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Absence of repellents in *Ustilago maydis* induces genes encoding small secreted proteins

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Abstract The *rep1* gene of the maize pathogen *Ustilago maydis* encodes a pre-pro-protein that is processed in the secretory pathway into 11 peptides. These so-called repellents form amphipathic amyloid fibrils at the surface of aerial hyphae. A SG200 strain in which the *rep1* gene is inactivated (Δ*rep1* strain) is affected in aerial hyphae formation. We here assessed changes in global gene expression as a consequence of the inactivation of the *rep1* gene. Microarray analysis revealed that only 31 genes in the Δ*rep1* SG200 strain had a fold change in expression of ≥2. Twenty-two of these genes were up-regulated and half of them encode small secreted proteins (SSPs) with unknown functions. Seven of the SSP genes and two other genes that are over-expressed in the Δ*rep1* SG200 strain encode proteins that can be classified as secreted cysteine-rich proteins (SCRPs). Interestingly, most of the SCRPs are predicted to form amyloids. The SCRP gene *um00792* showed the highest up-regulation in the Δ*rep1* strain. Using GFP as a reporter, it was shown that this gene is over-expressed in the layer of hyphae at the medium-air interface. Taken together, it is concluded that inactivation of *rep1* hardly affects the expression profile of *U. maydis,* despite the fact that the mutant strain has a strong reduced ability to form aerial hyphae.

Keywords Aerial hypha · Repellent · Hydrophobin-like protein · *Ustilago maydis* · SSP · SCRP

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Introduction

The life cycle of the pathogenic heterobasidiomycete *Ustilago maydis* is characterized by distinct morphological and nuclear states. Fusion of compatible yeast-like sporidia results in the formation of a pathogenic filamentous dikaryon. Upon formation of an appressorium the host (*Zea mays* [maize] and *Euchlaena mexicana* [Mexican teosinte]) is invaded. The fungus proliferates and branches inside the plant tissue, resulting in the formation of diploid teliospores. These spores are dispersed into the environment. After germination, meiosis occurs resulting in the formation of haploid sporidia (Banuett and Herskowitz 1988; Banuett 1992; Christensen 1963).

Only when two partners harbor different alleles of the *a*- and *b*-loci, mating and pathogenic development can occur. Cell fusion is controlled by the *a1* and *a2* mating-type loci (Bölker et al. 1992). These loci encode a pheromone and a receptor for the pheromone of the opposite mating type. The multi-allelic *b* locus regulates the post-mating processes of filament formation and pathogenic development. The *b* locus

encodes two unrelated homeodomain proteins, bE and bW, that form active hetero-dimers when derived from different alleles (Gillissen et al. 1992; Kämper et al. 1995).

Rep1 is one of the genes that is regulated by the bE/bW heterodimer (Bohlmann 1996; Brachmann et al. 2001; Romeis et al. 2000; Wösten et al. 1996). The *rep1* gene encodes a pre-pro-protein, which consists of a signal sequence for secretion and 12 repeated sequences. Processing at KEX2 recognition sites in the secretory pathway results in 10 peptides of 34–53 amino acids and one larger protein of 229 amino acids (Wösten et al. 1996). These cleavage products that are collectively known as repellents are secreted into the cell wall of filaments. Here they form amyloid fibrils (Teertstra et al. 2009), which are involved in attachment to hydrophobic surfaces and in formation of hydrophobic aerial hyphae (Teertstra et al. 2006, 2009; Wösten et al. 1996).

Strains in which the *rep1* gene has been inactivated form colonies with only few aerial hyphae (Teertstra et al. 2006; Wösten et al. 1996). We here show that expression of only 31 genes is changed at least two-fold in the Δ*rep1* strain when compared to the parental SG200 strain under conditions of aerial growth. Most of these genes are up-regulated and encode for small secreted proteins without a predicted enzymatic function.

