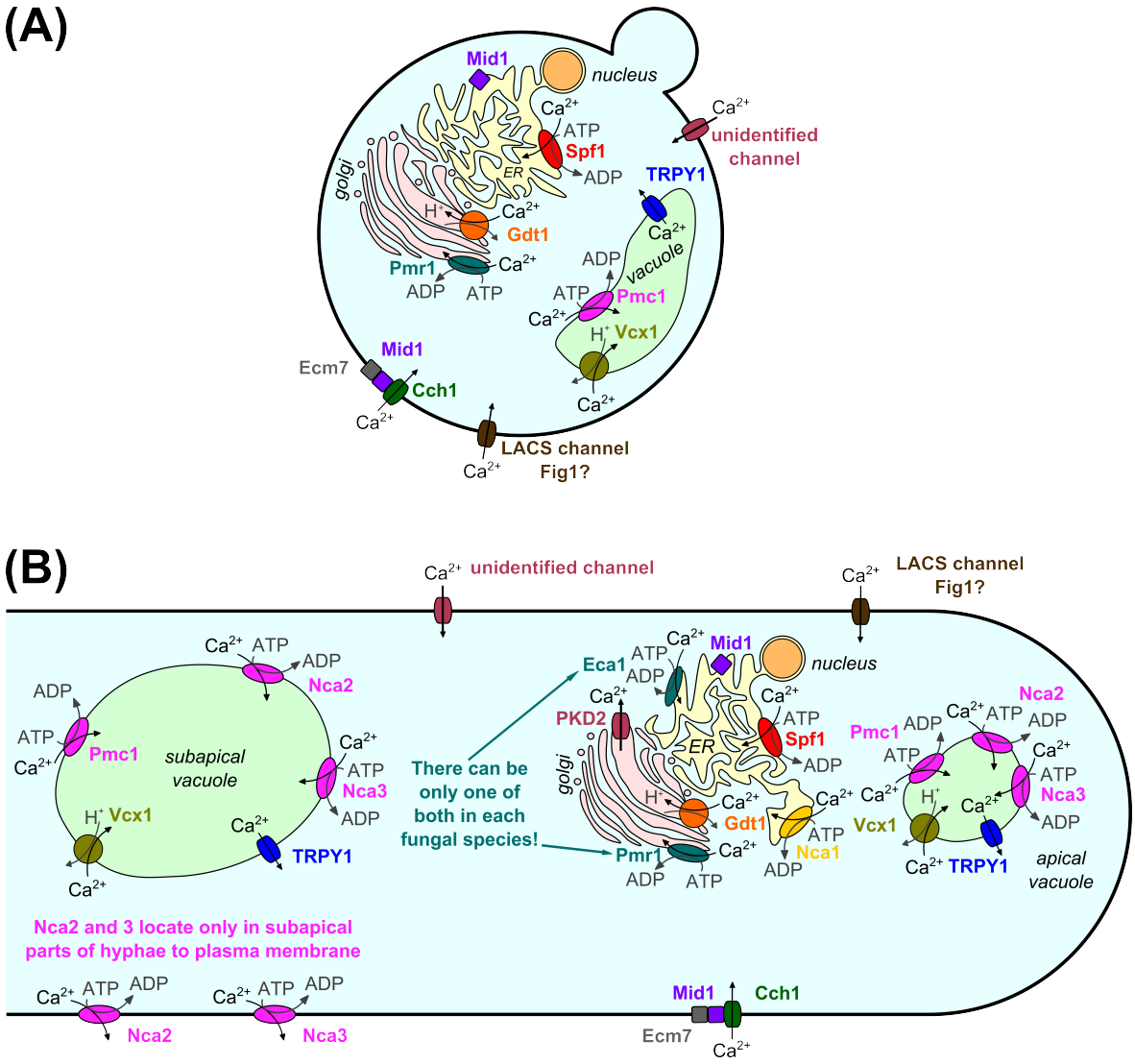

Fig. 1.1: *Subcellular localization of Ca^{2+} channels, Ca^{2+}/H^+ exchangers, and Ca^{2+} ATPases in* S. cerevisae *(A) and other fungi (B). Homologs are depicted in identical colors. Subcellular localizations are tshown as described or assumed in the literature. Note the more complex localization pattern and larger number of protein family members in non-yeast fungi.*

relies on HACS [Brand et al., 2007; Lew et al., 2008].

The relevance of HACS components for stress resistance is variable: Ionic stress tolerance is independent of *Mid1* in *A. fumigatus* [Jiang et al., 2014b] and *F. graminearum* [Cavinder et al., 2011], in contrast to *S. cerevisiae* and *Cryptococcus neoformans* [Liu et al., 2006]. Contrarily, both *Cch1* and *Mid1* are important to withstand oxidative stress in *A. fumigatus*, especially in media with low [Ca^{2+}] [de Castro et al., 2014; Jiang et al., 2014b]. A deletion of *Mid1* leads to increased sensitivity to cell wall stress in *C. purpurea* [Bormann and Tudzynski, 2009], while in *A. fumigatus* [Jiang et al., 2014b] there is no effect. In *A. nidulans* there is even an increased resistance to cell wall stress when *Cch1* or *Mid1* are deleted [Wang et al., 2012]. Deletions in *Cch1* or *Mid1* render *C. albicans* hypersensitive towards the fungicides ketoconazol and tebuconazol, Ecm7 deletion has less impact [Xu et al., 2015]. In *A. fumigatus*, the importance of *Cch1* and *Mid1* for resistance to a number of fungicides ranges from beneficial to adverse, with *Cch1* tending to be more important [de Castro et al., 2014].

The importance of HACS for sporulation and virulence also varies: *Cch1* and Mid1 are important for sporulation in *A. nidulans* [Wang et al., 2012] and *F. graminearum* [Cavinder et al., 2011; Hallen and Trail, 2008]. In *Magnaporthe oryzae*, *Cch1* is pivotal for sporulation, while a knock down of Mid1 shows only moderate defects [Nguyen et al., 2008]. In contrast, in *B. cinerea* both genes are dispensable for sporulation [Harren and Tudzynski, 2013]. In *N. crassa* the impact of *Mid1* on sporulation is debated [Cavinder et al., 2011; Lew et al., 2008]. With respect to virulence, the effect of deletion of HACS genes ranges from apathogenicity in *C. purpurea mid1*Δ [Bormann and Tudzynski, 2009] and *C. albicans cch1*Δ or *mid1*Δ [Yu et al., 2012a] via reduced virulence in *C. albicans ecm7*Δ [Yu et al., 2012a] and *A. fumigatus cch1*Δ or *mid1*Δ [de Castro et al., 2014], slight reduction in *M. oryzae cch1*Δ or *mid1*Δ [Nguyen et al., 2008] to no effect of *cch1*Δ or *mid1*Δ in *B. cinerea* [Harren and Tudzynski, 2013] and *F. graminearum* [Cavinder et al., 2011; Hallen and Trail, 2008].

In summary, the role of HACS for growth in low [Ca^{2+}] environments is widely conserved, but other functions vary widely between fungal species and the HACS genes, *Cch1*, *Mid1* and *Ecm7*.

1.2.3.2 The low-affinity Ca^{2+} uptake system (LACS): a stepchild compared to HACS

The molecular identity of LACS is still unclear. The PM protein Fig1 has been proposed to be either the LACS Ca^{2+} channel or an important regulator of it, and is needed for Ca^{2+} influx during mating and cell fusion in *S. cerevisiae* and *C. albicans* [Alby et al., 2010; Muller et al.,

2003; Yang et al., 2011].

Fig1 is important for vegetative growth and asexual reproduction in the filamentous fungi *F. graminearum* [Cavinder and Trail, 2012] and *A. nidulans* [Palma-Guerrero et al., 2015], but not in *N. crassa* [Zhang et al., 2014]. In these three fungi, Fig1 is needed for sexual fertility and for cell fusion of spore germlings [Cavinder and Trail, 2012; Palma-Guerrero et al., 2015; Zhang et al., 2014]. Hence, sexual reproduction is a core function of Fig1, while others are species-specific.

The PM protein Rch1 is a negative regulator of LACS, but not HACS in *C. albicans* [Alber et al., 2013], while it is also suggested as a suppressor of Ca^{2+} uptake in *S. cerevisiae* and *C. albicans* via HACS [Zhao et al., 2016], and shown to be important for growth at high $[Ca^{2+}]_{medium}$ [Alber et al., 2013; Zhao et al., 2016]. In *C. albicans*, Rch1 is essential for full virulence [Xu et al., 2015].

1.2.3.3 Ca^{2+} signals may be sourced from intracellular stores

The by far best-characterized intracellular Ca^{2+} channel of *S. cerevisiae* is TRPY1 (synonym Yvc1), a member of the Transient Receptor Potential family [Palmer et al., 2001]. TRPY1 locates to the vacuolar membrane [Denis and Cyert, 2002], is activated by stretch [Zhou et al., 2003], and amplifies hyperosmotic shock-triggered Ca^{2+} signals by CICR [Su et al., 2009]. In these respects, TRPY1 homologs of Kluyveromyces lactis [Zhou et al., 2005], *C. albicans* [Zhou et al., 2005], as well as of the filamentous fungus *F. graminearum* [Ihara et al., 2013] resemble ScTRPY1. However, the channel of *F. graminearum*, but not of *S. cerevisiae*, is negatively regulated by inositol phosphates [Ihara et al., 2013]. In *C. albicans* TRPY1 is needed to survive oxidative stress [Yu et al., 2014b], while the opposite is true for *S. cerevisiae* [Popa et al., 2010]. In *C. albicans* [Yu et al., 2014a] and *A. fumigatus* [de Castro et al., 2014], TRPY1 is also needed for biofilm formation and virulence, but not for growth on agar. In contrast to all other fungal species analysed so far, *M. oryzae* requires TRPY1 not only for virulence, but also for axenic growth on agar [Nguyen et al., 2008].

In summary, the involvements of TPRY1 homologs are highly diverse in the species studied so far.

1.2.4 Ca^{2+}/H^{+} antiporters: High-capacity, low-affinity Ca^{2+} sequestration

1.2.4.1 Ca^{2+}/H^{+} exchange over the vacuolar membrane

Vcx1 is a low-affinity high-capacity Ca^{2+}/H^{+} antiporter in vacuolar membrane of *S. cerevisiae* that can also sequester Mn^{2+}, allowing cells to grow at very high extracellular concentrations of these ions [Cunningham and Fink, 1996; Pozos et al., 1996]. Vcx1 serves to recover basal $[Ca^{2+}]_{cyt}$ after a peak, while the vacuolar Ca^{2+} ATPase Pmc1 (see 1.2.5.1) maintains the basal $[Ca^{2+}]_{cyt}$ prior to a signal [Denis and Cyert, 2002]. This recovery is slowed down by a repression of Vcx1 by Ca^{2+}-activated CN [Rusnak and Mertz, 2000].

Vcx1 of *C. neoformans* also localizes to the vacuolar membrane [Kmetzsch et al., 2010]. The division of work between Vcx1 and Pmc1 into a mainly pre- and post-Ca^{2+} signal activity seems not to exist in this fungus. Here, both the antiporter and the ATPase maintain the $[Ca^{2+}]_{cyt}$ baseline and sequestrate cytosolic Ca^{2+} after a peak [Kmetzsch et al., 2013]. Coherently, *vcx1*Δ and *pmc1*Δ lead to a reduced virulence of this fungus [Kmetzsch et al., 2013, 2010]. A knockdown of *M. oryzae Vcx1* leads to slight reduction in growth speed and a clear reduction in sporulation and appressorium formation, but there is no effect on pathogenicity [Nguyen et al., 2008]. The filamentous fungus *Beauveria bassiana* has five *Vcx1* homologs. Deletion of individual genes results in an increased expression of remaining family members. This redundancy makes detailed functional analysis difficult and may explain the relatively weak reduction in virulence of the deletion strains of this fungus [Hu et al., 2014].

As the studies of *Vcx1* homologs of *S. cerevisiae* and *C. neoformans* indicate, there are conserved functions of fungal Ca^{2+}/H^{2+} antiporters. However, with respect of pathogenicity there are striking differences between species.

1.2.4.2 Ca^{2+}/H^{+} exchange over Golgi membranes

Gdt1 is a putative Ca^{2+}/H^{2+} antiporter of *S. cerevisiae* localized to membranes of the cis- and medial-Golgi, which is believed to be important for both, supplying the Golgi with Ca^{2+}, as well as for sequestration of high $[Ca^{2+}]_{cyt}$ [Colinet et al., 2016; Demaegd et al., 2013]. A Gdt1 homolog of *C. albicans* complements the respective deletion in *S. cerevisiae* [Wang et al., 2015]. Gdt1 has been suggested to remove Ca^{2+} from the cytosol also in this fungus, and the mutant shows a reduced virulence [Wang et al., 2015].

There is clearly more research required on the function and the physiological roles of the newly identified Gdt1 family in fungi.

1.2.5 Ca^{2+} ATPases: Keeping the cytosolic free Ca^{2+} concentration at a low basal level and supplying organelles with Ca^{2+}

1.2.5.1 Sequestering Ca^{2+} into the vacuole

The Ca^{2+} ATPase Pmc1 locates to the vacuolar membrane and mediates Ca^{2+} sequestration, which is essential for growth of *S. cerevisiae* [Cunningham and Fink, 1994], *S. pombe* [Furune et al., 2008], and *Hansenula polymorpha* [Fokina et al., 2012] in high (30 to 200 mM) [Ca^{2+}] media. In *H. polymorpha* [Fokina et al., 2012], but not in *S. cerevisiae* [Pozos et al., 1996], Pmc1 is also responsible to withstand SDS stress. Pmc1 activity is partially inhibited through protein-protein-interaction with Nyv1 at basal $[Ca^{2+}]_{cyt}$. Under conditions of high $[Ca^{2+}]_{cyt}$, expression of *Nyv1* stays constant, while *Pmc1* expression is induced via CaM-CN-Crz1 signaling to keep $[Ca^{2+}]_{cyt}$ stable [Takita et al., 2001].

A. fumigatus harbors three *Pmc1* homologs (*PmcA*, *PmcB*, *PmcC*), whereby only *PmcA* is essential for spore germination, growth at high [Ca^{2+}] in rich (but not in minimal) media, and virulence [Dinamarco et al., 2012]. Similarly, Pmc1 of *C. neoformans* is needed for normal growth on Ca^{2+}-supplemented rich media and for replication in its host [Kmetzsch et al., 2013]. The non-pathogenic filamentous fungus *N. crassa* has two Pmc-type Ca^{2+} ATPases, Nca2 and Nca3 (for Nca1 see 1.2.5.2). Both locate to the vacuolar membrane network (VMN) and subapically also to the PM, as their mammalian homolog. Nca2 is needed to supply the VMN and export Ca^{2+} out of the cell; it is beneficial for growth in minimal media with low [Ca^{2+}] and essential when $[Ca^{2+}]_{ext}$ is high. Female spores of *nca2*Δ strains are infertile and both genders produce less spores. Nca3 seems to be dispensable for growth, sporulation, and stress tolerance [Bowman et al., 2011].

In yeasts, Pmc1 is dispensable for normal growth but important under stressful conditions, whereas in other fungi this protein is important during the normal life cycle. The notion that Pmc1 homologs have evolved specific roles in their lifestyle may be supported by the localization pattern of Nca2 and Nca3. In *A. fumigatus* and *C. neoformans* mutants for Pmc1 homologs, the impact of high $[Ca^{2+}]_{ext}$ stress is greatly increased in richer media, the mechanism for which is unclear [Dinamarco et al., 2012; Kmetzsch et al., 2013]. Both, lifestyle and medium differences demand for a functional analysis of Pmc1 in further species.

1.2.5.2 Ca^{2+} ATPases supplying the Golgi or the ER with Ca^{2+} and Mn^{2+} and removing excessive amounts of these ions from the cytosol

The phylogeny of another group of Ca^{2+} ATPases is separated in two clades: The Golgi-localized Pmr branch and the ER-borne Eca arm [Adamíková et al., 2004; Antebi and Fink, 1992]. Fungi bear either Pmr or Eca members in their genomes [Fan et al., 2007].

Pmr1 is needed for growth of *S. cerevisiae* in high- [Ca^{2+}] media [Cunningham and Fink, 1994; Szigeti et al., 2005], and for growth of *H. polymorpha* [Agaphonov et al., 2007], *K. lactis* [Uccelletti et al., 1999], *Pichia pastoris* [Dux and Inan, 2006], *S. cerevisiae* [Antebi and Fink, 1992] and *Yarrowia lipolytica* [Park et al., 1998] in low- [Ca^{2+}] media. In *S. cerevisiae* it allows vitality of stationary phase cells independently of medium [Ca^{2+}] [Rudolph et al., 1989]. Furthermore, it is known to be essential for mannosylation of secreted proteins in *H. polymorpha* [Agaphonov et al., 2007] and *S. cerevisiae* [Antebi and Fink, 1992]. *pmr1*Δ strains of *C. albicans*, similar to yeasts, grow poorly on low- [Ca^{2+}] media, show mannosylation defects, a weaker cell wall, and therefore a massively reduced virulence in mice [Bates et al., 2005]. In contrast to *S. cerevisiae*, reduced vitality is recovered by high external [Ca^{2+}] [Bates et al., 2005].

Pmr1 of the plant-pathogenic filamentous fungus *B. cinerea* functions similar as in yeasts and Candida and is needed for normal sporulation and virulence [Plaza et al., 2015]. *A. fumigatus* bears three *Pmr1* homologs of which the most similar gene to *ScPmr1*, that complements a *pmr1Delta* yeast strain, was studied further [Pinchai et al., 2010]. The deletion mutant is affected in cell wall integrity and growth on standard medium, even more so when Ca^{2+} is limited, but displays only a minimal reduction in virulence [Pinchai et al., 2010]. In contrast to *S. cerevisiae*, osmotic stabilization completely rescues the growth defects, but Ca^{2+} and Mn^{2+} are ineffective [Pinchai et al., 2010]. Deletion of *AnPmrA* in the non-pathogenic fungus *A. nidulans* caused a less prominent growth defect that is partially recovered in osmotically stabilized media [Jiang et al., 2014a]. In the non-pathogenic fungus *N. crassa* and the insect pathogen *Beauveria bassiana*, deletion of *pmr1* again resulted in reduced colony growth and, in *B. bassiana*, a clearly reduced virulence. In contrast to *A. fumigatus*, the growth defect of the *pmr1*Δ strains of both fungi fully recovered on medium supplemented with Mn^{2+}, while Ca^{2+} was less efficient [Bowman et al., 2012; Pinchai et al., 2010; Wang et al., 2013].

The Eca1 branch of this Ca^{2+} ATPase family was first revealed in *Ustilago maydis*. Eca1 resides, similar to mammalian SERCAs, in the ER [Adamíková et al., 2004]. It was also found in *C. neoformans* [Fan et al., 2007]. In both fungi it is responsible for removing excessive Ca^{2+}

from the cytosol and for supplying the ER lumen with essential Ca^{2+} [Adamíková et al., 2004; Fan et al., 2007]. *Eca1* deletion therefore leads to reduced tolerance to ER stress and perturbations in cytosolic Ca^{2+} signaling due to aberrant $[Ca^{2+}]_{cyt}$ [Adamíková et al., 2004; Fan et al., 2007]. *Eca1* deletion causes aberrant cell division due to defects in cytoskeleton regulation, but no virulence defect, in *U. maydis* [Adamíková et al., 2004], whereas virulence of an *eca1*Δ strain was reduced in *C. neoformans* [Fan et al., 2007]. In *N. crassa*, the SERCA-type ATPase Nca1 also locates to the ER, but deletion causes no phenotype [Bowman et al., 2011]. In contrast, in *M. oryzae* knock down of *Nca1* results in a slight reduction of colony growth and, more importantly, a complete blockage of sporulation, rendering the fungus apathogenic [Nguyen et al., 2008].

In spite of the bipartite phylogenetic relationship of the Pmr and Eca Ca^{2+} ATPase family members, their molecular functions are quite similar. Moreover, there are only subtle functional differences within the Pmr branch between yeast and filamentous fungi. However, their relevance for virulence greatly differs between fungal species. These differences may be owing to differences in alternative Ca^{2+} uptake mechanisms into the Golgi, as discussed in section 1.2.4.2.

1.2.5.3 Spf1 - an emerging family of ER-localized Ca^{2+} ATPases

Spf1, another Ca^{2+}/Mn^{2+}-ATPase is localized to the ER membrane of *S. cerevisiae*, supplies the ER lumen with Ca^{2+}/Mn^{2+}, and removes excessive cytosolic amounts of these ions [Cohen et al., 2013; Cronin et al., 2000; Suzuki and Shimma, 1999]. Deletion of *Spf1* results in reduced growth on high $[Ca^{2+}]$ media [Cronin et al., 2000], and Ca^{2+}/Mn^{2+} deficiency in the ER lumen, which provokes protein missfolding and reduced resistance to ER stress [Cohen et al., 2013; Cronin et al., 2000]. The *Spf1* homolog of *S. pombe*, *Cta4*, has similar functions as ScSpf1 [Lustoza et al., 2011]. In *C. albicans*, *Spf1* deletion causes the same defects as in *S. cerevisiae* and also represses the formation of the more pathogenic hyphal state. It is therefore important for virulence [Yu et al., 2013, 2012b].

An RNAi knockdown screen on the filamentous plant pathogen *M. oryzae* revealed that in this fungus, *Spf1* is important for colony growth, sporulation, spore germination, appressorium formation, and virulence [Nguyen et al., 2008].

The function of Spf1 is, as known so far, very similar in different fungal species as well as compared to the Pmr1/Eca1 family. This is contrasted by the clearly more diverse functions of Pmc1 in different species (see 1.2.5.1). Therefore, phylogenetic similarity does not necessarily

correlate with biological function.

1.2.6 Conclusions and Outlook

A wealth of new data on the proteins that shape Ca^{2+} signals in fungi has been gained over the last years. However, the fact that pharmacological Ca^{2+} signaling modulators lead to clear effects on Ca^{2+} signals in fungi [Lange and Peiter, 2016], despite the canonical targets of these chemicals are not present in the respective fungal species, indicates that we are still missing some fundamental parts of the fungal Ca^{2+} signalosome. Mathematical modeling of experimental evidence indicated that at least two additional Ca^{2+} channels (next to Cch1-Mid1) must exist in the PM [Cui et al., 2009]. One of the missing channels may be the LACS component Fig1, but this needs confirmation. The existence of a novel PM Ca^{2+} channel was also indicated by a pharmacological study; however its molecular identity remains unclear [Gonçalves et al., 2014]. Moreover, in a fraction of analysed fungal genomes there are homologes to P2X receptor channels [Cai, 2012] and Mitochondrial Calcium Uniporters, which have not been functionally characterized yet [Prole and Taylor, 2012].

The physiological role of some proteins involved in shaping Ca^{2+} peaks (e.g., Pmr1, Eca1, and Spf1) appears to be quite conserved over fungal evolution, while in others there are striking differences between species. Therefore, the ”seen one, seen ’em all” principle should be applied very cautiously, especially in the development of antifungal drugs. An ensuing question is still not answered however: What are the causes for this evolutionary divergence? To answer this question and to find the missing pieces of the Ca^{2+} puzzle, future work now needs proceed from the description of phenotypes to the deciphering of mechanisms.

1.2.7 Funding

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1.3 Aims of this thesis

C. graminicola is a devastating maize pathogen, rendering it an agronomically important pest [Bergstrom and Nicholson, 1999]. Furthermore, this fungus has been established as a hemibiotrophic model organism. It can be genetically modified [Panaccione et al., 1988], and its genome sequence was available on supercontig level at the start of this project [O'Connell et al., 2012].

The control of fungal pathogens in the field is done by applying fungicides. However, there is increasing resistance to the currently used substances [Lucas et al., 2015]. New molecular targets for fungicides may help to overcome this problem. One possible array of targets may be signal transduction pathways, as they are also important targets for pharmaceuticals [Saltiel, 1995]. Calcium (Ca^{2+}) is a pivotal second messenger in intracellular signalling cascades. Despite its ubiquitous occurrence, Ca^{2+} signalling is utilized differentially in different organisms [Plattner and Verkhratsky, 2015]. Therefore, the overarching aim of this study is to analyze Ca^{2+} signalling-related processes in *C. graminicola*, which may present a novel target for fungicides.

Since hyphal growth is a universal feature of all filamentous fungi, this thesis aims to analyze Ca^{2+} signals in growing hyphae. For this purpose, the genetically-encoded Ca^{2+} probes AequorinS and Yellow Chameleon 3.6 shall be used. The kinetics of Ca^{2+} signals in growing hyphae shall be correlated to growth patterns. Furthermore, a pharmacological survey shall be performed to examine the role of Ca^{2+} in determining growth.

Ca^{2+} signals are generated by the opening of Ca^{2+} channels that allow Ca^{2+} to enter the cytosol, followed by a response of Ca^{2+} pumps and antiporters to decrease of cytosolic Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) to basal level. As Ca^{2+} channels are more directly coupled to the triggering stimulus they were chosen as target of this thesis. In the model yeast fungus *Saccharomyces cerevisiae* there are two genetically and electrophysiologically well-characterized Ca^{2+} channels, *viz.* the Cch1-Mid1 complex in the plasma membrane and TRPY1 in the vacuolar membrane (1.2.3). In a high-throughput screen of the model pathogen *Magnaporthe oryzae* the *TRPY1* homologue was clearly more important for hyphal growth and pathogenicity then *Cch1* or *Mid1* [Nguyen et al., 2008]. Therefore, an in-depth analysis of genes homologous to the *Yeast Transient Receptor Potential Channel 1* (*Trpy1*) shall be done in *C. graminicola*. *TRPY1* homologues will be identified by bioinformatics and RACE-PCR. To analyze the expression pattern of the candidate genes, expression studies are planned during the infection cycle. To analyze the function of these genes, they shall be heterologously

expressed in a *trpy1*Δ yeast strain transformed with the Ca^{2+} probe *Apoaequorin*. These yeast strains will be used for $[Ca^{2+}]_{cyt}$ measurements after stressing them with high osmolarity. As TRP channels can locate to the plasma membrane as well as to intracellular membranes, the subcellular localization of the candidates is planned to be analyzed by fusion to fluorescent proteins. If a localization of the candidates to the same organelle is likely, this shall be proved by co-localization of the proteins. To allow efficient co-localization studies, an appropriate plasmid system shall be designed. Deletion of the candidate genes and a phenotypical characterization of the generated mutants shall identify the candidates' possible physiological roles in $[Ca^{2+}]_{cyt}$ homeostasis. Measurements of $[Ca^{2+}]_{cyt}$ in growing hyphae and with osmotic stress are intended. Next, growth assays under Ca^{2+} starvation and with a number of carbon sources are planned. To determine the role of the candidates during pathogenesis, germination assays *in vitro* and *in planta* as well as virulence assays on maize shall be done. To overcome possible functional redundancy of the identified *TRPY1* homologues, it is planned to perform multiplex RNAi of several or all candidates. For an efficient analysis of the generated RNAi strains a low-resource-requiring and highly reproducible culture method shall be established.

2 Membrane-assisted culture of fungal mycelium on agar plates for RNA extraction and pharmacological analyses

Lange M, Müller C, Peiter E. 2014.
Analytical Biochemistry 453: 58–60

2.1 Abstract

Fungal mycelium grown in liquid culture is easy to harvest for RNA extraction and gene expression analyses, but liquid cultures often develop rather heterogeneously. In contrast, growth of fungal mycelium on agar plates is highly reproducible. However, this biological material cannot be harvested easily for downstream analyses. This article describes a PVDF (polyvinylidene difluoride) membrane-assisted agar plate culture method that enables the harvest of mycelium grown on agar plates. This culture method leads to a strongly reduced variation in gene expression between biological replicates and requires less growth space as compared with liquid cultures.

Keywords

Colletotrichum graminicola, Fungal growth, Gene expression, PVDF membrane, RNA extraction

2.2 Full text link

The original paper has been published in the journal *Analytical Biochemistry*. Due to copyright restrictions, this paper and all supplementary material is not printed here but available online:

http://www.sciencedirect.com/science/article/pii/S0003269714000803

3 A modular plasmid system for protein co-localization and bimolecular fluorescence complementation in filamentous fungi

Lange M, Oliveira-Garcia E, Deising HB, Peiter E. 2014.
Current Genetics 60: 343–350

3.1 Abstract

To elucidate the function of a protein, it is crucial to know its subcellular location and its interaction partners. Common approaches to resolve those questions rely on the genetic tagging of the gene-of-interest (GOI) with fluorescent reporters. To determine the location of a tagged protein, it may be co-localized with tagged marker proteins. The interaction of two proteins under investigation is often analysed by tagging both with the C- and N-terminal halves of a fluorescent protein. In fungi, the tagged GOI are commonly introduced by serial transformation with plasmids harbouring a single tagged GOI and subsequent selection of suitable strains. In this study, a plasmid system is presented that allows the tagging of several GOI on a single plasmid. This novel double tagging plasmid system (DTPS) allows a much faster and less laborious generation of double-labelled fungal strains when compared with conventional approaches. The DTPS also enables the combination of as many tagged GOI as desired and a simple exchange of existing tags. Furthermore, new tags can be introduced smoothly into the system. In conclusion, the DTPS allows an efficient tagging of GOI with a high degree of flexibility and therefore accelerates functional analysis of proteins *in vivo*.

Keywords

Bimolecular fluorescence complementation, Co-localization, *Colletotrichum graminicola*, Plasmid, Protein localization

3.2 Full text link

The original paper has been published in the journal *Current Genetics*. Due to copyright restrictions, this paper and all supplementary material is not printed here but available online:

http://link.springer.com/article/10.1007%2Fs00294-014-0429-y

4 Cytosolic free calcium dynamics as related to hyphal and colony growth in the filamentous fungal pathogen *C. graminicola*

Lange M, Peiter E. 2016.
Fungal Genetics and Biology 91: 55–65

4.1 Abstract

Tip growth of pollen tubes and root hairs of plants is oscillatory and orchestrated by tip-focussed variations of cytosolic free calcium ($[Ca^{2+}]_{cyt}$). Hyphae of filamentous fungi are also tubular tip-growing cells, and components of the Ca^{2+} signalling machinery, such as Ca^{2+} channels and Ca^{2+} sensors, are known to be important for fungal growth. In this study, we addressed the questions if, as in plants, tip-focussed $[Ca^{2+}]_{cyt}$ transients govern hyphal and whole-colony growth in the maize pathogen *Colletotrichum graminicola*, and whether colony-wide $[Ca^{2+}]_{cyt}$ dynamics rely on external Ca^{2+} or internal Ca^{2+} stores. Ratiometric fluorescence microscopy of individual hyphae expressing the Ca^{2+} reporter Yellow Cameleon 3.6 revealed that Ca^{2+} spikes in hyphal tips precede the re-initiation of growth after wounding. Tip-focussed $[Ca^{2+}]_{cyt}$ spikes were also observed in undisturbed growing hyphae. They occurred not regularly and at a higher rate in hyphae growing at a medium-glass interface than in those growing on an agar surface. Hyphal tip growth was non-pulsatile, and growth speed was not correlated with the rate of spike occurrence. A possible relationship of $[Ca^{2+}]_{cyt}$ spike generation and growth in whole colonies was assessed by using a codon-optimized version of the luminescent Ca^{2+} reporter Aequorin. Depletion of extracellular free Ca^{2+} abolished $[Ca^{2+}]_{cyt}$ spikes nearly completely, but had only a modest effect on colony growth. In a pharmacological survey, some inhibitors targeting Ca^{2+} influx or release from internal stores repressed growth strongly. However, although some of those inhibitors also affected $[Ca^{2+}]_{cyt}$ spike generation, the effects on both parameters were not correlated. Collectively, the results indicate that tip growth of C. graminicola is non-pulsatile and not mechanistically linked to tip-focused or global $[Ca^{2+}]_{cyt}$ spikes, which are likely a response to micro-environmental parameters, such as the physical properties of the surface.

