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Interaction of a *Blumeria graminis* f. sp. *hordei* effector candidate with a barley ARF-GAP suggests that host vesicle trafficking is a fungal pathogenicity target

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SUMMARY

Filamentous phytopathogens, such as fungi and oomycetes, secrete effector proteins to establish successful interactions with their plant hosts. In contrast with oomycetes, little is known about effector functions in true fungi. We used a bioinformatics pipeline to identify *Blumeria* effector candidates (BECs) from the obligate biotrophic barley powdery mildew pathogen, *Blumeria graminis* f. sp. *hordei* (*Bgh*). *BEC1–BEC5* are expressed at different time points during barley infection. *BEC1*, *BEC2* and *BEC4* have orthologues in the *Arabidopsis thaliana*-infecting powdery mildew fungus *Golovinomyces orontii*. Arabidopsis lines stably expressing the *G. orontii BEC2* orthologue, *GoEC2*, are more susceptible to infection with the non-adapted fungus *Erysiphe pisi*, suggesting that *GoEC2* contributes to powdery mildew virulence. For *BEC3* and *BEC4*, we identified thiopurine methyltransferase, a ubiquitin-conjugating enzyme, and an ADP ribosylation factor-GTPase-activating protein (ARF-GAP) as potential host targets. Arabidopsis knockout lines of the respective *HvARF-GAP* orthologue (*AtAGD5*) allowed higher entry levels of *E. pisi*, but exhibited elevated resistance to the oomycete *Hyaloperonospora arabidopsidis*. We hypothesize that ARF-GAP proteins are conserved targets of powdery and downy mildew effectors, and we speculate that BEC4 might interfere with defence-associated host vesicle trafficking.

Keywords: ARF-GAP, *Blumeria graminis* f. sp. *hordei*, effector, *Erysiphe pisi*, *Golovinomyces orontii*, powdery mildew, vesicle trafficking.

INTRODUCTION

The barley powdery mildew fungus, *Blumeria graminis* f. sp. *hordei* (*Bgh*), was recently voted the sixth most important plant-pathogenic fungus (Dean *et al*., 2012). It is an obligate biotroph and is completely dependent on living host tissue for growth and proliferation. Following spore germination on the leaf surface, *Bgh* penetrates the plant cell wall at the site of appressorium formation. From the appressorium, a penetration peg emerges that extends through the host epidermal cell wall and differentiates into a highly specialized infection structure, the haustorium (reviewed in O'Connell and Panstruga, 2006). Haustoria are not truly intracellular structures, because they remain separated from the host cytoplasm through an invaginated derivative of the host plasma membrane, the extrahaustorial membrane (Koh *et al*., 2005; Micali *et al*., 2011). Among plant parasites, apart from powdery mildew fungi, only the obligate biotrophic rust fungi and some (hemi-) biotrophic oomycetes are known to form haustoria (Kamoun, 2006; Panstruga, 2003). Haustoria are thought to be the major interfaces for nutrient acquisition by these plant parasites (Hahn and Mendgen, 2001; Panstruga, 2003). In addition, the haustorium has been proposed as a major site of effector biosynthesis and delivery (Catanzariti *et al*., 2006; Panstruga and Dodds, 2009; Stergiopoulos and de Wit, 2009).

Effectors are molecules, e.g. secondary metabolites or proteins, which are synthesized by the pathogen to target host cellular processes and modify their function for successful plant colonization (reviewed in Kamoun, 2007). Plant-pathogenic bacteria use the type III secretion system to inject proteinaceous effectors into host cells (Block *et al*., 2008). Individual type III effectors target multiple host pathways and affect transcription, RNA stability, mitogen-activated protein kinase (MAPK) cascades and protein turnover, which often results in the suppression of plant immune responses (Block *et al*., 2008). Much less is known about the pathways targeted by haustoria-forming plant pathogens. The function of effectors from these eukaryotic pathogens has been mainly studied in the context of gene-for-gene-type interactions with plant resistance proteins. Effectors that are either directly or indirectly recognized by plant resistance proteins, thereby triggering a boosted resistance response, are also termed avirulence factors. In the case of the biotrophic flax rust fungus, *Melampsora lini*, four effectors with avirulence activity have been cloned (Catanzariti *et al*., 2006). AvrL567 and AvrM are directly recognized by the corresponding flax resistance proteins (Catanzariti *et al*., 2010; Dodds *et al*., 2004, 2006). This direct

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interaction with intracellular plant proteins indicates that the flax rust effectors are translocated from the haustoria into the host cell. How they are delivered inside host cells remains elusive (Panstruga and Dodds, 2009). In oomycete effectors, two classes of consensus sequences, the RXLR and LXFLAK amino acid motifs, are important for protein delivery inside the host cell (Kale *et al*., 2010; Schornack *et al*., 2010; Whisson *et al*., 2007). Similarly, for powdery mildew fungal effectors, a common amino acid motif was identified among 107 putatively secreted proteins whose transcripts were present in a haustorial *Bgh* cDNA library (Godfrey *et al*., 2010). However, so far, there is no experimental evidence to indicate that this Y/F/WxC motif is required for translocation.

To date, only a few *Bgh* effectors have been studied functionally. The atypical effectors AVR_{A10} and AVR_{K1} are recognized by the barley resistance proteins MLA10 and MLK1, respectively. The corresponding genes encode small proteins of unknown function that differ from other known effectors by lacking N-terminal signal peptides (SPs) for secretion. It has been hypothesized that these two effectors are secreted from the pathogen via an alternative, endoplasmic reticulum (ER)-independent secretory pathway (Ridout *et al*., 2006). Another unusual feature of AVR_{A10} and AVR_{K1} is that they belong to a large gene family with more than 1300 paralogues in the *Bgh* genome, which are physically associated and co-evolving with LINE-1 retrotransposons (Sacristan *et al*., 2009; Spanu *et al*., 2010). Cell biological analyses suggest that the recognition of AVR_{A10} triggers nuclear association of MLA10 and the transcriptional regulator WRKY2, which is believed to de-repress plant defence responses (Shen *et al*., 2007). Both AVR_{A10} and AVR_{K1} also appear to be important for the virulence of *Bgh* (Ridout *et al*., 2006). However, it is currently unknown which host proteins or pathways are targeted by AVR_{A10} and AVR_{K1} to support virulence.

In an extended analysis of the recently published *Bgh* (isolate DH14) genome (Spanu *et al*., 2010), Pedersen *et al*. (2012) identified 491 candidate secreted effector proteins (CSEPs) by searching for genes encoding secreted proteins without sequence similarity to proteins outside the powdery mildews. Most CSEPs were grouped into 72 gene families. Among them were two major types of effector families: one comprising short proteins with a high relative expression level in haustoria compared with epiphytic fungal structures, and one consisting of longer proteins with lower levels of differential expression (Pedersen *et al*., 2012). Most of the genes are physically dispersed between retrotransposons (Pedersen *et al*., 2012). One of the 491 CSEPs, CSEP0055, interacts with the barley pathogenesis-related proteins PR1 and PR17 (Zhang *et al*., 2012). The expression profile of *CSEP0055* suggests that the protein encoded by this gene contributes to sustained fungal growth at the penetration site (Zhang *et al*., 2012). Recently, a group of eight *Blumeria* effector candidates (BECs) was functionally characterized via expression analysis combined with host-induced gene silencing. Two of these show similarity to microbial secreted ribonucleases, but have not been studied in detail to date (Pliego *et al*., 2013).

Here, we report the identification and characterization of five additional *BEC* genes. Three belong to the set of 491 CSEPs and two more were identified from expressed sequence tags (ESTs) derived from *Bgh*-infected barley leaves. We demonstrate their expression during *Bgh* infection of barley leaves via quantitative real-time polymerase chain reaction (PCR). For three BECs, we identified sequence-related polypeptides encoded by the genome of *Golovinomyces orontii*, a powdery mildew pathogen colonizing *Arabidopsis thaliana*. We employed stably transformed Arabidopsis plants to demonstrate the virulence-promoting function of one of these effector orthologues. Furthermore, using a yeast two-hybrid approach, we identified host interactors of two BEC proteins and confirmed these interactions by bimolecular fluorescence complementation (BiFC) *in planta*. We employed Arabidopsis knockout mutants of one of the target genes (*AtAGD5*) to verify its contribution to plant immunity.

RESULTS

Bgh effector candidates are differentially expressed during infection

The majority of known fungal and oomycete effector proteins are characterized by their small size (typically less than 300 amino acids), N-terminal SP for secretion and unrecognizable biochemical activity, typically associated with the lack of any significant BLAST hit (van den Burg *et al*., 2006; Catanzariti *et al*., 2006; Dodds *et al*., 2004; Kemen *et al*., 2005). Guided by these criteria, we employed a bioinformatics approach to identify *Bgh* effector candidates from the COGEME phytopathogen EST database [Soanes and Talbot, 2006; Figs 1 and S1 (Supporting Information)]. Five candidate effectors were expressed during plant infection and were selected for further analysis. We named these *Blumeria* effector candidates (BECs) BEC1 to BEC5 (Table 1).