Methods

Strains and growth conditions

Ustilago maydis strain SG200 (*a1mfa2 bE1/bW2*) (Bölker et al. 1995) and its derivatives (see below) were used in this study. SG200 is a haploid FB1 (*a1b1*) strain (Banuett and Herskowitz 1989), which harbors an active b mating type complex. Consequently, the strain grows yeast-like in liquid medium and forms filaments at the water–air interface without the need for mating. *U. maydis* strains were routinely grown at 25°C using liquid YEPSL medium (0.4% yeast extract, 0.4% peptone, 2% sucrose) or potato dextrose agar (PDA, Sigma) that had either or not been supplemented with 1% (w/v) charcoal. For isolation of RNA for microarray analysis, cells were grown at 22°C on nitrate minimal medium (NM^+; Holliday 1974) containing 2% agar (w/v), 1% charcoal (w/v), 20 mg l^{-1} histidine, 380 mg l^{-1} leucine, 20 mg l^{-1} tryptophan, 50 mg l^{-1} uracil and 76 mg l^{-1} Yeast Synthetic Drop-out Medium Supplements (Sigma, Y2001). For GFP expression analysis, 1 µl of cell suspension (2×10^7 cells ml^{-1}) was seeded on each side of a 0.25 mm thin 20 × 20 mm square of solidified (1.5% agarose) NM^+ medium that was sandwiched between a glass slide and a cover slip. Cells were grown at 25°C under humid conditions.

Constructs

SG200Δ*rep1* was generated as described (Müller et al. 2008a). The PCR fragment that was used to inactivate *rep1* was amplified with oligonucleotide primers RepMUF-fw and RepMDF-rev (Table 1) using Phusion™ polymerase (Finnzymes). The resulting PCR fragment, consisting of a nourseothricin resistance cassette (Brachmann et al. 2004) flanked by the upstream and downstream sequences of the *rep1* gene, was used to transform the SG200 strain.

Vector pUC19-Rep-c was used to complement the SG200Δ*rep1* strain. A 3674 bp PCR fragment was amplified for its construction. This fragment consisted of a 1680 bp *rep1* promoter region and the coding sequence of this gene. For this, Phusion™ polymerase (Finnzymes) was used with genomic FB1 DNA as a template and oligonucleotide primers pRep-fw and cRep-rev (Table 1). The latter primer introduces a *Not*I-site at the 3′ end. The 3674 bp PCR-fragment was introduced in the *Sma*I-site of pUC19. In the next step, a *Not*I fragment encompassing a carboxin-resistance cassette (Brachmann et al. 2004) was introduced into the respective site of the pUC19 derivative, resulting in pUC19-Rep-c.

The promoter region of gene *um00792* was amplified from genomic DNA of FB1 using Phusion™

Table 1 Primers used in this study

Primer name	Sequence
RepMUF-fw	TTTGCGTATTCCACCTGCAGTAGCC
RepMDF-rev	CAACTACTGGGAAAAGTATGGAGCGG
pRep-fw	GGTACCGCAGCAATCACAGAG
cRep-rev	GCGGCCGCATGAGGAAACCCTAAC
pr792-fw	AAACTTGGGCCCGCTACCAG
pr792-rev	GGAGGAACAACGAGGATGAC
RepUF-fw	GGATGTAGCTGTCGTGCTTCCA
RepUF-rev	GGCCATCTAGGCCGTGATAATGT

polymerase (Finnzymes) with oligonucleotide primers pr792-fw and pr792-rev (Table 1). The resulting 1837 bp fragment was introduced in the *Sma*I site of pUC19, resulting in vector pUC19-pr792. The 1791 bp *Apa*I/*Nco*I promoter fragment of *um00792* was digested from pUC19-pr792 and introduced in the corresponding sites of pMF3c (Brachmann et al. 2004). As a result, enhanced GFP (*eGFP*) was placed under control of the *um00792* promoter. The resulting construct pMF3c-pr792 was linearized in the carboxin resistance cassette with *Age*I, thus targeting the DNA to the *sdh2*-locus of *U. maydis* upon transformation.