Keywords

Aequorin luminometry, Calcium signalling, *Colletotrichum graminicola*, Hyphal tip growth, Yellow cameleon imaging

4.2 Full text link

The original paper has been published in the journal *Fungal Genetics and Biology*. Due to copyright restrictions, this paper and all supplementary material is not printed here but available online:

http://www.sciencedirect.com/science/article/pii/S1087184516300354

5 The Transient Receptor Potential (TRP) Channel Family in *Colletotrichum graminicola*: A Molecular and Physiological Analysis

Lange M, Weihmann F, Schliebner I, Horbach R, Deising HB, Wirsel SGR, Peiter E. 2016.
PLOS ONE 11: e0158561

5.1 Abstract

Calcium (Ca^{2+}) is a universal second messenger in all higher organisms and centrally involved in the launch of responses to environmental stimuli. Ca^{2+} signals in the cytosol are initiated by the activation of Ca^{2+}_{cyt} channels in the plasma membrane and/or in endomembranes. Yeast (*Saccharomyces cerevisiae*) contains a Ca^{2+}-permeable channel of the TRP family, TRPY1, which is localized in the vacuolar membrane and contributes to cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevations, for example in response to osmotic upshock. A TRPY1 homologue in the rice blast fungus is known to be important for growth and pathogenicity. To determine the role of the TRP channel family in the maize pathogen *Colletotrichum graminicola*, proteins homologous to TRPY1 were searched. This identified not one, but four genes in the *C. graminicola* genome, which had putative orthologs in other fungi, and which we named emphCgTRPF1 through *4*. The topology of the CgTRPF proteins resembled that of TRPY1, albeit with a variable number of transmembrane (TM) domains additional to the six-TM-domain core and a diverse arrangement of putatively Ca^{2+}-binding acidic motifs. All *CgTRPF* genes were expressed in axenic culture and throughout the infection of maize. Like TRPY1, all TRPF proteins of *C. graminicola* were localized intracellularly, albeit three of them were found not in large vacuoles, but co-localized in vesicular structures. Deletion strains for the *CgTRPF* genes were not altered in processes thought to involve Ca^{2+} release from internal stores, i.e. spore germination, the utilization of complex carbon sources, and the generation of tip-focussed $[Ca^{2+}]_{cyt}$ spikes. Heterologous expression of *CgTRPF1* through *4* in a *tryp1*Δ mutant revealed that none of the channels mediated the release of Ca^{2+} in response to osmotic upshock. Accordingly, aequorin-based $[Ca^{2+}]_{cyt}$ measurements of *C. graminicola* showed that in this fungus, osmotic upshock-triggered $[Ca^{2+}]_{cyt}$ elevations were generated entirely by influx of Ca^{2+} from the extracellular space. *Cgtrpf* mutants did not show pathogenicity defects in leaf infection assays. In summary, our study reveals major differences between different fungi in the contribution of TRP channels to Ca^{2+}-mediated signal transduction.

Keywords

Colletotrichum graminicola; TRP channels; calcium; calcium channels; Yellow Cameleon; filamentous fungi

Full text link

The original paper has been published in the open access journal *PLOS ONE*. Therefore, it is printed here:

5.2 Introduction

Like any organism, fungi must perceive and respond to their environment to survive and propagate. For example, spores of plant pathogenic fungi perceive certain features of the host surface, which initiates a developmental programme that may culminate in an appressorium. This highly specialized cell allows for a pressure-mediated penetration of intact host cuticle and epidermal cell wall. This pressure, which may reach values of 5.5 MPa in *Colletotrichum graminicola*, is generated by the accumulation of osmotically active compounds [Bechinger et al., 1999] and needs to be sensed and tightly controlled to ensure successful penetration while preventing a bursting of the appressorium [Bergstrom and Nicholson, 1999]. *C. graminicola* is a hemibiotrophic pathogen of maize, which, inside its host, passes through a short biotrophic and a longer necrotrophic phase, characterised by the controlled expression of subsets of genes [Krijger et al., 2008; O'Connell et al., 2012]. Again, perception of and response to the environment within the host is important for an effective colonization [Albarouki et al., 2014; Oliveira-Garcia and Deising, 2013]. One of the fungus' environmental parameters that may change abruptly, within a wide range, and throughout the fungal life cycle is the osmotic potential. Osmotic shock situations occur, for example, during exposure to rainwater or during the lysis of host cells.

The coupling of stimulus perception by a fungus and its responses on transcriptional or post-transcriptional levels involves numerous interacting signalling networks, including, for example, G-proteins, MAP kinases, and cyclic nucleotides [Li et al., 2007; Xue et al., 2008]. Calcium Ca^{2+} is another ubiquitous second messenger in all higher organisms and plays a central role in the initiation of responses to external stimuli, including osmotic shock, and to internal cues [Berridge et al., 2003; Zelter et al., 2004]. In the cytosol, Ca^{2+} binds to target proteins, such as calcineurin and calmodulin (CaM), resulting in conformational changes that modulate their activity or their interaction with other proteins. In fungi, the Ca^{2+}- and CaM-activated protein phosphatase calcineurin dephosphorylates the transcription factor Crz1 allowing it to enter the nucleus and triggering transcription [Stathopoulos-Gerontides et al., 1999]. Deletions of either gene in filamentous fungi result in growth retardation and reduced virulence [da Silva Ferreira et al., 2007; Schumacher et al., 2008].

Ca^{2+} signals are generated by the passive diffusion of Ca^{2+} into the cytosol, facilitated by Ca^{2+}-permeable channels. The elevation of cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) is terminated by the activity of Ca^{2+}/H^{+} antiporters and Ca^{2+}-ATPases which transport Ca^{2+} out of the cytosol [Bowman et al., 2011; Dinamarco et al., 2012; Hu et al., 2014]. Hence, Ca^{2+} channels are

actively regulated by a signal transduction pathway, while Ca^{2+}/H^{+} antiporters and Ca^{2+}-ATPases respond to the increased $[Ca^{2+}]_{cyt}$. Ca^{2+}-permeable channels may be activated by a number of ligands, such as inositol phosphates, cyclic nucleotides or amino acids, and by physical parameters, such as voltage or stretch of the membrane [Peiter, 2011]. They may be either located in the plasma membrane or in membranes of intra-cellular compartments, hence mediating the entry of extracellular Ca^{2+} into the cytosol or Ca^{2+} release from internal stores, respectively. Albeit this diversity of Ca^{2+} conductances suggests a number of underlying genes, in fungi the molecular identity has been resolved for only very few channel systems. Comparative genomic analyses indicated that some fungi bear mitochondrial calcium uniporters, and some basal fungi also have genes encoding putative P2X receptors in their genomes [Cai, 2012; Prole and Taylor, 2012]. However, none of these putative fungal Ca^{2+} channel classes has been functionally analysed so far. The plasma membrane of the yeast *Saccharomyces cerevisiae* harbours a homologue of animal voltage-gated Ca^{2+} channels, Cch1, which physically interacts with another membrane protein, Mid1 [Fischer et al., 1997; Iida et al., 1994; Locke et al., 2000; Paidhungat and Garrett, 1997]. The Cch1Mid1 complex forms a high-affinity Ca^{2+} uptake system (HACS), which is activated by multiple stimuli, such as osmotic, iron, cold, and alkali stress [Matsumoto et al., 2002; Peiter et al., 2005a; Viladevall et al., 2004]. Deletion of either *Cch1* or *Mid1* leads to an increased sensitivity of yeast cells to these stresses, in particular if the Ca^{2+} concentration in the medium is low. In filamentous fungi, deletion of *Cch1* or *Mid1* homologues causes reduced hyphal growth, albeit fungal species differ in their requirement of this channel system [Harren and Tudzynski, 2013; Liu et al., 2006; Wang et al., 2012]. Next to the HACS, there also exists a low-affinity Ca^{2+} uptake system (LACS) of unclear genetic identity [Locke et al., 2000].

The vacuole represents the largest intracellular store for Ca^{2+} in yeast. The vacuolar membrane harbours a Ca^{2+}-permeable channel, initially named Yeast Vacuolar Channel 1 (Yvc1), which is related to Transient Receptor Potential (TRP) channels of animals [Palmer et al., 2001]. Animal TRP channels group into seven subfamilies (TRPC, TRPV, TRPA, TRPM, TRPP, TRPML, and TRPN), and most of them are permeable for Ca^{2+} [Nilius and Szallasi, 2014]. Animal TRP channels locate to various endomembranes as well as to the plasma membrane. All TRP channels are supposed to contain 6 transmembrane (TM) domains and a pore loop between TM domain 5 and 6. The C- and N-termini of TRP channels are highly diverse. TRP channels are often activated in a polymodal way, i.e. a single channel

integrates different stimuli, such as temperature, voltage, and ligands [Nilius and Szallasi, 2014]. In analogy to the animal TRP nomenclature, the TRP channel of yeast, Yvc1, was also denominated TRPY1. This channel is activated by cytosolic Ca^{2+} and by osmotic upshock leading to mechanical force on the vacuolar membrane [Denis and Cyert, 2002; Palmer et al., 2001; Su et al., 2009; Zhou et al., 2003]. Heterologous expression of the TRPY1 homologues of *Kluyveromyces lactis* and *Candida albicans*, as well as the filamentous plant pathogenic fungus *Fusarium graminearum* (teleomorph *Gibberella zeae*), in *S. cerevisiae* demonstrated a mechanosensitivity and a responsiveness to osmotic upshock, similar to TRPY1 [Ihara et al., 2013; Zhou et al., 2005]. In a comparative RNAi knock-down study [Nguyen et al., 2008] examined the importance of homologues of the yeast Ca^{2+} channels *Cch1*, *Mid1*, and *TRPY1* in the rice blast fungus *Magnaporthe oryzae*. Interestingly, in this pathogen *TRPY1* was clearly more important than *Cch1* and *Mid1* for growth and virulence [Nguyen et al., 2008]. Similarly, hyphal growth and virulence were strongly impaired in a *trpy1* mutant of the dimorphic fungus *C. albicans* [Yu et al., 2014a].

Despite the obvious importance of TRPY1-like channels in filamentous fungi, their functioning has been rarely analysed, with the notable exception of *TRPGz* from *F. graminearum* [Ihara et al., 2013]. We therefore searched for TRPY1 homologues in the maize pathogen *C. graminicola*. Intriguingly, we identified four genes with similarity to *TRPY1* in this organism, which were functionally characterised by heterologous expression in yeast and by sub-cellular localization. Deletion strains were analysed for $[Ca^{2+}]_{cyt}$ signal generation, germination, growth rates, tolerance to osmotic stress and Ca^{2+} starvation, as well as virulence. Surprisingly, our results differed considerably from data obtained previously on *TRPY1* homologues in other fungal species, indicating that fungi vary largely in their employment of Ca^{2+}-channel types.

5.3 Materials and Methods

5.3.1 Bioinformatic analyses

A tBLASTn search of the *C. graminicola* whole genome sequence hosted at the Broad Institute was carried out using default parameters. This identified four fragments with similarity to the TRPY1 channel (synonym Yvc1; systematic name: YOR087W) of *Saccharomyces cerevisiae*. The corresponding genes were denominated *CgTRPF1* through *CgTRPF4*. Full-length sequences of the *CgTRPF* genes were obtained by RACE-PCR as described below.

Membrane topology of the CgTRPF proteins was analysed with TOPCONS (accessed at http://topcons.net/) using default settings. The putative pore loop was inferred from previous analyses [Prole and Taylor, 2012]. Canonical Ca^{2+}-binding sites were searched with PFAM (accessed at http://pfam.xfam.org/) using default settings. TRPY1 is known to contain no classical Ca^{2+}-binding sites, but binds Ca^{2+} by a tetra-aspartate motif (DDDD) [Su et al., 2009]. Therefore, motifs with four or more consecutive acidic amino acid residues (Asp and Glu) were searched in the CgTRPF protein sequences. Homologous sequences of other fungi were obtained by tBLASTn searches of their annotated genomes. Multiple sequence alignments of the full-length proteins, including 43 sequences from 20 fungal species, were performed using ClustalW [Larkin et al., 2007]. Resulting alignments were trimmed with Jalview [Waterhouse et al., 2009] (S1 File). Subsequent phylogenetic analyses were performed by neighbour joining (10,000 bootstrap replicates) using Phylip hosted at http://www.es.embnet.org/Services/. A consensus tree was created by using plottree (http://www.bioinformatics.nl/tools/plottree.html). Amino acid identity and similarity was calculated with the help of the trimmed alignments that were used for the phylogenetic analysis at http://imed.med.ucm.es/Tools/sias.html with standard settings.

5.3.2 Expression analysis

Expression of *CgTRPF1* through *4* was examined by amplifying the full-length CDS from cDNA obtained from OMA-grown falcate conidia and hyphae grown on modified Leach's Complete Medium (mLCM) overlaid with a PVDF membrane [Lange et al., 2014a]. Transcript abundance of *CgTRPF* genes during infection of maize was analysed by qRT-PCR and RNA-Seq. For qRT-PCR experiments, maize (cv. Mikado, KWS Saat AG, Einbeck, Germany) plants were cultivated on compost soil in a greenhouse [Ludwig et al., 2014].Detached segments of the middle of the third leaf of 17-d-old plants were infected with one 10-µL drop per segment containing 10^4 conidia in a 0.04% Tween 20 (Carl Roth) solution. Leaf segments were incubated in moist chambers at 23 °C in darkness. After the indicated time, leaf discs of 8 mm diameter were excised and immediately frozen in liquid nitrogen. Four leaf discs were pooled for each point in time. Infection assays were performed in three biological replicates in consecutive weeks. Leaf discs were ground in liquid nitrogen. The resulting powder was suspended in RLT buffer (Qiagen, Venlo, The Netherlands) and processed in aliquots for RNA extraction using the PeqGold Plant RNA kit with on-column DNase I treatment according to the manual (PeqLab, Erlangen, Germany). qRT-PCR was performed using the

Power SYBR Green RNA-to-C_T 1-step kit (Applied Biosystems - Thermo Fisher Scientific, Waltham, MA, USA). Reactions comprising volumes of 20 µL were set up according to the manufacturer's instructions using 0.2 µM of each oligonucleotide and 50 ng RNA and executed in a MyiQ real-time detection system (Bio-Rad Laboratories, Hercules, USA). After reverse transcription at 48 °C for 30 min, the resulting cDNA was denaturated for 10 min at 95 °C and amplified in 50 cycles (95 °C for 15 seconds, 60 °C for 1 min). Calculation of the results was done according to [Liu and Saint, 2002]. The oligonucleotides used are listed in S5.1 Table. As reference for normalisation, transcript levels of HistonH3, Actin, and ILV5 were used. RNA-Seq data were obtained from the study of [Schliebner et al., 2014].

5.3.3 Media and culture conditions

To induce the production of falcate conidia, the fungus was grown on oat meal agar (OMA) [Lange and Peiter, 2016]. Colony growth assays were performed as described before [Lange and Peiter, 2016]. Colony diameters were recorded daily for 4 to 10 days. Vegetative hyphae of *C. graminicola* were grown in liquid mLCM or on mLCM solidified with 1.5% agar (Carl Roth, Karlsruhe, Germany) [Lange et al., 2014a]. For osmotic stress treatments, glycerol was added to mLCM agar prior to autoclaving at the indicated final concentrations. For assays testing growth on different carbon sources, mLCM, potato dextrose agar (PDA), and minimal medium supplemented with the respective carbon source were used. PDA was made of 2.4% potato dextrose broth (Formedium, Hunstanton, UK) and 1.5% agar. Minimal medium contained salt solutions as used in mLCM, 1.5% agar, and 2% of the respective carbon source (glucose, sucrose, sorbitol, mannitol, raffinose, cellulose, malate, or pectate). Sodium malate and sodium pectate were obtained by neutralizing DL-malic acid and pectic acid to pH 7.0 with NaOH, respectively. Ca^{2+}-depleted SC medium was prepared as described before [Lange and Peiter, 2016].

5.3.4 RNA extraction for cloning and RACE-PCR

C. graminicola RNA was extracted from fungal mycelium grown for four days at 23 °C and 30 rpm in mLCM medium, and *S. cerevisiae* RNA was extracted from a log-phase culture using the Spectrum Plant Total RNA Kit (Sigma). An on-column DNase I digest was performed with the RNase-free DNase I set (Omega bio-tek, Norcross, GA, USA) according to the manufacturer's instructions.

RACE-PCR was performed with the BD SMART RACE cDNA amplification kit (BD,

Franklin Lakes, NJ, USA) according to the manufacturer's recommendations. PCR products were purified from agarose gel slices using the Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA). RACE-PCR products were sequenced using the BigDye Terminator v1.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and gene-specific oligonucleotides (S5.1 Table). All oligonucleotides were purchased from Eurofins MWG Operon (Ebersberg, Germany).

5.3.5 Targeted gene deletion and Southern Blotting

A hygromycin B resistance cassette was amplified from pAN7-1 (GenBank No. Z32698) [Punt et al., 1987] using the oligonucleotides unihyg-F1 and unihyg-R1 (S5.1 Table) [Lingner et al., 2011]. Deletion cassettes were generated by double-joint PCR using the oligonucleotides listed in S5.1 Table [Yu et al., 2004]. Transformation of the *C. graminicola* M2 (M1.001) wild type isolate was performed as described previously [Werner et al., 2007]. Transformants were screened by PCR for the presence of the deletion cassette. For this purpose, DNA was isolated by using a quick extraction protocol [Lange et al., 2014b]. PCR-positive clones were assayed by Southern blotting for homologous integration of the deletion cassette and ectopic integration events. The probe was generated using the oligonucleotides Hph-5'-South-for and Hph-5'-South-rev (S5.1 Table) as described elsewhere [Werner et al., 2007]. DNA extraction and blotting were performed as described before [Horbach et al., 2009].

5.3.6 Yeast complementation analysis and $[Ca^{2+}]_{cyt}$ measurements

The *Saccharomyces cerevisiae trpy1* deletion strain CEN.SR36-3C (Acc. No. B0257A; genotype: CEN.PK; YOR087w/088w::HIS3) was obtained from Euroscarf (Frankfurt, Germany). *trpy1* cells were transformed with the plasmid pEVP11-AEQ encoding apoaequorin [Batiza et al., 1996]. as described elsewhere [Elble, 1992]. Full-length coding sequences of the four *CgTRPF* genes and the *TRPY1* gene were cloned into the *Not*I-site of the pFL61 plasmid [Minet et al., 1992]. The pEVP11-AEQ-containing *trpy1*Δ strain was transformed with these pFL61 descendants. An RT-PCR with RNA from log-phase liquid cultures was performed to confirm the presence of full-length mRNA of the *CgTRPF* genes in these strains. For luminometric analysis of$[Ca^{2+}]_{cyt}$, yeast cultures were grown on a rotating shaker in liquid SC-Leu-Ura medium containing 2 μM coelenterazine (Carl Roth) at 30 °C to a final density of 1 to 5×10^7 cells per mL and diluted to 1×10^7 cells per mL with fresh coelenterazine-containing medium. For $[Ca^{2+}]_{cyt}$ measurements in a tube luminometer (Sirius-1, Berthold

Detection Systems, Pforzheim, Germany), 20 µl of the cell suspension were used per experiment. After 1 min baseline recording, cells were treated with 200 µL of a solution (pH 7.0) containing 1.5 M NaCl, 50 mM MES, and 25 mM EGTA. Total aequorin luminescence was discharged at the end of the experiment by injecting 220 µL of a solution containing 2 M $CaCl_2$ and 20% ethanol. $[Ca^{2+}]_{cyt}$ was calculated as described by [Allen et al., 1977], which normalized differences in Aequorin expression (i.e., total aequorin luminescence) in different strains, and which is the most commonly used equation in the mycology community. Albeit absolute $[Ca^{2+}]_{cyt}$ values obtained by this procedure may be offset from the calculated values due to the cytosolic aequorin environment, this effect should be similar in all yeast studies employing this formula.

5.3.7 $[Ca^{2+}]_{cyt}$ measurements in *Colletotrichum graminicola*

To test $[Ca^{2+}]_{cyt}$ responses of *C. graminicola* wild type and *Cgtrpf*Δ deletion strains to osmotic upshock, the strains were transformed with the pAEQS1-G418 plasmid [Lange and Peiter, 2016] which encodes a codon-optimized Apoaequorin [Nelson et al., 2004]. Transformants were selected by using G418 (geneticin; 600 µg mL^{-1} during transformation, 150 µg mL^{-1} during selection and single-spore isolation). For $[Ca^{2+}]_{cyt}$ measurements, 1 mL mLCM agar supplemented with 10 µM coelenterazine was poured into sterile polystyrene cylinders with slip lid (36 mm diameter × 29 mm height; neoLab, Heidelberg, Germany). 300 washed macroconidia were inoculated onto the solidified medium, and the polystyrene cylinders were covered loosely to allow for gas exchange. The fungal cultures were incubated in darkness for 3 d at 23 °C and 65% relative humidity. Prior to $[Ca^{2+}]_{cyt}$ measurements, cultures were overlaid with 3 mL of a solution (pH 7.0) containing 50 mM MES-KOH and 0 or 25 mM EGTA. Cylinders were covered with parafilm and incubated for 30 min in the chamber of the Sirius-1 luminometer. After 1 min baseline recording, 4 mL of a solution (pH 7.0) containing 50 mM MES-KOH and 0 or 3 M NaCl plus 0 or 25 mM EGTA were injected through the parafilm cover. Aequorin luminescence was recorded for 30 min. To discharge total aequorin at the end of the experiment, 8 mL discharge solution containing 2 M CaCl2 and 100 µM digitonin (Applichem, Darmstadt, Germany) were injected, and recording continued for 60 min. Data shown represent three biological repetitions performed on different days. $[Ca^{2+}]_{cyt}$ was calculated as described above.

For $[Ca^{2+}]_{cyt}$ analyses in individual hyphae, wild type and *Cgtrpf*Δ deletion strains were transformed with a pGEM-T-PtrpC-nptII-TtrpC-PtoxB-YC3.6-TtrpC plasmid [Lange and

Peiter, 2016], encoding the FRET-based ratiometric Ca^{2+} reporter protein Yellow Cameleon 3.6 (YC3.6) [Nagai et al., 2004]. Measurements of tip-focussed $[Ca^{2+}]_{cyt}$ spikes during hyphal growth were performed as described before [Lange and Peiter, 2016].

5.3.8 Subcellular localization

For subcellular localization and co-localization of the CgTRPF proteins, a dual-tagging plasmid system was employed, following the cloning workflow described by [Lange et al., 2014b]. Oligonucleotides used for the cloning of localization plasmids are listed in S5.1 Table. Transformation and microscopic analyses were performed as described before [Lange et al., 2014b].

5.3.9 Germination assays

Germination and appressorium formation were assayed on polystyrene and on onion epidermis. Ten-µL droplets of washed macroconidia (see above) containing 100 and 1000 spores were inoculated onto 90-mm polystyrene Petri dishes (Greiner Bio One) and the hydrophobic face of onion epidermal strips, respectively. After incubation for 24 h in a humid chamber at 23 °C in darkness, infection structures were counted by phase contrast microscopy using an Axiovert 40 CFL inverted microscope (Carl Zeiss, Jena, Germany) equipped with a 10×/0.25 Ph1 objective for germination assays on polystyrene and an Axioskop upright microscope (Zeiss) equipped with a 20×/0.45 Ph2 objective for germination assays on onion epidermis.

5.3.10 Leaf segment infection assays

Segments of the third leaf of 14-day-old maize (*Zea mays* cv. Golden Jubilee) plants cultivated in an air-conditioned greenhouse were excised and incubated to assess virulence of *C. graminicola* as published previously [Behr et al., 2010].

5.4 Results

5.4.1 The *C. graminicola* genome contains four genes with similarity to the *S. cerevisiae* calcium channel *TRPY1*

The *C. graminicola* genome was searched by tBLASTn for an orthologue to *TRPY1* of *S. cerevisiae*. Unexpectedly, not one, but four genes were identified with E-values below 10^{-3}.

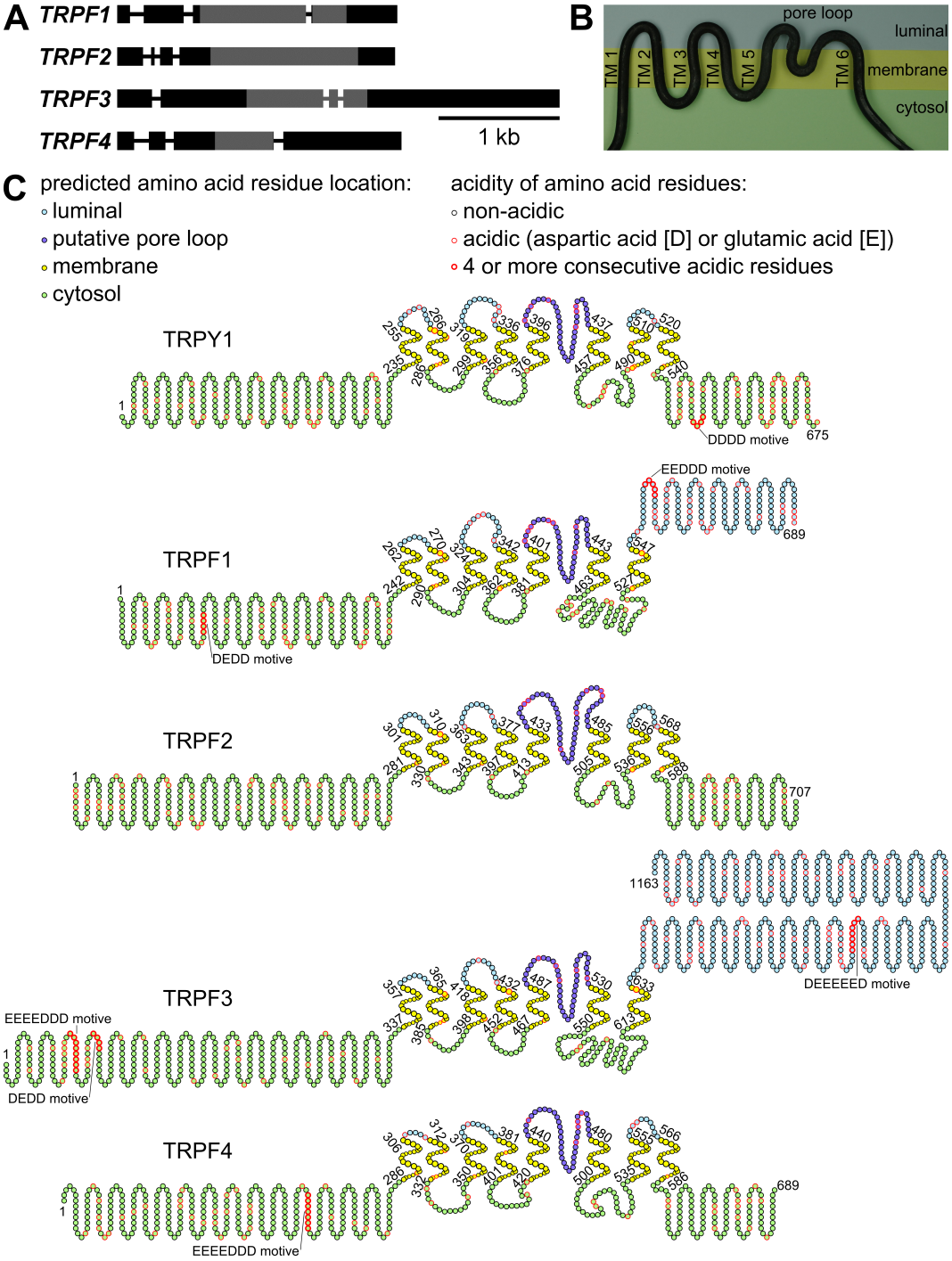

Fig. 5.1: Gene structures of C. graminicola TRPF *genes and predicted membrane topologies of yeast TRPY1 and* C. graminicola *TRPF proteins. (A) Gene structures of* CgTRPF *genes. Boxes: exons, lines: introns; grey: match of the initial tBLASTn search against TRPY1, black: regions identified by RACE-PCR and verified by cloning PCR. (B) Artistic representation (forged steel) of the TRP channel core structure containing six transmembrane (TM) domains and a pore loop between TM domain 5 and 6. (C) Predicted membrane topology of TRPY1 and CgTRPFs. Cytosolic amino acid residues are indicated in light green, TM domains are shown in yellow, luminal amino acid residues are depicted in light blue, and the predicted pore loop is marked in dark blue. Acidic amino acid residues [Asp (D) or Glu (E)] are indicated by a red edge, which is boldfaced in motifs of 4 or more consecutive acidic amino acid residues. One circle represents one amino acid residue. The first and the last amino acid of the whole protein, as well as of each TM domain, are enumerated.*

According to the animal TRP nomenclature, we named these genes *CgTRPF1* through *4*, standing for *C. graminicola TRP of Fungi*. Full-length cDNA sequences were obtained by RACE-PCR and verified by cloning and RNA-Seq. In Fig 5.1A the regions detected by the tBLASTn search are indicated in grey, the completed sequences in black. Comparison with the genomic regions revealed that each of the genes comprises four exons of varying length (Fig 5.1A). The CDSs of *CgTRPF1*, *2*, *3* and *4* consist of 2070, 2124, 3492, and 2073 bp from start to stop codon, respectively. The genomic sequences and the gene numbers can be found in S2 File. The amino acid similarities (and identities) of *CgTRPF1*, *2*, *3* and *4* compared to *TRPY1* are 60% (46%), 40% (24%), 38% (22%), and 34% (21%), respectively, based on the trimmed alignments. The gene structures that we obtained by tBLASTn searches and RACE-PCR perfectly matched the most recent genomic and transcriptomic data generated by RNA-Seq [Schliebner et al., 2014].

The membrane topology of yeast TRPY1 shows a Shaker-like core of six TM domains (Fig 5.1B), a pore loop between TM domain 5 and 6, and, in addition, two predicted additional TM domains after the sixth TM domain (Fig 5.1C), which were previously described as hydrophobic patches [Su et al., 2009]. All predicted TM domains of TRPY1 and CgTRPF1 through 4 are exactly 21 amino acids long (see Tab S5.2). There is also a high degree of conservation in the length of the luminal and cytosolic linkers of the TM domains as well as of the putative pore loop (see Tab S5.2) [Prole and Taylor, 2012]. CgTRPF2 and CgTRPF4 of *C. graminicola* have the same predicted topology as TRPY1, i.e. a six-TM-domain core and two additional TM domains. CgTRPF1 and CgTRPF3 also have the six-TM-domain core, but only one additional TM domain. Hence, the C-terminus of those proteins is predicted to reside in the lumen. In coincidence with this luminal C-terminus, CgTRPF1 and CgTRPF3 have a longer cytosolic linker between TM domain 6 and 7, as compared to TRPY1, CgTRPF2, and CgTRPF4, which have a predicted cytosolic C-terminus.

Like TRPY1, all TRPF proteins of *C. graminicola* have no EF-hands or other canonical Ca^{2+}-binding sites detected by PFAM. However, an acidic DDDD motif in the C-terminus of TRPY1 has been shown to be important for high-affinity Ca^{2+} binding and channel activation [Su et al., 2009]. The luminal C-terminus of CgTRPF1 and CgTRPF3 harbours acidic EEDDD and DEEEEED motifs, respectively, that may bind Ca^{2+} (Fig 5.1C). Additionally, acidic motifs, containing at least four consecutive amino acid residues, are present in the cytosolic N-termini of CgTRPF1, CgTRPF3, and CgTRPF4, with CgTRPF3 possessing two such stretches (Fig 5.1C). In CgTRPF2 there are no areas with least four consecutive acidic amino acid

residues. However, the cytosolic N- and C- termini of CgTRPF2 contain many densely clustered acidic amino acid residues that may also confer an ability to bind Ca^{2+}.

