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Receptor-like cytoplasmic kinases as downstream effectors of RAC/ROP GTPases in the interaction of *Arabidopsis* and barley with powdery mildew fungi

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List of abbreviations

ABP1	auxin-binding protein 1
ADH1	alcohol dehydrogenase 1
ASK	*Arabidopsis* SKP1-like
At	*Arabidopsis thaliana*
ATP	adenosine triphosphate
Avr	avirulence protein
BAK1	brassinosteroid insensitive 1-associated kinase 1
Bgh	*Blumeria graminis* f. sp. *hordei*
BI-1	bax inhibitor-1
BIK1	botrytis-induced kinase 1
Ca	*Capsicum annuum*
CA	constitutively active
Cdc42	cell division cycle 42
CEBiP	chitin elicitor binding protein
CERK1	chitin elicitor receptor kinase 1
CFP	cyan fluorescent protein
COI1	coronatine insensitive 1
CPR1	constitutive expressor of PR genes 1
CRIB	CDC42/RAC-interactive binding motif
CRL	cullin-RING
CSEP	candidate for secreted effector protein
CUL1	cullin 1
CWA	cell wall apposition
CYP83A1	cytochrome P450 83A1
Cys	cysteine
DAMP	damage-associated molecular pattern
DN	dominant negative
DNA	deoxyribonucleic acid
EBF1	ein3 binding F-box1
EDR1	enhanced disease resistance 1
EHM	extrahaustorial membrane
EIN3	ethylene insensitive 3
ELMO	engulfment and motility domain
ETI	effector-trigged immunity
ETS	effector-triggered susceptibility
F	filamentous
f. sp./ff.spp	*forma specialis/formae speciales*
FLS2	flagellin-sensing 2
FRET	fluorescence resonance energy transfer
GAP	GTPase activating protein
GDI	guanine nucleotide dissociation inhibitors
GDP	guanidine diphosphate
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GTP	guanidine triphosphate
GTPase	guanine triphosphatase
H_2O_2	hydrogen peroxide
HECT	homologous to the E6AP carboxyl terminus
HR	hypersensitive response
Hs	*Homo sapiens*
Hv	*Hordeum vulgare* L.
HVR	hypervariable region
ICR1	interactor of constitutively active ROP1
JA	jasmonic acid
JAZ	jasmonate ZIM-domain
KTN1	katanin 1
LRR	leucine-rich repeat
Lys	lysine
MAGAP1	microtubule-associated ROP-GTPase activating protein
MAMP	microbe-associated molecular pattern
MAPK	mitogen-activated protein kinase
MIDD1	microtubule depletion domain 1
MLO	mildew resistance locus O
NB	nucleotide binding
NCRK	novel cysteine-rich receptor kinase
Os	*Oryza sativa*
OSK	*Oryza sativa* SKP1-like
PAK	p21- activated kinase
PAMP	pathogen-associated molecular pattern
PBL1	PBS1-like 1
PBS1	AvrPphB
PEN1	penetration 1
PIK	pathogen-induced protein kinase
PIN1	pinformed 1
PMR1	powdery mildew-resistant 1
PR	pathogenesis-related

	protein
PRONE	ROP nucleotide exchanger
PRR	pattern recognition receptor
PTI	PAMP-triggered immunity
R	resistance protein
RAC	ras-related C3 botulinum toxin substrate
RBK1	ROP binding kinase 1
RBX1	RING-Box 1
REN1	ROP1 enhancer 1
Rho	rat sarcoma oncogene product homologue
RIC	ROP-interactive crib motif-containing protein
RING	really interesting new gene
RIP1	ROP-interactive partner 1
RIPK	RPM1-induced protein kinase
RLCK	receptor-like cytoplasmic kinase
RLP	receptor-like protein
RNAi	RNA-interference
ROP	rho of plants (synonyme to plant RAC)
ROR2	required for *mlo*-specified resistance
ROS	reactive oxygen species
RPS5	resistant to *P. syringae*
S	susceptibility gene
SA	salicylic acid
SCF	SKP1-cullin1-F-box
Ser	serine
SGT1	G2 allele of SKP1
SKP1	Spike 1
SKP1	s-phase kinase associated protein 1
SNARE	soluble n-ethylmaleimide-sensitive factor attachment protein receptor
TBL	trichome-birefringence-like
T-DNA	transfer DNA
Thr	threonine
TIGS	transient induced gene silencing
TIR1	transport inhibitor response 1
TMK	transmembrane kinase
WT	wild-type

Summary

The RAC/ROP GTPase HvRACB is a susceptibility factor in the interaction of barley (*Hordeum vulgare*) with the biotrophic barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*). HvRACB is required for fungal invasion and supports the establishment of haustoria in barley epidermal cells. Although several interacting proteins of HvRACB were identified the HvRACB downstream signaling cascades are barely known. Here, the previously suggested interaction was verified *in planta* of the receptor-like cytoplasmic kinase (RLCK) ROP binding kinase 1 (HvRBK1) with its activating RAC/ROP HvRACB. Transient induced gene silencing (TIGS) of *HvRBK1* affected microtubule stability in barley epidermal cells and supported the penetration success of *Bgh*. It was unexpected that HvRBK1 as a potential downstream effector of HvRACB played a role in basal resistance rather than in susceptibility. Consequently an HvRBK1-dependent negative feedback regulation mechanism of HvRACB was proposed.

Moreover a barley type II SKP1-like protein (HvSKP1-like) that presumably acts as part of an E3 ubiquitin ligase complex in ubiquitin-mediated proteolysis has been identified as an interacting protein of HvRBK1. TIGS of *HvSKP1-like* rendered barley epidermal cells more susceptible to the fungus. In addition, TIGS of either *HvSKP1-like* or *HvRBK1* increased protein abundance of HvRACB in barley epidermal cells. Consequently it is suggested that HvSKP1-like together with HvRBK1 controls abundance of HvRACB, influencing at the same time the outcome of the barley-*Bgh* interaction.

Characterization of AtRLCK VI_A3, the predicted *Arabidopsis* orthologue of HvRBK1, revealed a conserved function in the interaction of *Arabidopsis* with its adapted powdery mildew fungus *Erysiphe cruciferarum* (*E. cruciferarum*). AtRLCK VI_A3 had been identified as a molecular interactor of AtRAC/ROPs using biochemical and cell biological approaches. Similar to HvRBK1 in the barley-*Bgh* interaction, *Atrlck VI_A3* mutant plants showed enhanced susceptibility to *E. cruciferarum*. A retarded growth phenotype as well as an increase in trichome branch number was observed in *Atrlck VI*_A3 mutant plants. Therefore results indicate a function of the AtRAC/ROP-activated AtRLCK VI_A3 in basal resistance to *E. cruciferarum* as well as in plant growth and polar cell differentiation during trichome morphogenesis.

Taken together, these results indicate conserved functions of RAC/ROP-activated RLCKs in the expression of disease in the barley-*Bgh* and the *Arabidopsis*-*E.cruciferarum* interaction. In addition the HvRBK1-interacting HvSKP1-like might add ubiquitin-mediated proteolysis to regulation of HvRACB. This could explain the findings that HvRACB causes susceptibility whereas HvRBK1 and HvSKP1-like have a role in resistance against *Bgh.*

Zusammenfassung

In der Interaktion von Gerste (*Hordeum vulgare*) mit dem biotrophen Echten Gerstenmehltaupilz *Blumeria graminis* f. sp. *hordei* (*Bgh*) stellt die RAC/ROP GTPase HvRACB ein Anfälligkeitsfaktor dar. HvRACB unterstützt das Eindringen und die Etablierung der pilzlichen Infektionsstrukturen in Epidermiszellen von Gerste. Obwohl einige Proteine, die mit HvRACB interagieren identifiziert werden konnten, sind die HvRACB-nachgeschalteten Signaltransduktionskaskaden wenig verstanden. In der vorliegenden Arbeit wurde die vorbeschriebene Interaktion von HvRACB mit der HvRACB-aktivierbaren Rezeptor-ähnlichen zytoplasmatischen Kinase HvRBK1 (ROP binding kinase 1) in der Pflanze bestätigt. Der transiente Knock-down von *HvRBK1* durch RNA-Interferenz beeinflusste die Stabilität der Mikrotubuli in Epidermiszellen von Gerste und erhöhte den Penetrationserfolg von *Bgh*. Für ein dem Anfälligkeitsfaktors HvRACB nachgeschaltetes Protein ist eine Funktion in der basalen Resistenz ungewöhnlich. Dies führte zu der Hypothese, dass HvRACB über einen HvRBK1-abhängigen Feedback-Mechanismus negativ reguliert werden könnte.

Weiterhin wurde in Gerste ein SKP1-ähnliches Protein des Typs II (HvSKP1-like) als Interaktionspartner von HvRBK1 identifiziert. HvSKP1-like stellt eine mögliche Untereinheit von E3 Ubiqutin Ligasen dar, die im Ubiquitin-vermittelten Proteinabbau eine wichtige Rolle spielen. In dieser Arbeit wurde die Interaktion von HvRBK1 und HvSKP1-like in Hefe und in der Pflanze bestätigt. Der transiente Knock-down von *HvSKP1-like* durch RNA-Interferenz in Epidermiszellen von Gerste resultierte in einer erhöhten Anfälligkeit von Gerste gegenüber *Bgh*. Ebenso ist die Proteinmenge von HvRACB bei Knock-down von *HvRBK1* oder *HvSKP1-like* in Epidermiszellen von Gerste gesteigert.

Um eine konservierte Funktionsweise von RLCKs als RAC/ROP Effektoren zu überprüfen, wurde das vorhergesagt orthologe Gen von HvRBK1 aus *Arabidopsis thaliana*, AtRLCK VI_A3, in der Interaktion von *A. thaliana* mit dem Echten Mehltaupilz *Erysiphe cruciferarum* funktionell charakterisiert. AtRLCK VI_A3 ist kürzlich mit Hilfe von biochemischen und zellbiologischen Methoden als molekularer Interaktor von AtRAC/ROPs identifiziert worden. Ähnlich wie HvRBK1 in der HvRBK1-*Bgh* Interaktion, zeigten *Atrlck VI_A3* KO-Mutanten eine erhöhte Anfälligkeit gegenüber *E. cruciferarum*. Ein verlangsamtes Wachstum sowie eine Zunahme an Trichomverzweigungen konnte ebenfalls in *Atrlck VI_A3* Mutanten festgestellt werden. Diese Ergebnisse deuten darauf hin, dass die AtRAC/ROP-aktivierte AtRLCK VI_A3 eine Rolle in der basalen Resistenz gegenüber *E. cruciferarum* sowie im Pflanzenwachstum und der polaren Zelldifferenzierung während der Trichomentwicklung inne hat.

Zusammenfassend deuten die Ergebnisse darauf hin, dass Funktionen von RAC/ROP-aktivierten RLCKs in der Pathogeninteraktion von Gerste und *Bgh* sowie *Arabidopsis* und *E. cruciferarum* konserviert sind. Weiterhin stellt das HvRBK1-interagierende HvSKP1-like Protein

eine Verbindung zur Ubiquitinierungsmaschinerie her. Ein HvRBK1-abhänigiger Ubiquitin-vermittelter Abbau von HvRACB könnte einen zusätzlichen negativen Regulationsmechanismus von HvRACB darstellen, der die gegensätzliche Funktionsweise von HvRACB und HvRBK1 sowie HvSKP1-like in der Pathogeninteraktion von Gerste und *Bgh* erklärt.

1. Introduction

1.1. Plant Immunity

As plants are sessile organisms, they have to continuously adapt to changing environmental conditions. They are permanently exposed to pathogens like viruses, bacteria, fungi and nematodes but due to their efficient defense strategies disease development is rare. Besides preformed physical and chemical barriers including rigid cell walls, wax layers and antimicrobial compounds plants come up with complex inducible defense responses to ward off pathogens (Hückelhoven, 2007). Unlike mammals, plants do not have mobile defender cells but rather rely on the ability of each cell to autonomously recognize and respond to pathogens (Jones and Dangl, 2006). A pathogen that overcomes the preformed barriers is perceived by the plant through its pathogen- or microbe-associated molecular patterns (P/MAMPs). These are conserved molecular structures typical of a certain class of microbes like bacterial flagellin or fungal chitin which are not present in plants (Boller and Felix, 2009). Recognition of P/MAMPs is based on specific cell-surface pattern recognition receptors (PRRs) that are either receptor-like kinases (RLKs) or receptor-like proteins (RLPs) (Macho and Zipfel, 2014). Besides P/MAMPs, there are plant-derived signals called damage-associated molecular patterns (DAMPs) that are released upon pathogen damage and recognized in a similar way as P/MAMPs (Boller and Felix, 2009). P/MAMPs recognition by PRRs leads to a variety of defense responses including production of reactive oxygen species (ROS), changes in cytosolic Ca^{2+} levels, activation of pathogenesis-related (PR) proteins and of mitogen-activated protein kinases (MAPKs), and callose depositions (Hückelhoven, 2007, Boller and Felix, 2009, Dodds and Rathjen, 2010). This first layer of defense is called PAMP-triggered immunity (PTI) and can prevent further colonization of the pathogen (Jones and Dangl, 2006). Pathogens, in turn, try to evade recognition or suppress P/MAMP-triggered signal transduction by secretion of effector molecules resulting in effector triggered susceptibility (ETS). To counteract these effector proteins plants have evolved cytoplasmic resistance (R) proteins. R proteins represent intracellular receptors, most often of the nucleotide-binding leucine-rich repeat (NB-LRR) class of proteins, which directly recognize the effector or detect effector-triggered cellular changes (guard hypothesis)(Dangl and Jones, 2001, Jones and Dangl, 2006). This second layer of defense is referred to as effector-triggered immunity (ETI). ETI is typically associated with a form of localized cell death called hypersensitive response (HR) (Jones and Dangl, 2006, Dodds and Rathjen, 2010). Due to selection pressure, pathogens modify their effectors or evolve novel ones to circumvent or suppress ETI. In turn, plants develop novel R genes that are able to recognize these modified or novel effectors, resulting again in ETI. This ongoing co-evolutionary arms race between pathogens and plants is illustrated in the ‘zigzag’ model by Jones and Dangl (2006). In contrast to PTI, that provides immunity to a broad spectrum of invaders, ETI, formerly