Transformation of *U. maydis*

Ustilago maydis was transformed according to Brachmann et al. (Brachmann et al. 2004). Transformants were selected on PDA plates supplemented with nourseothricin (150 μg ml^{-1}) and carboxin (2 μg ml^{-1}), respectively. Deletion of the *rep1* gene was confirmed by Southern analysis. Chromosomal DNA of *U. maydis* was isolated as described (Hoffman and Winston 1987) and blotted onto Hybond N+ (Amersham). Hybridization was performed at 60°C in 0.5 M phosphate buffer, pH 7.2, containing 7% SDS and 10 mM EDTA. A 936 bp ^{32}P-α-dCTP labeled PCR-fragment was used as a probe, which was derived from the *rep1* promoter region of FB1 using the primers RepUF-fw and RepUF-rev (Table 1).

Northern analysis

Ustilago maydis cultures were homogenized with a microdismembrator (B.Braun). RNA was extracted with Trizol (Invitrogen) according to the manufacturer's protocol. Hybridization was performed using standard protocols (Sambrook et al. 1989). ^{32}P-α-dCTP labeled fragments of genes *um00792*, *um00913* and *um03817* were used as a probe.

Microarray analysis

2×10^7 cells of exponentially growing cultures of strains SG200 and SG200Δ*rep1* were seeded on solid NM^+-charcoal medium and grown for 48 h. Cells were harvested, frozen in liquid nitrogen, and homogenized with a dismembrator (Retsch). RNA was isolated with Trizol, after which double stranded cDNA was made according to the Affymetrix protocol (Affymetrix). The cDNA was purified over a cDNA clean up spin column, after which biotin labeled antisense cRNA was obtained using the Enzo BioArray HighYield RNA transcript labeling kit (ENZO Diagnostics). Concentration of the cRNA was determined using the NanoDrop N-1000 (Thermo Scientific) and quality of the cRNA was determined using the 2100 Bioanalyzer (Agilent Technologies). cRNA was fragmented according to the Affymetrix protocol and the resulting 30–200 bp fragments were hybridized to the Affymetrix custom array (MIPIUstilagoA). Array data have been deposited in NCBI's Gene Expression Omnibus (Edgar et al. 2002) and are accessible through GEO Series accession number GSE21490 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE21490). Data analysis of biological triplicates was performed as described previously (Eichhorn et al. 2006). Genes with a P value of < 0.01, whose mean expression was at least 2-fold changed, were filtered in Excel.

Analysis of protein sequences

Protein sequences of *U. maydis* were obtained from the MUMDB database (http://mips.gsf.de/genre/proj/ustilago) and analyzed for functional domains with SCOP (Murzin et al. 1995), SMART (Ponting et al. 1999), PFAM (Bateman et al. 2002), SignalP (Bendtsen et al. 2004), TANGO (Fernandez-Escamilla et al. 2004) and Waltz (Maurer-Stroh et al. 2010).

Fluorescence microscopy

Fluorescence microscopy was carried out using a Zeiss Axioscope 2PLUS equipped with a HBO 100 W mercury lamp and a Leica LFC 420C camera (2592 × 1944 pixels). GFP fluorescence was monitored using a FITC filter set.

Results

Absence of repellents has a minor effect on overall gene expression in *U. maydis*

Gene *rep1* was deleted in strain SG200 by introducing a PCR-fragment consisting of a nourseothricin resistance cassette flanked by upstream and downstream sequences of *rep1* (see Methods). Five out of

12 transformants showed loss of surface hydrophobicity and reduction of aerial hyphae formation on PDA charcoal. Southern analysis confirmed deletion of *rep1* in these strains. Introduction of pUC19-Rep-c encompassing the *rep1* gene restored formation of aerial hyphae, showing that the phenotype was solely caused by the gene deletion (Data not shown).