We investigated the expression pattern of the *BEC* genes by quantitative reverse transcription-polymerase chain reaction (qRT-PCR) in a time-course experiment during *Bgh* infection [for details, see experimental procedures in File S1 (Supporting Information)]. Transcript levels of *BEC1* decreased during the infection process until the relative levels were very low at 48 h post-inoculation (hpi; Fig. 2). Similarly, the abundance of *BEC2* and *BEC3* transcripts decreased at this late infection time point (48 hpi). *BEC3* and *BEC4* transcript levels increased transiently two-fold during the penetration process (12 hpi) compared with time point 0 hpi (Fig. 2), whereas the transcript abundance of *BEC2* was only elevated at the time point of appressorium emergence (6 hpi; four-fold compared with time point 0 hpi, Fig. 2). By contrast, *BEC5* transcript levels increased at the later stages of infection. When haustoria were fully developed (24 hpi), *BEC5*

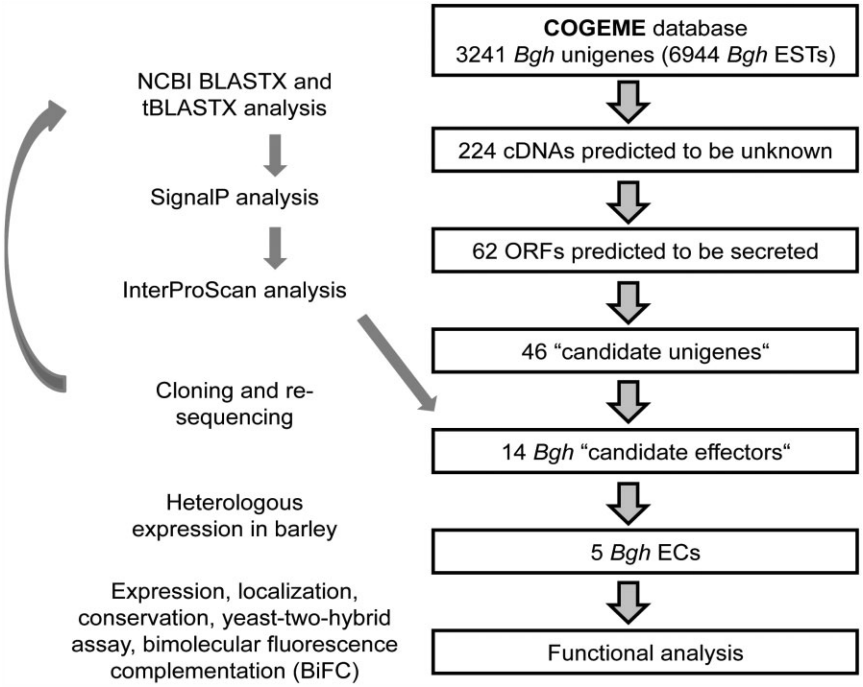


Fig. 1 Bioinformatic and experimental pipeline for *Blumeria* effector candidate (BEC) identification. BECs were identified bioinformatically from expressed sequence tags (ESTs) deposited in the COGEME database based on the presence of a full-length open reading frame (ORF), a predicted signal peptide (SP) for secretion and the absence of sequence relatedness to known proteins. For further details, see File S1. *Bgh*, *Blumeria graminis* f. sp. *hordei*.

transcription levels were dramatically higher (60-fold compared with time point 0 hpi) and increased further when secondary hyphae developed at 48 hpi (80-fold compared with time point 0 hpi) (Fig. 2). Consistent with this expression profile, the *BEC5* gene product is present in the *Bgh* haustorial proteome (Bg3; Bindschedler *et al*., 2009).

Transient expression of BECs in barley leaf epidermal cells

We tested the candidate effectors for their capacity to promote virulence of the *Bgh* fungus on barley. To date, no reliable transformation protocol is available for *Bgh*, precluding targeted gene knockout of the candidate effector genes as an experimental route. Instead, we opted for transient expression of the candidate effector genes in single barley leaf epidermal cells following bombardment of DNA-coated gold particles (Panstruga, 2004). Using this approach, we expressed constructs encoding the effector candidates, both as full-length variants and as N-terminally truncated variants lacking the SP, together with a *β-glucuronidase* (*GUS*) reporter gene construct in barley leaves (Fig. S2A, see Supporting Information). The baseline susceptibility of the epidermal cells in a compatible interaction is reflected by the mean *Bgh* haustorium index, set to 100% on expression of the *GUS* reporter construct alone. Co-expression of *GUS* and *MLO*, a compatibility factor for powdery mildew fungi (Eichmann and Hückelhoven, 2008; Jørgensen, 1992), led to super-susceptibility of barley and, on average, increased the haustorium index to 163% (compared with the GUS control set at 100%; Fig. S2B). The expression of all full-length *BEC*s, as well as the expression of the cloned *Bgh* effector AVR_{A10} (Ridout *et al*., 2006), elevated the mean haustorium indices (Fig. S2B). However, this elevation was only statistically significant for *BEC3* (135%), *BEC5* (115%) and AVR_{A10} (116%; Fig. S2B).

BEC protein sequences are highly homomorphic among the six *Bgh* isolates

To assess allelic variation and to identify potential polymorphic sites in the *BEC* genes, we amplified the genes from the genomic DNA of six *Bgh* strains that differed with regard to their virulence/avirulence spectra (see Experimental procedures), and determined the nucleotide sequence of the respective PCR amplicons by direct sequencing (Fig. S1, Table 1). Remarkably few nucleotide polymorphisms were found in the *BEC* genomic DNA. *BEC2* and *BEC4* were identical among the six isolates, whereas single polymorphic sites were identified at the C-termini of *BEC1*, *BEC3* and *BEC5* [Figs 3 and S3 (Supporting Information)]. In *BEC3*, only silent nucleotide substitutions were detected. In conclusion, the five *BEC* genes are largely homomorphic among the six tested *Bgh* isolates, indicating that they are not subject to diversifying selection. Their gene products are thus unlikely to be recognized by cognate barley resistance proteins.

Most BEC proteins are encoded by single-copy genes and are in part conserved among powdery mildew species

Next, we assessed whether the *BEC* genes are members of multigene families and whether they are specific to *Bgh*. We

Table 1 *Blumeria* effector candidates identified in the COGEME database.

COGEME contig*	BEC	GenBank accession no.	BLASTX(nr)	*E* value†	Organism	SignalP score‡	Protein signature§	Size (amino acids)	MW¶ (kDa)
BgCon0039	BEC1	HQ435746	Hypothetical protein	9e-09	*Branchiostoma floridae*	0.977	SP**	155	16
BgCon0062	BEC2	HQ435747	Predicted protein	4e-06	*Botryotinia fuckeliana*	1.000	SP, CFEM††	133	14
BgCon0034	BEC3	HQ435748	–	n.a.	n.a.	0.999	SP	114	11
BgCon0254	BEC4	HQ435749	Predicted protein	6e-19	*Botryotinia fuckeliana*	0.998	SP, CFEM	235	24
BgCon1160	BEC5	HQ435750	–	n.a.	n.a.	1.000	SP	139	16

*http://cogeme.ex.ac.uk/index.html (Soanes and Talbot, 2006).
†*E* value cut-off: 10^{-5}.
‡Translated sequences were analysed with the SignalP Hidden Markov Model (HMM) and the SignalP Neural Network (not shown) algorithms.
§The protein signature was assigned using InterProScan (http://www.ebi.ac.uk/InterProScan/index.htm).
¶Molecular weight.
**Signal peptide.
††Eight cysteine-containing domain present in fungal extracellular membrane proteins.
n.a., not applicable.

performed BLAST searches in the draft genome sequences of four powdery mildew species [*Bgh*; *Blumeria graminis* f. sp. *tritici* (*Bgt*), virulent on wheat (Wicker *et al*., 2013); *G. orontii*, virulent on the dicotyledonous reference species *A. thaliana*; and *Erysiphe pisi*, virulent on pea (*Pisum sativum*; Spanu *et al*., 2010)]. For all five *BEC*s, single matching genomic DNA sequence contigs were identified in the *Bgh* (isolate DH14) genome, indicating that they are single-copy genes in the barley pathogen (Table 2). *BEC1*, *BEC2* and *BEC5* are also present in the CSEP dataset (Pedersen *et al*., 2012; Table 2). Furthermore, for each *BEC*, we identified one matching *Bgt* contig, indicating conservation of all *BECs* in the closely related wheat powdery mildew pathogen. Moreover, *BEC2* and *BEC4* have corresponding gene sequences in the *G. orontii* and *E. pisi* genomes (Table 2). *BEC1* only matches a weakly similar sequence in the *G. orontii* genome, and *BEC3* and *BEC5* lack any related sequences in the genomes of the latter powdery mildew species. This could be a result of incomplete coverage of these genomes, assembly mistakes or may indicate that these genes are recent innovations of the grass powdery mildew species.

