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A barley powdery mildew fungus non-autonomous retrotransposon encodes a peptide that supports penetration success on barley

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Abstract

Pathogens overcome plant immunity by means of secreted effectors. Host effector targets often act in pathogen defense, but might also support fungal accommodation or nutrition. The barley ROP GTPase HvRACB is involved in accommodation of fungal haustoria of the powdery mildew fungus Blumeria graminis f.sp. hordei (Bgh) in barley epidermal cells. We found that HvRACB interacts with the ROP-interactive peptide 1 (ROPIP1) that is encoded on the active non-long terminal repeat retroelement Eg-R1 of Bgh. Overexpression of ROPIP1 in barley epidermal cells and host-induced post-transcriptional gene silencing (HIGS) of ROPIP1 suggested that ROPIP1 is involved in virulence of Bgh. Bimolecular fluorescence complementation and co-localization supported that ROPIP1 can interact with activated HvRACB in planta. We show that ROPIP1 is expressed by Bgh on barley and translocated into the cytoplasm of infected barley cells. ROPIP1 is recruited to microtubules upon co-expression of MICROTUBULE ASSOCIATED ROP GTPase ACTIVATING PROTEIN (HvMAGAP1) and can destabilize cortical microtubules. The data suggest that Bgh ROPIP targets HvRACB and manipulates host cell microtubule organization for facilitated host cell entry. This points to a possible neo-functionalization of retroelement-derived transcripts for the evolution of a pathogen virulence effector.

Keywords: Blumeria graminis, effector-triggered susceptibility, Hordeum vulgare, microtubule, retrotransposon, ROP GTPase, susceptibility factor, virulence.

Introduction

Considerable effort is invested in the understanding of plant immunity against infection by pathogens (Spoel and Dong, 2012) and the underlying genes such as resistance genes (R-genes) or quantitative trait loci (QTLs) that might be used in breeding for crops with improved resistance. In the general model, plant immunity towards invading pathogens is made up of two main layers, namely pattern-triggered immunity (Macho and Zipfel, 2014) and effector-triggered immunity (Spoel and Dong, 2012). Adapted pathogens evolved means to overcome host immunity, which is mainly attributed to secreted effector proteins that manipulate host cellular processes for the benefit of the pathogen. Plant hosts on the other hand evolved resistance proteins that directly or indirectly recognize the presence of a corresponding effector, or the action of effector proteins...
on their host targets, or on host decoy proteins that molecularly mimic host targets. Resistance protein signaling accelerates and increases defense responses typically resulting in the hypersensitive cell death response, thereby restricting further proliferation of biotrophic and hemibiotrophic pathogens. The exerted mutual selection pressure drives co-evolution of host R-genes and pathogen effectors (Jones and Dangl, 2006).

The investigation of host factors that allow susceptibility against a pathogen is an alternative approach to searching for factors of host immunity. The products of susceptibility (S) genes might function in the regulation of plant defense responses or cell death. Alternatively, S-factors can be part of essential cellular processes from which the pathogen profits or that are co-opted by pathogens. The loss of function of S-gene products creates the chance for durable pathogen control due to the loss of a cellular function required for compatibility, given that possible pleiotropic effects are not detrimental for plant cultivation (for a review, see van Schie and Takken, 2014). A paradigm example for making use of the loss of S-gene functionality is the MLO gene, that represents a negative regulator of basal resistance against powdery mildews. Loss of MLO function is associated with powdery mildew resistance in diverse commercially important crop plant species (Kusch and Panstruga, 2017).

The ascomycete Blumeria graminis f.sp. hordei (Bgh) grows and reproduces on living host tissue where it causes barley powdery mildew. Bgh forms an appressorium and an infection peg for penetration of the host epidermis at 10–15 h after inoculation (hai). This penetrates and differentiates into a mature haustorium up to 48 hai. Haustoria stay separated from the host cell cytoplasm by the extrahaustorial membrane and a surrounding extrahaustorial membrane. In addition to expanding the surface for absorption of carbohydrates and amino acids (Voegele et al., 2001), haustoria may serve for effector delivery into host cells. Penetration of the host cell is a prerequisite for further epidermal development and asexual reproduction of Bgh.

The genomes of Bgh and of the close relative Blumeria graminis f.sp. tritici (Bgt) have been sequenced (Spanu et al., 2010; Wicker et al., 2013). Effector proteins of B. graminis are identified either via their avirulence (Avr) function if they are recognized by corresponding R-proteins or because of canonical characteristics of secreted effector proteins. Bgh encodes >500 candidate secreted effector proteins (CSEPs) (Pedersen et al., 2012) identified by defined criteria for effector architecture. Some CSEPs are alternatively called BECs for Blumeria effector candidates, if they have been found to be expressed in Bgh-infected barley tissue (Bindschedler et al., 2009; Pliego et al., 2013). Recently, several CSEP proteins were shown to act as Avr factors in race-specific resistance of wheat and barley (Bourras et al., 2015; Lu et al., 2016; Praz et al., 2017). Bgh also encodes 1350 paralogous copies of the second class of Bgh effector candidates, EKAs (effectors homologous to AvrK1 and AvrRgr10), which do not encode N-terminal signal peptides. The EKAs AvrT10 and AvrK1 are reported to be recognized by the corresponding barley R–proteins MLA10 and MLK1, respectively (Ridout et al., 2006; Shen et al., 2007; Nowara et al., 2010). AvrT10 and AvrK1 evolved from 3’-truncated ORF1 proteins of Bgh long-interspersed element (LINE) retrotransposons (Amselem et al., 2015). The ~120 Mb genome of Bgh and other powdery mildews is highly enlarged in comparison with the ascomycete mean, which was attributed to a high abundance of transposable elements (TEs). The genome of Bgh was estimated to be composed of ~65% TEs, and ~75% repetitive DNA content in total (Spanu et al., 2010); >90% repetitive DNA content was estimated for Bgt (Wicker et al., 2013). Both species show a substantial loss in gene number including genes for enzymes of primary and secondary metabolism. This might reflect their adaption to their obligate biotrophic lifestyle with a reduced gene set and some biological functions provided by the host.

The bulk of TE content in the Bgh genome are class I retrotransposons. Of these, non-long terminal repeat (LTR) retrotransposons are more abundant than the retrovirus-related LTR retrotransposons. Within non-LTR retrotransposons, autonomous LINEs are more abundant than non-autonomous short-interspersed elements (SINEs) that typically need LINE assistance for retrotransposition as they do not encode the required proteins. The SINE-classified non-LTRs Eg-R1 (Wei et al., 1996) and Egh24 (Rasmussen et al., 1993), for example, cover ~10% of the Bgh genome space (Spanu et al., 2010).

The Hordeum vulgare (Hv) small monomeric Rho of plants (ROP) GTpase HvRACB has been shown to support Bgh haustorial ingrowth into barley epidermal cells when expressed as a constitutively activated (CA) mutant (Schultheiss et al., 2003; Scheler et al., 2016). Vice versa, RNAi-mediated silencing of HvRACB restricts haustorial invasion (Schultheiss et al., 2002; Hoefle et al., 2011; Scheler et al., 2016). The activated GTP-bound HvRACB protein may thus support susceptibility. Two HvRACB-interacting barley proteins negatively regulate GTP-bound HvRACB. HvMAGAP1 is a microtubule- (MT) associated ROP-GTpase-activating protein (ROP-GAP) that apparently stimulates GTP hydrolysis depending on the catalytic arginine finger of its GAP domain (Hoefle et al., 2011). Barley ROP-binding kinase1 (HvRBK1) is an active cytoplasmic receptor-like kinase, whose activity is stimulated by CA HvRACB in vitro and that directly binds to CA HvRACB in planta (Huesmann et al., 2012). HvRBK1 in turn interacts with components of an E3 ubiquitin ligase complex and controls protein abundance of activated HvRACB (Reiner et al., 2016).

