Supplementary Material

Supplementary Table 1. Primers used in this study.

Primersequence $5' \rightarrow 3'$	Description (Name)			
	UMAG_03180 (wco1) LF fwd			
	UMAG_03180 (wco1) LF fev			
ATATAATATTCTTATTACTTACTACCACAGTGC	UMAG_03180 (wco1) RF rev			
ATATAATATTGAGCACGCTTCCGGTCGACGCG	UMAG_05732 (phy1) LF fwd			
ATATGGCCATCTAGGCCGCGATCCGCCTAGACGTATAGC	UMAG_05732 (phy1) LF rev			
ATATGGCCTGAGTGGCCTGTTGTCTGTGTTGAAGAATGG	UMAG_05732 (phy1) RF fwd			
ATATAATATTGGTTCGAGCTTGGTTGCGATTCG	UMAG_05732 (phy1) RF rev			
GTAACGCCACCGTTTTTCCCACTCACCACCGTTTAAATTTACACCAACCTCCTCCTCCTCAAACACC	UMAG 02629 (ops1) LF fwd			
CAATTGTCACGCCATGGTGGCCATCTAGGCCATCATTTTCCAGTCACCAACGCGAGAATTTTTT	UMAG 02629 (ops1) LF rev			
AC	_ (1)			
GTGCGGCCGCATTAATAGGCCTGAGTGGCCGTTGAGAAGCTTCACGGATCAAGGAGGTGACACG	UMAG_02629 (ops1) RF fwd			
GCGGATAACAATTTCACACAGGAAACAGCATTTAAATCACGAGTGTCGCACGAGGCAGTAGAAC	UMAG_02629 (ops1) RF rev			
	LIMAC 00271 (ops2) LE fud			
	$UMAG_00371$ (ops2) LF Twu $UMAG_00371$ (ops2) LF rev			
GTGCGGCCGCATTAATAGGCCTGAGTGGCCACCACTCGATTACCGCGTTGCACTCGCCCCTTG	UMAG_00371 (ops2) RF fwd			
GCGGATAACAATTTCACACAGGAAACAGCAATATTATCGATCTGGAAGCCAAGTTTGCGCCG	UMAG 00371 (ops2) RF rev			
ATGGTACCATGAACGTCGTATCCGAGCTGC	UMAG_02629 fwd for p1747			
ATGGATCCCTGGGTAACGGTGTGCATTTGG	UMAG_02629 rev for p1747			
	LIMAC 00271 find for p1742			
	UMAG_00371 rev for p1742			
GAACTTCAAGCTCTCGCAC	UMAG_03726 (cpr1) qRT-PCR fwd			
TCTCGATAGCCTTGACGATG	UMAG_03726 (cpr1) qRT-PCR rev			
ACTCTTCGCTCGTTCTCACC	UMAG_02629 (ops1) qRT-PCR			
CCTCGTAGTCTGAAAACTTGCC	UMAG_02629 (ops1) qRT-PCR rev			
TTTGCTCGATACATTGACTGG	UMAG_00371 (ops2) qRT-PCR fwd			
GGATGAACACCAGCAATCAC	UMAG_00371 (ops2) qRT-PCR rev			
	onsi fivid for			
ACT <u>AAGCTT</u> ATGAACGTCGTATCCGAGCTGCTG	pcDNA ^{TM5} /FRT/TO [©]			
TGGCGGCCGCCTGGGTAACGGTGTGCATAAG	ops1 rev for pcDNA ^{TM5} /FRT/TO [©]			
ACTAAGCTTATGAACTCGTTCTACTACGACAGC	ops2 fwd for pcDNA ^{TM5} /FRT/TO [©]			
TGGCGGCCGCGCGTCTTCAGCGCGGGGTTC	ops2 rev for pcDNA ^{TM5} /FRT/TO [©]			
ACTAAGCTTATGTTCACAAACATCTTGCTCAAACGC	ops3 fwd for pcDNA ^{TM5} /FRT/TO [©]			
TGGCGGCCGCGGTAACAAGAGTGTGTGGAACG	ops3 rev for pcDNA ^{TM5} /FRT/TO [©]			
	107. CDV D			
CCATCTTTTCGATTGGGTCATGATCATCACC	187t_SDM_Primer_L149W_Um Ops1_fwd			
GGTGATGATCATGACCCAATCGAAAAAGATGG	188t_SDM_Primer_L149W_Um Ops1_rev			
CCTGCTCCTCGACATCCTGCTTG	205t_UmOps1_E129D_fwd			
	206t_UmOps1_E129D_rev			
	2071_UMOps1_D225E_IWd 208t_UmOps1_D225E_rev			
	2001_Onopsi_D225L_IO			

