A barley SKP1-like protein controls abundance of the susceptibility factor RACB and influences the interaction of barley with the barley powdery mildew fungus

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SUMMARY

In an increasing number of plant–microbe interactions, it has become evident that the abundance of immunity-related proteins is controlled by the ubiquitin–26S proteasome system. In the interaction of barley with the biotrophic barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*), the RAC/ROP [RAT SARCOMA-related C3 botulinum toxin substrate/RAT SARCOMA HOMOLOGUE (RHO) of plants] guanosine triphosphatase (GTPase) HvRACB supports the fungus in a compatible interaction. By contrast, barley HvRBK1, a ROP-binding receptor-like cytoplasmic kinase that interacts with and can be activated by constitutively activated HvRACB, limits fungal infection success. We have identified a barley type II S-phase kinase 1-associated (SKP1)-like protein (HvSKP1-like) as a molecular interactor of HvRBK1. SKP1 proteins are subunits of the SKP1-cullin 1-F-box (SCF)–E3 ubiquitin ligase complex that acts in the specific recognition and ubiquitination of protein substrates for subsequent proteasomal degradation. Transient induced gene silencing of either *HvSKP1-like* or *HvRBK1* increased protein abundance of constitutively activated HvRACB in barley epidermal cells, whereas abundance of dominant negative RACB only weakly increased. In addition, silencing of *HvSKP1-like* enhanced the susceptibility of barley to haustorium establishment by *Bgh*. In summary, our results suggest that HvSKP1-like, together with HvRBK1, controls the abundance of HvRACB and, at the same time, modulates the outcome of the barley–*Bgh* interaction. A possible feedback mechanism from RAC/ROP-activated HvRBK1 on the susceptibility factor HvRACB is discussed.

Keywords: *Blumeria graminis* f.sp. *hordei*, *Hordeum vulgare*, HvRACB, ROP-binding kinase, SCF–E3 ubiquitin ligase, SKP1.

INTRODUCTION

The ubiquitin–26S proteasome system is a major regulator of protein stability. Being responsible for controlled protein degra-

dation, the system is involved in almost all processes of plant biology, including hormone signalling, cell division, plant development and responses to abiotic and biotic stresses (Dreher and Callis, 2007; Sadanandom *et al.*, 2012; Smalle and Vierstra, 2004, Vierstra, 2009). The substrates of the ubiquitin–26S proteasome system are post-translationally modified through the attachment of ubiquitin chains prior to their degradation by the 26S proteasome. In a three-step enzymatic cascade, ubiquitin is activated by an E1 ubiquitin-activating enzyme, transferred from an E1 enzyme to an E2 ubiquitin-conjugating enzyme and attached to the target protein via an E3 ubiquitin ligase. To obtain polyubiquitinated proteins, which are subjected to proteasomal degradation, this mechanism is repeated several times (Vierstra, 2009). Although E1 and E2 enzymes are poorly specific, E3 ubiquitin ligases confer substrate specificity to the ubiquitination pathway. One type of E3 ubiquitin ligase, the CRL (cullin-RING) ligases, is subdivided into several groups with the S-phase kinase-associated protein 1-cullin 1-F-box (SCF)–E3 ubiquitin ligases representing one large family (Hua and Vierstra, 2011, Vierstra, 2009). In this family, cullin 1 is linked through the adaptor protein S-phase kinase-associated protein 1 (SKP1) to an F-box protein, which specifically recognizes the target protein (Hua and Vierstra, 2011). Plants have a large number of SKP1 and F-box subunits ensuring highly variable SCF complexes (Hua and Vierstra, 2011). A closer look at *SKP1* genes in plants revealed that there are 21 genes in *Arabidopsis thaliana*, named *Arabidopsis SKP1-like* (ASKs), and 32 in rice, named *Oryza sativa SKP1-like* (OSKs) (Kahloul *et al.*, 2013; Kong *et al.*, 2007). Based on their gene and protein structure, plant *SKP1-like* genes are divided into three types of genes and two types of proteins (Kong *et al.*, 2007). Type I proteins, encoded by type Ia and type Ib genes showing either one or no intron in their genomic sequence, contain two conserved domains (SKP1_POZ and SKP1). The type II protein structure is similar to that of type I proteins, except that they show a carboxy-terminal elongation. In addition, the type II-encoding genes contain several introns (Kong *et al.*, 2007). The function of *SKP1-like* genes in plants has not been characterized in detail. For ASK1 and ASK2 proteins, it has been shown that they are involved in flower development, male sterility, embryogenesis and seedling development (Liu

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et al., 2004; Zhao *et al.*, 2003). Furthermore, ASK1 and ASK2 are part of the SCF-E3 ubiquitin ligase complexes CORONATINE INSENSITIVE 1 (SCF^{COI1}), TRANSPORT INHIBITOR RESPONSE 1 (SCF^{TIR1}) and EIN3 BINDING F-BOX 1/EIN3 BINDING F-BOX 2 (SCF^{EBF1/EBF2}), which function in the derepression of jasmonate, auxin and ethylene signalling, thereby linking the regulation of defence gene expression via hormone signalling to ubiquitin-mediated proteolysis (Potuschak *et al.*, 2003; Trujillo and Shirasu, 2010). It has also been speculated that ASK1 and ASK2, together with SUPPRESSOR OF G2 ALLELE OF SKP1 (SGT1), SUPPRESSOR OF *rps4-RLD1* (SRFR1) and CONSTITUTIVE EXPRESSOR OF PR GENES 1 (CPR1), controls the accumulation of the intracellular plant resistance (R) protein homologue SUPPRESSOR OF *npr1-1*, CONSTITUTIVE 1 (SNC1) (Duplan and Rivas, 2014; Gou *et al.*, 2009, 2012). Within recent years, it has also become evident that E3 ubiquitin ligases play a role in plant immunity and that ubiquitin-mediated protein degradation is involved in many aspects of plant-microbe interactions (Delaure *et al.*, 2008; Dielen *et al.*, 2010; Dreher and Callis, 2007; Duplan and Rivas, 2014; Marino *et al.*, 2012; Zeng *et al.*, 2006).