described as gene-for-gene resistance is race-specific (Flor, 1942). Furthermore, ETI-triggered immune responses are stronger and more robust than those in PTI (Tsuda and Katagiri, 2010). Although PTI and ETI are described as two distinct phases of plant immunity, they partially share the same downstream signaling machinery (Tsuda and Katagiri, 2010). Moreover there exist microbial defense activators that do not fit in with the common classification of P/MAMPs and effectors, hence proposing a continuum between PTI and ETI (Thomma *et al.*, 2011). Generally, pathogens need to suppress plant immunity in order to establish a compatible interaction in which a disease is manifested. In the case of fungal biotrophic pathogens that establish feeding structures within living host cells the pathogen has to ensure a failure of the plant immune system throughout the whole infection process. In addition, the active cooperation of the host in non-immunity related processes such as changes in host membrane and cytoskeleton organization during fungal accommodation is needed for a compatible interaction with biotrophic pathogens (Hückelhoven *et al.*, 2013a, Lapin and Van den Ackerveken, 2013). Plant genes that are required for compatible interactions are called susceptibility genes (S-genes) (Eckardt, 2002, Pavan *et al.*, 2010, van Schie and Takken, 2014). S-genes either encode for negative regulators of plant defense or for genes that are required for fungal establishment. The latter are rather called susceptibility factors and fulfill the metabolic or structural needs of the fungus during its development process. As susceptibility factors play a role in plant-pathogen interactions as well as in other aspects of plant physiology, their loss is often accompanied by pleiotropic effects (Pavan *et al.*, 2010, Hückelhoven *et al.*, 2013a). Although for a long time the identification of R- and defense genes played a major role in plant immunity research, there is an increasing interest in identification of S-genes and in the elucidation of their mechanisms in controlling disease susceptibility (Pavan *et al.*, 2010, Hückelhoven *et al.*, 2013a)

1.2. Powdery mildew fungi

Powdery mildew fungi are obligate biotrophic plant pathogenic fungi that establish long lasting interactions with their living host cells by forming haustoria. They belong to the order Erysiphales within the Ascomycetes and are able to colonize around 10 000 different monocotyledonous and dicotyledonous plant species (Glawe, 2008). Characteristic symptoms of the so-called powdery mildew disease are whitish pustules that appear on the surface of aerial organs of infected plants (Glawe, 2008). Powdery mildew infection occurs in temperate and humid climates and reduces yield as well as product quality of agronomic important plants like cereals, grapevine and ornamental plants (Micali *et al.*, 2008). Control of this plant disease is managed by the use of fungicides and disease-resistant plant varieties (Dean *et al.*, 2012, Acevedo-Garcia *et al.*, 2014). In the research field of plant pathology, powdery mildews serve as well-established models to investigate plant-pathogen interactions on a cellular- and molecular level (Acevedo-Garcia *et al.*, 2014). In the present work the interaction of the monocotyledonous

barley (*Hordeum vulgare* L.) with the adapted powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) and the interaction of the dicotyledonous model plant *Arabidopsis thaliana* (*A.thaliana*) with *Erysiphe cruciferarum* (*E. cruciferarum)* is highlighted. While powdery mildew fungi of the genus *Blumeria* only affect Poacea and show strict host specialization displayed by its different *formae speciales* (ff. *spp.*), many dicot powdery mildews have a broader host range (Micali *et al.*, 2008, Troch *et al.*, 2014). Accordingly, *A. thaliana* can be colonized by powdery mildews of cucurbits and crucifers (*E. cruciferarum, Golovinomyces orontii, Golovinomyces cichoracearum*) as well as by the tomato powdery mildew fungus *Oidium neolycopersici* (Micali et al., 2008). The interaction of powdery mildews with their host plants as well as their life cycles have been extensively reviewed (Green *et al.*, 2002, Zhang *et al.*, 2005, Eichmann and Hückelhoven, 2008, Micali *et al.*, 2008). Usually, an infection starts with the landing of an asexual conidiospore on the leaf surface of a susceptible host plant. Within the next hours the conidiospore germinates and forms depending on the genera one (*E. cruciferarum*) or two (*B. graminis*) germ tubes. The formation of a primary germ tube that is involved in first water uptake and host-sensing is a unique characteristic of *B. graminis*. Since primary germ tubes do not form haustoria, a second germ tube evolves from the conidiospore of *B. graminis* and subsequently develops like the single germ tube from all other powdery mildew fungi into an appressorium at its tip (Eichmann and Hückelhoven, 2008, Glawe, 2008, Micali *et al.*, 2008). This specialized infection structure marks the site where the fungus tries to break through the cuticle and cell wall of an underlying epidermal cell using additional means like mechanical pressure or a mixture of cell wall degrading enzymes (Green *et al.*, 2002). During the penetration effort of the fungus, the host usually responses with the production of cell wall appositions (CWA, also named papillae) which are assumed to be physical and chemical barriers to stop fungal invasion (Hückelhoven, 2005). Successful penetration of the fungus leads to the invagination of the host plasma membrane and the formation of the primary feeding structure of the fungus, the haustorium. The haustorium is part of the haustorial complex that comprises besides the haustorium the extrahaustorial matrix and the plant-derived extrahaustorial membrane (EHM) (Koh *et al.*, 2005). In addition to its function in nutrient uptake, it is assumed that haustoria deliver effectors to suppress plant immune responses (O'Connell and Panstruga, 2006, Micali *et al.*, 2008, Giraldo and Valent, 2013). Haustoria of diverse powdery mildew differ significantly in their morphology. While the haustoria from *B. graminis* typically have a digitate shape, the haustoria from *E. cruciferarum* are more ovoid and elongated in shape (Eichmann and Hückelhoven, 2008, Micali *et al.*, 2008). Interestingly, growth of powdery mildew fungi is typically restricted to the epidermal layer of plant tissues and they do not colonize underlying plant tissues like mesophyll cells. After haustorial establishment, powdery mildew fungi form secondary hyphae and continue their growth epiphytically. Alongside the hyphae, the fungi can penetrate further epidermal cells and establish haustoria. Finally, the asexual life cycle of the pathogen is completed by the formation of conidiophores

from which conidiospores emerge and are dispersed by wind to infect other plants (Green *et al.*, 2002, Micali *et al.*, 2008). The genome of several powdery mildews have been sequenced and it turned out that in comparison to the genomes of related Ascomyota the genomes of *B. graminis* and other powdery mildews are with over 120 Mb much larger (Spanu *et al.*, 2010). Simultaneously, partial loss of genes involved in secondary metabolism, carbohydrate degradation and nitrate uptake was observed. This might reflect the biotrophic lifestyle of *B. graminis* in which the host is able to take over conserved metabolic pathways thereby compensating the loss of certain fungal genes (Spanu *et al.*, 2010, Hückelhoven *et al.*, 2013a). In addition genome analysis of *Bgh* revealed candidates for secreted effector proteins (CSEPs) of which several could also be identified in the genomes of *Erysiphe pisi* and *Golovinomyces orontii* (*G. orontii*) (Spanu *et al.*, 2010). Especially in the barley-*Bgh* interaction the discovery and characterization of effectors is progressing during the past years (Zhang *et al.*, 2012, Pliego *et al.*, 2013, Schmidt *et al.*, 2014). However, until recently most research of the plant-powdery mildew interaction has been done on the plant side of the interaction. Hence, several host genes have been identified that either contribute to powdery mildew resistance or susceptibility (Vogel and Somerville, 2000, Xiao *et al.*, 2001, Consonni *et al.*, 2006, Acevedo-Garcia *et al.*, 2014, Douchkov *et al.*, 2014).

1.3. Susceptibility genes in the plant-powdery mildew interaction

S-genes are required for disease susceptibility. In the plant-powdery mildew interaction it has been shown that the lack of S-genes results somewhere between reduced disease severity and up to broad-spectrum resistance against powdery mildew fungi (Pavan *et al.*, 2010, van Schie and Takken, 2014). In *A. thaliana* the *enhanced disease resistance1* (*edr1*), *edr2* and *edr3* mutants display growth inhibition of *Golovinomyces cichoracearum* (*G. cichoracearum*) at a late stage of the infection process. Compared to wild-type plants, this salicylic acid (SA)-dependent powdery mildew resistance goes along with a more rapid induction of host defenses including programmed cell death (Frye and Innes, 1998, Tang *et al.*, 2005, Tang *et al.*, 2006). Furthermore, a collection of *A. thaliana* mutants called *powdery mildew-resistant 1-4* (*pmr1-4*) that affect normal growth of the powdery mildew pathogen has been identified and later completed with the mutants *pmr5* and *pmr6* (Vogel and Somerville, 2000, Vogel *et al.*, 2002, Vogel *et al.*, 2004). PMR4 encodes for a callose synthase (independently identified as glucan synthase-like 5) that is responsible for wound and papilla-associated callose deposition (Jacobs *et al.*, 2003, Nishimura *et al.*, 2003). *pmr4* mutants are more resistant against G. *cichoracearum* and show enhanced SA-dependent defense signaling consequently indicating PMR4 as negative regulator of SA-dependent defense (Nishimura *et al.*, 2003). *pmr5* and *pmr6* mutants likewise show reduced growth of powdery mildew fungi but in contrast to *pmr4* mutants, resistance is mediated independently from well-described defense pathways through the alteration of cell wall composition (Vogel *et al.*, 2002, Vogel *et al.*, 2004). While PMR6 encodes

a pectate lyase-like protein and represents the very first described susceptibility factor, PMR5 belongs to the plant specific trichome-birefringence-like (TBL) protein family (Eckardt, 2002, Vogel *et al.*, 2002, Vogel *et al.*, 2004, Chandran *et al.*, 2013). It turned out recently that powdery mildew-resistant *pmr5* and *pmr6* mutant plants exhibit reduced ploidy level in the mesophyll cells that directly underlie the feeding site of *G. orontii* (Chandran *et al.*, 2013). It is assumed that an increase in mesophyll ploidy at the feeding site of the fungus is mediated through powdery mildew-induced endoreduplication in order to support the metabolic demands of the fungus, hence promoting its growth (Chandran *et al.*, 2013). This hypothesis is supported by the transcription factor MYB3R4 that is required for the regulation of endoreduplication. *Arabidopsis myb3r4* mutant plants show reduced reproduction of *G. orontii* (Chandran *et al.*, 2010, Chandran *et al.*, 2013). One of the best-studied S-genes against powdery mildew is the *mildew resistance locus O* (*MLO*) that was originally discovered in barley. Loss-of-function mutations in barley *MLO* confer durable broad-spectrum resistance of barley against all *Bgh* isolates. On these plants, fungal pathogenesis is stopped at the early stage of cell wall penetration involving papillae formation and H_2O_2 accumulation on the host side (Aist *et al.*, 1988, Jörgensen, 1992, Büschges *et al.*, 1997, Hückelhoven *et al.*, 1999). Powdery mildew resistant *mlo* mutants have been identified in various monocotyledonous and dicotyledonous plant species including *A. thaliana* in which simultaneous loss-of-function mutation of the genes *AtMLO2* (allelic to *PMR2*, *6* and *12* mediates resistance to *G. orontii* (Consonni *et al.*, 2006, Acevedo-Garcia *et al.*, 2014, van Schie and Takken, 2014). In *Arabidopsis* and barley, *mlo*-mediated resistance partially depends on the respective syntaxins penetration 1 (PEN1) and the barley homolog required for *mlo*-specified resistance in barley (ROR2). Together, these genes are suggested to have a function in the formation of ternary soluble N-ethylmaleimide-sensitive factor attachment protein receptor (SNARE) protein complexes delivering defense associated compounds at the cell periphery. In addition, PEN2 and PEN3 that encode a myrosinase and an ATP-binding cassette multidrug transporter respectively are needed for full *mlo*-mediated resistance in *A. thaliana* (Humphry *et al.*, 2006, Humphry *et al.*, 2010, Acevedo-Garcia *et al.*, 2014). A recent study points out that MLO proteins regulate plant defenses rather directly than being manipulated by the fungus for its colonization success (Humphry *et al.*, 2010). Moreover, the negative regulator of programmed cell death BAX Inhibitor-1 (BI-1) is a susceptibility factor in the interaction of barley with *Bgh*. BI-1 seems to act independently or downstream of MLO as its expression is modulated by this protein (Eichmann *et al.*, 2010). *Arabidopsis* BI-1 interacts with the monooxygenase CYP83A1 *in planta*. Loss of function mutants of *cyp83a1* showed reduced growth of *E. cruciferarum* which is possibly due to their altered chemical composition (Weis *et al.*, 2013, Weis *et al.*, 2014). Lifeguard proteins that are similar to BI-1 proteins likewise limit susceptibility of *Arabidopsis* and barley powdery mildew fungi when silenced. In contrast they support development of fungi when overexpressed (Weis *et al.*, 2013). It is also shown that a barley alcohol dehydrogenase 1 (HvADH1) modulates susceptibility to *Bgh* (Pathuri *et al.*,

2011). Finally, members of the plant specific Rho family of GTPases such as HvRACB in barley or AtROP6 in *A. thaliana* support establishment of powdery mildew fungi and will be discussed in more detail in the following.