Supplementary Table 2. Overview of various *U. maydis* strains expressing eGFP-tagged UmOps1 or UmOps2.

UmOps1 (UMAG_02629)								
Name	Description	Resistance	Microscopic evaluation					
FB1 ΔUmOps1 <i>pcrg</i> - UmOps1::eGFP #1	genomic deletion of UMAG_02629 + single genomic insertion of pcrg- UMAG_02629::eGFP into <i>ip</i> -locus (pCRG-GFP-MXN)	Hyg, Cbx	See Figure 5.					
FB1 <i>pcrg</i> - UmOps1::eGFP #A	multiple genomic insertions of pcrg- UMAG_02629::eGFP into <i>ip</i> -locus (pCRG-GFP-MXN)	Cbx	Localization in plasma membrane; in addition accumulation in the endoplasmic reticulum while the promoter is active.					
UmOps2 (UMAG_003	71)							
FB1 ΔUmops2 <i>petef</i> - UmOps2::eGFP #1	genomic deletion of UMAG_00371 + multiple genomic insertions of <i>petef-UMAG_00371::eGFP</i> into <i>ip</i> - locus (pETEF-GFP-MXN)	Hyg, Cbx	Localization in vacuolar membrane; in addition accumulation in the endoplasmic reticulum.					
FB1 ΔUmOps2 <i>petef</i> - UmOps2::eGFP #9	genomic deletion of UMAG_00371 + single genomic insertion of <i>petef-UMAG_00371::eGFP</i> into <i>ip</i> -locus (pETEF-GFP-MXN)	Hyg, Cbx	See Figure 5.					
FB1 <i>pcrg</i> - UmOps2::eGFP #1	multiple genomic insertions of <i>pcrg-UMAG_00371::eGFP</i> into <i>ip</i> -locus (pCRG-GFP-MXN)	Cbx	Localization in vacuolar membrane; in addition accumulation in the endoplasmic reticulum.					
FB1 <i>pcrg</i> - UmOps2::eGFP #7	multiple genomic insertions of <i>pcrg-UMAG_00371::eGFP</i> into <i>ip</i> -locus (pCRG-GFP-MXN)	Cbx	See supplementary Figure 8. Localization in vacuolar membrane; in addition accumulation in the endoplasmic reticulum when the promoter is active.					
FB1 ΔUmOps2 <i>pcrg</i> - UmOps2::eGFP #B	genomic deletion of UMAG_00371 + multiple genomic insertions of <i>pcrg-UMAG_00371::eGFP</i> into <i>ip</i> - locus (pCRG-GFP-MXN)	Hyg, Cbx	Localization in vacuolar membrane; in addition accumulation in the endoplasmic reticulum also several hours after inactivation of the promoter.					

