

1 **Identification and characterization of two transmembrane proteins** 2 **required for virulence of *Ustilago maydis***

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11 **Abstract**

12 Smut fungi comprise a large group of biotrophic phytopathogens infecting important crops such as
13 wheat and corn. Through the secretion of effector proteins, the fungus actively suppresses plant
14 immune reactions and modulates its host's metabolism. Consequently, how soluble effector proteins
15 contribute to virulence is already characterized in a range of phytopathogens. However, membrane-
16 associated virulence factors have been much less studied to date. Here, we investigated six
17 transmembrane (TM) proteins that show elevated gene expression during biotrophic development of
18 the maize pathogen *Ustilago maydis*. We show that two of the six proteins, named Vmp1 and Vmp2
19 (**v**irulence-associated **m**embrane **p**rotein), are essential for the full virulence of *U. maydis*. The deletion
20 of the corresponding genes lead to a substantial attenuation in the virulence of *U. maydis*. Furthermore,
21 both are conserved in various related smuts and contain no domains of known function. Our
22 biochemical analysis clearly shows that Vmp1 and Vmp2 are membrane-associated proteins,
23 potentially localizing to the *U. maydis* plasma membrane. Mass photometry and light scattering suggest
24 that Vmp1 mainly occurs as a monomer, while Vmp2 is dimeric. Notably, the large and partially
25 unstructured C-terminal domain of Vmp2 is crucial for virulence while not contributing to
26 dimerization. Taken together, we here provide an initial characterization of two membrane proteins as
27 virulence factors of *U. maydis*.

28 **1 Introduction**

29 An increasing number of infectious diseases are threatening agricultural and natural systems. This
30 development results in large crop losses, with up to 20 % of maize harvest loss caused by fungal
31 pathogens such as *Ustilago maydis* (Fisher et al. 2012). Despite the high number of fungal species
32 infecting plants, only a few fungal plant pathogen systems allow the physiological, molecular, and
33 biochemical investigation of both host and parasite (Dean et al. 2012; Giraldo and Valent 2013).
34 Among those, the smut fungus *U. maydis* represents an excellent case to study the infection process.
35 Smut fungi are a large group of biotrophic parasites with currently more than 1500 described species
36 infecting mostly grasses, including important cereal crops such as maize, wheat, barley, and sugar cane
37 (Zuo et al. 2019). The host of *U. maydis* is the sweet corn *Zea mays*, where it can infect all aerial parts
38 of the plant and establishes a biotrophic interface with its host cells.

39 Biotrophy implies the formation of a tight interaction zone between host and fungal intruder that allows
40 for the exchange of signals and nutrients without initiating apoptosis of host cell tissue. Biotrophic
41 pathogens need to maintain their respective host's viability in order to complete their life cycle.
42 Therefore, *U. maydis* suppresses defense responses, manipulates the metabolism of host cells, and
43 alters their proliferation-rate, ultimately leading to the formation of large spore-filled tumors in the
44 infected tissue (Zuo et al. 2019). The secretion of a variety of effector proteins plays a critical role
45 during this process (Lanver et al. 2017). Effector proteins can be grouped in apoplastic effectors, which
46 remain in the apoplastic space between plant and fungal cells, and cytoplasmic effectors that are further
47 translocated into the host cells' cytoplasm (Mueller et al. 2008).

48 This molecular warfare is not restricted to the apoplastic space or the cytosol of host cells. Instead,
49 pathogenic development and tumor formation are accompanied by a thorough remodeling of both plant
50 and fungal cell walls (Matei et al. 2018). These processes support fungal development as the
51 breakdown and import of carbohydrates derived from the host are important sources of carbon for the
52 fungus during growth (Sosso et al. 2019). Sugar sensing and its uptake have thus gained more attention
53 in *U. maydis* in recent years, leading to the identification of several transporters essential for virulence
54 (Wahl et al. 2010; Schuler et al. 2015). The genome of *U. maydis* encodes more than 19 sugar
55 transporters, and most of them are upregulated during pathogenic development (Sosso et al. 2019).
56 Consequently, plants have evolved mechanisms to detect and deplete apoplastic sugar concentrations
57 to hinder fungal growth and activate immune responses (Lemoine et al. 2013; Morkunas and Ratajczak
58 2014). While these examples are among the first transmembrane proteins studied in the infection
59 context, they also highlight the relevance of membrane-embedded proteins during virulent growth of
60 smut fungi.

61 However, there is little known on specialized membrane proteins involved in signaling, stimuli
62 recognition, and thus establishing a compatible interaction with the respective host plants. In one case,
63 the membrane protein Pit1, encoded within the pit (**protein important for tumors**) gene cluster, is
64 required for tumor formation (Doehlemann et al. 2011). It has been reported to localize to hyphal tips,
65 although the precise molecular function remains unclear.

66 Here, we have analyzed a set of six genes showing elevated expression levels during pathogenic
67 development of *U. maydis* (Lanver et al. 2018) encoding proteins that harbor predicted transmembrane
68 helices. Of those two show a strong attenuation in virulence upon deletion of their respective genes.
69 Therefore, we name these proteins Vmp1 and Vmp2 for virulence-associated **m**embrane **p**rotein and
70 present a biochemical characterization giving insights into their molecular architecture and suggesting
71 a potential role during virulence of *U. maydis*.

72 **2 Material and Methods**

73 *Molecular cloning of expression plasmids.* For the plasmid constructions, standard molecular cloning
74 strategies and techniques were applied (Sambrook J, Fritsch EF 1989). All plasmids and primers used
75 in this study are listed in tables **S1** and **S2**. For the overproduction of the C-terminal domain (CTD) of
76 Vmp2, the plasmid pEMGB1-*vmp2*_{CTD} was generated. The overproduced protein will be fused to the
77 solubility-tag GB1 (56 amino acids), including a hexahistidine tag (Huth et al. 1997). To do so, the
78 region encoding the Vmp2_{CTD} was amplified by PCR from genomic DNA of *U. maydis* SG200 and
79 inserted into the *NcoI/XhoI* sites of the vector pEMGB1. For the overproduction of the full-length
80 constructs, the genes encoding Vmp1 and Vmp2 were amplified from genomic DNA of *U. maydis*
81 SG200 without the signal peptide and subsequently ligated into the pEMstX1 vector using *BsaI*
82 restriction sites. The protein constructs will be fused to a Mistics-tag (110 amino acids), including a

83 hexahistidine tag (Roosild et al. 2005). In both plasmids, a tobacco etch virus (TEV) cleavage site is
84 located between expression tag and cloned gene.

85 *Strains, growth conditions, and plant infection assays.* The *Escherichia coli* strain Dh5 α (New England
86 Biolabs) was used for cloning purposes. The *E. coli* strain OverExpress™ C43 (DE3) (Sigma-Aldrich)
87 was used to express the full-length constructs of Vmp1 and Vmp2. The *E. coli* strain BL21 (DE3)
88 (Novagen) was used to express the CTD of Vmp2. *E. coli* strains were grown under constant shaking
89 in a temperature-controlled incubator. *Zea mays* cv. Early Golden Bantam (EGB, Urban Farmer,
90 Westfield, IN, USA) was used for infection assays with *Ustilago maydis* and grown in a temperature-
91 controlled greenhouse (light and dark cycles of 14 hours at 28 °C and 10 hours at 20 °C, respectively).
92 *U. maydis* strains used in this study are listed in table **S3**. *U. maydis* strains were grown in YEPS_{light}
93 medium (1 % (w/v) yeast extract, 0.4 % (w/v) peptone and 0.4 % (w/v) sucrose) and subsequently
94 adjusted to an OD₆₀₀ of 1.0 using sterile double-distilled water. For the infection of maize plants 500 μ l
95 of *U. maydis* cultures were injected into the stem of 7-day-old maize seedlings using a syringe as
96 described by Kämper and coworkers (Kämper et al. 2006).

97 *Gene knockout in U. maydis.* The plasmid pMS73 was digested with *Acc65I* to integrate the respective
98 sgRNA expression cassette via Gibson Assembly, according to Schuster and coworkers (Schuster et
99 al. 2018). The PCR obtained a double-stranded DNA fragment containing the respective target
100 sequences, scaffold, terminator, and the corresponding overlapping sequences. The fragments were
101 cloned into pMS73 yielding pFA001 and pFA003-pFA007 (**Tab. S1**). The target sequences (**Tab. S2**)
102 were designed using the E-CRISP tool (Heigwer, Kerr, and Boutros 2014). The inserts in all plasmids
103 were validated by sequencing.