In the metazoan kingdom, small monomeric RAC (RAT SARCOMA-related C3 botulinum toxin substrate) guanosine triphosphatases (GTPases) are substrates of the 26S proteasome (Lerm *et al.*, 2002; Zhao *et al.*, 2013). Plant RAC/ROP [RAT SARCOMA-related C3 botulinum toxin substrate/RAT SARCOMA HOMOLOGUE (RHO) of plants] GTPases are regulators in plant-microbe interactions (Kawano *et al.*, 2014), but nothing is known about the post-translational regulation of protein abundance of plant RAC/ROPs. In the interaction of barley (*Hordeum vulgare*) with the adapted powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*), the RAC/ROP protein HvRACB is a susceptibility factor needed for the accommodation of haustoria in intact epidermal cells (Hoefle *et al.*, 2011; Pathuri *et al.*, 2008; Schultheiss *et al.*, 2002, 2003). Barley ROP BINDING KINASE1 (HvRBK1) interacts with and becomes activated by the constitutively active (CA) form of HvRACB (HvRACB^{G15V}). Transient induced gene silencing (TIGS) of *HvRBK1* reduces microtubule stability and enhances the infection success of *Bgh*. By contrast, TIGS of *HvRACB* limits fungal infection, whereas the expression of CA *HvRACB* supports it (Schultheiss *et al.*, 2003). Hence, a mechanism has been suggested which involves a negative feedback from HvRBK1 on HvRACB (Huesmann *et al.*, 2012). Here, we show that HvRBK1 interacts with a barley type II SKP1-like protein (HvSKP1-like) in yeast and *in planta*. TIGS of either *HvSKP1-like* or *HvRBK1* in barley epidermal cells increased the protein abundance of CA HvRACB, but abundance of inactive, dominant negative (DN) HvRACB (HvRACB^{T20N}) hardly changed. TIGS of *HvSKP1-like* supported fungal infection success. These findings suggest a role of the E3 ubiquitin ligase component HvSKP1-like in the control of HvRACB abundance, thereby modulating interaction outcome in the barley-*Bgh* interaction.

RESULTS

Isolation and sequence analysis of *HvSKP1-like*

To identify barley proteins that interact with HvRBK1 (Huesmann *et al.*, 2012), we performed a yeast two-hybrid screening in which HvRBK1 was used to screen against a cDNA library derived from a pool of powdery mildew-infected and non-infected barley plants (Hoefle *et al.*, 2011). A partial cDNA fragment of 200 bp was isolated in the screening, for which we identified a corresponding genomic contig sequence of 6716 bp in the barley genome sequence (morex_contig_1577381, <http://webblast.ipk-gatersleben.de/barley/>) (Mayer *et al.*, 2012). BLAST and intron-exon predication tools (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>; <http://www.ncbi.nlm.nih.gov/spidey/spideyweb.cgi>) revealed a gene model on barley chromosome 4H consisting of eight introns and nine exons. The identified full-length open reading frame of 1038 bp was cloned from a barley cDNA pool and encodes a protein of 345 amino acids. Analysis of the deduced amino acid sequence indicated an SKP1 domain (SM000512, <http://smart.embl-heidelberg.de/>) in the protein, and a sequence similarity search identified *Arabidopsis thaliana* S-phase kinase-associated like 21 (ASK21; 62% identity) and *Oryza sativa* SKP1-like 31 (OSK31; 91% identity) as the most similar proteins in *Arabidopsis* and rice, respectively (Figs 1 and S1, see Supporting Information). The barley SKP1-like protein was classified as a type II SKP1 protein as its encoding gene contains more than one intron (Kong *et al.*, 2007). Phylogenetic analysis of plant SKP1-like proteins revealed that HvSKP1-like clustered with type II OSK31, ASK20 and ASK21 (Fig. 1) (Kahloul *et al.*, 2013; Kong *et al.*, 2007). BLAST searches for barley HvSKP1-like resulted in no further matches, suggesting that the corresponding gene is the only type II *SKP1* gene in barley. As a result of these findings, we named the protein *Hordeum vulgare* type II SKP1-like (in the following HvSKP1-like; accession number, LN714776).

HvSKP1-like interacts with HvRBK1 in yeast and *in planta*

In independent targeted yeast two-hybrid assays, the interaction of the partial (amino acids 156–222, data not shown) and full-length sequence of HvSKP1-like with HvRBK1 (Fig. 2a) was confirmed. No interaction was detected between HvSKP1-like and variants of HvRACB in the yeast two-hybrid assay (Fig. 2b), although these variants of HvRACB can interact with diverse barley proteins in yeast (Hoefle *et al.*, 2011; Huesmann *et al.*, 2012; Schultheiss *et al.*, 2008). Because HvSKP1-like interacted with HvRBK1 in yeast, we wanted to determine whether these proteins can physically interact *in planta*. Subcellular co-localization of proteins can give a first hint towards protein-protein interaction. Hence, we transiently co-expressed green fluorescent protein (GFP)-tagged HvSKP1-like

Fig. 1 Phylogenetic relationships of plant S-phase kinase 1-associated (SKP1)-like proteins. The evolutionary history was inferred using the maximum likelihood method based on the Poisson correction model (Zuckerkandl and Pauling, 1965). The tree with the highest log likelihood (−2113.4302) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches. Initial tree(s) for the heuristic search were obtained automatically by applying neighbour-joining and BioNJ algorithms to a matrix of pairwise distances estimated using a Jones, Taylor and Thornton model, and then selecting the topology with a superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 52 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 30 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013).

with red fluorescing mcherry-tagged HvRBK1 in barley epidermal cells. Both proteins similarly localized in the cytoplasm and nucleoplasm 24 h after bombardment (hab) with plasmid-coated gold particles (Fig. 3a). Single optical sections confirmed that signals of GFP-HvSKP1-like largely overlapped with those of mcherry-HvRBK1. However, co-localization was not perfect, perhaps suggesting enrichment of the respective proteins in cytoplasmic subdomains (Fig. S2a, see Supporting Information). In addition, GFP-HvSKP1-like largely co-localized with the cytoplasmic and nucleoplasmic marker mcherry on transient co-expression in barley epidermal cells (Figs 3b and S2b). Furthermore, we conducted fluorescence resonance energy transfer (FRET) measurements using the acceptor photobleaching method to determine whether HvSKP1-like interacts with HvRBK1 *in vivo*. For this purpose, yellow fluorescent HvRBK1-YFP was co-expressed with either cyan fluorescent CFP-HvSKP1-like or non-fused CFP in barley epidermal cells. Regions of interest for bleaching were selected at the cell periphery because cytoplasmic movement, which can dilute FRET efficiency after acceptor bleaching, was reduced in this area. We measured 15.6% FRET efficiency by 24 h after transformation in cells expressing HvRBK1-YFP and CFP-HvSKP1-like, whereas control cells expressing HvRBK1-YFP and CFP showed only 2.4% of background energy transfer (Fig. 3c). These results suggest that HvRBK1 and HvSKP1-like can interact *in planta*.