To monitor the effect of the *rep1* deletion on overall gene expression during aerial hyphae formation, DNA microarrays (representing ~90% of the genes of *U. maydis*) were hybridized with RNA from SG200 and a SG200Δ*rep1* strain. To this end, both strains had been grown for 2 days on a synthetic medium containing charcoal. SG200 formed abundant aerial hyphae, whereas aerial hyphae formation in the mutant strain was severely affected (Fig. 1b). Thirty-one genes showed a fold change ≥2 when expression of the two strains was compared. Twenty-two of these genes were up-regulated, whereas 9 were down-regulated in the SG200Δ*rep1* strain (Table 2). The microarray data were confirmed with Northern analysis by probing for 3 genes (Fig. 1a). This showed that expression of genes *um00792* and *um00913* was increased in the SG200Δ*rep1* strain compared to the parental strain at least 6-fold and 3-fold, respectively, whereas expression of gene *um03817* was decreased at least 10-fold. Expression of genes *um00792* and *um00913* was higher on PDA than on NM+-charcoal, which correlated with the number of aerial hyphae that were formed. In contrast, expression of *um03817* was more pronounced on solidified NM^{+}-charcoal. This indicates that expression of at least *um03817* is not only influenced by the *rep1* deletion, but also by the composition of the medium.

Of the genes that were down-regulated in the SG200Δ*rep1* strain, 2 encode secreted proteins (Table 2). One of these genes is predicted to have enzymatic activity. This gene, *um11112*, is related to genes encoding Versicolorin B synthase. The *U. maydis* gene is proposed to encode a protein with aryl-alcohol oxidase activity (Müller et al. 2008b). The other gene, *um04248*, encodes a repetitive protein with no similarity to known proteins (Müller et al. 2008a). Eighteen out of the 22 genes with an increased expression in the SG200Δ*rep1* strain encode secreted proteins (Table 2), as predicted by SignalP and TargetP (see Müller et al. 2008b). Several of these genes have already been deleted in *U. maydis*, either as a single or part of a multiple gene deletion (Table 2; Kämper et al. 2006; Leuthner et al. 2005; Vraneš 2006). Two up-regulated genes encode secreted proteins with an enzymatic function. Gene *um00913* encodes glyoxaloxidase 2 (Leuthner et al. 2005), whereas *um04422* is predicted to encode an endo-xylanase (Müller et al. 2008b). Remarkably, the

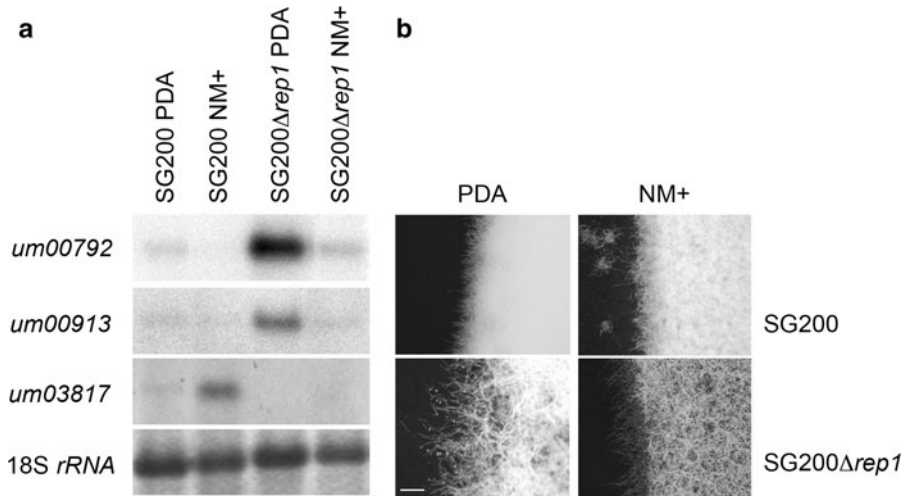

Fig. 1 Inactivation of *rep1* results in reduced aerial hyphae formation and in changes in gene expression. **a** Northern analysis of genes *um00792*, *um00913* and *um03817* in strains SG200 and SG200Δ*rep1,* 18S *rRNA* serving as a control. Cultures were grown on PDA-charcoal and NM+-charcoal for 48 h. **b** Detail of colonies of the SG200 and SG200Δ*rep1* strain grown on PDA and NM+-charcoal plates. Colonies of strain SG200 form abundant aerial hyphae, whereas in the *rep1* deletion strain aerial hyphae formation is severely reduced. Bar represents 0.5 mm

Table 2 Genes that change their expression 2-fold or more when *rep1* is inactivated ($P < 0.01$)