Besides its role as an S-factor, HvRACB appears to function in polar cell growth processes (Hoefle et al., 2011; Scheler et al., 2016). Other plant ROP GTpases act in plant immunity (Kawano et al., 2014). However, HvRACB apparently does not influence the ability of barley to express canonical PTI responses such as generation of reactive oxygen species (ROS) and phosphorylation of mitogen–activated protein kinases (Scheler et al., 2016).

Here, we report on the HvRACB-interacting Bgh ROP–interactive peptide 1 (ROP1P1) that is encoded on the Bgh SINE–like retroposon Eg–R1. Our study suggests that ROP1P1 acts as a secreted intracellular virulence factor of Bgh.

Materials and methods

Plant growth and pathogen infection

Barley (Hordeum vulgare L.) cultivar ‘Golden Promise’ was grown at 18 °C, 60% relative humidity under a photoperiod of 16 h and a photon flux of 150 μmol s⁻¹ m⁻². Blumeria graminis (DC) Speer f.sp. hordei Em. Marchal,
race A6 (Waber, 1974) was propagated on barley cultivar ‘Golden Promise’ under the same conditions. For protein extraction, 7-day-old barley plants were inoculated with ~150 conidia mm⁻² and left to grow until 10 days after inoculation (dai). The first leaves were inoculated with ~150 conidia mm⁻² for reverse transcription–PCR (RT–PCR) and harvested at the indicated time points, or were inoculated with ~300 conidia mm⁻² and left to grow until 3 dai for immunogold labeling and TEM. Transiently transformed detached 7-day-old primary leaves kept on 0.5% water–agar were inoculated with ~150 conidia mm⁻² at 24 h after transformation (hat).

**Targeted Y2H**

ROP1IP1 was identified by DNA sequencing of positive prey clones from a yeast two-hybrid (Y2H) screen using HvRACB, CA HvRACB, and CA HvRAC1 as bait against a cDNA library prepared from Bgh-infected barley leaves, as in Hoefle et al. (2011). For targeted Y2H assays, yeast strain AH109 MATa was co-transformed with pGBK7 bait plasmids and pGADT7 prey plasmids following the small-scale LiAc yeast transformation procedure (Clontech, Heidelberg, Germany).

ROP1IP1-Nter was PCR-amplified from pGADT7-ROP1IP1 using primers V42A_SmaI_F and R_V42A_Nter_BamHI (Supplementary Table S3 at JXB online), and Smal/BamHI-cloned into pGADT7. ROP1IP1-Cter was PCR-amplified from C-ROP1IP1 using primers F_V42ACter_Sma and R_V42ACter_Bam, and SmaI/BamHI-cloned into pGADT7. Cloning of barley ROP proteins into the pGBK7 vector is described in Schultheiss et al. (2008). Transformed cells were selected on SD medium lacking Leu and Trp (–L–W), resuspended in ultrapure water and spotted on SD–L–W and on interaction selective SD medium lacking Ade, His, Leu and Trp (–A–H–L–W), 3-Amino-1,2,4-triazole (3-AT) and spotted on SD-L-W and on interaction selective SD medium lacking Leu and Trp (–L–W), resuspended in ultrapure water and spotted on SD–L–W and on interaction selective SD medium lacking Ade, His, Leu and Trp (–A–H–L–W), 3-Amino-1,2,4-triazole (3-AT) was optionally added in concentrations from 0.5 mM to 2.5 mM to the SD-A–H–L–W medium to increase selectivity.

**Transient transformation of barley leaf epidermal cells**

Primary leaves of 7-day-old barley plants were cut and placed on solid 0.5% water–agar. Plasmids were coated to 1.0 µm gold particles (BioRad) and bombarded into barley epidermal cells using the PDS-1000/He (Bio-Rad) system as described earlier (Douchkov et al., 2005; Eichmann et al., 2010).

**Transient overexpression and HIGS**

For transient overexpression, ROP1IP1 and ROP1IP1-Cter were PCR-amplified from cDNA using 5’-oligos V20A,V42ABamHI_fwd and V42A,V20B_BamHI_kurz, respectively, and 3’-oligo V42A,V20B_Brev. A/T cloned into pGEM-T (Promega), and Smal I/BamHI cleavage site, resulting in pGY1-GFP-ROP1IP1-RNAi-rescue. For the HIGS experiment, 1.0 µg per shot of pGY1T30N-ROP1IP1, or empty pGY1T30N (control) plus either 1.0 µg per shot of pGY1-ROP1IP1-RNAi-rescue or empty pGY1 and 0.5 µg per shot of pGY1-GFP each were bombarded into barley epidermal leaf cells. Assessment of fungal development on GFP-expressing cells took place at 48 h, as described above for the overexpression experiment.

**Western blot**

Total protein extracts from heavily Bgh-infected barley primary leaves or mock-treated control leaves were prepared using the Plant Total Protein Extraction kit (Sigma-Aldrich) following the manufacturer’s instructions. Around 200 µg of liquid N₂-ground barley leaf powder was used for 250 µl of Protein Extraction Reagent Type 4. The protein concentration was determined by a Bradford assay. An aliquot of 50–100 µg of total protein per lane was separated by SDS–PAGE on hand-cast mini-gels (15% resolving gel, 4% stacking gel) using the Mini-PROTEAN Tetra Cell (Bio-Rad) in the Laemmli (Laemmli, 1970) buffer system; 200 V were applied for up to 45 min. Separated proteins were blotted onto 0.2 µm nitrocellulose membranes using a Fastblot B43 (Biometra) semi-dry blot system. A current of 5 mA cm⁻² was applied for 25 min. Successful protein transfer was checked by Ponceau S staining. Nitrocellulose membranes were destained by two rounds of washing in 1× phosphate-buffered saline (PBS) for 10 min, before blocking in 5.0% non-fat dry milk in PBS for 1 h at room temperature. The blot was incubated with diluted primary antibodies (total barley protein extracts, 1:100; recombinant *Eichhornia crassipes* crude lysates, 1:10 000) in blocking buffer overnight at 4°C. After three rounds of washing in PBS-T each for 15 min, blots were incubated with anti-rabbit-horseradish peroxidase (Sigma-Aldrich) secondary antibodies diluted 1:80 000 in blocking buffer for 2 h at room temperature and washed again for three rounds. The SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific) was used as the ECL substrate. Chemiluminescence was detected with a Fusion-SL4 system operated with FusionCapture Advanced Solo 4 (version 16.06) software. The custom antipeptide antibody α-ROP1IP1 (Pineda Antibody Service, Berlin, Germany) was raised against the synthesized peptide NH₂-IPSRLRDLYRLHF-COOH in rabbits in a 145 d course protocol and purified to ≥95% by affinity chromatography.

**Heterologous expression of recombinant ROP1IP1**

ROP1IP1 was PCR amplified from plasmid using primers B8B21B_H1fwd and V42A,V20B_Bsalrev (Supplementary Table S3), and BamHI/SalI cloned into the pET28b(+) vector. The pET28b–ROP1IP1–6His plasmid was further digested with Ndel/BamHI to excise additional ATG start codons in the multiple cloning site (MCS). Sticky ends were blunted and the plasmid religated. The resulting pET28b–6His-ROP1IP1–6His plasmid was transformed into chemically competent Rosetta (DE3) E. coli.