Hal.sal.BR Fus.fuj.CarO Neu.cra.Nop1 Lep.mac.ops1 Ust.may.ops1 Ust.may.ops2 Ust.may.ops3 Clustal Cons	MLELHL MADHL MIHPEQVADM MIVD-QFEEV MNVVSEL MNSF-YYDSV MFTNIL *	YAR-KN LRFTST LMKTSQLFPL LKRAGN LKRAGN	DAL- TSSHVP- PTATQSAQPT MAL- FKKPE- RAL-	LPTAVE NVNPDIVN GPVPTVVP HVAPVPTVLP SSNPT VGIPPIPP DVNPP *	GVSQAQITGR GQRSDINITV TPTEYQTLGE DTPIYETVGD VADIDITT HLEHPIVSTQ NADIHLGR	PEWIWLALGT RGSDWYWAVC TGHRTLWVTF SGSKTLWVVF PGSDWLWAVF WGHTYLWVVF WGSDWGWVVF	ALMGLGTLYF AVMTVSTFAF ALMVLSSGIF VLMLIASAAF SVMAATGLGT FIMAGFSLLF CVMAASTLGL :*
Hal.sal.BR Fus.fuj.CarO Neu.cra.Nop1 Lep.mac.ops1 Ust.may.ops1 Ust.may.ops2 Ust.may.ops3 Clustal Cons	LVKGMGVSDP LGLGMRKPR- ALLSWNVPT- TALSWKIPV- MVWSLKVSR- FISSQRASY- LVWTFILPR-	DAKKFYAITT TDRIFHYITA SKRLFHVITT MRRLYHVITT GERAFHYLSA RYRLMHTTTF KRRTFHYLLT : :	LVPAIAFTMY GITMIASIAY LITVVASLSY IITLTAALSY AILATASVAY FITAIAALSY AVLAISTISW : : :	LSMLLGYGLT FTMASNLGWT FAMATGHATT FAMATGHGVA FSMASDLGAT FAMATGVGKT YSQASDLGAT	MVPFG PIAVEF-QRS FNCDTAWDHH LNKIVIRTQH PVRVEF-TNY LSTIG PITTQF-LHN	G NHRVAG KH-VPDTSHQ DH-VPDTYET GPN-EVNGLR GGHH SQRGGGYGGY	EQNPIYWARY IYREIFYARY VCRQVFWGRY VYRQVYYARY PTRSIWYVRY APREFYFARY PTRQIWYSBY
Hal.sal.BR Fus.fuj.CarO Neu.cra.Nop1 Lep.mac.ops1 Ust.may.ops1 Ust.may.ops2 Ust.may.ops3 Clustal Cons	ADWLFTTPLL IDWFLTTPLL IDWALTTPLL IDWALTTPLL IDWALTTPLL IDWALTTPLL ** ** ** 85 86 89 93	LLDLALLVDA LTDLLLTAGM LLELCLLAGV LLDLGLLAGM LLEILLVSGL LFDLTLLAGL LLSLLLITGL * * 96	DQGTILALVG PWPTVLWVIL DGAHTLMAIV SGAHIFMAIV PLSTVFITIF PVAEIIVLVL PLSIIFITLF : :	ADGIMIGTGL VDWVMIVTGL ADVIMVLCGL ADIIMVLTGL FDIVMIITGL ADEVMIVTGV