BEC2 virulence-promoting function is conserved in the barley and Arabidopsis powdery mildew fungus

Pairwise alignment of the deduced amino acid sequences of BEC2 and its corresponding *G. orontii* counterpart, designated *Go*EC2, indicates that the positions of the predicted SP as well as the CFEM (cysteine-rich fungal extracellular membrane) domain are conserved (Fig. 4A). As *BEC2* has a characteristic expression pattern with markedly high transcript levels at the time of appressorium formation (6 hpi; Fig. 2), we examined the expression of *GoEC2* during *G. orontii* pathogenesis by qRT-PCR at the same time points post-inoculation as for the barley–*Bgh* interaction. Similar to *BEC2*, *GoEC2* transcript levels decreased at later time points (24 and 48 hpi) (Fig. 4B), but, in contrast with *BEC2*, there was no pronounced peak in *GoEC2* expression at 6 hpi (Fig. 4B). Notably, however, *GoEC2* transcripts were highly represented in a haustorial cDNA library of *G. orontii* (Weßling *et al*., 2012; GoEST_c268). To explore the function of *GoEC2* during the infection of Arabidopsis, we stably transformed *A. thaliana* Col-0 plants with binary vector constructs, leading to the heterologous expression of haemagglutinin (HA)-tagged *Go*EC2 variants. Similar to the set of experiments carried out in barley (Fig. S2B), either full-length *GoEC2* or a truncated *GoEC2* version lacking the sequence coding for the N-terminal SP was used. Immunoblot analysis revealed abundant steady-state levels of the full-length *Go*EC2 variant, whereas the version lacking the N-terminal SP ($GoEC2_{18–154}$) accumulated to considerably lower levels (Fig. 4C). To determine whether transgenic Arabidopsis plants expressing either construct were more susceptible to fungal infection, we challenged plants with the pea powdery mildew fungus, *E. pisi*, which shows low levels of invasion on *A. thaliana* wild-type plants

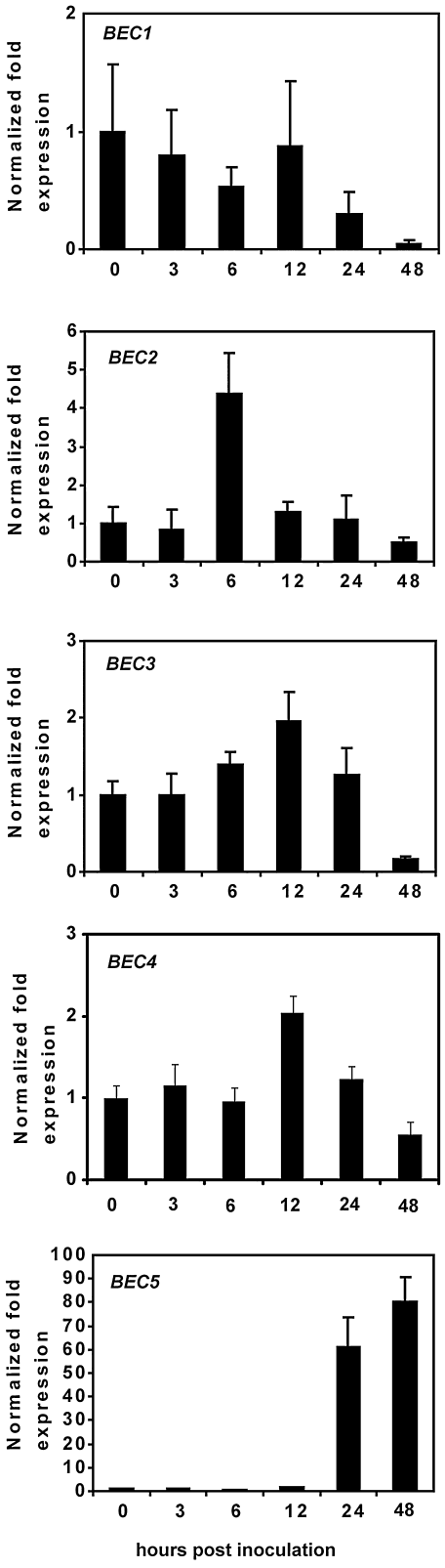


Fig. 2 *Blumeria effector candidate* (*BEC*) genes are differentially expressed during *Blumeria graminis* f. sp. *hordei* (*Bgh*) infection. The expression levels of *BEC* genes were monitored during the compatible interaction of *Bgh* isolate K1 with *Hordeum vulgare* (cultivar Golden Promise) at 0, 3, 6, 12, 24 and 48 h post-inoculation (hpi). Total RNA was isolated from barley leaves at the indicated time points. Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed for the *BEC* genes indicated in the respective panel. *Bgh β-tubulin* expression was used to normalize the expression value in each sample, and expression values were normalized against the average expression value at time point 0 hpi (set as unity). The standard deviation was calculated from three to four technical replicates each. Experiments were repeated twice with independent biological material, yielding similar results.

(Lipka *et al*., 2005), and with the adapted powdery mildew pathogen, *G. orontii*. Two independent transgenic lines were used to test full-length *Go*EC2 and one for the truncated *Go*EC2$_{18–154}$ construct. Strikingly, transgenic Arabidopsis plants expressing either full-length or truncated *GoEC2* conferred higher entry rates of *E. pisi* (Fig. 4D), whereas the entry rates and sporulation of *G. orontii* remained unaltered (Fig. S4, see Supporting Information). Consistent with the differential protein accumulation (Fig. 4C), we found that the transgenic Arabidopsis line expressing *GoEC2*$_{18–154}$ showed lower *E. pisi* entry rates than transgenic lines expressing full-length *GoEC2* (*GoEC2*$_{1–154}$). Taken together, these experiments suggest that *Go*EC2 has the potential to enhance host susceptibility by promoting fungal entry. This effect might be masked in the interaction with the adapted powdery mildew pathogen by the already very high entry rates of *c*. 70% (Fig. S4).

Identification of potential host targets of powdery mildew effector proteins

To obtain first insights into the host pathways targeted by the BEC proteins, we searched for barley protein interactors by performing yeast two-hybrid screens. In these, LexA-BEC protein fusions served as baits and a cDNA library from *Bgh*-infected barley leaves as the prey. All LexA-BEC fusion proteins were expressed in yeast (Fig. S5A, see Supporting Information), except for BEC1, which could not be cloned as a fusion to LexA, and BEC2, which auto-activated the reporter system (Fig. S5B). We did not identify any interacting protein for BEC5. With the LexA-BEC3 and LexA-BEC4 bait constructs, we identified 36 prey cDNAs representing three different proteins for BEC3, and 14 prey cDNAs representing six different proteins for BEC4. Only two barley interactors for BEC3 and BEC4, respectively, were represented by multiple cDNAs encoding length variants of the protein's N-termini. In the case of BEC3, these cDNAs encoded an *S*-adenosylmethionine synthetase (SMS; eight cDNA clones) and a thiopurine methyltransferase (TPMT; 27 cDNA clones). SMS synthesizes *S*-adenosyl-L-methionine (SAM), an important product in methionine metabolism. Methyltransferases, such as TPMT, depend on SAM as a cofactor for activity, because they serve as the methyl donor in the

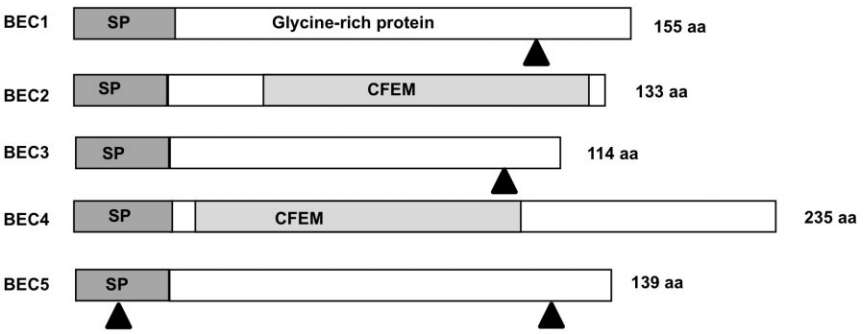


Fig. 3 *Blumeria* effector candidate (BEC) proteins are structurally diverse, but are not polymorphic amongst different *Blumeria graminis* f. sp. *hordei* (*Bgh*) isolates. The scheme (not drawn to scale) depicts the predicted BEC protein signatures. Amino acid polymorphisms were identified by sequencing the *BEC* cDNAs of six different *Bgh* isolates and are indicated by black arrowheads. aa, amino acid; CFEM, cysteine-rich fungal extracellular membrane; SP, signal peptide.

transfer of the methyl group to the target molecule (Wang and Weinshilboum, 2006). As SMS also interacted with BEC4 in reciprocal testing (Table S2, see Supporting Information), we reasoned that SMS is probably a non-specific interactor and excluded it from further analysis. *TPMT* genes are conserved among species from bacteria to mammals, but their cellular functions remain largely elusive (Scheuermann *et al*., 2003). A bacterial TPMT converts selenium-and tellurium-containing compounds to less toxic, volatile, methylated derivatives (Favre-Bonté *et al*., 2006; Ranjard *et al*., 2004).