The proteasomal inhibitor MG132 and RNA interference (RNAi) of *HvRBK1* enhance protein abundance of CA HvRACB in protoplasts

Recent findings in the animal research field have revealed that SCF-E3 ubiquitin ligase complexes target HvRACB-related GTPases for their proteasomal degradation (Wei *et al.*, 2013; Zhao *et al.*, 2013). As HvSKP1-like interacts with HvRBK1, which again interacts with the CA form of HvRACB (Huesmann *et al.*, 2012), we speculated that HvRACB might be a target for proteasomal degradation. To test whether CA HvRACB is degraded via the ubiquitin–26S proteasome pathway, mcherry-CA HvRACB and GFP were co-transformed into barley protoplasts that were treated with the proteasome inhibitor MG132. The fluorescence intensity of mcherry-CA HvRACB was measured at the cell periphery and normalized to that of co-expressed GFP in the nucleus. Fluorescence intensity in dimethylsulfoxide (DMSO) solvent control-treated protoplasts was set to 100%. The fluorescence intensity of mcherry-CA HvRACB increased by about 60% after MG132 treatment, indicating that mcherry-CA HvRACB might be a target for proteasomal degradation (Fig. 4a). To test whether the presence of HvRBK1 is relevant for the degradation of CA HvRACB, a gene construct encoding a cytosolic version of CA HvRACB, GFP-CA HvRACBΔCSIL, lacking the C-terminal prenylation motif CSIL responsible for membrane association (Schultheiss *et al.*, 2003), was transformed into barley leaf mesophyll protoplasts, together

Fig. 2 Targeted yeast two-hybrid assay of barley type II S-phase kinase 1-associated (SKP1)-like protein (HvSKP1-like) with barley ROP BINDING KINASE1 (HvRBK1) or different variants of HvRACB. (a) HvSKP1-like interacts with HvRBK1. (b) HvSKP1-like shows no interaction with any variant of HvRACB. Positive protein–protein interaction is shown on selective medium lacking leucine, tryptophan, adenine and histidine (SD-LWAH). Yeast growth on selective medium lacking leucine and tryptophan (SD-LW) indicates successful co-transformation. Yeasts in (a) and (b) were dropped in parallel on the same plate, but the figure was split for better labelling. Expression of proteins was driven by the ADH1 promoter. CA, constitutively active, DN, dominant negative, WT, wild-type.

with an *HvRBK1*-RNAi construct (Huesmann *et al.*, 2012) or the empty RNAi vector pIPKTA30N. The use of cytosolic GFP-CA HvRACB Δ CSIL was necessary instead of full-length GFP-CA HvRACB to enhance the amount of immuno-detectable tagged CA HvRACB protein that could be extracted from barley protoplasts in a reproducible manner. Western blot analysis showed that GFP-CA HvRACB Δ CSIL was more abundant in protoplasts in which *HvRBK1* was silenced than in protoplasts with the empty RNAi vector (Fig. 4b). However, silencing of *HvSPK1-like* did not lead to a consistent and reproducible increase in GFP-CA HvRACB Δ CSIL signal on Western blots (data not shown). To address the question of whether *HvRBK1*-RNAi limits specifically the protein abundance of GFP-CA HvRACB Δ CSIL, we repeated the experiment with free GFP. In contrast with GFP-CA HvRACB Δ CSIL, the protein abundance of GFP was not affected in the presence of the *HvRBK1*-RNAi construct (Fig. 4b). These findings suggest that HvRBK1 could be involved in the specific degradation of HvRACB.

CA HvRACB abundance increases on silencing of *HvRBK1* or *HvSKP1-like* in barley epidermal cells

Because *HvRBK1*-RNAi had an effect on the protein abundance of GFP-CA HvRACB Δ CSIL in protoplasts, we wondered whether silencing of *HvRBK1* or *HvSKP1-like* in barley epidermal cells would lead to similar results. For this purpose, barley epidermal

cells were transiently transformed with CFP-CA *HvRACB*, the appropriate RNAi construct for TIGS and *mcherry* by particle bombardment. The fluorescence intensity of CFP-CA *HvRACB* was measured at the plasma membrane at 48 hab and normalized to *mcherry* in the nucleus. The fluorescence intensity of CFP-CA *HvRACB* was more than three times higher in cells transformed with CFP-CA *HvRACB* and the silencing construct of either *HvRBK1* or *HvSKP1-like* than in cells transformed with CFP-CA *HvRACB* and the empty RNAi vector or CFP-CA *HvRACB* alone (Fig. 5a). We also repeated this experiment with CFP instead of CFP-CA *HvRACB*. The fluorescence intensity of CFP was unaffected by *HvRBK1*- or *HvSKP1-like*-RNAi constructs, indicating the specific influence of both RNAi constructs on the protein stability of HvRACB (Fig. 5b). To investigate whether the increase in CA HvRACB abundance is associated with its activation state, CFP-WT (wild-type) *HvRACB* and CFP-DN *HvRACB* were transiently transformed into barley epidermal cells, together with the respective RNAi construct for TIGS and *mcherry* for normalization. The fluorescence intensity of CFP-WT *HvRACB* increased on silencing of either *HvRBK1* or *HvSKP1-like* by more than two- or three-fold, respectively, compared with cells transformed with CFP-WT *HvRACB* alone (Fig. S3, see Supporting Information). The increase in fluorescence intensity of CFP-WT *HvRACB* was less pronounced when compared with that of CFP-CA *HvRACB* (Fig. 5a). Co-transformation of CFP-DN *HvRACB* with silencing constructs of

Fig. 3 Subcellular localization of barley type II S-phase kinase 1-associated (SKP1)-like protein (HvSKP1-like) and *in planta* interaction of HvSKP1-like with barley ROP BINDING KINASE1 (HvRBK1). (a) GFP-HvSKP1-like largely co-localizes with mCherry-HvRBK1 in cytoplasmic strands in transiently transformed barley epidermal cells 24 h after bombardment (hab). (b) GFP-HvSKP1-like shows a cytoplasmic localization similar to that of soluble mCherry that is co-transformed as a cytoplasmic and nucleoplasmic marker by 24 hab. Expression of fusion proteins was driven by the cauliflower mosaic virus (CaMV) 35S promoter. Confocal images show maximum projection of 23–26 optical sections at 2- μ m increments. Scale bar, 20 μ m. (c) Quantitative analysis of fluorescence resonance energy transfer (FRET) by acceptor photobleaching in transiently transformed barley epidermal cells at 24 hab confirmed the interaction of CFP-HvSKP1-like and HvRBK1-YFP *in planta*. All constructs were under the control of the CaMV 35S promoter. Data show mean $\pm$ standard error of the mean (SEM) of three independent experiments. In each experiment, 10 cells were analysed. *** $P \leq 0.01$ according to an unpaired Student's *t*-test. CFP, cyan fluorescent protein; GFP, green fluorescent protein; YFP, yellow fluorescent protein.

either *HvRBK1* or *HvSKP1-like* increased the fluorescence intensity of inactive CFP-DN HvRACB by around 40% or 50%, respectively, compared with the controls (Fig. 5c). Although this increase was statistically significant, it was weak when compared with that of wild-type or activated HvRACB. This suggests similar functions of HvSKP1-like and HvRBK1 in the preferential regulation of the abundance of the active HvRACB protein.

