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C Hoefle, R Hückelhoven

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A barley ENGULFMENT and MOTILITY domain containing protein modulates Rho GTPase activating protein HvMAGAP1 function in the barley powdery mildew interaction

Caroline Hoefle[1], Ralph Hückelhoven[1*]

[1] Lehrstuhl für Phytopathologie, Technische Universität München, Emil-Ramann-Straße 2, D-85350 Freising-Weihenstephan, Germany

*corresponding author: Ralph Hückelhoven

Phone: +498161-713682

Fax: +498161-714538

e-mail: hueckelhoven@wzw.tum.de

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Abstract

ENGULFMENT AND MOTILITY (ELMO) proteins are involved in the regulation of small GTPase activity in eukaryotic organisms, but little is known about ELMO proteins in plants. We isolated the barley ELMO DOMAIN CONTAINING protein, HvELMOD_C, in a yeast two hybrid screen for proteins interacting with HvMAGAP1 (MICROTUBULE ASSOCIATED ROP-GTPase ACTIVATING PROTEIN 1). HvMAGAP1 is considered as an antagonist of barley RACB, a member of the RHO of plant (ROP) family GTPases, which functions as susceptibility factor in the interaction of barley with barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei*. HvELMOD_C interacts with the central RHO-GAP domain of HvMAGAP1. Cytoplasmic HvELMOD_C translocates to microtubules (MTs) on co-expression of HvMAGAP1 but not on co-expression of HvMAGAP1-R185G, a mutant of the catalytically active arginine R185 in the RHO-GAP domain. HvELMOD_C, when simultaneously expressed with HvMAGAP1, abolished the resistance-inducing effect of HvMAGAP1 to *B. graminis* f.sp. *hordei.* Therefore, HvELMOD_C might function as a new modulator of HvMAGAP1 and thus ROP activity in barley.

Introduction

The RAS superfamily of small monomeric GTP binding proteins comprises a group of highly conserved proteins that are subdivided into five families RAS, RHO, RAB, ARF and RAN. RHO family GTPases function in animal and plant cells as molecular switches in diverse cellular processes. Animal RHO GTPases act as regulators of the actin cytoskeleton, microtubule dynamics, vesicular trafficking and gene transcription, controlling cell cycle progression, cell morphology and polarization in phagocytosis and migration (Etienne-Manneville and Hall 2002; Raftopoulou and Hall 2004; David et al. 2012). In plants, a specific subfamily of RHO GTPases exists, the 'Rho of plants' (ROP also called RAC), which control cytoskeleton dynamics, vesicle trafficking, hormone signaling and polarization, division and morphology of cells during growth and reaction to external stimuli (Bloch et al. 2005; Fu et al. 2005; Opalski et al. 2005; Lavy et al. 2007; Hoefle et al. 2011; Chen et al. 2012; Lin et al. 2012). Several pathogens of animals co-opt host RHO functions to facilitate cell entry (Gruenheid and Finlay 2003; Cote and Vuori 2007). Similarly, barley HvRACB is required for entry of the powdery mildew fungus *Blumeria graminis* f.sp. *hordei* into epidermal host cells, and a constitutively activated (CA) variant of HvRACB supports fungal entry (Schultheiss et al. 2003; Pathuri et al. 2008; Hoefle et al. 2011).

The switch function of RHO GTPases is achieved via binding of GTP, rendering the protein active, and hydrolysis of the nucleotide leading to an inactive GDP-bound status. In their active form, RHO GTPases interact with a wide range of so-called effector proteins with distinct downstream functions. Many effector proteins contain a CRIB (for CDC42/RAC-interactive binding) motive that mediates binding to active RHO GTPases (Burbelo et al. 1995; Wu et al. 2001).

Strict spatiotemporal regulation of RHO GTPase activity is very important for the cell, e.g. to ensure polar transport processes. Consequently, a series of GTPase regulating proteins exist. GDP/GTP EXCHANGE FACTORS (GEFs), GTPase ACTIVATING PROTEINS (GAPs) and GUANINE NUCLEOTIDE DISSOCIATION INHIBITORS (GDIs) regulate the activity of RHO family proteins in animals and plants (Bos et al. 2007; Cote and Vuori 2007; Berken and Wittinghofer 2008; Kost 2008; Garcia-Mata et al. 2011). RHO-GEFs bind to GDP-bound RHO GTPases and facilitate the exchange of GDP for GTP, activating the RHO GTPase switch. Mammalian RHO-GEFs are divided in two families based on their distinct domain structures, i. the DbI-related GEFs and ii. the DEDICATOR OF CYTOKINESIS PROTEIN (DOCK) GEFs (Cote et al. 2005; Cote and Vuori 2007; Rossman et al. 2005; David et al. 2012). Few GEFs were identified in plants that fit into the two GEF families of mammals: *Arabidopsis thaliana* SPIKE1 (SPK1) is a DOCK family protein (Qiu et al. 2002), and more recently, SWAP70A and SWAP70B were identified as representatives of the DbI-related GEF family in rice (*Oryza sativa*) (Yamaguchi et al. 2012). In addition, plants possess proteins with a plant-specific ROP nucleotide exchanger (PRONE) domain, the PRONE-GEFs (Berken et al.