For crude cell lysate preparation, 50 ml of LB Kan (50 µg ml⁻¹ kanamycin) were inoculated with a 1:100-diluted overnight culture. Small-scale cultures were grown until they reached an OD₆₀₀ of 0.8–1.0. Non-induced aliquots were taken. Recombinant protein expression was induced by addition of isopropyl-β-D-galactopyranoside (IPTG) to a final concentration of 1 mM. Induced and parallel non-induced cultures
were grown at 37 °C for an additional 1–3 h. Crude cell lysates were prepared by resuspending bacterial pellets in 100 µl of Lysis Buffer (50 mM NaH2PO4-H2O, 300 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mg ml-1 lysozyme, pH 8.0) per 1 ml of culture volume and incubation on ice for 30 min. Three rounds of ultrasonic bath incubation for 10 s each, followed, placing the lysates on ice in between each round. Viscosity of lysates was reduced by addition of 50 U of Benzonase (Merck Millipore) per 1 ml of culture volume and a further incubation on ice for 15 min. Up to 10 µl of heat-denatured crude lysate were loaded per lane onto SDS–polyacrylamide gels. Non-induced control samples and IPTG-induced samples were run as duplicates on the same gels followed by western blotting. Afterwards, one half of the nitrocellulose membrane was incubated with α-RoPIP1 as primary antibody and the duplicate half was incubated with anti-His-Hrp (Carl Roth).

RecRoPIP1 was purified with the ProtoNi TiTED 2000 packed columns kit (Macherey Nagel) following the batch gravity–flow purification protocol under native conditions (User Manual, version Rev.04, protocol 5.5).

**Immunocytohistochemical detection of a-RoPIP1**

Sample preparation for TEM and immunogold labeling was performed according to a modified version described previously (Redkar et al., 2015). Briefly, samples were fixed with 2.5% (v/v) paraformaldehyde and 0.5% (v/v) glutaraldehyde in 0.06 M Sørensen phosphate buffer, then rinsed in buffer, dehydrated in acetone, and embedded in LR–White resin (London Resin). Immunogold labeling of α-RoPIP1 was performed on ultrathin sections with an automated immunogold labeling system (Leica EM 1110, Leica Microsystems). The sections were blocked for 20 min with 2% (w/v) BSA (Sigma–Aldrich) in PBS, pH 7.2, and then treated with the primary antibody α-RoPIP1 against RoPIP1 for 90 min diluted 1:100 in PBS containing 1% (w/v) BSA. After sections were washed twice for 5 min with PBS containing 1% (w/v) BSA, they were treated with a 10 nm gold-conjugated secondary antibody (goat anti-rabbit IgG, British BioCell International) diluted 1:100 in PBS containing 1% (w/v) BSA for 90 min. After a short wash in PBS (3 × 5 min), labeled grids were post-stained with 2% uranyl acetate aqueous solution for 15 s and then investigated with a Philips CM10 transmission electron microscope. The ideal dilutions and incubation times of the primary and secondary antibodies were determined in preliminary studies by evaluating the labeling density after a series of labeling experiments. The final dilutions used in this study showed a minimum background labeling outside the sample with a maximum specific labeling in the sample. Various negative controls were performed to confirm the specificity of the immunocytohistochemical approach. Gold particles were absent on sections when (i) no primary antibody; (ii) a non-specific secondary antibody (goat anti-mouse IgG); and (iii) pre-immune serum instead of the primary antibody was used.

**Live cell imaging**

Transiently transformed barley epidermal leaf cells expressing fluorescent fusion proteins were imaged with a Leica TCS SP5 confocal laser scanning microscope using standard wavelengths for excitation and emission. Barley epidermal cells were scanned as z-stacks in 2 µm increments in sequential scan mode. Maximum projections were exported from the Leica LAS AF software (version 2.5.1) in jpeg or tiff format.

**Quantification of GFP–RoPIP1 MT localization and destruction**

GFP was cloned in-frame with RoPIP1 into the 5’-BamHI restriction site of pGY1-RFP-HvHvMAGAP1 and variants is described in Hoele et al. (2011). Barley epidermal cells were transiently transformed with 0.5 µg per shot of pGY1-GFP or 0.75 µg per shot of pGY1-GFP-RoPIP1 plus 1.0 µg per shot of pGY1-RFP-HvHvMAGAP1 or 1.0 µg per shot of pGY1-RFP-HvHvMAGAP1-Cter and imaged as whole–cell scans with 2 µm increments at 12–24 h. For quantification of MT localization of GFP–RoPIP1, cells were categorized into GFP signal present at MT’s or absent from MTs. The numbers of categorized cells were compared between cells co-expressing red fluorescent protein (RFP)–HvHvMAGAP1 or RFP–HvHvMAGAP1-Cter together with GFP–RoPIP1 in a χ² test with df=1. For quantification of the MT network organization, maximum projections were categorized into intact, disordered, or fragmented MTs. The distribution of the absolute cell numbers per category was compared between cells co-expressing GFP or GFP–RoPIP1 along with RFP–HvHvMAGAP1 in a χ² test with df=2.

**Bimolecular fluorescence complementation**

RoPIP1 was PCR–amplified from plasmid using 5’-oligo V20A, V42ABamHI fwd and 3’-oligo V42A, V20Bsalrev, and BamHI/SulI cloned into the MCS of pUC–SPYNE (Walter et al., 2004) which translated into ROPIP–YFPSP. The cloning of pUC–SPYCE–CA HvRACB and pUC–SPYCE–DN HvRACB, both translating into an N-terminal fusion of YFPC to CA/DN (dominant negative) HvRACB, is described in Schultheiss et al. (2008).

Barley leaf epidermal cells (7 d old) were transiently co-transformed with 0.75 µg per shot of pUC–SPYNE–RoPIP1 plus 0.75 µg per shot of pUC–SPYCE–CA HvRACB and pUC–SPYCE–DN HvRACB, 0.5 µg per shot of pGY1–CFP, and 1.0 µg per shot of pGY1–RFP–HvHvMAGAP1–R185G. Transformed cells were identified by cyan fluorescent protein (CFP) fluorescence and imaged by confocal laser scanning microscopy at 36 h. Each fluorophore was excited and detected in an individual scan by sequentially scanning between frames. All hardware and software settings were kept identical for all cells and repetitions.

The bimolecular fluorescence complementation (BiFC) signal was analyzed in a quantitative manner using maximum projections of transformed cells and the Leica LAS AF (version 2.5.1.6757) ‘Quantify’ tool. The first region of interest (ROI 1) was put at the cell periphery of the transformed cell. The second, copy-pasted, ROI 2, was placed into the surrounding background close to the cell. The mean values of fluorescence intensity of the ROIs (mean fluorescence intensity, MFI) of the yellow fluorescent protein (YFP) and the CFP detector were read out from the quantification reports. The background fluorescence MFI (ROI 2) was subtracted from ROI 1. The corrected MFI of the YFP detector was divided by the corrected MFI of the CFP detector. The obtained YFP/CFP MFI ratios of YFP–CA HvRACB and YFP–DN HvRACB-co-expressing cells were compared in a two-sided Student’s t-test. The corrected CFP MFIs were also compared in a two-sided Student’s t-test and did not differ.