5.4.2 The *C. graminicola* TRPF genes have putative orthologs in other fungi

To examine whether other fungi may also possess multiple predicted proteins with similarities to TRPY1, a phylogenetically diverse set of annotated genomes of Ascomycota and Basidiomycota was searched by tBLASTn (S5.3 Table). The obtained sequences were aligned, trimmed, and a phylogenetic tree was generated that comprised six major branches (Fig 5.2). Species belonging to the Saccharomycotina harboured only one putative TRP protein and

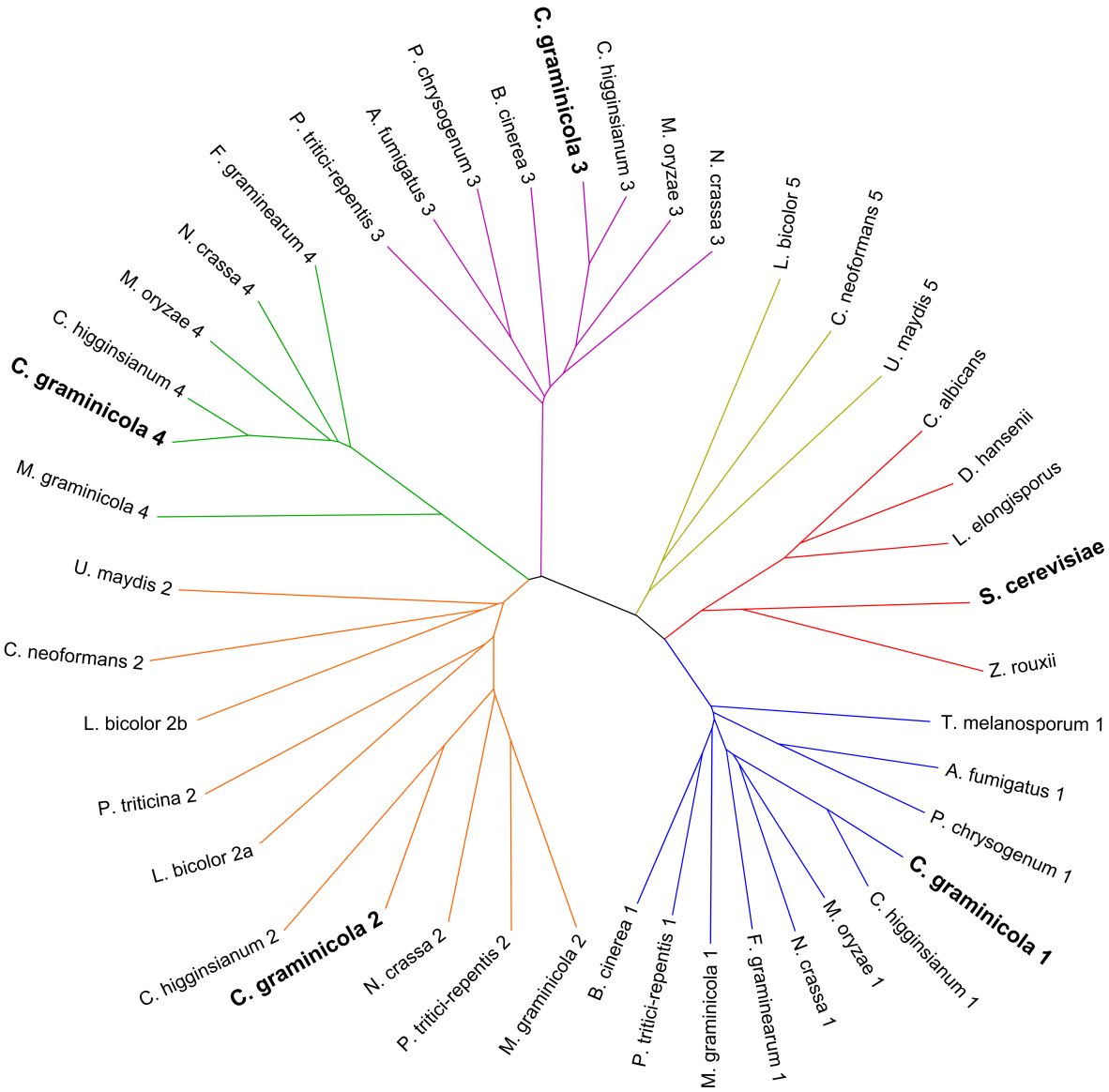

Fig. 5.2: *Phylogenetic tree of fungal TRP proteins. Red: yeast TRPYs; blue, orange, violet and green: TRPF groups 1, 2, 3, and 4, each containing a* C. graminicola *homologue; yellow: TRPF group not containing a* C. graminicola *homologue.*

clustered on a distinct branch. *C. graminicola* paralogs are present in four of the five remaining branches. The fifth branch comprises only predicted proteins from Basidiomycota. In general, all examined filamentous fungi carry one to four predicted TRPF proteins. *Tuber melanosporum* contains just one TRPF which is most closely related to CgTRPF1; *Puccinia triticina* has one TRPF that co-groups with CgTRPF2. Two predicted TRPF proteins, grouping with CgTRPF1 and CgTRPF3, were found in *Aspergillus fumigatus*, *Botrytis cinerea* and *Penicillium chrysogenum*. *Fusarium graminearum* carries two TRPFs homologous to CgTRPF1 and CgTRPF4. *Cryptococcus neoformans* and *Ustilago maydis* also bear two TRPFs; one homologous to CgTRPF2, with the other one not grouping with any of the CgTRPFs. *Laccaria bicolor* has two homologues to CgTRPF2 and one not grouping with a CgTRPF. Three TRPFs were found in *Mycosphaerella graminicola* (homologous to CgTRPF1, 2, and 4), *Magnaporthe oryzae* (homologous to CgTRPF1, 3 and 4), *Pyrenophora tritici-repentis* (homologous to CgTRPF1, 2 and 3). *Colletotrichum higginsianum* and *Neurospora crassa* are the only of the analyzed species containing predicted proteins with similarity to all four CgTRPFs. There is no apparent correlation between nutritional lifestyle or pathogenicity and the number of TRPF proteins per species.

5.4.3 The *C. graminicola* TRPF genes are expressed in axenic culture and throughout infection

To determine whether the four CgTRPF genes may play a role during growth, we first determined their expression in spores and in colonies cultivated on a membrane overlying mLCM agar, as described by Lange and co-workers (2014) [Lange et al., 2014a]. Full-length transcripts for all four genes were detectable in vegetative hyphae and conidial spores (Fig 5.3A). To determine transcript abundance throughout the infection process on maize, two independent methods were applied on two different cultivars (Mikado, Golden Jubilee). Transcript levels were assessed from 0 to 120 hours post inoculation (hpi). Irrespective of the cultivar, both, qRT-PCR and RNA-Seq experiments indicated that transcripts of all *CgTRPF* genes were clearly detectable from conidial to necrotrophic stage of infection (Fig 5.3B, C). Compared to 0 hpi, transcript levels of *CgTRPF1* were induced from 12 hpi onward by up to 13 fold in both data sets. Transcript abundances of *CgTRPF4* were also consistently elevated throughout earlier stages of the infection process, albeit to a lesser extent (Fig 5.3B, C). The transcriptomic data indicate that all four *CgTRPF* genes may play a role in all stages of growth and infection.

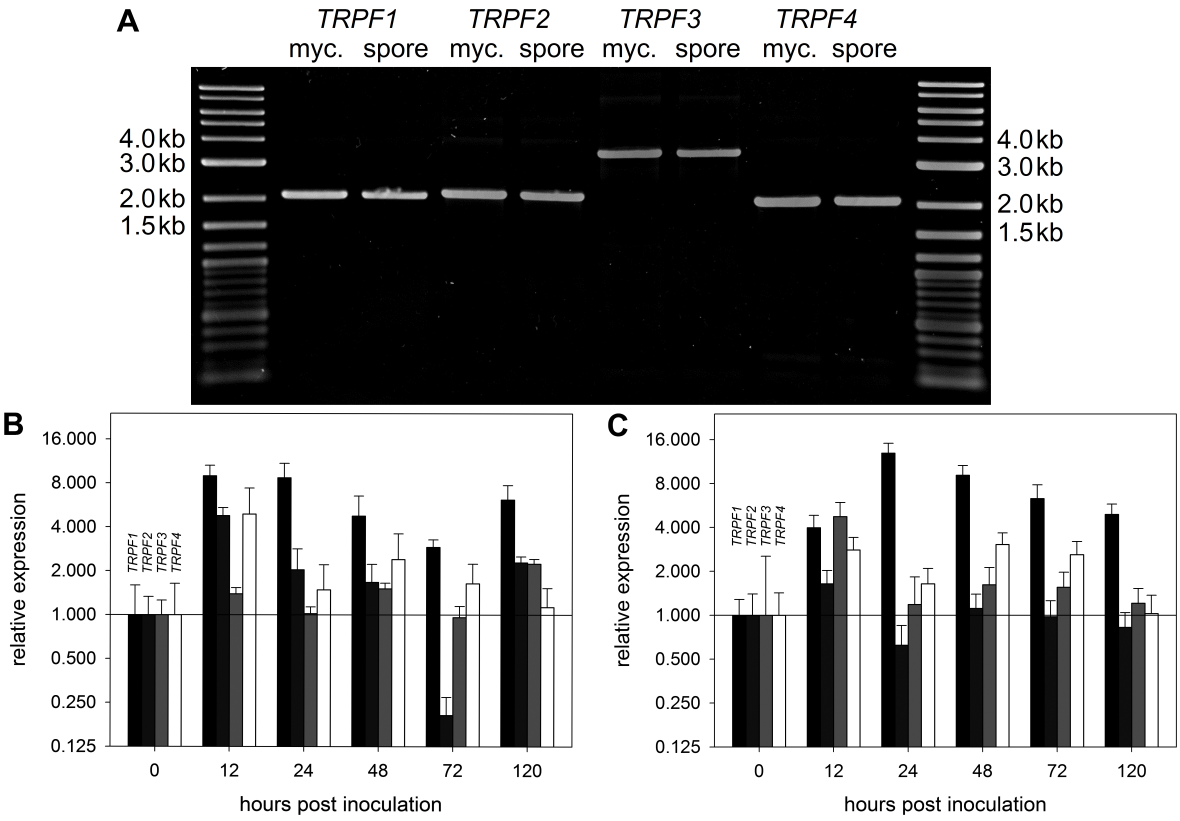

Fig. 5.3: *Expression profiles of the* CgTRPF *genes in axenic culture and during infection of* C. graminicola *on maize. (A) Full-length cDNA of* CgTRPF *genes was amplified from RNA extracted from* in vitro *grown mycelium and spores. Products were expected at 2098, 2152, 3522, and 2101 bp for* CgTRPF1, CgTRPF2, CgTRPF3, *and* CgTRPF4, *respectively. (B, C) Expression during the infection process relative to the expression in spores (0 hours post infection, hpi); black:* CgTRPF1, *dark grey:* CgTRPF2, *light grey:* CgTRPF3, *white:* CgTRPF4. *(B) Detached third leaves of 2.5-week-old drop-infected plants (cv. Mikado); assayed by qRT-PCR. Data are means ±SE (N=3). (C) Third leaf of intact two-week-old drop-infected plants (cv. Golden Jubilee); assayed by RNA-Seq. Data are means ± SE (N = 3).*

5.4.4 The *C. graminicola* TRPF proteins localize at intracellular membranes

TRP channels of animals localize either at the plasma membrane or at membranes of intracellular organelles, while the *S. cerevisiae* TRPY1 channel localizes at the membrane of the central vacuole. To determine the sub-cellular localization of the CgTRPF proteins, fusions of their genes, including native promoters, to the *EGFPf* gene were created, and the *C. graminicola* wild type was transformed with the fusion constructs. All CgTRPF-EGFPf proteins localized at intracellular organelles (Fig 5.4A). However, only CgTRPF4 resided in membranes delineating large vacuoles, similar to TRPY1 in *S. cerevisiae*, whereas CgTRPF1, CgTRPF2, and CgTRPF3 were found in small vesicular structures. Because CgTRPF1 through 3 exhibited a similar localization pattern, we investigated whether they are present in the same compartment. To this end, the respective genes were fused to *mCherry* and combined with *EGFPf* fusion constructs in a dual-tag plasmid system [Lange et al., 2014b]. The *mCherry* and

EGFPf fusion constructs were co-expressed in *C. graminicola*. Fluorescence microscopy of the resulting transformants strongly suggested that CgTRPF1, CgTRPF2, and CgTRPF3 do indeed co-localize in the same cellular compartment and may thus share a common function (Fig 5.4B).

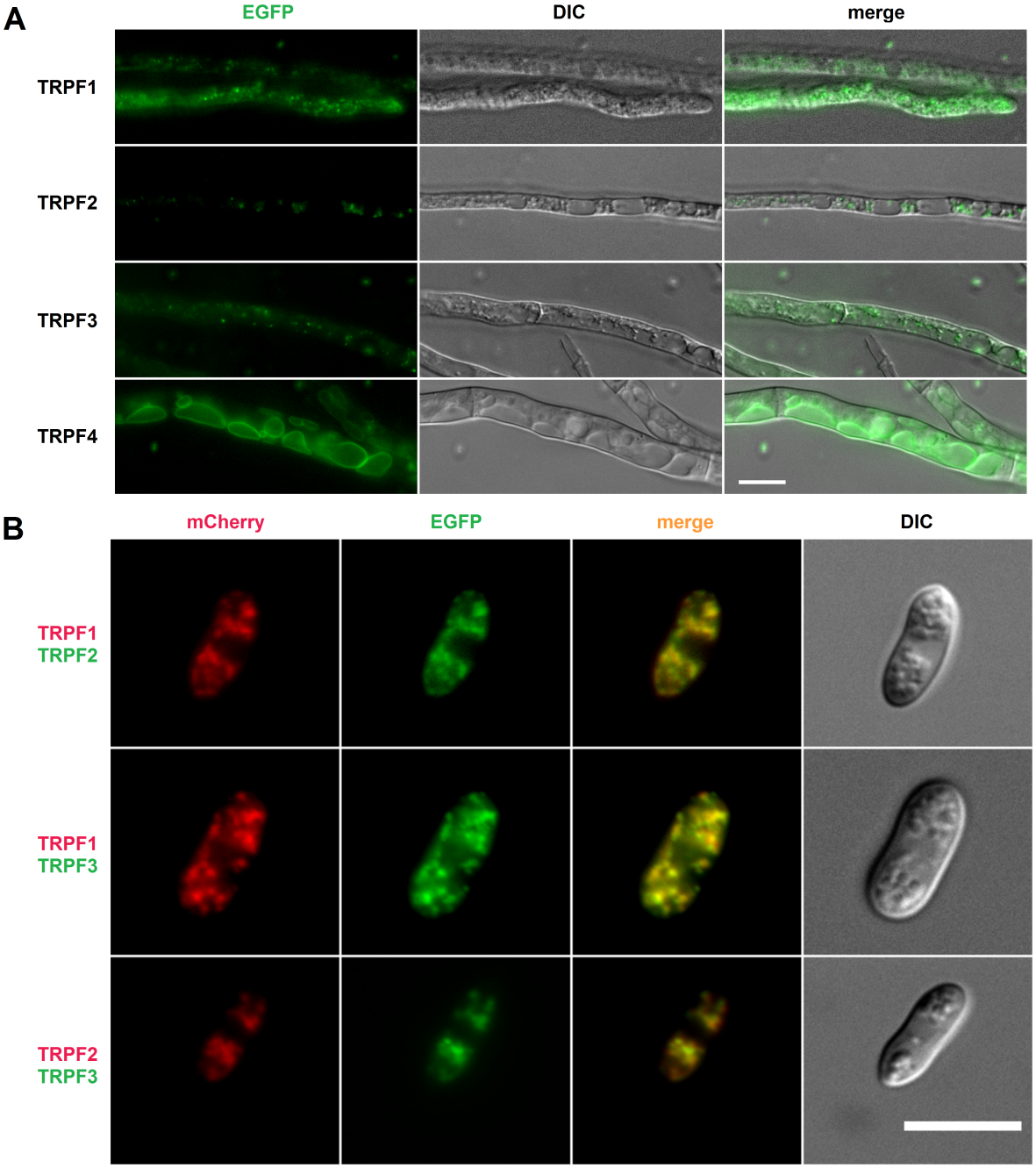

Fig. 5.4: *Sub-cellular localization of* Colletotrichum graminicola *TRPF proteins. (A) Hyphae expressing* CgTRPF1 *through* 4 *fused to* EGFPf *driven by the respective native* CgTRPF *promoters. (B) Co-localisation of TRPF1 through 3 with each other. Oval conidia expressing* CgTRPF1 *through* 3 *genetically fused to* mCherry *and* EGFPf *driven by the respective native* CgTRPF *promoters. Each strain was transformed with a* mCherry*-tagged TRPF gene and another* EGFPf*-tagged* CgTRPF *gene. Upper panel:* CgTRPF1-mCherry *and* CgTRPF2-EGFPf*, middle panel:* CgTRPF1-mCherry *and* CgTRPF3-EGFPf*, bottom panel:* CgTRPF2-mCherry *and* CgTRPF3-EGFPf*. Bars: 10* µm*.*

5.4.5 Deletion strains for all four *CgTRPF* genes were obtained

As all *CgTRPF* genes were expressed in spores, plate cultures, and throughout the infection process, and since mutants of other fungi for *TRPY1* homologues show severe defects [Nguyen et al., 2008; Yu et al., 2014a], we analysed the role of *CgTRPF1* through *4* in growth and pathogenicity. To this end, we created deletion strains for each gene by homologous recombination. One strain for Δ*Cgtrpf1* and Δ*Cgtrpf3* and two strains for Δ*Cgtrpf2* with the desired single integration of the deletion cassette were obtained (Fig. S5.1). For Δ*Cgtrpf4* only strains with several integrations were obtained. For further analysis of this gene, three individual strains were chosen that showed different Southern blot patterns for one additional integration event (Fig. S5.1).

As there are four *TRPY1* homologs in the genome of *C. graminicola*, a functional redundancy of the genes is not unlikely. This may be indicated by an increased expression of the remaining *CgTRPF* genes in the deletion strains. We therefore performed qRT-PCR experiments on wild type and *Cgtrpf* deletion strains, which are shown in (see Tab S5.2). In the deletion strains, an occasional weak upregulation of other family members was observed. However, this alteration was always well below two-fold, which does not indicate a strong compensatory response.

5.4.6 Spore germination is not altered in *Cgtrpf* deletion mutants

Germination of spores and appressorium formation are initial steps in the infection process of *Colletotrichum* species, which have been reported to be dependent on Ca^{2+} release from internal stores [Warwar and Dickman, 1996]. As all *CgTRPF* genes are expressed in spores

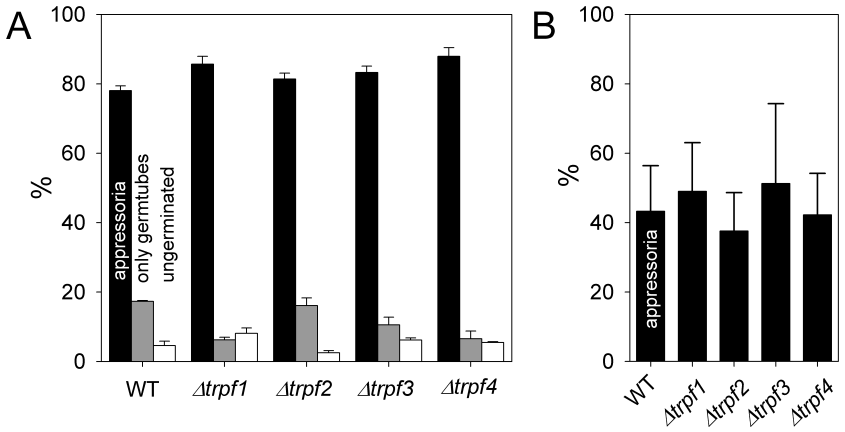

Fig. 5.5: *Germination of* C. graminicola *spores on (A) polystyrene and (B) onion epidermis. Black: germ tubes with appressoria, grey: germ tubes without appressoria, white: ungerminated conidia. On onion epidermis, only appressoria were counted. Data are means ± SE (N = 3; > 100 spores per replicate).*

(Fig 5.3) and as all CgTRPF proteins are localized to endomembranes (Fig 5.4), a role of those proteins in spore germination appeared likely. Therefore, germination was tested on polystyrene (Fig 5.5A) and on onion epidermis (Fig 5.5B). In both types of assay, germination of the deletion strains was not reduced compared to the wild type, indicating either no role of the *CgTRPF* genes in this process or a functional redundancy of the genes.

5.4.7 *Cgtrpf* deletion mutants are not defective in the utilization of complex carbon sources

C. graminicola is able to grow on culture media and plant tissues containing complex carbon sources. To utilize those, the fungus has to secrete hydrolytic enzymes by exocytosis, allowing the uptake of low-molecular compounds. Since exocytosis is known to be dependent on locally elevated $[Ca^{2+}]_{cyt}$, intracellular Ca^{2+} channels may have a potential impact on the secretion of enzymes that hydrolyse carbohydrates. To test whether CgTRPFs may function in this process or in the utilization of diverse carbon sources, wild type and deletion strains for all of the four genes were assayed for growth on mLCM and PDA, as well as on minimal media supplemented with glucose, sucrose, raffinose, sorbitol, mannitol, malate, pectate, or cellulose. The strains did not show any consistent and reproducible growth differences on any of the tested media (Fig 5.6). Hence, *CgTRPF* genes are either not required for those secretion events, or the genes are functionally redundant in this process.

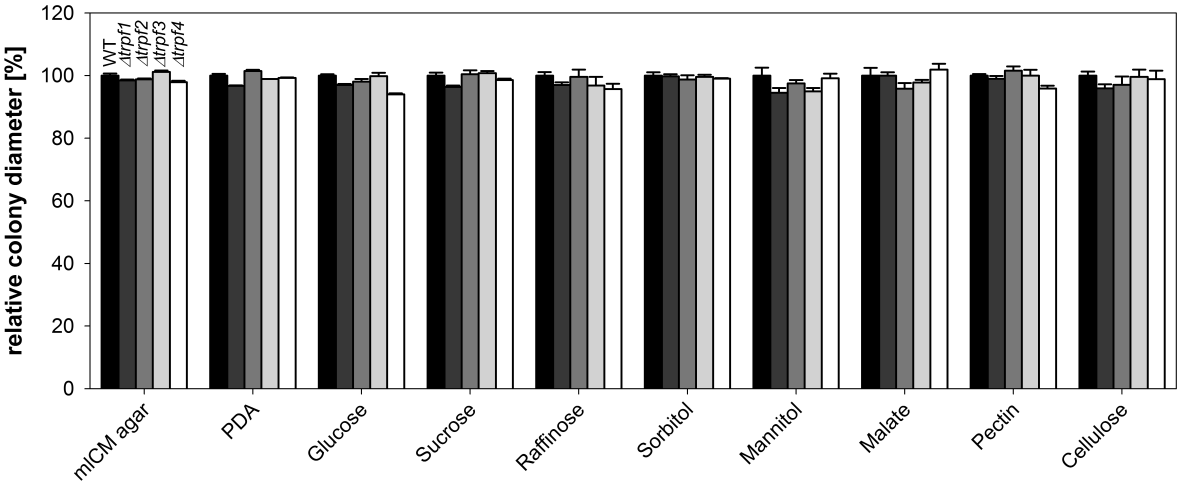

Fig. 5.6: *Growth of* C. graminicola *colonies on different carbon sources. Growth was assessed on mLCM agar, PDA, and minimal media administered with 2% of the respective carbon source. All values were normalized to the growth of the wild type on the respective medium. Colony diameter was measured 117 hours post inoculation. Data are means ± SE (N = 3).*

5.4.8 *Cgtrpf* deletion mutants are not defective in growth on low-Ca^{2+} media and in the generation of tip-focussed $[Ca^{2+}]_{cyt}$ spikes

As the CgTRPF proteins have similarities to the Ca^{2+}-permeable TRPY1, which is known to regulate $[Ca^{2+}]_{cyt}$ homeostasis in yeast, we tested if they play a role for growth under Ca^{2+}-limited conditions. To this end, the wild type and deletion strains were cultivated on Ca^{2+}-depleted SC agar medium, containing 1.7 µM total Ca^{2+}, and on Ca^{2+}-replete SC medium supplemented with 900 µM Ca^{2+} [Lange and Peiter, 2016]. As previously reported [Lange and Peiter, 2016], the wild type showed a growth reduction by around 20% on low-Ca^{2+} medium (Fig 5.7). This decrease in growth was also apparent, but not exacerbated, in the *Cgtrpf* mutant strains (Fig 5.7). Therefore, a role of CgTRPF proteins during Ca^{2+} starvation is unlikely.

To directly analyse a possible role of the CgTRPF proteins in the generation of $[Ca^{2+}]_{cyt}$ signals, wild type and *Cgtrpf* deletion mutants were transformed with the Ca^{2+} reporter Yellow Cameleon 3.6, and individual hyphae were examined by ratiometric fluorescence microscopy. As reported previously [Lange and Peiter, 2016], the *C. graminicola* wild type showed tip-focused spikes of high $[Ca^{2+}]_{cyt}$ during hyphal growth in a highly variable manner (Fig 5.8). It has been suggested that in *N. crassa*, a tip-focussed $[Ca^{2+}]_{cyt}$ gradient is generated by Ca^{2+} release from intracellular vesicles [Silverman-Gavrila and Lew, 2002]. We therefore tested whether any of the CgTRPFs may contribute to the apical $[Ca^{2+}]_{cyt}$ spikes of *C. graminicola*. However, all mutants still showed $[Ca^{2+}]_{cyt}$ spiking at the hyphal tip (Fig

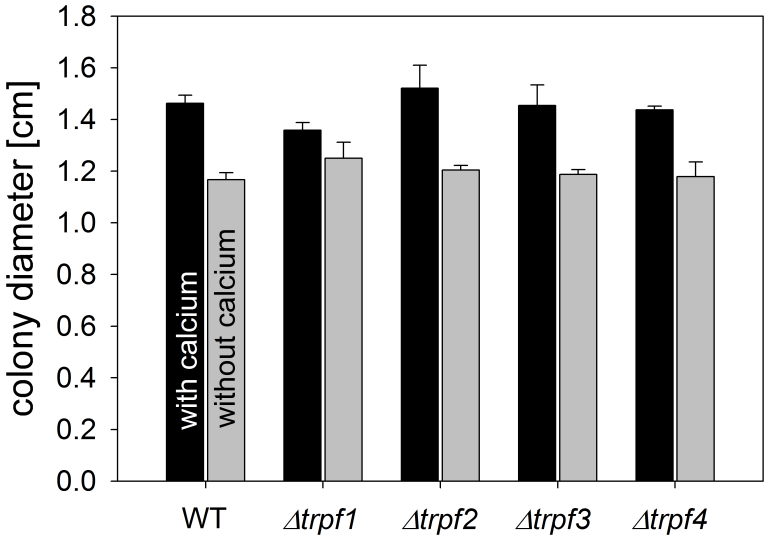

Fig. 5.7: *Colony growth of* C. graminicola *on low-Ca^{2+} media. Wild type and deletion strains were grown for 144 h on Ca^{2+}-depleted SC media, and colony diameter was determined. Data are means ± SE (N = 3).*

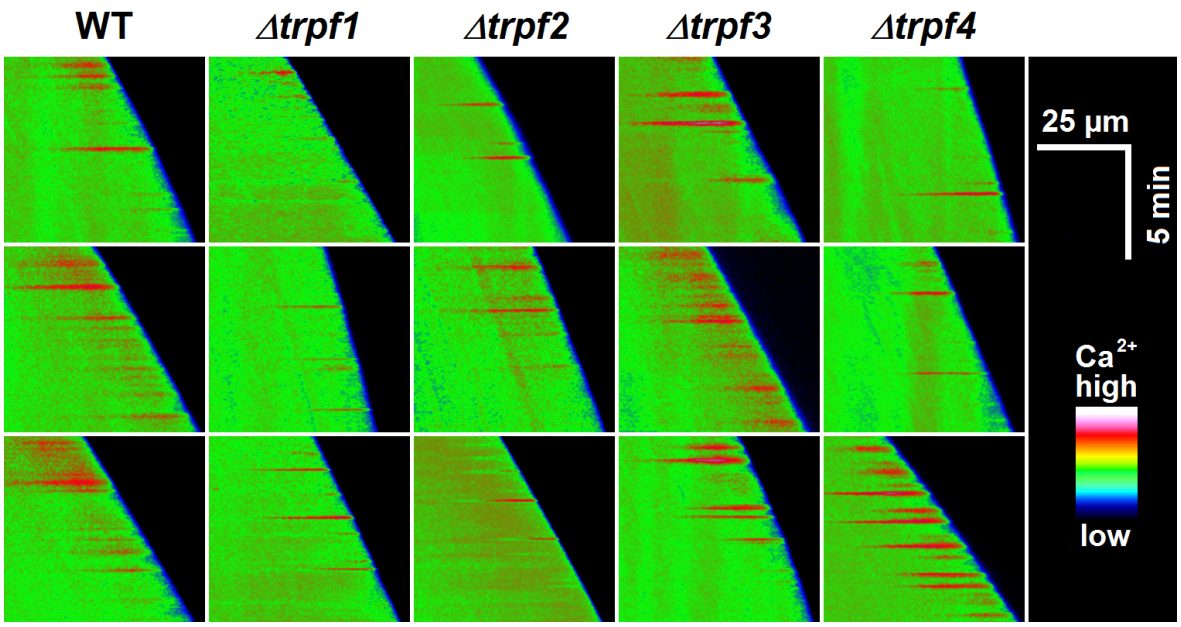

Fig. 5.8: *Yellow Cameleon-based measurements of* $[Ca^{2+}]_{cyt}$ *in individual hyphae. Kymographs of individual hyphae of wild type and deletion mutants. Each horizontal pixel line represents the mean* $[Ca^{2+}]_{cyt}$ *of a 5-pixel-wide ROI in the middle of each hypha. ROIs of subsequent images, acquired every 2 s, were plotted one below the other. The slope of the kymographs, read from top to bottom, thus indicates the growth rate. Relative* $[Ca^{2+}]_{cyt}$ *is displayed in false-colour using the RGB rainbow scale. All measurements were performed on hyphae growing at an mLCM agar-glass interface [Lange and Peiter, 2016].*

5.8), with the spike occurrence being highly variable between individual hyphae.

To observe $[Ca^{2+}]_{cyt}$ spike occurrence in undisturbed whole colonies, we developed a protocol based on aequorin luminometry [Lange and Peiter, 2016]. Similar to our previous study [Lange and Peiter, 2016], the wild type generated distinct $[Ca^{2+}]_{cyt}$ spikes during growth, which had a similar duration as the spikes observed in individual tips (not shown). The rate of spike occurrence was not reduced in any of the deletion strains (S5.3 Fig).

5.4.9 CgTRPF proteins do not function as osmotic stress sensors

TRPY1 is known to act as a sensor for osmotic disturbance in *S. cerevisiae*. To analyse whether CgTRPFs of *C. graminicola* may share this function, deletion mutants were analysed for growth under osmotic stress conditions. As expected, growth of the *C. graminicola* wild type strain on mLCM agar was increasingly inhibited by increasing glycerol concentrations (Fig 5.9A). All deletion mutants of the four *CgTRPF* genes were inhibited similar to the wild type on medium containing 0.5 M glycerol (Fig 5.9B).