1.4. RAC/ROP GTPase signaling

The Rho family of monomeric G proteins belongs to the Ras superfamily of small GTPases and regulates a variety of signaling pathways in all eukaryotic kingdoms. While in animals the Rho family is subdivided into Rho, Cdc42 and Rac proteins, plants contain a unique subfamily of Rho GTPases termed Rho of plants (ROPs). Since the primary amino acid sequence of ROPs is most closely related to the animal Rac subfamily they are also termed RACs (Brembu *et al.*, 2006, Berken and Wittinghofer, 2008). Members of the RAC/ROP family share five highly conserved G-box-motifs (G1 or P-loop, G2 or switch region I, G3 or switch region II, G4 and G5) that play a role in GDP/GTP and Mg^{2+} binding as well as GTP hydrolysis. Upon GTP hydrolysis, the flexible switch regions G2 and G3 undergo conformational changes thereby regulating the interaction with downstream effector proteins (Berken, 2006, Berken and Wittinghofer, 2008). At their C-terminus RAC/ROP proteins display a hypervariable region (HVR) consisting of a polybasic stretch which can be post-translationally modified in order to target RAC/ROPs to the plasma membrane where their activation takes place (Berken and Wittinghofer, 2008). Depending on their posttranslational lipid modification RAC/ROPs are divided into two phylogenetic subgroups (Winge *et al.*, 2000). While type I RAC/ROPs have a C-terminal CAAX motif that is prenylated, type II RAC/ROPs possess conserved Cys residues at their C-terminus for palmitoylation (Yalovsky *et al.*, 2008). Additionally RAC/ROPs have a so-called Rho insert region consisting of 9-11 conserved amino acids which is supposed to be involved in the recognition of specific effector or regulatory molecules (Berken and Wittinghofer, 2008). In plants, the RAC/ROP family is particularly involved in signal transduction pathways that influence growth, development and immunity (Berken, 2006, Nibau *et al.*, 2006, Kawano *et al.*, 2014). In doing so, RAC/ROP proteins fulfill their roles as molecular switches and transduce extracellular stimuli into intracellular responses via shuttling between an inactive GDP-bound and an activated GTP-bound state (Fig. 1). The ratio between these two states depends on the activity of regulatory proteins that spatiotemporally fine-tune the outcome of RAC/ROP signaling. Guanine nucleotide exchange factors (ROPGEFs) stimulate the release of GDP from RAC/ROPs and favor the binding of GTP, hence activating RAC/ROPs. Plants possess a unique family of ROPGEFs marked by a plant-specific ROP nucleotide exchanger (PRONE) domain (Berken *et al.*, 2005). Activity of ROPGEFs in turn seems to be regulated by RLKs that represent potential upstream regulators for RAC/ROP signaling (Duan *et al.*, 2010). GTPase activating proteins (ROPGAPs) interfere specifically with the GTP-bound state of RAC/ROPs and increase their low intrinsic GTPase activity supporting hydrolysis of GTP and thus RAC/ROP inactivation. Like their non-plant homologues, ROPGAPs contain an arginine residue

in their catalytic active GAP domain which is frequently involved in the reaction mechanism (Berken, 2006). Additionally, depending on their family ROPGAPs either have a CDC42/RAC-interactive binding (CRIB) domain for G-protein binding or a pleckstrin homology domain for lipid binding (Eklund *et al.*, 2010, Hoefle *et al.*, 2011). A third class of regulators of RAC/ROP proteins present guanine nucleotide dissociation inhibitors (ROPGDIs). ROPGDIs bind the C-terminal lipid modifications of GDP-bound RAC/ROPs and sequester them into the cytosol (Berken and Wittinghofer, 2008). Indications that RAC/ROPs are additionally regulated via ubiquitin-mediated proteasomal degradation of themselves or of their regulatory proteins like it is discussed in the animal research field were most recently shown in rice (Nethe and Hordijk, 2010, Visvikis *et al.*, 2010, Liu *et al.*, 2015).

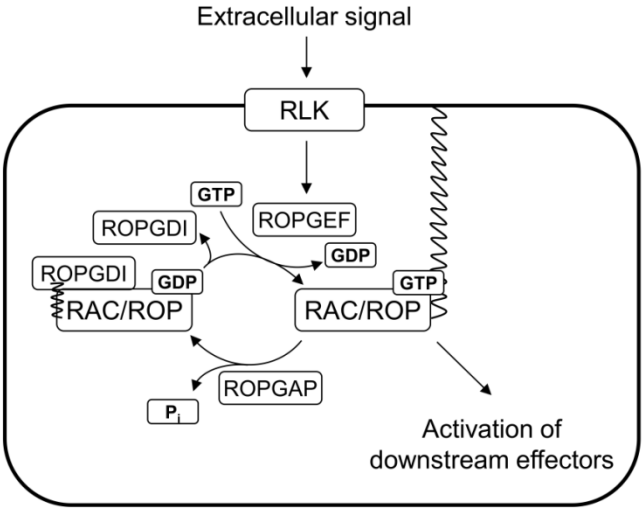

Fig 1. RAC/ROP signaling switch
In response to extracellular stimuli most likely receptor-like kinases (RLKs) activate guanine nucleotide exchange factors (ROPGEFs) that in turn mediate the conversion of the GDP-bound inactive form of RAC/ROP proteins into their active GTP-bound form. GTPase activating proteins (ROPGAPs) stimulate the intrinsic GTPase activity of RAC/ROP proteins, thereby returning their active into their inactive form. Guanine nucleotide dissociation inhibitors (ROPGDIs) negatively regulate RAC/ROPs via supporting their dissociation from the cell membrane into the cytosol inhibiting the exchange of GDP for GTP.

Members of the RAC/ROP family are omnipresent in all monocotyledonous and dicotyledonous plant species including mosses and conifers. In each investigated species RAC/ROP proteins comprise a closely related multigenic family. For example there are 11 RAC/ROP genes described in *A. thaliana* (Yang, 2002), six in barley (Schultheiss *et al.*, 2003) and seven in rice (Chen *et al.*, 2010). The presence of multiple RAC/ROPs highlight their involvement in numerous pathways. This is supported by the observation that expression of several RAC/ROPs is tissue specific and often depends on the developmental stage of the plant (Winge *et al.*, 1997, Schultheiss *et al.*, 2003, Brembu *et al.*, 2005). However due to the fact that RAC/ROP proteins are functionally redundant and are often involved in signaling crosstalk it is difficult to assign specific functions to individual members of one RAC/ROP family.

1.4.1. RAC/ROP signaling in polarized cell growth

RAC/ROP proteins are central regulators of polarized cell growth. They control the establishment of cell polarity through the regulation of the cytoskeleton and vesicular trafficking. In the last years, polarized cell growth involving RAC/ROP-dependent microtubule and filamentous (F)-actin reorganization as well as membrane trafficking has been extensively studied using model systems like root hairs, pollen tubes, leaf pavement cells and trichomes (Yalovsky *et al.*, 2008, Yang, 2008).

Root hairs and pollen tubes rapidly elongate at a single end by polarized tip growth. Polarized tip growth is mediated through RAC/ROP proteins that accumulate at the apical region where they induce polarized cell growth (Lin *et al.*, 1996, Molendijk *et al.*, 2001). In *Arabidopsis*, overexpression of CA AtRAC/ROPs abolish polar growth and induces root hair swelling and ballooning at the tip of pollen tubes (Kost *et al.*, 1999, Li *et al.*, 1999, Molendijk *et al.*, 2001, Jones, 2002). Shortening and ballooning of root hairs was also observed in transgenic barley plants expressing CA HvRAC/ROPs (Pathuri *et al.*, 2008). By contrast overexpression of dominant negative (DN) AtRAC/ROPs inhibits elongation of pollen tubes and transgenic barley lines containing a *HvRACB*-RNAi construct were impaired in root hair outgrowth (Kost *et al.*, 1999, Li *et al.*, 1999, Jones, 2002, Hoefle *et al.*, 2011). Periodic pollen tube tip growth is based on the oscillatory activity of apical AtROP1 signaling that controls F-actin dynamics via two counteracting downstream pathways (Gu *et al.*, 2005, Hwang *et al.*, 2005). Apical AtROP1 is activated and distributed as an apical cap that defines the site of exocytosis before it interacts with its effectors Rop-interactive CRIB motif-containing protein 4 (RIC4) and RIC3. While the CA AtROP1-RIC4 pathway promotes F-actin assembly and the accumulation of exocytic vesicles to the tip, the CA AtROP1-RIC3 pathway causes a tip focused Ca^{2+} gradient that induces F-actin disassembly and facilitates exocytosis. In addition the CA AtROP1 effector ROP-interactive partner 1 (RIP1)/interactor of constitutively active ROP1 (ICR1) targets the SEC3 exocyst subunit and triggers polar exocytosis. Hence, polar exocytosis is crucial for tip growth as it provides membrane and cell wall materials (Qin and Yang, 2011, Craddock *et al.*, 2012, Guan *et al.*, 2013). Presenting a self-organizing mechanism, AtROP1 signaling in pollen tube tip growth includes positive and negative feedback regulations. Via the CA AtROP1-RIC4 pathway upstream components of AtROP1 signaling like ROPGEFs and receptor kinases are probably targeted to increase the area of active CA AtROP1, hence presenting a positive feedback loop. A negative feedback loop is caused by the ROPGAP ROP1 enhancer 1 (REN1) that is targeted to the apical plasma membrane upon polarized exocytosis where it inhibits AtROP1 (Hwang *et al.*, 2008, Qin and Yang, 2011, Craddock *et al.*, 2012, Guan *et al.*, 2013). To investigate polarity involving cell-cell coordination, the jigsaw-puzzle shaped diffuse growing leaf pavement cells of *Arabidopsis* represent a well-established model system. *Arabidopsis* leaf pavement cells are formed through the interplay of two antagonizing RAC/ROP pathways that are controlled through the auxin-dependent activation of membrane-localized transmembrane kinases (TMK)

via auxin-binding protein 1 (ABP1) (Fu *et al.*, 2002, Fu *et al.*, 2005, Fu *et al.*, 2009, Xu *et al.*, 2010, Xu *et al.*, 2014). ABP1 and TMKs act upstream of AtROP2 and AtROP6 that are responsible for the formation of complementary lobes and indentations respectively. Functionally redundant AtROP2 and AtROP4 promote via their effector RIC4 local accumulation of F-actin at sites of lobe outgrowth and simultaneously inactivate RIC1. This results in lobe formation. By contrast, in the indenting zone, AtROP6 activates RIC1 which leads to the stabilization of microtubules and the formation of neck regions (Fu *et al.*, 2005, Fu *et al.*, 2009, Zhao *et al.*, 2013). Recently, the microtubule severing protein katanin (KTN1) was identified as downstream element of the AtROP6-RIC1 signaling pathway. KTN1 detaches branched microtubules and promotes ordering of parallel cortical microtubules to maintain indentations (Lin *et al.*, 2013a). Interestingly, the interdigitated growth of pavement cells underlies a self-organizing mechanism in which localized extracellular auxin is generated through its AtROP2-dependent export via lobe localized auxin-transporter proteins called pinformed 1 (PIN1) (Xu *et al.*, 2010). Moreover lobe outgrowth of *Arabidopsis* epidermal cells is also restricted by knock-out of the AtRAC/ROP effector ICR1. ICR1 interacts with CA AtRAC/ROPs and the exocyst vesicle-tethering complex subunit SEC3 thereby providing a link between RAC/ROPs, cell polarity and vesicle trafficking (Lavy *et al.*, 2007). In addition, ICR1 seems to be required for plasma membrane recruitment and polar localization of PIN proteins (Hazak *et al.*, 2010). Interestingly, expression and subcellular localization of ICR1 is regulated by auxin, indicating a self-organizing mechanism that underlies auxin distribution (Hazak *et al.*, 2010, Hazak *et al.*, 2014). While AtRAC/ROPs are crucial for the development of the jigsaw puzzle shaped leaf pavement cells in *Arabidopsis* the influence of HvRAC/ROPs on the shape of the brick-like formed barley epidermal cells is less obvious. Transgenic barley plants expressing CA HvRAC/ROPs showed slightly induced epidermal cell expansion and abnormal developed stomata indicating that HvRAC/ROPs are also involved in the development of barley epidermal cells (Pathuri *et al.*, 2008, Pathuri *et al.*, 2009).