HvSKP1-like modulates fungal infection success

To investigate the possible function of the HvRBK1-interacting HvSKP1-like in the barley–*Bgh* interaction, we analysed transcript abundance by reverse transcription quantitative real-time polymerase chain reaction (PCR). A time course analysis of gene expression during fungal development showed a slight, but statistically non-significant, accumulation of transcripts 12 h after inoculation (hai) with *Bgh* when compared with non-inoculated controls (Fig. 6a). The transcript abundance of the *Bgh*-responsive *HvPR-1b* transcripts (Bryngelsson *et al.*, 1994) strongly accumulated after inoculation with *Bgh* in the same samples (Fig. 6a). Next, we tested whether HvSKP1-like in barley epidermal cells is important for the interaction outcome of barley with *Bgh*. Therefore, we scored the frequency of established haustoria after inoculation of leaf segments in cells, in which we silenced *HvSKP1-like*. The infection success of *Bgh* increased on transformed single cells after TIGS of *HvSKP1-like* from 33% to 47% by 48 hai, corresponding to a relative increase of 42% (Fig. 6b). To assess the efficacy of TIGS on the expression of *HvSKP1-like*, we quantified the ability of the *HvSKP1-like*-RNAi construct to silence the expression of *GFP-HvSKP1-like* in transiently transformed barley epidermal cells. Therefore, we counted cells co-expressing the non-target mCherry and target GFP-HvSKP1-like on co-transformation of the *HvSKP1-like*-RNAi construct. Co-transformation of the *HvSKP1-like*-RNAi construct reduced the co-expression rate of GFP-HvSKP1-like in mCherry-expressing cells from 86% to 38% when compared with the empty RNAi vector pIPKTA30N control (Fig. 6c). Together, this suggests a function of HvSKP1-like in limiting the susceptibility of barley to powdery mildew infection.

DISCUSSION

In this study, we identified a barley type II SKP1-like protein, which is a *bona fide* SCF–E3 ubiquitin ligase subunit, as a molecular interactor of the receptor-like cytoplasmic kinase HvRBK1. Our findings suggest that HvSKP1-like might regulate the abundance of HvRACB through proteasomal degradation, thereby influencing the outcome of the interaction of barley with *Bgh*. HvSKP1-like does not seem to interact directly with the putative SCF substrate HvRACB, which, however, interacts with HvRBK1. This is consistent with the fact that SKP1 proteins interact with the substrates of the

Fig. 4 Effect of the proteasome inhibitor MG132 and *HvRBK1*-RNAi (*barley ROP BINDING KINASE1*-RNA interference) on protein abundance of constitutively active (CA) HvRACB in barley protoplasts 17 h after transformation. (a) mCherry-CA HvRACB and green fluorescent protein (GFP)-expressing barley protoplasts were incubated for 3 h with 10 μ M MG132 or dimethylsulfoxide (DMSO) as a control. To quantify fluorescence intensity, the mean pixel intensity of mCherry-CA HvRACB was measured at the plasma membrane and normalized against the mean pixel intensity of GFP in the nucleus. The fluorescence intensity of MG132-treated protoplasts is significantly increased relative to that of control DMSO-treated protoplasts. All constructs are under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Data show the mean $\pm$ standard error of the mean (SEM) of four independent experiments. In each experiment, 10 protoplasts were analysed. * $P \leq 0.05$ according to an unpaired Student's *t*-test. (b) Immunoblot with an anti-GFP antibody of protein extracts from barley protoplasts expressing cytosolic GFP-CA HvRACB Δ CSIL or GFP and either the empty RNAi vector pIPKTA30N or *HvRBK1*-RNAi, respectively. The protein abundance of GFP-CA HvRACB Δ CSIL is increased in protoplasts co-expressing *HvRBK1*-RNAi relative to protoplasts co-expressing the empty RNAi vector. The protein abundance of GFP is not changed in protoplasts co-expressing pIPKTA30N or *HvRBK1*-RNAi, respectively. All constructs are under the control of the CaMV 35S promoter. Ponceau S staining of the Western blot served as a loading control.

SCF complex indirectly. *In silico* analysis classified HvSKP1-like into the type II class of SKP1-like proteins (Kong *et al.*, 2007) and predicted nuclear and cytoplasmic localization of HvSKP1-like. This was supported by the observation that GFP-HvSKP1-like largely co-localized with the cytoplasmic and nucleoplasmic marker mCherry and with cytoplasmic mCherry-HvRBK1 (Huesmann *et al.*, 2012) on transient co-expression. HvRACB appears to be a possible target of an HvSKP1-like-containing SCF complex, because CA HvRACB abundance increased on treatment with the proteasome inhibitor MG132 in protoplasts and on silencing of either *HvSKP1-like* or *HvRBK1* in barley leaf epidermal cells. In leaf mesophyll protoplasts, an increase in CA HvRACB protein was also detected on silencing of *HvRBK1*, but was inconsistent on silencing of *HvSKP1-like*. This might be a result of the different plant tissues in which CA HvRACB abundance was investigated and the different times at which protein abundance was analysed. Tissue-specific SCF-E3 ubiquitin ligase-mediated protein degradation was recently also proposed for the RAC/ROP effector INTERACTOR of CONSTITUTIVELY active ROP1 (ICR1) (Hazak *et al.*, 2014).

To form an active SCF-E3 ubiquitin ligase complex, F-box proteins are recruited by the adaptor protein SKP1 (Petroski and Deshaies, 2005). In addition to their F-box domain, F-box proteins contain, in most cases, a recognition domain composed of kelch repeats, leucine-rich repeats (LRRs) or other motifs for ligand binding at their C-terminal end (Hua and Vierstra, 2011; Hua *et al.*, 2011). Neither the HvSKP1-like interacting F-box protein nor its interacting cullin have yet been identified. Although HvRBK1 can

interact with both HvSKP1-like and HvRACB, we currently do not consider HvRBK1 as an F-box protein, because it lacks the above-mentioned protein interaction domains.

In the barley–barley powdery mildew interaction, HvRACB is a susceptibility factor towards haustorium establishment by *Bgh* (Hoefle *et al.*, 2011; Schultheiss *et al.*, 2002, 2003). Although HvRBK1 can be activated by CA HvRACB, TIGS of *HvRBK1* enhances the infection success of *Bgh* (Huesmann *et al.*, 2012). Hence, Huesmann *et al.* (2012) hypothesized a negative feedback regulation of HvRACB involving HvRBK1. Our results now suggest that HvSKP1-like acts as a subunit of an SCF complex that negatively regulates the abundance of active HvRACB in concert with HvRBK1, and this limits susceptibility to *Bgh*. A proteasome-dependent negative feedback mechanism would explain the findings that HvRACB has a function in susceptibility, whereas HvRBK1 and HvSKP1-like limit the success of haustorium establishment. Phosphorylation of the target protein is often essential for recognition by an F-box protein (Gagne *et al.*, 2002; Petroski and Deshaies, 2005; Skaar *et al.*, 2013). It might thus be possible that HvRBK1, in its kinase function, triggers the interaction of HvRACB and an unknown F-box protein, e.g. via phosphorylation of HvRACB. This would be similar to the mechanism observed in SCF^{FBXL19}-mediated degradation of human RAC1 (HsRAC1), in which phosphorylation of HsRAC1 by AKT kinase acts as a signal for the association of the F-box protein FBXL19 with phosphorylated HsRAC1 (Zhao *et al.*, 2013). Hence, potential phosphorylation of HvRACB by HvRBK1 needs to be investigated