FNILMIVCGL : :*: *: 116	VGALTKVY VGALVKS FAALGEGGNT FAAFGSE-GT IGSLVES IAGVHPTK LGALTRT	SYRFVWWAIS SYKWGYFAFG AQKWGWYTIG PQKWGWYTIA TYKWGYYTMG TGKWGFFTFS RYKWGYFAFA :: ::::	TAAMLYILYV CAALAYIVYV CFSYLFVIWH CIAYIFVVWH CVAMFYVFWV CIAFAWVLLQ CAALGYVLYH : ::.
Hal.sal.BR Fus.fuj.CarO Neu.cra.Nop1 Lep.mac.ops1 Ust.may.ops1 Ust.may.ops2 Ust.may.ops3 Clustal Cons	LFFGFTSKAE LAWEARLHAK VALHGSRTVT LVLNGGANAR IYGPGLKSAS LVTSGRSTAF VFASGLRSSR :	SMRPEVASTF HVGPDVGRTF AKGRGVSRLF VKGEKLRSFF HLGADFKKAY LRSPKVGGLY RLGSRFGRAF	KVLRNVTVVL VMCGSLTAVV TGLAVFALLL VAIGAYTLIL LYSSLVLTIL NQVSLALVVV LAASLLLAII :: 178	NSAMPVVMLI WILMPIAMGV WTAMPIIMGI WTAMPIVMGL WTLMPVAMGL WTAMPVAFAL WPMPICMGL * **::: 182 185 189	GS2GAGIVPL -CPGGNLIAP -AGGARRTNV -ADGARKIGV -ADGSNTISP -CPGTGKLNP -SPGGNVIGV . * 194	NIETLLFMVL DSEAVFYGIL DTEILIYTVL DGEIIAYAVL NAEMIFYGVL DAEILFFAIL SAEFLWYGLL * : : * 204	DVSAKVGFGL DLIAKPVFGA DLLAKPVFGF DVLAKGVFGA DLLAKPVFAL DVIAKPLWGA DIFSKPIFAF *::*: 212 216 219
Hal.sal.BR Fus.fuj.CarO Neu.cra.Nop1 Lep.mac.ops1 Ust.may.ops1 Ust.may.ops2 Ust.may.ops3 Clustal Cons	ILLRSRAIF- LLLWGHRNI- WLLLSHRAM- WLLVTHANL- FHLWSLRRC- WLLLATPDEG LFLGMLKKC- *	GEAEAP DPARLG PETNID RESDVE NYSSLH HVLVPESLCA DYGALR	EP-SA LRIRDIDERI LP-GYW LN-GFW LKSGKFSD PA-GSP LRSGKRSE	FPDGPN YEDLSGNHYR -EDSD	-GDG -SHG ANG SMEGGKASEA -ASGV	ANKVASG-HGA NKVASG-HGA L GVTGAGVNGV GYGA HEPR	AATS RNDTATAS-G ATEGR NREGA -ATGVNTTGA ISQEP -RENMEKD-H
Hal.sal.BR Fus.fuj.CarO Neu.cra.Nop1 Lep.mac.ops1 Ust.may.ops1 Ust.may.ops2 Ust.may.ops3 Clustal Cons	SNVNPNA- IRIGEED- IRIGEDDGA- DVTGANVLHP RAEDA- ERIG	APPTQMHTVT	- - Q				