2005). Many GEFs show auto-inhibition by intramolecular interactions that blocks access of the corresponding GTPase (Lu et al. 2005; Gu et al 2006; Cote and Vuori 2007; Patel et al. 2011b).

RHO-GAPs inactivate the RHO GTPases by stimulation of the otherwise low RHO-intrinsic GTPase activity. Many RHO-GAPs of animals are multidomain proteins assumed to integrate signals from several signaling pathways by interaction with a plethora of specific effectors and regulatory proteins and their RHO-deactivating activity (Tcherkezian and Lamarche-Vane 2007). The structure of plant ROP-GAPs is more simple with a conserved RHO-GAP domain, harboring the catalytic arginine residue, and for most ROP-GAPs an additional, more N-terminal, Pleckstrin-homology (PH) domain or a CRIB-motif (Borg et al. 1999; Klahre and Kost 2006; Berken and Wittinghofer 2008). Recently, we identified a ROP-GAP of barley that has the ability to associate with microtubules (MT), the MICROTUBULE ASSOCIATED ROP-GTPase ACTIVATING PROTEIN 1 (HvMAGAP1). MT association of HvMAGAP1 is mediated by the C-terminal part of the protein. Apart from the MT association domain, HvMAGAP1 contains a central ROP-GAP domain, N-terminally flanked by a CRIB motif. HvMAGAP1 interacts with HvRACB in *planta*, and is recruited by the constitutively activated CA HvRACB to the plasma membrane. During powdery mildew attack, HvMAGAP1 promotes focal polarization of the cortical MTs and supports basal resistance to fungal entry presumably via negative regulation of the susceptibility factor HvRACB (Hoefle et al. 2011). When we screened for barley proteins that interact with HvMAGAP1 in yeast, we identified an ELMO domain containing protein, we named HvELMOD_C. In barley epidermal cells, the majority of yellow fluorescing protein-tagged YFP-HvELMOD_C was found in the cytoplasm. However, co-expression of red fluorescing protein-tagged RFP-HvMAGAP1 sequestered HvELMOD_C at cortical MTs. Over-expression of HvELMOD_C alone had no effect on the interaction with powdery mildew but abolished the resistance-inducing effect of HvMAGAP1, when both proteins were co-expressed. We suggest a role for HvELMOD_C as a modulator of HvMAGAP1 function.

Materials and methods

Plant growth, pathogens and inoculation conditions

Barley (*Hordeum vulgare* L.) plants of the cultivar 'Golden Promise' were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h with 150 µmol $m^{-2}sec^{-1}$. *Blumeria graminis* (DC) Speer f.sp. *hordei* Em. Marchal was maintained on Golden Promise under the above described conditions.

In transient transformation experiments, detached primary leaf segments of barley were placed on 0.5% (w/v) H_2O-agar seven days after germination, and inoculated with >100 conidia mm^{-2}.

Yeast two Hybrid Screening and Targeted Yeast two Hybrid Assay

Yeast two hybrid (Y2H) screening and transformation of yeast was performed according to the yeast protocols handbook and the Matchmaker library construction & screening kits manual (Clontech, Mountain View, USA, www.clontech.com). For the Y2H screening the full length sequence of HvMAGAP1 was fused to the DNA-binding domain in the yeast two hybrid vector pGBKT7 to obtain pGBKT7-*HvMAGAP1*. The construction of the Y2H library from a pool of barley plants inoculated with *Blumeria graminis* f. sp. *hordei* is described in detail in Hoefle et al. (2011). We made use of this library to screen 19 mill. mating events.

For the targeted Y2H assay, pGBKT7-*HvMAGAP1* and similar constructs with the mutant variant *HvMAGAP1R185G* were produced. *HvELMOD_C* was isolated by RT-PCR with the primers HvELMOD_C_EcoRIf 5'AGAATTCATGCCACCCGGGCCGGTGGC 3' and HvELMOD_C_BamHIr 5'AGGATCCAACAGATATGACCCCTAGCAAA 3' from a barley cDNA pool and fused with the GAL4 activation domain (GAL4 AD) via the *Eco*RI and *Bam*HI restriction sites in the Y2H vector pGADT7. Co-transformed yeast cells were selected on SD-medium lacking leucine (-Leu) and tryptophan (-Trp). Selection of yeast expressing interacting proteins was performed on SD-medium lacking leucine (-Leu), tryptophan (-Trp), histidine (-His) and adenine (-Ade).

Protein Localization and Protein-Protein Interaction *in planta*

For localization studies, an N-terminal fusion of HvELMOD_C was produced by amplification of the coding sequence with the primers HvELMOD_C_SalI 5'GTCGACCATGCCACCCGGGCCGGTGGC 3' and HvELMOD_C_PstI_2 5'ACGATTCATCTGCAGCTCGAGC 3' and insertion in frame with *YFP* in the *Sal*I and *Pst*I restriction sites of the expression vector pGY-1-*YFP* (Schweizer et al. 1999). Leaves of Golden Promise were transiently transformed with the *YFP-HvELMOD_C* fusion construct under control of the 35S promoter via particle bombardment. As cytoplasmic and transformation marker soluble *RFP* in pGY-1 under the control of the P35S promoter was co-transformed. Each shot delivered 1 µg of the fusion construct and 0.5 µg of the transformation marker. Protein interactions were tested in transient co-expression experiments, where each shot delivered 1 µg *YFP-HvELMOD_C* fusion construct together with 1 µg of pGY-1-*RFP-HvMAGAP1*or pGY1-*RFP-HvMAGAP1* R185G. Leaves were inspected 24 h or 48 h after bombardment by confocal laser scanning microscopy (CLSM). Pictures were generated by sequential scanning to avoid channel crosstalk. YFP was excited by a 514 nm laser line and detected at 524-550 nm, while RFP was excited by a 561 nm laser line and detected at 571-610 nm.