104 *Generation of U. maydis complementation constructs.* To generate complementation strains of
105 SG200 Δ vmp1 and SG200 Δ vmp2, the constructs pFA511 and pFA512 were generated (**Tab. S1**).
106 Genomic DNA from *U. maydis* SG200 containing promoter and open reading frame (ORF) of the
107 respective gene was amplified by PCR using the primers listed in table **S2**. The amplified fragments
108 were introduced into the *KpnI/NotI* sites of plasmid p123 (Aichinger et al. 2003). Prior to
109 transformation, the plasmids were linearized using the restriction enzyme *SalI*.

110 *Generation of U. maydis strains.* The genes encoding the six putative transmembrane proteins were
111 disrupted in *U. maydis* SG200 using the CRISPR-Cas9 approach recently described for genetic
112 manipulation of *U. maydis* (Schuster et al. 2016). A donor DNA was supplied during transformation
113 to delete the respective ORF from the genome without further disruption of neighboring genes (**Fig.**
114 **S2**). Isolated *U. maydis* transformants were confirmed for deleting the respective genes by colony PCR
115 using the primers listed in table **S2** and sequencing (**Fig. S2**). To complement the phenotypes of
116 SG200 Δ vmp1 and SG200 Δ vmp2, plasmids pFA511 and pFA512 were integrated into the *ip* locus of
117 SG200. Isolated *U. maydis* transformants were confirmed by Southern-blot analysis to ensure single
118 integration events in the *ip* locus (Keon, White, and Hargreaves 1991).

119 *Production and purification of soluble Vmp2_{CTD}.* The CTD of Vmp2 was produced in *E. coli* BL21
120 (DE3) (Novagen). *E. coli* BL21 (DE3) was transformed with pFA508 to produce Vmp2_{CTD} fused to an
121 N-terminal GB1 tag including a hexahistidine tag. The protein production was performed in auto-
122 inductive Luria-Miller broth (Roth) containing 1 % (w/v) α -lactose (Roth). The cells were grown for
123 20 h at 30 °C and 180 rpm. The cultures were harvested by centrifugation (4,000 xg, 15 min, 4°C),
124 resuspended in HEPES buffer (20 mM HEPES, 200 mM NaCl, 20 mM KCl, 40 mM imidazole, pH
125 8.0), and subsequently disrupted using a microfluidizer (M110-L, Microfluidics). The cell debris was
126 removed by centrifugation (50,000 xg, 20 min, 4 °C). The supernatant was loaded onto Ni-NTA FF-

127 HisTrap columns (GE Healthcare) for affinity purification via the hexahistidine tag. The columns were
128 washed with HEPES buffer (10x column volume) and eluted with HEPES buffer containing 250 mM
129 imidazole. Prior to size exclusion chromatography (SEC), the GB1-tag was cleaved off by adding 0.8
130 mg purified TEV protease directly to the eluate and incubating under constant rotation at 20 °C for 3
131 hours. Cleaved His-tagged GB1 and remaining TEV protease were removed via a second Ni-NTA
132 purification after buffer exchange to HEPES buffer containing 40 mM imidazole using an Amicon
133 Ultra-10K centrifugal filter (Merck Millipore). The tag-free protein was subjected to SEC using a
134 Superdex S75 Increase 10/300 column equilibrated in HEPES buffer without imidazole and a pH of
135 7.5. The peak fractions were analyzed using a standard SDS-PAGE protocol, pooled, and concentrated
136 with Amicon Ultra-10K centrifugal filters.

137 *Production and Purification of membrane proteins.* The plasmids pFA659 and pFA670 encoding full-
138 length Vmp2 and Vmp1 were transformed in *E. coli* OverExpress™ C43 (DE3) (Sigma-Aldrich).
139 Transformants were grown in Terrific-Broth medium (24 g/l yeast extract, 20 g/l tryptone, 4 ml/l
140 glycerol, buffered with 10 % phosphate buffer pH 7.4 (0.17 M KH₂PO₄, 0.72 M K₂HPO₄)) under
141 constant shaking at 180 rpm and 37 °C to an OD₆₀₀ of 0.5 – 0.6. The cultures were then cooled to 20 °C,
142 induced with 0.2 M Isopropyl-β-D-thiogalactopyranosid (IPTG), and incubated for 20 h at 20 °C and
143 180 rpm. The cultures were harvested by centrifugation (4,000 xg, 15 min, 4 °C), resuspended in Tris-
144 buffer (50 mM Tris-Base, 300 mM NaCl, 40 mM imidazole, pH 8.0), and subsequently disrupted using
145 a microfluidizer (M110-L, Microfluidics). The cell debris was removed by centrifugation (8,000 xg,
146 20 min, 4 °C) and the supernatant was centrifuged (115,000 xg, 1 h, 4 °C) using a fixed-angle rotor (70
147 Ti, Beckmann) in an ultracentrifuge (Optima XPN-80, Beckmann). The pellet was resuspended in 10
148 ml Tris-Buffer using a Dounce-homogenizer (Carl Roth). The homogenized pellet was mixed with 10
149 ml Tris-Buffer containing either 2 % (w/v) Lauryldimethylamine-N-Oxide (LDAO) or 2 % (w/v)
150 Dodecyl-β-D-maltosid (DDM) for Vmp1 and Vmp2, respectively, and incubated for 2.5 h at 4 °C under
151 constant rotation. The solubilized membrane was again centrifuged (115,000 xg, 1 h, 4 °C). The
152 supernatant was loaded onto 1 ml Ni-NTA FF-HisTrap columns (GE Healthcare) for affinity
153 purification via the hexahistidine tag. The detergent concentration was lowered to 0.1 % (w/v) during
154 the Ni-NTA purification of both proteins. Prior to SEC, the Mistics-tag was cleaved off by adding 0.8
155 mg purified TEV directly to the eluate and incubating under constant rotation at 20 °C for 3 hours.
156 Cleaved His-tagged Mistics and remaining TEV protease were removed via a second Ni-NTA
157 purification after buffer exchange to Tris buffer containing 40 mM imidazole in an Amicon centrifugal
158 filter (Merck Millipore) with adequate cutoff. The protein was subjected to SEC using a Superdex 200
159 Increase 10/300 column equilibrated in HEPES-buffer (20 mM HEPES, 200 mM NaCl, 20 mM KCl,
160 pH 7.5) containing either 0.1 % (w/v) LDAO or 0.03 % (w/v) DDM for Vmp1 and Vmp2, respectively.
161 The peak fractions were analyzed using a standard SDS-PAGE protocol, pooled, and concentrated with
162 appropriate Amicon centrifugal filters.

163 *Multi-angle light scattering (MALS).* Multi-angle light scattering coupled size-exclusion
164 chromatography (SEC-MALS) was performed using an Äkta PURE system (GE Healthcare) with a
165 Superdex 200 Increase 10/300 column attached to a MALS detector 3609 (Postnova Analytics) and a
166 refractive index detector 3150 (Postnova Analytics). The column was equilibrated with 0.2 μm filtered
167 HEPES buffer (20 mM HEPES, 200 mM NaCl, 20 mM KCl, pH 7.5) containing either 0.1 % (w/v)
168 LDAO or 0.03 % (w/v) DDM for Vmp1 and Vmp2, respectively. For each measurement, 100 μl of a
169 50 μM protein solution was injected.

170 *Mass photometry (MP).* Mass photometry experiments were performed using a OneMP mass
171 photometer (Refeyn Ltd, Oxford, UK). Data acquisition was performed using AcquireMP (Refeyn Ltd.
172 v2.3). Mass photometry movies were recorded at 1 kHz, with exposure times varying between 0.6 and

173 0.9 ms, adjusted to maximize camera counts while avoiding saturation. Microscope slides (70 x 26
174 mm) were cleaned 5 minutes in 50 % (v/v) isopropanol (HPLC grade in Milli-Q H₂O) and pure Milli-
175 Q H₂O, followed by drying with a pressurized air stream. Silicon gaskets to hold the sample drops were
176 cleaned in the same manner fixed to clean glass slides immediately prior to measurement. The
177 instrument was calibrated using NativeMark Protein Standard (Thermo Fisher) immediately prior to
178 measurements. Immediately prior to mass photometry measurements, protein stocks were diluted
179 directly in HEPES buffer. Typical working concentrations of Vmp1 and Vmp2 were 25-50 nM for the
180 actual measurement. Each protein was measured in a new gasket well (i.e., each well was used once).
181 To find focus, 18 µl of fresh room temperature buffer was pipetted into a well, the focal position was
182 identified and locked using the autofocus function of the instrument. For each acquisition, 2 µL of
183 diluted protein was added to the well and thoroughly mixed. The data were analyzed using the
184 DiscoverMP software.