Fig. 5 Effect of *HvRBK1* (barley ROP BINDING KINASE1)- and *HvSKP1-like* [barley type II S-phase kinase 1-associated (SKP1)-like protein]-RNAi on the fluorescence intensity of CFP-CA HvRACB, CFP-DN HvRACB or CFP in transiently transformed barley epidermal cells 48 h after bombardment. (a) The fluorescence intensity of CFP-CA HvRACB is significantly increased on co-expression with *HvRBK1*-RNAi or *HvSKP1-like*-RNAi, but not on co-expression with the empty RNAi vector pPKTA30N. (b) The fluorescence intensity of CFP remains similar on co-expression with the empty RNAi vector pPKTA30N, *HvRBK1*-RNAi or *HvSKP1-like*-RNAi. (c) The fluorescence intensity of CFP-DN HvRACB is significantly increased on co-expression with *HvRBK1*-RNAi or *HvSKP1-like*-RNAi, but the increase in fluorescence intensity is lower than that observed for CFP-CA HvRACB. In all experiments, confocal laser scanning microscopy was used to measure the mean pixel intensity of CFP, CFP-CA HvRACB or CFP-DN HvRACB at the plasma membrane and the mean pixel intensity of the co-expressed soluble mcherry in the nucleus. CFP, CFP-CA HvRACB and DN-HvRACB fluorescence was normalized against mcherry. Data represent the mean $\pm$ standard error of the mean (SEM) of three to four independent experiments. In each experiment, 10 cells per combination were analysed. Different letters indicate significant difference [analysis of variance (ANOVA) Duncan test, $P \leq 0.05$]. All constructs contain the cauliflower mosaic virus (CaMV) 35S promoter. CA, constitutively active; CFP, cyan fluorescent protein; DN, dominant negative; RNAi, RNA interference.

in the future. Interestingly, phosphomimetic mutations of RAC/ROP GTPases also influence their activity via accessibility for ROPGEFs (Fodor-Dunai *et al.*, 2011).

In contrast with human SCF^{FBXL19}, which mediates the ubiquitination and degradation of both active and inactive forms of HsRAC1, the E3 ubiquitin ligases HACE1 and IAPs only target the active form of HsRAC1 for degradation (Torrino *et al.*, 2011; Zhao *et al.*, 2013). As *HvRBK1* interacts with and is activated by CA *HvRACB*, but does not interact with DN *HvRACB* (Huesmann *et al.*, 2012), we speculate that the active form of *HvRACB* is preferentially targeted for degradation. This is confirmed because the abundance of CFP-DN *HvRACB* only increased slightly on silencing of *HvRBK1* or *HvSKP1-like*. DN *HvRACB* might be less sensitive to *HvRBK1*- and

SCF-mediated degradation, because it cannot neither activate *HvRBK1* nor subsequent negative feedback on its own abundance. However, we cannot exclude the possibility that *HvRBK1* and *HvSKP1-like* also act in constitutive protein turnover of *HvRACB*, because the inactive form DN of *HvRACB* was also slightly sensitive to the silencing of these factors.

Inactivation of *HvRACB* signalling via proteasome-dependent degradation of *HvRACB* would describe an additional mechanism to the regulation of *HvRACB* signalling by the barley MICROTUBULE-ASSOCIATED ROP-GTPASE ACTIVATING PROTEIN 1 (*HvMAGAP1*) (Hoeftle *et al.*, 2011). *HvMAGAP1* interacts with *HvRACB* and limits the susceptibility of barley to *Bgh*, hence reflecting the regulatory function of ROPGAPs in controlling the

Fig. 6 Functional analysis of barley type II S-phase kinase 1-associated (SKP1)-like protein (HvSKP1-like) in the barley–*Blumeria graminis* f.sp. *hordei* (*Bgh*) interaction. (a) Reverse transcription quantitative real-time polymerase chain reaction analysis of *HvSKP1-like* and *HvPR-1b* gene expression in response to *Bgh* infection 12, 24 and 48 h after inoculation (hai). Gene expression was normalized to the housekeeping gene *HvUBC2* and relative expression was compared with the non-inoculated samples at the same time point. Data show the mean $\pm$ standard error of the mean (SEM) of three independent experiments. Expression of *HvSKP1-like* did not change significantly. (b) Transient-induced gene silencing (TIGS) of *HvSKP1-like* by RNAi results in enhanced haustoria frequencies of *Bgh* in transformed barley epidermal cells compared with cells expressing the empty RNAi vector pIPKTA30N. Barley leaves were inoculated 48 h after bombardment with *Bgh* and a minimum of 50 interaction sites per experiment were analysed at 48 hai with the fungus. Data show the mean $\pm$ SEM of six independent experiments. * $P \leq 0.05$ according to an unpaired Student's *t*-test. All constructs contain the cauliflower mosaic virus (CaMV) 35S promoter. (c) Efficiency of *HvSKP1-like*-RNAi directed against the *GFP-HvSKP1-like* expression construct in transiently transformed barley epidermal cells 48 h after bombardment. In three independent experiments, a minimum of 50 mcherry fluorescence displaying cells were analysed for the accumulation of GFP-HvSKP1-like. The number of cells expressing both mcherry and GFP-HvSKP1-like was significantly reduced on co-transformation with *HvSKP1-like*-RNAi compared with cells co-transformed with the empty RNAi vector pIPKTA30N. All constructs were under the control of the CaMV 35S promoter. Data show the mean $\pm$ SEM. ** $P \leq 0.01$ according to an unpaired Student's *t*-test. GFP, green fluorescent protein; RNAi, RNA interference.