MUMDB um number	Description	Fold change	Amino acids	TargetP secretion prediction	Secretome* enzymatic function	Secretome* SCRP cys class	TANGO[b] number of β-aggregation regions	Waltz[c] number amylogenic regions	Gene deletion mutant	Phenotype deletion mutant
um03924	Repellent precursor	0.01	652	RC2	No		1	14	Single gene	Reduced aerial hyphae, virulence unaffected[d]
Genes up	**Secreted**									
um00792	Cons hyp U-spec protein	8.76	174	RC1	No	cys II	2	2	Single gene	Virulence unaffected[e]
um00913	Glyoxaloxidase	5.28	625	RC2	Yes				Single gene	Virulence unaffected[f]
um12258	Hypothetical protein	4.60	142	RC1	No	cys V	2	1		
um01239	Cons hyp U-spec protein	4.41	283	RC1	No		4	2	Cluster2a	Increased virulence[g]
um01377	Hyp pr expansin	4.03	532	RC1	No		1	2		
um00793	Cons hyp U-spec protein	3.72	160	RC1	No	cys II	2	1		
um03614	Cons hyp pr	3.01	489	RC4	No	cys III	6	6	Cluster 9A	Virulence unaffected[g]
um10418	Hypothetical protein	2.74	146	RC1	No	cys III	4	2		
um05495	Cons hyp pr expansin	2.70	385	RC2	No		2	4		
um01375	Hypothetical protein	2.55	118	RC2	No		0	2		
um01820	Hypothetical protein	2.47	240	RC2	No		1	3	Single gene	Virulence unaffected[e]
um06126	Mig2-6	2.38	404	RC2	No	cys IX	1	0		
um01240	Cons hyp U-spec protein	2.32	279	RC1	No		4	1	Single gene/ cluster2A	Virulence unaffected/ increased virulence[eg]
um04422	Rel xylanase	2.23	631	RC5	Yes					
um03202	Cons hyp U-spec protein	2.15	126	RC1	No	cys VI	0	1	Cluster 8A	Virulence unaffected[g]
um00081	Put protein GPI	2.04	261	RC1	No	cys X	5	5		
um01300	Cons hyp U-spec protein	2.02	136	RC2	No	cys V	1	2	Cluster 2B	Virulence unaffected[g]
um05781.2	Hypothetical protein	2.00	329	RC1	No		1	1		

Table 2 continued

MUMDB um number	Description	Fold change	Amino acids	TargetP secretion prediction	Secretome* enzymatic function	Secretome* SCRP cys class	TANGO[b] number of β-aggregation regions	Waltz[c] number amylogenic regions	Gene deletion mutant	Phenotype deletion mutant
	Non-secreted									
um01374	Hypothetical protein	2.88	435	No						
um03881	Rel Hsp80	2.83	215	No						
um11883	Put pr mitochondrion	2.37	621	RC4/no						
um11935	Cons hyp U-spec protein	2.37	571	No						
Genes down	**Secreted**									
um11112	rel.versicolorin B synthase	0.27	599	RC3	Yes					
um04248	Putative protein	0.34	407	RC1	No		1	1		
	Non-secreted									
um03817	Putative protein	0.16	185	No						
um04106	Transferase	0.20	465	No						
um02050	Cons hyp protein	0.29	3,743	No						
um03398	Rel esterase	0.41	401	No						
um03117-2	Cons hyp protein	0.43	615	No						
um04482	Cons hyp protein	0.47	460	No						
um06459	Rel Cyt P450	0.48	589	No						

Properties of the secreted proteins are indicated

[a] Secretome: classification according to Müller et al. (2008b)

[b] TANGO β-aggregation regions at pH 7.0 (Fernandez-Escamilla et al. 2004): http://tango.crg.es/

[c] Waltz amylogenic regions at pH 7.0 (Maurer-Stroh et al. 2010): http://waltz.switchlab.org/

[d] Teertstra et al. (2006)

[e] Vraneš (2006)

[f] Leuthner et al. (2005)