**5’-RACE-PCR**

The Dynabeads mRNA Direct Kit (Thermo Scientific) was used according to the manufacturer’s instruction for isolation of poly(A) RNA from Bgl2-infected barley primary leaves. After Dnase I digestion, the isolation process was repeated. A 0.5–1.0 µg aliquot of poly(A) RNA was reverse-transcribed into first-strand cDNA following the instructions of the 5’/3’ RACE kit, 2nd Generation, version 12 (Roche) and using the oligo TW42A_R as the cDNA synthesis primer (Supplementary Table S3). The resulting 5’-tailed cDNA was used as template for PCR amplification using Phusion High-Fidelity DNA Polymerase (Thermo Scientific) and V42A–V20B as the gene-specific primer. V42A–V20B was used as the nested gene-specific primer in a second PCR run. PCR products were gel purified, A-tailed, cloned into pGEM-T (Promega), and sequenced.

**Semi-quantitative RT–PCR**

Bgl2-infected and mock-treated barley primary leaves (7 d old) were cut and immediately frozen in liquid N2. Total RNA was prepared (Chomczynski and Sacchi, 1987), precipitated by NaAc/ethanol to achieve greater purity, and digested with DNase I (Thermo Scientific). First-strand cDNA was synthesized with RevertAid Reverse Transcriptase (Thermo Scientific) using oligo(dt)15 primer (Promega). The barley Ubiquitin Conjugating Enzyme 2 (HvUBC2; AY220735.1) gene was amplified using the oligo pair HvUBC2_fwd and HvUBC2_rev. The barley Basic PR-1-Type Pathogenesis Related Protein (HvPR1b; X74940.1) gene
was amplified using the oligo pair T-PR1b/5’-2 and T-PR1b/3’-2. The Bgh TUB2 Gene For Beta Tubulin (Bgh tub2; AJ313149) gene was amplified using the oligo pair Bgh_beta-tub_F and Bgh_beta-tub_R. Bgh ROPIP1 transcript was amplified using the oligo pair V42fwd and V42rev.

Results

ROP1P1 is encoded by the retrotransposable element Eg-R1 of Bgh

We performed Y2H screens using the barley ROPs HvRACB (GenBank accession number: AJ344223), CA HvRACB, and CA HvRAC1 (GenBank accession number: AJ518933) as baits against a cDNA library prepared from Bgh-infected barley leaves. Besides the barley proteins, HvMAGAP1 and HvRBK1 (Hoefle et al., 2011; Huesmann et al., 2012), a Bgh-derived cDNA was repeatedly isolated (twice with HvRACB, four times with CA HvRACB, and twice with CA HvRAC1). Sequencing of the respective plasmids isolated from yeast retrieved a polyadenylated transcript and fragments of the same transcript that aligned to its 5’ region. Initial BLAST searches against the NCBI nucleotide database identified the transcript as the non-LTR retroelement Eg-R1 (Wei et al., 1996) (GenBank accession number: X86077.1) of Bgh. The Eg-R1 5’ sequence as obtained from the fragments in-frame with the activation domain of the prey vector would give rise to a 74 amino acids peptide (Supplementary Fig. S1) which interacted with HvRACB, CA HvRACB, and CA HvRAC1 in the bait vectors. Later on, we named this peptide ROP-INTERACTIVE PEPTIDE 1 (ROP1P1) of Bgh.

A BLAST search of the Eg-R1 nucleotide sequence against the assembled Bgh reference genome [BGH DH14 Genome v3b (contigs); https://genome.jgi.doe.gov/Blugr1/Blugr1.home.html] of race DH14 suggested >3000 genomic insertions of the Eg-R1 element and similar numbers in other Bgh races (Hacquard et al., 2013). This number is probably underestimated as, for example, only half of the genome of Bgh race A6 was assembled due to the high repeat content (Hacquard et al., 2013). We randomly selected 53 full-length Eg-R1 genomic insertions for inspection of the direct genomic environment. Interestingly, eight of the 53 insertions showed 5’-elongated ORFs including the 74 amino acids sequence was found encoded on Eg-R1, which is probably a functional since Eg-R1 is expressed as polyadenylated RNA (Wei et al., 1996; Supplementary Fig. S1; see Supplementary Fig. S2 for Eg-R1 architecture). This supports protein-coding gene-like transcription of Eg-R1 by RNA polymerase III. The ROP1P1 nucleotide sequence was amplifiable from cDNA prepared from total RNA extracts as well as from poly(A) mRNA preparations of Bgh-inoculated barley leaves but not from the non-inoculated control (Supplementary Fig. S3). Wei and colleagues (1996) detected Eg-R1 on a northern blot of poly(A) RNA (Wei et al., 1996). Expression of ROP1P1 and Eg-R1 was further supported by BLAST searches against ESTs of Bgh (BGH DH14 All ESTs database) of race DH14 (https://genome.jgi.doe.gov/Blugr1/Blugr1.home.html) and RNAseq data of Bgh race A6 grown on the immunocompromised Arabidopsis thaliana (Hacquard et al., 2013). Genomic insertions of Eg-R1 were found located in the close spatial vicinity of CSEP, where Eg-R1 was suggested to contribute to unequal crossing over events (Pedersen et al., 2012). This might be supported by our finding of truncated Eg-R1 genomic insertions not being reflected by preferential insertion of an Eg-R1 partial sequence, which could have arisen from, for example, incomplete insertion of the element (Supplementary Fig. S4A, B). Eg-R1 is deposited at Repbase (Repbase Report 2011, Volume 11, Issue 9; Jurka et al., 2005) as one member of a family of eight B. graminis non-LTR retrotransposons (BG_Non-LTRs), which were found to be conserved in its 5’ region (Supplementary Fig. S4C, D). In summary, the ROP1P1 sequence was found encoded on Eg-R1, which is probably a member of a class of as yet not well characterized, non-autonomous, RNA polymerase II-transcribed retroelements.

ROP1P1 interacts with barley susceptibility factor HvRACB in yeast

We next verified the ROP1P1–HvRACB protein interaction in yeast by independent targeted Y2H assays. Besides the wild-type and CA HvRACB, the dominant negative mutant DN HvRACB and HvMAGAP1 (GenBank accession number: AK371854) were additionally included as bait proteins. Yeast colony growth of the prey–bait combinations ROP1P1–HvRACB and ROP1P1–CA HvRACB exceeded all other combinations on interaction-selective media (Fig. 1A; Supplementary Fig. S5A). Weak background growth of the ROP1P1 prey was