signalling output of ROP GTPases. Similar to that described for barley, T-DNA insertion lines of *Arabidopsis AtROPGAP1* and *AtROPGAP4*, and of the orthologue *AtRLCK VI_A3*, show enhanced susceptibility to the powdery mildew fungus *Erysiphe cruciferarum* (Hoefle *et al.*, 2011; Huesmann *et al.*, 2011; Reiner *et al.*, 2015). In the barley–*Bgh* interaction, the CRIB motif protein HvRIC171 is supposed to be a downstream effector of HvRACB as it interacts with activated, but not inactive, HvRACB, and supports the accommodation of fungal haustoria, similar to CA HvRACB (Schultheiss *et al.*, 2008). Therefore, HvMAGAP1, HvRBK1 and the E3 ligase subunit HvSKP1-like could limit susceptibility to *Bgh* by control of the HvRACB–HvRIC171 pathway. Control of HvRACB activity by HvMAGAP1 and of HvRACB abundance by HvRBK1 and HvSKP1-like would allow for spatio-temporal fine tuning of the signalling output of RAC/ROPs and their downstream effectors. Such complex regulation further underpins the central regulatory position of RAC/ROPs in response to plant endogenous and exogenous signals. However, we cannot exclude additional functions of HvRBK1 as a downstream effector of HvRACB or of other RAC/ROP GTPases. Future studies should provide more evidence on the mechanism and function of ubiquitination of HvRACB. Because distinct RAC/ROP pathways are strongly interlinked and individual RAC/ROPs can fulfil redundant, distinct and antagonistic functions in plant–microbe interactions (Chen *et al.*, 2010) and in plant development (Yang, 2008), a better understanding is needed of the post-translational regulation of RAC/ROPs in these processes. In this regard, it is interesting that recently a connection has been reported between ubiquitin-mediated protein degradation and the rice OsRAC1-associated defensome acting in plant immunity (Liu *et al.*, 2015). The current findings show that the ROPGAP SPIN6 (SPL11-interacting protein 6) is ubiquitinated via the U-box E3 ubiquitin ligase SPL11, thereby influencing the signalling output of OsRAC1 in rice. This is reminiscent of results from the animal research field, in which the regulation of RHO GTPases and their regulatory proteins by ubiquitin-mediated proteasomal degradation is widely discussed as an additional mechanism to the control of RHO GTPases by RHOGEPs and RHOGAPs (Liu *et al.*, 2015; Nethe and Hordijk, 2010; Visvikis *et al.*, 2010).

EXPERIMENTAL PROCEDURES

Plants, pathogen and inoculation

Barley (*Hordeum vulgare* L.) cultivars Golden Promise and Pallas were grown in a growth chamber at 18 °C, 60% relative humidity and a 16-h photoperiod at 150 $\mu\text{mol}/\text{m}^2/\text{s}$. *Blumeria graminis* (DC) Speer f.sp. *hordei* EM. Marchal (*Bgh*) was maintained on Golden Promise under the above-described conditions. For gene expression analysis, 7-day-old barley leaves of the cultivar Pallas were inoculated with the fungus. Inoculation for

transient transformation experiments was performed as described in Huesmann *et al.* (2012).

Cloning procedures

The complete coding sequence of *HvSKP1-like* was amplified from a barley cDNA pool of cultivar Pallas using the primers HvK2_fw (5'GCATTGGCAAGAATGTCAGAAAGTGAG3') and K2potStopp (5'CTGTATGTGCACAAATTTCCTAGGC3'), ligated into the pGEM-T easy vector (Promega, Madison, WI, USA) and sequenced. All overexpression constructs for microscopic analysis and protoplast transformation were derived on the pUC18-based plant expression vector pGY1 which contains the cauliflower mosaic virus (CaMV) 35S promoter and CaMV 35S terminator site (Schweizer *et al.*, 1999). To produce pGY1-*HvSKP1-like*, a fragment encoding the full-length *HvSKP1-like* was amplified from pGEM-T easy-*HvSKP1-like* using the primers HvK2fw_Smal (AACCCGGGATGTCAGAAAGTGAG) and M13rev (AACAGCTATGACCATGA), and introduced into pGY1 via *SmaI* and *SalI* restriction sites. The fluorescence tag containing constructs pGY1-*GFP*, pGY1-*mcherry* and pGY1-*CFP* have been described elsewhere (Hoeftle *et al.*, 2011; Huesmann *et al.*, 2012). For FRET measurements, *CFP* was amplified using primers CFP *SmaI* fw + 1 (5'ACCCGGGTATGGTGA GCAAGGCGAGG3') and CFP *SmaI* rev - 1 (5'ACCCGGGTACAGCT CGTCCATGCCGA3'), and inserted into the *SmaI* restriction site of pGY1-*HvSKP1-like* to obtain an N-terminal fusion of *HvSKP1-like* with *CFP*. The generation of pGY1-*HvRBK1-YFP* has been described previously (Huesmann *et al.*, 2012). pGY1-*GFP-HvSKP1-like* was obtained in the same way as pGY1-*CFP-HvSKP1-like*, except that *GFP* instead of *CFP* was amplified. pGY1-*mcherry-HvRBK1* was obtained by amplification of *mcherry* with primers GFP5'*BamHI* (5'GGATCCATGGTGAGCAAGG GCGAG3') and GFP3'noStop+2 (5'CCTTGTACAGCTCGTCCAT3'), and subsequent blunt-end ligation of *mcherry* into *SmaI*-digested pGY1-*HvRBK1*. Via *BamHI* restriction sites, *mcherry* was cloned into *BamHI*-digested pGY1-*CA HvRACB* (Schultheiss *et al.*, 2003) to obtain an N-terminal fusion of *mcherry* with CA *HvRACB*. The construction of pGY1-*CFP CA HvRACB* and pGY1-*CFP DN HvRACB* has been described in Hoeftle *et al.* (2011). Constructs for RNAi-mediated TIGS were generated using the modified Gateway entry vector pIPKTA38 and the destination vector pIPKTA30N, according to the method described in Douchkov *et al.* (2005). For the entry vector construct, a 200-bp-long cDNA fragment of *HvSKP1-like* (bp 466–666 of the coding sequence) was inserted into antisense orientation into pIPKTA38. Off-target analysis for the final pIPKTA30N-*HvSKP1-like* RNAi construct using the Si-Fi program (<http://labtools.ipk-gatersleben.de/>; Nowara *et al.*, 2010) suggested *HvSKP1-like* as the only target in the barley transcriptome. The TIGS construct of *HvRBK1* has been described elsewhere (Huesmann *et al.*, 2012). The construction of pGY1-*GFP CA HvRACBΔCSIL* has been described elsewhere (Schultheiss *et al.*, 2003). Constructs for the yeast two-hybrid assays were based on the vectors pGBKT7 and pGADT7 (Clontech, Mountain View, CA, USA). The *HvSKP1-like* gene was amplified using the primers HvK2 *NdeI* fw (5'ACATA TGATGTCAGAAAGTGAGTTATC3') and M13fwd (-40) (5'GTTTCCCA GTCACGAC3'). Via the restriction sites *NdeI* and *SalI* (*XhoI* for pGADT7), the open reading frame of *HvSKP1-like* was cloned into pGADT7. The generation of the different barley *RAC/ROP* constructs based on the vector pGBKT7, as well as the generation of the construct pGADT7-*HvRBK1*, has been described previously (Hoeftle *et al.*, 2011; Huesmann *et al.*, 2012).