A possible redundancy of the CgTRPF proteins in osmotic sensing was investigated by heterologous expression in yeast harbouring the $[Ca^{2+}]_{cyt}$ reporter apoaequorin. *S. cerevisiae* responds to hyperosmotic stress with a Ca^{2+} influx from the extracellular medium and a

concomitant release of Ca^{2+} from the vacuole mediated by TRPY1 [Denis and Cyert, 2002]. Chelation of extracellular Ca^{2+} renders the osmotic upshock-triggered $[Ca^{2+}]_{cyt}$ elevation absolutely dependent on TRPY1. Under those conditions, a *trpy1*Δ deletion mutant carrying the empty vector pFL61 did not show any $[Ca^{2+}]_{cyt}$ response to an osmotic upshock exerted by 1.5 M NaCl (Fig 5.9C, red line). As expected, the transient increase in $[Ca^{2+}]_{cyt}$ after application of osmotic stress was restored in transformants complemented with the native *TRPY1* from *S. cerevisiae* (Fig 5.9C, green line). To test if any of the *C. graminicola* TRPF

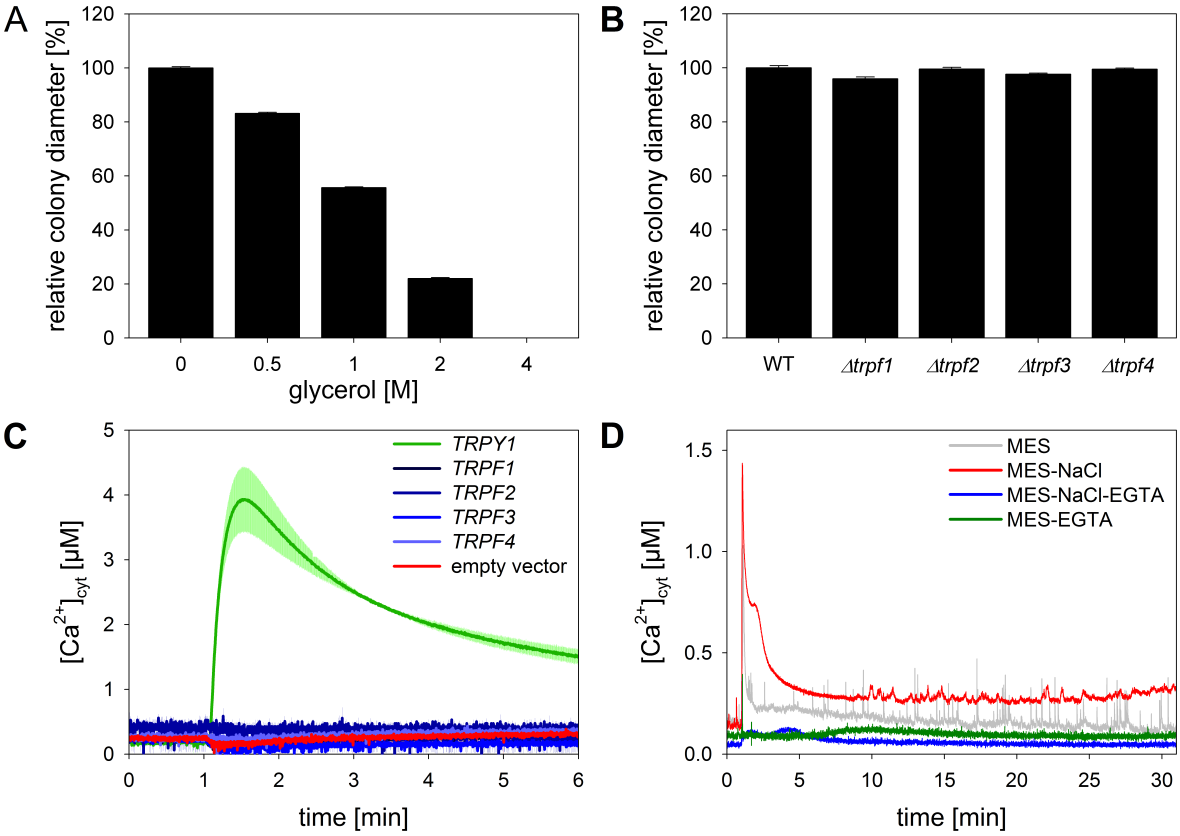

Fig. 5.9: *Response of mycelial growth of* C. graminicola *and* $[Ca^{2+}]_{cyt}$ *of* C. graminicola *and yeast to hyperosmotic stress. (A, B) Mycelial growth of* C. graminicola *osmotically stressed with glycerol. (A) Colony diameter of wild type stressed with varying concentrations of glycerol, normalized to unstressed colonies. (B) Wild type and deletion strains stressed with 0.5 M glycerol, normalized to the wild type. Colony diameters were determined 122 hours post inoculation. Data are means ± SE (N = 3). (C, D)* $[Ca^{2+}]_{cyt}$ *response of yeast and* C. graminicola *to NaCl measured by aequorin luminescence. (C) Response of* trpy1Δ *yeast mutant cells transformed with the indicated vectors to a solution (pH 7.0) containing 1.5 M NaCl, 50 mM MES-KOH, and 25 mM EGTA. Treatment was started at 1 min. Red line: empty pFL61 vector (negative control); green line: pFL61-ScTRPY1 (positive control); blue lines: yeast strains transformed with pFL61 containing* CgTRPF1 *through* 4. *Data are means ± (N = 3) per strain. (D)* $[Ca^{2+}]_{cyt}$ *measurements on* C. graminicola *wild type colonies. Whole colonies were pre-treated with 50 mM MES-KOH (pH 7.0) for 30 min prior to recording, followed by treatment with a solution (pH 7.0) containing 50 mM MES-KOH and no NaCl (grey line) or 1.5 M NaCl (final concentration; red line). To abolish the influx of extracellular* Ca^{2+}*, colonies were pre-treated with a solution (pH 7.0) containing 50 mM MES-KOH and 25 mM EGTA for 30 min prior to measurement, followed by treatment with a solution (pH 7.0) containing 50 mM MES-KOH, 25 mM EGTA, and no NaCl (green line) or 1.5 M NaCl (final concentration) (blue line). Treatment solutions were added after 1 min of recording. Traces show individual measurements in order to demonstrate* $[Ca^{2+}]_{cyt}$ *spikes in the MES-KOH control treatment. Replicate measurements can be found in S5.4 Fig.*

proteins mediates a Ca^{2+} flux in response to osmotic upshock, the full-length cDNAs of *CgTRPF1* though *4* were constitutively expressed in the *trpy1*Δ mutant. Expression of the CgTRPF genes was confirmed by RT-PCR (S5.5 Fig). Surprisingly, none of the *CgTRPF* genes complemented the $[Ca^{2+}]_{cyt}$ response of the yeast mutant (Fig 5.9C, blue lines), suggesting that they may either not be sensitive to osmotic shock or not active, e.g. due to problems with heterologous expression.

Since the *CgTRPF* genes did not complement the *trpy1*Δ yeast strain, we asked whether this fungus, like *S. cerevisiae*, responds to hyperosmotic shock with a $[Ca^{2+}]_{cyt}$ transient that is partially generated by Ca^{2+} release from internal stores. To this end, we employed the apoaequorin-harbouring wild type strain. Treatment of whole colonies with buffer alone caused a short and small response that phased out entirely after about 3 min (Fig 5.9D, grey line). This baseline was stable until the end of the measurement at 30 min except for very short $[Ca^{2+}]_{cyt}$ spikes in the range of a few seconds, as described above (S5.3 Fig). Treatment with a solution containing 1.5 M NaCl evoked a large initial peak in $[Ca^{2+}]_{cyt}$ that was followed by a shoulder and a sustained elevation at a level well above the baseline (Fig 5.9D, red line). This strong response was nearly completely prevented by the addition of EGTA, which chelates extracellular Ca^{2+} (Fig 5.9D, blue line). This indicates that, in contrast to *S. cerevisiae*, the hyperosmotic stress-triggered $[Ca^{2+}]_{cyt}$ response of *C. graminicola* is sourced nearly entirely from the external medium. EGTA alone provoked no discernible response (Fig 5.9D, green line).

5.4.10 Cgtrpf deletion mutants are unaffected in pathogenicity

As all assays failed to identify a possible involvement of *C. graminicola* TRPF proteins in various aspects of growth and environmental responses, and as all *CgTRPF* genes were expressed during infection of maize (Fig 5.3), we tested the deletion strains for virulence, which integrates a large array of sensing and signalling mechanisms. In a detached leaf segment assay, none of the mutants exhibited symptoms that differed noticeably from those of the wild type (Fig 5.10).

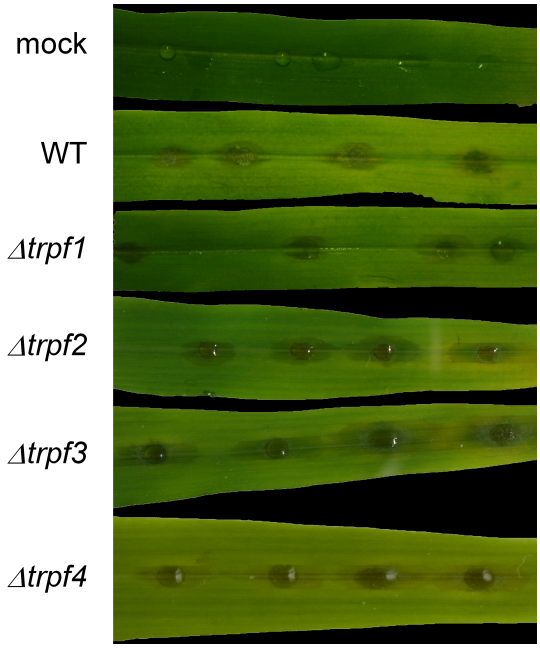

Fig. 5.10: *Detached-leaf infection assay. Sections of the third leaf of two-week-old maize plants (cv. Golden Jubilee) were infected with 10^4 spores of* C. graminicola *wild type or* Cgtrpf1 *through* 4 *deletion mutants per 10-µL drop, or mock-infected with 10 µL of 0.02% Tween 20 in bidistilled water. Representative images of three biological replicates are shown; the experiment was repeated twice with similar results.*

5.5 Discussion

A number of pharmacological studies have suggested a role of Ca^{2+} release from internal organelles in the regulation of various developmental processes in fungi, such as germination [Warwar and Dickman, 1996] and hyphal tip elongation [Silverman-Gavrila and Lew, 2002], as well as in responses of fungi to environmental stimuli, such as changes in osmolarity [Denis and Cyert, 2002; Matsumoto et al., 2002; Nelson et al., 2004]. In *S. cerevisiae*, the Transient Receptor Potential channel homologue TRPY1 is a Ca^{2+}-permeable channel in endomembranes that has been demonstrated to contribute to the generation of $[Ca^{2+}]_{cyt}$ signals [Palmer et al., 2001]. In support of a pivotal role of this class of ion channels in fungi, the knock-down of a *TRPY1* homologue in *M. oryzae* resulted in a drastically reduced colony growth [Nguyen et al., 2008].

We therefore considered this family as a potential target for plant protection strategies against the devastating maize pathogen *C. graminicola*, and analysed its involvement in growth, environmental responses, and pathogenicity of this fungus. Our initial database queries revealed that this gene family was expanded in most filamentous fungi, with up to four members in the examined genomes, while the genomes of all examined yeasts contain only a single *TRPY1* homologue. This is in good agreement with a comparative genomic

analysis pointing to a possible expansion of the TRP family in filamentous fungi [Prole and Taylor, 2012]. The members in filamentous fungi, which we denominate *TRPF*s, cluster in five subgroups. In the genome of *C. graminicola*, four *TRPF* genes were found. All were expressed throughout development and infection, and all encoded proteins were localized in intracellular organelles. Further on, the proteins display a membrane topology similar to the yeast TRPY1 channel, albeit with a variable number of TM domains additional to the core of six TM domains. CgTRPF1, 3, and 4 also have acidic motifs that are similar to the Ca^{2+}-binding tetra-aspartate motif of TRPY1 [Su et al., 2009]. These motifs are predicted to reside in cytosolic or luminal termini. Interestingly, a luminal Ca^{2+}-binding site and regulation of channel activity by luminal Ca^{2+} have been demonstrated for the Two Pore Channel 1 (TPC1) [Dadacz-Narloch et al., 2011], a vacuolar cation channel in plants [Peiter et al., 2005b]. CgTRPF1 and CgTRPF3 may thus sense and be regulated by luminal $[Ca^{2+}]$. Collectively, an important role of these genes in Ca^{2+}-related processes, in particular those involving Ca^{2+} release from internal stores, seemed likely, but could not be unveiled in our experiments.

5.5.1 *C. graminicola* TRPFs are not activated by osmotic upshock

In *S. cerevisiae*, the only TRP member, TRPY1 (Yvc1), forms a stretch-activated Ca^{2+}-permeable cation channel in the vacuolar membrane ([Palmer et al., 2001; Zhou et al., 2003]) that contributes to the generation of the hyperosmotic-shock-triggered $[Ca^{2+}]_{cyt}$ signal [Denis and Cyert, 2002]. By complementation analysis of the *trpy1*Δ mutant, mechanosensitivity and osmotic response were also shown for the *TRPY1* homologues of the yeasts *K. lactis* and *C. albicans* [Zhou et al., 2005], as well as of the filamentous fungus *Fusarium graminearum* [Ihara et al., 2013]. In contrast, none of the *CgTRPF* genes from *C. graminicola* complemented the defective $[Ca^{2+}]_{cyt}$ response of the *S. cerevisiae trpy1*Δ mutant to osmotic upshock. This might indicate that technical problems of the heterologous expression system (e.g. incorrect folding, missing interaction partners, insufficient stabilization against protein degradation, or mislocalization of the CgTRPF proteins) have prevented yeast complementation. However, we consider this as not very likely because other TRP channels are functional in this system. Alternatively, the failure to complement the *trpy1*Δ response to osmotic upshock may indicate that not all TRPF proteins act in the perception of osmotic stress. To further test this presumption, the response of *C. graminicola* to hyperosmotic shock was analysed. In *C. graminicola*, 1.5 M NaCl triggered a massive Ca^{2+} influx via the plasma membrane, but,

unlike in yeast, a negligible release of Ca^{2+} from internal stores. Since TRPF channels of *C. graminicola* were localized to intracellular membranes, these results further substantiate the idea that in *C. graminicola* TRP proteins are not involved in Ca^{2+} release into the cytosol upon hyperosmotic stress.

5.5.2 *C. graminicola* TRPFs are dispensable for hyphal growth in axenic culture

A further process that we considered likely to be regulated by TRPF proteins is the utilization of complex carbon sources, which relies on the exocytosis of hydrolytic enzymes. In yeast, membrane fusion, a prerequisite of exocytosis, is dependent on the activation of calmodulin by Ca^{2+} release from the fusing vesicles [Peters and Mayer, 1998]. Furthermore, tricalbins, which contain three Ca^{2+}-binding C2 domains, and which are homologous to Ca^{2+}-activated synaptotagmin in animals, have been linked to membrane fusion in yeast [Schulz and Creutz, 2004]. However, despite the presumed $[Ca^{2+}]_{cyt}$ dependence of enzyme secretion, the *Cgtrpf* deletion strains did not differ from the wild type in their growth on complex carbon sources.

Growth of *C. graminicola* colonies is very sensitive to the inhibitors 2-APB and capsazepine, which block a range of TRP channels in animals [Lange and Peiter, 2016]. However, none of the *Cgtrpf* deletion stains showed a diminished growth potential on various standard media, or on media with complex carbon sources, all containing high amounts of Ca^{2+}. Since release of Ca^{2+} from intracellular stores might become important for growth under Ca^{2+}-limiting conditions, we cultivated the strains on Ca^{2+}-depleted medium, which causes a moderate growth depression in the wild type. However, growth depression in *trpf* deletion strains was not more severe than in the wild type. Hence, individual CgTRPF proteins are dispensable for growth in Ca^{2+}-limiting environments.

During undisturbed growth, filamentous fungi, including *C. graminicola*, generate short tip-focussed $[Ca^{2+}]_{cyt}$ pulses that can be visualized by fluorescence ratio imaging microscopy of the Yellow Cameleon (YC) reporter protein [Kim et al., 2012, 2015; Lange and Peiter, 2016]. Unlike in other tip-growing systems, such as pollen tubes [Steinhorst and Kudla, 2013], these $[Ca^{2+}]_{cyt}$ pulses are apparently not related to growth kinetics, but may rather be involved in environmental sensing [Lange and Peiter, 2016]. $[Ca^{2+}]_{cyt}$ spikes of short duration are also detectable on whole-colony level as luminescence of the Ca^{2+} reporter aequorin [Lange and Peiter, 2016]. In *N. crassa*, a tip-focussed Ca^{2+} gradient has been suggested to be maintained by Ca^{2+} release from intracellular vesicles [Silverman-Gavrila and Lew, 2002]. However,

in *Cgtrpf* deletion mutants, $[Ca^{2+}]_{cyt}$ spike generation was affected neither on single-hypha level nor on whole-colony level. This corresponds well to the fact that on whole-colony level, spike occurrence was nearly completely abolished by chelation of extracellular Ca^{2+} [Lange and Peiter, 2016], suggesting that those $[Ca^{2+}]_{cyt}$ spikes depend on Ca^{2+} influx rather than Ca^{2+} release from internal stores.

5.5.3 Deletion of *C. graminicola TRPFs* does not impede pathogenicity

The *TRPF* genes were expressed throughout the infection process of *C. graminicola* on maize plants. Interestingly, there was a transcriptional regulation of the genes by up to 13-fold during the course of infection. This is unexpected for ion channels, which are primarily regulated on post-translational level. However, regulation of TRP channels on mRNA level is also known for a number of mammalian family members, such as *TRPC1*, *TRPC3*, *TRPV4*, and *TRPV6* [Jung et al., 2009; Pigozzi et al., 2006; Walters et al., 2006]. Leaf segment infection assays were performed to integrate all pathogenic processes from spore germination to leaf necrosis [Münch et al., 2011]. These assays did not indicate pathogenicity defects in any of the *Cgtrpf* deletion strains. This is in stark contrast to the phenotype of a *trpf1* RNAi knockdown mutant of *M. oryzae*, which showed a severely repressed virulence [Nguyen et al., 2008]. However, the *M. oryzae* genome bears only two *TRPF* genes, so that in *C. graminicola*, there may be higher degree of functional redundancy between the family members, albeit their topologies and structures vary. To resolve this question, we attempted a quadruple RNAi knockdown approach, employing a vector system that has previously been successfully used to diminish expression of a *C. graminicola* β1,3-Glucan Synthase-encoding gene [Oliveira-Garcia and Deising, 2013]. Unfortunately, RNAi-based knockdown of *CgTRPF* genes was not very efficient, with their expression being decreased by not more than 50% (data not shown). Quadruple-RNAi strains did not show any phenotypic differences to the wild type during the strain selection process and were still pathogenic in leaf segment assays (data not shown). Unfortunately, it was therefore not possible to analyse a functional redundancy of the four *CgTRPF* genes, which might be the cause of the absence of phenotypical alterations in any of the single *Cgtrpf* mutants. Albeit a redundancy was not obvious on transcriptional level in the deletion strains, a redundancy may also occur on the level of protein activity. It is also possible that the *TRPF* genes of *C. graminicola* are important under conditions not tested in this study, but occurring in its native habitat, such as extreme temperatures, high light intensities, or interaction with other microorganisms.

5.5.4 Evidence for a functional diversification of orthologous Ca^{2+} channels in fungi

In contrast to *M. oryzae*, TRPF proteins in *C. graminicola* are not essential for colony growth and virulence. Furthermore, these channels do not mediate a $[Ca^{2+}]_{cyt}$ elevation after osmotic upshock, as shown for homologues from other fungal species. This indicates a functional diversification of this ion channel family in fungi. A change in functionality can be similarly observed in an animal TRP family member, TRPV1, the receptor for capsaicin (the spicy component of hot chilli pepper): Rabbits are about 100 times less sensitive to capsaicin than humans and rats, and birds are completely insensitive. A molecular basis for these drastic differences lies in just two point mutations in the *TRPV1* gene [Nilius and Szallasi, 2014]. Hence, even small variations may also render the TRPF orthologs functionally highly diverse in different fungal species.

Species-specific differences in the regulation of fungal growth have also become apparent for the Cch1/Mid1 complex that mediates Ca^{2+} uptake across the plasma membrane. In *Aspergillus nidulans* a deletion of *Cch1* and/or *Mid1* results in drastically reduced colony growth even on complete media [Wang et al., 2012], whereas in *Cryptococcus neoformans* and *Botrytis cinerea* the deletion of *Cch1* and/or *Mid1* affects growth only under severe Ca^{2+} limitation, and deletion strains of *B. cinera* are phenotypically indifferent from the wild type inside their native host [Harren and Tudzynski, 2013; Liu et al., 2006]. These examples support the notion that components of the Ca^{2+} signalling toolbox may be employed differently in different fungi and that results may not always be simply extrapolated from one species to another.

5.6 Acknowledgments

We thank Liane Freitag and Tina Peiter-Volk for excellent technical assistance and Elke Vollmer for skilful plant husbandry.

6 General Discussion

6.1 Measurements of cytosolic free Ca^{2+} concentration ($[Ca^{2+}]_{cyt}$) in *C. graminicola*

Calcium concentrations in fungi can be determined by a number of techniques. Steady state concentrations of whole cell Ca^{2+} can be measured by inductively coupled plasma atomic emission spectroscopy (ICP-AES) [Colinet et al., 2016]; radioactive measurements of $^{45}Ca^{2+}$ can be used to easily quantify cellular Ca^{2+} uptake. However, for signalling $[Ca^{2+}]_{cyt}$ is more important than total Ca^{2+} [Borle, 1990]. $[Ca^{2+}]_{cyt}$ can be determined by Ca^{2+}-sensitive dyes or proteins. Dyes don't need genetic transformation of organisms, but dye loading bears the risk to induce artefacts [Takahashi et al., 1999]. As genetic engineering is possible in *C. graminicola*, transgenic Ca^{2+} reporter proteins are the method of choice. In this study, the luminescent aequorin probe was used in the *AeqS* version optimized for filamentous fungi [Nelson et al., 2004]. Aequorin is an enzyme that converts its substrate coelenterazine in a Ca^{2+}-dependent manner to coelenteramide in a light-releasing reaction. Aequorin can report $[Ca^{2+}]_{cyt}$ over very long periods, up to days. Successful aequorin measurements need either extremely strong signals from individual cells or a large number of cells generating the signal in a synchronized way to detect smaller Ca^{2+} signals [Créton et al., 1999]. The second reporter in use was Yellow Cameleon 3.6 (YC3.6). This reporter consists of a yellow and a cyan fluorescent moiety linked by a Ca^{2+}-sensitive motile spacer. Therefore, $[Ca^{2+}]_{cyt}$ is translated into FRET efficiency. YC3.6 can detect Ca^{2+} signals of single cells. However, YC3.6 is photo-bleached during measurements, limiting measurement duration [Krebs et al., 2012]. It was attempted to measure $[Ca^{2+}]_{cyt}$ in all relevant stages of *C. graminicola*'s hemi-biotrophic lifecyle by employing these Ca^{2+} reporters. In the infection process of *C. graminicola*, spores germinate, a short germ tube emerges, and an appressorium is formed at the germ tube's apex. Next, penetration of the host and biotrophic growth occur, followed by necrotrophic growth and formation of new spores [Bergstrom and Nicholson, 1999]. It was initially attempted to determine Ca^{2+} signals during spore germination, the

first developmental stage in the lifecycle of *C. graminicola*. The germination process is non-synchronized and occurs between 3 to 9 h after contact with the maize leaf surface [Mercure et al., 1994]. Unfortunately, the aequorin signals of individual germinating spores were below the detection limit. Chimera of aequorin and EGFP were reported to be more sensitive than aequorin alone [Baubet et al., 2000] and were therefore tested in preparation of this project. However, in *C. graminicola* this lead to an aggregation of the reporter which did not allow any $[Ca^{2+}]_{cyt}$ measurement (Mario Lange, unpublished). YC3.6 measurements of *C. graminicola* were limited to approximately 1 h before bleaching became too strong. Therefore, a large number of spores measured at different stages of the germination process would have been necessary to reconstruct the entire germination process. To acquire such a data set, good germination efficiency would be needed. Indeed, falcate spores matured for 3 to 4 weeks showed high germination efficiency in the measurement chambers used for YC3.6 microscopy. However, spores matured for just 2 weeks, which is common for virulence assays, germinated inefficiently under the conditions of YC3.6 measurements. In addition, whereas YC3.6 was well-detectable in the spores aged 2 weeks, it degraded quickly afterwards. Unfortunately, stronger expression of YC3.6 resulted in the formation of clumpy fluorescent aggregates, as described above for the aequorin-EGFP chimera. In conclusion, with recent technologies it was not possible to measure $[Ca^{2+}]_{cyt}$ in *C. graminicola* during germination and appressorium formation.

In *C. graminicola* growing *in planta*, $[Ca^{2+}]_{cyt}$ spikes were measureable with aequorin. However, a localization of these signals within the colony was not possible due to low signal intensity. This is a typical limit of the aequorin technique in small- to medium-sized cells [Créton et al., 1999]. To overcome this limitation, YC3.6 was tested under these conditions. Now, the yellow fluorescence of YC3.6 was clearly detectable, but the cyan fluorescence was lower than the autofluorescence of maize and onion tissues. For those reasons, *in planta* $[Ca^{2+}]_{cyt}$ measurements of *C. graminicola* were not possible with the employed technologies.

C. graminicola can also grow as a saprophyte [Bergstrom and Nicholson, 1999]. Therefore, it was attempted to measure $[Ca^{2+}]_{cyt}$ during growth on and in culture medium. The autofluorescence of culture medium was much lower than that of plant tissues and allowed YC3.6 measurements of hyphal tips (see chapter 4). These measurements showed (I) that $[Ca^{2+}]_{cyt}$ spikes occur prior to re-initiation of hyphal growth and (II) that $[Ca^{2+}]_{cyt}$ spikes are not correlated to continuous hyphal growth in *C. graminicola*. The first result indicates an involvement of Ca^{2+} signalling in the hyphal apex during growth re-initiation. These signals

have not been observed in fungi before. The process of hyphal growth re-initiation is rarely described in literature and mostly treated as technical problem [Sampson et al., 2003]. Its physiology has not been analyzed so far. This lack of literature data may be due to the fact that hyphal growth re-initiation is very variable and poorly predictable.

In tubular growing plant cells, i.e. pollen tubes and root hairs, $[Ca^{2+}]_{cyt}$ oscillations at the apex and oscillations in growth are closely linked [Monshausen et al., 2008; Steinhorst and Kudla, 2013]. In a study by Kim and colleagues, apical $[Ca^{2+}]_{cyt}$ spikes were detectable in growing hyphae of two *Fusarium* species and of *Magnaporthe oryzae* [Kim et al., 2012]. These authors speculated about a possible correlation of $[Ca^{2+}]_{cyt}$ spikes and oscillations in growth in fungi, but due to technical and biological limitations they were unable to resolve this issue. In this PhD thesis, it was possible to answer this question for *C. graminicola*: Hyphal growth was non-oscillatory, and no correlation between hyphal growth and apical $[Ca^{2+}]_{cyt}$ spikes was found. In young pollen tubes, there are also no apical $[Ca^{2+}]_{cyt}$ oscillations or oscillations in growth, and it was reported that older pollen tubes may switch from oscillatory to linear growth [Messerli and Robinson, 1997; Sampson et al., 2003]. Therefore, the model of oscillations in $[Ca^{2+}]_{cyt}$ being linked to an oscillatory growth rate in tubular plant cells is limited to some stages and is not (at least not generally) applicable in fungi.

Spatially resolved $[Ca^{2+}]_{cyt}$ measurements on a whole-colony level were impossible because the signal intensity of YC3.6 in older hyphae declined below the useful range, and the signal intensity of aequorin was too low for a spatially resolved analysis. However, as saprophytic growth is less complex compared to growth *in planta*, even spatially non-resolved aequorin measurements allow some insight into processes on a whole-colony level. Hence, the pharmacological analysis showed that there is also no correlation of whole-colony growth rate and $[Ca^{2+}]_{cyt}$ spikes detected from whole fungal colonies (see chapter 4).

Kim and colleagues were able to image $[Ca^{2+}]_{cyt}$ dynamics in spores and in hyphae, but they could not correlate them to growth [Kim et al., 2012]. They were also unable to statistically analyze $[Ca^{2+}]_{cyt}$ signatures during spore germination, but rather showed data from individual spores. Although they were able to image *Fusarium oxysporum* hyphae growing in the root of *Arabidopsis thaliana*, measurements in leaves were very problematic *Kim2012*. Taken together with the experience gained in this thesis, there are technical limitations that restrict the examination of certain questions and processes to different fungal species. In this respect, the next generation of $[Ca^{2+}]_{cyt}$ sensors may help. Autofluorescence problems could be overcome by YC derivatives with modified spectral characteristics, such as green-

orange Cameleons. However, the ratio-change of these spectral variants is below that of YC3.6 [Pérez Koldenkova and Nagai, 2013]. A highly sensitive alternative to YC3.6 is the non-ratiometric Ca^{2+} sensor R-GECO1. Unfortunately, the current version of this sensor is not only sensitive to Ca^{2+} but also to pH, while YC3.6 is almost insensitive to changes in pH [Keinath et al., 2015]. The pH sensitivity is especially problematic, as changes in $[Ca^{2+}]_{cyt}$ and pH can happen simultaneously [Monshausen et al., 2008]. This pH sensitivity may be reduced in future versions e.g. by mutated versions of the fluorophore [Griesbeck et al., 2001]. Measurements using aequorin can be performed for much longer time than those with YC3.6. However, despite the very large dynamic range of aequorin, its signal intensity is very low. The sensitivity of aequorin can be enhanced by fusing to a green-fluorescent protein [Baubet et al., 2000]. Unfortunately, in *C. graminicola* high expression levels of EGFP and its derivates lead to the formation of clumpy fluorescent aggregates. This phenomenon was even more problematic with earlier versions of GFP [Tsumoto et al., 2003]. Therefore, this problem of aggregation may be overcome with future GFP versions or a green-fluorescent protein that is not derived from jellyfish and may have an improved solubility in *C. graminicola*. Such an improved aequorin-green-fluorophore pair may then allow not only temporally but also spatially resolved $[Ca^{2+}]_{cyt}$ measurements. Another alternative to aequorin is the recently developed bright Nano-lantern(Ca^{2+}) reporter. At the moment, this reporter suffers from a low dynamic range as compared to aequorin [Saito et al., 2010]. This limit might be partially overcome by using new synthetic Ca^{2+}-sensitive moieties, e.g. from D3 (Ca^{2+} sensor design version 3) [Palmer et al., 2006]. In addition, the yellow fluorescing moiety of Nano-lantern(Ca^{2+}) may be replaced by a more soluble non-jellyfish counterpart, which would allow a high expression of this sensor. In summary, far better $[Ca^{2+}]_{cyt}$ sensors than those available at present can be expected in the future. These sensors likely will allow much deeper insights into Ca^{2+} signalling in fungi and beyond.