Barley *BEC4* prey cDNAs encode a ubiquitin-conjugating enzyme (UBC; 12 cDNA clones) and an ADP ribosylation factor (ARF)-GTPase-activating protein (GAP) (ARF-GAP; six cDNA clones). UBC proteins (also termed E2 proteins) transfer a ubiquitin moiety from a ubiquitin-activating enzyme (E1) to a ubiquitin ligase (E3), which then attaches the ubiquitin moiety to a target protein (Passmore and Barford, 2004). ARF-GAPs belong to one of two families that are key regulators of membrane trafficking in yeast, animal and plant cells by modulating the activity and localization of ARF proteins during vesicle formation and cargo recruitment (D'Souza-Schorey and Chavrier, 2006; Inoue and Randazzo, 2007; Nielsen *et al*., 2008).

Mutual testing of BEC3 and BEC4 bait–prey combinations revealed that, within these tested controls, the interactions are specific (Fig. 5A,B). Interaction of BEC3 with *Hv*TPMT$_{108–247}$ and interaction of BEC4 with both *Hv*ARF-GAP$_{245–476}$ and *Hv*UBC$_{60–196}$ was further analysed by testing respective full-length cDNA sequences in the yeast two-hybrid assay (Fig. 5A,B). Only full-length *Hv*ARF-GAP$_{1–476}$ interacted with BEC4 in yeast, whereas no interaction could be demonstrated between full-length *Hv*UBC$_{1–196}$ and BEC4 or full-length *Hv*TPMT$_{1–247}$ and BEC3 (Fig. 5A,B).

In planta validation of protein–protein interactions

Next, we employed BiFC to monitor the BEC3–*Hv*TPMT, BEC4–*Hv*ARF-GAP and BEC4–*Hv*UBC interactions *in planta*. For the BiFC assay (Walter *et al*., 2004), non-fluorescent N-terminal and C-terminal segments of yellow fluorescent protein (YFP) (YFP^N and YFP^C, respectively) were fused to the N- and C-termini of the proteins to be tested (i.e. BEC3 and BEC4 lacking the SP, full-length *Hv*TPMT, *Hv*UBC and *Hv*ARF-GAP). Interactions between the respective fusion proteins were tested in various constellations (N- and C-terminal fusions) in barley epidermal cells using transient gene expression via particle bombardment. On co-expression of BEC3-YFP^C–*Hv*TPMT-YFP^N and YFP^N-BEC4–*Hv*UBC-YFP^C protein pairs, fluorescence was observed in the nucleus and at the cell periphery, probably the cytosol (Fig. 5C). Likewise, co-expression of BEC3-YFP^N–HvTPMT-YFP^C and BEC3-YFP^N–HvTPMT$_{108–247}$-YFP^C led to YFP fluorescence (data not shown). Co-expression of different variants of tagged *Hv*ARF-GAP and BEC4 variants did not result in visible YFP fluorescence, indicating that these proteins either do not interact in this assay or the proteins do interact, but the YFP halves are positioned in an unfavourable orientation preventing formation of intact YFP. However, as we could not obtain *Hv*ARF-GAP fusion constructs with a C-terminal tag, we cannot exclude the possibility that the addition of the N-terminal tag interfered with the interaction of *Hv*ARF-GAP and BEC4. YFP-tagged *Hv*ARF-GAP partly co-localizes with a Golgi marker, consistent with its function as an ARF activator and regulator of vesicle budding at the Golgi. However, *Hv*ARF-GAP is also present in the cytoplasm, where the mature form of BEC4 is localized (Fig. 5D), and here the proteins can potentially interact. Taken together, the BiFC data show that BEC4 interacts with *Hv*UBC and BEC3 interacts with *Hv*TPMT *in planta*, indicating that these protein pairs probably do not require other fungal proteins to physically interact inside barley cells.

The Arabidopsis *At*AGD5 orthologue of *Hv*ARF-GAP is required for full resistance to powdery mildew infection

ARF-GAPs belong to a medium-sized protein family encoded by 15 genes (*AGD*) in the *A. thaliana* genome (Vernoud *et al*., 2003). Phylogenetic analysis indicates that *Hv*ARF-GAP, which was identified as an interactor of BEC4, clusters with a distinct subgroup consisting of only two family members, *At*AGD5 and *At*AGD15 (Fig. 6A). The highest sequence similarity was found for *Hv*ARF-GAP and *At*AGD5, and therefore *At*AGD5 (At5g54310) probably represents the Arabidopsis orthologue of *Hv*ARF-GAP.

Table 2 BEC conservation in other powdery mildew species.

BEC	Length (amino acids)	Gene ID in BLUGEN	CSEP*	*Bgt* JIW2 contig†	E value	Identities	Positives	*Go* contig‡	E value	Identities	Positives	*Ep* contig§	E value	Identities	Positives
BEC1	155	bgh02536	CSEP0052	contig_78336_1	2e-35	132/151	134/151	Go_V1_Contig3372.1	1e-09	26/49	30/49	–	–	–	–
BEC2	133	bgh04522	CSEP0214	contig_27469_1	1e-48	130/161	133/161	Go_V1_Contig6387.1	4e-28	40/68	50/68	Ep_V2_contig14308	2e-16	32/67	37/67
												Ep_V2_contig10908	1e-15	19/37	26/37
BEC3	114	bgh02534	–	contig_29824_1	0.003	60/71	63/71	–	–	–	–	–	–	–	–
BEC4	235	bgh02531	–	contig_27403_1	2e-43	128/150	133/150	Go_V1_Contig8164.1	1e-26	37/56	41/56	Ep_V2_contig03185	6e-32	36/58	43/58
BEC5	139	bgh00016	CSEP0004	contig_78207_1	3e-36	54/92	65/92	–	–	–	–	–	–	–	–

*The draft genome sequence of *Blumeria graminis* f. sp. *hordei* isolate DH14 was searched at http://www.blugen.org using tBLASTN.

†The *Blumeria graminis* f. sp. *tritici* (*Bgt*) JIW2 genome deposited at the Whole-genome shotgun contigs (wgs) database at the National Center for Biotechnology Information (NCBI) was searched for *Bgt* homologues using tBLASTN.

‡The draft genome sequence of *Golovinomyces orontii* was searched at http://www.mpipz.mpg.de/23693/Powdery_Mildews using tBLASTN.

§The draft genome sequence of *Erysiphe pisi* was searched at http://www.mpipz.mpg.de/23693/Powdery_Mildews using tBLASTN.

To explore the biological significance of ARF-GAP proteins in plant–powdery mildew interactions, we took advantage of the genetic resources available for Arabidopsis and tested a panel of T-DNA insertion lines for their susceptibility to powdery mildew infection. We identified three different mutant alleles of *AtAGD5* that are each predicted to encode truncated, non-functional protein variants (Fig. 6B). These mutations are considered as strong loss-of-function alleles, because they appear to completely disrupt ARF-GAP function (Liljegren *et al*., 2009). Phenotypically, the knockout lines were smaller than wild-type Col-0 plants and occasionally exhibited spontaneous chlorotic lesions in the absence of any pathogen (Fig. S6A, see Supporting Information).

We inoculated the *Atagd*5 knockout lines with spores of the non-adapted powdery mildew pathogen *E. pisi*, which exhibits a limited extent of host cell entry and haustorium formation on *A. thaliana* (Fig. 4D; Lipka *et al*., 2005), and microscopically evaluated host cell entry rates at 7 days post-inoculation. After pathogen challenge, rosette leaves of the *Atagd5* knockout lines were more chlorotic than those of Col-0 wild-type plants (Fig. S6B). Interestingly, all three tested knockout lines showed higher entry levels than wild-type Col-0 plants following inoculation with the non-adapted powdery mildew fungus (Fig. 6C). Penetration rates of the adapted powdery mildew pathogen *G. orontii* on Col-0 wild-type plants are high (*c*. 70%) and were not increased further on the *Atagd5* T-DNA insertion lines (Fig. S4). We also challenged seedlings of the *Atagd5* mutant lines with a virulent isolate of the oomycete pathogen *Hyaloperonospora arabidopsidis*, the causal agent of downy mildew disease, and found decreased sporulation (*c*. 50%–60% compared with Col-0) on all three lines (Fig. 6D). In summary, *At*AGD5 appears to function antagonistically in defence against (non-adapted) powdery mildews and the adapted downy mildew pathogen in Arabidopsis.