Interaction of HvELMOD_C and MAGAP1 was verified by fluorescence resonance energy transfer (FRET) analysis *in planta*. Leaves of 7 days-old barley plants were co-transformed by particle bombardment with 1 µg pGY-1-YFP-*HvELMOD_C* together with 1 µg pGY-1-RFP-*MAGAP1*. To calculate the correction factors in the FRET analysis, indicated amounts of every construct were also transformed alone. FRET analysis was performed 40 h after bombardment by the sensitized emission (SE) method using the Leica Application Suite, Advanced Fluorescence 1.8.0 software (Leica Microsystems, Mannheim, Germany). FRET efficiencies were calculated according to the method of Wouters et al. (2001). In all experiments YFP was excited by a 514 nm laser line and detected at 524-550 nm. RFP was excited by a 561 nm laser line and detected at 571-610 nm.

Transient over-expression experiments

Construction of the pGY-1-*RFP-HvMAGAP1* expression construct was described previously in Hoefle et al. (2011). To obtain the over-expression construct pGY-1-*HvELMOD_C* of HvELMOD_C the same primers and restriction sites as for the *YFP* fusion were used to clone the coding sequence in the over-expression vector pGY1.

Leaves of 7 days-old barley plants were transiently transformed as described earlier (Douchkov et al. 2005; Eichmann et al. 2010) with the PDS-1000/He System (Bio-Rad Laboratories GmbH, Munich, Germany, www.bio-rad.com) plant transformation gun with the single-adapter. In all over-expression experiments, each shot delivered 1 µm gold particles (25 mg/ml) coated with 1 µg of the respective expression construct pGY-1-*RFP-HvMAGAP1*, pGY-1-*HvELMOD_C* or the empty vector control pGY-1 and pGY-1-*RFP*, and 0.5 µg of the reporter plasmid pGY-1-*GFP*. For microscopic evaluation of the *B. graminis* f.sp. *hordei* penetration success, we worked according to Eichmann et al. (2010).

Statistical analysis

All experiments were repeated at least four times and the mean over all experiments was shown. In each experiment 80-100 transformed, powdery mildew attacked cells per construct were counted. The numeric data were subjected to statistical analysis using the Student's t-test in Excel 2010.

Accession Numbers

Sequence data from this article can be found in the GenBank data libraries under accession number: *Hordeum vulgare*: *HvRACB* (AJ344223), *HvHvMAGAP1* (AK371854), ELMO domain proteins of barley and other organisms: *Hordeum vulgare*: *HvELMOD_C* (submitted to EMBL); *Oryza sativa*: BAG91897; *Arabidopsis thaliana*: At1g67400; *Zea maydis*: ACF83115*; Homo sapiens*: HsELMOD2 NP714913, HsELMO2 NP_877496.

Results

Isolation of barley *HvELMOD_C*

HvMAGAP1 binds HvRACB in planta and may regulate HvRACB activity (Hoefle et al. 2011). To identify potential interaction partners of HvMAGAP1, we performed a yeast two hybrid (Y2H) screening with HvMAGAP1 as the bait and a pooled prey cDNA library of barley. One of the candidates, we isolated in the screening, was an ELMO (ENGULFMENT and MOTILITY) domain containing protein that we named HvELMOD_C according to the nomenclature proposed by East et al. (2012) for the most identical *Arabidopsis* ELMO domain containing protein. The complete cDNA of *HvELMOD_C* encoded for a protein of 254 amino acids with a central ELMO domain covering nearly two thirds of the protein sequence (aa 56-226). Analysis of available barley sequences revealed further five complete and two partial sequences of potential ELMO domain containing proteins in barley. In Database searches of publicly available sequences of *Arabidopsis*, rice and corn, we identified six ELMO domain containing proteins in Arabidopsis and eight and nine in rice and corn respectively. The protein identity of ELMO proteins was most pronounced in the central ELMO domain of the proteins with the highest identity to HvELMOD_C in rice (BAG91897) with 92%, followed by corn (90%, ACF83115) and *Arabidopsis* (74%, At1g67400).