6.2 Development of new techniques to study fungal *TRP* genes

There are four TRPF genes in the *C. graminicola* genome. The gene products of these genes might display a certain degree of redundancy. To elucidate this redundancy, a simultaneous knock-down approach of all four *TRPF* genes was performed. Many clones needed to be screened to find a clone with strongly reduced expression of all *TRPF* genes. The existing methods to culture mycelium of *C. graminicola* for RNA extraction were insufficient with respect to throughput and replicate-to-replicate reproducibility. The PVDF (polyvinylidene

difluoride) membrane-assisted agar plate (PAAP) culture, developed in this thesis, clearly improved both aspects (see chapter 2). Unfortunately, both designs of the quadruple RNAi constructs didn't induce a strong enough knock down. However, the PAAP culture protocol turned out to be very useful also in experiments that required a transfer of intact fungal colonies between different media (see chapters 4 and 5).

As the localization of a protein largely determines its potential function, the TRPF proteins were localized by GFP-tagging. TRPF1 through 3 showed a similar pattern of subcellular localization. Therefore, the encoding genes were also tagged with the red-fluorescent marker *mCherry*. To analyze potential co-localization, the stably transformed fungal *TRPF-GFP* strains should be transformed in a second round with *TRPF-mCherry*. However, after transformation the *TRPF-GFP* strains had lost their GFP-fluorescence (data not shown), albeit a PCR analysis showed that the *GFP*-tagged *TRPF* genes were still present in the strains. To overcome this problem of potential gene silencing, the tagging plasmid system was developed further (see chapter 3). This new version enabled the simultaneous transformation of *GFP*- and *mCherry*-tagged genes. Hence, it was named double tagging plasmid system (DTPS). Now microscopic analysis of co-localization became possible before gene silencing happened. The DTPS was not only used in this study, but also in a cooperation to analyze protein-protein interactions via split YFP (Ely-Oliveira Garcia and Holger Deising, unpublished).

6.3 Analysis of *TRPY1* homologues of *C. graminicola*

Pharmacological studies pointed to an important role of intracellular Ca^{2+} release for fungal pathogenicity [Magalhaes et al., 1991; Silverman-Gavrila and Lew, 2002; Singh et al., 2001]. Accordingly, a comparative RNAi study in *M. oryzae* revealed that an intracellular Ca^{2+}-permeable *TRP* channel homologue is more important for pathogenicity than the plasma membrane Ca^{2+} channel Cch1-Mid1 [Nguyen et al., 2008]. In most filamentous fungi there are several *TRP* channel homologues, which we named *TRPF* (for *TRP in Fungi*), but only one *Cch1* and *Mid1* homologue. In *C. graminicola*, four *TRPF* members exist. Therefore, an important role of this channel family in *C. graminicola* was assumed (see chapter 5).

In *S. cerevisiae* TRPY1 releases Ca^{2+} from the vacuole into the cytosol upon hyperosmotic shock [Palmer et al., 2001]. This is also true for the TRPY1 homologues of the yeast fungus *K. lactis*, the dimorphic fungus *C. albicans*, as well as the filamentous fungus *F. graminearum* when expressed in a *trpy1*Δ strain of *S. cerevisiae* [Ihara et al., 2013; Zhou et al., 2005].

However, none of the *C. graminicola TRPF* genes expressed in a *trpy1*Δ strain of *S. cerevisiae* complemented the deletion phenotype. It was found that the Ca^{2+} signal upon hyperosmotic shock in *C. graminicola* is indeed generated via influx over the plasma membrane, and not by a release from intracellular stores. This result and the intracellular localization of the TRPF proteins point to a function different from an osmotic sensor. TRPY1 resides in the vacuolar membrane of *S. cerevisiae* [Palmer et al., 2001]. However, TRPF1 through 3 co-locate to small vesicles, and only TRPF4 is a vacuolar membrane protein. All TRPF genes were expressed throughout the entire infection cycle of *C. graminicola* on maize as well as in axenic culture. This indicated novel functions of *TRPF*s in *C. graminicola*. To elucidate them, deletion strains were generated and physiologically analyzed. However, no difference to the wild type was detected in many tested parameters believed to involve intracellular Ca^{2+} release, i.e. colony growth under Ca^{2+} starvation; with complex carbon sources; as well as under osmotic stress; $[Ca^{2+}]_{cyt}$ spikes in hyphal tips and on whole-colony level; spore germination *in vitro* and *in vivo*; and virulence. Since a redundancy of the genes cannot be ruled out, albeit their structure and topology are diverse, a quadruple-RNAi approach was attempted, and the mutants were screened using the PAAP culture method. Unfortunately, two tested quadruple-RNAi constructs did not induce a strong knock-down of the *TRPF* genes. In subsequent studies, the generation of multiple deletion strains should be attempted. New genome editing approaches, e.g. by CRISPR-Cas9 technology, may also be considered in this respect.

6.4 General conclusions

In this study $[Ca^{2+}]_{cyt}$ signals were shown to be unrelated to continuous growth of *C. graminicola*, both on single-hypha as well as on colony level. This is in contrast to tubular growing plant cells. The $[Ca^{2+}]_{cyt}$ signals in fungal tips may instead be related to the sensing of environmental conditions. Moreover, the re-initiation of hyphal growth is tightly linked to apical $[Ca^{2+}]_{cyt}$ spikes in *C. graminicola*. Current $[Ca^{2+}]_{cyt}$ sensors have a restricted usability in *C. graminicola* and other fungi, rendering measurements of spatially resolved $[Ca^{2+}]_{cyt}$ signals on whole-colony level, *in planta* and during spore germination problematic. In future, more sensitive $[Ca^{2+}]_{cyt}$ sensors may overcome these limitations.

In the course of this project the PAAP culture method with lower culture-to-culture variability and higher throughput, as well as a new double tagging plasmid system allowing efficient protein co-localization and interaction studies in fungi were developed.

Despite the expression of all four *TRPF* genes throughout the lifecycle of *C. graminicola*, no deletion phenotype of individual mutants was found. In contrast to *TRP* homologues of other fungi, there was also no complementation of a *trpy1*Δ yeast strain. Further on, in *C. graminicola* there is practically no release of intracellular Ca^{2+} upon hyperosmotic shock. Altogether, these results point to a strong functional diversification of *TRP* genes in different fungi.

7 Summary / Zusammenfassung

7.1 Summary

Calcium (Ca^{2+}) is a universal second messenger in all higher organisms and centrally involved in the launch of responses to environmental stimuli. Numerous reports have shown that genetic or pharmacological interference with Ca^{2+} signalling affects growth and pathogenicity of filamentous fungi. A better understanding of Ca^{2+} signalling and Ca^{2+} signal generation may thus indicate new fungicide targets. The present thesis analyses aspects of Ca^{2+} signalling in *Colletotrichum graminicola*, a pathogen of maize that can cause drastic yield losses.

Tip growth of pollen tubes and root hairs of plants is oscillatory and orchestrated by tip-focussed variations of cytosolic free calcium ($[Ca^{2+}]_{cyt}$). Hyphae of filamentous fungi are also tubular tip-growing cells, and components of the Ca^{2+} signalling machinery, such as Ca^{2+} channels and Ca^{2+} sensors, are known to be important for fungal growth. This thesis addressed the questions if, as in plants, $[Ca^{2+}]_{cyt}$ transients govern hyphal and whole-colony growth in *C. graminicola*, and whether colony-wide $[Ca^{2+}]_{cyt}$ dynamics rely on external Ca^{2+} or internal Ca^{2+} stores. Ratiometric fluorescence microscopy of individual hyphae expressing the Ca^{2+} reporter Yellow Cameleon 3.6 revealed that Ca^{2+} spikes in hyphal tips precede the re-initiation of growth after wounding. Tip-focussed $[Ca^{2+}]_{cyt}$ spikes were also observed in undisturbed growing hyphae. They occurred not regularly and at a higher rate in hyphae growing at a medium-glass interface than in those growing on an agar surface. Hyphal tip growth was non-pulsatile, and growth speed was not correlated with the rate of spike occurrence. A possible relationship of $[Ca^{2+}]_{cyt}$ spike generation and growth in whole colonies was assessed by using a codon-optimized version of the luminescent Ca^{2+} reporter Apoaequorin. Depletion of extracellular free Ca^{2+} abolished $[Ca^{2+}]_{cyt}$ spikes nearly completely, but had only a modest effect on colony growth. In a pharmacological survey, some inhibitors targeting Ca^{2+} influx or release from internal stores repressed growth strongly. However, although some of those inhibitors also affected $[Ca^{2+}]_{cyt}$ spike generation,

the effects on both parameters were not correlated. Collectively, the results indicate that tip growth of *C. graminicola* is non-pulsatile and not mechanistically linked to tip-focused or global $[Ca^{2+}]_{cyt}$ spikes, which are likely a response to micro-environmental parameters, such as the physical properties of the surface.

Ca^{2+} signals in the cytosol are initiated by the activation of Ca^{2+} channels in the plasma membrane and/or in endomembranes. Yeast (*Saccharomyces cerevisiae*) contains a Ca^{2+}-permeable channel of the TRP family, TRPY1, which is localized in the vacuolar membrane and contributes to cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevations, for example in response to osmotic upshock. A *TRPY1* homolog in the rice blast fungus is known to be important for growth and pathogenicity. To determine the role of the TRP channel family in *C. graminicola*, proteins homologous to TRPY1 were searched. This identified not one, but four genes in the *C. graminicola* genome, which have putative orthologs in other fungi, and which were named *CgTRPF1* through *4*. All of them were expressed in axenic culture and throughout the infection of maize. In order to elucidate the localization of the TRPF proteins, a plasmid system was developed that allows the tagging of several genes-of-interest on a single plasmid. This novel "Double Tagging Plasmid System" allows a much faster and less laborious generation of double-labeled fungal strains compared with conventional approaches. Like TRPY1, all CgTRPF proteins were localized intracellularly, albeit three of them were found not in large vacuoles, but co-localized in vesicular structures.

Deletion strains for the *CgTRPF* genes were not altered in processes thought to involve Ca^{2+} release from internal stores, i.e. spore germination, the utilization of complex carbon sources, and the generation of tip-focussed $[Ca^{2+}]_{cyt}$ spikes. Heterologous expression of *CgTRPF1* through *4* in a *tryp1*Δ yeast mutant revealed that none of the channels mediated the release of Ca^{2+} in response to osmotic upshock. Accordingly, aequorin-based $[Ca^{2+}]_{cyt}$ measurements of *C. graminicola* showed that in this fungus, osmotic upshock-triggered $[Ca^{2+}]_{cyt}$ elevations were generated entirely by influx of Ca^{2+} from the extracellular space. Therefore, it is extremely likely that *C. graminicola* TRPFs are not involved in osmo-sensing but may have other functions. *Cgtrpf* deletion mutants did not show a pathogenicity defect in leaf infection assays. In summary, this study reveals major differences between different fungi in the contribution of TRP proteins to Ca^{2+}-mediated signal transduction.

The lack of phenotypes in *trpf* single deletion mutants may possibly be due to functional redundancy of the genes. This was attempted to be tested by a quadruple-RNAi-based knockdown approach. For this purpose, a novel PVDF membrane-assisted agar plate culture

method was developed, which enables the harvest of mycelium grown on agar plates for gene expression analyses. This culture method lead to a strongly reduced variation in gene expression between biological replicates and requires less growth space as compared to conventional liquid cultures. Unfortunately, the RNAi constructs did not result in a sufficient suppression of *TRPF* expression, and RNAi strains did not show an apparent phenotype.

This thesis underlines that there is a rather strong variability in the physiological relevance of Ca^{2+} signalling-related proteins in different fungal species. Therefore, many Ca^{2+} signalling proteins, including TRPFs, are not considered as a reliable target for broad-spectrum anti-fungal strategies. However, in agricultural systems, mutualistic and pathogenic fungi occur side-by-side, and hence, specific anti-pathogen treatments are highly desirable. In this scenario, specific Ca^{2+} signalling proteins may indeed be interesting fungicide targets.

7.2 Zusammenfassung

Calcium (Ca^{2+}) ist allen höheren Organismen ein universeller sekundärer Botenstoff. Dabei ist es auch von zentraler Bedeutung bei der Weiterleitung von Umweltreizen. Die physiologische Relevanz der Ca^{2+}-vermittelten Signalleitung für das Wachstum und die Pathogenität von filamentösen Pilzen wurde in zahlreichen genetischen und pharmakologischen Studien gezeigt. Daher könnten neue Ziele für Fungizide durch die Untersuchung der Ca^{2+}-Signal erzeugenden und verarbeitenden Prozesse aufgedeckt werden. In der vorliegenden Arbeit wurden daher Aspekte der Ca^{2+}-vermittelten Signalleitung in *Colletotrichum graminicola*, einem phytopathogenen filamentösen Pilz, der zu drastischen Ernteverlusten bei Mais führen kann, untersucht.

Die Geschwindigkeit des apikalen Wachstums von Pollenschläuchen und Wurzelhaaren höherer Pflanzen besitzt eine oszillatorische Kinetik. Koordiniert wird dies durch phasenverschobene Oszillationen der Konzentration von cytosolischem Ca^{2+} ($[Ca^{2+}]_{cyt}$) in der Spitze der wachsenden Zelle. Ebenso wie Pollenschläuche und Wurzelhaare sind auch die Hyphen von filamentösen Pilzen langgestreckt und durch Spitzenwachstum gekennzeichnet. Elemente der Ca^{2+}-Signalleitungsmaschinerie, wie Ca^{2+}-Kanäle und Ca^{2+}-Sensoren, sind wichtig für das Wachstum von filamentösen Pilzen. Daher wurde in dieser Arbeit untersucht, ob -ebenso wie in Pflanzen- Konzentrationsänderungen der $[Ca^{2+}]_{cyt}$ das Wachstum von *C. graminicola* auf Einzelhyphen- und Ganzkolonieebene regulieren. Weiterhin wurde untersucht, ob Veränderungen der $[Ca^{2+}]_{cyt}$ auf Ganzkolonieebene aus extrazellulären Quellen oder intrazellulären Speichern gespeist werden. Messungen der $[Ca^{2+}]_{cyt}$ auf Einzelhy-

phenebene wurden unter Zuhilfenahme des Ca^{2+}-sensitiven Fluoreszenzproteins Yellow Cameleon 3.6 (YC3.6) durchgeführt. Dabei wurden vorübergehende Erhöhungen der spitzenorientierten $[Ca^{2+}]_{cyt}$ vor dem Wiederanwachsen von zuvor verwundeten Hyphen gezeigt. Auch in unverwundeten Hyphenspitzen konnten vorrübergehende Erhöhungen der spitzenorientierten $[Ca^{2+}]_{cyt}$ nachgewiesen werden. Hierbei war die Frequenz dieser Erhöhungen statistisch signifikant höher bei Hyphen, die an der Grenze von Medium und Deckglasglas wuchsen, als bei jenen, die auf dem Medium wuchsen. Die Hyphenwachstumsgeschwindigkeit oszillierte nicht und korrelierte auch nicht mit der Frequenz der spitzenorientierten $[Ca^{2+}]_{cyt}$ Erhöhungen. Auf Ganzkolonieebene wurde eine mögliche Beziehung Frequenz der $[Ca^{2+}]_{cyt}$ Erhöhungen unter Anwendung einer codonoptimierten Version des lichterzeugenden Reporters Aequorin untersucht. Dabei führte eine drastische Reduktion der extrazellulären Ca^{2+}-Konzentration einerseits zu einem fast völligen Verschwinden der Erhöhungen der $[Ca^{2+}]_{cyt}$, andererseits kam es zu einer nur moderaten Verringerung des Koloniewachstums. In einer pharmakologischen Studie hatten einige der verwendeten Inhibitoren der Ca^{2+}-Signalleitungsmaschinerie, welche zur Blockade des Einstroms von extrazellulärem Ca^{2+} oder der Freisetzung von intrazellulärem Ca^{2+} eingesetzt wurden, eine massive Unterdrückung des Koloniewachstums zur Folge. Einige dieser Inhibitoren beeinflussten auch die Frequenz der $[Ca^{2+}]_{cyt}$-Erhöhungen. Es zeigte sich jedoch keine Korrelation der Beeinflussung des Koloniewachstums und der Frequenz der $[Ca^{2+}]_{cyt}$-Erhöhungen. Insgesamt deuten diese Ergebnisse darauf hin, dass die Geschwindigkeit des Spitzenwachstums von *C. graminicola* nicht oszilliert und es keine mechanistische Verknüpfung von spitzenorientierten wie auch globalen Erhöhungen der $[Ca^{2+}]_{cyt}$ mit der Wachstumsgeschwindigkeit gibt. Ein Zusammenhang der Frequenz der spitzenorientierten Erhöhungen der $[Ca^{2+}]_{cyt}$ und der Mikroumgebung, wie z. B. der physikalischen Oberflächeneigenschaften, ist hingegen wahrscheinlich.

Cytosolische Ca^{2+}-Signale werden durch die Aktivierung von Ca^{2+}-Kanälen in der Plasmamembran und/oder in Endomembranen ausgelöst. Hefe (*Saccharomyces cerevisiae*) hat einen Ca^{2+}-durchlässigen Kanal aus der TRP-Familie, TRPY1. Dieser ist in der vakuolären Membran lokalisiert und entlässt in Reaktion auf Stimuli, wie zum Beispiel hyperosmotischen Schock, Ca^{2+} aus der Vakuole ins Cytosol. Es ist bekannt, dass ein Homolog von TRPY1 wichtig für Wachstum und Pathogenität von *Magnaporthe oryzae* ist, einem filamentösen Pilz, welcher Reis befällt. Zur Analyse der Funktion der TRP-Kanalfamilie in *C. graminicola* wurde nach Homologen von TRPY1 gesucht. Hierbei wurden im Genom von *C. graminicola* nicht nur

ein, sondern insgesamt vier Familienmitglieder gefunden, welche auch putative Orthologe in anderen Pilzen haben und als *CgTRPF1* bis *4* benannt wurden. Alle *CgTRPF*-Gene wurden sowohl in axenischer Kultur wie auch im gesamten Infektionsverlauf exprimiert. Zur Aufklärung der Lokalisation der TRPF-Proteine wurde ein Plasmidsytem entwickelt, welches die gleichzeitige Fusion verschiedener zu untersuchender Gene mit unterschiedlichen Fluoreszenzreportergenen ermöglicht. Im Vergleich zu konventionellen, seriellen Ansätzen beschleunigt dieses neuartige "*Double Tagging Plasmid System*" die Erzeugung von Pilzstämmen, welche zwei zu untersuchende Gene tragen, die mit verschiedenen Fluoreszenzreportergenen fusioniert sind, bei gleichzeitiger Reduktion des Arbeitsaufwands. Wie auch TRPY1, befinden sich alle TRPF-Proteine in intrazellulären Membranen. Jedoch zeigte sich, dass nur TRPF4 in der Membran von großen Vakuolen lokalisiert war, während TRPF1 bis 3 in kleinen vesikulären Strukturen kolokalisierten.

CgTRPF-Deletionsstämme zeigten keine Unterschiede im Vergleich zum Wildtyp bei Prozessen, in welchen eine Beteiligung einer Freisetzung intrazellulären Ca^{2+} vermutet wurde, d. h. Sporenkeimung, Nutzung komplexer Kohlenstoffquellen und Erzeugung von spitzenorientierten Erhöhungen der $[Ca^{2+}]_{cyt}$. Eine heterologe Expression von *CgTRPF1* bis *4* in einem *trpy1*Δ Hefestamm führte nicht zu einer Wiederherstellung der Freisetzung von Ca^{2+} ins Cytosol nach einem hyperosmotischen Schock. In Übereinstimmung mit diesem Ergebnis zeigte sich in Aequorin-basierten Messungen der $[Ca^{2+}]_{cyt}$ in *C. graminicola*, dass es nach einem hyperosmotischen Schock lediglich zu einem Einstrom von Ca^{2+} aus dem Medium, nicht aber zu einer Freisetzung von Ca^{2+} aus intrazellulären Speichern kam. Daher ist es sehr wahrscheinlich, dass CgTRPF-Proteine nicht an der Reizerfassung bei hyperosmotischem Schock beteiligt sind, sondern andere Aufgaben wahrnehmen. *CgTRPF*-Deletionsstämme zeigten in Blattinfektionsversuchen keine Pathogenitätsdefekte. Zusammengenommen deckte diese Arbeit wesentliche Unterschiede in der Beteiligung von TRP-Proteinen in der Ca^{2+}-vermittelten Signalweiterleitung in unterschiedlichen Pilzen auf.

Das Fehlen von phänotypischen Unterschieden zwischen den *CgTRPF*-Deletionsstämmen und dem Wildtyp könnte möglicherweise auf einer funktionalen Redundanz beruhen. Daher wurde ein vierfach-RNAi-Ansatz zur simultanen Unterdrückung der Expression aller *CgTRPF*-Gene unternommen. Zur effizienten Expressionsanalyse der erzeugten RNAi-Stämme wurde eine PVDF-Membran-basierte Kulturmethode entwickelt, welche die Ernte von auf festem Agarmedium gewachsenem Myzel zur anschließenden Expressionsanalyse erlaubt. Diese Kulturmethode führte zu einer signifikant reduzierten Replikat-zu-Replikat-

Variation der Genexpression bei gleichzeitiger Erhöhung des Durchsatzes im Vergleich zur herkömmlichen Kultur in Flüssigmedien. Leider führten zwei unabhängige RNAi-Konstrukte nicht zu einer hinreichenden Unterdrückung der Genexpression der *CgTRPF*-Gene.

Diese Arbeit weist auf eine deutliche Spezies-zu-Spezies-Variabilität bezüglich der physiologischen Relevanz von Proteinen, welche an der Ca^{2+}-vermittelten Signalleitung beteiligt sind, hin. Daher sind an der Ca^{2+}-vermittelten Signalleitung beteiligte Proteine nicht als geeignetes Ziel für Fungizide mit breitem Wirkungsspektrum einzustufen. In landwirtschaftlichen Systemen treten jedoch mutualistische und pathogene Pilze simultan auf. Daher ist eine gezielte Bekämpfung pathogener Pilze bei gleichzeitiger Verschonung mutualistischer Pilze wünschenswert. In Anbetracht dieser Tatsache könnten spezifische mit der Ca^{2+}-vermittelten Signalleitung assoziierte Proteine interessante Ziele für selektive Fungizide darstellen.

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9 Appendix

9.1 Supplementary material for chapter 5

9.1.1 Supplementary figures for chapter 5

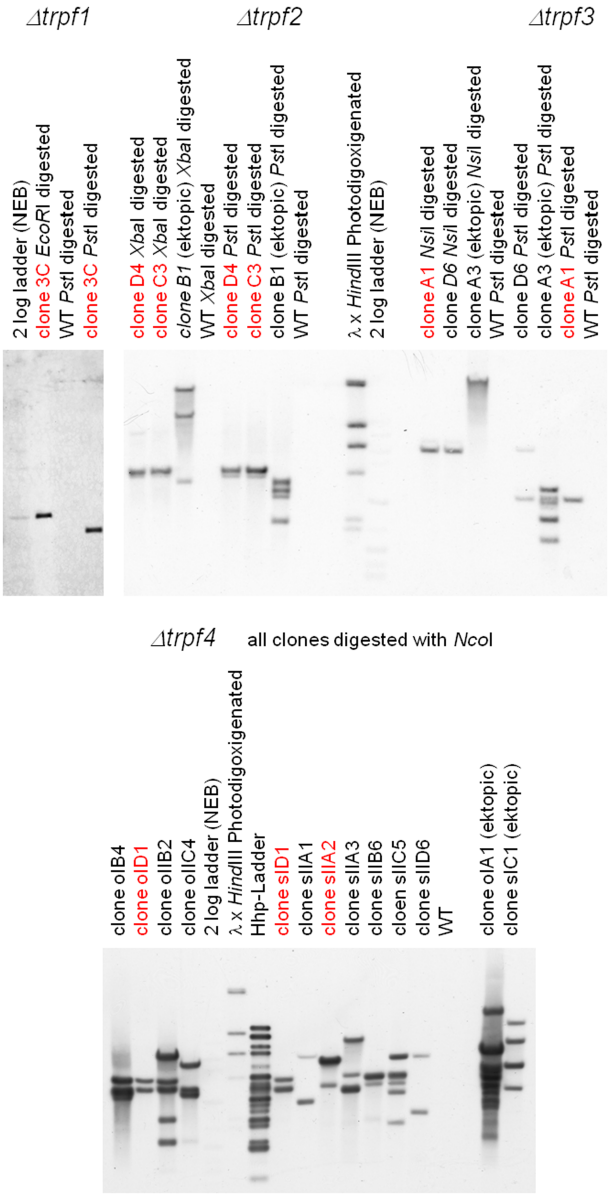

Fig. S5.1: *Genomic Southern Blots of the deletion mutants for* TRPF1 *through* 4. *Genomic DNA was digested using the indicated restriction endonucleases and probed with a digoxigenin-labelled probe binding to the 5′ region of the* hygromycinB phosphotransferase *gene. Clones used in this study are indicated in red.*

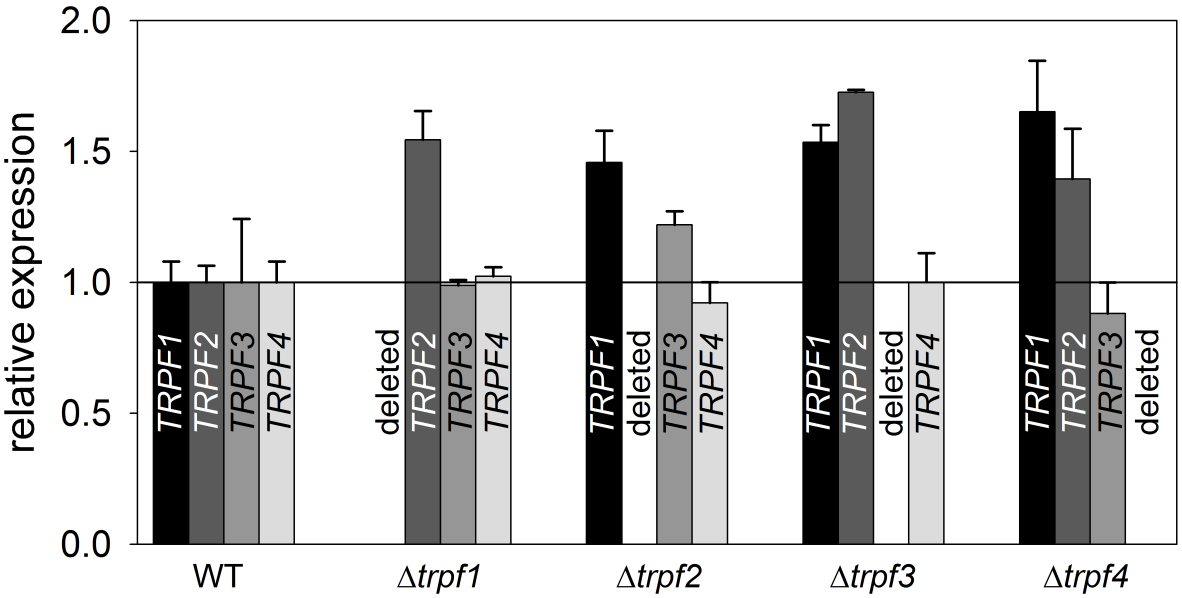

Fig. S5.2: *Relative expression of the* CgTRPF *genes in* C. graminicola *wild type and in strains deleted for individual* CgTRPF *genes. Strains were cultivated for 3.5 days on mLCM agar using the PAAP protocol [Lange et al., 2014a] and assayed by qRT-PCR. Black:* CgTRPF1*, dark grey:* CgTRPF2*, middle grey:* CgTRPF3*, light grey:* CgTRPF4*. Data are means ± SE (N=3).*

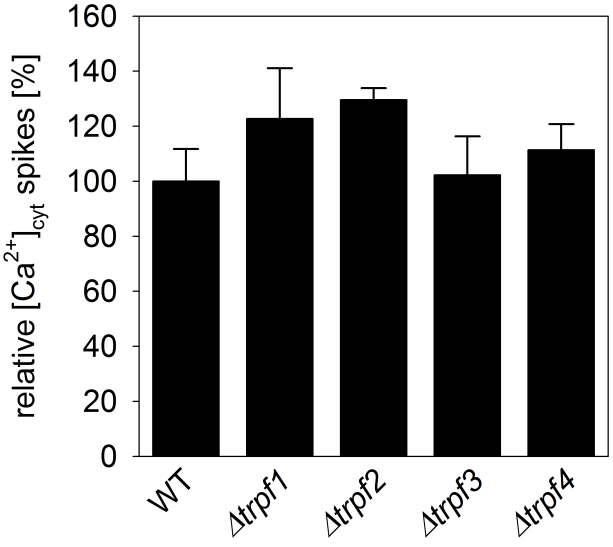

Fig. S5.3: *Relative* $[Ca^{2+}]_{cyt}$ *spiking rate during colony growth of* C. graminicola*. Colonies of* C. graminicola *wild type and* Cgtrpf1 *through* 4 *deletion strains expressing apoaequorin were grown for 80 h in 35-mm Petri dishes on mLCM agar supplemented with 10 μM coelenterazine.* $[Ca^{2+}]_{cyt}$*-dependent luminescence was detected for 20 min. Data are means ± SE (N = 4).*

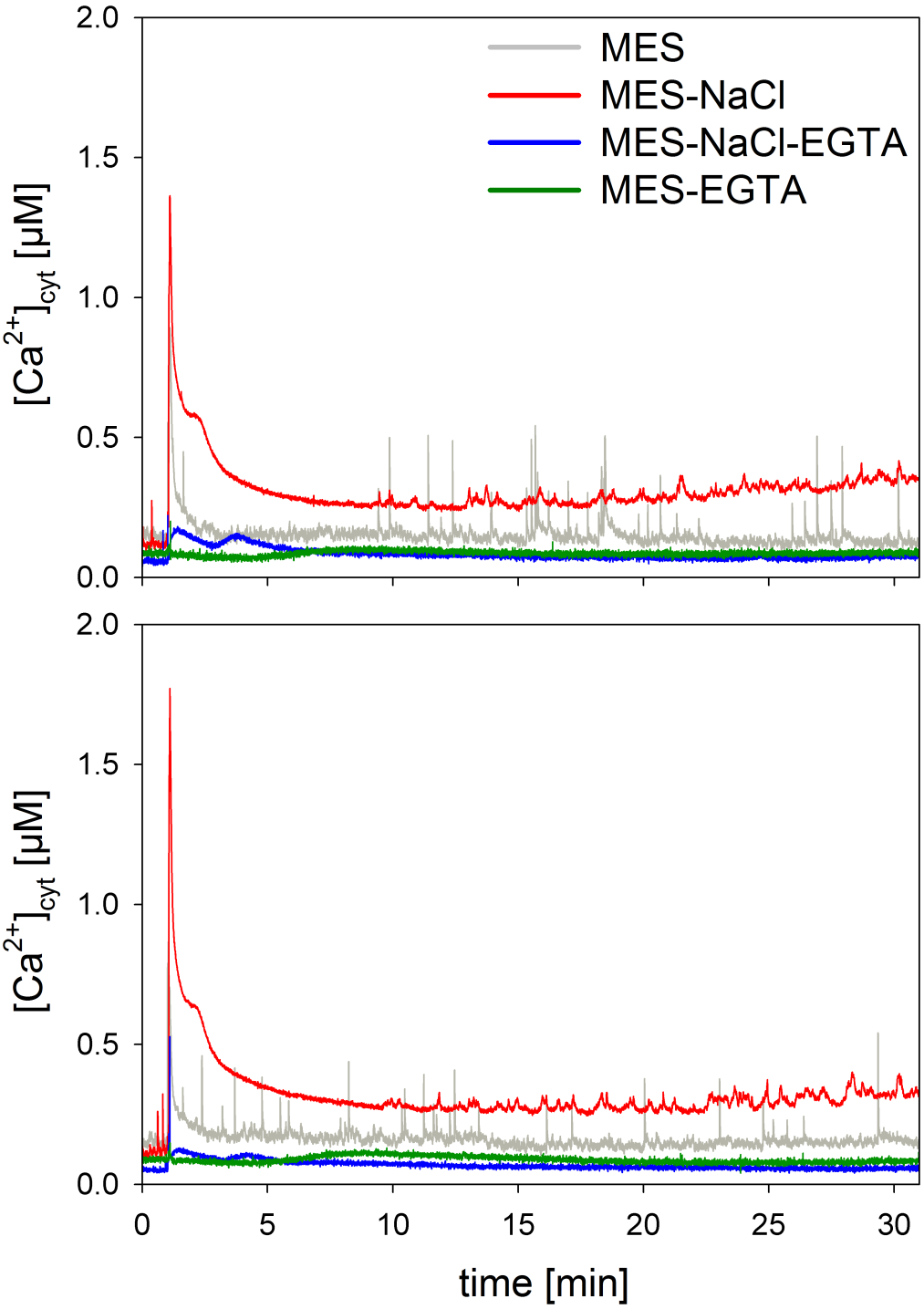

Fig. S5.4: *Additional repeats of the aequorin luminescence measurements of* $[Ca^{2+}]_{cyt}$ *responses of* C. graminicola *to NaCl. Whole colonies were pre-treated with 50 mM MES-KOH (pH 7.0) for 30 min prior to recording, followed by treatment with a solution (pH 7.0) containing 50 mM MES-KOH and no NaCl (grey line) or 1.5 M NaCl (final concentration; red line). To abolish the influx of extracellular* Ca^{2+}*, colonies were pre-treated with a solution (pH 7.0) containing 50 mM MES-KOH and 25 mM EGTA for 30 min prior to measurement, followed by treatment with a solution (pH 7.0) containing 50 mM MES-KOH, 25 mM EGTA, and no NaCl (green line) or 1.5 M NaCl (final concentration; blue line). Treatment solutions were added after 1 min of measurement. Traces show single measurements in order to demonstrate* $[Ca^{2+}]_{cyt}$ *spikes in the MES-KOH control treatment.*