185 *Confocal light microscopy.* The proliferation of *U. maydis* in infected maize leaf tissue was visualized
186 by confocal microscopy as described previously (Tanaka et al. 2014). A leaf area of 1 cm² located 2
187 cm below the injection site was excised 2 days post-infection (dpi). The leaf samples were destained
188 with ethanol and treated with 10 % (w/v) potassium hydroxide at 85°C for 4 h. The fungal hyphae were
189 stained with Wheat Germ Agglutinin-Alexa Fluor 488 (WGA-AF488, Invitrogen). The plant cell walls
190 were stained with propidium iodide (Sigma-Aldrich) by incubating decolorized samples in staining
191 solution (1 µg/ml propidium iodide, 10 µg ml⁻¹ WGA-AF488) and observed with a TCS-SP8 confocal
192 laser-scanning microscope (Leica Microsystems) under the following conditions: WGA-AF488:
193 excitation at 488 nm and detection at 500–540 nm; propidium iodide: excitation at 561 nm and
194 detection at 580–660 nm.

195 *Fungal stress assays.* Fungal strains were grown in YEPS_{light} medium (1 % (w/v) yeast extract, 0.4 %
196 (w/v) peptone and 0.4 % (w/v) sucrose) to an OD₆₀₀ of 1.0. The cells were pelleted and resuspended in
197 sterile double distilled H₂O to an OD₆₀₀ 0.1. For the induction of filament formation, 10 µl of serial
198 dilutions were spotted on potato-dextrose charcoal plates (Holliday 1974). The stress assays were
199 performed on CM plates (Holliday 1974) supplemented with 750 µM calcofluor white (Sigma-
200 Aldrich), 3 mM hydrogen peroxide (H₂O₂), 1 M NaCl or 1 M sorbitol. Images were taken after over-
201 night incubation at 28 °C.

202 *Statistical analysis.* Disease symptoms of infected plants were scored at 12 dpi using the previously
203 established scoring scheme by Kämper and colleagues (Kämper et al. 2006). Disease symptoms were
204 quantified based on three biological replicates and are presented as stacked histograms. Significant
205 differences among disease symptoms within individual disease categories were determined by
206 Student's t-test. The raw data of all infection assays and the statistical analysis can be found table S5.

207 *Accession numbers.* The genes and encoding protein sequences from *U. maydis* are available at NCBI
208 under the following accession numbers.: *vmp1* (UMAG_00032), XP_011386009.1; *vmp2*
209 (UMAG_01689), XP_011387666.1; UMAG_01713, XP_011387687.1; UMAG_04185,
210 XP_011390672.1; UMAG_10491, XP_011390314.1; UMAG_03474, XP_011389930.1.

211 **3 Results**

212 *Identification of membrane proteins critical for pathogenic development of U. maydis.*

213 To identify membrane proteins that show an increase in transcript abundance during infection stages
214 associated with biotrophic development of *Ustilago maydis*, we analyzed the transcriptomic data
215 obtained by Lanver and coworkers (Lanver et al. 2018). Highly upregulated protein-encoding genes

216 were then examined for the presence of potential transmembrane helices (TMs) using the Consensus
217 Constrained TOPology prediction web server CCTOP (**Fig. S1**) (Dobson, Reményi, and Tusnády
218 2015). By this approach, we could identify six genes strongly elevated during infection and their
219 respective proteins containing at least one predicted TM. They show their strongest expression two to
220 four days post-infection while not induced in axenic culture under non-infective conditions (**Fig. 1A**).
221 These proteins are UMAG_00032, UMAG_01689, UMAG_01713, UMAG_03474, UMAG_04185,
222 and UMAG_10491.

223 To evaluate these protein's impact on virulence, we deleted their respective genes in the solopathogenic
224 *U. maydis* strain SG200 (Kämper et al. 2006). The gene deletion was performed using a CRISPR-Cas9-
225 based approach as described by Schuster and coworkers (Schuster et al. 2016). A donor DNA was
226 supplied to delete the respective open reading frames (ORF's) from the genome while keeping the
227 surrounding genetic environment intact (**Fig. S2**). The deletion of four genes resulted in a wildtype-
228 like behavior during maize infection experiments (*UMAG_01713*, *UMAG_03474*, *UMAG_04185*, and
229 *UMAG_10491*), the two other genes (*UMAG_00032* and *UMAG_01689*) lead to attenuation in
230 virulence (**Fig. 1B**). To investigate whether the differences in phenotypical symptoms between the
231 deletion strains of *UMAG_00032*, *UMAG_01689*, and SG200 are significant, we scored the disease
232 symptoms of each infected plant using a previously established scoring scheme (Kämper et al. 2006).
233 For the significance-analysis, we performed a two-sided Student's t-test. Our analysis for each category
234 confirmed that the differences are significant, with p-values below 0.05 for several categories (**Tab.**
235 **S5**).

236 Our results reveal two TM proteins that strongly impact the virulence of *U. maydis* during maize
237 infection. Therefore, we named both genes *vmp1* (*UMAG_00032*) and *vmp2* (*UMAG_01689*) for
238 virulence-associated membrane protein 1 and 2.

239 *Vmp1 and vmp2 are conserved among related smut species*

240 *Vmp1* encodes a protein of 142 amino acids (aa), whereas *vmp2* encodes a 335 aa long protein. Both
241 proteins contain an N-terminal signal peptide (SP) of 25 aa, as predicted by SignalP-5.0 (Almagro
242 Armenteros et al. 2019). Our *in silico* analyses indicate that both proteins harbor one TM helix spanning
243 the residues 60 to 77 in *Vmp1* and residues 100 to 115 in *Vmp2* (**Fig. S1**). The N-terminal domain
244 (NTD) of both proteins is predicted to be extracellular (**Fig S1**).

245 In a next step, we analyzed the genetic context of both proteins in *U. maydis* and compared it to related
246 smut fungi. Using the Basic Local Alignment Search Tool (BLAST) we identified *Vmp1* orthologs in
247 the genomes of *Pseudozyma hubeiensis* SY62, *Kalmanozyma brasiliensis* GHG001, *Sporisorium*
248 *reilianum* SRZ2, *Ustilago trichophora*, *Sporisorium scitamineum*, *Moesziomyces antarcticus*,
249 *Moesziomyces aphidis* DSM 70725, and *Testicularia cyperi* with identities ranging from 58 % to 34 %
250 (determined by CLUSTAL2.1) (**Fig. S3A**). However, it was absent in *Ustilago hordei* or *Ustilago*
251 *bromivora* with the genetic context being similar to *U. maydis* (**Fig. 2A**). A protein related to *Vmp1*
252 was also identified in the genome of *T. cyperi* a pathogen of *Rhynchospora* spp. (Kijpornyongpan et
253 al. 2018). The genetic context showed differences to the closely related species due to the ancestral
254 nature of *T. cyperi* (**Fig. 2A**).

255 The neighboring genes encode a proline dehydrogenase (*UMAG_00030*), a TM protein of unknown
256 function (*UMAG_00031*), a Zn₂-C6 fungal-type transcription factor (*UMAG_10009*), and a putative
257 MFS transporter (*UMAG_00034*) (**Fig. 2A**). These genes are also induced during axenic growth and
258 might thus not be directly related to virulence. However, *UMAG_00034* shows elevated transcript

259 levels between 24 h and 48 h post-infection while not induced during axenic growth (Lanver et al.
260 2018).

261 We also identified orthologs of Vmp2 in a variety of related smut fungi (**Fig. S3B**). Namely, *P.*
262 *hubeiensis* SY62, *U. bromivora*, *Sporisorium graminicola*, *S. reilianum* SRZ2, *U. hordei*, *K.*
263 *brasiliensis* GHG001, *U. trichophora*, *M. antarcticus*, *S. scitamineum*, and *T. cyperi*. Here, the
264 sequence identities ranged from 43 % to 36 % (**Fig. S3B**). Notably, Vmp2 is highly conserved from
265 amino acid 82 to 195 (within the Vmp2 sequence from *U. maydis*), while the C-terminus shows a
266 higher degree of deviation in the investigated orthologs (**Fig. S3B**). In Ustilaginaceae, the loci of *vmp2*
267 are similarly to *vmp1* highly syntenic although the intergenic region towards *UMAG_01690* and its
268 orthologs shows some length differences (**Fig. 2B**). The neighbouring genes include an OBG-type G-
269 domain-containing protein (*UMAG_01687*), a putative nuclear transport factor (*UMAG_01688*), a
270 secreted effector protein of unknown function (*UMAG_01690*) and a DNA helicase (*UMAG_01691*)
271 (**Fig. 2B**).