[g] Kämper et al. (2006)

other 16 genes encode secreted proteins with no apparent enzymatic function. Eleven of them belong to the small secreted proteins (SSPs), which have an arbitrary size limit of 300 amino acids. Of these genes, 7 encode secreted cysteine-rich proteins (SCRPs), whereas another 2 up-regulated SCRPs exceed 300 aa (Table 2; Müller et al. 2008b). The TANGO and Waltz algorithms were used to assess whether the 16 secreted proteins that are predicted not to have an enzymatic function have the potential to form amyloid fibrils. The TANGO algorithm predicts regions within a protein that are prone to intermolecular β-sheet formation. These intermolecular β-sheets can result in protein aggregation (Fernandez-Escamilla et al. 2004). It was found that five consecutive residues with a TANGO score above 5% are a good predictor of aggregation (Fernandez-Escamilla et al. 2004). Using standard settings (see http://tango.crg.es/), 14 out of the 16 mature proteins had one or more of these aggregation prone regions and 5 of them had 4 or more. The Waltz algorithm is a position-specific scoring matrix that has been developed to distinguish between amyloid sequences and amorphous β-sheet aggregates (Maurer-Stroh et al. 2010). Using standard settings (http://waltz.switchlab.org/), it was shown that 15 out of the 16 proteins had between 1 and 6 amylogenic regions. Only Mig2-6 that is encoded by *um06126* (Zheng et al. 2008) was found not to have such a region. Taken together, the TANGO and Waltz algorithms predict that most of the proteins that are encoded by the up-regulated genes within the *rep1* mutant have the propensity to form amyloids.

Gene *um00792* is highly induced in filaments of the SG200Δ*rep1* strain that grow at the water–air interface.

The SCRP gene *um00792* shows the highest change in expression when strains SG200 and SG200Δ*rep1* are compared by micro-array analysis (Table 2). Its expression is 8.75 fold increased in the SG200Δ*rep1* strain. Expression of this gene was monitored using GFP as a reporter. To this end, strains SG200 and SG200Δ*rep1* were transformed with construct pMF3c-pr792. This vector contains the *eGFP* gene, which is placed behind the *um00792* promoter. Transformants uG114 and uG115, which are derivatives of SG200 and SG200Δ*rep1*, respectively, showed a fluorescence pattern representative for the majority of the fluorescent transformants. These strains were therefore selected for further study. They were grown along a thin slab of nitrate minimal medium, sandwiched between an object glass and a cover slip. Aerial hyphae and hyphae growing in the substrate showed similar GFP fluorescence in strains uG114 and uG115 (Fig. 2a). However, at the medium-air interface, a 10-fold increase in fluorescence was shown in the SG200Δ*rep1* derivative uG115 when compared to the wild-type derivative uG114. This layer consists of yeast cells and filaments that have not yet escaped into the air (Fig. 2b, c). Both cell types