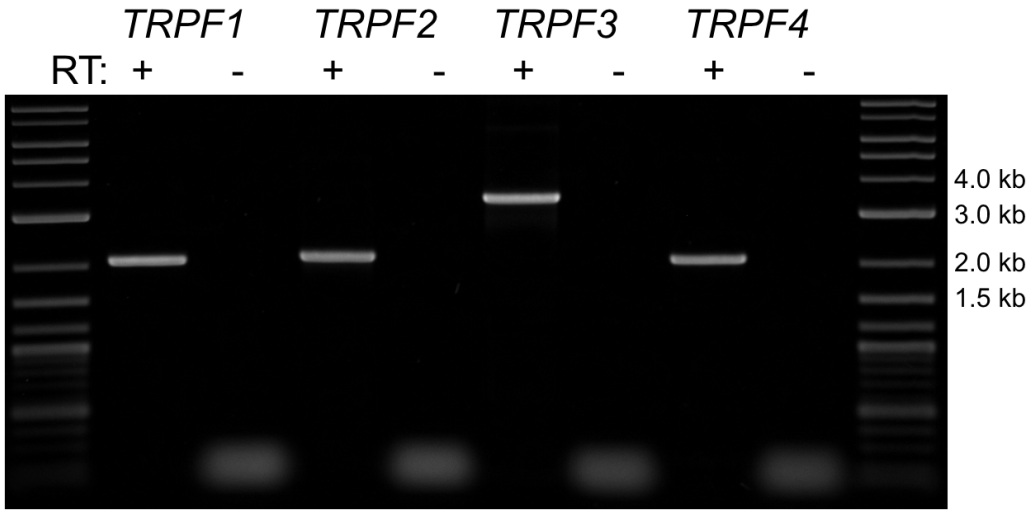

Fig. S5.5: *Expression of* CgTRPF *genes in transformed* S. cerevisiae trpy1Δ *strains. Full-length cDNAs of the* TRPF *genes were amplified from RNA extracted from log-phase cultures of* S. cerevisiae trpy1Δ *transformed with pFL61-CgTRPF1 through pFL61-CgTRPF4. Products were expected at 2098, 2152, 3522, and 2101 bp for* CgTRPF1, CgTRPF2, CgTRPF3, *and* CgTRPF4, *respectively. RT: + reverse transcriptase added in cDNA synthesis, - reverse transcriptase omitted in cDNA synthesis..*

9.1.2 Supplementary tables for chapter 5

Tab. S5.1: *Oligonuleotides used in this study. Underlined regions in the oligonucleotides for subcellular localization indicate restriction enzyme sites for* PacI *and* FseI*. Italicized* CC *in reverse oligonucleotides for subcellular localization indicate a part of a five amino acid linker between the TRPF genes and the fluorescent tag not covered by the* FseI *site. Underlined regions in the oligonucleotides for the generation of deletion cassettes indicate universal overhangs for fusion PCR. Italicized region in the dual purpose oligonucleotide TRPF4_5′Flank/Loc_f indicate the part needed for subcellular localization only. Underlined regions in the oligonucleotides for Yeast complementation indicate restriction enzyme sites for* NotI*. References: ⋆[Krijger et al., 2008]; # [Abou Ammar et al., 2013].*

Purpose	Name	Sequence	Ref.
RACE	TRPF1_3′RACE_1	TGGGTTTCATCTACTCGCCTGTCCTCG	
	TRPF1_5′RACE_1	GCCGATGTAGACCAGGGAGATGATGACC	
	TRPF2_3′RACE_1	CCACCCCCATTCAACCTCCTTGCTCT	
	TRPF2_5′RACE_1	GCGGTGCCTGGTGCGGTTCATAAAG	
	TRPF2_5′RACE_2	GCGAAGAGGCAGTAGACGACAGCAG	
	TRPF3_3′RACE_1	CCAACGCAGCACCATCCTCATCACGG	
	TRPF3_5′RACE_1	GATACGGGGAAGCAGCAGGATGGC	
	TRPF3_5′RACE_2	AGTGCTTTGCGTCTACGAGGAATACCC	
	TRPF4_3′RACE_1	CGAGCAGAACACCGAACGCATCACCCAC	
	TRPF4_5′RACE_1	CGTGTGGGCGTTGTAGAAGCGGGGC	
	TRPF4_5′RACE_2	GGCTTCGCAGAGACCCTTGTTGGAC	
qRT-PCR	Real_TRPF1_for	CCGACTTTAGCGGGTTCGAC	
	Real_TRPF1_rev	TGTCCTCGTACGCCGAGTTG	
	Real_TRPF2_for	CGGATTCGGCGTCTCGTA	
	Real_TRPF2_rev	CAGGGTCGTCGGGGTCAT	

	Real_TRPF3_for	CTTGTCTTGGCACGGATGAA	
	Real_TRPF3_rev	GCCTTGTGGTGGGAGCAGT	
	Real_TRPF4_for	TGTTCTTAGGGTCATCGGTATTG	
	Real_TRPF4_rev	GGATTAGCGTGCCCCAGTAG	
	Real_Act_for	TCCTACGAGCTTCCTGACGG	*
	Real_Act_rev	CCGCTCTCAAGACCAAGGAC	*
	Real_HistH3_for	CGAGATCCGTCGCTACCAGA	*
	Real_HistH3_rev	GGAGGTCGGACTTGAAGTCCT	*
	Real_ILV5_for	GCTGCCTCATGGGTGGTATC	
	Real_ILV5_rev	CCTCGACGGTCTCGTTGAAG	
Subcellular	TRPF1_Loc_for	TTAATTAACTCTTCGGGGTGTTCCTGTGTG	
localization	TRPF1_Loc_rev	GGCCGGCCCCCTCTTCGTCACTCGAGCTAGAGCC	
	TRPF2_Loc_for	AAAAAATTAATTAACGGTGTTACCAGCAAGACAAGTGAG	
	TRPF2_Loc_rev	AAAAAAGGCCGGCCCCCAGCCTCCTTACAGGTGACCCAG	
	TRPF3_Loc_for	AAAAAATTAATTAACCTGACAACGGCGACGATGG	
	TRPF3_Loc_rev	AAAAAAGGCCGGCCCCCATCGAGCTCCGTTTAATCGGC	
	TRPF4_5'Flank/Loc_f	AAAAAATTAATTAAACAGTAGTTGGGGAGAGACATTACG	
	TRPF4_Loc_rev	AAAAAAGGCCGGCCCCAGAGGGCGATCCGGGC	
Generation	uni-hyg.F1	TGTACGACTGTCAGTTGCACTGACCGGTGCCTGGATCTTC	#
of deletion	uni-hyg.R1	AAGCTAGTGAGACTCCAGACGGTCGGCATCTACTCTATTCC	#
cassettes	TRPF1_5'Flank_f	GAGTTGATTGGATGGGACGATG	

TRPF1_5′Flank_r	GTGCAACTGACAGTCGTACACCTTCCTATGCTATGGGCGAC
TRPF1_3′Flank_f	TCTGGAGTCTCACTAGCTTGACCCGTGCTGAATGATGTG
TRPF1_3′Flank_r	GTCTCACTTTCACTCTTCCACTCG
TRPF1_5′Flank_n	GGTCTGGCACTGAAGTAGCAATAAC
TRPF1_3′Flank_n	TTGCTTCGGGCTATTGATGG
TRPF2_5′Flank_f	GAACCAACCTTAGAAGAATGTCGG
TRPF2_5′Flank_r	GTGCAACTGACAGTCGTACAGAAGTCGGGATTTGATGATGGTAG
TRPF2_3′Flank_f	TCTGGAGTCTCACTAGCTTCAACGCCAGCATCTCCAATAC
TRPF2_3′Flank_r	GGTCTGCTCTTTCGTAGTGTTCTTC
TRPF2_5′Flank_n	AACCTTCTCAGCATCCATTCTCTATC
TRPF2_3′Flank_n	AGCACTTTGGAGTATCTTTGGCAG
TRPF3_5′Flank_f	TGCCTTGCCTTGCCGTG
TRPF3_5′Flank_r	GTGCAACTGACAGTCGTACAGCTTTTGCCTACAGGAGAATGG
TRPF3_3′Flank_f	TCTGGAGTCTCACTAGCTTGGGTTACGCCTGGAGCATTGTTC
TRPF3_3′Flank_r	CCCTCCACACCCCCGAGAATC
TRPF3_5′Flank_n	TAGCCTCGTCTTGCCTCCTTG
TRPF3_3′Flank_n	CATCGTAGAACACCGCAGATAACC
TRPF4_5′Flank/Loc_f	*AAAAAATTAATTAA*ACAGTAGTTGGGGAGAGACATTACG
TRPF4_5′Flank_r	GTGCAACTGACAGTCGTACAACGGGAAGAAGAGGGCGAC
TRPF4_3′Flank_f	TCTGGAGTCTCACTAGCTTATGATGGTTCTTGAAAAGGTAGATTGC
TRPF4_3′Flank_r	AGGCTATGCGATGACTGTCTCACTTA

	TRPF4_5'Flank_n	GTTTTGTTGTAGACTGCGACGG
	TRPF4_3'Flank_n	TCTCAGCCAATCCAAGCCAC
Probe generation	Hph-5'-South-for	CTAAAATCCGCCGCCTCCAC
for Southern Blot	Hph-5'-South-rev	CGGACAGACGGGGCAAAGC
Yeast	TRPF1_Yeast_for	AAAAAAGCGGCCGCATGGCCGCCTTCAACTGGG
Complementation	TRPF1_Yeast_rev	AAAAAAGCGGCCGCTCACTCTTCGTCACTCGAGCTAGAGC
	TRPF2_Yeast_for	AAAAAAGCGGCCGCATGGAAGAAGCCGAATGTCCG
	TRPF2_Yeast_rev	AAAAAAGCGGCCGCTCACAGCCTCCTTACAGGTGACC
	TRPF3_Yeast_for	AAGCGGCCGCATGTTTTCCTCCCTGCTGCG
	TRPF3_Yeast_rev	AAAAAAGCGGCCGCTTACATCGAGCTCCGTTTAATCG
	TRPF4_Yeast_for	AAAAAAGCGGCCGCATGCCCATCCGCGTCAGGT
	TRPF4_Yeast_rev	AAAAAAGCGGCCGCGATGTGATGTATTTTATTCCCAGTTCGC
	TRPY1_Yeast_for	AAAAAAGCGGCCGCATGGTATCAGCCAACGGCG
	TRPY1_Yeast_rev	AAAAAAGCGGCCGCTTACTCTTTCTTATCCTTTATGTCTAATTTTTC

Tab. S5.2: *Predicted TRP protein topology. Topology prediction was performed with TOPCONS (http://topcons.net/) using standard settings and the full length translated protein sequences of TRPY1, TRPF1, TRPF2, TRPF3 and TRPF4.*

	amino acid position					element size				
	TRPY1	TRPF1	TRPF2	TRPF3	TRPF4	TRPY1	TRPF1	TRPF2	TRPF3	TRPF4
cytosol	001 - 234	001 - 241	001 - 280	001 - 336	001 - 285	234	241	280	336	285
TM1	235 - 255	242 - 262	281 - 301	337 - 357	286 - 306	21	21	21	21	21
vesicular	256 - 265	263 - 269	302 - 309	358 - 364	307 - 311	10	7	8	7	5
TM2	266 - 286	270 - 290	310 - 330	365 - 385	312 - 332	21	21	21	21	21
cytosol	287 - 298	291 - 303	331 - 342	386 - 397	333 - 349	12	13	12	12	17
TM3	299 - 319	304 - 324	343 - 363	398 - 418	350 - 370	21	21	21	21	21
vesicular	320 - 335	325 - 341	364 - 376	419 - 431	371 - 380	16	17	13	13	10
TM4	336 - 356	342 - 362	377 - 397	432 - 452	381 - 401	21	21	21	21	21
cytosol	357 - 375	363 - 380	398 - 412	453 - 466	402 - 419	19	18	15	14	18
TM5	376 - 396	381 - 401	413 - 433	467 - 487	420 - 440	21	21	21	21	21
putative pore region	397 - 436	402 - 442	434 - 484	488 - 529	441 - 479	40	41	51	42	39
TM6	437 - 457	443 - 463	485 - 505	530 - 550	480 - 500	21	21	21	21	21
cytosol	458 - 489	464 - 526	506 - 535	551 - 612	501 - 534	32	63	30	62	34
TM7	490 - 510	527 - 547	536 - 556	613 - 633	535 - 555	21	21	21	21	21
vesicular	511 - 519	548 - 689	557 - 567	634 - 1163	556 - 565	9	142	11	530	10
TM8	520 - 540	not exsistent	568 - 588	not exsistent	566 - 586	21		21		21
cytosol	541 - 675	not exsistent	589 - 707	not exsistent	587 - 689	135		119		103

Tab. S5.3: *TRPF protein sequences used to generate the phylogenetic tree. Organisms were ordered by* phylum *and* class*. The sequenced strain and the* NCBI Taxid *of each species are given in the indicated columns. To link the proteins of this table with the tree see column* TRPY1 Homologue No*. For the distinct identification of the proteins the locus tag may be used. E-values were calculated on NCBI.* [1)] *In the cases where no appropriate NCBI database entry was available,* E-values *were calculated locally using appropriate databases downloaded from* Broad Institute *as indicated in column* Source*. References:* [2)] *[Zhou et al., 2005];* [3)] *[Palmer et al., 2001];* [4)] *this study;* [5)] *[Ihara et al., 2013];* [6)] *[Nguyen et al., 2008].*

Phylum	Class	Species	Strain	NCBI Taxid	TRPY1 Homologe No.	E-value	Locus tag	Source
Ascomycota	Dothideomycetes	*Mycosphaerella graminicola*	IPO323	336722	1	2e-141	MYCGRDRAFT_76604	NCBI
					2	2e-15	MYCGRDRAFT_20435	
					4	4e-12	MYCGRDRAFT_75841	
		Pyrenophora tritici-repentis	Pt-1C-BFP race 1	426418	1	4e-134	PTRG_05238.1	Broad institute[1)]
					2	9e-12	PTRG_06414.1	
					3	9e-16	PTRG_08905.1	
	Eurotiomycetes	*Aspergillus fumigatus*	Af293	330879	1	1e-131	Afu3g13490	NCBI
					3	8e-19	Afu6g11300	
		Penicillium chrysogenum	Wisconsin 54-1255	500485	1	6e-122	Pc13g15030	NCBI
					3	3e-24	Pc21g21930	
	Leotiomycetes	*Botrytis cinerea*	B05.10	40559	1	1e-137	BC1T_03504	Broad institute[1)]
					3	1e-15	BC1T_03018	
	Pezizomycetes	*Tuber melanosporum*	Mel28	656061	1	1e-138	GSTUM_00011244001	NCBI
	Saccharomycetes	*Candida*	SC5314	237561	1	0	CaO19.2209	NCBI

	albicans			TRPY3[2)]			
	Debaryomyces hansenii	CBS767	284592	1	0	DEHA2C08228g	NCBI
	Lodderomyces elongisporus	NRRL YB-4239	379508	1	0	LELG_01988	NCBI
	Saccharomyces cerevisiae	S288c	559292	1 TRPY1[3)]	0	YOR087W	NCBI
	Zygosaccharomyces rouxii	CBS 732	559307	1	0	ZYRO0C15510g	NCBI
Sordariomycetes	*Colletotrichum graminicola*	M1.001	31870	1 TRPF1[4)]	3e-138	GLRG_09114.1	NCBI
				2 TRPF2[4)]	2e-17	GLRG_10771.1	
				3 TRPF3[4)]	6e-18	GLRG_08368.1	
				4 TRPF4[4)]	9e-14	GLRG_09848.1	
	Colletotrichum higginsianum	IMI 349063	80884	1	6e-135	CH063_11552.1	NCBI
				2	3e-10	CH063_03277.1	
				3	1e-18	CH063_02273.1	
				4	1e-15	CH063_07357.1	

		Fusarium graminearum	PH-1	5518	1 TRPGz[5)]	1e-123	FGSG_04178.3	NCBI
					4	2e-15	FGSG_05259.3	
		Magnaporthe oryzae	70-15	242507	1[6)]	2e-137	MGG_09828.6	NCBI
					3	4e-16	MGG_01538.6	
						1e-21	MGG_06118.6	
		Neurospora crassa	OR74A	367110	1	4e-106	NCU16725.7	Broad institute[1)]
					2	2e-16	NCU04465.7	
					3	5e-13	NCU08283.7	
					4	2e-16	NCU06601.7	
Basidiomycota	Agaricomycetes	*Laccaria bicolor*	S238N-H82	486041	1	1e-53	LACBIDRAFT_311591	NCBI
					2a	5e-11	LACBIDRAFT_305275	
					2b	5e-08	LACBIDRAFT_297283	
	Pucciniomycetes	*Puccinia triticina*	BBBD Race 1	630390	2	1e-9	PTTG_08032T0	Broad institute[1)]
	Tremellomycetes	*Cryptococcus neoformans*	grubii H99	235443	1	2e-54	CNAG_07368.2	NCBI
					2	8e-07	CNAG_03844.2	
	Ustilaginomycetes	*Ustilago maydis*	521	237631	1	4e-67	UM03685.1	NCBI
					2	1e-12	UM04857.1	

9.1.3 Supplementary files for chapter 5

Supplementary file 5.1 Tree alignments.

```
>M._graminicola_1
 --IDATNFIT KNMLNAIMGA AEFDG--WDN FAPPFGLILY YIYNFVIVVI LLNILVALYN
 SAYEDITGNA IDEYLALYA- ---------Q KTMQFVRAPD ENVYIAPFNL VEIVF---LV
 LPFEWWMPSH RYEQLNDAVM GIVYSPVLVL TALLETRTAR MVKFNRSRNE SDD
>M._graminicola_2
 ---HTIITIS KWMLWIWFGL DGTGIQRSVE MHWFLGPLLM IMFAFLGNTL FMTILVAMLS
 NTYASLSKNA TAEIEFRRA- ---------V LTFEGVKSDA LFAYRPPFNV LALVILLPLK
 FVLT----PR WFHKVNITAV RILNAPILLL ISWYERRYLW KRPTLLSPP- ---
>M._graminicola_3
 --HYTLGRMN WILIKVFFGS SYLGFVSP-- QIPILGPPLM FLFVAMTNIL LITSLTSLLS
 NKLDKVMDHA RDEYLFVYA- ---------V YVLEASTSNR LTYFLPPLNL IPLLLR-PLR
 LVLP----NQ QLRTLRISLL KATHFPLVGL ILAYENGRLW IHDRRTTTPG DKI
>P._tritici-repentis_1
 -LANDTWFVI QAMLNAVMQS PEFEG--FDN FSPPFGIILY YIFTFVVMVI LLNILIALYN
 SAYEDITENA IDEYMALFS- ---------Q KTMQFVRAPD ENVFIAPFNL IEMVF---LI
 LPFEWWMDSA RYDKLNDMVM AVIYSPLLLV TALLEQQEAW VVKENRRRGE DDE
>P._tritici-repentis_2
 --ELGASDAA YAIFQLVMGF TPAAWDLWGE MN-FLGKTLL ALFLFICHFL IVTILITVLT
 NSFMAVVKNA DEEHQFLFA- ---------V NTISMVKSDA LFSYIAPTNI IGWLLIP-LK
 YTMP----FK KFLRMNRLVI KLTHLPILFL IFIYERVLLS HLSYGPTDLV EQR
>P._tritici-repentis_3
 EDSPNTITIS KWMLWIWFGL DGTGIDEAPG FHEVLGPTLM VIYAFLGNTL FLTVLVSILS
 NTFSKIAADA TAEIQFRRA- ---------V LTFEGVKSDS LFAYRPPFNL LAFVILLPLK
 FLLS----PR WFHKINVTAV RALNFPILLG ITHYERRYLW KPRRQLSVSG ---
>A._fumigatus_1
 --LALTKGII QGMANSVMQS PDFGV--FEK LAFPFGLVLY YLFNFIVMIV LLNILIALYN
 SAYEDISGNA VDEYMAIFA- ---------L KTMQFVRAPD ENVFIPPFNL LEIIF---LV
 LPFEWWMPRE HYAKLNEVIM GIIYSPLLLI IATLETREAH RIRWNRRQGE EDD
>A._fumigatus_2
 -EEHTPASVA YALFQMLMGF TPAAWTLWDD YN-ILGKIIL TLFLFICHFV VVTILITVLT
 NSFMAIVQNA NQEHQFLFA- ---------V NTISMVKSDA LFSYVAPTNI IAWLVTP-LR
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 YFMP----LR QFIRLNRTMI KITHFPVLFT ICLYEKAILS PRVNEPIDLI DHS
>P._chrysogenum_1
 --VPIHRNIL QGMVNSIMQS PEFDT--FQD FAFPFGIILY YVFNFIVMTV LLNILIALYN
 SAYEDISGNA TDEYMAIFA- ---------Q KTMQFVRAPD ENVFIPPFNL VEIVF---LV
 APFEWWLPRE TYARLNELVM GVIYSPLLIV TAWIENRQAH RIRWNRRHGE EDD
>P._chrysogenum_2
 -DNKSPKVVA YGLFQMLMGF TPTAWAMWDE YD-FLGKTIL TVFLFICHFV VVTILITVLT
 NSFMRIVQNA NQEHQFLFA- ---------V NTISMVKSDA LFSYVAPTNV IAWLVTP-FR
 YLMP----FR EFIRLNRTII KITHLPILFT ICLYEKTILC SRVVQPMDLI EPA
>B._cinerea_1
 --VDSATFIL QAMANAVMQS PDFSG--FDN FAPPFGIILY YIFTFLIMVV LLNILIALYN
 SAYEDITDNA IDEYLALFS- ---------Q KTMQFVRAPD ENVFIAPTNL IEIFF---LI
 LPCEWWMPRK QYAKLNDYVM AVVYSPLLLI AAWFEQRSAI KVKGNRQRGD DDD
>B._cinerea_2
 --EYDAAGVA YKIFQILMGF TPAAWEAWET YN-FLGKALL VLFLFICHFL VITILITVLT
 NSFMSIVSNA NEEHQFVFA- ---------V NTISMVKNDA LFSYVAPTNI LAWVLTP-CR
 FFFP----FR TFIKMNRTVI KMTHFPLLFS ICAYEKIFLA RSVFEPTDLV EKV
>T._melanosporum_1
 -KKDASFFIL KSMTKAILQS PEFEG--FDN FAHPFGIVLY YLFTFVVMVI LLNVLIALYN
 QAYTDITENA VDEYLALFA- ---------G KTLQFVRAPD ENVFLPPFNL IEIFF---LV
 IPFEWWMERR KYQKLNDYVM TVLYSPFMLL IAFLESREAR RVSRNRARGE QDD
>C._albicans_1
 --NEATQRIL ISLVKAVIGG SSFED--MGN LVPPYASVLY YFYQFMLTVI LMNILIALYS
 TAYAAIVENA TDEYFALVA- ---------H KTLRYIRAPD QNLYVPPFNL IELLI---TP
 IG--WFVSTS TWKNINYYVM LVIYSPLLAY ITSDELSNAR RIQYNRFKGV PDD
>D._hansenii_1
 --NEATKRIL ISLVSAVIGE ASFDD--LAS LDPPYASILY YIYSFLLSVI LMNILIALYS
 TAYANIVENA TDEYFALVA- ---------Q KTLRYVRAPD QELYVPPLNL IELII---VP
 FS--FVVSSS TFRLINHFIM LIIYSPLLTY ITIDELQNAR RIQYNRFKGI PDD
>L._elongisporus_1
 --SDATKKIL ISLMKAVIGG SDFGD--MGK LVPPYASILY YCYQFLLSVI LMNILIALYS
 TAYAAVVENA TDEYFALVA- ---------Q KTLRYVRAPD ENIYVPPFNL IELAM---TP
 LG--WVLSEA AYKDLNYYVM LVIYSPLLLY ITTEELTTAR RISYNRFKGL PDD
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>S._cerevisiae_TRPY1
 --RDITGPIL GNLTITVLGL GSFDV--FEE FAPPYAAILY YGYYFIVSVI LLNILIALYS
 TAYQKVIDNA DDEYMALMS- ---------Q KTLRYIRAPD EDVYVSPLNL IEVFM---TP
 IF--RILPPK RAKDLSYTVM TIVYSPFLLL ISVKETREAR RIKYNRMKRL NDD
>Z._rouxii_1
 --IEIAGPIM RNLVMTVVGA GGFDT--FKS FAPPYAAILW YFYCFIVTVI LLNILIALYS
 NAYQKVVENA SDEYMALMC- ---------E KTLRFIRAPD EYVFVPPLNI IELAL---KP
 LE--YIISEK NSDRLTHFIM CAIYSPTLLI VAILEVRTAK RIQYNRMTKL QDD
>C._graminicola_TRPF 1
 -VAEDITFIM SAMANAIMQS PDFSG--FDK FSPPFGIILY YCFTFIVMVV LLNILIALYN
 SAYEDIYDNA NDEYLTLFA- ---------Q KTMQFVRAPD ENVYIPPFNL IEMVV---IG
 L--FWWMEKS KFERMSDFIM GFIYSPVLVF AAVFETRSAA EIRSNRARGE EDD
>C._graminicola_TRPF 2
 KAALTWYETC KWLIWTWFGL DGTGIDRSDE FHPILGPALM IAFAFLGNIL FLTILVAILS
 NTFSKLISDA PAEIQFRRA- ---------V LTFAGVKSDS IFSYPPPFNL LALAALLPLK
 SMLS----PR SFHDVNTSLI RALNAPALMI ISALERRRVA HPRRARSQS- ---
>C._graminicola_TRPF 3
 ---NDAGDIA FRIFQILMGF TPAAWEVWPG YN-WLERGLM AFFLILCHFV IVTILITVLT
 NSFMKIASNA REEHQFLFA- ---------I NTISMVKNDA LFSYVAPGNI FAWLLMP-MR
 YCMP----LK QFVKLNRTII KVTHFPLLFC IFLYEKYWLA PSMYEPTDLV ENH
>C._graminicola_TRPF 4
 --AYNFSHMT MILTKIFFGS AYVGIEIMDD IDKVFGPPLM IIFIILSSFL LMGSLTGMLS
 NSFSRVITHA REEYLYVYS- ---------V YVLEASTSNR LTHFYPPFNV IALVIFRPLR
 LFLPS---DD KFRQSRILLL KATHLPIVGL IRMYESIRR- RVMPDEYAGF KGP
>C._higginsianum_1
 -VADDILFII SAMANAIMQS PDFDG--FDR FSPPFGIILY YCFTFIVMVV LLNILIALYN
 SAYEDIYDNA NDEYLALFA- ---------Q KTMQFVRAPD ENVYIPPFNL IEMVV---IA
 L--FWWMEKS KFERMSDIIM GILYSPVLVV AAVFETQSAA EIRSNRARGE EDD
>C._higginsianum_2
 ---NDAADIA FRIFQILMGF TPAAWEVWPG YN-WLERGLM AFFLIICHFV VVTILITVLT
 NSFMKIASNA REEHQFLFA- ---------I NTISMVKNDA LFSYVAPGNI FAWLLMP-LR
 YCMP----LR QFVKLNRTII KATHFPLLLF IFLYEKYWLA PSMYEPTDLV ENH
>C._higginsianum_3
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 --AYTFSHMT MILTKIFFGS SYVGIEIMDD IDKVFGPPLM IIFIILSSFL LMGSLTGMLS
 NSFSRVITHA REEYLYVYS- ---------V YVLEASTSNR LTHFYPPFNL IALVIFRPLR
 LFLPS---DD KFRHSRIVLL KATHLPIVGI IQLYENIRR- RVMPDEYAGF KGP
>C._higginsianum_4
 KAALTWYETC KWLIWTWFGL DGTGIDRSGE FHPVLGPALM IAFAFLGNIL FLTILVAILS
 NTFSKLIADA PAEIQFRRA- ---------V LTFAGVKSDS IFAYPPPFNX XXLAALLILF
 NVPQ----RR L--------- ---------- ---------- ---------- ---
>F._graminearum_1
 -ILGDTYFII ESMLKAIMQS PEFDG--FEN FGHPFGLILY YCFTFVVMII LLNILIALYN
 SAYEGIYENA DDEYLALFA- ---------Q KTMQFVRAPD ENVYIAPFNL VEIVV---SG
 L-LEWWMPKP TYEFINDCVM AILYSPLLFV AAWFEKRDAR KIRSNRSRGE EDD
>F._graminearum_2
 --VFAFKSMA WILTKIFYGS APIGFEVMNQ IDPFFGPPLM IMFITLSSFL LMGSLTGMLS
 NSFSRVITHA KEEYLYVYS- ---------V YVLEASTSNR LTHFYPPFNL LALVIFRPWR
 LIFTA---DE KFRAGRIILL KATHWPIVGI IKLYEMYRKP GALGDEFAGF KGP
>M._oryzae_1
 -ALSDTGFII QSMANALMQS PDFSG--FEK FSHPFGIILY YFFTFIVMVI LLNVLIALYN
 SAYEDIYDNA DDEFLALFA- ---------Q KTMQFVRAPD ENVYIAPLNL IEVFL---VA
 LPFEWWMEKR MYERLNDIVM AVLYSPLLLF TAMFEMRAAG EIRANRSRGE EDD
>M._oryzae_2
 --RFGLANMT MFLTKIFFGS SYVGFDIMRD IDPVFGPPLM LTFITLSSIL LMGSLTGMLS
 NSFSRVISHA REEYLYVYS- ---------V YVLEASTSNR LTHFYPPFNL IALVIFRPLR
 LFLPS---DH KFRAARIALL KATHWPIVGV IQLYELIRG- KFRPTDKFRG PVP
>M._oryzae_3
 ---GDKADIA YRIFQILMGF TPAAWDVWPH YD-VLGKMVI GLFLTICHFV VVTILITVLT
 NSFMTIASNA REEHQFLFA- ---------I NTISMVKNDA LFSYIAPGNI LAWLLTP-LR
 FILP----LR QFVYLNRFVI KLTHFPLLFC IYLYERFFLA PSMYAPTDLV ENP
>N._crassa_1
 -VVEDTMFIV QSMANALMQS PDFSG--FEK FNHPFGLLLY YCFTFVVMVI LLNILIALYN
 SAYEDIYDNA NDEYLGMFA- ---------Q KTMQFVRAPD ENVFIPPFNL IEIFL---LA
 LPLEWWMNKT TYEKINDWCM ATIYSPLLLV SAFIEVKTAQ EIRANRARGD GDD
>N._crassa_2
 EDRPGWATIG KWLLWIWFGL DGTGIERTAD FHTILGPALA VTFAFLGNTL FLTILVAMLT
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 NTFSKIISNA QAEIYFRRA- ---------V LTFQGVKSDS IFAYPPPFNL LALAVMLPLK
 LVVG----PA MFHHINVAAI KVINFPVLLM IALYERRRVW AKSATPSAG- ---
>N._crassa_3
 --SFPLRDMT WFLTKIFFGS TFIGFDIMRQ IDPVFGPPLM ILFVMLSSIL LMGSLTGMLS
 NSFSRVITHA REEYLFVYS- ---------I YVLEASTSNR LTHFYPPFNL LALVIFRPLR
 LFLPS---DD KFRRARIILL KATHLPIVFI IAMYEWIRGT SLRGATYSAV KAP
>N._crassa_4
 ---DDPSVVA YSMFQILVGY SPAAWEIWPD HN-WMGKTLL GLFLIICHFV IVTLLVTVLT
 NSFMAIALNA KQEHQFLFA- ---------I NTISMVKNDS LFSYVAPVNI LAWALTP-LR
 FLMP----MR QFVWLNRTVI KVTHFPLLLI IFIYERFILA PSVYEPTDLV ENP
>L._bicolor_1
 --TEPPSTVV NVLVQALLGS PDFG----KF DASTAGLLLY YLWNTVTAIV LLNVLISLFS
 SAYSDVVDDA EAQYLAFFA- ---------S KTVEMIRAPD SYVYPAPFNL IEAIFVAPFE
 FVPLAKLSPA HYARLNRVVM GGIFLIPLAT IAFYESTFDR RKHKWMNAWF RGN
>L._bicolor_2
 PHKQINRTIA WWMLDLWFGL DASGFDRSTE FHPIFGPVLM VTYACLSNTL LLTVLVSILS
 NTFATINEDA AAELYRRSRG MILQSYRDHT QRIFRVKADS LFSYLPPVNL IALCIMLPAS
 YLLS----PR WFHKVNVFMI RLTSFPVLLS ISLYERQAKM AGTTGFYETV S--
>L._bicolor_3
 -----ISSIA WLMVQIWFGN TSLSFDQATS FHPILGPILM TIFAALSNTL LLTILICILS
 NTVARIDSNA TQEHLYQFT- ---------I STIEGVKSDA LFSYQPPFNL VALIILKPAS
 WFLS----PR ALHSTNVFLI KLTSLPQLIV IGLYERHLCS CRRFRSSGK- ---
>P._triticina_1
 QAPYSLGEIA IWMVYIFFGL DATGISNAHE FHRILGPAIF IIYACLSNTL LVTVLVSILS
 KIFSDISNDS IAETLYRKA- ---------V LTFEGVKSDA LFSFQPPFNI PAMMILIPLK
 PFLT----PR KFHTVVVFLC RMFGCPVLLT ISFFERYVVG PFDKDRNSVG NSL
>C._neoformans_1
 --SGGRGIVI NNLIQALLGA PDFDSP-SER FGYPFGLIIF YGWNFVATII LVNVLIALFG
 SAYSDVTDNE TDEYLVFFA- ---------H KTIDLIRAPD SYVYSAPFNL IEAFLIAPLT
 QY-RWILPRD MYIALNRYTM TILFFVPLTF IVLFETNISH SKNRSISAYF NEP
>C._neoformans_2
 ------VFIH WYLFLSLDPG TCYMDCEADC MADGSDLVWI LIPRALCNVL LITMLIAILS
 NKFAAINQNA QQEHLFQRV- ---------V KTVEGVKTDA LFSYFPPINI LAFAILVPLS
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 WTVS----AR TLHRINVFAI RLTSFPILIA ISAYERYTYR AKQRAIHLGA STM
>U._maydis_1
 -VDNVLVKIV DLLTQSVLGG ADFDLTSEDA FGYPYGHILY YAYCFVTAVI LLNILIAFFG
 TAYSDVVDSA DEVYAAFFC- ---------Q KVIAMVRAPD QFVYLPPFNL LEAFVIAPLE
 WV----TSHE TYARINHTVQ SVLFAVPLMW IAFYESRMAR NGGSIRLPML ETE
>U._maydis_2
 -----FKAVL WLMLKVWFGS SYLGFDEAQS FSLTFGPPLM IIYTIMSNTL LLTVLISLLS
 NTFQVVAMNA NEEAMFQFA- ---------V KTMSGITTDA IFSYQPPLNL LAVAIVMPLS
 FLVS----PR WLHKINVFLI RSTSFHVLLI IHFAELNVFG HGISLTAEKG RGL
```