272 *Vmp1 hinders fungal infection after penetration of the plant epidermis*

273 The *vmp1* deletion strain showed the strongest reduction in virulence with tumor formation being
274 entirely abolished in infected plants (**Fig. 3A, B**). Anthocyanin production was observed in the vicinity
275 of the infection site, a universal sign of infections and thus the presence of infectious hyphae (Tanaka
276 et al. 2014). The deletion strain SG200Δ*vmp1* was complemented by integrating a single copy of *vmp1*
277 into the *ip* locus (SG200Δ*vmp1*-*vmp1*, **Fig. 3A**). The complementation did not fully restore
278 SG200Δ*vmp1*, leading mainly to the formation of smaller tumors and larger ones only to a lesser extent
279 (**Fig. 3A, B**). Thus, we wanted to know whether SG200Δ*vmp1* remains able to grow inside vascular
280 bundles and elicits a plant defense response or whether fungal growth is arrested after penetration of
281 the epidermal layer.

282 To detect differences in host colonization, we visualized fungal hyphae by staining with WGA-AF488
283 at 2 and 6 days post-infection (dpi) (**Fig. 3C**). It became apparent that SG200Δ*vmp1* has a reduced
284 number of fungal hyphae on the plant leaf surface combined with less proliferation (**Fig. 3C**). However,
285 hyphae could still penetrate the epidermal layer and grow inside the vascular bundles (**Fig. 3C**). Fungal
286 growth was seemingly arrested at this stage as the amount of fungal material inside the plant leaves
287 was not drastically increased at 6 dpi (**Fig. 3C**). To rule out that the reduced virulence was due to
288 reduced growth and stress sensitivity, we grew SG200Δ*vmp1* in the presence of NaCl, sorbitol,
289 calcofluor white, and H₂O₂. However, mutant strains were indistinguishable from SG200 (**Fig. S4**).

290 In conclusion, we show that the TM protein encoded by *vmp1* is essential for full virulence and might
291 be important for establishing the biotrophic interface. It is conserved among related smut fungi (**Fig.**
292 **S3A**) indicating that its function might also be conserved among these relatives.

293 *Vmp2 leads to reduced tumor formation*

294 The deletion of *vmp2* led to a strong reduction in virulence of *U. maydis*, with solely small tumors
295 being formed (**Fig. 4A**). We complemented SG200Δ*vmp2* by integrating a single copy of *vmp2* into
296 the *ip* locus (SG200Δ*vmp2*-*vmp2*, **Fig. 4A, B**). This complementation could fully restore the phenotype
297 of SG200Δ*vmp2*. To rule out that the deletion of *vmp2* leads to altered growth of *U. maydis* under
298 stress conditions, we grew SG200Δ*vmp2* in the presence of NaCl, sorbitol, calcofluor white and H₂O₂
299 and did not detect differences from SG200 (**Fig. S4**).

300 In the next step, we aimed to understand how deleting the two predicted soluble domains would impact
301 the function of Vmp2 *in vivo* (**Fig. S1**). We generated two constructs deleting either the predicted
302 extracellular NTD or the cytosolic CTD and transformed *U. maydis* SG200 to perform infection assays
303 (**Fig. S2D**). Our experiments show that SG200vmp2 Δ CTD phenocopies SG200 Δ vmp2, while
304 SG200vmp2 Δ NTD is less attenuated in virulence (**Fig. 4A, B**).

305 Taken together, we can show that *vmp2* is an essential player for the infection process in *U. maydis*
306 and potentially related organisms. Additionally, our infection experiments indicate that the CTD of
307 Vmp2 is important for full virulence.

308 *Vmp1 shows concentration-dependent oligomerization*

309 To allow for a biochemical investigation of Vmp1, we cloned the open reading frame without the signal
310 peptide (residues 1-20) for heterologous protein production in *E. coli* (see materials & methods). First
311 expression and solubility tests did not allow to purify the full-length protein in amounts sufficient for
312 biochemical analysis. Thus, we generated a construct that includes an N-terminal Mstics-tag (MstX)
313 separated by a TEV protease cleavage site. This 110 amino acid long protein tag forms four
314 transmembrane helices and inserts autonomously in the membrane. It has been used to improve the
315 expression of membrane proteins in several cases (Roosild et al. 2005). In our case, the production of
316 MstX-Vmp1 was drastically enhanced compared to protein production without the fusion-tag.
317 Attempts to solubilize MstX-Vmp1 from the membrane fraction using Dodecyl- β -D-maltosid (DDM)
318 failed and thus we tested a variety of commercially available detergents. Solubilization was only
319 achieved employing Lauryldimethylamine-N-Oxide (LDAO). Notably, all attempts to cleave the MstX
320 tag via TEV cleavage only resulted in inefficient and partial cleavage. It is likely that the spacing
321 between the membrane-embedded MstX and the membrane spanning helix within Vmp1 (residues 60
322 – 77) might not allow for a proper TEV recognition and cleavage. Consequently, we used the full-
323 length fusion protein for biochemical analysis.

324 Purified MstX-Vmp1 was subjected to size-exclusion chromatography coupled multi-angle light
325 scattering (SEC-MALS) using a Superdex 200 Increase 3.2/300 column equilibrated with SEC buffer
326 including 0.1% LDAO (see materials & methods). The protein eluted in a single peak at 1.62 ml
327 corresponding to 90 kDa according to the calibration calculation for this column (**Fig. 5A**). Our
328 analysis with MALS and refractive index resulted in a mass of 113 ± 17 kDa and thus yielded a slightly
329 higher molecular weight (**Fig. 5A**). The calculated mass of the MstX-Vmp1 fusion protein is around
330 31 kDa. MALS allowed us to clearly distinguish between empty micelles and the membrane protein-
331 detergent complexes. Notably, the molecular weight of free LDAO micelles was found to be 40 ± 5
332 kDa in our experiments and thus a bit larger than 16-20 kDa reported in literature (Timmins et al.
333 1988). As membrane proteins are likely not embedded into detergent micelles but rather form
334 membrane protein-detergent complexes (Chaptal et al. 2017), our results indicate that two or three
335 Vmp1 molecules would be encaged by LDAO detergent molecules.

336 To achieve a better resolution of Vmp1 oligomerization, we employed mass photometry, a method that
337 became recently available and allows rapid and reliable determination of the dynamic molecular weight
338 of macromolecules in solution (Olerinyova et al. 2021; F et al. 2020). We firstly used a final
339 concentration of 25 nM MstX-Vmp1 for mass photometric analysis which was achieved by rapid 1:10
340 dilution of a 250 nM solution into SEC buffer without detergent. Approximately 60 % of MstX-Vmp1
341 had a measured mass of 42 kDa (**Fig. 5B**) suggesting a monomer of MstX-Vmp1 and ~50 LDAO
342 detergent molecules (11 kDa). A subfraction higher molecular weight assemblies was also visible,
343 however gaussian fitting was not possible at this concentration. When using 50 nM of MstX-Vmp1, a

344 second gaussian could be fitted additionally to the 60 % of molecules with a mass of 42 kDa indicating
345 the presence of a 118 kDa species containing 20 % of all molecules (**Fig. 5C**). To rule out that no
346 empty LDAO micelles were detected, we subjected a buffer containing no protein and only LDAO at
347 the working concentration of 0.01% to mass photometry. However, no events were detectable
348 suggesting that micelles are not formed at this detergent concentration.

349 Taken together, we conclude that Vmp1 mainly occurs as a monomer but forms higher oligomeric
350 species at higher concentrations.

351 *Vmp2 is a dimeric membrane protein*

352 In a next step, we aimed to investigate Vmp2 after heterologous protein production in *E. coli*. Similar
353 to Vmp1, the expression of full-length Vmp2 was insufficient for biochemical analyses and only the
354 fusion of an N-terminal Mistics-tag allowed to obtain adequate amounts of membrane-bound protein.
355 Vmp2 could be solubilized with DDM and was purified using a Superose 6 Increase 10/300 column
356 (GE Healthcare) equilibrated with SEC buffer and 0.03 % DDM (see materials & methods). The
357 protein eluted at 17.22 ml corresponding to a molecular weight of approx. 83 kDa (**Fig. 6A**).