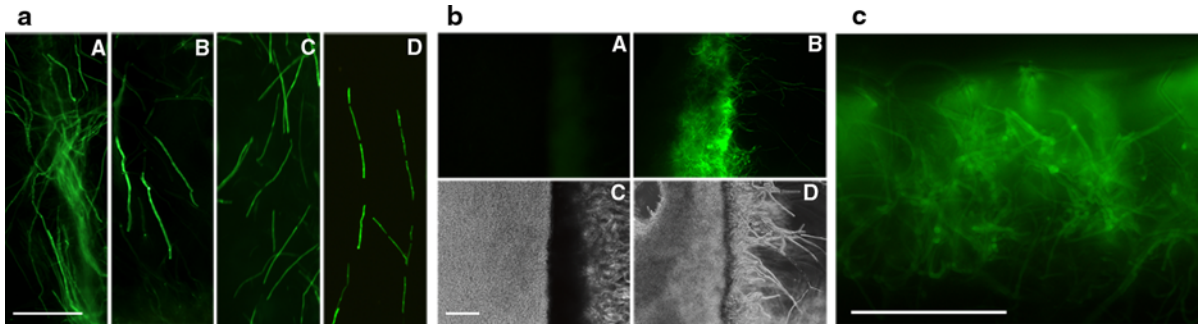

Fig. 2 Localization of expression of *um00792* in the SG200 derivative uG114 and the SG200Δ*rep1* derivative uG115 using eGFP as a reporter. Cells were seeded along a thin layer of solidified medium between a glass slide and a cover slip and grown for 40 h. Cells were monitored by phase contrast and fluorescence microscopy. **a** Individual hyphae of strain uG114 (*A, B*) and uG115 (*C, D*) growing in the substrate (*A, C*) or in the air (*B, D*) show similar fluorescence. **b** Strain uG114 (*A, C*) and strain uG115 (*B, D*) growing along the solidified medium visualized by fluorescence (*A, B*) and light (*C, D*) microscopy. Total fluorescence of the top layer, consisting of yeasts and developing hyphae, is highly increased in the uG115 strain (*B*). **c** Detail of (**b**, *B*). Bar represents 100 μm

within this layer showed fluorescence. In contrast, gene expression was not detected in yeast cells in liquid shaken cultures (data not shown).

Discussion

Strains in which the *rep1* gene is inactivated form few aerial hyphae (Teertstra et al. 2006; Wösten et al. 1996). We here assessed global changes in gene expression as a consequence of the inactivation of the *rep1* gene. It is shown that deletion of this gene has a minor effect on global gene expression under conditions of aerial growth in the wild-type. Interestingly, a majority of the genes that are up-regulated at least two-fold encode small secreted proteins that are predicted to form amyloids.

Gene expression at the stage of aerial hyphae formation was monitored with microarray analysis. Only 31 genes had a fold change in expression of at least two when expression profiles of strains SG200 and SG200Δ*rep1* were compared. Of these genes, 7 had a fold change of at least 4 and only 1 gene had a fold change of at least 8. This is a striking difference with gene regulation in the basidiomycete *Schizophyllum commune* and the filamentous bacterium *Streptomyces coelicolor*. These microorganisms produce repellent-like proteins; i.e. the hydrophobins in *S. commune* (Wösten et al. 1993, 1994a, b, 1999; Wösten and Willey 2000) and the chaplins in *S. coelicolor* (Claessen et al. 2003, 2004). Like repellents, hydrophobins and chaplins form functional amyloid fibrils at the hyphal surface (Butko et al. 2001; Claessen et al. 2003; Wösten and de Vocht 2000; de Vocht et al. 2002; Scholtmeijer et al. 2009). Inactivation of the genes encoding these proteins results in a phenotype similar to that of the Δ*rep1* strain (Claessen et al. 2003; van Wetter et al. 1996, 2000; Wösten et al. 1994b). Over 4000 genes had changed their expression at least two-fold under conditions of aerial growth when expression of a wild-type *S. commune* strain was compared to that of the hydrophobin knockout strain Δ*SC3*Δ*SC4* (R.A. Ohm, H.A.B. Wösten, unpublished results). In *S. coelicolor* 244 genes had a ≥2-fold changed expression in the Δ*chp* strain (de Jong et al. 2009; Claessen et al. 2006). Transcriptional regulators were among the genes with an affected expression in both *S. commune* and *S. coelicolor*. In contrast, genes encoding transcriptional activators were not affected in the Δ*rep1* strain. How can we explain this result? In *S. commune* and *S. coelicolor,* aerial hyphae formation is the first step in a differentiation process leading to the formation of fruiting bodies and spore chains, respectively. In contrast, aerial hyphae of *U. maydis* seem to represent vegetative hyphae that happen to grow into the air.

Remarkably, most of the genes that changed their expression in the Δ*rep1* strain encode secreted proteins without an enzymatic function. Eleven out of 22 genes that were up-regulated encode small secreted proteins (SSPs) and 9 of them belong to the class of secreted cysteine-rich proteins (SCRPs) (Müller et al. 2008b). These proteins harbor no common protein domains. For these proteins, similarity in three-dimensional structure may be more important than sequence similarity. This is in agreement with the results obtained with the TANGO and Waltz algorithms (Fernandez-Escamilla et al. 2004; Maurer-Stroh et al. 2010). These algorithms predict β-aggregation and amylogenic regions in more than 80% of the SSPs and SCRPs that are up-regulated in the Δ*rep1* strain. The algorithms also predict amylogenic regions in the repellents of *U. maydis* and in the *S. commune* hydrophobin SC3, which are known to form amyloids (Wösten and de Vocht 2000; de Vocht et al. 2002; Scholtmeijer et al. 2009; Teertstra et al. 2009).