The initially identified ELMO proteins in humans and *Caenorhabditis elegans* are multidomain proteins that were described to promote DOCK-GEF activity and subsequent reorganization of the actin cytoskeleton via RAC1. These ELMO proteins are associated to DOCK-GEFs. They can release the autoinhibition of the DOCK-GEF switch, when binding to small GTPases such as RHOG and ARF LIKE4 (ARL4) via the Ras-Binding Domain (RBD), located on the extreme N-terminus of these ELMO proteins (Katho et al. 2006; Cote and Vuori 2007; Patel et al. 2011a, 2011b). In contrast to the first discovered multidomain ELMO proteins human ELMO DOMAIN CONTAINING proteins (ELMOD), like plant ELMOs, possess only the ELMO domain and no further conserved domains recognized so far (Bowzard et al. 2007; Brzostowski et al. 2009). We further compared the ELMO domain of barley HvELMOD_C to those of mammalian ELMO and ELMOD proteins. This positioned

HvELMOD_C closer to the ELMOD1 and 2 proteins (identity 23% and 27 % respectively, in the ELMO domain) than to the ELMOs (identity in the ELMO domain 17-18%). Correspondingly, East et al. (2012) placed the six *Arabidopsis* ELMO domain containing proteins into the ELMOD clade after phylogenetic analysis. No function was discovered for the ELMO domain in the ELMO proteins yet, but in the human ELMOD proteins the ELMO domain can act as a GTPase activating domain. This GAP activity appears to be directed towards ADP RIBOSYLATION FACTOR (ARF) and ARF LIKE (ARL) GTPases (Bowzard et al. 2007; East et al. 2012). The corresponding consensus motif $WX_3G(F/W)QX_3PXTD(F/L)RGXGX_3LX_2L$ contains a conserved arginine residue that is essential for GAP activity (East et al., 2012). This consensus motive is fully conserved in barley HvELMOD_C (Fig. 1). Hence the barley HvELMOD_C protein belongs to the family of ELMO domain containing proteins such as the human ELMODs.

HvELMOD_C interacts with HvMAGAP1 in yeast and *planta*

To test, whether the ROP-GAP domain of HvMAGAP1 might be involved in the interaction between HvELMOD_C and HvMAGAP1, we used a targeted Y2H assay based on co-transformation. In this assay we used either HvMAGAP1 or HvMAGAP1-R185G, a mutant version in which the catalytically active arginine within the ROP-GAP domain was replaced by glycine. HvMAGAP1-R185G can be considered an inactive protein, which, however, can still interact with HvRACB (Hoefle et al. 2011; Fig. 2a). In this assay, HvELMOD_C interacted with HvMAGAP1 in yeast but not with the mutant version HvMAGAP1-R185G (Fig. 2a).

To determine whether HvELMOD_C co-localizes with HvMAGAP1 *in planta* and where the proteins might interact, we transiently transformed barley epidermal cells with *YFP-HvELMOD_C*. Expression of the YFP-HvELMOD_C fusion protein alone revealed localization in the cytoplasm and the nucleoplasm (Fig. 2b). Pixel intensity measurements in transversal sections over the cells showed near perfect co-localization with soluble RFP that was co-transformed as nucleoplasmic and cytoplasmic marker (Fig. 2b, c). By contrast, most of the YFP-HvELMOD_C fusion protein co-localized with HvMAGAP1 at the cortical MTs on co-expression of *YFP-HvELMOD_C* with *RFP-HvMAGAP1* and only a small proportion of HvELMOD_C remained in the cytoplasm (Fig. 2d and 2g). Hence, HvMAGAP1 apparently recruited HvELMOD_C to the MTs, suggesting *in planta* protein-protein interaction. YFP-HvELMOD_C signals remained in the cytoplasm, when the MT-associating C-terminus of HvMAGAP1 was removed from RFP-MAGAP1 (RFP-HvMAGAP1ΔCter, Fig. 2e). In this situation, both proteins largely co-localized in the cytoplasm (Fig. 2h). Recruitment of YFP-HvELMOD_C and co-localization with the RFP signal was also abolished, if only the MT associating C-terminal domain of HvMAGAP1 (aa 319 - 484) was tagged by RFP without the rest of

the protein (RFP-HvMAGAP1-Cter, Fig. 2f and 2i). Because the Y2H experiments and the inability of HvMAGAP1-Cter to recruit YFP-HvELMOD_C hint to a possible function of the ROP-GAP domain of HvMAGAP1 in binding HvEMLOD_C, we tested the ability of RFP-tagged HvMAGAP1-R185G to recruit YFP-HvELMOD_C to MTs *in planta*. Obviously, YFP-HvELMOD_C remained in the cytoplasm in epidermal cells co-expressing RFP-HvMAGAP1-R185G (Fig. 2j and 2k). We further confirmed the dependency of the interaction of HvELMOD_C with HvMAGAP1 on the intact ROP-GAP domain by fluorescence resonance energy transfer experiments based on in *planta* sensitized emission. Co-transformation of YFP-HvELMOD_C and RFP-HvMAGAP1 resulted in strong FRET signals at the microtubules. By contrast, co-expression of YFP-HvELMOD_C with RFP-HvMAGAP1-R185G resulted in very weak FRET (Fig 3). Together, data suggest that binding of HvELMOD_C and recruitment by HvMAGAP1 to the MTs depends on the presence of an intact GAP domain of HvMAGAP1.

HvELMOD_C interferes with the function of HvMAGAP1 in the interaction with powdery mildew

HvMAGAP1 supports basal resistance of barley in the interaction with *B. graminis* f.sp. *hordei.* HvMAGAP1 also promotes polarization of MT arrays at the site of fungal attack (Hoefle et al. 2011). As HvELMOD_C binds HvMAGAP1, we tested whether HvELMOD_C can influence the effect of HvMAGAP1 during powdery mildew interaction of barley. In transient transformation experiments, *HvELMOD_C* and *RFP-HvMAGAP1* were expressed in epidermal barley cells either alone or together. Over-expression of *HvELMOD_C* alone had no effect on the interaction of barley with *B. graminis* f.sp. *hordei*, whereas sole over-expression of *HvMAGAP1* reduced the relative penetration efficiency of the fungus by about 20%. When we co-expressed both proteins, the effect of HvMAGAP1 on the penetration success of *B. graminis* f.sp. *hordei* was abolished and no differences to the control could be detected any more (Fig. 4a). In fungus-attacked cells YFP-HvEMLOD_C and RFP-HvMAGAP1 co-localized at MTs, forming radial arrays around the site of attack (Fig. 4b). Together this suggests that HvELMOD_C possibly modulates functions of HvMAGAP1 at the MTs.