358 We again employed mass photometry to accurately determine the molecular weight of Vmp2 and
359 investigate whether different oligomeric species might be visible even at nanomolar concentrations.
360 However, using DDM as detergent, 0.03 % (w/v), which is equivalent to 600 μ M and thus generated a
361 strong detergent background that did not allow us to distinguish between empty micelles and Vmp2.
362 Thus, we investigated whether Vmp2 would be stable in LDAO or lauryl maltose neopentyl glycol
363 (LMNG), a detergent that contains two DDM moieties and has a very low CMC at 10 μ M which is
364 perfectly suited for mass photometry. We thus solubilized Vmp2 using DDM and exchanged the
365 detergent during Ni-ion affinity chromatography and applied the protein to a Superose 6 Increase
366 10/300 column equilibrated in 0.1 % LDAO or 0.001 % LMNG, respectively.

367 Firstly, Vmp2 purified in the presence of 0.1 % LDAO was measured (**Fig. 6B**). To remove excess
368 detergent micelles during mass photometry, a stock solution at 1 μ M of Vmp2 was rapidly diluted 1:10
369 in SEC buffer without detergent. A Gaussian fit of the peak fraction contained 92 % of all measured
370 molecules at a MW of 81 kDa. In a second approach, we used Vmp2 solubilized in 0.001 % LMNG
371 and again rapidly diluted it 1:10 in SEC buffer containing no detergent. Here, we could fit 84 % of all
372 counts resulting in a MW of approximately 94 kDa (**Fig. 6C**). The mass differences between the LDAO
373 and LMNG solubilized Vmp2 likely is a result from the different protein-detergent complex sizes
374 formed by the two detergent molecules. As Vmp2 has a theoretical molecular weight of 32 kDa, the
375 81 kDa would correspond to a dimer of Vmp2 and ~75 LDAO (17 kDa) detergent molecules, while
376 the 94 kDa suggest a Vmp2 dimer and ~30 LMNG (30 kDa) detergent molecules.

377 In summary, our mass photometry results are in agreement with the MW calculated from size exclusion
378 chromatography and indicate the presence of a Vmp2 dimer.

379 *The CTD of Vmp2 is largely unstructured and does not contribute to dimerization*

380 Next, we investigated the predicted cytosolic CTD of Vmp2. We subjected purified Vmp2_{CTD} to a
381 Superdex 75 Increase 10/300 column. The protein eluted at 9.28 ml which corresponds to a molecular
382 weight of 45 kDa (**Fig. 6D**). However, multi-angle light scattering coupled SEC (SEC-MALS)
383 unambiguously revealed a MW of $25 \pm 1,5$ kDa of Vmp2_{CTD} (**Fig. 5D**). Our secondary structure and
384 disorder prediction through PSIPRED indicated that residues 200 to 335 are potentially disordered
385 (**Fig. S5**). As disordered or non-globular proteins show a different migration behavior than the SEC-

386 standard, this would explain the discrepancy between SEC and MALS MW calculation. In conclusion,
387 we can show that Vmp2 is dimeric membrane protein with a CTD that is largely unstructured and does
388 not contribute to dimerization.

389 4 Discussion

390 In this study, we have identified six genes that are strongly induced between 0.5 and 2 days post-
391 infection (dpi) and remain upregulated until 12 dpi (**Fig. 1A**), while not being expressed in axenic
392 culture. This expression pattern correlates with establishing and maintaining biotrophy, a critical
393 feature of pathogenic development in smut fungi (Lanver et al. 2018). Our *in silico* analysis suggested
394 that all of them harbor at least one transmembrane spanning helix, rendering them interesting targets
395 as proteins associated with virulence in smut fungi are predominantly soluble effectors (Lanver et al.
396 2017). The deletion of two of them, subsequently named Vmp1 and Vmp2 (virulence associated
397 **membrane protein**), resulted in a strong attenuation of virulence during maize infection, while growth
398 of the deletion strains was neither affected in axenic liquid culture nor in the presence of various stress
399 causing agents (**Fig. S4**). We can thus conclude that both Vmp1 and Vmp2 are important during
400 pathogenic but not axenic growth of *U. maydis*. Attempts to reveal a potential function of these TM
401 proteins by the prediction of functional domains yielded no results for Vmp1 and Vmp2 using the
402 DomPred server embedded in the PSIPRED algorithm (Buchan and Jones 2019).

403 To shed light on the function of Vmp1, we inspected the deletion strains in more detail. Deletion of
404 Vmp1 led to a strong attenuation of fungal growth that was arrested after epidermal penetration (**Fig.**
405 **2C**) although some hyphae were still visible growing inside vascular bundles. Notably, tumor
406 formation on maize leaves inoculated with *vmp1* mutant strains was not observed in infection
407 experiments. Vmp1 thus plays a critical role during the early infection stages. Notably, *vmp1* mutant
408 strains still elicited a plant defense response as anthocyanin production could still be observed on
409 infected plant leaves.

410 Our biochemical analysis suggested that Vmp1 predominantly occurs as a monomer (**Fig. 5B**) as the
411 cellular concentrations of Vmp1 will most likely be low. This is further supported by the gene
412 expression data as *vmp1* shows the lowest expression of all six transmembrane protein encoding genes
413 investigated (**Fig. 1A**). During investigation of the genomic context of *vmp1*, it became apparent that
414 the gene *UMAG_00031* is found in the same orientation upstream of *vmp1* in several related species.
415 A recent study demonstrated that *UMAG_00031* encodes a putative transmembrane protein potentially
416 involved in pH regulation (Cervantes-Montelongo et al. 2020). In contrast to *SG200Δvmp1*,
417 *UMAG_00031* mutant strains showed reduced growth under pH stresses as well as in the presence of
418 sorbitol and NaCl (Cervantes-Montelongo et al. 2020). The study suggested *UMAG_00031* to be a
419 member of the Pal/Rim pathway in *U. maydis*, a widely conserved signaling pathway involved in pH
420 adaptation (Selvig and Alspaugh 2011; Fonseca-García, León-Ramírez, and Ruiz-Herrera 2012).
421 However, our data indicate that Vmp1 is most likely not directly involved in pH adaptation or
422 regulation. It might still play an accessory role in these processes serving e.g. as adaptor protein. Here,
423 future research might identify a connection towards pH related regulation to during plant infection.

424

425 Vmp2 (UMAG_01689) has already been identified to contribute to virulence in *U. maydis* (Uhse et al.
426 2018). In their study, the authors also showed that the fungal biomass is strongly reduced in infected
427 plant leaves. However, as the knockout was only delivered as a proof-of-concept of their method to
428 identify genes essential for virulence, no further information on Vmp2 was provided. Our data confirm
429 the phenotype observed by Uhse and coworkers (**Fig. 4A**). Furthermore, we can show that Vmp2 has
430 a short N-terminal (NTD) and a long C-terminal domain (CTD). While deletion of the CTD
431 phenocopies SG200Dvmp2, strains deleted for the NTD cause slightly more severe symptoms on
432 infected plants. This suggests that the CTD is indispensable for virulence. Sequence alignments to
433 homologs from other smut fungi show that the C-termini is highly variable, while the region
434 surrounding the membrane spanning helix is conserved (**Fig. S3**). Our analysis by SEC-coupled MALS
435 confirmed that the CTD is largely unstructured. Proteins containing unstructured regions have been
436 characterized in the context of many scenarios and can make up substantial amounts of the total protein
437 content (Van Der Lee et al. 2014). A possible scenario is that the unstructured region of Vmp1 becomes
438 ordered in the context of an interaction partner. Here, the sequence variability in related organisms
439 suggests that this interface is species-specific. Another possible explanation might be that the
440 unstructured domain is involved in membrane shaping or impacts the local membrane heterogeneity
441 (Fakhree, Blum, and Claessens 2019). A thorough investigation of the interactome of Vmp2 *in planta*
442 might deliver an explanation for the role of CTD of Vmp2 during maize infection of *U. maydis*.

443

444 In conclusion, we here present two membrane proteins that act as virulence factors during maize
445 colonization of *U. maydis*. While we deliver an initial characterization of the two proteins expanding
446 the current knowledge on virulence associated membrane proteins of smut fungi, future research needs
447 to address their precise functions.

448 **5 Conflict of Interest**

449 The authors declare that the research was conducted in the absence of any commercial or financial
450 relationships that could be construed as a potential conflict of interest.

451 **6 Author Contributions**

452 F.A. conceived of the project and designed the study. F.A. and P.W. performed experiments,
453 analysed data and wrote the paper.

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460 subsequent assistance with data collection.