Supplementary Figure 1. Alignment of the amino acid sequences of selected microbial rhodopsins. The alignment includes BR (*Halobacterium salinarum*, AAA72504), CarO (*Fusarium fujikuroi*, CAD97459), Nop1 (*Neurospora crassa*, XP_959421), LR (*Leptosphaeria maculans*, AAG01180), and the three *Ustilago maydis* rhodopsins UmOps1 (UMAG_02629), UmOps2 (UMAG_00371), and UmOps3 (UMAG_04125). Functionally and structurally important residues are highlighted by colored boxes: red (proton donor/-acceptor), green (ion transport), blue (Schiff base), orange (structural importance), grey (putative interaction site in CarO-like rhodopsins). Numbers below the alignment represent the respective position of the BR sequence.



Supplementary Figure 2. Pump activity of UmOps1 (A) and UmOps2 (B) in absence of intracellular sodium. I-V-plots of the pump current in CsCl-based pipette solution at different membrane potentials and extracellular pH values as indicated. Measurements in TEA-Cl were similar but did not yield satisfying signal-to-noise ratios. In both conditions, the pump characteristics of both rhodopsins were hardly influenced by the absence or presence of sodium ions. For comparison see Figure 3B and 3C.



Supplementary Figure 3. Pump activity of UmOps1 (A) and UmOps2 (B) in absence of extracellular chloride. I-V-plots of the pump current in NaCl pH 7.4 and sodium gluconate-based bath solutions at different membrane potentials and extracellular pH values as indicated. In general, the pump characteristics of UmOps1 and UmOps2 were similar in gluconate- as in chloride-based (Fig. 3 B, C) solutions but slightly enhanced. C. Relative pump activity at 0 mV at different extracellular conditions. The presence of sodium gluconate in general leads to a slight enhancement of the pump activity when compared with NaCl. The supporting effect is very pronounced in UmOps1 at pH 5.0.



Supplementary Figure 4. Supporting effect of sodium acetate on UmOps1 pump activity at different concentrations and intra- and extracellular pH 7.4. The pipette solution was either untreated (A) or supplemented with 2.8 mM sodium acetate (B). Though the pump activity of UmOps1 is much lower in neutral environment than in pH 5.0 (see Fig. 4C), the supporting effect of sodium acetate is still present. The behavior is essentially the same when sodium acetate is absent or present in the pipette solution, suggesting that the interaction of WOAs and UmOps1 occurs in the extracellular part of the protein.



Supplementary Figure 5. Increase of pump activity of UmOps1 in presence of Indole-3-acetic acid (IAA, A) and Indole-3-propionic acid (IPA, B). I-V-plots of the pump currents in extracellular NaCl pH 7.4 and NaCl pH 5.0 with different concentrations of IAA / IPA at different membrane potentials as indicated.



Supplementary Figure 6. Whole cell patch-clamp analysis of UmOps1 mutants L149W and D225E in comparison with WT. A. Typical traces showing the light-induced charge transfer by UmOps1 as indicated by the green bars. The pump intensity of UmOps1 WT and L149W strongly increased in the presence of WOAs at external pH 5.0 (lower raw) compared to NaCl pH 7.4 (upper raw), this behavior is less pronounced in D225E, which also exhibits a transient increase in pump activity after illumination indicating an electrogenic dark reaction. B. Relaxation of the UmOps1 pump activity after replacement of NaCl pH 5.0 + 7 mM sodium acetate by NaCl pH 9.0. Every 10 seconds the pump current was recorded. Using an exponential decay function, time constants of 89.2 s (WT), 56.5 s (L149W), and 89.7 s (D225E) were obtained. The D225E mutant exhibits a transient increased pump activity in pH 9.0, similar as described for CarO from *F. fujikuroi* (Adam et al., 2018).



Supplementary Figure 7: Heterologous expression of eYFP-tagged UmOps1 (A) and UmOps2 (B) in HEK293 cells. Confocal laser scanning micrographs show the location of the respective rhodopsin (cyan) and the nucleus stained with SYTO® 59 (magenta). In accordance with our observations in sporidia (Fig. 5, main part), we found distinct eYFP mediated fluorescence in the plasma membrane of HEK293 UmOps1::eYFP but mainly in endomembranes in HEK293 UmOps2::eYFP cells. The low amount of UmOps2 in the plasma membrane is in accordance with small pump currents of this rhodopsin. Scale bars represent 10 μ m.



Supplementary Figure 8: CLSM analysis of the localization of UmOps2::eGFP in *U. maydis* sporidia in dependence of the expression time. The strain FB1 *pcrg*-UmOps2::eGFP with an arabinose-inducible promoter was used for this analysis. Protein localization after promoter on-times between 30 min and 180 min were analyzed as indicated (time of exposure to arabinose). The samples were fixed with 1% formaldehyde either 25 min (A) or 195 min (B) after the removal of arabinose allowing to trace the protein expression after stop of transcription. Note that the UmOps2::eGFP protein was never observed in the plasma membrane but only found in vacuolar membranes or after longer expression times also in the ER. All images were obtained using the same parameters for illumination and recording (4% laser power (488 nm), photomultiplier: 700). White bars represent 5 μ m. Arrows highlight fluorescence in the vacuolar membranes.