Discussion

Rho GTPases regulate a plethora of processes in animals and plants. Understanding of RHO-dependent signalling greatly depends on knowledge on how RHO GTPase activity is spatiotemporally regulated. The ELMO-DOCK180 complex induces polymerization of actin and membrane ruffling via activation of RAC1 in metazoan cells. This finally defines the polarity in cell migration and phagocytosis (Cote and Vuori 2007; Ridley 2011). Polar localisation of the ELMO-DOCK180 complex occurs by binding to phosphatidylinositol (3,4,5)-bisphosphate (PtdIns(3,4,5)P_3) and by

binding of GTPases. Studies on the regulation of the ELMO-DOCK180 complex revealed that the RBD of ELMO is a versatile domain, able to bind different GTPases and placed the ELMO-DOCK complex in the centre of cross talk between diverse signalling pathways. Actin polymerization in *Arabidopsis thaliana* also involves the DOCK-GEF SPIKE1 (SPK1) and active ROP via interaction with the heteromeric WAVE and actin related protein (ARP) 2/3 complex (Qiu et al. 2002; Basu et al. 2008). Furthermore, SPK1 regulates polar PIN2 distribution by auxin mediated activation of AtROP6 (Lin et al. 2012), but no interaction of SPK1 with ELMO proteins was reported.

Here we identified the barley ELMO domain containing protein HvELMOD_C as a direct interaction partner of HvMAGAP1, which is an antagonist of HvRACB (Fig. 5). All known plant ELMO proteins belong to the ELMOD family. Mammalian ELMOD proteins show ARF- and ARL-GAP activity, although they do not contain the canonical ARF-GAP motif ($CX_2CX_{16-18}CX_2CX_4R$). Instead, an alternative GAP consensus motif was identified by East et al. (2012) in the ELMOD domain, and the catalytic activity of the arginine residue (compare Fig. 1) confirmed by creation of mutated protein variants. ARF GTPases are regulators of vesicle biogenesis and trafficking at the endoplasmatic reticulum (ER), the Golgi apparatus and in the peripheral vesicle transport (Memon 2004; Naramoto et al. 2010). A more diverse function is proposed for the ARL GTPases that localize to cytosol, nucleus, mitochondria and cytoskeleton. Protein function is only known for ARL2 and ARL3 in the regulation of microtubule dynamics and for ARL1 at the trans Golgi network in vesicle tethering (Burd et al. 2004).

The presence of a conserved ARF-GAP domain in HvELMOD_C indicates potential diverse functions of the protein, which may be balanced in response to fungal attack. Recently a function for ARF GTPases was established for basal resistance of barley to *B. graminis* f.sp. *hordei* interaction (Böhlenius et al. 2010). HvELMOD_C functions might be found in crosstalk between ARF and ROP GTPases. Crosstalk between RHO and ARF is well established in the metazoan field and is postulated for plants (Xu and Scheres 2005). In metazoans, it involves ARF6 and the ELMO-DOCK180 complex (Myers and Casanova 2008). ELMO complexes were predominantly reported to influence actin dynamics, but recently a complex of the spectraplakin ACTIN CROSSLINKING FAMILY 7 (ACF7) and HsELMO1 was found to promote microtubule capture and stability during cell migration (Margaron et al. 2013).

HvMAGAP1 is associated to MTs and influences MT arrays (Hoefle et al. 2011). Therefore, HvELMOD_C might participate in the regulation of MT dynamics. However, so far we found no direct evidence for such a function of HvELMOD_C, because HvELMOD_C interfered HvMAGAP1 function without an obvious effect on MT structure or arrays.

Human ELMOD2 participates in the antiviral response through interferon induction and is essential for the Toll-like-receptor 3 activated antiviral response in human alveolar epithelial A549 cells (Pulkkinen et al. 2010). Further ELMO proteins and GTPases are involved in effector triggered entry of bacteria into metazoan cells (Gruenheid and Finlay 2003). *Yersinia* triggers RHOG activation via integrins and subsequent RAC1 activation with the ELMO-DOCK180 complex in the invasive phase, followed by inactivation of RHOG by the type III effector YOPE during the immune suppressive phase (Roppenser et al. 2008). The *Shigella* type III effector IpgB1 directly targets the ELMO-DOCK180 complex promoting membrane ruffles to facilitate bacterial entry by mimicking the function of RHOG (Handa et al. 2007).