EG-R1 was originally described as a repetitive element that shares some features with SINEs but which is also distinct from classical SINEs (Wei et al., 1996). SINEs typically share sequence similarities with tRNAs, 7SL RNA, or 5S rRNA from which they may derive (Kramarov and Vassetzky, 2011). All these are transcribed by RNA polymerase III. As reported by Wei and colleagues (1996), Eg-R1 lacks A-box and B-box RNA polymerase III transcription initiation sites within its 5’ region. Furthermore, internal poly(T) stretches would act as RNA polymerase III termination signals such that a RNA polymerase III transcript would be truncated, which renders transcription by RNA polymerase III very unlikely. Genomic insertions of Eg-R1 lacked genomic poly(A)-coding stretches at their 3’ ends but comprised a 5’-AAUAAA-3’ polyadenylation signal, which is obviously functional since Eg-R1 is expressed as polyadenylated RNA (Wei et al., 1996; Supplementary Fig. S1; see Supplementary Fig. S2 for Eg-R1 architecture). This supports protein-coding gene-like transcription of Eg-R1 by RNA polymerase II. The ROP1P1 nucleotide sequence was amplifiable from cDNA prepared from total RNA extracts as well as from poly(A) mRNA preparations of Bgh-inoculated barley leaves but not from the non-inoculated control (Supplementary Fig. S3). Wei and colleagues (1996) detected Eg-R1 on a northern blot of poly(A) RNA (Wei et al., 1996). Expression of ROP1P1 and Eg-R1 was further supported by BLAST searches against ESTs of Bgh (BGH DH14 All ESTs database) of race DH14 (https://genome.jgi.doe.gov/Blugr1/Blugr1.home.html) and RNAseq data of Bgh race A6 grown on the immunocompromised Arabidopsis thaliana (Hacquard et al., 2013). Genomic insertions of Eg-R1 were found located in the close spatial vicinity of CSEP, where Eg-R1 was suggested to contribute to unequal crossing over events (Pedersen et al., 2012). This might be supported by our finding of truncated Eg-R1 genomic insertions not being reflected by preferential insertion of an Eg-R1 partial sequence, which could have arisen from, for example, incomplete insertion of the element (Supplementary Fig. S4A, B). Eg-R1 is deposited at Repbase (Repbase Report 2011, Volume 11, Issue 9; Jurka et al., 2005) as one member of a family of eight B. graminis non-LTR retrotransposons (BG_Non-LTRs), which were found to be conserved in its 5’ region (Supplementary Fig. S4C, D). In summary, the ROP1P1 sequence was found encoded on Eg-R1, which is probably a member of a class of as yet not well characterized, non-autonomous, RNA polymerase II-transcribed retroelements.

ROP1P1 interacts with barley susceptibility factor HvRACB in yeast

We next verified the ROP1P1–HvRACB protein interaction in yeast by independent targeted Y2H assays. Besides the wild-type and CA HvRACB, the dominant negative mutant DN HvRACB and HvMAGAP1 (GenBank accession number: AK371854) were additionally included as bait proteins. Yeast colony growth of the prey–bait combinations ROP1P1–HvRACB and ROP1P1–CA HvRACB exceeded all other combinations on interaction-selective media (Fig. 1A; Supplementary Fig. S5A). Weak background growth of the ROP1P1 prey was
abolished when plating yeast on 2.5 mM 3-AT (Fig. 1B). No colony growth was observable when ROPIP1 was combined with either DN HvRACB or HvMAGAP1. ROPIP1 also did not interact (for CA HvRACD, CA HvROP6, and CA HvRAC3) or weakly interacted with other barley ROP baits (HvRAC1 and CA HvRAC1) (Supplementary Fig. S5A). There is no obvious ATG start at the very 5' end of the Eg-R1 nucleotide sequence we found in the Y2H screening. However, there is an ORF in the same reading frame of the ROPIP1 sequence, which translates into a shorter peptide of 44 amino acids and which we refer to as ROPIP1-Cter (Supplementary Fig. S1). Interestingly, the ATG start and TGA stop codons of ROPIP1-Cter are present in the majority of Eg-R1 full-length genomic insertions (see, for example, Supplementary Table S2 for the Eg-R1 consensus sequence) but not conserved in the other Bg-non-LTRs. In order to delimit the HvRACB-interacting part, ROPIP1 was split into ROPIP1-Cter and the remaining N-terminal part (ROPIP1-Nter) which did not interact with the baits. A total of 10^6 cells of each combination were dropped in parallel on SD-Leu,-Trp (-L-W) as transformation control and on SD-Ade,-His,-Leu,-Trp (-A-H-L-W) selection medium. (B) Serial dilution of 10^5–10^7 yeast cells transformed with pGADT7-ROPIP1 as prey vector and pGBK77-HvRACB WT as bait vector or pGBK77-empty as empty vector control. Left panel: transformation control medium (SD-L-W). Right panel: selection medium (SD-A-H-L-W) supplemented with 2.5 mM 3-AT to increase selectivity. (This figure is available in colour at JXB online.)

**Fig. 1.** *Bgh* ROPIP1 and ROPIP1-Cter interacted with barley HvRACB and CA HvRACB in yeast. (A) ROPIP1 of *Bgh* was tested as prey in targeted Y2H assays for interaction with the barley small GTPase HvRACB in three different variants: WT, wild-type protein; CA, constitutively activated mutant (HvRACB G15V); DN, dominant negative mutant (HvRACB T20N) and with the HvRACB-interacting protein HvMAGAP1. The ROPIP1 sequence was additionally split into its small inherent C-terminal ORF (ROPIP1-Cter) which was sufficient for protein interaction with WT HvRACB and CA HvRACB and the remaining N-terminal part (ROPIP1-Nter) which did not interact with the baits. A total of 10^5 cells of each combination were dropped in parallel on SD-Leu,-Trp (-L-W) as transformation control and on SD-Ade,-His,-Leu,-Trp (-A-H-L-W) selection medium. (B) Serial dilution of 10^5–10^7 yeast cells transformed with pGADT7-ROPIP1 as prey vector and pGBK77-HvRACB WT as bait vector or pGBK77-empty as empty vector control. Left panel: transformation control medium (SD-L-W). Right panel: selection medium (SD-A-H-L-W) supplemented with 2.5 mM 3-AT to increase selectivity. (This figure is available in colour at JXB online.)

ROPIP1 enhances virulence of *Bgh*

As ROPIP1 interacted with the S-factor HvRACB, we checked whether ROPIP1 can affect the susceptibility of barley against *Bgh*. Therefore, we transiently expressed ROPIP1 in barley epidermal cells by microprojectile bombardment prior to inoculation with *Bgh* conidial spores at 24 hai and microscopic analysis of fungal development at 48 hai. To express the full ROPIP1 sequence including the ROPIP1-Nter and ROPIP1-Cter *in planta*, we equipped the sequence with an additional ATG start codon at its very 5' end (Supplementary Tables S1, S2). Transformed cells were identified by co-bombarded GFP. Overexpression of ROPIP1 led to a significant increase (P<0.05, Student's t-test) in susceptibility to fungal penetration of transformed barley leaf epidermal cells. This was evident from an enhanced frequency of attacked cells with fungal haustoria. Hence, ectopic expression of ROPIP1 promoted virulence of *Bgh* (Fig. 2A). The relative penetration
Co-expression of a ROPIP1-RNAi-rescue construct (RNAi rescue) significantly complemented HIGS of the native transcript. Bars represent the mean values of six independent experiments in (A) and four independent experiments in (B). Error bars are ±SE. * \( t \leq 0.05 \) (Student’s \( P \)-test).

Fig. 2. ROPIP1 modulated susceptibility of barley epidermal cells towards \( Bgh \). (A) Transient overexpression of ROPIP1 and ROPIP1-Cter in barley epidermal cells significantly increased the relative penetration rate of \( Bgh \) in comparison with the control. (B) Host-induced gene silencing (HIGS) of native ROPIP1 by transient expression of ROPIP1 as dsRNA (ROPIP1-RNAi) in barley epidermal cells significantly decreased the relative penetration rate of \( Bgh \). Co-expression of a ROPIP1-RNAi-rescue construct (RNAi rescue) significantly complemented HIGS of the native ROPIP1 transcript. Bars represent the mean values of six independent experiments in (A) and four independent experiments in (B). Error bars are ±SE. * \( P \leq 0.05 \) (Student’s \( t \)-test).

rate increased thereby by ~40%. Ectopic overexpression of ROPIP1-Cter in barley epidermal cells had an effect comparable with albeit somewhat weaker than that of ROPIP1. This added to the view of ROPIP1-Cter being the part of ROPIP1 that promotes virulence of \( Bgh \).