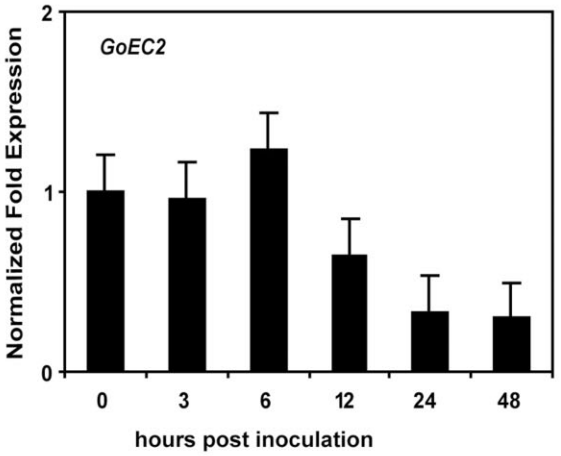
DISCUSSION

Powdery mildew fungi are obligate biotrophic phytopathogens that form haustoria inside host plant cells for nutrient acquisition and host cell manipulation. Although biological information pertaining to their pathogenicity mechanisms is sparse, the recent publication of the genomes of four powdery mildew species has been a valuable source of information regarding the molecular basis of the life style of these cryptic pathogens (Spanu *et al*., 2010; Spanu and Panstruga, 2012; Wicker *et al*., 2013). The *Bgh* genome encodes 491 CSEPs, which are small, secreted proteins of unknown function, primarily expressed in haustoria (Pedersen *et al*., 2012). These candidate effector genes appear to be largely species-specific, reflecting plant–host co-evolution. Thus, in addition to the non-canonical effectors, AVR_{A10} and AVR_{K1} (Ridout *et al*., 2006), *Bgh* probably also possesses effectors that are secreted via the classical ER pathway, similar to those identified in other plant-pathogenic fungi. During a compatible interaction,

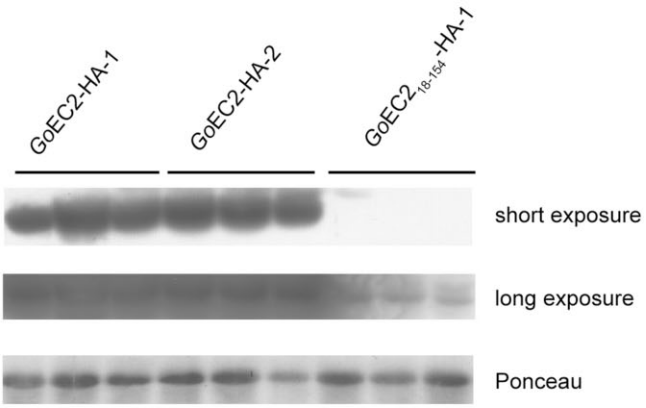
A

```
                    10         20         30         40         50         60
BghBEC2     MQLIRLLVLA FAAFVAASPY STLSHTEGGK GGKGGKGDDD GDDDGPQTCN DALQTLPKCA
GoEC2       MQFTKLIVLA FVACVTATP- SGLNFYVRRT DG-------- GDDEAPLTCT EALQEIPKCA
Consensus    **: :*:*** *.* *:*:*  * *..    . .*         ***:.* **. :*** :****

                   70         80         90        100        110        120
BghBEC2     LQCVVQPPID AGCKSAADFA CTCEHDTNKA ITKQETPCVL DHCSLQDSIG ALKAGRLICD
GoEC2       LSCITQPPID AGCKSAADFA CSCEKKTQKQ ITKAETPCVL KACGLKEALA ASRAGKDVCG
Consensus   *.*:.***** ********** *:**:.*:*  *** ****** . *.*::::. * :**: :*.

                  130        140        150        160
BghBEC2     LCNVPAN--- -------DTD SYN------- ---------- ---
GoEC2       ACNTSPNEPS DGHGDDGDDD SYEKSVDAEK TPEIPNSMQN SDV
Consensus    **...*          * * **:
```

B



C



D

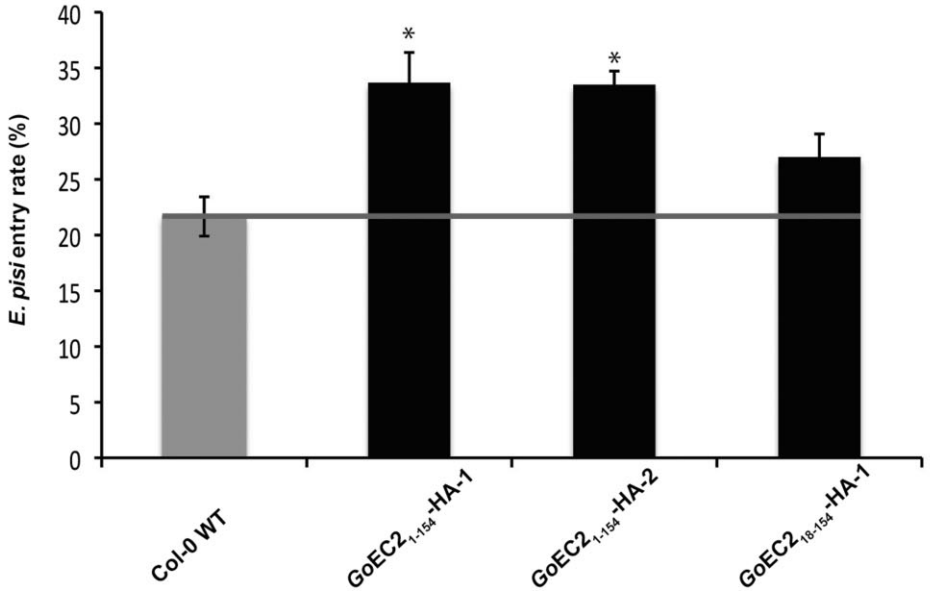


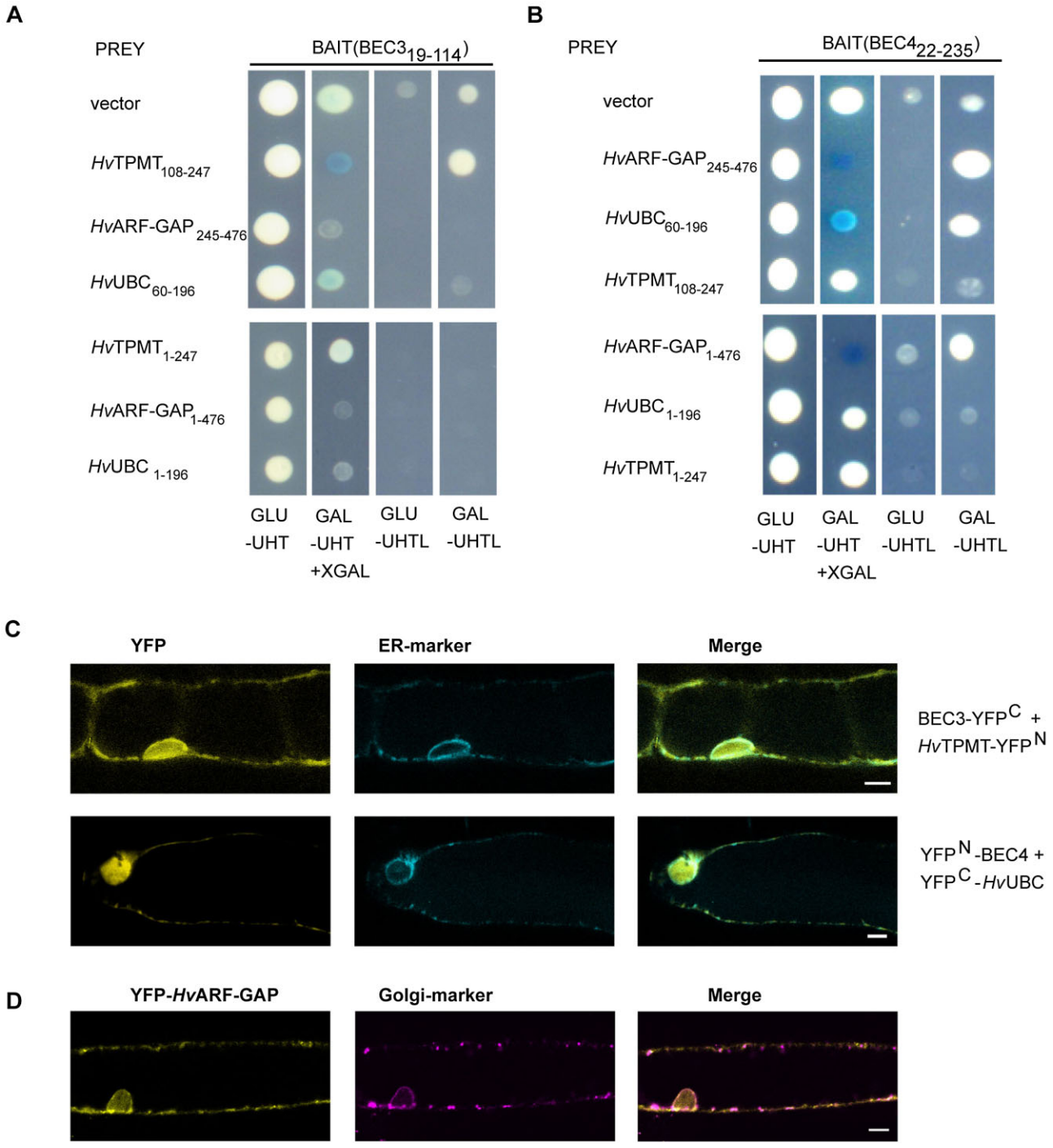
Fig. 4 Constitutive expression of a *Golovinomyces orontii BEC2* orthologue enhances powdery mildew entry in Arabidopsis. (A) *BEC2* has a presumptive orthologue in the *G. orontii* genome. Pairwise alignment of the deduced protein sequences of *BEC2* and its presumed *G. orontii* orthologue (*Go*EC2). The *GoEC2*-containing DNA contig was identified by BLAST searches against the draft genome sequence of the Arabidopsis powdery mildew pathogen. The predicted signal peptide (SP) is boxed. Cysteine residues forming the CFEM (cysteine-rich fungal extracellular membrane) domain are conserved in both species (shaded in grey). Identical residues are indicated by '*', conservative substitutions by ':' and semi-conservative substitutions by '.' in the consensus sequence. (B) *GoEC2* is constitutively expressed during the early phase of *G. orontii* infection. The expression level was monitored during the compatible interaction with *Arabidopsis thaliana* Col-0 as described in Fig. 2. Expression of *G. orontii β-tubulin* was used to normalize the expression value in each sample. The standard deviation was calculated from three to four technical replicates each. Experiments were repeated twice with independent biological material, yielding similar results. (C) $GoEC2_{18–154}$ expression levels are lower than $GoEC2_{1–154}$ expression levels in stably transformed Arabidopsis plants. Total protein extracts of transgenic and wild-type Arabidopsis leaves were analysed by Western blot to determine the abundance of the *Go*EC2 haemagglutinin (HA)-tagged protein variants. (D) Transgenic Arabidopsis plants expressing full-length *Go*EC2 or $GoEC2_{18–154}$ allow higher entry rates of the non-adapted powdery mildew fungus *Erysiphe pisi*. Host cell entry was determined quantitatively at 7 days post-infection with *E. pisi* spores on wild-type Col-0 and *Go*EC2-expressing plants. Two independent lines expressing $GoEC2_{1–154}$ and one line expressing $GoEC2_{18–154}$ (T_3 generation) were tested. Plants were tested individually for protein expression before inoculation with fungal spores. Results represent mean ± standard error of three independent experiments. Asterisks indicate $P \leq 0.05$ (Student's *t*-test) compared with the wild-type.