The interaction of barley HvELMOD_C with HvMAGAP1 suggests a distinct mechanism of ELMOD function. Binding of HvELMOD_C to the ROP-GAP domain of HvMAGAP1 provides the potential for a regulatory role of HvELMOD_C on activity of HvMAGAP1. Accordingly, HvELMOD_C neutralized the resistance-inducing effect of HvMAGAP1 during the interaction of barley with *Blumeria graminis* f.sp. *hordei* possibly by down-regulation of HvMAGAP1 activity on HvRACB. Over-expression of HvELMOD_C alone, however, had no influence on the interaction. Hence, HvEMLOD_C function on HvMAGAP1 might be limited by the HvMAGAP1 expression level. Transient induced gene silencing of *HvELMOD_C* also did not result in significantly altered resistance to *B. graminis* f. sp. *hordei*. Hence, although HvEMLOD_C binds HvMAGAP1 *in planta* and interferes with its function, when ectopically expressed, the intrinsic function of HvELMOD_C remains to be elucidated. The lack of an effect of over- and under-expression, however, does not exclude a function in plant-microbe interaction.

Replacement of the catalytic arginine by glycine in the ROP-GAP domain of HvMAGAP1 abolished interaction of HvELMOD_C with HvMAGAP1. HvMAGAP-R185G can still bind to HvRACB in yeast and *in planta* suggesting that HvMAGAP-R185G folds normally (Fig. 2a; Hoefle et al. 2011). This supported the possibility that HvELMOD_C regulates function of HvMAGAP1 by binding to its GAP domain. HvEMLOD_C might thus compete with HvRACB for access to the GAP domain of HvMAGAP1. This competition may prevent deactivation of HvRACB, thus rendering HvRACB in the active status (Fig. 5). The ELMO-DOCK complex activates RAC1 and RAC homologs in animal cells. In both cases more active RHO signalling might result from EMLO protein function albeit via another mechanism.

Characterization of HvELMOD_C as interaction partner of HvMAGAP1 opens the field for new mechanisms of regulating GTPase activity in plants. We suggest for HvELMOD_C a function in the regulation of the ROP-GAP activity of HvMAGAP1 (Fig. 5). Further studies may reveal if HvELMOD_C is involved in the control of cytoskeleton dynamics or of ARF functions in plant cells.

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Figure Legends

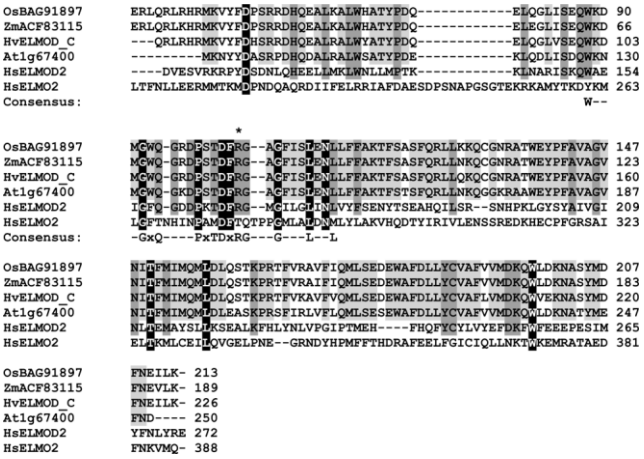

Fig. 1 Alignment of selected ELMO domains from predicted ELMO proteins of barley, *Arabidopsis*, corn, rice and humans. ELMO domains from barley (*Hordeum vulgare*: Hv), rice (*Oryza sativa*: Os), *Arabidopsis thaliana* (At) and corn (*Zea maydis*: Zm) including the human ELMOD protein HsELMOD2 (Acc. no.: NP714913) and the human ELMO protein HsELMO2 (Acc. no.: NP_877496) are compared. Accession numbers of the plant ELMO domain containing proteins are as indicated. Sequences were aligned using ClustalW2 (www.ebi.ac.uk/Tools/msa/clustalw2/). The ARF-GAP consensus sequence is given below the sequences, whereas the catalytically important arginine is marked by an asterisk above the sequences. Conserved amino acids in all sequences are shaded in black, conserved amino acids of ELMOD proteins are shaded in dark grey and conserved amino acids in plant ELMOD proteins are shaded in light grey.