Alignment and phylogenetic analysis

Multiple protein sequence alignments were generated using the MUSCLE algorithm (<http://www.ebi.ac.uk/Tools/msa/muscle/>) (Edgar, 2004). The maximum likelihood method was used to construct a phylogenetic tree with MEGA6 software (Tamura *et al.*, 2013). Phylogeny was tested with a bootstrap of 500 (Poisson's correction model). Protein sequences of the 21 ASKs were retrieved from The Arabidopsis Information Resource (TAIR) (<http://www.arabidopsis.org/>). According to the criteria and nomenclature of Kahloul *et al.* (2013), 30 OSKs were selected for the phylogenetic analysis. The protein sequences of OSKs were retrieved from the rice genome annotation project (<http://rice.plantbiology.msu.edu/>).

Yeast two-hybrid assays

A yeast two-hybrid screen, in which pGBKT7-*HvRBK1* was used as bait protein to screen against a cDNA library derived from a pool of powdery mildew-infected and non-infected barley plants, was performed as described previously (Hoeftle *et al.*, 2011). In total, 12 million transformants were screened. For targeted yeast two-hybrid interaction studies, the *Saccharomyces cerevisiae* strain AH109 was used. Yeast transformation was carried out following the yeast protocols handbook (Clontech). For targeted yeast two-hybrid assays, the selection of yeast transformants was conducted on selective medium lacking leucine and tryptophan (SD-LW). Selection for protein interaction was performed on selective medium lacking leucine, tryptophan, adenine and histidine (SD-LWAH). Several transformants of each combination were resuspended in water, diluted to an optical density at 600 nm (OD₆₀₀) = 1 and 10-fold serial dilutions were spotted in parallel on SD-LW and SD-LWAH media.

Protein localization, protein–protein interaction and fluorescence intensity measurements *in planta*

For subcellular localization, protein–protein interaction studies and subcellular fluorescence intensity measurements, barley epidermal cells were transiently transformed using microprojectile bombardment (Schweizer *et al.*, 1999). Leaves of 7-day-old barley plants were bombarded with DNA-coated gold particles (25 mg/mL, Ø = 1.0 µm) using a PDS-1000/He biolistic delivery system equipped with a single adapter (Bio-Rad, Hercules, CA, USA). Bombardment was performed under a vacuum of 26 mmHg and a gas pressure of 900 psi. For subcellular localization studies, each shot delivered 1.0 µg of pGY1-*GFP-HvSKP1-like* and 1.0 µg of pGY1-*mcherry-HvRBK1* or 0.5 µg of pGY1-*mcherry* into barley epidermal cells. Protein localization was analysed at 24 h using confocal laser scanning microscopy (CLSM; Leica TCS SP5, Leica Microsystems, Mannheim, Germany). GFP fluorescence of tagged *HvSKP1-like* protein was excited with a 488-nm laser. Emission was detected at 500–550 nm. *mcherry* fluorescence of tagged *HvRBK1* was excited at 561 nm and detected at 570–610 nm. Images were obtained using LAS AF 1.8.0 software (Leica Microsystems) and processed using PhotoImpact X3 (Ulead, Corel, Ottawa, ON, Canada). For FRET measurements, each shot delivered 1.5 µg of pGY1-*CFP-HvSKP1-like* or 0.8 µg of pGY1-*CFP* and 1.3 µg of pGY1-*HvRBK1-YFP* into barley epidermal cells. FRET efficiency between CFP-*HvSKP1-like* or CFP, and *HvRBK1-YFP* was measured by the acceptor

photobleaching method at 24 h and analysed by CLSM using LAS AF 1.8.0 software (Leica Microsystems). For bleaching, a region of interest was selected at the cell periphery with little cytoplasmic streaming, in which both acceptor and donor were detected. In the bleaching process, the region was scanned 30 times with 100% relative laser intensity of the 514-nm laser line (20% of 10-mW laser power at 514 nm). Before and after the bleaching process, a photograph with identical settings and sample position was taken. The FRET efficiency ($FRET_{eff}$) in the bleached area was calculated from the donor fluorescence intensity before (D_{pre}) and after (D_{post}) bleaching according to the following equation: $FRET_{eff} = (D_{post} - D_{pre})/D_{post}$. In total, 10 cells per experiment and variant were analysed. Experiments were repeated three times for statistical analysis. CFP fluorescence was excited by a 458-nm laser and detected with a hybrid detector (HyD) at 465–504 nm. YFP fluorescence was excited at 514 nm and detected at 524–550 nm.

To quantify the fluorescence intensity, 1.0 µg of pGY1-*CFP-CA HvRACB*, pGY1-*CFP WT HvRACB*, pGY1-*CFP* or 1.5 µg of pGY1-*CFP DN HvRACB*, together with 0.5 µg of pGY1-*mcherry* and 1.0 µg of the respective RNAi construct pIPKTA30N, pIPKTA30N-*HvRBK1* or pIPKTA30N-*HvSKP1-like*, were delivered to the barley leaves per shot. The mean pixel intensity of the CFP-tagged proteins was measured at the plasma membrane and normalized against the mean pixel intensity of soluble mcherry in the nucleus 48 h after transformation. The fluorescence intensity was plotted relative to CFP-CA HvRACB, CFP-WT HvRACB, CFP or CFP-DN HvRACB. In each experiment, the microscopic settings remained constant and the fluorescence intensity of 10 cells per combination was measured. CFP and mcherry fluorescence were detected with the above-described laser settings, but only CFP-DN HvRACB was detected with HyD.

TIGS and functionality testing of the *HvSKP1-like* RNAi construct

TIGS was performed as described previously (Huesmann *et al.*, 2012); 7.0 µg per shot of the empty vector pIPKTA30N or pIPKTA30N-*HvSKP1-like* and 3.5 µg of the transformation marker pGY1-*GFP* were used. Barley leaf segments were bombarded 48 h prior to inoculation with *Bgh*. Another 48 h later, fungal structures were stained with 0.3% calcofluor (w/v in water) for 30 s, and haustoria frequency of *Bgh* on GFP-expressing epidermal cells was defined as the number of haustoria-containing cells divided by the total number of attacked transformed cells using light and fluorescence microscopy. For each experiment, a minimum of 50 interaction sites were analysed. Functionality of the pIPKTA30N-*HvSKP1-like* construct was tested as previously described (Hoeftle *et al.*, 2011). Per shot, 1.0 µg of pGY1-*GFP-HvSKP1-like*, together with 1.0 µg pIPKTA30N-*HvSKP1-like* or 1.0 µg of the empty vector pIPKTA30N and 0.5 µg of pGY1-*mcherry* were transiently transformed into barley epidermal cells.