CSEP0055 has been shown to interact with the barley proteins PR1 and PR17 (Zhang *et al*., 2012). PR17 is secreted and accumulates in papillae that are formed underneath *Bgh* penetration sites. As *CSEP0055* is only expressed at a later stage during infection, this protein has been implicated in sustaining the fungus at the infection site (Zhang *et al*., 2012). Here, we present the identification and functional characterization of five additional *Bgh* effector candidates that were identified from EST databases of *Bgh*-infected barley leaves.

BEC1, *BEC2* and *BEC5* correspond to *CSEP0052*, *CSEP0214* and *CSEP0004*, respectively. *BEC3* and *BEC4*, however, are not yet represented in the CSEP dataset (Pedersen *et al*., 2012). Most short CSEPs (100–150 amino acids) are expressed during haustoria formation, whereas longer CSEPs (300–400 amino acids) do not display a distinct expression profile (Pedersen *et al*., 2012). In our analysis, we were most interested in effector proteins that act during the early stage of infection, including germ tube formation, fungal cell wall penetration and the onset of haustoria formation. Of the five tested candidates, *BEC2* expression peaks earliest, at 6 hpi during appressorium formation (Fig. 2). *BEC2* is one of the very few CSEPs with a recognizable orthologue in the *G. orontii* genome. *GoEC2* was able to elevate the entry levels of the non-adapted fungus *E. pisi*, a pathogen on pea, when stably expressed in Arabidopsis plants (Fig. 4), suggesting that *Go*EC2 promotes virulence in *G. orontii*. Given the early stage of *BEC2* expression and its cysteine richness, BEC2 may, like other cysteine-rich effectors, reside and operate in the apoplastic space, where its interaction with plant targets may prepare host cells for fungal entry.

BEC3 and *BEC4* expression levels peak at 12 hpi, indicating that they might add to plant penetration and haustorium formation (Fig. 2). For these newly described effector candidates, we have identified potential plant targets. BEC4, a CFEM domain-containing protein, interacts with $HvUBC_{60–196}$ and $HvARF\text{-}GAP_{1–476}$ in yeast (Fig. 5A,B). We found that the C-terminus of BEC4 is necessary for the interaction with *Hv*UBC and *Hv*ARF-GAP in yeast, as deletion of the C-terminal 90 amino acids was sufficient to abolish the interaction of BEC4 with the C-termini of the two barley proteins (data not shown). We propose that BEC4 is a modular protein, in which the N-terminus with the SP and the CFEM domain direct the protein to its subcellular destination, where it can then interact with barley target proteins via its C-terminus.

The presumptive host targets of BEC4 are involved in two different pathways. UBCs are components of the proteasomal ubiquitin-mediated degradation pathway. They function between the ubiquitin-activating enzymes that activate ubiquitin by conjugating the ubiquitin chain and ubiquitin ligases that transfer ubiquitin to target proteins for degradation by the host proteasome (Passmore and Barford, 2004). BEC4 only interacted with $HvUBC_{60–196}$ in yeast (Fig. 5B), whereas it interacted with the full-length $HvUBC_{1–196}$ *in planta* (Fig. 5C), indicating that the interaction of BEC4 with its presumptive barley targets is different inside the host cell. The N-terminus might hinder the interaction with BEC4 in yeast, but not *in planta*, which might point to a conformational change of the *Hv*UBC protein *in planta*, e.g. through interaction with another protein. Exploitation of the host ubiquitin pathway has also been demonstrated for other plant pathogens. The *Pseudomonas syringae* effector HopM1 marks the Arabidopsis protein *At*MIN7 for proteasomal degradation (Nomura *et al*., 2006). Interestingly, *AtMIN7* encodes an Arabidopsis ARF-GEF, i.e. one of the ARF-GAP counter players in regulating intracellular vesicle trafficking. One explanation for the observation that BEC4 interacts strongly with *Hv*ARF-GAP in yeast, but not *in planta*, may be that it causes instability or degradation of *Hv*ARF-GAP inside the barley cell. This degradation could be facilitated through the interaction of BEC4 with $HvUBC_{60–196}$. This concept is further supported by our failure to observe the co-expression of all three fluorophore-tagged proteins in the same cell in either barley or tobacco (data not shown). In the case of *P. syringae*, the HopM1-mediated proteasomal degradation of *At*MIN7 has been suggested to interfere with host vesicle trafficking. Vesicle trafficking has been convincingly implicated in defence against powdery mildew fungi in both barley and Arabidopsis. The barley syntaxin *Hv*ROR2 and its Arabidopsis orthologue PEN1 are



both required to restrict the entry of powdery mildew into plant cells (Collins *et al*., 2003). Here, we have demonstrated that another component of host vesicle trafficking, the Arabidopsis orthologue of *HvARF-GAP*, *AtAGD5*, is also required for powdery mildew defence, as knockout of this gene renders Arabidopsis plants more accessible to invasion by a non-adapted powdery mildew fungus (Fig. 6C). The only moderate increase in *E. pisi* entry rates seen in these mutant plants could be caused by some functional redundancy of *AtAGD5* and its closest relative, *AtADG15* (Fig. 6A). Pathogen-triggered accumulation of the syntaxin *Hv*ROR2 underneath fungal attack sites has recently been shown to depend on two members of the barley ARF family (Böhlenius *et al*., 2010). ARF proteins regulate vesicle budding by recruiting coat proteins to specific membrane patches in their

Fig. 5 BEC3 and BEC4 interact with different barley proteins. (A) BEC3 interacts with the C-terminus of *Hv*TPMT in yeast. Yeast strain EGY48-pLexA-BEC3$_{19–114}$ (BAIT) was independently transformed with the following PREY constructs: pB42AD-GWY (vector) alone or pB42AD-GWY containing either the N-terminally truncated or full-length coding sequences of *Hv*TPMT, *Hv*ARF-GAP and *Hv*UBC. Strains were spotted onto non-inducing (GLU-UHT and GLU-UHTL) and inducing (GAL-UHT+X-Gal and GAL-UHTL) medium and incubated for 2 days at 30 °C. (B) BEC4 interacts with full-length *Hv*ARF-GAP and the C-terminus of *Hv*UBC in yeast. Yeast strain EGY48-pLexA-BEC4$_{22–235}$ (BAIT) was independently transformed with either pB42AD-GWY alone or with pB42AD-GWY containing either the N-terminally truncated or full-length coding sequences of *Hv*ARF-GAP, *Hv*UBC and *Hv*TPMT. Strains were plated on media as described in (A). (C) BEC3 and BEC4 interact *in planta* with full-length *Hv*TPMT and *Hv*UBC, respectively. Bimolecular fluorescence complementation (BiFC) of the interaction between *Blumeria* effector candidate (BEC) proteins and their respective barley targets was examined by confocal laser scanning microscopy after bombardment of the respective gene constructs into single barley epidermal cells. To identify transformed cells prior to yellow fluorescent protein (YFP) analysis, a control plasmid encoding an endoplasmic reticulum (ER) marker fused to mCherry (ER-rk CD3-959) was co-bombarded. Yellow fluorescence indicates interaction of BEC3$_{19–114}$ and *Hv*TPMT and BEC4$_{22–235}$ and *Hv*UBC. The coding regions of the *BEC4*, *BEC3*, *HvTPMT* and *HvUBC* genes were fused with the N-terminal (YFP^N; amino acids 1–155) and C-terminal (YFP^C; amino acids 156–239) segments of YFP, respectively. (D) *Hv*ARF-GAP accumulates in barley epidermal cells and partially co-localizes with the Golgi network. *Hv*ARF-GAP$_{1–476}$ was translationally fused to YFP and transiently co-expressed in single barley epidermal cells with a Golgi marker fused to mCherry (G-rk CD3-967). Please note that the Golgi marker also weakly labels the ER (Nelson *et al*., 2007). Two days after the bombardment, we examined fluorescence by confocal laser scanning microscopy. Micrographs are false coloured. Size bars indicate 10 μm.