Ectopic expression of double-stranded RNAi constructs in barley epidermal cells proved to be a valuable tool for silencing \( Bgh \) transcripts in a process called HIGS (Nowara et al., 2010; Zhang et al., 2012; Pliego et al., 2013; Ahmed et al., 2015). ROPIP1 was hence cloned as an inverted repeat into the plant RNAi vector pPKTA30N (Douchkov et al., 2005). Off-target prediction using the SI-FI software (Nowara et al., 2010) did not reveal further targets in \( Bgh \) or in barley. For the HIGS experiment, the transformed leaves were inoculated at 24 h with \( Bgh \) conidia followed by microscopic analysis of fungal development at 48 hai. HIGS of ROPIP1 significantly (\( P \leq 0.05 \), Student’s \( t \)-test) reduced the relative penetration rate of \( Bgh \) on transformed cells by 38% (Fig. 2B). We included a synthetic ROPIP1 RNAi-insensitive rescue construct (Supplementary Fig. S6C) in the experiment to ensure that the observed drop in virulence of \( Bgh \) was due to post-transcriptional silencing of ROPIP1. The functionalities of the ROPIP1-RNAi and ROPIP1-RNAi-rescue constructs were tested in advance by transient co-expression experiments and silencing of GFP–ROPIP fusion constructs (Supplementary Fig. S6A). Accordingly, ROPIP1-RNAi-rescue partially but significantly (\( P \leq 0.05 \), Student’s \( t \)-test) rescued the ROPIP1-RNAi-mediated decrease in fungal penetration success (Fig. 2B).

ROPIP1 protein is detectable in \( Bgh \)-infected barley leaf protein extracts

We next investigated whether a native ROPIP1 protein is detectable. A custom rabbit polyclonal antibody, \( \alpha \)-ROPIP1, was raised against a synthesized epitope peptide derived from ROPIP1-Cter. The monospecific IgG fraction was purified to \( \geq 95\% \) by affinity chromatography using the epitope peptide as antigen. Total protein extracts were prepared from heavily \( Bgh \)-infected and non-inoculated barley primary leaves. A unique band in the protein extract of the \( Bgh \)-inoculated sample was repeatedly observable in a series of western blots (Fig. 3A). The band was never seen in the protein extract prepared from non-inoculated samples. Recombinant, \( E. \ coli \)-expressed His-tagged ROPIP1 (recROPIP1) was run as a positive control on the same gel and was detected by \( \alpha \)-ROPIP1 (Fig. 3A). Further, \( \alpha \)-ROPIP1 specifically detected recROPIP1 in crude cell lysates of \( E. \ coli \) cell cultures following induction of recombinant protein expression with isopropyl-\( \beta \)-d-1-thiogalactopyranoside (IPTG). The identity of the signal was confirmed by, first, the absence of the band in the non-induced control, and secondly by probing aliquots of the same crude cell lysates with an independent \( \alpha \)-His antibody, which resulted in an identical signal pattern (Fig. 3B).

\( \text{TEM localizes ROPIP1 in } Bgh \text{ structures and in the host cell cytoplasm.} \)

Next, we analyzed the localization of the protein labeled by \( \alpha \)-ROPIP1 in situ. We used immunogold labeling and TEM. Ultrathin cuts of heavily \( Bgh \)-infected (3 dai) barley primary leaves were incubated with \( \alpha \)-ROPIP1 or an unspecific anti-\( \alpha \)-ROPIP1 antibody as primary antibodies. Primary antibodies were detected by anti-rabbit secondary antibodies conjugated to 10 nm gold particles.

Fungal intra- and extracellular structures, the extracellular space, the cell wall, and the barley epidermal cell interior were almost free from gold particles in the unspecific antibody control (Fig. 4A, and detail in B). In contrast, gold particles labeled fungal and host cell structures when using of \( \alpha \)-ROPIP1 as primary antibody. In a barley epidermal cell, showing a host
In sum, immunogold labeling with α-ROPIP1 detected a secreted Bgh protein that translocated from the fungus into barley epidermal cells, where it could interact with HvRACB.

HvRACB binding HvMAGAP1 recruits ROPIP1 to microtubules

With ROPIP1 being a potential intracellular effector of Bgh, we progressed to live cell imaging of GFP-tagged ROPIP1 by confocal laser scanning microscopy. Transient expression of GFP–ROPIP1 in barley epidermal cells did not show a distinct subcellular localization of ROPIP1. GFP–ROPIP1 labeled the cytoplasm and the nucleus (Fig. 5A). This was in line with the ROPIP1 sequence not showing any predictable cellular localization signatures or protein domains. As HvRACB-interacting proteins associate with MTs or function in regulation of MT network stability, we expressed GFP–ROPIP1 together with the putative HvRACB regulator HvMAGAP1 that has a unique localization at MTs (Hoefle et al., 2011). Although ROPIP1 did not interact with HvMAGAP1 in yeast (Fig. 1), GFP–ROPIP1 was recruited to MTs under co-expression of red fluorescing RFP–HvMAGAP1 (Fig. 5B). The C-terminus of HvMAGAP1 (HvMAGAP1-Cter) mediates MT association of HvMAGAP1 but does not interact with HvRACB because it lacks the ROP-interacting CRIB motif and the GAP domains (Hoefle et al., 2011). Quantification of subcellular fluorescence of GFP–ROPIP1 at 12–24 h revealed that full-length RFP–HvMAGAP1 recruited GFP–ROPIP1 to MTs whereas RFP–HvMAGAP1-Cter hardly co-localized with GFP–ROPIP1 at MTs (P ≤ 0.001, χ², Fig. 5C, D). Instead, GFP–ROPIP1 labeled the cytoplasm, as did soluble GFP upon co-expression of RFP–HvMAGAP1 or RFP–HvMAGAP1-Cter (Fig. 5C, D). Hence, GFP–ROPIP1 localization at cortical MTs depended on RFP–HvMAGAP1 with its corresponding HvRACB–binding domains.

ROPIP1 and CA HvRACB interact in planta and can co-localize with HvMAGAP1

To support that ROPIP1 can interact with activated HvRACB in planta, we performed ratiometric BiFC (Fig. 6A–C) (Miller et al., 2015). Therefore, we fused the N-terminal part of YFP (YFP N) to ROPIP and the C-terminal part (YFP C) to different versions of HvRACB. ROPIP1–YFP N was transiently co-expressed with either YFP C–CA HvRACB or YFP C–DN HvRACB, RFP–HvMAGAP1–R185G, a mutant lacking the catalytic arginine finger of GAP domains (Hoefle et al., 2011), and CFP. The RFP–HvMAGAP1 R185G mutant was chosen as its co-expression with ROPIP1 was seen to influence the organization of the cortical MT network less than co-expression of RFP–HvMAGAP1, which destabilized MTs in the presence of ROPIP1. However, RFP–HvMAGAP1 R185G interacts with CA HvRACB in planta (Hoefle et al., 2011), and GFP–ROPIP1 was recruited to MTs by RFP–HvMAGAP1–R185G (Supplementary Fig. S8). Ratiometric measurement of YFP versus CFP signals showed fluorescence complementation of ROPIP1–YFP N with YFP C–CA HvRACB but only weakly with YFP C–DN HvRACB or YFP C–HvMAGAP1 (Fig. 6A:

Fig. 3. Western blot of barley leaf protein extracts using α-ROPIP1 antibody. (A) Affinity-purified anti-peptide antibody α-ROPIP1 was used as the primary antibody in western blots of total protein extracts prepared from barley leaves inoculated (+Bgh) or non-inoculated (–Bgh) with Bgh. His-tag purified recombinant ROPIP1 (recROPIP1) was run as a positive control on the same gel. RecROPIP1 and a protein exclusive to the +Bgh lane. (B) Controls for α-ROPIP1 specificity. Escherichia coli Rosetta cells were transformed with the IPTG-inducible vector pET28b:ROPIP1. Crude cell lysates were prepared from small-scale cell cultures with (+) or without (–) IPTG induction. Recombinant His-tagged α-ROPIP1 in the IPTG-induced sample (+) but ROPIP1 was detected by α-ROPIP1 in the recROPIP1 lane in (A). MW, molecular weight protein ladder; PE, protein extract. (This figure is available in colour at JXB online.)

cell wall apposition (CWA; also called a papilla), gold particles were found in the epicuticular fungal hyphae, the appressorium, inside the host cell wall, and the host CWA (Fig. 4C, and were found in the epicuticular fungal hyphae, the appressorium, and the host CWA (also called a papilla), gold particles were detectable in mesophyll cells of Bgh-infected barley leaves. Very few gold particles were occasionally observed in plastids (Supplementary Fig. S7).