Trichomes which represent another model system to study cell polarity are specialized epidermal cells that are found on the surface of most land plants. In *Arabidopsis* (Columbia-0) these unicellular structures are evenly distributed on leaves and stems and typically consist of a stalk and three to four branches (Hülskamp *et al.*, 1994, Schellmann and Hülskamp, 2005). Trichome development and branching is regulated through the microtubule and actin cytoskeleton (Mathur, 2006). In regard to cytoskeleton rearrangement, trichome morphogenesis resembles the RAC/ROP involving development of leaf pavement cells in which RAC/ROPs are prominent modulators for microtubule dynamics. Hence, it is speculated that the activation of katanins via the AtROP6-RIC1 pathway is also involved in trichome branch formation as mutations in the katanin complex reduces trichome branching (Burk *et al.*, 2001, David Marks, 2014). Another AtRAC/ROP that is involved in the regulation of cytoskeleton dynamics is AtROP11. AtROP11 interacts with the microtubule depletion domain1 (MIDD1) protein that in

turn associates with AtKinesin-13A (Mucha *et al.*, 2010) and depolymerizes microtubules in xylem cells (Oda and Fukuda, 2012, Oda and Fukuda, 2013, Oda and Fukuda, 2014). *Arabidopsis* AtKinesin-13A T-DNA insertion lines show four instead of three trichome branches thereby indicating a function of AtKinesin-13A in blocking branch initiation by destabilizing microtubules. This observation might provide another link between RAC/ROP signaling and trichome morphogenesis (Lu *et al.*, 2005, David Marks, 2014). Furthermore, transgenic *Arabidopsis* plants expressing CA AtROP2 show altered trichome morphology in comparison to wild type plants although the number of branches is not affected (Fu *et al.*, 2002). It is also shown that changes in RAC/ROP activity mediated by bacterial toxin or mutations in a ROPGEF protein influences trichome branching (Qiu *et al.*, 2002, Basu *et al.*, 2008, Singh *et al.*, 2013). In summary it is noticeable that in cell polarity establishment of plants RAC/ROP signaling networks coordinate multiple pathways. Thereby RAC/ROP proteins can act as branching points for multiple signaling pathways or different RAC/ROP signaling pathways can be interlinked through their inhibitory function on each other. These complex signaling networks often includes feedback loops and are able to integrate RAC/ROP signaling pathways with antagonizing functions.

1.4.2. RAC/ROP signaling in plant immunity

Within the last years, it became evident that RAC/ROPs are key regulators of plant immunity (Kawano *et al.*, 2014). RAC/ROP proteins are involved in both resistance and susceptibility to fungal pathogens reflecting the complex function of RAC/ROP signaling. In rice the best studied RAC/ROP protein *Oryza sativa* RAC1 (OsRAC1) acts as positive regulator for resistance against the rice blast fungus *Magnaporthe oryzae* and the bacterial pathogen *Xanthomonas oryzae* pv*. oryzae*. The chitin-receptor complex chitin elicitor binding protein (OsCEBiP)/ chitin elicitor receptor kinase (OsCERK1) transmits the fungal-derived chitin signal via OsRACGEF1 to OsRAC1. This in turn triggers PTI signaling including ROS production, lignin accumulation and MAPK signaling-dependent activation of PR genes, phytoalexins and cell death induction (Akamatsu *et al.*, 2013, Kawano *et al.*, 2014). In addition, it is assumed that OsRAC1 functions downstream of several R proteins contributing to ETI (Kawano *et al.*, 2010, Kawano and Shimamoto, 2013, Kawano *et al.*, 2014). In contrast, OsRAC4 and OsRAC5 together with OsRAC6 (also known as OsRACB) act as susceptibility factors and negatively regulate blast resistance (Jung *et al.*, 2006, Chen *et al.*, 2010, Kawano *et al.*, 2014). Among the RAC/ROP proteins in barley, HvRACB, HvRAC1 and HvRAC3 are able to support susceptibility to the barley powdery mildew fungus *Bgh* (Schultheiss *et al.*, 2002, Schultheiss *et al.*, 2003, Pathuri *et al.*, 2008). While all active forms of these HvRAC/ROPs promote penetration success of *Bgh*, only active HvRAC1 supports callose deposition and whole cell accumulation of H_2O_2 in *Bgh* attacked but non-penetrated barley epidermal cells (Pathuri *et al.*, 2008). In recent years most of the research on barley RAC/ROPs has been concentrated on HvRACB highlighting its

function as susceptibility factor in the barley-*Bgh* interaction (Schultheiss *et al.*, 2002, Schultheiss *et al.*, 2003, Hoefle *et al.*, 2011). While stable transgenic barley lines expressing CA HvRACB are more susceptible to penetration and haustoria formation of *Bgh*, stable transgenic knock-down of HvRACB limits fungal accommodation success (Schultheiss *et al.*, 2005, Pathuri *et al.*, 2008, Hoefle *et al.*, 2011). As first interacting protein of HvRACB a ROP-interactive CRIB (CDC42/RAC interactive binding) motif containing protein of 171 amino acids (HvRIC171) was identified by Schultheiss et al. (2008). CA HvRACB interacts with HvRIC171 in yeast and *in planta* and similar to CA HvRACB, transient overexpression of HvRIC171 in barley epidermal cells enhances penetration success of *Bgh* (Schultheiss *et al.*, 2002, Schultheiss *et al.*, 2003, Schultheiss *et al.*, 2008). Since co-expression of CA HvRACB and HvRIC171 did not further increase this effect, HvRIC171 is suggested to act in the same pathway as HvRACB as potential downstream effector (Schultheiss *et al.*, 2008). In addition, accumulation of HvRIC171 at the entry site of *Bgh* was observed assuming that local activity of HvRAC/ROPs play a role in susceptibility to fungal invasion (Schultheiss *et al.*, 2008). Interaction of HvRACB with the cytoskeleton thereby modulating fungal penetration success has long been suggested as underlying mechanism of HvRACB function. It was shown that transient overexpression of CA HvRACB in barley epidermal cells inhibits F-actin polarization towards the site of fungal attack while transient RNAi-mediated knockdown of HvRACB supports F-actin polarization (Opalski *et al.*, 2005). A direct link between fungal invasion success and HvRACB-signaling-dependent microtubule organization was first suggested by Hoefle et al., 2011. A barley microtubule-associated ROP-GTPase activating protein (HvMAGAP1) interacts with CA HvRACB in yeast and *in planta.* HvMAGAP1 promotes polar organisation of cortical microtubules during the attack of *Bgh* and reduces penetrations success of the fungus upon transient overexpression in barley epidermal cells. Limiting susceptibility to penetration by *Bgh* reflects the general function of ROPGAPs to negatively regulate RAC/ROP downstream signaling. Hence, the results indicate an antagonistic role of HvRACB and HvMAGAP1 in cytoskeleton organization for fungal entry (Hoefle *et al.*, 2011). Recently, an engulfment and motility (ELMO) domain containing protein (HvELMOD_C) was identified that interacts with HvMAGAP1 in yeast and *in planta* (Hoefle and Hückelhoven, 2014). HvELMOD_C counteracts the resistance-inducing effect of HvMAGAP1 in the barley-*Bgh* interaction when both proteins are simultaneously expressed. Hence, HvELMOD_C might act as a regulator of HvMAGAP1 and thereby indirectly control HvRACB activity (Hoefle and Hückelhoven, 2014). While in barley and rice, the function of RAC/ROP proteins in plant immunity is better and better understood, little is known about the function of RAC/ROPs in immunity of the model *Arabidopsis*. Until now, only for AtROP6 a function in susceptibility against *G. orontii* was suggested (Poraty-Gavra *et al.*, 2013). In addition, AtROPGAP1 and AtROPGAP4 which are most similar to HvMAGAP1 interact with CA AtROP4 and CA AtROP6 in yeast and *in planta* and seem to be involved in plant immunity. Similar to what was observed for transient knock-down of HvMAGAP1 in the barley-*Bgh* interaction,

Arabidopsis T-DNA insertion lines of AtROPGAP1 and AtROPGAP4 show enhanced susceptibility to *E. cruciferarum* (Hoefle *et al.*, 2011, Huesmann *et al.*, 2011).

1.5. Receptor-like cytoplasmic kinases and their function in plant immunity

Receptor-like cytoplasmic kinases (RLCKs) represent a subfamily of the large and diverse receptor-like kinase (RLK) family that is involved in plant growth, development and immune response (Afzal *et al.*, 2008). Like all members of the RLK superfamily, RLCKs have a conserved serine/threonine (Ser/Thr) kinase domain but the extracellular and transmembrane domains are lacking. This effects the cytoplasmic localization of RLCKs (Afzal *et al.*, 2008, Gish and Clark, 2011). In rare cases, RLCKs are still anchored to the plasma membrane via myristoylation motifs (Murase *et al.*, 2004, Veronese *et al.*, 2006, Tang *et al.*, 2008). So far, 379 and almost 200 *RLCK* genes have been identified in rice and *Arabidopsis* respectively (Shiu *et al.*, 2004, Jurca *et al.*, 2008, Vij *et al.*, 2008). Based on phylogenetic clades RLCKs are divided into 13 subfamilies (RLCKs I-XIII) (Shiu *et al.*, 2004). In general, RLCKs activate or inactivate target proteins through phosphorylation thereby transmitting intracellular signals. In addition, RLCKs can form complexes with RLKs to pass cellular signaling via transphosphorylation events. As signal transducers, RLCKs are either alone or in concert with RLKs involved in the embryonic patterning process (Bayer *et al.*, 2009), self-incompatibility (Murase *et al.*, 2004), organ separation (Burr *et al.*, 2011), ethylene (Laluk *et al.*, 2011) and brassinosteroid signaling (Tang *et al.*, 2008, Sreeramulu *et al.*, 2013). Several studies highlight the importance of RLCKs in plant immunity in which especially RLCKs of the subfamily VII are involved (Lin *et al.*, 2013b). In *Arabidopsis*, Botrytis-induced kinase 1 (BIK1), a member of the RLCK VII subfamily that was originally identified as a component in plant defense against necrotrophic pathogens is involved in PTI signaling. BIK1 associates with the flagellin-sensing 2 (FLS2)/ brassinosteroid 1-associated kinase 1 (BAK1) receptor complex, gets phosphorylated upon flagellin perception and induces downstream signaling after transphosphorylation of the FLS2/BAK1 complex (Veronese *et al.*, 2006, Lu *et al.*, 2010, Zhang *et al.*, 2010). AvrPphB susceptible (PBS1) and PBS1-like1 (PBL1) also belonging to the RLCK VII subfamily mediate flagellin-dependent PTI signaling similar to BIK1 (Lu *et al.*, 2010, Zhang *et al.*, 2010). In rice, the RLCK VII subfamily protein OsRLCK185 is phosphorylated upon recognition of chitin by the OsCERK1 complex possibly connecting PRR signals to MAP kinase activation and defense responses (Yamaguchi *et al.*, 2013). Similar, the *Arabidopsis* ortholog of OsRLCK185, PBL27, was identified as a downstream component of *Arabidopsis* CERK1 and is involved in chitin-induced immune responses (Shinya *et al.*, 2014). Furthermore the pepper (*Capsicum annuum*) RLCK VII protein named pathogen-induced protein kinase 1 (CaPIK1) is involved in the activation of defense responses against microbial pathogens (Kim and Hwang, 2011). Several members of the RLCK VII subfamily are targets of pathogen effectors. The function of BIK1, PBL1 and PBS1 is inhibited by the *Pseudomonas syringae* (*P. syringae*) effector AvrPphB that proteolytically

cleaves these RLCKs. The *Arabidopsis* NB-LRR immune receptor resistant to *P. syringae* 5 (RPS5) in turn monitors PBS1 cleavage and initiates ETI (Zhang *et al.*, 2010). Another RLCK VII, RPM1-induced protein kinase (RIPK) is a target of the *P. syringae* effector AvrB. RIPK subsequently phosphorylates the NB-LRR immune receptor RPM1 that in turn triggers ETI (Liu *et al.*, 2011). In addition *Xanthomonas campestris* pv. *campestris* effector AvrAC possess uridylyl transferase activity and targets BIK1 and RIPK thereby reducing their kinase activity and blocking subsequent activation of immune responses (Feng *et al.*, 2012). In rice, the recently identified *Xanthomonas oryzae* pv. *oryzae* effector Xoo1488 targets OsRLCK185 and blocks OsRLCK185-mediated immune responses (Yamaguchi *et al.*, 2013). The very first characterized R gene in plants, tomato Pto, encodes a RLCK that interacts with *P. syringae* effectors AvrPto and AvrPtoB. This interaction results in the activation of ETI signaling and confers resistance to *P. syringae* (Kim *et al.*, 2002). Furthermore several *Arabidopsis* RLCKs of the VI subfamily whose 14 members are classified into group A and B according to their domain structure are described as RAC/ROP interactors (Jurca *et al.*, 2008, Molendijk *et al.*, 2008, Dorjgotov *et al.*, 2009). Molendijk et al (2008) identified two *Arabidopsis* RLCK VI_As named ROP binding protein kinase1 (AtRBK1) and AtRBK2 as RAC/ROP effectors. AtRBK1 interacts with CA AtROP4 in yeast and *in planta* and its expression is upregulated upon exposure to *Phytophtora infestans* and *Botrytis cinerea* (Molendijk *et al.*, 2008). The observation that RLCK VI_As of *Arabidopsis* and *Medicago trunculata* (*M. trunculata*) are activated by active RAC/ROPs *in vitro* further supports the assumption that RLCK VI_As act as downstream effectors of RAC/ROP signaling (Dorjgotov *et al.*, 2009).

1.6. Ubiquitination

Ubiquitin, a highly conserved small protein of 76 amino-acid residues, is found in all eukaryotes. Post-translational modification of proteins by ubiquitin occurs in a process called ubiquitination. As basic mechanism, ubiquitination regulates the function and stability of proteins and is therefore involved in almost all plant cellular processes including hormone signaling, cell division, plant development and responses to abiotic and biotic stress (Smalle and Vierstra, 2004, Dreher and Callis, 2007, Vierstra, 2009, Sadanandom *et al.*, 2012). The covalent attachment of ubiquitin to its target protein is mediated through a three-step enzymatic cascade (Fig. 2a). First ubiquitin is activated by an E1 ubiquitin activating enzyme in an ATP-dependent manner and subsequently transferred from the E1 enzyme to an E2 ubiquitin-conjugating enzyme. Finally ubiquitin is attached to a lysine residue of the target protein via an E3 ubiquitin ligase (Vierstra, 2009). Target proteins can be either mono- or poly-ubiquitinated through the reiterative binding of ubiquitin to one of the seven lysines within the previously attached ubiquitin molecule. Mono-ubiquitination is mainly involved in non-proteolytic processes like regulation of protein activity, localization, protein-protein interactions or in histone modification (Delauré *et al.*, 2008). Most poly-ubiquitinated proteins are targeted for degradation. Especially

those proteins that are modified with Lys48-linked poly-ubiquitin chains of at least four ubiquitin molecules are favoured substrates for the proteolysis by the cytoplasmic and nucleoplasm localized 26S proteasome complex (Delauré *et al.*, 2008).