We previously showed that repellents form amyloids in the cell wall of hyphae of *U. maydis* (Teertstra et al. 2009). Interestingly, filaments of the SG200Δ*rep1* strain also stained with ThT, from which it was concluded that other secreted proteins also have the capability to form amyloid fibrils. The SSPs that are up-regulated in SG200Δ*rep1* could be candidates for such proteins. These results are in line with previous findings that amyloids can be generally found on microbial surfaces (Claessen et al. 2003; Chapman et al. 2002; Larsen et al. 2007; Otoo et al. 2008; Rauceo et al. 2004; Gebbink et al. 2005). Our results indicate that a variety of proteins can form such structures at a particular cell surface. It may well be that only part of them (e.g. the chaplins, hydrophobins and repellents) form an amphipathic amyloid film, thus enabling aerial growth by lowering the water surface tension. The SSPs of *U. maydis* may not be able to do so, which would explain why aerial growth in the Δ*rep1* strain is strongly affected.

Twelve clusters are found in the genome of *U. maydis* that contain genes encoding SSPs (Kämper

et al. 2006). Deletion of five of them changed virulence (Kämper et al. 2006). Four of these clusters contain SSPs that are up-regulated in the Δ*rep1* strain (see Table 2). The symbiotic basidiomycete *Laccaria bicolor* also harbors a high number of genes encoding SSPs. Ten percent of the secreted proteins belong to this class, many of which are classified as SCRPs. A part of these genes is up-regulated during mycorrhizal interactions, whereas others are down-regulated. This suggests an important role for these proteins in the symbiosis (Martin et al. 2008). SCRPs of fungi and oomycetes have been described to function as apoplastic effectors (Kamoun 2006). Such effectors include toxins, elicitors, virulence and avirulence proteins. The cysteine residues have been proposed to enhance stability of the proteins and yield protection to plant proteases (Joosten et al. 1997; Luderer et al. 2002). Whether (some of) the *U. maydis* SSP and SCRP genes are also apoplastic effectors remains to be investigated.

The highest up-regulated gene in the Δ*rep1* strain is gene *um00792*. It encodes a SCRP of 144 amino acids with 7 cysteines. The gene is clustered in the genome with genes *um00793*, *um00794* and *um00795*. It has 28% sequence homology with gene *um00793*, which is also up-regulated in the SG200Δ*rep1* strain. Deletion of gene *um00792* has no influence on pathogenicity (Vraneš 2006) but its expression depends on *biz1*. This gene encodes a transcription factor, which functions at the stage of filament and appressorium formation (Flor-Parra et al. 2006). It down-regulates *clb1*, the mitotic cyclin, (Flor-Parra et al. 2006) and thereby causes G2 cell cycle arrest prior to plant invasion. Of interest, expression of *um06126, um01240, um01820* and *um01377* is also increased in the absence of repellents and decreased in the absence of Biz1 (This study; Vraneš 2006). The latter indicates a role for these genes in the early stages of pathogenic development.

Expression of gene *um00792* was followed using GFP as a reporter. Expression of this gene was detected in strains SG200 and SG200Δ*rep1* in aerial hyphae and hyphae growing in the substrate. Gene expression was not detected in yeast cells growing in liquid culture, but yeast cells growing in and on the solid substrate, where hyphal growth is initiated, did show fluorescence. Strikingly, over-expression of *um00792* was only detected in the Δ*rep1* strain in the layer of seeded cells that lay on the solid substrate. This layer contains many hyphae that seem unable to escape from the extracellular matrix. Possibly, the fungus senses absence of the repellents in the extracellular matrix, causing up-regulation of gene *um00792*. The protein encoded by this gene, however, is not able to complement repellents in formation of aerial hyphae (see above). Future studies should reveal the role of the SSPs and SCRPs that are up-regulated in *U. maydis* upon inactivation of the *rep1* gene. It may well be that the genes are redundant, requiring multiple knock-outs before a phenotype will be observed.

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