In sum, immunogold labeling with α-ROPIP1 detected a secreted Bgh protein that translocated from the fungus into barley epidermal cells, where it could interact with HvRACB.
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The mean YFP/CFP ratio of YFP<sup>C</sup>–CA HvRACB-co-expressing cells was significantly different from that in YFP<sup>DN</sup> HvRACB-co-expressing or YFP<sup>C</sup>–HvMAGAP1 cells (P≤0.01 or 0.001, respectively Student’s t-test; Fig. 6C; Supplementary Fig. S9). The BiFC signal of ROPIP1–YFP<sup>N</sup> and YFPC–CA HvRACB was predominantly observed at the cell periphery and as filamentous strings at the cell cortex, probably representing cortical MTs (Fig. 6B, D). Localization at the cell periphery is indicative for the plasma membrane, as activated HvRACB is partially plasma membrane associated (Schultheiss et al., 2003). This supported a direct protein–protein interaction of ROPIP1–YFP<sup>N</sup> and YFPC–CA HvRACB, but not with YFP<sup>DN</sup> HvRACB or YFP<sup>C</sup>–HvMAGAP1 in planta. Localization of the BiFC signal at filamentous structures suggested that ROPIP1, activated HvRACB, and HvMAGAP1 are simultaneously present at MTs, when co-expressed. This was supported by co-localization of GFP–ROPIP1, CFP–CA HvRACB, and RFP–HvMAGAP1 at both MTs and the cell periphery (Fig. 6D).

ROPIP1 causes microtubule network destabilization

MTs reorganize towards the site of attempted entry by Bgh (Hoefle et al., 2011). We hence asked whether the recruitment of ROPIP1 to MTs by HvMAGAP1 could influence MT organization. RFP–HvMAGAP1 was co-bombarded into barley epidermal cells with either GFP–ROPIP1 or GFP as control. We scored MT organization in three categories: intact MT network, disordered MT network, or fragmented MT network (Fig. 7A). Co-expression of GFP–ROPIP1 together with RFP–HvMAGAP1 led to a highly significant change (P≤0.001, χ² test) in the distribution of the three categories when compared with control cells (Fig. 7B). The relative amount of category 3 cells exhibiting a fragmented MT network tripled from 15% in control cells to ~45% in cells co-expressing GFP–ROPIP1 and RFP–HvMAGAP1.

Discussion

We identified the retroelement-encoded peptide ROPIP1 of Bgh that shows the potential to interact with the barley S-factor HvRACB and to promote fungal penetration success on barley. Some B. graminis effectors have recently been characterized. Direct interaction with potential host target proteins has been reported for CSEP0055 that interacts with the barley pathogenesis-related protein PR17c (Zhang et al., 2012) and for CSEP0105 and CSEP0162 that interact with the small heat shock proteins 16.9 and 17.5 (Ahmed et al., 2015). Bgh BEC3 and BEC4 were shown to interact with the host proteins thiopurine methyltransferase, an ubiquitin-conjugating enzyme, and an ADP ribosylation factor–GTPase-activating protein (Schmidt et al., 2014). Additionally, in a combination of protein pull-down and Y2H experiments, CSEP0064 interacted with a glutathione S-transferase, a malate dehydrogenase, and a pathogenesis-related-5 protein isoform (Pennington et al., 2016). Some B. graminis effector candidates do not...
possess N-terminal signal peptides for secretion, though they are thought to act intracellularly. This is the case for the class of EKA effectors (Ridout et al., 2006) and candidate effector proteins (CEPs) of the wheat powdery mildew Bgt (Wicker et al., 2013). EKA effector genes are evolutionarily and transcriptionally linked with autonomous non-LTR retroelements (Ridout et al., 2006; Sacristán et al., 2009), whereas CSEP genes are surrounded by non-autonomous non-LTRs such as Eg-R1 and Egh24 (Pedersen et al., 2012). Recent findings suggest that EKAs evolved from insertions of premature stop codons in LINE ORF1 protein (ORF1p), which subsequently underwent positive selection (Amselem et al., 2015). This further supports potential neo-functionalization of Bgh retroelements as a genetic resource for the evolution of novel effector proteins.

The ROPIP1 sequence is distributed in the genome of Bgh by Eg-R1 but does not encode an N-terminal signal peptide. The N-terminal ROPIP1 sequence part is not equipped with a canonical start codon on Eg-R1, whereas ROPIP1-Cter could be translated from an internal ATG. This raises the future question of whether there might be a gain of function through formation of chimeric ORFs or whether the C-terminal peptide ORF ROPIP1-Cter represents the actual effector. Inspection of the Bgh genome readily revealed the presence of several chimeric ORFs which encoded extended stretches of amino acids and partially N-terminal signal peptides that are in-frame with ROPIP1 (Supplementary Table S1). Due to the repetitive nature of ROPIP1 and the consequent presence of thousands of copies, the genomic origin of the detected polyadenylated RNAs and the corresponding protein remain unresolved and need further investigations. Our western blot experiment suggested that a ROPIP1-related sequence indeed is translated into protein, because the antibody used against a ROPIP1-Cter peptide detected both recombinant ROPIP1 and a single protein which was only present in Bgh-infected leaves. The apparent shift in mobility of E. coli-expressed recROPIP1 and the native ROPIP1 signal might be explained in different ways. Possibly, a single 5’-extended chimeric ORF is translated in Bgh and detected here. The higher molecular weight could also be explained by post-translational