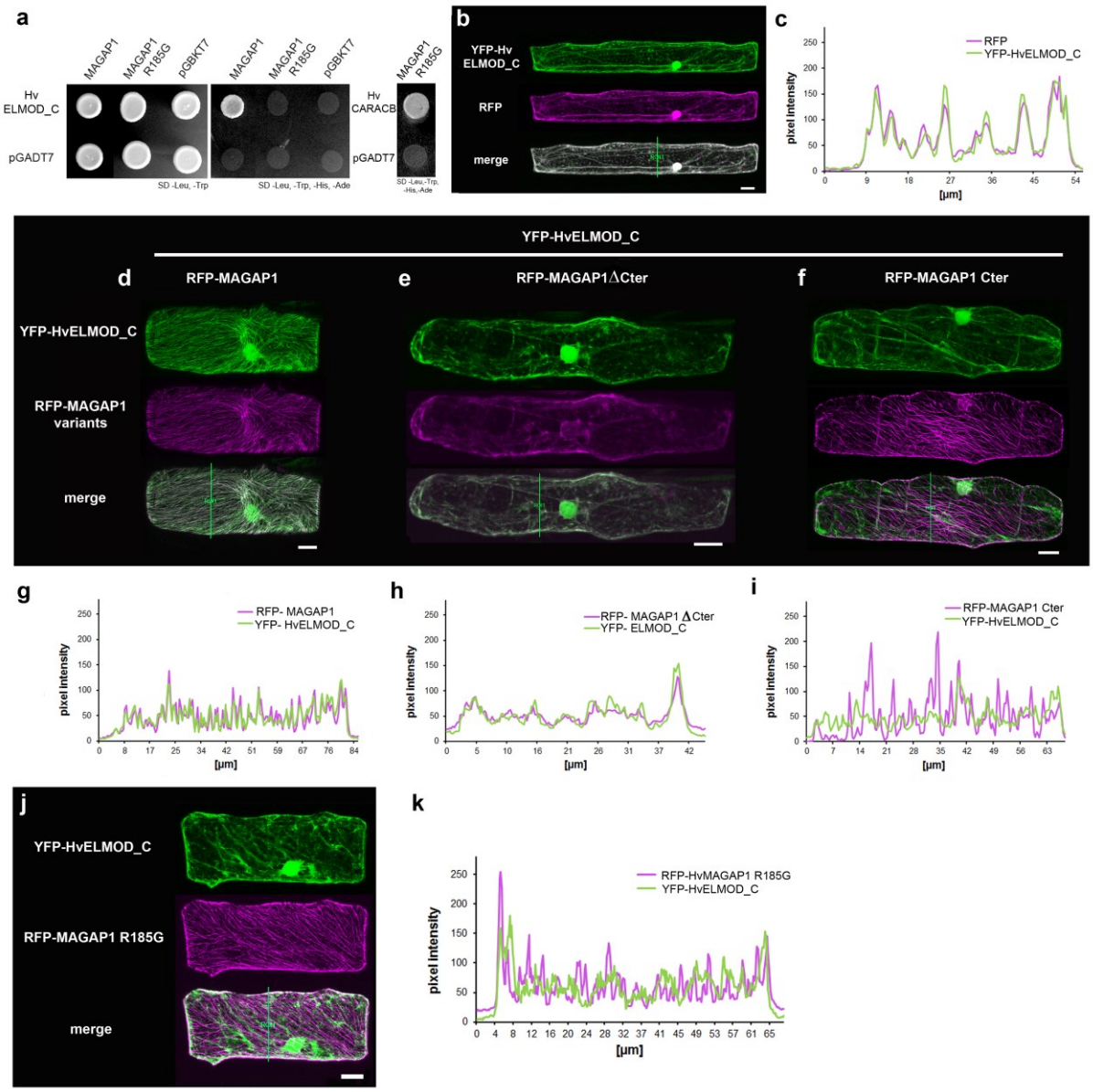

Fig. 2 HvELMOD_C interacts with HvMAGAP1 at the microtubules. **a** Targeted yeast two hybrid assay of HvELMOD_C and HvMAGAP1 wild type and mutant protein HvMAGAP1 R185G in the yeast strain AH109. Yeast was co-transformed with fusion constructs of HvMAGAP1 and HvMAGAP1R185G in pGBKT7 and HvELMOD_C in pGADT7. Transformants were adjusted to the same optical density and dropped in parallel on SD-medium lacking leucine (leu) and tryptophan (trp) as control for positive transformation and on SD-medium lacking leucine (leu), tryptophan (trp), histidine (his) and adenine (ade) for selection of positive interactions. No growth was observed when the empty vector was co-transformed with the fusion constructs as control. Interaction of constitutively activated CA RACB with HvMAGAP-R185G served as a positive control for proper folding of HvMAGAP-R185G in yeast. **b** The YFP-HvELMOD_C fusion protein revealed cytoplasmic localization similar to the soluble RFP that was co-transformed as cytoplasmic and nucleoplasmic

marker. **c, g, h, i** and **k** Intensity plots of the transversal sections in the YFP-HvELMOD_C and RFP or RFP-HvMAGAP1 variants expressing barley cells are indicated in the respective pictures. **d** Upon transient co-expression in barley cells with RFP-HvMAGAP1, YFP-HvELMOD_C co-localized with the RFP-HvMAGAP1 fusion protein on cortical MTs. **e** Cytoplasmic co-localization of the both fusion proteins was observed when YFP-HvELMOD_C was co expressed with RFP-HvMAGAP1ΔCter (AAs 1-328). No recruitment of YFP-HvELMOD_C to the MTs was detected in barley cells transiently co-transformed with RFP-HvMAGAP1 Cter (AAs 319-484) lacking the N-terminus, the CRIB and the GAP domain **f,** or RFP-HvMAGAP1 R185G **j**.

Barley cells were transiently transformed with YFP-HvELMOD_C (green) and respective RFP or RFP fusion constructs of full length and mutant HvMAGAP1 or HvMAGAP1 domains (magenta). Co-localization of green and magenta pixels are indicated by pink or white color. Pictures are maximum projections of 20-30 optical sections with 2 µm increment. The scale bar is 20 µm in all pictures. Pixel intensity was measured along transversal sections across the cell axis indicated in the pictures.