GTP-bound state. After vesicle budding, the ARF GTPase activity is activated by an ARF-GAP to release the protein from the vesicle. Absence of the ARF-GAP should result in the same phenotype as an amino acid substitution in the ARF protein that locks it in its GTP-bound state. This is actually the case: transient expression of *Hv*ARFA1b/1c harbouring an amino acid substitution that eliminates GTPase activity resulted in increased *Bgh* haustorium formation (Böhlenius *et al*., 2010). We speculate that BEC4 might target *Hv*ARF-GAP to degradation, via its interaction with *Hv*UBC, to interfere with host vesicle trafficking. Interestingly, *Atagd5* knockout plants exhibit reduced sporulation on challenge with the Arabidopsis downy mildew pathogen, *H. arabidopsidis*, and thus show a phenotype that opposes that seen with powdery mildew (Fig. 6D, see above). This outcome may indicate that *At*AGD5 also represents a target for secreted effectors of the downy mildew pathogen, but that the mode of action of these effectors is antagonistic to the activity of BEC4. The latter may reflect the differential needs of a pathogen exclusively colonizing epidermal cells relative to a pathogen preferentially attacking leaf mesophyll cells.

In this study, we not only identified five *Bgh* effector candidates that are expressed at different stages of barley infection, but also unravelled host vesicle trafficking as a pathway that might be targeted by the invading pathogen. Mining for further *Bgh* effector candidates has been greatly expedited by the recent publication of the *Bgh* genome (Pedersen *et al*., 2012; Spanu *et al*., 2010). However, genetic inaccessibility of *Bgh* will still require functional assays, such as transient gene expression (Panstruga, 2004), host-induced gene silencing (Nowara *et al*., 2010) and yeast two-hybrid screens, to establish virulence functions and unveil plant targets. It will be interesting to determine whether more powdery mildew effectors target the host vesicle trafficking machinery.

EXPERIMENTAL PROCEDURES

A detailed description of the experimental procedures can be found in File S1.

Plant and fungal material and pathogen assays

Barley seedlings (*Hordeum vulgare* cultivar Golden Promise) were grown at 20 °C and 16 h light/8 h darkness in a protected environment. The barley powdery mildew (*Bgh*) isolates K1 (*AvrMla1*, *virMla6*, *virMla10*, *virMla12*, *virMlg*), A6 (*virMla1*, *AvrMla6*, *AvrMla10*, *AvrMla12*, *AvrMlg*), DH14 (*AvrMla1*, *AvrMla6*, *virMla10*, *AvrMla12*, *virMlg*), CC146 (*AvrMla1*, *AvrMla6*, *virMla10*, *AvrMla12*, *AvrMlg*), CC148 (*AvirMla1*, *virMla6*, *AvrMla10*, *virMla12*, *virMlg*) and CC52 (*AvrMla1*, *AvrMla6*, *virMla10*, *virMla12*, *virMlg*) were propagated on barley lines 'I10' (*Mla12*) and 'P01' (*Mla1*) for mutual exclusion. Plants or detached leaves were kept at 20 °C, 60% relative humidity and 16 h light/8 h darkness after inoculation with *Bgh* conidiospores. The pea powdery mildew (*E. pisi*) isolate *Birmingham* was maintained on 3-week-old pea plants, cultivar Linga. Pea and inoculated *A. thaliana* plants (Col-0) were kept at 22 °C, 70% humidity, 100 μE/m^2/s and 12 h light/12 h darkness in a protected environment. To visualize epiphytic fungal structures, specimens were stained with Coomassie Brilliant Blue. For the quantification of fungal host cell entry of *E. pisi* and *G. orontii*, the proportion of germinated fungal sporelings that developed secondary hyphae served as an approximation of penetration success. The *H. arabidopsidis* (isolate Noco) pathogen assay and quantification of *G. orontii* conidiation were essentially performed as described previously (Stuttmann *et al*., 2011; Weßling and Panstruga, 2012) by scoring asexual spore formation on Arabidopsis seedlings using a haemocytometer.

cDNA synthesis and RT-PCR analysis

Barley and *Bgh* cDNAs were synthesized from total RNA using the SuperScript® II Reverse Transcriptase kit (Life Technologies, http://www.lifetechnologies.com). qRT-PCR analysis was carried out on the basis of SYBR Green chemistry.

Plasmid constructs

Gateway® cloning technology (Life Technologies) was used to create the destination vectors employed in this study (Table S1, see Supporting Information). Briefly, PCR products were recombined into the vector pDONR201 (Life Technologies) by Gateway® BP recombination, and sub-