Supplementary file 5.2 DNA sequences of *TRPF* genes in *C. graminicola*. The core of the sequences was identified by tBLASTn searches. Full-length sequences were obtained by RACE-PCR and verified by cloning and RNA-Seq.

Exons are indicated in uppercase letters, introns are written in lowercase letters.

>TRPF1_GLRG09114

ATGGCCGCCT TCAACTGGGT TCCCGTATGC TTGGCTGGGA CCGACACTCA AGACATCACC
ACGACTTCCA TTCGGACTGG ATTCGCGATG ACCGTAGACG CCgtacgtaa ctcttcttag
tcttgcattc acttgcgctc ctccacctgc taccttcgcg atgcctgttc ttcaatcgct
tcccccttgct tacaagaggg tctactgtcc cgcaccagTC CTGCCCCAGT ACCGATCCGA
AGACCTCGAG TCCGCCATCC CTGCCCCCGA AGTCACAAAG ATCGCTCTGA AGCTCCGCCA
TCTGGTTGAG CTGGCCGTGC CCTGCGAGCT GGACGAGGAC GACATCACAA AAGCCCACAG
CACCATCATC ACTAAGAAGG TCATCAAGGC CGCCAAGGAG GCTGGCGGCT CCCACTACGG
CTCCTGTGTC GTCTTCTGCC TCATCGTCTG CAAGAGGTGG TTCAAGCACC AGGCACTGAC
AGAGCTGTGG GATGCCGACC TGCATCGCGT GCGCGCCGTG GCCTGTGAGG TCATTGCTAA
GCAGATgtga gttagagaat cagccagaac aatactgttg gagaaaagac aagtcctgcc
tagcgctaac cgcaactgct gcgcagCATC GAGGGAGAAG AGGACCTCCA ATACCTCATG
CACTCAGTTC TGCTCCGCCG CTACTCCATC ATCGTCGACG GCAAGTCCAC TCCTCCCGCC
AACGTCATCG AAAAGGCCGT CGATCTTCAC GCCGTTAGGG TTATTGGTTC ATCGGGCTAC
CAGAAGTGTA TCTCCTACCT GTGGAAGGGA TGGCTGGTTC AGGACGAGAA CGACCCCTCC
GTCTTCGTCG ACTACAAAGA CAAGACTAAC CCCTCCTTCC TTGTTCACAT GGACCCCGAC
CGTATTCGTG CTCCCATGTA CCAGAACGCG ACGCAGGTCA TCATCTCCCT GGTCTACATC
GGCCTATACA CGGCCGTGAT CAATTCGGTC AACGCCAAGG GTGTCTTGGA CTCGGCCGAG
GTGTTGTTAT ACATCTTCAC GCTCGGCTTC ATCTGTGAAG AGGTCACCAA GTTCTGGAAG
GCCGGCTACC ATATCCTGGG TTTCTGGAAT GCCTTCAACG GCATTCTCTA CTCCTTTATC
TCGCTGTCCC TGATCCTGCG CATCATCGGC CTGACCCATG GCGAGGGCGA AGATGCGCGG
AAGTACTACA GCGAGCTCAG CTATAACTTC CTGGCCTTCA GTGCGCCAAT GATCTGGTCT
CGCCTGTTGT TGTACCTCGA CAGCTTCCGC TTCTTCGGCG CCATGCTCGT CGTGCTCAAG
GTCATGATGA AGGAGTCCAT CATCTTCTTC GCCTTGCTGG CCGTGTTGAT CATTGGCTTT
CTCCAGGCAT TTGTCGGCCT TGATCTGGCC GACGATCTTG TCGCCGAAGA CATTACCTTC
ATCATGTCCG CCATGGCCAA CGCCATCATG CAGAGTCCCG ACTTTAGCGG GTTCGACAAG
TTTTCGCCGC CGTTTGGCAT CATACTCTAC TACTGCTTTA CCTTTATCGT CATGGTaggc
ctttcccgtt catgggatat ctgttgcagt cgctaaccac aacgcaggtC GTTCTGCTCA
ACATACTCAT TGCGTTGTAC AACTCGGCGT ACGAGGACAT CTACGACAAC GCCAACGACG

AGTACCTTAC GCTGTTTGCG CAAAAGACTA TGCAGTTCGT CCGCGCGCCC GATGAGAACG
TTTACATCCC GCCTTTCAAC CTGATCGAAA TGGTCGTTAT TGGCCTCTTC TGGTGGATGG
AAAAGTCAAA GTTCGAGCGC ATGAGCGACT TCATCATGGG TTTCATCTAC TCGCCTGTCC
TCGTTTTTGC AGCCGTCTTC GAGACGCGAT CGGCCGCCGA GATTAGAAGT AACCGGGCTC
GAGGCGAGGA AGACGACGAT ACCATCGAGG AATGGGAGCA GATGATGGAC CAGGTCGACT
TTGAGTCTGA CGGTTGGAAC AAGGTGTGCA TCACGGCCAA GACGGACCTA GATGTCGACC
CGACGATTTC CGAGGTGCAG AAGCTGCGCA GCGAGGTCGA GGAGCTCAAG AAAATGCTGG
TGGACATCTC AAAGGCCGTC TCGGCTGGAA ACGCCGGCAA CACGCAAGCA ACCAATAACC
TGATTGACCT CGGCGAGACC ACCGCTCCTG CCGCTGCCGG GGGCTCGGAC AAGAACAAGA
AGAGGAGCAA GAAGGGAAAC AAGAACAAGA ACAACCAACA GCCGACAGGC GACGACGGCA
AGGCACCAGG GGGCTCTAGC TCGAGTGACG AAGAGTGA

>TRPF2_GLRG10771

ATGGAAGAAG CCGAATGTCC GCGGACCCCG GACTTGATCG ACTGGAGTCG CCCGCTGTCG
CCCTGGGCTG ATGAAAACCC GGCGACGCGT ACCGGTGATG CCAATGGCGG CATGGACCCC
GACTTGGAGG CGGACCGACA TGTTTCTGCC AGCGCTACAG GAACCGTGAG CACGGAAGTG
CCGCCGTCTG TGTATTTCAC GATACATCGG tgagtctggt ctaccacagc ttttgctccc
acctaaatga cacgtactga tcgcaaacaa aactgcaggA TTCGGCGTCT CGTACTCGCA
AGTATAGgta catggctctt atatctgttc tcatgtcatt ttctaacaga tcccagATGA
CCCGTACACT CTGGACCAGC TCCGTGAGCC GCGGATGAAC CTACTGGTTG TAAGACCTCT
TGTGGATCGT TTATATGACC CCGACGACCC TGCTGTCGgt ctgtttctcc gcggataatg
cttcttcatc gacacctttg gctgacgagg ctacagTCTA CTGCCTCTTC GCGAACCGGA
TCCAATTTCT GAGGCAACAC ACTAGCACCG CGCGTCAGAC CGTCAATGTC GCCCGCGCTA
CCCTATGCGA GCTTGTCGCC AGTCGCGTGC TGCGCCGATA TCATGAGGAT CATCCCGGTC
AGATAGGCCT CCTCATTTTG GCACATATAC TTGTCGAAGG GTTTGATCCC TTCCAGGGCG
CACCCGAGGA GGTTGAGAGC GAGTGTCGCC AGCTGCAATG GCCCATCCAA CGACGCGACG
GGCATGAGAG GAAGCTCACA ACCTTGGAGC TGGCAATCAT TTCGGAGAGC AAACTCTTTC
TGAGTTCATC GGCATGCCAG AGGTTGGTTG ACGCCGTTTG GAACGGAGTG GTAACCTACA
CCCCTCTGTC CTTTGTCGAT ATCCTTCCAG ATCATTACGA ATACGCCTCG GTTTCTCTTT
ATGAACCGCA CCAGGCACCG CTGCTTGACG TGGGACGTCT ATTGGTCCCA TGGATCCGAC
AGATGATAGA GCTGTTCCAG TTCATTACCC TGGTCATCTT ATACATATTG ACAATGGTGA
ACCGGAGCAG TCCCACTCTT AGTGGGTGGG AATGCGTCTT TGCCATATAC ACTGCGGGAT
GGACTCTCAA TGAATTCGCA GCCATCATCG AACACGGCTG GGTTGTCCAC TCTCAGACCC

TGTGGTCGTT CCTCGATATT ACGTTCAGCC TCATCTTTAG CTGTTACATT TTCGCACGCA
TTTACGATAC CATGGGCTAT CAAGGGGTTG TTGCCGACGG ATACGGCGTG CACATTCTCT
GCGTTGCCGC TCCTGTTCTT CTCACCCGCC TCGCCTTCAC CCTTTTACCT GACAACATTG
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AGTTTCGCCG CGCCGTACTA ACGTTTGCGG GCGTGAAGAG CGACTCAATA TTCTCATATC
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TGATCATCAG CGCTCTTGAG CGGCGTCGTG TAGCACACCC GCGCCGTGCA AGGTCGCAGT
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TTGGCTTCGC GGAAAGCGAA CTGGGCGACA GAGAACCATG GGACGTTGGC CGGTCAATGC
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TGTTCCCCCT GCCAAGTGTG CAGCGTGCTG GCACAAATGA GTTTGAGCCA TTGTCTGGGT
CACCTGTAAG GAGGCTGTGA

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GACGATGGCT CAGGCCACTC TGGGGACGAC AACGAACATG TAGACGACGA GGACCTTAGC
CGCCGCTCTA TGCCCGTTCT TCCTCTCTTC TCGTCAACAT ACCTTGgtac gctcagccca
tctagcctcc tatggctgcg cccattctcc tgtaggcaaa agctgacacc ggtttctagA
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AACGACGTTA ACATGGGACC AAATACGCTC CCCCCAGGTA TCCCAGTTCC TGGTCAAGCC
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TACGAGGGCT TTGGTCTGCG AACTCCTCGC TCTCAAGCTC CTCAAGGAAT ACAATACCCG
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GCCTGCCAGC ACTCCCAAGG CAGACCCGAG AAACAAGGCG CGCCTCACCG CGTCACGAAC

CTCTACACTC GAAGTCGCCA TCCGCGCCTC CGCCAAGCAC TTCTTGGCCC ATCCTTTGGT

CGTGCAACAG CTCGAGGCTA TCTGGAACGG CGCCATTAGC TTTTATTCGT CCGCAGACAG

CCTGCACCGC GAGCCACCTC CTTCGCCTAC TGGCATCAGA CCCGATGCGA GAACGCCACT

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TCTTTCTCAA CGCTCGGCCC GCATTAGCTC GCTGGAATTG ATATTTTGGT TTTGGAGTGC

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GAGCTTTTGG AACGCTTTCG ATCTGGGGAT CCTGCTGTTG TTGATCGTCT ACTACTTCAT

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GTACTTCTCT CAGCTGCTCA TCGCCTTCCG CTTGATGGCG GTTGACCTCG CAGCCGTTTG

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CAATGATGCG GGCGACATCG CCTTCAGGAT CTTCCAGATC TTGATGGGCT TTACTCCAGC

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GATCCTTTGT CACTTCGTCA TCGTgtaggc ctgtctcgtc tcctgaaaca gccgtctttt

gctgaccaac gcagCACCAT CCTCATCACG GTCTTGACAA ATTCCTTCAT GAAGATTGCC

TCCAATGCTA GGGAGGAACA TCAGTaagtt tgatcccgaa tttccctgca atcactctgc

tcacataacc aggtTTCTCT TTGCCATCAA TACCATCTCC ATGGTCAAGA ACGATGCTCT

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CATGCCCCTG AAACAGTTTG TAAAGCTGAA CAGGACCATC ATCAAAGTGA CACATTTCCC

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CCCGGCGAGC CGGCCTGCTT TGTTCAGCCC CAACATCCGG GTCCGCGAAG AATCCGTCGC

TGGGTACCAG AAGGATCATG CTCTTGAAGA GGTCTTCCGG CGCGGCCCCG GTTTTGCTAC

TCTGCGCAAT CAGCGGCGCA ACGAGAGGAA GAAAACCCAG CACGCGGTCC GGAACTGGAT

GGATCGGAAC GACGAAGCCA GCGCTTCTCC GCAACTCTCC CAGTGGCCAA CTTTGGATGG

CAGCCAGGGC GTTCGACCGG ATTGGCTTAG GAGGCTGAGT CTGTCCAGGG AGCTTAGGAC

GCGCCGGTCA CGTCAGATGT CAGATGTCAG ATCAGCGGCT AGCGACCCAG CAGATCTTAT

CTCGCAGCCT GCTATCGGGT CTTTCTTCAG GGGTATAGCC GCGGCCACCA CAGCCAAAGA

TGAGGAGAAC CAGCAAACGG ACGCCGACGG GGATGACGAG CTCGTCACAA ACGACGAGGA

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GCGAGGTCCC CCCGTCCCGA GGAGCGCCTT GAGTCTCGCC ATTCGTCACG CCCCCAGCAC
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CTCAACGACA AGATTGACAA GCTCACGGCG CTTGTCGAGG CCCTCCAGCA GCGGCCCGGA
TCGCCCTCTT GA

Supplementary file 5.3 *C. graminicola* TRPF predicted amino acid sequences. Cytosolic amino acid residues are highlighted in yellow, luminal amino acid residues are highlighted in light blue, amino acid residues in the putative pore loop are highlighted in dark blue. Acidic amino acid residues are indicated in red.

>TRPY1 8TM

MVSANGDLHL PISNEQCMPE NNGSLGFEAP TPRQILRVTL NLKYLIDKVV PIVYDPNDIV CDHSEILSPK
VVKLAYEACG GNPKDKANKR KYQSVIIFSL LKVCEWYSIL ATMEVHNAKL YETRNLASQQ LCKLLIEREE
TRDLQFLFMQ LLLRRYVINE NDEDQEPLNA LELATDMHCT TVIGSSGFQR CLKWIWRGWI VQNGLDPTTF
IKDDSLAEVS LISHFNPVRL KAPVYQNYLQ MIFSFLFLGL YTLVVNGKDS ERVQSFDLLE SIFYVFNTGF
ILDELTKLYY IGYAHLSFWN LFNDTTYLII TFAMGFRAMS VTPLNAKYSS EDWDKISYRV LSCAAPFVWS
RLLLYLESQR FIGIMLVILK HMMKESIVFF FLLFLIMIGF TQGFLGLDSA DGKRDITGPI LGNLTITVLG
LGSFDVFEEF APPYAAILYY GYYFIVSVIL LNILIALYST AYQKVIDNAD DEYMALMSQK TLRYIRAPDE
DVYVSPLNLI EVFMTPIFRI LPPKRAKDLS YTVMTIVYSP FLLLISVKET REARRIKYNR MKRLNDDANE
YDTPWDLTDG YLDDDDGLFS DNRNSGMRAT QLKNSRSLKL QRTAEQEDVH FKVPKKWYKN VKKCSPSFEQ
YDNDDTEDDA GEDKDEVKEL TKKVENLTAV ITDLLEKLDI KDKKE

>TRPF1 7TM

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AKQIIEGEED LQYLMHSVLL RRYSIIVDGK STPPANVIEK AVDLHAVRVI GSSGYQKCIS YLWKGWLVQD
ENDPSVFVDY KDKTNPSFLV HMDPDRIRAP MYQNATQVII SLVYIGLYTA VINSVNAKGV LDSAEVLLYI
FTLGFICEEV TKFWKAGYHI LGFWNAFNGI LYSFISLSLI LRIIGLTHGE GEDARKYYSE LSYNFLAFSA
PMIWSRLLLY LDSFRFFGAM LVVLKVMMKE SIIFFALLAV LIIGFLQAFV GLDLADDIVA EDTTFIMSAM
ANAIMQSPDF SGFDKFSPPF GIILYYCFTF IVMVVLLNIL IALYNSAYED IYDNANDEYL TLFAQKTMQF
VRAPDENVYI PPFNLIEMVV IGLFWWMEKS KFERMSDFIM GFIYSPVLVF AAVFETRSAA EIRSNRARGE
EDDDTIEEWE QMMDQVDFES DGWNKVCITA KTDLDVDPTI SEVQKLRSEV EELKKMLVDI SKAVSAGNAG
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>TRPF2 8TM

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IRRLVLASID DPYTLDQLRE PRMNLLVVRP LVDRLYDPDD PAVVYCLFAN RIQFLRQHTS TARQTVNVAR
ATLCELVASR VLRRYHEDHP GQIGLLILAH ILVEGFDPFQ GAPEEVESEC RQLQWPIQRR DGHERKLTTL
ELAIISESKL FLSSSACQRL VDAVWNGVVT YTPLSFVDIL PDHYEYASVS LYEPHQAPLL DVGRLLVPWI
RQMIELFQFI TLVILYILTM VNRSSPTLSG WECVFAIYTA GWTLNEFAAI IEHGWVVHSQ TLWSFLDITF
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IWCFMGFFLA LHWLVGSNDD TSAVAGGKAA LTWYETCKWL IWTWFGLDGT GIDRSDEFHP ILGPALMIAF
AFLGNILFLT ILVAILSNTF SKLISDAPAE IQFRRAVLTF AGVKSDSIFS YPPPFNLLAL AALLPLKSML
SPRSFHDVNT SLIRALNAPA LMIISALERR RVAHPRRARS QSLLNWSFSG FNPHGDIQAV FKVEPPPAVE
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>TRPF3 7TM

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IRTQQFNRAT LYCLMANCLQ FNKEGHMYPG NAGTSYTRAL VCELLALKLL KEYNTRELID ALSYDFYPLQ
GLPGAQPAST PKADPRNKAR LTASRTSTLE VAIRASAKHF LAHPLVVQQL EAIWNGAISF YSSADSLHRE
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GLFLAVLSQR SARISSLELI FWFWSAGFML DELVGFNEQG FSLYIMSFWN AFDLGILLLL IVYYFMRVYG
VFLVDAKHWN DSAYDVLAAN AILLLPRIFS VLDHYQYFSQ LLIAFRLMAV DLAAVCILIL ICCSGFFVFF
TLADTNNDAG DIAFRIFQIL MGFTPAAWEV WPGYNWLERG LMAFFLILCH FVIVTILITV LTNSFMKIAS
NAREEHQFLF AINTISMVKN DALFSYVAPG NIFAWLLMPM RYCMPLKQFV KLNRTIIKVT HFPLLFCIFL
YEKYWLAPSM YEPTDLVENH HHHGRDRTIS FADPASRPAL FSPNIRVREE SVAGYQKDHA LEEVFRRGPG
FATLRNQRRN ERKKTQHAVR NWMDRNDEAS ASPQLSQWPT LDGSQGVRPD WLRRLSLSRE LRTRRSRQMS
DVRSAASDPA DLISQPAIGS FFRGIAAATT AKDEENQQTD ADGDDELVTN DEEEEEDIAT NADDSRHGPV
IHQTSIDDYF NTTPSTKRFV PPTSSSLGSS GRPSVSATPR SARRVPHSRT MSTNTILYAP QELRRPPSSS
THSPDPPPTR ARVQNGRPAV VEPAVASGHR SPRRQTYVAT TKPRPIMPPR DAAQTAPNRA GLVSVDQRPR
PNPLRRLSSV DLSVLSDNIV PDDPNGGGIS SSFQTQMAMA LMKDARLGGA GRGTDSGDSN RMSKLVLARM
KTLEESFADV AKELRELKTT SSSTAPTTRR NSSGEELQKM TSLMSAEATR HKDGGSERPK LGGRRTTGKR
PASRKSLLDP KSERARGKGK EPALSSGDDS DSESMSPIKR SSM

>TRPF4 8TM

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PDPKEPPTPP PNADLESNED SPLLSGGSWS GNGSRRSSVR PGSSAKRYQL LSSLSRLTMS MHADQEEEED
DDPTSPFTNL NALEIAAVAD AKRFLSQHVV QKIITGIWNG DIIFWDSLSV HAQKKPRFYN AHTTDIFSRL
RVPKYLKAWE AFFFFLFLCL YYSVLVEQNT ERITHNEIVL YIWLAAFLYD ELSEWADAGT IFYATDVWNL
FDMIMIGIGI AFAVLRVIGI ANNDRHITNT AFNVFALEAL FMVPRVFSLL SLSPYWGTLI PCLKEMGKDF
VKFMVLVVVI YLGFLTTFSL IGQDAYNFSH MTMILTKIFF GSAYVGIEIM DDIDKVFGPP LMIIFIILSS
FLLMGSLTGM LSNSFSRVIT HAREEYLYVY SVYVLEASTS NRLTHFYPPF NVIALVIFRP LRLFLPSDDK
FRQSRILLLK ATHLPIVGLI RMYESIRRRV MPDEYAGFKG PRGATGATRQ RSRGAFQANR PSSGYRPARS
PRPEERLESR HSSRPQHRAI EADEADAPSA VEVRISELND KIDKLTALVE ALQQRPGSP

Supplementary file 5.4 Raw data of this study.

Relative qRT-PCR

t [hpi]	TRPF1-mean	TRPF1-SE	TRPF2-mean	TRPF2-SE	TRPF3-mean	TRPF3-SE	TRPF4-mean	TRPF4-SE
0	1	0,595548048	1	0,330157339	1	0,256485439	1	0,636999613
12	8,961745317	1,619770498	4,789204887	0,628616582	1,393021376	0,140237061	4,904049109	2,473936816
24	8,685009497	2,239813202	2,035626251	0,793152801	1,018999409	0,114089804	1,483370412	0,72305696
48	4,756943507	1,775359355	1,671201949	0,55226315	1,511112394	0,138753611	2,395526543	1,197971651
72	2,906206771	0,374249406	0,204726859	0,067284279	0,960334444	0,185069769	1,639868308	0,593456737
120	6,156049376	1,557232894	2,281410554	0,224089237	2,236043687	0,172431777	1,127431777	0,393387811

Raw qRT-PCR

Raw F0 Values

	TRPF1_Repeat_1	TRPF1_Repeat_2	TRPF1_Repeat_3	TRPF2_Repeat_1	TRPF2_Repeat_2	TRPF2_Repeat_3	TRPF3_Repeat_1	TRPF3_Repeat_2	TRPF3_Repeat_3	TRPF4_Repeat_1	TRPF4_Repeat_2	TRPF4_Repeat_3
0	1,25609E-08	1,43487E-06	6,27184E-07	5,43896E-05	0,00019287	0,000221402	5,95428E-05	0,00012485	0,000160071	1,58993E-06	9,77995E-08	4,38397E-07
12	4,25357E-06	8,12759E-06	6,67573E-06	0,000868417	0,000571696	0,000860507	0,000183449	0,000175391	0,000133	1,81291E-07	6,29024E-06	4,21571E-06
24	4,44022E-06	4,47546E-06	1,75355E-06	0,000187361	6,26459E-05	0,000314907	5,965E-05	6,52412E-05	8,29548E-05	1,09065E-07	1,15469E-06	6,03759E-07
48	3,98667E-06	9,29841E-06	2,82399E-06	0,000284785	0,000705797	0,000287896	0,000318508	0,000292424	0,000238728	5,04044E-06	3,00191E-06	2,71345E-07
72	3,78461E-05	5,59394E-05	5,73396E-05	0,0004608	0,000627004	0,00131715	0,00180493	0,003613839	0,002872833	2,18814E-05	1,56873E-05	4,98231E-05
120	0,00046009	0,000217617	0,000242205	0,029571931	0,026169651	0,021272362	0,020924223	0,018173779	0,016381194	4,29788E-05	3,25575E-05	9,71212E-05

Normalized F0 Values (F0_Raw/Normalization_Factor=Relative_mean_Expression_of_HistonH3_Actin_and_ILV5)

Normalizat	TRPF1_Repeat_1	TRPF1_Repeat_2	TRPF1_Repeat_3	TRPF2_Repeat_1	TRPF2_Repeat_2	TRPF2_Repeat_3	TRPF3_Repeat_1	TRPF3_Repeat_2	TRPF3_Repeat_3	TRPF4_Repeat_1	TRPF4_Repeat_2	TRPF4_Repeat_3
1,00	1,25609E-08	1,43487E-06	6,27184E-07	5,43896E-05	0,00019287	0,000221402	5,95428E-05	0,00012485	0,000160071	1,58993E-06	9,77995E-08	4,38397E-07
1,02	4,149855E-06	7,92939E-06	6,51294E-06	0,00084724	0,000557755	0,000839522	0,000178975	0,000171114	0,000129756	1,7687E-07	6,13685E-06	4,1129E-06
0,59	7,498577E-06	7,5581E-06	2,96137E-06	0,000316413	0,000105796	0,000531811	0,000100736	0,000110179	0,000140093	1,84188E-07	1,95002E-06	1,01962E-06
1,63	2,442344E-06	5,69644E-06	1,73005E-06	0,000174466	0,000432389	0,000176372	0,000195126	0,000179146	0,000146251	3,08791E-06	1,83905E-06	1,66233E-07
25,07	1,5099E-06	2,23175E-06	2,28761E-06	1,8384E-05	2,50148E-05	5,25488E-05	7,20092E-05	0,000144177	0,000114614	8,72976E-07	6,25856E-07	1,98773E-06
72,03	6,38758E-06	3,02124E-06	3,36261E-06	0,000410557	0,000363322	0,000295331	0,000290498	0,000252312	0,000227425	5,96689E-07	4,52006E-07	1,34836E-06

Relative fpkm

t [hpi]	TRPF1-mean	TRPF1-SE	TRPF2-mean	TRPF2-SE	TRPF3-mean	TRPF3-SE	TRPF4-mean	TRPF4-SE
0	1	0,281333105	1	0,398556427	1	1,535981754	1	0,423278061
12	3,980573092	0,860338522	1,643572775	0,384670611	4,74533278	1,185401169	2,801196616	0,61527058
24	12,94197555	2,159404915	0,623078241	0,227867681	1,185314614	0,646103984	1,645197538	0,450609814
48	9,113745735	1,525444036	1,114680558	0,281606758	1,618821337	0,505591243	3,05680203	0,612675535
72	6,321450167	1,525444036	0,976371008	0,282240512	1,554406112	0,415992582	2,601284427	0,601784079
120	4,917362778	0,872847631	0,825650527	0,216819107	1,209468498	0,318082837	1,027665037	0,344095673