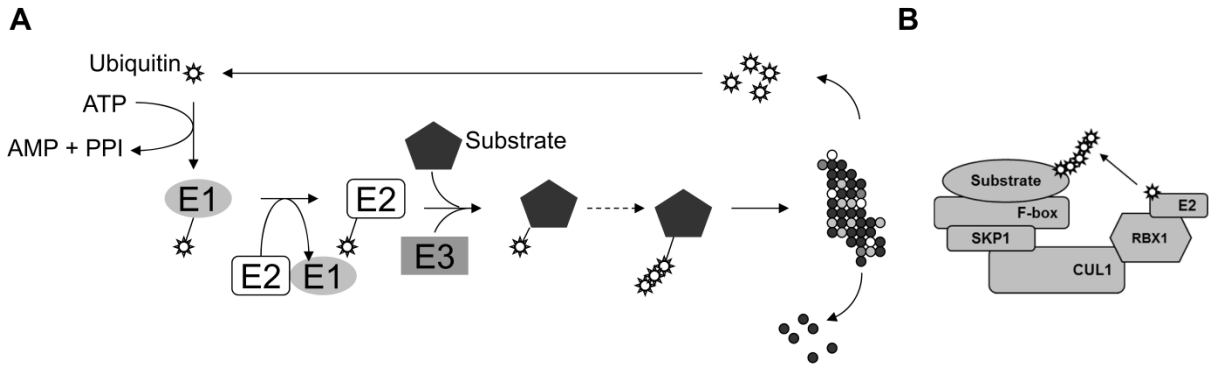

Fig 2. The ubiquitin-26S proteasome system
(A) Ubiquitin gets activated by an E1 activating enzyme in an ATP-dependent manner followed by its transfer to an E2 ubiquitin conjugating enzyme. An E3 ubiquitin ligase finally mediates the transfer from E2 to a lysine residue in the target protein. Additional ubiquitin molecules are ligated to form poly-ubiquitin chains. Proteins with poly-ubiquitin chains are targeted to the 26S-proteasome for degradation. (B) General composition of an SCF-E3 ubiquitin ligase complex. The S phase kinase-associated protein 1 (SKP1) links cullin 1 (CUL1) to an F-box protein which in turn specifically recognizes the target protein. CUL1 also associates with RING-BOX1 (RBK1) that presents a docking platform for E2 ubiquitin conjugating enzymes and transfer their bound ubiquitin to the substrate.

Among the enzymes that participate in the ubiquitination cascades, E3 ubiquitin ligases confer substrate specificity to the ubiquitination pathway. Based on their subunit composition and their mechanism of action, E3 ubiquitin ligases can be classified into HECT (homologous to E6-associated protein C-Terminus), RING (really interesting new gene), U-box and CRL (cullin-RING) ligases (Vierstra, 2009). The multimeric CRLs are further subdivided into four groups of which the S-phase kinase associated protein 1-cullin 1-F-box (SCF) E3 ubiquitin ligase represents the one best-described (Fig. 2b). SCFs are composed of four different polypeptides. The N-terminus of the scaffold protein Cullin 1 (CUL1) is linked through the adaptor protein S-phase kinase associated protein 1 (SKP1) to an F-box protein which in turn specifically recognizes the target protein. The C-terminus of CUL1 associates with RING-BOX 1 (RBX1) that presents a docking platform for E2s (Hua and Vierstra, 2011).

1.7. SKP1 proteins and their function in plant immunity

SKP1 proteins are scaffolding proteins for multiple SCF-E3 ubiquitin ligase complexes. While humans or fungi only have one or a few SKP1 proteins and a little number of F-box proteins, plants encode a large number of SKP1 and F-box subunits allowing the formation of highly variable SCF-complexes (Hua and Vierstra, 2011). In *Arabidopsis* there are 21 genes that encode for *Arabidopsis* SKP1-like (ASKs) proteins and in rice 32, named *Oryza sativa* SKP1-like (OSKs) (Kong *et al.*, 2007, Kahloul *et al.*, 2013). According to Kong et al. (2007), plant

SKP1-like proteins are classified into three types of genes and two types of proteins. Type Ia and Ib genes contain a single or no intron respectively and code for type I proteins that show two characteristic conserved domains (SKP1_POZ and SKP1). Besides a carboxy-terminal elongation type II proteins, encoded by type II genes are structurally similar to type I proteins. Furthermore, type II genes have multiple introns (Kong *et al.*, 2007). Expect for ASK1 and ASK2 in *Arabidopsis*, the individual function of SKP1-like proteins is hardly characterized. Based on expression studies in rice and *Arabidopsis* a function of SKP1-like proteins in a broad spectrum of physiological processes is suggested (Zhao *et al.*, 2003, Dezfulian *et al.*, 2012, Kahloul *et al.*, 2013). In addition, their interaction with a variety of F-box proteins was investigated (Risseeuw *et al.*, 2003, Kong *et al.*, 2004, Takahashi *et al.*, 2004, Kong *et al.*, 2007, Dezfulian *et al.*, 2012, Kuroda *et al.*, 2012, Kahloul *et al.*, 2013). For ASK1 and ASK2 a function in flower development, male sterility, embryogenesis and seedling development is proposed (Zhao *et al.*, 2003, Liu *et al.*, 2004). As part of SCF-E3 ubiquitin ligase complexes, ASK1 and ASK2 are able to associate with transport inhibitor response 1 (SCF^{TIR1}), coronatine insensitive 1 (SCF^{COI1}), and ein3 binding F-box1/ein3 binding F-box2 ($SCF^{EBF1/EBF2}$) which play a respective role in auxin-, jasmonate- and ethylene signaling. The jasmonic acid pathway is induced upon attack of necrotrophic pathogens and leads to ubiquitin-mediated proteolysis of jasmonate ZIM-domain (JAZ) transcriptional repressors resulting in a derepression of JA-responsive genes involved in pathogen defense (Trujillo and Shirasu, 2010, Robert-Seilaniantz *et al.*, 2011). Ethylene signaling is linked to resistance against necrotrophic pathogens and its biosynthesis is induced upon PAMP perception of various biotic stresses (Trujillo and Shirasu, 2010). The central transcription factor ethylene insensitive 3 (EIN3) positively regulates ethylene responses and is controlled through the $SCF^{EBF1/EBF2}$ E3 ubiquitin ligase (Trujillo and Shirasu, 2010). Hence, several pathogen-induced defense responses that are triggered trough hormone signaling are regulated via ubiquitin-mediated proteolysis (Delauré *et al.*, 2008, Trujillo and Shirasu, 2010). Furthermore, ASK1 and ASK2 appear to control in cooperation with the G2 allele of SKP1 (SGT1), suppressor of rps4-RLD1 (SRFR1) and constitutive expressor of PR genes 1 (CPR1) the accumulation of the R protein suppressor of *npr1-1* constitutive 1 (SNC1) (Gou *et al.*, 2009, Gou *et al.*, 2012, Duplan and Rivas, 2014). However, there is increasing evidence that E3 ubiquitin ligases are involved in all layers of plant-immunity and that ubiquitin-mediated protein degradation is responsible for the regulation of a variety of immune signaling pathways (Zeng *et al.*, 2006, Dreher and Callis, 2007, Delauré *et al.*, 2008, Dielen *et al.*, 2010, Marino *et al.*, 2012, Duplan and Rivas, 2014).

1.8. Objectives and methodology

In the interaction of barley with the barley powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*) the barley small monomeric G protein HvRACB is known as susceptibility factor. However, knowledge about the signaling mechanisms controlling HvRACB-dependent disease susceptibility in the barley-*Bgh* interaction is incomplete. The general aim of this study was to functionally characterize the receptor-like cytoplasmic kinase HvRBK1, a putative HvRACB-interactor and the barley type II S-phase kinase 1 associated protein HvSKP1-like which was found to interact with HvRBK1 in the barley-*Bgh* interaction. Similarly, the putative *Arabidopsis* orthologue of HvRBK1, AtRLCK VI_A3 was functionally characterized in the interaction of *Arabidopsis* and the adapted powdery mildew fungus *E. cruciferarum.*

In a yeast-two hybrid assay HvRBK1 was identified as possible HvRACB- and HvRAC1-interacting protein (Holger Schultheiss, University of Giessen; Caroline Hoefle, TU München, personal communications). Since subcellular localization and functional characterization studies (Huesmann, 2011) suggested HvRBK1 as HvRACB effector, one of the objectives of this study was to verify the interaction of HvRACB and HvRBK1 *in planta* by fluorescence resonance energy transfer (FRET) measurements. Functional characterization of HvRBK1 in the barley-*Bgh* interaction was continued using a transient gene-silencing approach. To verify previous observations (Huesmann, 2011), recruitment of green fluorescent protein (GFP)-tagged HvRBK1 by active HvRAC/ROPs as well as different arrangements of fluorescent labelled microtubules after silencing of *HvRBK1* were quantified using confocal laser scanning microscopy.

HvSKP1-like that represents a part of an SCF-E3 ubiquitin ligase complex was originally identified as putative HvRBK1-interactor in a yeast-two hybrid assay (Caroline Hoefle, TU München, personal communication). This study aimed to isolate the full length sequence of HvSKP1-like from a barley cDNA pool and to demonstrate the interaction of HvRBK1 and HvSKP1-like in yeast and *in planta* by independent yeast-two hybrid assays and FRET measurements. The subcellular localization of HvSKP1-like and its co-localization with HvRBK1 was investigated using fluorescently tagged proteins in conjunction with confocal laser scanning microscopy. Gene expression studies employing reverse-transcription quantitative real-time PCR should reveal transcript changes of HvSKP1-like in the barley-*Bgh* interaction. Gene function of HvSKP1-like in the barley-*Bgh* interaction was further addressed by transient induced gene silencing of *HvSKP1-like* in barley epidermal cells. Since HvRACB was assumed as potential target of the HvSKP1-like containing SCF-E3 ubiquitin ligase complex changes of HvRACB protein abundance were analyzed. For this purpose protein abundance of different fluorescent-tagged versions of HvRACB were measured after treatment with the proteasome inhibitor MG132 or silencing of *HvRBK1* or *HvSKP1-like*. This was performed in transiently transformed barley mesophyll protoplasts or barley epidermal cells by fluorescence intensity

measurements using confocal laser scanning microscopy or western-blotting analysis. Overall, the biological significance of HvSKP1-like in the barley-*Bgh* interaction and in the regulation of HvRAC/ROP abundance was studied using molecular-, cellular- and biochemical approaches.

As putative orthologue of barley HvRBK1, AtRLCK VI_A3 was identified in *A. thaliana*. A function of AtRLCK VI_A3 in the interaction of *Arabidopsis* and *E. cruciferarum* was likewise assumed after first analysis of fungal disease progression on AtRLCK VI_A3 T-DNA insertion lines. Interaction of AtRLCK VI_A3 with different *Arabidopsis* RAC/ROP proteins was also observed (Huesmann, 2011). Here, transgenic mutant complementation lines of AtRLCK VI_A3 were generated by *Agrobacterium*-mediated transformation and molecularly and phenotypically characterized together with *Atrlck VI_3* mutant lines. Microscopic and quantitative real-time PCR-based quantification of *E. cruciferarum* infection on *Atrlck_VIA3* mutant and mutant complementation lines provided insight into the function of AtRLCK VI_A3 in the *Arabidopsis-E. cruciferarum* interaction. Through *in vitro* kinase activity assays a function of AtRLCK VI_A3 as AtRAC/ROP effector was further validated (in collaboration with Dalma Ménesi, Attila Fehér, Biological research centre, Szeged, Hungary).

2. Results

2.1. Barley ROP binding kinase1 is involved in microtubule organization and in basal penetration resistance to the barley powdery mildew fungus

2.1.1. Summary and contribution to publication Huesmann and Reiner *et al.*, 2012

Small monomeric G proteins of the plant specific Rho family called `Rho of plants` (RAC/ROPs) are involved in a multitude of signaling processes including plant development, cytoskeleton remodeling and pathogen defense. Active RAC/ROPs are reported to interact with receptor-like cytoplasmic kinases (RLCKs or ROP binding kinases, RBKs) *in planta* and regulate their activity *in vitro*. The barley RAC/ROPs HvRACB and HvRAC1 are both involved in the establishment of powdery mildew disease caused by the biotrophic fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*). In yeast-two hybrid screenings in which HvRACB and HvRAC1 were used as bait proteins a barley RAC/ROP interacting ROP binding kinase (HvRBK1) was identified as potential interactor of HvRAC/ROPs. Protein interaction of either constitutively active (CA) HvRACB or CA HvRAC1 with HvRBK1 was verified in yeast. *In planta* CA HvRACB and CA HvRAC1 but not their respective dominant negative (DN) forms recruited the cytoplasmic located green fluorescent protein (GFP)-tagged HvRBK1 to the cell periphery. A function of HvRBK1 as HvRAC/ROP effector was further confirmed by *in vitro* kinase activity assays. HvRBK1 showed basal kinase activity which was increased in a dose-dependent manner upon presence of CA HvRACB or GTP-loaded HvRAC1. Transient-induced gene silencing (TIGS) of *HvRBK1* in barley epidermal cells resulted in an increased penetration success of *Bgh* and indicates a role of HvRBK1 in basal resistance. Furthermore, TIGS of *HvRBK1* had a destabilizing effect on microtubules in barley epidermal cells. Taken together, HvRBK1 was verified as HvRAC/ROP effector and is supposed to be involved in microtubule organization and penetration resistance against the barley powdery mildew fungus.