Protoplast transformation

The lower epidermis of primary leaves of 7-day-old barley plants was removed and the leaves were floated for up to 1.5 h on enzyme solution [0.45 M mannitol, 10 mM MES, pH 5.7, 10 mM CaCl₂, 0.3% (w/v) Gamborg B5, 0.5% (w/v) Cellulase R10, 0.5% (w/v) Macerozyme R10, 0.5% (w/v) Driselase] at room temperature in the dark. After digestion, the enzyme–protoplast solution was diluted with an equal amount of W5 solution

(154 mM NaCl, 125 mM CaCl₂, 5 mM KCl, 2 mM MES, pH 5.7) and filtered through a 40-µm nylon mesh. Protoplasts were collected by centrifugation at 120 g for 3 min at room temperature and washed once with W5 solution. After resuspension in 1–2 mL of W5 solution, protoplasts were incubated for 30 min on ice. The supernatant was discarded and the protoplasts were resuspended at a density of 2×10^6 protoplasts/mL in MMG solution (0.4 M mannitol, 15 mM MgCl₂, 2 mM MES, pH 5.7). For each transformation, 20 µg of plasmid DNA was mixed with 200 µL of protoplasts and 220 µL of polyethylene glycol (PEG) solution [40% (w/v) PEG 4000, 0.2 M mannitol, 0.1 M CaCl₂]. After incubation for 10 min at room temperature in the dark, 880 µL of W5 solution was added and the protoplasts were collected by centrifugation at 120 g for 3 min at room temperature. Transformed protoplasts were resuspended in 2 mL of W1 solution (0.5 M mannitol, 20 mM KCl, 4 mM MES, pH 5.7) in each well of a six-well tissue culture plate, and incubated under very dim light at room temperature for 16–20 h prior to analysis.

Protein extraction, immunoblotting and antibodies

Protoplasts were harvested 16–20 h after transformation by centrifugation at 900 g for 3 min. Protoplasts were lysed with 50 µL IP buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), 0.1% Nonidet P-40, 50 µM MG132, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% protease inhibitor cocktail, Sigma-Aldrich, St. Louis, MO, USA] and incubated for 30 min on ice. Extracts were centrifuged at 20 000 g for 10 min at 4 °C. Concentrations of total protein extracts were determined by the Bradford assay (Bradford, 1976) using the Bio-Rad protein assay reagent (Bio-Rad). Afterwards, the supernatant was diluted with 6 × Laemmli buffer, heated at 95 °C for 10 min and about 40 µg of total protein per sample were subjected to immunoblot analysis. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), immunoblotting and Ponceau S staining were performed according to standard methods. The antibodies used for immunoblotting were anti-GFP [3H9] (Chromotek, Planegg-Martinsried, Germany) and horseradish peroxidase-conjugated anti-rat (Sigma-Aldrich). Signals were detected by chemiluminescence detection using SuperSignal West Femto Chemiluminescent substrate (Thermo Scientific, Pierce Biotechnology, Rockford, IL, USA).

Drug treatment and quantification of fluorescence intensities in protoplasts

Protoplasts were transformed with pGY1-*mcherry-CA HvRACB* and pGY1-*GFP* according to the above-described PEG-mediated transformation method; 17 h after transformation, protoplasts were treated for 3 h with 10 µM MG132 dissolved in DMSO or DMSO alone. Afterwards, the fluorescence intensity of mcherry-CA HvRACB was measured at the plasma membrane and normalized to the fluorescence intensity of soluble GFP in the nucleus using CLSM. The results were plotted relative to those of the DMSO-treated protoplasts which served as a control. In each experiment, 10 protoplasts per treatment were analysed.

Gene expression analysis

Total RNA was extracted from inoculated and mock-inoculated barley leaves of the cultivar Pallas at 0, 12, 24 and 48 h. Five individual leaves

per time point were pooled and RNA was extracted from frozen plant material using Ribozol™ RNA Extraction Reagent (Amresco, Solon, OH, USA) according to the manufacturer's protocol. One microgram of total RNA was reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Quantitative real-time PCR was performed to analyse the expression of *HvSKP1-like* in inoculated and mock-inoculated barley leaves using Maxima SYBR Green/ROX qPCR Master Mix (2×) (Thermo Scientific); 100 ng of DNA and a final primer concentration of 100 µM were used in each triplicate reaction. Cycling conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 55 °C and 1 min at 72 °C. A dissociation curve analysis was finally conducted as follows: 1 min at 95 °C, 30 s at 55 °C and 30 s at 95 °C. As a standard gene for normalization, barley *Ubiquitin conjugating enzyme 2* (*UBC2*, AY220735) was used and amplified with the primers Ubc2 fwd (5'TCTCGTCC CTGAGATTGCCACAT3') and Ubc2 rev (5'TTTCTCGGGACAGCAACA CAATCTTCT3'). *HvSKP1-like* was amplified with the primers qPCR-HvK2-fw (5'GGAGCGCCAGAACTAAAGG3') and qPCR-HvK2-rev (5'CCAGAACCTCCATCCCCATT3'). In addition, *HvPR1b* was amplified using primers HvPR1b (5'TGGTATAGAGCAGGCCATAGAA3') and HvPR1b (5'GCTAGGGCGAGCAAGATGAC3'). All primers were tested for similar amplification efficiency and expression of *HvSKP1-like*, and the data were evaluated using MxPro QPCR software (Stratagene, Agilent Technologies, Santa Clara, CA, USA).

Accession numbers

HvRACB (AJ344223), HvRBK1 (HE611049), *HvSKP1-like* (LN714776).

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SUPPORTING INFORMATION

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

Fig. S1 Amino acid alignment of HvSKP1-like and related plant SKP1-like proteins. Alignment was generated using MUSCLE (<http://www.ebi.ac.uk/Tools/msa/muscle/>) (Edgar, 2004) and visualized using GeneDoc (Nicholas *et al.*, 1997). The sequences used are ASK21 (At3g61415), OSK31 (Os03g01660), HvSKP1-like (LN714776) and predicted SKP1-like proteins from *Brachypodium distachyon* (BdXP_003559016) and *Zea mays* (ZmXP_008675156).

Fig. S2 Subcellular localization of HvSKP1-like in transiently transformed barley epidermal cells 24 h after bombardment (hab). (a) GFP-HvSKP1-like largely co-localizes with mcherry-HvRBK1. (b) GFP-HvSKP1-like co-localizes with the cytoplasmic and nucleoplasmic marker mcherry. The expression of fusion proteins was driven by the cauliflower mosaic virus (CaMV) 35S promoter. The confocal image shows selected single optical sections. GFP, green fluorescent protein. Scale bar, 20 µm.

Fig. S3 Effect of *HvRBK1*- and *HvSKP1-like*-RNAi on the fluorescence intensity of CFP-WT HvRACB in transiently transformed barley epidermal cells 48 h after bombardment. The fluorescence intensity of CFP-WT HvRACB is significantly increased on transformation of CFP-WT HvRACB with *HvRBK1*-RNAi or *HvSKP1-like*-RNAi. Data represent the mean ± standard error of the mean (SEM) of four independent experiments. In each experiment, 10 cells per combination were analysed. Different letters indicate significant difference [analysis of variance (ANOVA) Duncan test, $P < 0.05$]. CFP, cyan fluorescent protein; RNAi, RNA interference; WT, wild-type.