Raw fpkm

t [hpi]	TRPF1-Rep_0	TRPF1-Rep_1	TRPF1-Rep_2	TRPF2-Rep_0	TRPF2-Rep_1	TRPF2-Rep_2	TRPF3-Rep_0	TRPF3-Rep_1	TRPF3-Rep_2	TRPF4-Rep_0	TRPF4-Rep_1	TRPF4-Rep_2
0	0,0000	0,0000	17,9102	14,5222	15,7707	16,9879	2,1140	18,4293	0,0000	3,9564	16,2005	0,0000
12	14,2502	27,9402	29,1024	24,8949	20,3354	32,4792	39,2723	38,4823	19,7301	20,1922	12,5236	23,7476
24	99,9933	75,3018	56,4985	10,2067	4,47517	14,7778	8,8278	0,0000	15,5225	9,0570	9,18685	14,9183
48	59,8093	60,1105	43,3092	13,8475	12,2378	26,618	13,9470	8,06166	11,2471	20,3830	17,8141	23,4183
72	34,0935	47,1715	31,9535	21,1263	9,54345	15,494	13,1692	7,05011	11,7133	25,3707	12,2443	14,8186
120	25,6494	27,3512	35,0703	12,451	13,1652	13,4214	7,8009	8,1417	8,90383	9,24147	5,57468	5,89833

Fig. S5.6: *Raw Data of Fig. 5.3*

Relative Polystyrene						
Strain	Appressoria mean	Appressoria SE	Germtubes mean	Germtubes SE	Ungerminated mean	Ungerminated SE
WT	78,0799	1,362	17,3446	0,204	4,5755	1,2571
D*trpf1*	85,6757	2,2937	6,2187	0,7613	8,1056	1,5443
D*trpf2*	81,3916	1,7111	16,1131	2,2303	2,4953	0,6273
D*trpf3*	83,2707	1,8542	10,5382	2,2303	6,1911	0,5856
D*trpf4*	87,9675	2,4631	6,5366	2,2327	5,4959	0,2304
Raw Polystyrene						
Strain	Drop	Appressoria	Germtubes	Ungerminated	Sum	
WT	1	112	24	3	139	
WT	2	107	25	9	141	
WT	3	105	23	7	135	
Δ*trpf1*	1	147	14	20	181	
Δ*trpf1*	2	187	12	16	215	
Δ*trpf1*	3	183	11	12	206	
Δ*trpf2*	1	108	24	2	134	
Δ*trpf2*	2	101	24	3	128	
Δ*trpf2*	3	116	16	5	137	
Δ*trpf3*	1	96	17	6	119	
Δ*trpf3*	2	119	9	9	137	
Δ*trpf3*	3	130	17	11	158	
Δ*trpf4*	1	153	13	11	177	
Δ*trpf4*	2	189	7	10	206	
Δ*trpf4*	3	174	18	11	203	
Relative Onion						
Strain	Appressoria mean	Appressoria SE				
WT	43,3122	13,1192				
c	49,0262	14,0196				
D*trpf2*	37,6016	11,0404				
D*trpf3*	51,3121	22,9598				
D*trpf4*	42,2775	11,9195				
Raw Onion						
Strain	Drop	Appressoria	Ungerminated	Sum		
WT	1	89	47	136		
WT	2	5	97	102		
WT	3	111	21	132		
WT	4	16	110	126		
WT	5	54	130	184		
WT	6	71	41	112		
Δ*trpf1*	1	21	90	111		
Δ*trpf1*	2	54	52	106		
Δ*trpf1*	3	41	61	102		
Δ*trpf1*	4	111	16	127		
Δ*trpf2*	1	35	120	155		
Δ*trpf2*	2	101	11	112		
Δ*trpf2*	3	50	108	158		
Δ*trpf2*	4	44	83	127		
Δ*trpf2*	5	17	114	131		
Δ*trpf2*	6	45	88	133		
Δ*trpf3*	1	114	10	124		
Δ*trpf3*	2	90	11	101		
Δ*trpf3*	3	31	114	145		
Δ*trpf3*	4	5	172	177		
Δ*trpf4*	1	29	89	118		
Δ*trpf4*	2	116	19	135		
Δ*trpf4*	3	57	74	131		
Δ*trpf4*	4	58	81	139		
Δ*trpf4*	5	21	108	129		

Fig. S5.7: *Raw Data of Fig. 5.5*

Relative										
Strain	mICM agar mean	mICM agar SE	PDA mean	PDA SE	Glucose mean	Glucose SE	Raffinose mean	Raffinose SE	Saccharose mean	Saccharose SE
WT	100	0,3575	100	0,3771	100	0,4056	100	0,9381	100	0,509
Δ*trpf1*	98,4848	0,3514	96,723	0,2276	97,0228	0,8112	96,9981	1,2225	96,395	0,4702
Δ*trpf2*	101,7316	0,2919	101,4799	0,3123	106,3047	0,6574	99,6248	1,0415	100,4702	0,6733
Δ*trpf3*	101,1905	0,2973	98,9429	7,45E-07	99,8249	0,7943	96,8105	1,4131	100,7837	0,4305
Δ*trpf4*	97,9437	0,4366	99,26	0,3171	94,0455	0,3771	95,6848	1,1257	98,5893	0,4888
Strain	Malate mean	Malate SE	Pectin mean	Pectin SE	Cellulose mean	Cellulose SE	Mannitol mean	Mannitol SE	Sorbitol mean	Sorbitol SE
WT	100	1,4317	100	1,0309	100	1,5852	100	1,5706	100	0,6675
Δ*trpf1*	100	0,9853	98,9691	0,7614	95,941	1,3717	94,5607	1,21	99,8066	0,7559
Δ*trpf2*	113,8365	1,7276	101,5464	1,1938	97,048	1,4885	104,6025	0,8368	107,9304	0,8806
Δ*trpf3*	97,7987	1,1804	100	1,6739	99,631	1,5891	94,9791	1,1493	99,6132	0,7803
Δ*trpf4*	101,8868	1,1378	95,8763	1,4244	98,893	1,5734	99,1632	1,0925	99,0329	0,8248
Raw										
C-Source	Strain	Mean Plate 1	Mean Plate 2	Mean Plate 3		C-Source	Strain	Mean Plate 1	Mean Plate 2	Mean Plate 3
mICM agar	WT	3,8	3,8625	3,8875		Sorbitol	WT	2,2	2,125	2,1375
mICM agar	Δ*trpf1*	3,7875	3,775	3,8125		Sorbitol	Δ*trpf1*	2,125	2,15	2,175
mICM agar	Δ*trpf2*	3,925	3,9	3,925		Sorbitol	Δ*trpf2*	2,3375	2,3125	2,325
mICM agar	Δ*trpf3*	3,8625	3,925	3,9		Sorbitol	Δ*trpf3*	2,175	2,125	2,1375
mICM agar	Δ*trpf4*	3,75	3,8	3,7625		Sorbitol	Δ*trpf4*	2,1375	2,125	2,1375
PDA	WT	3,95	3,975	3,9		Raffinose	WT	2,2625	2,225	2,175
PDA	Δ*trpf1*	3,825	3,8125	3,8		Raffinose	Δ*trpf1*	2,1875	2,125	2,15
PDA	Δ*trpf2*	3,975	4	4,025		Raffinose	Δ*trpf2*	2,2125	2,3	2,125
PDA	Δ*trpf3*	3,9	3,9	3,9		Raffinose	Δ*trpf3*	2,225	2,2	2,025
PDA	Δ*trpf4*	3,9125	3,925	3,9		Raffinose	Δ*trpf4*	2,075	2,2	2,1
Malate	WT	1,3125	1,275	1,3875		Pectin	WT	0,8125	0,8125	0,8
Malate	Δ*trpf1*	1,35	1,325	1,3		Pectin	Δ*trpf1*	0,8	0,8125	0,7875
Malate	Δ*trpf2*	1,5	1,55	1,475		Pectin	Δ*trpf2*	0,825	0,8375	0,8
Malate	Δ*trpf3*	1,3	1,3125	1,275		Pectin	Δ*trpf3*	0,7875	0,8	0,8375
Malate	Δ*trpf4*	1,325	1,325	1,4		Pectin	Δ*trpf4*	0,7625	0,7875	0,775
Sucrose	WT	2,6125	2,7	2,6625		Glucose	WT	2,3625	2,375	2,4
Sucrose	Δ*trpf1*	2,5375	2,575	2,575		Glucose	Δ*trpf1*	2,325	2,3	2,3
Sucrose	Δ*trpf2*	2,725	2,675	2,6125		Glucose	Δ*trpf2*	2,5	2,5625	2,525
Sucrose	Δ*trpf3*	2,675	2,65	2,7125		Glucose	Δ*trpf3*	2,4	2,35	
Sucrose	Δ*trpf4*	2,6375	2,625	2,6		Glucose	Δ*trpf4*	2,2375	2,25	2,225
Manitol	WT	2	1,9	2,075		Cellulose	WT	2,2	2,3	2,275
Manitol	Δ*trpf1*	1,925	1,9	1,825		Cellulose	Δ*trpf1*	2,125	2,225	2,15
Manitol	Δ*trpf2*	2,1	2,075	2,075		Cellulose	Δ*trpf2*	2,275	2,225	2,075
Manitol	Δ*trpf3*	1,925	1,9	1,85		Cellulose	Δ*trpf3*	2,35	2,225	2,175
Manitol	Δ*trpf4*	2,025	1,925	1,975		Cellulose	Δ*trpf4*	2,35	2,2	2,15

Fig. S5.8: *Raw Data of Fig. 5.6*

Relative				
Strain	With Ca2+ mean	With Ca2+ SE	Without Ca2+ mean	Without Ca2+ SE
WT	1,4625	0,0315	1,1667	0,0273
Δ*trpf1*	1,3583	0,03	1,25	0,0617
Δ*trpf2*	1,5208	0,0897	1,2042	0,0182
Δ*trpf3*	1,4542	0,0795	1,1875	0,0191
Δ*trpf4*	1,4375	0,0144	1,1792	0,0561
Raw				
Ca2+	Strain	Mean Plate 1	Mean Plate 2	Mean Plate 3
With	WT	1,5	1,4	1,4875
With	Δ*trpf1*	1,4	1,375	1,3
With	Δ*trpf2*	1,4375	1,425	1,7
With	Δ*trpf3*	1,3625	1,3875	1,6125
With	Δ*trpf4*	1,4375	1,4625	1,4125
Without	WT	1,1125	1,1875	1,2
Without	Δ*trpf1*	1,2375	1,3625	1,15
Without	Δ*trpf2*	1,175	1,2375	1,2
Without	Δ*trpf3*	1,2	1,15	1,2125
Without	Δ*trpf4*	1,2875	1,1	1,15

Fig. S5.9: *Raw Data of Fig. 5.7*

Relative, WT Glycerol increase					**Relative, WT vs. KO**			
mean	SE	Glycerol [M]			Strain	mean	SE	
100	0,4246	0			WT	100	0,77	
83,1715	0,3935	0,5			Δ*trpf1*	95,927	0,7233	
55,6634	0,277	1			Δ*trpf2*	99,5567	0,6041	
22,0065	0,3184	2			Δ*trpf3*	97,6563	0,3682	
0	0	4			Δ*trpf4*	99,4918	0,3941	
Raw, WT Glycerol increase					**Raw, WT vs. KO**			
Glycerol [M]	Mean Plate 1	Mean Plate 2	Mean Plate 3		Strain	Mean Plate 1	Mean Plate 2	Mean Plate 3
0	3,8375	3,9125	3,8375		WT	3,9	3,95	3,8
0,5	3,2625	3,1625	3,2125		Δ*trpf1*	3,775	3,7625	3,6
1	2,1375	2,175	2,1375		Δ*trpf2*	3,7875	3,825	3,9
2	0,825	0,875	0,85		Δ*trpf3*	3,7625	3,7375	3,8
4	0	0	0		Δ*trpf4*	3,85	3,8375	3,825

Fig. S5.10: *Raw Data of Fig. 5.9*

Raw								
Pos	Name	Ct SYBR	Amount SYBR [Copies]		Pos	Name	Ct SYBR	Amount SYBR [Copies]
C6	WT_HH3	18,57	167		A2	TRPF1_WT	23,52	215
B6	WT_HH3	18,60	163		B2	TRPF1_WT	23,68	187
A6	WT_HH3	18,50	176		C2	TRPF1_WT	23,37	244
F6	Dtrpf1_HH3	18,63	158					
E6	Dtrpf1_HH3	18,77	143					
D6	Dtrpf1_HH3	18,65	156					
G6	Dtrpf2_HH3	18,62	160		G2	TRPF1_Dtrpf2	23,23	274
G7	Dtrpf2_HH3	18,67	154		G3	TRPF1_Dtrpf2	22,97	343
H6	Dtrpf2_HH3	18,61	161		H2	TRPF1_Dtrpf2	23,26	267
F7	Dtrpf3_HH3	18,67	155		D3	TRPF1_Dtrpf3	23,21	279
D7	Dtrpf3_HH3	18,65	156		E3	TRPF1_Dtrpf3	23,09	308
E7	Dtrpf3_HH3	18,67	154		F3	TRPF1_Dtrpf3	23,03	324
C7	Dtrpf4_HH3	18,93	126		A3	TRPF1_Dtrpf4	23,47	223
B7	Dtrpf4_HH3	18,78	142		B3	TRPF1_Dtrpf4	23,01	330
A7	Dtrpf4_HH3	18,92	127		C3	TRPF1_Dtrpf4	23,21	280
Pos	Name	Ct SYBR	Amount SYBR [Copies]		Pos	Name	Ct SYBR	Amount SYBR [Copies]
A5	TRPF2_WT	22,99	157		A11	TRPF4_WT	23,87	243
B5	TRPF2_WT	23,13	141		B11	TRPF4_WT	24,15	186
C5	TRPF2_WT	22,86	173		C11	TRPF4_WT	23,99	217
D5	TRPF2_Dtrpf1	22,49	232		D11	TRPF4_Dtrpf1	24,06	203
F5	TRPF2_Dtrpf1	22,48	234		E11	TRPF4_Dtrpf1	24,08	200
E5	TRPF2_Dtrpf1	22,74	191		F11	TRPF4_Dtrpf1	24,11	194
E6	TRPF2_Dtrpf3	22,41	248		G11	TRPF4_Dtrpf2	24,00	217
D6	TRPF2_Dtrpf3	22,40	250		G12	TRPF4_Dtrpf2	24,21	177
B6	TRPF2_Dtrpf4	22,71	196		H11	TRPF4_Dtrpf2	24,28	165
C6	TRPF2_Dtrpf4	22,74	191		D12	TRPF4_Dtrpf3	24,35	154
A6	TRPF2_Dtrpf4	23,27	126		E12	TRPF4_Dtrpf3	23,98	220
					F12	TRPF4_Dtrpf3	23,98	220
Pos	Name	Ct SYBR	Amount SYBR [Copies]		Pos	Name	Ct SYBR	Amount SYBR [Copies]
A5	HistonH3_WT	19,03	181		C8	TRPF3_WT	25,03	176
C5	HistonH3_WT	19,01	183		B8	TRPF3_WT	26,19	73,3
B5	HistonH3_WT	18,97	190		A8	TRPF3_WT	25,03	176
E5	HistonH3_Dtrpf1	19,27	151		F8	TRPF3_Dtrpf1	25,60	115
F5	HistonH3_Dtrpf1	19,27	150		E8	TRPF3_Dtrpf1	25,67	109
D5	HistonH3_Dtrpf1	19,31	147		D8	TRPF3_Dtrpf1	25,58	116
A6	HistonH3_Dtrpf2	19,00	185		C9	TRPF3_Dtrpf2	25,08	170
C6	HistonH3_Dtrpf2	18,93	195		B9	TRPF3_Dtrpf2	24,93	190
B6	HistonH3_Dtrpf2	19,00	185		A9	TRPF3_Dtrpf2	25,09	169
D6	HistonH3_Dtrpf4	19,39	138		F9	TRPF3_Dtrpf4	25,51	123
E6	HistonH3_Dtrpf4	19,21	158		E9	TRPF3_Dtrpf4	25,68	108
F6	HistonH3_Dtrpf4	18,83	211		D9	TRPF3_Dtrpf4	25,63	112

Fig. S5.11: *Raw Data of Fig. S5.2*

Relative				
Strain	mean	SE		
WT	100	11,7363		
Δ*trpf1*	122,7273	18,3702		
Δ*trpf2*	129,5455	4,3519		
Δ*trpf3*	102,2727	14,0713		
Δ*trpf4*	111,3636	9,3707		
Raw				
Strain	Repeat 1	Repeat 2	Repeat 3	Repeat 4
WT	10	8	14	12
Δ*trpf1*	17	13	8	16
Δ*trpf2*	13	15	14	15
Δ*trpf3*	11	7	13	14
Δ*trpf4*	15	12	10	12

Fig. S5.12: *Raw Data of Fig. S5.3*

9.2 Abbreviations used in this thesis

Tab. I: *Chemicals*

A	adenosine
A	alanine
Ala	alanine
2-APB	2-aminoethoxydiphenyl borate
Asp	aspartic acid
C	cysteine
C	cytidine
Ca	calcium
$^{45}Ca^{2+}$	calcium ions of the calcium isotope with 45 nucleons
Ca^{2+}	calcium ions
$CaCl_2$	calcium chloride
$Ca(NO_3)_2$	calcium nitrate
D	aspartic acid
DMSO	dimethyl sulfoxide
E	glutamic acid
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
FDA	fluorescein diacetate
F	phenylalanine
Fe	iron
G	glycine
G	guanosine
$GdCl_3$	gadolinium(III) chloride
Glu	glutamic acid
Gly	glycine
H	histidine
HCl	hydrogen chloride, solved in water: hydrochloric acid
HNO_3	nitric acid
H_2O_2	hydrogen peroxide
I	isoleucine

IP_3	inositol 1,4,5-trisphosphate
K	lysine
KH_2PO_4	monopotassium phosphate
KOH	potassium hydroxide
L	leucine
$La(NO_3)_3$	Lanthanum(III) nitrate
LiCl	lithium chloride
Leu	leucine
M	methionine
MES	2-(N-morpholino)ethanesulfonic acid
Mg	magnesium
$MgSO_4 \cdot 7H_2O$	magnesium sulfate heptahydrate
Mn^{2+}	manganese ions
N	asparagine
NaCl	sodium chloride
NH_4Cl	ammonium chloride
$(NH_4)_2SO_4$	ammonium sulfate
P	proline
PVDF	polyvinylidene difluoride
Q	glutamine
R	arginine
S	serine
SDS	sodium dodecyl sulfate
T	threonine
T	thymidine
TMB8·HCl	[3,4,5-trimethoxybenzoic acid 8-(diethylamino)octyl ester] hydrochlorid
Tris	tris(hydroxymethyl)aminomethane
U73122	1-[6-[((17β)-3-Methoxyestra-1,3,5[10]-trien-17-yl)amino]hexyl]-1H-pyrrole-2,5-dione
V	valine
W	tryptophan
W-7	N-(6-aminohexyl)-5-chloro-1-naphthalenesulfonamide hydrochloride
Y	tyrosine

Tab. II: *General*

3′	terminus of a nuclic acid molecule with a free hydroxy moiety
5′	terminus of a nuclic acid molecule with a free phosphate moiety
ApE	A plasmid Editor
Apo	apochromat
ATPase	adenylpyrophosphatase
BiFC	bimolecular fluorescence complementation
BLAST	basic bocal alignment search tool
bp	base pair
CA	California
Ca^{2+}_{cyt}	cytosolic calcium ions
$[Ca^{2+}]_{cyt}$	cytosolic calcium ion concentration
cDNA	complementary DNA
CDS	coding DNA sequence
CICR	calcium induced calcium release
CIP	calf intestinal phosphatase
CSM	complete supplement mixture
C-terminus	termius of a poly peptide or protein with a free carboxyl moiety
cv	cultivar
D3	desing 3
DIC	differential interference contrast microscopy
DL	racemate
DNA	deoxyribonucleic acid
DFG	Deutsche Forschungsgemeinschaft (German Research Foundation)
d. h.	das heißt, that means
DTPS	double tagging plasmid system
E. coli	Escherichia coli
e.g.	exempli gratia, for example
EMBL	European Molecular Biology Laboratory
ER	endoplasmic reticulum
For	forward
FOR	Forschergruppe (Research Unit)
FRET	Förster resonance energy transfer

GA	Georgia
HACS	high affinity calcium uptake system
HE	high efficiency
IAEW	Institut für Agrar- und Ernährungswissenschaften (Institute of Agricultural and Nutritional Sciences)
ICP-OES	inductively coupled plasma optical emission spectrometry
i.e.	id est, that is
IZN	Interdisziplinäres Zentrum für Nutzpflanzenforschung (Interdisciplinary Centre for Crop Plant Research)
LACS	low affinity calcium uptake system
L-type Ca^{2+} channel	long-lasting activation Ca^{2+} channel
MA	Massachusetts
MCS	multiple cloning site
mLCM	modified Leach's complete medium
MLU	Martin Luther University Halle-Wittenberg
M-MLV	murine leukemia virus
MSLC	membrane surface liquid culture
NCBI	National Center for Biotechnology Information
NEB	New England Biolabs
NJ	New Jersey
N-terminus	termius of a poly peptide or protein with a free amino moiety
OMA	oat meal agar
PAAP	PVDF membrane-assisted agar plate
PCR	polymerase chain reaction
PDA	potato dextrose agar
PE	Pflanzenernährung (Plant nutrition]
Ph	phase contrast
PM	plasma membrane
PMT	photomultiplier tube
qRT-PCR	quantitativ reverse transcription real time detection PCR
RACE	rapid amplification of cDNA ends
RE	restriction endonuclease
Rev	reverse

rh	relative humidity
RLT	RNeasy Lysis Buffer T
RNA	ribonucleic acid
RNAi	RNA interference
RNA-Seq	ribonucleic acid sequencing
ROI	region of interest
SC	synthetic complete medium
SC-Leu-Ura	synthetic complete medium without leucine and uracil
TA cloning	thymidine-adenosine-cloning
TM	transmembrane
TMD	transmembrane domain
tBLASTn	translated nucleotide BLAST
UK	United Kingdom
USA	United States of America
VMN	vacuolar membrane network
WI	Wisconsin
Z	depth
z. B.	zum Beispiel, for example

Tab. III: *Genes and proteins*

Aeq	apo-aequorin
AeqS	apo-aequorinS
bla	β-lactamase
CaM	calmodulin
Cch1	calcium channel homolog 1
CgTRPF	*Colletotrichum graminicola* TRP of Fungi
CN	calcineurin
CFP	cyan fluorescent protein
Crz1	calcineurin-responsive zinc finger 1
Eca1	endoplasmic calcium ATPase 1
Ecm7	extracellular mutant 7
EGFP	enhanced green fluorescent protein
EGFPf	enhanced green fluorescent protein in fungal codon usage

EYFP	enhanced yellow fluorescent protein
F1 ori	origin of replication of the bacteriophage F1
Fig1	factor-induced gene 1
Gcr1	glycolysis regulation 1
Gdt1	Gcr1 dependent translation factor
GLS1	β-1,3-glucan synthase 1
GOI	gene of interest
GpdA	glyceraldehyde 3-phosphate dehydrogenase A
HIS3	histidine 3
Hph	hygromycinB phosphotransferase
HXT1	hexose transporter 1
HXT5	hexose transporter 5
Ilv5	isoleucine-plus-valine requiring 5
MAP kinase	mitogen-activated protein kinase
mCherry	monomeric Cherry
Mid1	mating pheromone-induced death 1
Nca1	neurospora calcium P-type ATPase 1
Nca2	neurospora calcium P-type ATPase 2
Nca3	neurospora calcium P-type ATPase 3
NptII	neomycin phosphotransferase II
Nyv1	new yeast V-SNARE 1
ori	origin of replication
p	probability
Pkd2	polycystic kidney disease 2
Pmc1	plasma membrane calcium 1
PMCA	plasma membrane Ca^{2+} ATPase
Pmr1	plasma membrane ATPase related 1
PmrA	plasma membrane ATPase related A
Rch1	regulator of calcium homeostasis 1
R-GECO	red fluorescent genetically encoded Ca^{2+} indicator for optical imaging
RHO1	rat sarcoma homolog 1
RHO2	rat sarcoma homolog 2
SERCA	sarco/endoplasmic reticulum Ca2+-ATPase

Spf1	sensitivity to *Pichia farinosa* killer toxin
ToxB	toxin B6
TrpC	tryptophane synthase C
TRP	transient receptor potential
TRPA	transient receptor potential ankyrin
TRPC	transient receptor potential canonical
TRPF1	transient receptor potential of fungi 1
TRPF2	transient receptor potential of fungi 2
TRPF3	transient receptor potential of fungi 3
TRPF4	transient receptor potential of fungi 4
TRPGz	transient receptor potential gibberella zeae
TRPM	transient receptor potential melastatin
TRPML	transient receptor potential mucolipin
TRPN	transient receptor potential no mechanoreceptor potential C
TRPP	transient receptor potential polycystin
TRPV	transient receptor potential vanilloid
TRPV1	transient receptor potential vanilloid 1
TRPY1	transient receptor potential yeast 1
TRPY2	transient receptor potential yeast 2
TRPY3	transient receptor potential yeast 3
Vcx1	vacuolar H^+/Ca^{2+} exchanger 1
V-SNARE	vesicle synaptosome-associated protein receptor
YC	yellow chameleon
YC3.6	yellow chameleon 3.6
YFP	yellow fluorescent protein
Yvc1	yeast vacuolar conductance 1

Genes in general are written in italics with the first letter in uppercase. Deletions of genes are denoted in italics and lowercase letters, deletion is indicated by a Δ sign in front or after the gene name, according to the habits of the respective research community. Promoters are indicated by a P and the abbreviation of the gene that the promoter naturally drives. Terminators are indicated by a T and handled the same way. Proteins are written in normal font with the first letter in uppercase. Deviations from the standard are made when this is common in the research community the name originates from. In order to avoid confusion

the genus and species were indicated in front of gene or protein names when needed.

Tab. IV: *Enzymes*

*Apa*I	type II restriction endonuclease number 1 from *Acetobacter pasteurianus* sub. *pasteurianus* (ATCC 9432)
DNaseI	nonspecific DNA hydrolase
*Eco*RI	type II RE number 1 from *E. coli* RY13 (R.N. Yoshimori)
*Fse*I	type II RE number 1 from *Frankia* species Eul1b (NRRL 18528)
*Hind*III	type II RE number 3 from *Haemophilus influenzae* Rd (ATCC 51907)
*Not*I	type II RE number 1 from *Nocardia otitidis-caviarum* (ATCC 14630)
*Pac*I	type II RE number 1 from *Pseudomonas alcaligenes* (C. Polisson)
*Sbf*I	type II RE number 1 from *Streptomyces* species Bf-61 (S.K. Degtyarev)
*Sma*I	type II RE number 1 from *Serratia marcescens* (ATCC 49779)

Tab. V: *Units and prefixes*

c	prefix: centi 10^{-2}
°C	degree Celsius
d	day
d	prefix: deci 10^{-1}
E-value	expect value
g	gram
g	gravitational
h	hour
hpi	hours post inoculation
Hz	hertz
k	prefix: kilo 10^{3}
m	meter
m	prefix: milli 10^{-3}
M	molar
M	prefix: mega 10^{6}
µ	prefix: micro 10^{-6}
min	minute
n	prefix: nano 10^{-9}
n or N	sample number

l or L	litre
Pa	pascal
pH	potentia hydrogenii, negative decadic logarithm of H^+ concentration
%	percent
r^2	regression coefficient
RLU	relative light units
rpm	rotations per minute
s	second
W	watt

9.3 Curriculum Vitae

Personal Data

Name:	Mario Lange
Gender:	male
Academic degree:	Diploma in Biochemistry
Date of birth:	06th October 1983
Place of birth:	Zwickau
Nationality:	German
Family status:	Single, no children
Mailing address:	Dorotheenstraße 18, 06108 Halle (Saale)

Professional Experiences

2015	Postgraduate researcher
	Martin Luther University Halle-Wittenberg
	Institute of Agricultural and Nutritional Sciences,
	Phytopathology and Plant Protection Laboratory
2009 – 2014	Postgraduate researcher
	Martin Luther University Halle-Wittenberg
	Institue of Agricultural and Nutritional Sciences,
	Plant Nutrition Laboratory
2003 – 2004	Alternative civilian service

Education

2004 – 2009	Studies of Biochemistry, University Leipzig Degree: Diploma Diploma thesis: Alters- und jahreszeitabhängige Veränderung von Astroglia, Neurogenese und synaptischer Plastizität im Gehirn von Tenreks
1990 – 2003	Primary and Secondary School

Datum / Date

Unterschrift des Antragstellers / Signature of the applicant

9.4 List of own publications

9.4.1 Papers

Lange, M.; Weihmann, F.; Schliebner, I.; Horbach, R.; Deising, H.B.; Wirsel, S.G.R.; & Peiter, E. The Transient Receptor Potential (TRP) Channel Family in *Colletotrichum graminicola*: A Molecular and Physiological Analysis. *PLOS ONE* 11: e0158561, 2016

Lange, M.; & Peiter, E. Cytosolic free calcium dynamics as related to hyphal and colony growth in the filamentous fungal pathogen *Colletotrichum graminicola*. *Fungal Genet. Biol.* 91: 55 – 65, 2016

Lange, M.; Oliveira-Garcia, E.; Deising, H.B. & Peiter, E. A modular plasmid system for protein co-localization and bimolecular fluorescence complementation in filamentous fungi. *Curr. Genet.* 60: 343 – 350, 2014

Lange, M.; Müller, C. & Peiter, E. Membrane-assisted culture of fungal mycelium on agar plates for RNA extraction and pharmacological analyses. *Anal. Biochem.*, 453C, 58 – 60, 2014

Lange, M.; Arendt, T. & Härtig, W. An inexpensive technique for dot blotting in a 96-well format. *Anal. Biochem.*, 398: 129 – 131, 2010

9.4.2 Presentations

Calcium signals in growing tips of the filamentous fungus *Colletotrichum graminicola*. Annual meeting 2015 of the Mycology study-group of the German Phytomedical Society (DPG) (51. Jahrestagung 2015 des DPG AK Mykologie) 19 – 20th March 2015, Halle (Saale), Germany.

The generation of calcium signals in a hemibiotrophic plant pathogen. 3rd Sophia Antipolis Workshop on Compatibility Mechanisms in Plant-Microbe Interactions. INRA Centre Agrobiotech, 29 – 30th September 2011, Sophia-Antipolis, France.

9.4.3 Posters

Lange, M.; Peiter, E. (2014). Does calcium orchestrate tip growth in fungal hyphae? Presented at:

- Plant Nutrition 2014: International Conference of the German Society of Plant Nutrition, 10 – 12th September 2014, Halle (Saale), Germany.
- Plant Calcium Signaling 2014, 22 – 25th June 2014, Münster, Germany.

Lange, M.; Peiter-Volk, T.; Horbach, R.; Peiter, E. (2010). The generation of calcium signals in a hemibiotrophic plant pathogen. Presented at:

- 4th International Workshop Rauischholzhausen, Novel Molecular Targets for Improvement of Crop Resistance as a Measure against Global Famine, 30th September – 02nd October 2010, Rauischholzhausen, Germany.
- Plant Calcium Signaling 2010, 31th August – 04th September 2010, Münster, Germany.

9.5 Eidesstattliche Erklärung / *Declarationunder Oath*

Ich erkläre an Eides statt, dass ich die Arbeit selbstständig und ohne fremde Hilfe verfasst, keine anderen als die von mir angegebene Quellen und Hilfsmittel benutzt und die den benutzten Werken wörtlich oder inhaltlich entnommenen Stellen als solche kenntlich gemacht habe.

I declare under penalty of perjury that this thesis is my own work entirely and has been written without any help from other people. I used only the sources mentioned and included all the citations correctly both in word or content.

Datum / Date	Unterschrift des Antragstellers / Signature of the applicant