Own contributions:

Experimentation: Quantification of HvRBK1 recruitment; Quantification of GFP-HvRBK1 signal intensity in cells co-expressing different versions of HvRAC/ROPs; FRET measurements; TIGS of *HvRBK1* to assess fungal penetration rate; Influence of TIGS of *HvRBK1* on microtubule organization using DsRED-MBD as microtubule marker

Data analysis: analysis of the above data

Writing: critical reading of the manuscript

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

2.2.1. Summary and contribution to publication Reiner *et al.*, 2015a

During the last years it became evident that in a multitude of cases the abundance of immunity-related proteins involved in plant-microbe interactions is regulated by the ubiquitin-26S proteasome system. In a three-step enzymatic cascade ubiquitin is covalently attached to a target protein prior to its degradation. Here, we identified a barley type II SKP1-like protein (HvSKP1-like) as molecular interactor of the HvRACB-interacting HvRBK1. SKP1-like proteins are subunits within the SKP1-cullin 1-F-box (SCF)-E3 ubiquitin ligases that catalyze the last step in the ubiquitination cascade, specifically recognizing target proteins for proteasomal degradation. HvRBK1 and HvSKP1-like interacted in yeast and *in planta*. Transient induced gene silencing (TIGS) of either *HvSKP1-like* or *HvRBK1* increased protein abundance of fluorescence tagged CA HvRACB in barley protoplasts and epidermal cells. Furthermore TIGS of *HvSKP1-like* in barley epidermal cells led to increased penetration success of *Bgh*. Transcript level of *HvSKP1-like* also changed upon inoculation with the fungus. These findings suggest a function of the E3 ubiquitin ligase subunit HvSKP1-like together with HvRBK1 in controlling HvRACB abundance thereby modulating disease outcome in the barley-barley powdery mildew interaction. Such a negative feedback regulation mechanism of HvRACB involving HvRBK1 and HvSKP1-like might explain the contradicting findings that the susceptibility factor HvRACB is involved in susceptibility whereas HvRBK1 rather plays a role in penetration resistance against *Bgh*.

Own contributions:

Experimentation: design and performance of experiments except the initial yeast-two hybrid screen

Data analysis: data analysis including statistical analysis

Writing: design and preparation of figures; writing of the manuscript

2.3. The *Arabidopsis* ROP-activated receptor-like cytoplasmic kinase RLCK VI_A3 is involved in control of basal resistance to powdery mildew and trichome branching

2.3.1. Summary and contribution to publication Reiner *et al.*, 2015b

RLCKs represent a subgroup of the large family of RLKs that play a role in a multitude of cellular processes including plant growth, development and immunity. In several studies RLCKs were identified as downstream effectors of RAC/ROPs. In the present work the *A. thaliana* AtRLCK VI_A3 which is closely related to the barley RLCK HvRBK1 is described as molecular interactor of several AtRAC/ROPs. Interaction of AtRLCK VI_A3 with different AtRAC/ROPs was verified in yeast. In addition, cytoplasmic localized GFP-tagged AtRLCK VI_A3 was recruited to the plasma membrane upon transient co-expression of either CA AtROP4 or CA AtROP6 in *Arabidopsis* epidermal cells. *In vitro* kinase activity assays showed basal phosphorylation activity of AtRLCK VI_A3. Kinase activity of AtRLCK VI_A3 was further increased in the presence of WT or CA AtROP6 supporting the assumption that AtRLCK VI_A3 is a downstream signaling effector of AtRAC/ROPs. Compared to wild-type plants *Atrlck VI_A3* mutant lines displayed a retarded growth phenotype and showed an increased number of trichome branches. Furthermore they were more susceptible to the *Arabidopsis* powdery mildew fungus *E. cruciferarum*. All studied phenotypes could be restored after overexpression of *AtRLCK VI_A3* in the mutant background. These results suggest that AtRLCK VI_A3 has a function in basal resistance against *E. cruciferarum* hence reflecting the results obtained for the related HvRBK1 in the barley-*Bgh* interaction. Furthermore AtRLCK VI_A3 is involved in plant growth and cellular differentiation during trichome morphogenesis.

Own contributions:

Experimentation: generation of *Atrlck VI_A3* mutant complementation lines; molecular and phenotypical characterization of *Atrlck VI_A3* mutant and mutant complementation lines; microscopic and quantitative real-time PCR-based quantification of *E. cruciferarum* infection on *Atrlck_VIA3* mutant and mutant complementation lines; quantitative analysis of trichome branch number of *Atrlck VI_A3* mutant and mutant complementation lines; *in vitro* kinase activity assay was performed in cooperation with Dalma Ménesi and Attila Fehér (Biological research centre, Szeged, Hungary)

Data analysis: data analysis including statistical analysis

Writing: design and preparation of figures; writing of the manuscript

3. Discussion

In plants, remarkable forms of immunity have evolved to cope with a multitude of pathogens. During the last years it became evident that RAC/ROP proteins act as major molecular switches in plant immunity and cytoskeleton rearrangement (Yang, 2008, Kawano *et al.*, 2014). In a compatible interaction between plants and invasive biotrophic fungal pathogens RAC/ROP proteins are of particular interest. In such an interaction host cytoskeletal rearrangement might be needed for the establishment of fungal feeding structures inside living host cells. In the interaction of barley with the barley powdery mildew fungus *Bgh* there is accumulating evidence that HvRACB enables establishment of the fungus by rearrangement of the host cytoskeleton. It is further speculated that the fungus itself might target HvRACB and manipulate its function for its own colonization success (Hückelhoven *et al.*, 2013a, Hückelhoven *et al.*, 2013b). Due to the fact that HvRACB supports accommodation of *Bgh* in the barley-barley powdery mildew interaction it is described as susceptibility factor (Schultheiss *et al.*, 2002, Schultheiss *et al.*, 2003). Although several interacting proteins of HvRACB are described its complex signaling mechanism including downstream effectors in the barley-*Bgh* interaction is little understood. In the present work the barley RLCK HvRBK1 and its *Arabidopsis* orthologue AtRLCK VI_A3 which were both identified as respective HvRAC/ROP- or AtRAC/ROP-interacting proteins were functionally characterized. Characterization was performed in the interaction of barley and *Bgh* or *Arabidopsis* and *E. cruciferarum* respectively. A barley SKP1-like protein that interacts with HvRBK1 and modulates the abundance of the susceptibility factor HvRACB was further investigated in the barley-*Bgh* interaction.

3.1. RAC/ROPs interact and activate receptor-like cytoplasmic kinases

In 2008, Molendijk *et al*. showed that two *Arabidopsis* RLCKs of the subfamily VI_A, AtRBK1 and AtRBK2, interact with CA AtROP4 in yeast and *in planta*. Interaction of AtRBK1 and AtRBK2 with further AtRAC/ROPs in yeast was additionally demonstrated by Dorjgotov *et al*. (2009). A yeast-two hybrid matrix experiment from Dorjgotov *et al*. (2009) also revealed a specific interaction of AtRAC/ROPs with most members of the group A of the AtRLCK VI family. However, interaction of a novel cysteine-rich receptor kinase (NCRK), a RLCK of the subfamily VIII, with AtROP4 and AtROP11 was also reported (Molendijk *et al.*, 2008). Here, the interaction of the barley RLCK HvRBK1 which is most closely related to *Arabidopsis* AtRLCK VI_A3 with HvRAC1 as well as HvRACB was shown (Huesmann and Reiner *et al.*, 2012). Both HvRAC/ROPs are involved in the barley-*Bgh* interaction (Schultheiss *et al.*, 2002, Schultheiss *et al.*, 2003, Pathuri *et al.*, 2008). HvRBK1 interacted with either CA HvRACB or CA HvRAC1 but not with their respective DN forms in yeast and *in planta* (Huesmann and Reiner *et al.*, 2012). *In planta* protein-protein interaction is assumed to occur mainly at the plasma membrane where these active HvRAC/ROPs are predominantly located (Schultheiss *et al.*, 2003). This is

supported by the observation that CA HvRACB and CA HvRAC1 but not their respective DN forms were able to recruit the cytoplasmic localized GFP-tagged HvRBK1 to the plasma membrane upon transient co-expression in barley epidermal cells (Huesmann and Reiner *et al.*, 2012). Subcellular localization of CA HvRACB seems to be important for its function as impaired plasma membrane localization of CA HvRACB abolished the biological function of CA HvRACB (Schultheiss *et al.*, 2003). In a heterologous targeted yeast-two hybrid assay the putative *Arabidopsis* orthologue of HvRBK1, AtRLCK VI_A3 interacted with similar HvRAC/ROPs as HvRBK1 (Reiner *et al.*, 2015b). These results might indicate a possible functional conservation of HvRBK1 and AtRLCK VI_A3 as interacting partners of RAC/ROPs in *Arabidopsis* and barley. Furthermore AtRLCK VI_A3 interacted with several AtRAC/ROPs in yeast (Reiner *et al.*, 2015b). Due to their redundancy it is difficult to assign specific functions to distinct AtRAC/ROPs. However in the targeted yeast-two hybrid assay AtRLCK VI_A3 interacted with all AtRAC/ROPs (AtROP2, AtROP4, AtROP6 and AtROP11) that are known to influence microtubule stability and organization (Fu *et al.*, 2002, Fu *et al.*, 2005, Fu *et al.*, 2009, Xu *et al.*, 2010, Oda and Fukuda, 2012, Lin *et al.*, 2013a, Oda and Fukuda, 2013, Oda and Fukuda, 2014, Reiner *et al.*, 2015b). Transient co-expression of plasma membrane localized CA AtROP4 or CA AtROP6 in *Arabidopsis* epidermal cells resulted in recruitment of cytoplasmic GFP-tagged AtRLCK VI_A3 to the plasma membrane. In contrast cytoplasmic localization of GFP-AtRLCK VI_A3 was not changed upon transient co-expression of DN AtROP6 (Reiner *et al.*, 2015b). This suggests that protein-protein interaction of CA AtRAC/ROPs and AtRLCK VI_A3 also occurs *in planta*.

Previously it was reported that CA or GTP-loaded RAC/ROPs are able to activate *Arabidopsis* or *M. trunculata* kinases which belong to the RLCK subfamily VI_A *in vitro* (Dorjgotov *et al.*, 2009). Together with our cooperation partner Attila Fehér (Biological research centre, Szeged, Hungary) *in vitro* kinase activity assays with HvRBK1 and AtRLCK VI_A3 were performed (Huesmann and Reiner *et al.*, 2012, Reiner *et al.*, 2015b). HvRBK1 as well as AtRLCK VI_A3 showed basal kinase activity *in vitro*. In both cases kinase activity increased upon respective addition of CA or GTP-loaded *Arabidopsis* or barley RAC/ROPs in a dose-dependent manner. In contrast, kinase activity was not increased upon addition of the DN *Arabidopsis* or barley RAC/ROP proteins. These results further indicate that RLCKs act as RAC/ROP effectors in *Arabidopsis* and barley (Huesmann and Reiner *et al.*, 2012, Reiner *et al.*, 2015b).

Phosphorylation and dephosphorylation of proteins is a major mechanism in cellular signal transduction. In animal cells specific protein kinases phosphorylate RAC/ROP GTPases at various residues. This affects their activity as well as their signaling function (Loirand *et al.*, 2006). For human RAC1 (HsRAC1) it was shown that phosphorylation at residue S71YP by AKT kinase limits its GTP-binding activity without changing its GTPase activity (Kwon *et al.*, 2000). Phosphorylation of HsRAC1 at S71 further inhibited its interaction with the effector p21-

activated kinase 1 (PAK1) blocking subsequent downstream signaling (Rehani *et al.*, 2009, Fodor-Dunai *et al.*, 2011). Although the site at which HsRAC1 gets phosphorylated by AKT kinase is conserved in plant RAC/ROPs, until now it is not known whether plant RAC/ROPs undergo phosphorylation (Kwon *et al.*, 2000, Fodor-Dunai *et al.*, 2011). As protein kinases and RAC/ROP-interacting proteins AtRLCK VI_A3 as well as HvRBK1 might have the potential to phosphorylate RAC/ROPs. While *in vitro* kinase activity assays of AtRLCK VI_A3 showed no phosphorylation of AtROP6 preliminary results suggested that HvRACB might be phosphorylated by HvRBK1 *in vitro* (Attila Fehér, Biological research centre, Szeged, Hungary, personal communication). Targeted phosphorylation of RLCKs and subsequent RLCK-dependent transphosphorylation of immune receptors is a common mechanism of PTI-signaling and highlight the importance of RLCKs in plant immunity. However mainly RLCKs of the subfamily VII are involved in plant immunity (Veronese *et al.*, 2006, Lu *et al.*, 2010, Zhang *et al.*, 2010, Lin *et al.*, 2013b).

In summary, protein-protein interaction studies in yeast and *in planta* as well as *in vitro* kinase activity assays confirmed RLCKs as RAC/ROP interactors in plants.