461 **9 Contribution to the Field Statement**

462 Fungal phytopathogens are an increasing threat to important crops such as wheat, sugar cane, and
463 maize. The smut fungi *Ustilago maydis* specifically infects the sweet corn *Zea mays* and leads to
464 annual crop losses of up to 20 %. The pathogen needs to establish a biotrophic interaction with its
465 host in order to gain access to valuable resources for life cycle completion. Maintaining this tight
466 interaction, without triggering any immune responses of the host is mainly accomplished by the
467 secretion of a variety of effector proteins in the apoplastic space between host and parasite. Many of
468 those soluble effector proteins have recently been described to be involved in the attenuation of
469 defense mechanisms and the manipulation of host cell metabolism. However, we still don't fully
470 understand the underlying principles of important processes such as cellular host recognition and
471 attachment, the endosomal transport of proteins to the apoplastic space and host cells, or the uptake
472 of signals and nutrients. Membrane-bound proteins with elevated expression during the pathogenic
473 development of *U. maydis* are highly likely to be involved in these processes and should therefore
474 attract more attention in this research field. By delivering an initial characterization of two membrane
475 proteins required for virulence, we highlight the importance of membrane proteins for understanding
476 the infection process of *U. maydis*.

477 10 References

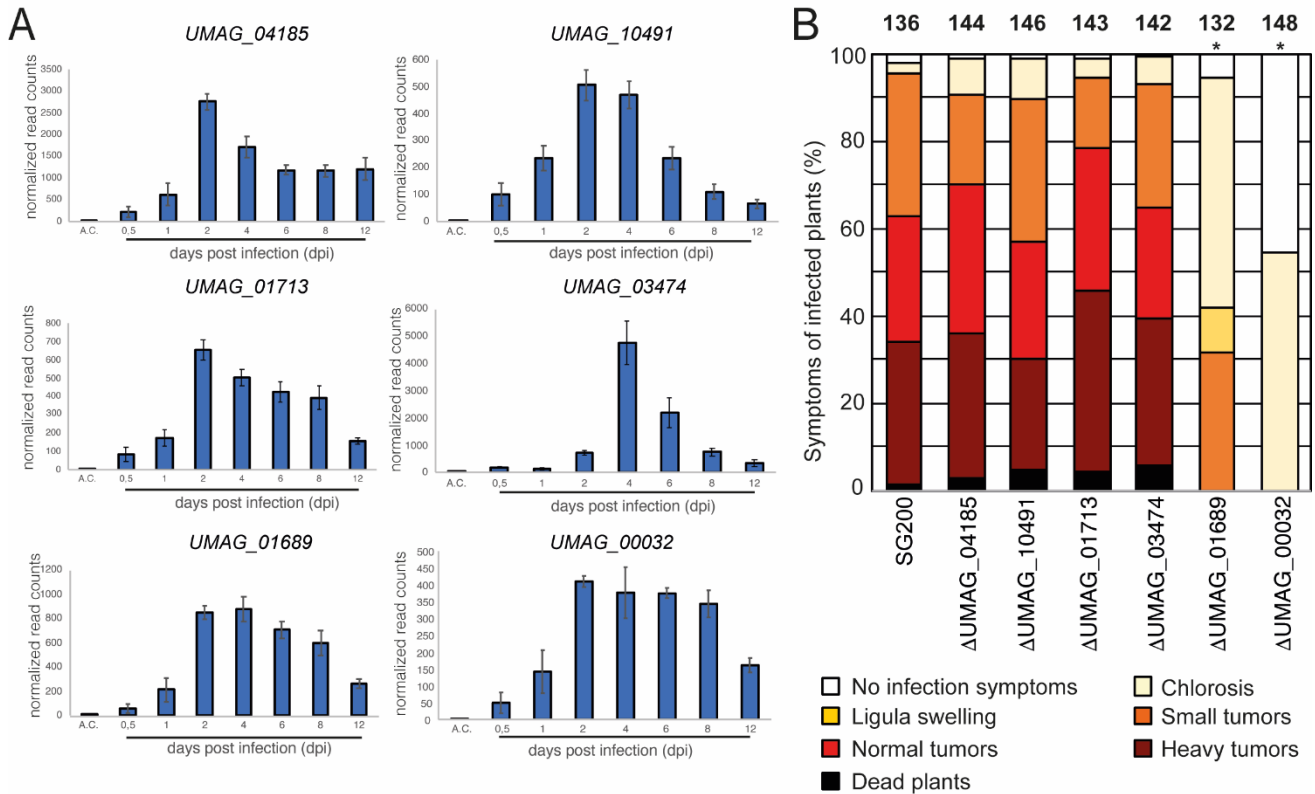
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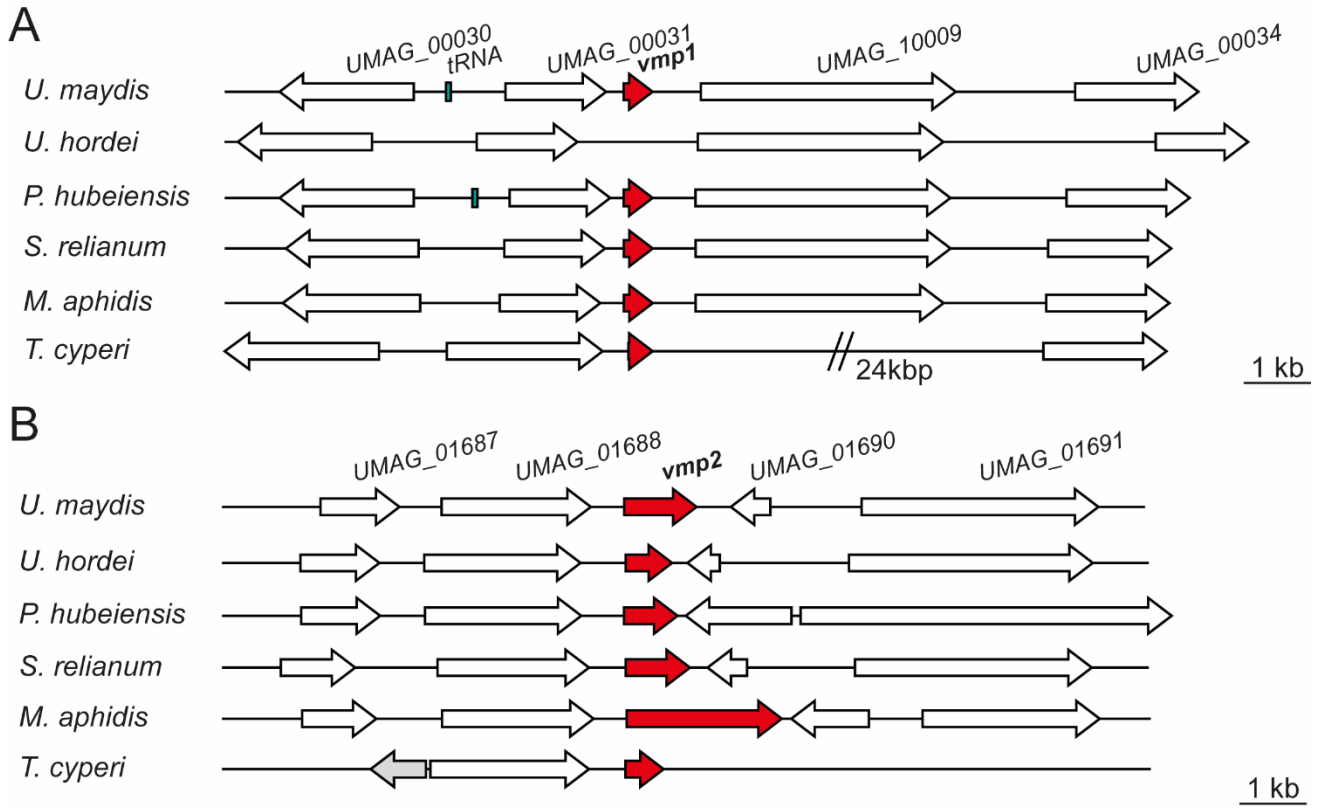
607 **Figures**