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**Fig. 5.** Recruitment of GFP–ROPIP1 to cortical microtubules (MTs) by RFP–HvMAGAP1. Barley leaf epidermal cells were transiently transformed by particle bombardment and imaged with confocal laser scanning microscopy as sequential whole-cell scans in 2 µm increments at 12–24 hat. (A) Whole-cell projection showing cytoplasmic and unspecific subcellular localization of GFP–ROPIP1. Co-localization with cytoplasmic and nucleoplasmic mCherry fluorescence is indicated by white pixels in the merge picture. The observation was consistently repeatable in more than three experiments. (B) Recruitment of GFP–ROPIP1 to cortical MTs upon co-expression of MT-associated RFP–HvMAGAP1. White pixels in the merge picture indicate co-localization. A maximum projection of 20 optical sections in 2 µm increments is shown. The observation was consistently repeatable in more than three experiments. (C) Visualization of co-expressed fusion protein combinations used for quantitative analysis. C-ter, truncation of HvMAGAP1 to the MT-associated C-terminus (HvMAGAP1-Cter); FL, full-length HvMAGAP1. Ten optical sections of the upper cell cortex were merged for the pictures. (D) Quantification of the combinations shown in (C). Bars are frequencies of cells with GFP fluorescence being located at MTs or in the cytoplasm only (CYT) derived from three independent experiments. The respective absolute numbers of the categories were compared in a χ² test. RFP–HvMAGAP1-Cter highly significantly reduced MT association of GFP–ROPIP1 (***P≤0.001, n=61, 60, 53, and 57 cells from left to right). Scale bars in (A), (B), and (C) are 20 µm.
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Modification of ROPIP1. Alternatively, Eg-R1 transcripts translate as ROPIP1-Cter only and form SDS-stable oligomers. Indeed, the HHpred server (Söding et al., 2005) for protein remote homology detection and 3D structure prediction detects that ROPIP-Cter shows weak similarity to functionally diverse YigF proteins from pro- and eukaryotes that have the ability to form homodimers or homotrimers (Deriu et al., 2003). Immunogold labeling and TEM further supported that this protein is secreted by the fungus and translocated into the host cell. Protein signal appeared in the infecting fungus and infected cells, but did not appear in either uninfected barley or the mesophyll of infected barley. Hence, a host-translocated and intracellularly acting protein of Bgh was detected by the α-ROPIP1 antibody. Since α-ROPIP1 also detected recombinant ROPIP1 expressed from E. coli, we suggest that ROPIP1 or a ROPIP1-related protein was detected in the fungus and the host cell cytoplasm. Further, ROPIP1 interacted with the barley S-factor HvRACB in yeast and in planta. Hence, the barley small GTPase HvRACB is probably the host target of a ROPIP1 effector. Some first insights into a possible mode of action of ROPIP1 were gained. GFP–ROPIP1 co-located with CFP–HvRACB and HvRACB–interacting RFP–HvMAGAP1 at cortical MTs in barley epidermal cells. Transient overexpression of GFP–ROPIP1 together with RFP–HvMAGAP1 promoted the breakdown of the cortical MT array. Although this dramatic effect may be attributed to overexpression of ROPIP1, we hypothesize that release of ROPIP1 from appressoria creates a sufficient concentration for spatially restricted effects on MTs. MTs are involved in penetration resistance to powdery mildew fungi, but MT structure is locally diffuse, where Bgh penetrates. Additionally, HvRACB and HvRACB-like ROP GTPases are key regulators of MTs (Kobayashi et al., 1997; Hoefle et al., 2011; Huesmann et al., 2012). The potential manipulation of host MT arrays by ROPIP1 could either inhibit polarized cell wall-associated defense or facilitate fungal invasion and membrane delivery for formation of the extrahaustorial membrane and matrix (Dörmann et al., 2014).

ROPIP1: a Bgh effector of retroelement origin

ROPIP1 does not fit pre-defined categories or definitions of secreted effector proteins of filamentous plant pathogens,
or prokaryotic or eukaryotic pathogens in general. However, there are recent published examples that expand the current model of plant pathogen effectors beyond strict definitions. The effectors PsIsc1 and VdIsc1 of the oomycete Phytophthora sojae and the phylogenetically distinct true fungus Verticillium dahliae, respectively, attenuate the PTI response by misdirecting the synthesis of the plant defense hormone salicylic acid. Neither protein encodes N-terminal signal peptides for secretion, and PsIsc1 can functionally replace the N-terminal signal peptide and the RXLR-deER host translocation motif of the effector Avr1b of P. sojae (Liu et al., 2014). This adds to the assumption that there should be an additional secretion pathway besides the conventional co-translational loading into the endomembrane route or a process of cytoplasm exchange with host cells in filamentous plant pathogens possibly involving exosome release from multivesicular bodies (Micali et al., 2011).

ROPIP1 constitutes an unconventional effector candidate whose evolution was possibly supported by the high repeat content of the Bgh genome. Sequences similar to ROPIP1 can be found in Bgt but no clear ROPIP1/Eg-R1 homologs are present in sequenced genomes of powdery mildew fungi from dicots. It would be of great interest to learn whether there are further repeat-encoded proteins being expressed in other species. The finding of long intergenic non-coding (linc) RNAs being translated in the human proteome provoked the view that presumably non-coding RNAs constitute an evolutionary playground (Wilhelm et al., 2014). Similarly, ribosome profiling identified 5' regions of ~10–100 codons of yeast long non-coding RNAs to be bound by ribosomes, which suggests their translation (Smith et al., 2014). By looking at ROPIP1, we are possibly observing the neo-functionalization of a non-coding retroelement into a new effector gene. The nature of the Eg-R1 element has to be characterized further as it shares some properties of SINEs but, different from SINEs, it is obviously transcribed by RNA polymerase II (Wei et al., 1996; this study).

The Bgh genome is largely composed of TEs, with genes being interspersed in small clusters. It is one of the biggest ascomycete genomes possibly due to the absence of a TE spread controlling the repeat-induced point mutation (RIP) mechanism (Spanu et al., 2010). The high repeat content may give myriads of options for non-allelic recombination, making the genome very dynamic. The current knowledge is too sparse to draw a clear conclusion on the evolution of a possibly virulence-promoting sequence being dispersed throughout the genome by a SINE-like retroelement. In any case, the experimental data suggest an effector function of a ROPIP1 sequence-containing protein. It further appears possible that ROPIP1 gained an N-terminal signal peptide by insertional formation of chimeric ORFs like those exemplarily identified in this study (Supplementary Table S1). Even if this should not be the case, ROPIP1 or ROPIP1-Cter yielded scores for predicted non-classical protein secretion comparable with those of PsIsc1 and VdIsc1 using the SecretomeP 2.0 server (Bendtsen et al., 2004) in analogy to Liu et al. (2014). Predicted protein folding (Supplementary Fig. S4) but absence of predictable functional domains in ROPIP1 is typical as many effector proteins represent novel folds which implies the possibility that they are not derived from sequence variation of pre-existing genes. Further, gene losses of the primary and secondary metabolism of B. graminis, probably due to high retrotransposon activity, reflect its extreme obligate biotrophic lifestyle (Spanu et al., 2010; Wicker et al., 2013) which is likely to enhance selective pressure. In a genome with a reduced gene set, non-gene transcripts may gain novel functionalities in virulence and in general.
Supplementary data

Supplementary data are available at JXB online.

Table S1. Genomic ROPIP1 sequence variants with signal peptide prediction.

Table S2. Nucleotide and amino acid sequences of ROPIP1 and Eg-R1.

Table S3. List of oligonucleotides used in this study

Fig. S1. Sequence alignments of Eg-R1, ROPIP1, and ROPIP1-Cter.

Fig. S2. Exemplary genomic insertion and hypothetical architecture of the Eg-R1 retroelement.

Fig. S3. Semi-quantitative reverse transcription–PCR of ROPIP1.

Fig. S4. Genomic insertion size distribution of Eg-R1, 5’ end similarity of BG_non-LTR elements, and secondary and tertiary structure prediction of ROPIP1.

Fig. S5. Targeted assays showing preferential and specific protein interaction of Bgh ROPIP1 with wild-type (WT) RACB and CA_RACB.

Fig. S6. Test of silencing capacity of ROPIP1-RNAi and sequence alignment of ROPIP1 and ROPIP1-RNAi-resuce.

Fig. S7. Immunogold labeling of α-ROPIP1 in mesophyll cells of Bgh-infected barley leaves.

Fig. S8. R185G mutation of HvMAGAP1 does not alter microtubule association of GFP–ROPIP1.

Fig. S9. HvMAGAP1 does not interact with ROPIP1 in a splitYFP complementation assay.

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