3.2. RAC/ROP-activated receptor-like cytoplasmic kinases in cytoskeleton organization

RAC/ROP proteins are central regulators of cell polarity thereby coordinating microtubule and F-actin organization (Yang, 2008). Via regulation of the actin and microtubule network RAC/ROPs control the shape of cells such as trichomes and epidermal pavement cells (Fu *et al.*, 2002, Fu *et al.*, 2005, Mathur, 2006, Fu *et al.*, 2009, David Marks, 2014). Here, TIGS of *HvRBK1* resulted in more fragmented microtubules in barley epidermal cells (Huesmann and Reiner *et al.,* 2012) and *Arabidopsis Atrlck VI_A3* knock-out lines showed increased trichome branch numbers (Reiner *et al.*, 2015b) suggesting a role of RAC/ROP-activated RLCKs in cytoskeleton organization of barley and *Arabidopsis*.

AtROP2 and its functional redundant AtROP4 as well as AtROP6, which all interacted with AtRLCK VI_A3, organize the jigsaw puzzle shaped *Arabidopsis* leaf pavement cells via an auxin-dependent antagonistic pathway (Fu *et al.*, 2002, Fu *et al.*, 2005, Fu *et al.*, 2009, Xu *et al.*, 2010, Lin *et al.*, 2013a, Xu *et al.*, 2014, Reiner *et al.*, 2015b). Local activation of AtROP2/4 activates RIC4-mediated assembly of actin beneath the plasma membrane promoting lobe outgrowth. Active AtROP2/4 also sequesters microtubule-associated RIC1 that promotes the formation of highly ordered cortical microtubule arrays in non-expanding regions between lobes when activated through AtROP6. In addition, RIC1 is able to reduce AtROP2/4 activity through a negative feedback. Hence microtubules aggregate into parallel bundles that are arranged transversely across future indentation regions in this AtRAC/ROP-dependent anisotropic cell expansion (Fu *et al.*, 2002, Fu *et al.*, 2005, Fu *et al.*, 2009). This reflects the common mechanism of cell expansion which preferentially occurs in the direction perpendicular to

microtubules at sites of F-actin accumulation (Fu *et al.*, 2005). A similar organization of microtubules and F-actin can be observed during trichome branching. In non-branched trichomes, cortical microtubules accumulate in an apical collar, which indicates the area for future bulge formation. Formation of a new branch occurs underneath this microtubule collar in a region where microtubules are absent and F-actin accumulates as soon as the bulge starts to growth (Sambade *et al.*, 2014). Due to the F-actin cap of the growing trichome, further expansion of the new branch resembles RAC/ROP-regulated tip growth in pollen tubes (Qin and Yang, 2011). Furthermore, it is suggested that the F-actin at the tips of the enlarging branches is needed to reorganize microtubules and to localize them correctly at the new tip (Sambade *et al.*, 2014). In the formation of leaf pavement cells as well as trichome development microtubules are suggested to play the most important role. Hence in leaf pavement cells the direction of cell growth is controlled through the stabilization of microtubules. In trichomes pharmacological and mutant analyses have been shown that stabilization of microtubules increased branch number of wild-type trichomes. In contrast disruption of microtubules resulted in unbranched trichomes (Mathur and Chua, 2000, David Marks, 2014, Sambade *et al.*, 2014). Interestingly the AtRLCK VI_A3 related HvRBK1 supported microtubule stability in barley epidermal cells (Huesmann and Reiner *et al.*, 2012). A similar function of AtRLCK VI_A3 on microtubules in *Arabidopsis* was not obvious (Huesmann, 2011) and would be contradicting to the findings that trichome branch number was increased in *Atrlck VI_A3* mutant plants (Reiner *et al.*, 2015b). This consideration is based on the observation that an increase in trichome branch number is associated with the stabilization of microtubules (Mathur and Chua, 2000). However, the AtRLCK VI_A3-interacting AtROP11 promotes depolymerization of cortical microtubules via the MIDD1-Atkinesin-13A pathway (Oda *et al.*, 2010, Oda and Fukuda, 2013). Similar to trichomes of *Atrlck VI_A3* mutant plant trichomes of AtKinesin-13A T-DNA insertion lines showed four instead of three trichome branches (Lu *et al.*, 2005, David Marks, 2014). Although the link between RAC/ROP signaling and trichome morphogenesis is still not understood in detail it was recently demonstrated that the activity of RAC/ROPs is able to influence trichome branching (Singh *et al.*, 2013). Application of *Clostridium difficile* toxin B, which blocks the interaction of RAC/ROPs with their effectors inhibited trichome branching (Singh *et al.*, 2013). Similar, mutations in the ROPGEF protein SPIKE1 (SKP1) prevented trichome branch initiation (Qiu *et al.*, 2002, Basu *et al.*, 2008). However, although AtRLCK VI_A3 is an effector of AtRAC/ROPs which are involved in cytoskeleton reorganization no direct influence was observed yet of AtRLCK VI_A3 on the cytoskeleton.

During their development process trichomes undergo several rounds of endoreduplication in which nuclear DNA is replicated without subsequent cell division. In several identified branching mutants an increase or reduction in trichome branch number comes along with an increase or reduction in ploidy level respectively (Schellmann and Hülskamp, 2005). The increased

trichome branch number of the *Atrlck VI_A3* mutant plants might thus point towards an altered cell cycle which leads to an enhanced ploidy level of cells. However endoreduplication is usually positively correlated with leaf size (Gegas *et al.*, 2014) and *Atrlck VI_A3* mutants showed reduced leaf rosette size in combination with enhanced trichome branching (Reiner *et al.*, 2015b).

In barley, HvRAC/ROPs modulate epidermal cell size and polarity in tip growing root hairs but little is known about their direct influence on microtubule and F-actin organization (Pathuri *et al.*, 2008, Hoefle *et al.*, 2011). Transgenic barley plants expressing CA HvRAC/ROPs showed shortening and ballooning of root hairs (Pathuri *et al.*, 2008). This is consistent with the root hair phenotype observed in *Arabidopsis* expressing CA AtRAC/ROPs (Molendijk *et al.*, 2001, Jones, 2002). In contrast transgenic barley lines containing an *HvRACB*-RNAi construct were impaired in root hair outgrowth (Hoefle *et al.*, 2011). These results indicate that the function of RAC/ROPs in polar growth processes is conserved in barley and *Arabidopsis*. In contrast to what was observed for AtRAC/ROPs in *Arabidopsis*, the influence of CA HvRAC/ROPs on cell shape formation of barley epidermal cells was less pronounced (Fu *et al.*, 2002, Fu *et al.*, 2005, Pathuri *et al.*, 2008, Fu *et al.*, 2009). Besides more irregular cell shapes of their epidermal cells, barley plants expressing CA HvRAC/ROPs showed less and abnormal developed stomata (Pathuri *et al.*, 2008, Pathuri *et al.*, 2009). In 2011, studies from Hoefle *et al*. showed for the first time that HvRAC/ROP signaling in barley is linked to microtubule organization. The identified HvMAGAP1 negatively regulates downstream signaling of HvRACB and promotes focusing of microtubules to sites of *Bgh* defense. Since HvMAGAP1 is associated at the microtubules a negative feedback from microtubules to HvRAC/ROPs to control their activity is suggested (Hoefle *et al.*, 2011, Dörmann *et al.*, 2014). Here, a function of HvRBK1 in microtubule stabilization was observed (Huesmann and Reiner *et al.*, 2012). This would be a further mechanism that counteracts the function of active HvRACB which is assumed to loosen cortical microtubules (Hückelhoven *et al.*, 2013a, Dörmann *et al.*, 2014). However it remains to be elucidated whether HvRBK1 targets microtubules through a not yet identified microtubule regulatory protein or whether it influences microtubule organization via its possible impact on HvRACB activity.

Together these findings suggest that HvRBK1 as well as AtRLCK VI_A3 link cytoskeleton reorganization to RAC/ROP signaling. While in barley a function of HvRBK1 on microtubule organization was observed, a function of AtRLCK VI_A3 on cytoskeleton organization was indirectly suggested as *Atrlck VI_A3* mutants showed aberrant phenotypes in trichome branching.

3.3. RAC/ROP-activated receptor-like cytoplasmic kinases in plant-powdery mildew interactions

RAC/ROP proteins play a major role in plant immunity (Kawano *et al.*, 2014). HvRACB as well as HvRAC1 are the best studied HvRAC/ROPs in the interaction of barley with the biotrophic barley powdery mildew fungus *Bgh* (Schultheiss *et al.*, 2002, Schultheiss *et al.*, 2003, Opalski *et al.*, 2005, Pathuri *et al.*, 2008, Hoefle *et al.*, 2011). In 2002, Schultheiss *et al*. identified HvRACB as susceptibility factor in the barley-*Bgh* interaction which is needed for successful invasion of the fungus and the establishment of fungal haustoria. HvRAC1 supports establishment of *Bgh* but it is also involved in callose deposition and H_2O_2 burst (Pathuri *et al.*, 2008). Most likely due to their functional redundancy, knowledge about AtRAC/ROPs involved in *Arabidopsis-Arabidopsis* powdery mildew interactions is rare. So far only for AtROP6 a possible function in susceptibility against the powdery mildew fungus *G. orontii* was reported (Poraty-Gavra *et al.*, 2013). In addition it was shown that CA AtROP6 localizes to the site of fungal entry (Hoefle and Hückelhoven, 2008). As effector proteins of these RAC/ROPs a function of HvRBK1 and AtRLCK VI_A3 in the respective plant-powdery mildew interaction was expected. Indeed TIGS of *HvRBK1* in barley epidermal cells rendered plants more susceptible to *Bgh* and the reproductive success of *E. cruciferarum* was increased on *Atrlck VI_A3* mutant plants (Huesmann and Reiner *et al.*, 2012, Reiner *et al.*, 2015b). A function of both RLCKs in basal resistance suggested a conserved function of RAC/ROP-activated RLCKs in plant-powdery mildew interactions. However, it was unexpected that HvRBK1 as well as AtRLCK VI_A3 were involved in basal resistance rather than susceptibility because both RLCKs interacted with RAC/ROPs that are involved in susceptibility to powdery mildew fungi (Schultheiss *et al.*, 2002, Schultheiss *et al.*, 2003, Pathuri *et al.*, 2008, Hoefle *et al.*, 2011, Huesmann and Reiner *et al.*, 2012, Poraty-Gavra *et al.*, 2013, van Schie and Takken, 2014, Reiner *et al.*, 2015b). There are several explanations for this observation which will be discussed in the following using the example of HvRACB and HvRBK1 in the barley-*Bgh* interaction. First it might be possible that a HvRBK1-involving feedback mechanism negatively regulates the function of the susceptibility factor HvRACB in the barley-*Bgh* interaction (Huesmann and Reiner *et al.*, 2012). A possible phosphorylation of HvRACB by HvRBK1 might influence its activity which would then lead to altered downstream signaling events like it is shown for HsRAC1 (Kwon *et al.*, 2000, Rehani *et al.*, 2009). In plants, a negative impact on ROPGEF-mediated activation was already observed upon phosphomimetic mutation of RAC/ROPs (Fodor-Dunai *et al.*, 2011). Absence of HvRBK1 would trigger enhanced downstream signaling of HvRACB including its effectors that are possibly involved in susceptibility. The HvRACB-interacting HvRIC171 might be such an effector as it supports like HvRACB entry of *Bgh* and accumulates at sites of fungal attack (Schultheiss *et al.*, 2008). A second explanation would be that HvRBK1 is activated by HvRAC/ROPs that are involved in basal resistance rather than susceptibility against *Bgh*. In rice for example there are OsRAC/ROPs that promote disease development of the rice blast fungus and

OsRAC/ROPs that are involved in resistance against the fungus (Chen *et al.*, 2010). Cross-talk of different RAC/ROP signaling pathways involving mutualistic inhibition is well-established in RAC/ROP-dependent development of *Arabidopsis* epidermal cells (Miyawaki and Yang, 2014). This might also be the case for barley HvRAC/ROPs. HvRAC1 which also interacted with HvRBK1 has a complex function in interaction with pathogenic fungi (Huesmann and Reiner *et al.*, 2012). It supports callose depositions and H_2O_2 accumulation at sites of fungal attack but it is not able to support basal resistance against *Bgh* (Pathuri *et al.*, 2008). However, similar to OsRAC1, CA HvRAC1 promotes basal resistance against the rice blast fungus *M. oryzae* (Pathuri *et al.*, 2008, Chen *et al.*, 2010, Kawano *et al.*, 2014). Finally, it appears possible that signaling of antagonistic HvRAC/ROP pathways converges at HvRBK1 (Huesmann and Reiner *et al.*, 2012).