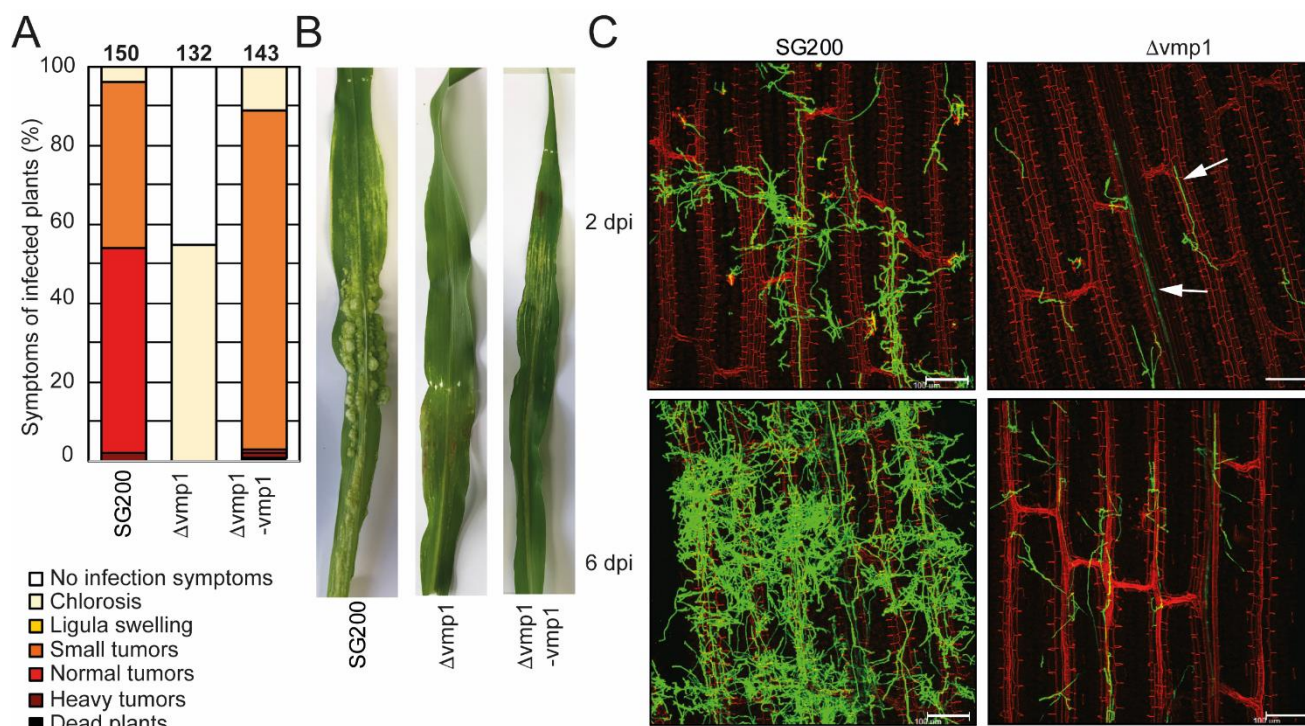
608

609 **Figure 1. Identification of a transmembrane protein important for virulence.** **A.** The expression
 610 pattern of genes encoding transmembrane proteins in *U. maydis* during plant infection re-analyzed
 611 from RNA sequencing data (Lanver et al. 2018). A.C., expression level in axenic culture. The
 612 numbers below the bars indicate the days post inoculation (dpi). Error bars indicate \pm SD. **B.**
 613 Virulence assay of genes encoding transmembrane proteins in the *U. maydis* SG200 background.
 614 Disease symptoms were quantified on maize leaves 12 days post infection. Similar results were
 615 observed in three independent experiments. Shown is the mean percentage of plants placed in a
 616 particular disease category. The number of infected plants is indicated above the bars. The asterisk
 617 indicates a significant difference in infection symptoms between SG200, SG200 Δ vmp1 and
 618 SG200 Δ vmp2.

Membrane-bound virulence factors

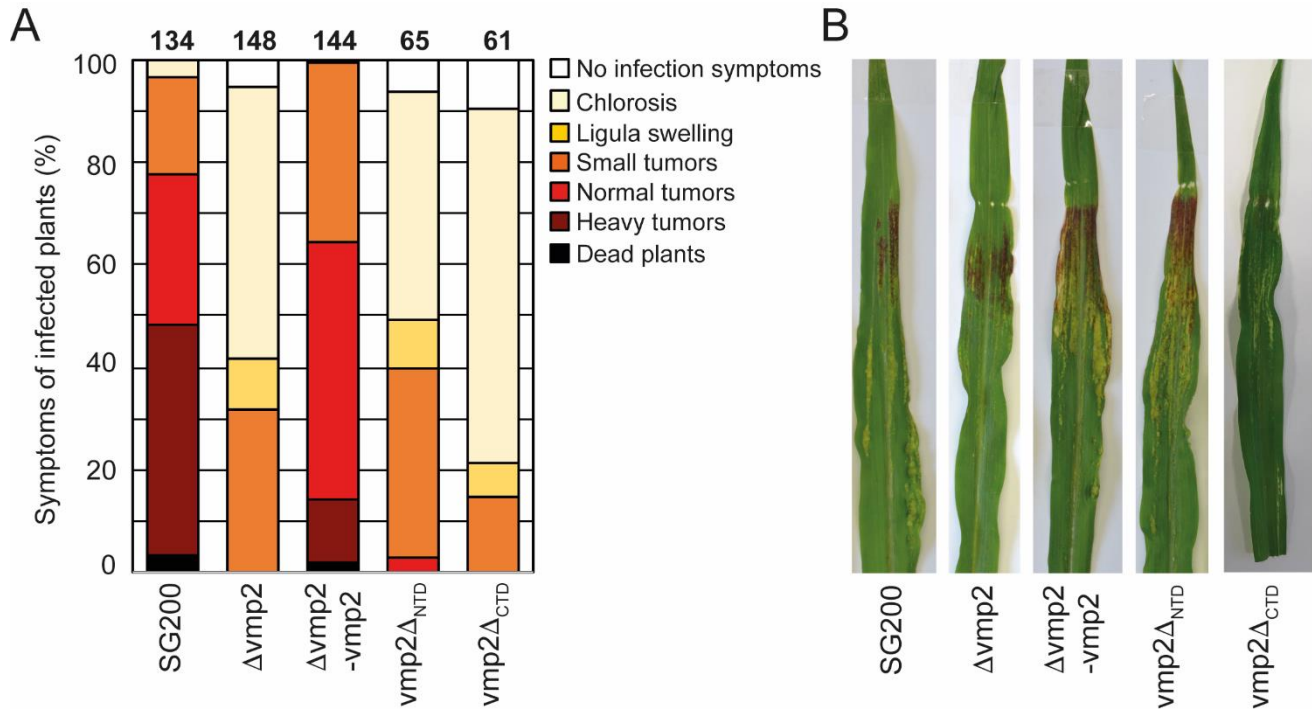


620 **Figure 2. Vmp1 and Vmp2 orthologs are conserved in related smut fungi.** Schematic picture of
 621 gene loci encoding Vmp1 (A) and Vmp2 (B) and orthologs in the related smut pathogens *Ustilago*
 622 *hordei*, *Pseudozyma hubeiensis*, *Sporisorium relianum*, *Moesziomyces aphidis* and *Testicularia*
 623 *cyperi*. White arrows indicate genes found in all of the respective species, while the grey gene was
 624 solely present in the genome of *T. cyperi*.