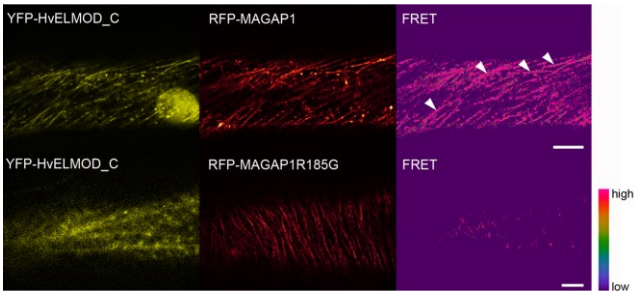

Fig. 3 FRET suggests interaction of HvMAGAP1 and HvELMOD_C *in planta*. Strong FRET signals on the microtubules (arrowheads) indicate interaction of RFP-MAGAP1 and YFP-HvELMOD_C in transiently transformed barley epidermal cells. No distinct FRET signal results from the co-transformation of barley cells with RFP-MAGAP1R185G and YFP-HvELMOD_C. Pictures represent single optical sections through the periclinal cell cortex. FRET intensity is shown with the pseudo colored scale at the right. The scale bar is 10 µm in all pictures of zoomed cell details.

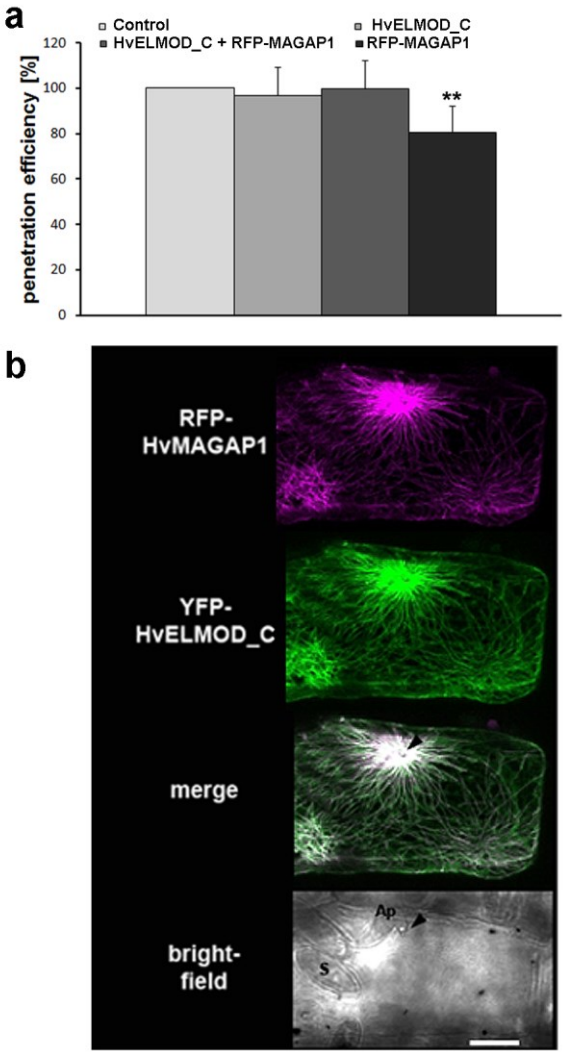

Fig. 4 HvELMOD_C abolished the resistance inducing effect of RFP-HvMAGAP1 in barley towards penetration of *Blumeria graminis* f.sp. *hordei.* **a** Columns show relative penetration success of *B. graminis* f.sp. *hordei* in barley cells transiently transformed with over-expression constructs of *HvELMOD_C* and *RFP-HvMAGAP1*. The empty expression vector or the non-fused RFP was used as control. *HvELMOD_C* expression alone had no effect on the fungal penetration success. Over-expression of *RFP-HvMAGAP1* resulted in a significant reduction of the fungal penetration success, whereas co-expression of *HvELMOD_C* compensated this effect, when co-expressed. Error bars present confidence intervals with alpha = 0.05. Results are significant after a two sided student's t-test $p < 0.01$. **b** *RFP-HvMAGAP1* and *YFP-HvELMOD_C* co-localize on MTs in a barley cell attacked by *Blumeria graminis* f.sp. *hordei* 18 hours after inoculation. Ap, appressorium; S, Spore arrow head shows the site of attack. Scale bar = 20 µm.

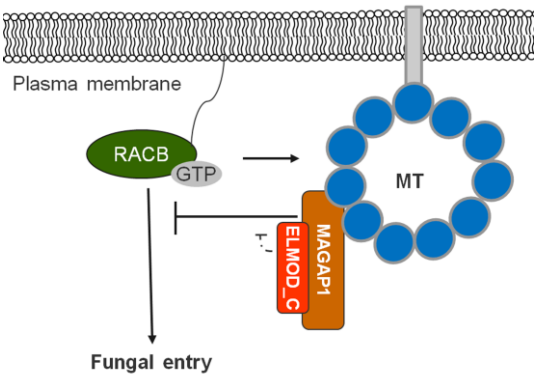

Fig. 5 Hypothetical interplay of HvEMLOD_C, HvMAGAP1 and HvRACB in barley susceptibility to fungal entry. Active GTP-bound HvRACB supports susceptibility to fungal entry and influences microtubule (MT, blue, cross-sectioned in the model) arrays and stability. By contrast, HvMAGAP limits fungal penetration success most likely antagonizing HvRACB downstream effects via activating GTPase activity of HvRACB, which leads to switch off. HvEMLOD_C function is limited by HvMAGAP1. This can be explained by binding of HvELMOD_C to the ROP-GAP domain of HvMAGAP1.