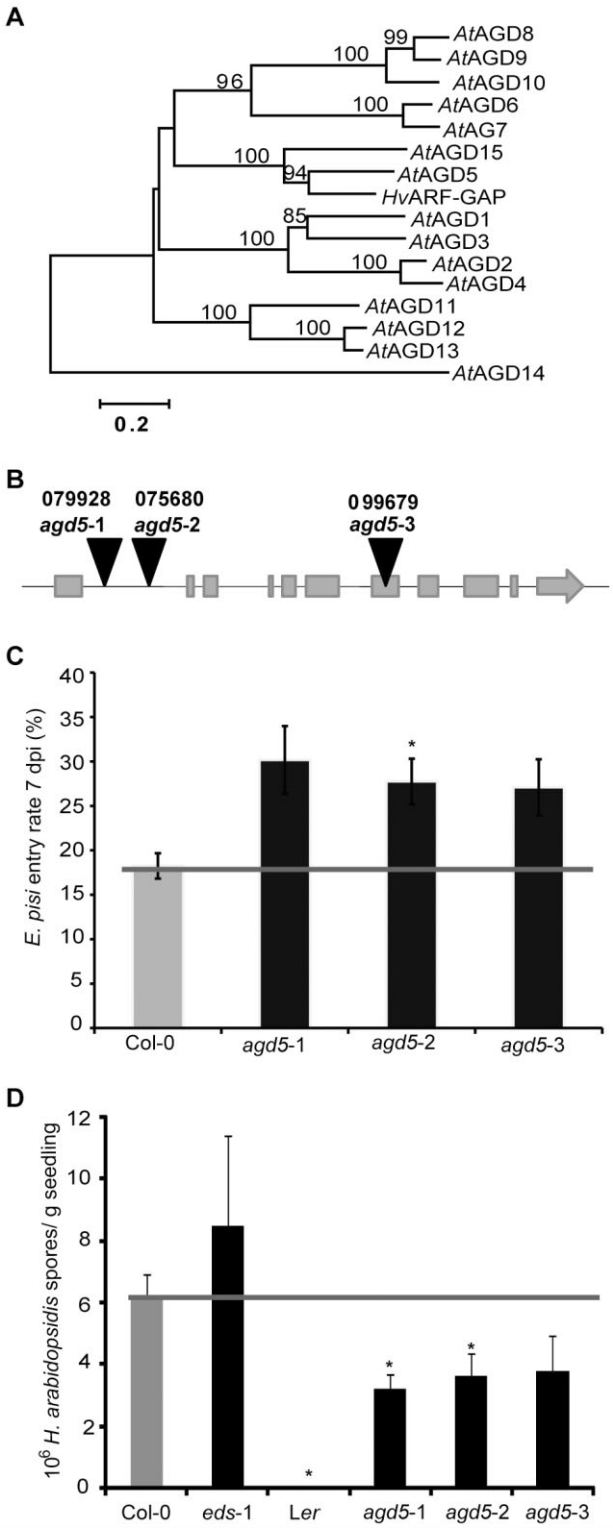


Fig. 6 *AGD5* plays different roles in powdery mildew and downy mildew disease progression. (A) *At*AGD5 is an orthologue of *Hv*ARF-GAP. An unrooted phylogenetic tree was generated by the neighbour-joining method using protein sequences of the 15 Arabidopsis ADP ribosylation factor-GTPase-activating protein (ARF-GAP) (*At*AGD1-15) members, as well as the barley ARF-GAP (*Hv*ARF-GAP, NCBI accession number AK250094). The pairwise deletion and Poisson correction options were used. Bootstrap values (given as % on the basis of 1000 replications) are indicated on the interior branches. The scale bar represents the number of amino acid substitutions per site. (B) Scheme depicting the *At5g54310* locus showing the sites of T-DNA insertion. Grey boxes indicate the exons of *At5g54310* coding for *At*AGD5. T-DNA insertion sites are marked with black arrowheads and the number of the T-DNA insertion line from the SALK collection, as well as the respective allele designation. (C) *Atagd5* plants allow higher entry rates of the non-adapted powdery mildew fungus *Erysiphe pisi*. Fungal entry rates were determined quantitatively at 7 days post-inoculation with *E. pisi* spores on wild-type Col-0 and *Atagd5* plants. Results represent mean ± standard error of three independent experiments. The asterisk indicates $P \leq 0.05$ (Student's *t*-test) compared with the wild-type. (D) *Atagd5* seedlings show reduced sporulation of the oomycete pathogen *Hyaloperonospora arabidopsidis* (isolate Noco). Genotypes *eds1*-2 (in the Col-0 background) and Landsberg *erecta* (L*er*) served as controls for enhanced and reduced sporulation, respectively. Graphs represent the average ± standard error of four independent experiments. Asterisks indicate $P \leq 0.05$ (Student's *t*-test) compared with the wild-type.

sequently shuttled into destination vectors by Gateway® LR recombination to create the expression constructs. The vector used for transient expression of the *BECs* (pUbi-GWY) drives transcription from a constitutive maize ubiquitin promoter. The pUCSPYNE, pUCSPYCE, pESPYNE and pESPYCE vectors harbouring N- and C-terminal segments of enhanced YFP were adapted to be Gateway® compatible and have been described previously (Waadt and Kudla, 2008; Walter *et al*., 2004).

Transient gene expression in single barley epidermal cells

Ballistic transformation of detached barley leaves (cultivar Golden Promise) was carried out as described previously (Schweizer *et al*., 1999; Shirasu *et al*., 1999). Briefly, gold particles of 1 μm diameter were coated with reporter plasmids plus effector plasmids, followed by biolistic delivery into 7-day-old barley leaves using a particle gun equipped with a Hepta adapter (Biolistic PDS-1000/He device, Bio-Rad Laboratories, Hercules, CA, USA). To determine the haustorium index after transient effector gene expression, bombarded leaves were inoculated with a high density of powdery mildew conidiospores (isolate K1) at 4 h after bombardment. Two days after inoculation, bombarded leaves were infiltrated with GUS staining solution (Schweizer *et al*., 1999). Epiphytic fungal structures were marked by Coomassie Brilliant Blue staining. Leaf epidermal cells attacked by the appressorial germ tube of powdery mildew sporelings were evaluated by light microscopy for the presence or absence of haustoria. The haustorium index was calculated as the percentage of transformed cells exhibiting one or multiple haustoria in relation to the total number of transformed cells attacked by powdery mildew sporelings.

Fluorescence microscopy

For subcellular localization studies, barley leaves (cultivar Golden Promise) were ballistically co-transformed with various BiFC fusion constructs and constructs of mCherry either fused to an ER-targeting and retention signal (ER-rk CD3-959) as ER marker or fused to the cytoplasmic tail and

transmembrane domain of the soybean α-1,2-mannosidase I (G-rk CD3-967) as Golgi marker (Nelson *et al*., 2007). Confocal microscopy of the transformed leaves was carried out using the Zeiss LSM510 META confocal microscope (Zeiss, http://www.zeiss.com).

Yeast two-hybrid screen

Interacting proteins of the effector candidates were identified by means of the LexA-based yeast two-hybrid assay. Bait proteins were expressed as LexA fusion proteins and tested for interaction by mating with a barley prey cDNA library and subsequent growth on selective media.

Transformation of *A. thaliana*

Full-length and truncated versions (lacking the N-terminal SP) of *Go*EC2 were cloned into the binary destination vectors pPAM-PAT-GWY and pPAM-PAT-GWY-3xHA, and ecotype Col-0 was transformed with the resulting constructs using the floral dip method, as described by Clough and Bent (1998). Transformants were selected in the T_1 generation by spraying with Basta®, and analysed in the T_3 generation.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Binding sites of polymerase chain reaction (PCR) primers used in this study shown on cDNA sequences of *BEC1–BEC5*.

Fig. S2 *Blumeria* effector candidate (BEC) proteins enhance *Blumeria graminis* f. sp. *hordei* (*Bgh*) haustorium formation. Single epidermal cells of 7-day-old barley leaves (cultivar Golden Promise) were biolistically transformed with either a *β-glucuronidase* (*GUS*) reporter construct alone or together with an overexpression construct of the *BEC*s. Subsequently leaves were inoculated with *Bgh* conidiospores and, at 48 h post-inoculation (hpi), were stained for GUS activity, which renders transformed cells greenish-blue. Epiphytic fungal structures were stained with Coomassie Brilliant Blue. (A) Successful penetration (S) results in the formation of an intracellular haustorium (h). Non-penetrated resistant cells (R) are marked by the presence of a papilla (p) that coincides with penetration arrest. (B) The ratio of transformed cells enabling haustorium formation divided by the total number of attacked transformed cells results in the haustorium index, and is a measure of the impact of the tested gene on the infection success. The *GUS* only control was set to 100%. The data shown represent the means ± standard error from at least nine independent experiments. For each experiment and tested gene construct, at least 100 transformed cells were evaluated. Truncated protein variants are indicated by subscripts denoting the respective amino acid range. Barley *MLO* was used as a positive control.

Asterisks indicate $P \leq 0.05$ (Student's *t*-test) compared with the negative control (*GUS* alone).

Fig. S3 Nucleotide sequence alignment of the *Blumeria effector candidate* (*BEC*) genes from six different *Blumeria graminis* f. sp. *hordei* (*Bgh*) isolates. Genomic DNA was isolated from barley leaves infected with one of the six *Bgh* isolates (K1, CC52, DH14, CC146, CC148, A6) and sequenced with primers BECF and BECR (Fig. S1) specific for the *BEC* genes. (A) *BEC1*. (B) *BEC2*. (C) *BEC3*. (D) *BEC4*. (E) *BEC5*.

Fig. S4 Transgenic Arabidopsis plants expressing the full-length *Golovinomyces orontii BEC2* orthologue ($GoEC2_{1\text{-}154}$), $GoEC2_{18–154}$ and *Atagd5* mutant plants show unaltered entry rates and sporulation on challenge with *G. orontii*. Host cell entry (A) and conidiation (B) were quantitatively assessed at 2 days (A) or 7 days (B) post-inoculation with *G. orontii* on Col-0 wild-type, *GoEC2*-expressing and *Atagd5* mutant plants. Two independent lines expressing $GoEC2_{1–154}$ and one line expressing $GoEC2_{18–154}$ (T_3 generation), as well as three different *Atagd5* T-DNA lines (see Fig. 6), were tested. Results represent the mean ± standard error of five independent experiments.

Fig. S5 Accumulation and autoactivation of bait proteins in the yeast two-hybrid assay. (A) BEC-LexA fusion proteins are stably expressed in yeast. *Blumeria* effector candidate (BEC) bait proteins fused to the LexA binding domain were expressed in yeast strain EGY48. Fusion proteins were detected by immunoblotting with a LexA antiserum in crude yeast protein extracts from four independent transformants per construct. Molecular marker masses are indicated on the left. (B) BEC2 autoactivates the LexA reporter system in yeast. Yeast strain EGY48 was independently transformed with the following constructs: pLexA-GWY (vector) alone or pLexA-GWY containing $BEC2_{25–133}$, $BEC3_{19–114}$, $BEC4_{22–235}$ and $BEC5_{20–139}$. Tenfold dilution series of the transformed strains were spotted onto inducing galactose-containing medium without uracil, histidine, tryptophan and leucine medium and incubated for 2 days at 30 °C. Four transformants were tested in each case.

Fig. S6 Phenotype of *Atagd5* plants. (A) *Atagd5* plants are chlorotic and smaller than Col-0 wild-type plants. Photographs were taken of 4-week-old *Arabidopsis thaliana* wild-type Col-0 and *Atagd5* plants before powdery mildew infection. (B) Rosette leaves of *Atagd5* plants are more chlorotic after powdery mildew challenge. Photographs were taken of 4-week-old *A. thaliana* Col-0 and *Atagd5* plants at 7 days post-infection with the non-adapted powdery mildew fungus *Erysiphe pisi*. The lower panel shows a close-up view of representative rosette leaves. Scale bars indicate 1 cm.

Table S1 Primers used in this study.

Table S2 Effector candidate (EC) interacting proteins from barley.

File S1 Detailed experimental procedures.