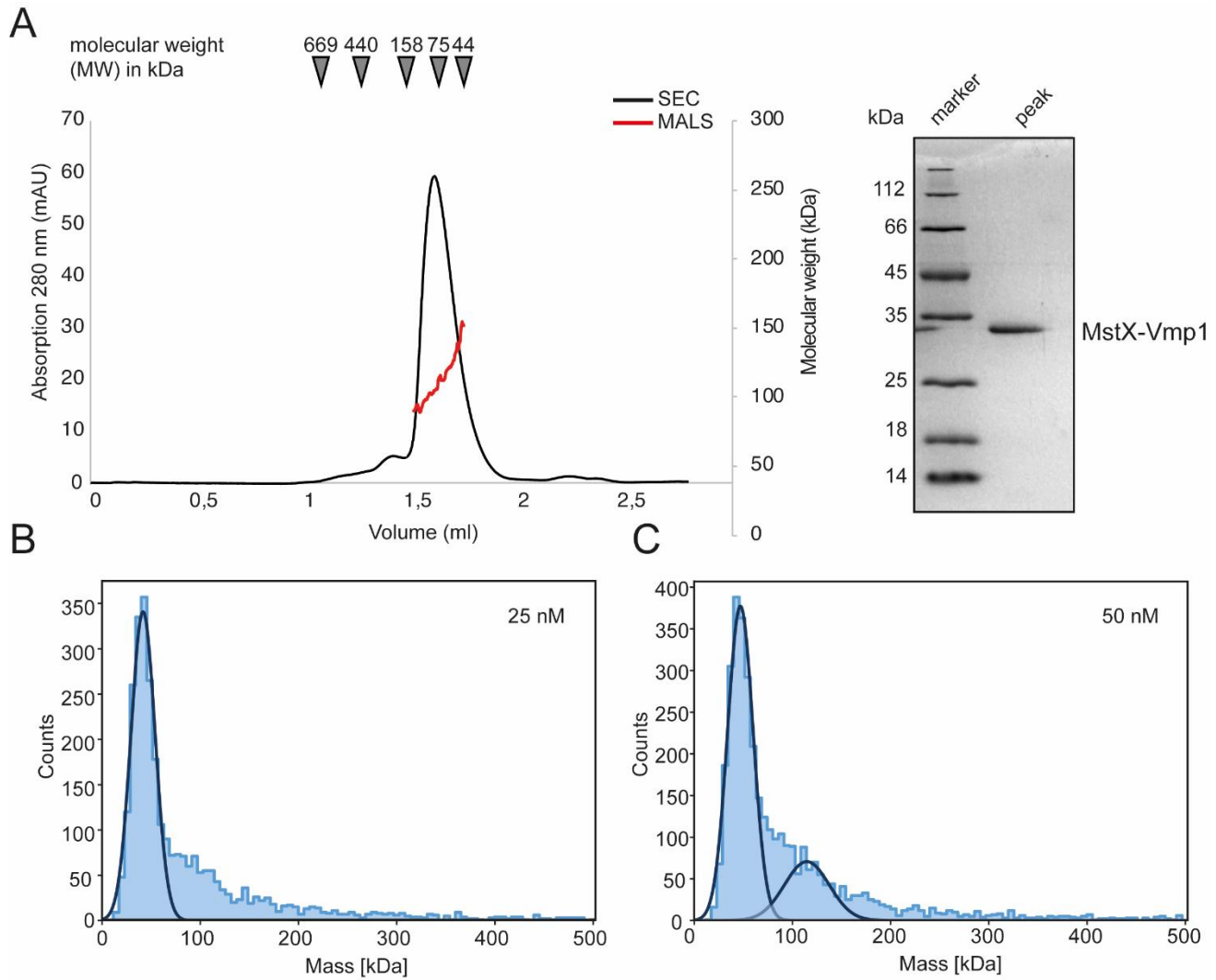
625

626 **Figure 3. Vmp1 is required for virulence.** **A.** Virulence assay of the SG200 $\Delta vmp1$ mutant strain
 627 and SG200 $\Delta vmp1$ -vmp1 complementation strain in an *U. maydis* SG200 background. The mean
 628 percentage of disease symptoms in the different categories is shown that were quantified based on
 629 three biological replicates. The number of infected plants is indicated above the bars. **B.** Macroscopic
 630 pictures of maize leaves 12 days post infection with *U. maydis* SG200, SG200 $\Delta vmp1$ and
 631 SG200 $\Delta vmp1$ -vmp1. **C.** Leaf tissues infected with SG200 and SG200 $\Delta vmp1$ were stained with
 632 WGA-AF488 and propidium iodide at 2 and 6 dpi. Green color indicates fungal hyphae and red color
 633 indicates leaf vascular bundles. Bar = 100 μ m.



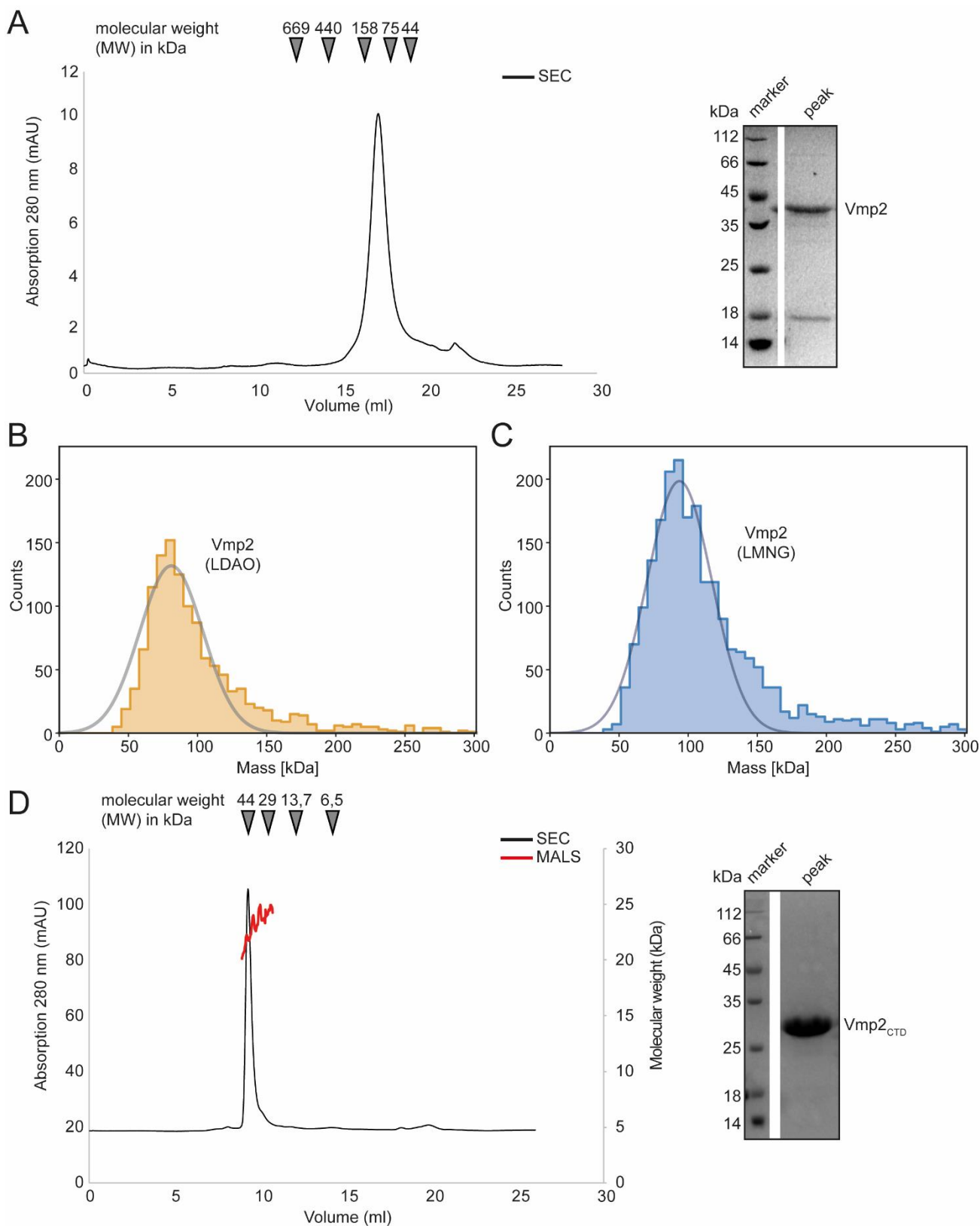
634

635 **Figure 4. Vmp2 is required for virulence A.** Virulence assay of the SG200Δvmp2 mutant strain
636 and SG200Δvmp2-vmp2 complementation strain in *U. maydis* SG200 background. Disease
637 symptoms were quantified based on three biological replicates. The number of infected plants is
638 indicated above the bars. **B.** Macroscopic pictures of maize leaves infected by *U. maydis* SG200,
639 SG200Δvmp2 and SG200Δvmp2-vmp2 at 12 dpi.



640

641 **Figure 5. Biochemical analysis of Vmp1.** A. Multi-angle-light scattering coupled size-exclusion
642 chromatography (SEC-MALS) of full length MstX-Vmp1. The black line depicts the absorption at
643 280 nm, while the red line corresponds to the molecular weight as determined by MALS. The inset
644 shows a SDS PAGE of the peak fraction. B and C. Mass photometry of Vmp2 in 0.1% LDAO at 25
645 and 50 nM concentration, respectively.



646

647 **Figure 6. Biochemical analysis of Vmp2.** **A.** SEC chromatogram of full length Vmp2. The inset
648 shows a SDS PAGE of the peak fraction. **B.** Mass photometry of Vmp2 in 0.1% LDAO. **C.** Mass
649 photometry of Vmp2 in 0.001% LMNG. **D.** Multi-angle-light scattering coupled size-exclusion
650 chromatography (SEC-MALS) shows that the C-terminal domain (CTD) of Vmp2 (aa 120 – 335) is

Membrane-bound virulence factors

651 monomeric with an apparent molecular weight (MW) of 24 kDa. The black line depicts the
652 absorption at 280 nm, while the red line corresponds to the molecular weight as determined by
653 MALS. The inset shows a SDS PAGE of the peak fraction.