Analysis of regulatory proteins that control the activity of RAC/ROPs might further contribute to the understanding of RAC/ROP-activated pathways in the plant-powdery mildew interaction. ROPGAPs for example negatively control activity of RAC/ROP proteins and their subsequent downstream signaling. Recently it was observed that *Atropgap1* and *Atropgap4* T-DNA insertion lines were more susceptible to *E. cruciferarum* (Hoefle *et al.*, 2011, Huesmann *et al.*, 2011). This would be best explained by enhanced activity of AtRAC/ROPs. Due to the fact that both AtROPGAPs broadly interacted with leaf-expressed AtRAC/ROPs in yeast it is not possible to assign a function in the *Arabidopsis*-*E. cruciferarum* interaction to a single AtRAC/ROP (Hoefle *et al.*, 2011). However both AtROPGAPs interacted with AtROP6 which in turn interacted with AtRLCK VI_A3 (Hoefle *et al.*, 2011, Reiner *et al.*, 2015b). In addition to their related function in the *Arabidopsis*-*E. cruciferarum* interaction and their similar yeast-two hybrid interaction pattern AtROPGAP1 and AtROPGAP4 were recruited to the plasma membrane upon coexpression of active AtROP6. This suggests a redundant function of AtROPGAP1 and AtROPGAP4 in *Arabidopsis* (Hoefle *et al.*, 2011, Huesmann, 2011). In barley it has been shown that HvMAGAP1 which is the most similar protein to AtROPGAP1 and AtROPGAP4 interacts with the susceptibility factor HvRACB. As negative regulator of HvRACB-activity HvMAGAP1 limits penetration success of *Bgh* in barley epidermal cells (Hoefle *et al.*, 2011). Interestingly, HvMAGAP1 is associated with microtubules and modulate their polar organization in the barley-*Bgh* interaction (Hoefle *et al.*, 2011). In cells that successfully defended fungal penetration attempts it has been shown that HvMAGAP1-labelled microtubules form a dense nest-like network around the cell wall apposition (Hoefle *et al.*, 2011). In this study, TIGS of the HvRACB-interacting *HvRBK1* resulted in the fragmentation of cortical microtubules in barley epidermal cells and increased susceptibility towards *Bgh* (Huesmann and Reiner *et al.*, 2012). This is consistent with the assumption that microtubules have a function in basal penetration resistance (Kobayashi *et al.*, 1997). One implication of these findings might be that in the barley-*Bgh* interaction active HvRACB locally loosens the microtubule cytoskeleton for better penetration

success of *Bgh* whereas HvMAGAP1 as well as HvRBK1 might act as HvRACB-antagonistic players in microtubule organization for fungal defense (Hoefle *et al.*, 2011, Huesmann and Reiner *et al.*, 2012, Hückelhoven *et al.*, 2013a). This reflects the fact that in the interaction of plants with biotrophic fungi reorganization of the microtubule cytoskeleton is important for the establishment of fungal haustoria in living plant cells (Hardham, 2013). Furthermore it is assumed that accommodation of the haustorium reflects the process of inverted cellular tip growth (Schultheiss *et al.*, 2003, Hoefle *et al.*, 2011). Accumulation of active HvRACB at the site of haustorial invasion at which microtubule density is low is similar to RAC/ROP localization in polar tip growth of plant cells such as root hairs and pollen tubes (Schultheiss *et al.*, 2008, Yang, 2008, Hoefle *et al.*, 2011). As RAC/ROP proteins are prominent regulators of the cytoskeleton network as well as of tip growth processes (Yang, 2008, Craddock *et al.*, 2012, Kawano *et al.*, 2014) they are ideal candidates to support fungal establishment in plants. In barley it is hypothesized that HvRACB might be a target for fungal effectors (Hückelhoven *et al.*, 2013b). Hence it is assumed that *Bgh* manipulates the function of HvRACB in microtubule rearrangement during in-growth into barley epidermal cells for its own purpose (Hoefle *et al.*, 2011, Hückelhoven *et al.*, 2013b). Furthermore HvRACB interferes with the actin cytoskeleton which is involved in defense mechanisms during fungal penetration (Schmelzer, 2002, Eichmann and Hückelhoven, 2008). In barley, focusing of F-actin towards sites of fungal attack is seen in cells with successful defense of *Bgh*. CA HvRACB restricts this polarization of F-actin towards sites of fungal attack whereas knock-down of HvRACB enhances actin focusing (Opalski *et al.*, 2005). Linkage of HvRACB-signaling with both the actin and microtubule cytoskeleton which are supposed to support defense and susceptibility mechanisms in the barley-*Bgh* interaction respectively is not yet understood in detail. It might be possible that besides destabilization of host microtubules fungal pathogens modulate the actin skeleton via HvRACB to facilitate their colonization success in barley. This would be similar to what is known for bacterial pathogens in humans (Radhakrishnan and Splitter, 2012). For example it is shown that destabilization of host microtubules by the bacterial pathogen *Shigella flexeneri* leads to the activation of HsRAC1. Activation of HsRAC1 involves modulation of the actin cytoskeleton and promotes the formation of membrane ruffles that facilitate internalization of the bacterium into the host cell (Yoshida *et al.*, 2002, Radhakrishnan and Splitter, 2012).

While several of the AtRLCK VI_A3-interacting AtRAC/ROPs have an effect on cytoskeleton organization (Fu *et al.*, 2005, Yang, 2008, Poraty-Gavra *et al.*, 2013) microtubule destabilization was not yet observed in *Atrlck VI_A3* mutant plants (Huesmann, 2011). Therefore microtubule reorganization is no explanation for the enhanced colonization success of *E. cruciferarum*.

Taken together both investigated RAC/ROP-interacting RLCKs seem to play a role in basal resistance against powdery mildew fungi. In barley a function of HvRBK1 in basal resistance is in accordance with its microtubule-stabilizing effect. However it remained to be elucidated why

both RLCKs are involved in basal resistance although their interacting RAC/ROPs play a role in susceptibility against powdery mildew fungi. One possible explanation comes from the molecular link of HvRBK1 with SCF-complexes (Reiner *et al.*, 2015a).

3.4. A barley SKP1-like protein interacts with the HvRAC/ROP-activated receptor-like cytoplasmic kinases HvRBK1

To further decipher downstream signaling of the HvRAC/ROP-interacting HvRBK1 a yeast-two hybrid assay in which HvRBK1 was used as bait protein was performed (Caroline Hoefle, TU München, personal communications). One HvRBK1-interacting protein was a barley type II SKP1-like protein. In this study, protein-protein interaction of HvSKP1-like and HvRBK1 was verified in yeast as well as *in planta* (Reiner *et al.*, 2015a). SKP1 proteins are part of the multisubunit SCF-E3 ubiquitin ligase complexes that catalyze the last step of the ubiquitination cascade (Vierstra, 2009). In such an SCF-complex SKP1 has a role as adaptor protein thereby linking CUL1 to an F-box protein that in turn specifically recognizes the target protein for subsequent ubiquitination and degradation by the 26S proteasome (Hua and Vierstra, 2011). According to this general SCF-complex composition the identified protein-protein interactions of HvRACB-HvRBK1 and HvRBK1-HvSKP1-like might point to HvRACB as possible target for ubiquitination (Reiner *et al.*, 2015a). In this case HvRBK1 might function as F-box protein. Characteristic for F-box proteins are their F-box domain. In addition they contain a variable recognition domain like for example leucine-rich (LRR), kelch or WD-40 repeats that bind to appropriate substrates for ubiquitination (Hua and Vierstra, 2011, Hua *et al.*, 2011). As HvRBK1 does not show any of these domains, it possibly represents a new kind of protein for recognizing SCF-targets or an additional maybe transient component of the complex. Thus, the composition of the complete HvSKP1-like containing SCF-E3 ubiquitin ligase complex remains unclear as neither the HvSKP1-like interacting F-box protein nor its interacting cullin has been identified until now. Furthermore HvSKP1-like did not interact with HvRACB in yeast (Reiner *et al.*, 2015a). This is in agreement with the fact that SKP1 proteins indirectly interact with the substrates of their SCF-complexes (Hua and Vierstra, 2011). However the mechanism of the tripartite interaction of HvRACB, HvRBK1 and HvSKP1-like is not yet understood in detail.

In transiently transformed barley epidermal cells GFP-tagged HvSKP1-like co-localized with mcherry-tagged HvRBK1 in the nucleoplasm and cytoplasm (Reiner *et al.*, 2015a). This is in agreement with the observed subcellular localization of selected type I ASKs in *Arabidopsis* epidermal cells (Dezfulian *et al.*, 2012). In addition most ubiquitinated proteins are subjected to the 26S proteasome for their breakdown. This protease complex is also present in both the cytoplasm and the nucleus (Vierstra, 2009).

To sum up, HvSKP1-like a presumable component of an SCF-E3 ubiquitin ligase complex was identified as interacting protein of the HvRAC/ROP-activated HvRBK1. HvSKP1-like and

HvRBK1 co-localized in the cytoplasm and nucleoplasm and their protein-protein interaction was confirmed in yeast and *in planta*.

3.5. Ubiquitination as potential additional regulatory mechanism of HvRACB

In the animal research field ubiquitin-mediated proteasomal degradation of RAC/ROP GTPases is discussed as additional regulatory mechanism for RAC/ROP activity and their subsequent signaling output (Nethe and Hordijk, 2010, Visvikis *et al.*, 2010). The finding that the HvRACB-interacting HvRBK1 in turn interacted with the SCF-E3 ubiquitin ligase subunit HvSKP1-like provides a possible link between HvRAC/ROP signaling and ubiquitin-dependent proteasomal degradation (Reiner *et al.*, 2015a).

Fluorescence intensity of mcherry-CA HvRACB in barley protoplast increased upon treatment with the proteasome inhibitor MG132. This supported the assumption that HvRACB is targeted for proteasomal degradation. In addition, silencing of either *HvRBK1* or *HvSKP1-like* in barley epidermal cells increased fluorescence intensity of CFP-CA HvRACB. Similar, protein abundance of GFP-CA HvRACBΔCSIL was increased in barley protoplasts when *HvRBK1* was silenced (Reiner *et al.*, 2015a). GFP-CA HvRACBΔCSIL lacks the C-terminal motif CSIL which is responsible for membrane association of HvRACB. Because this truncated form of CA HvRACB is localized in the cytoplasm (Schultheiss *et al.*, 2003) it was used to extract a sufficient amount of detectable GFP-CA HvRACB from barley protoplasts. However alterations in protein abundance of GFP-CA HvRACBΔCSIL in barley protoplasts when *HvSKP1-like* was silenced were inconsistent (data not shown) and did not reflect increased fluorescence intensity of CFP-CA HvRACB in epidermal cells when *HvSKP1-like*-RNAi was co-transformed (Reiner *et al.*, 2015a). A possible reason for this might be the different plant tissues in which CA HvRACB abundance was investigated and the different time-points at which protein abundance was analyzed. It might be possible that in the short living and fragile mesophyll protoplasts time was too short to silence *HvSKP1-like* efficiently and therefore no effect on fluorescence intensity of GFP-HvRACBΔCSIL was observed (Reiner *et al.*, 2015a). Furthermore it might be possible that in mesophyll protoplasts several additional barley SKP1 proteins exist which are absent from epidermal cells and functionally compensate silencing of *HvSKP1-like*. In *Arabidopsis* it is shown that although each type of F-box protein has a preference for certain ASKs different ASKs are able to interact with the same F-box protein thereby allowing the combinatorial diversity of SCF-complexes (Dezfulian *et al.*, 2012, Kuroda *et al.*, 2012).

Nevertheless data indicated that HvRBK1 as well as HvSKP1-like are involved in a possible degradation mechanism of CA HvRACB (Reiner *et al.*, 2015a). HsRAC1 which is more than 55% identical to HvRACB is targeted by different E3 ubiquitin ligases for degradation (Schultheiss *et al.*, 2002, Torrino *et al.*, 2011, Zhao *et al.*, 2013). The SCF-E3 ubiquitin ligase SCF^{FBXL19} mediates ubiquitination and degradation of active and inactive HsRAC1. Interestingly phosphorylation of HsRAC1 by AKT kinase is essential for the association of the F-box protein

FBXL19 with HsRAC1 (Zhao *et al.*, 2013). Phosphorylation of the target protein is often observed prior to its recognition by F-box proteins (Gagne *et al.*, 2002, Petroski and Deshaies, 2005, Skaar *et al.*, 2013). Therefore it is hypothesized that HvRBK1 phosphorylates HvRACB prior to its degradation in order to allow recognition of HvRACB by a so far unknown F-box protein (Reiner *et al.*, 2015a). Although preliminary data suggest that HvRACB gets phosphorylated by HvRBK1 (Attila Fehér, Biological research centre, Szeged, Hungary, personal communication), phosphorylation of HvRACB by HvRBK1 needs to be verified. Protein sequence comparison of HsRAC1 and HvRACB showed that the lysine residue which is needed for ubiquitination as well as the AKT phosphorylation site of HsRAC1 are conserved in HvRACB (Zhao *et al.*, 2013). This further strengthens the assumption that HvRACB is a candidate for ubiquitination. The active form of HsRAC1 is subjected for proteasomal degradation by two additional E3 ubiquitin ligases called HACE1 and inhibitors of apoptosis (IAPs) (Torrino *et al.*, 2011, Zhao *et al.*, 2013). Due to the observation that CA HvRACB but not DN HvRACB interacted with HvRBK1 which in turn interacts with HvSKP1-like it is suggested that preferably the active form of HvRACB is subjected to ubiquitin-dependent proteasomal degradation. However we cannot exclude that HvRBK1 together with HvSKP1-like also targets the inactive form of HvRACB for proteasomal degradation as fluorescence intensity of CFP-DN HvRACB was also slightly increased upon either silencing of *HvRBK1* or *HvSKP1-like* in barley epidermal cells (Reiner *et al.*, 2015a).

In summary data suggest that abundance of CA HvRACB is negatively regulated via an HvRBK1- and HvSKP1-like involving pathway possibly linking the ubiquitination pathway to RAC/ROP signaling. However so far, a direct proof that CA HvRACB is ubiquitinated is missing.

3.6. Negative regulation of CA HvRACB abundance via HvRBK1 and HvSKP1-like explains opposite functions of HvRACB and its interacting HvRBK1 in the barley-*Bgh* interaction

During the last years it became evident that E3 ubiquitin ligases are involved in nearly all layers of plant immunity. It turned out that the function of a variety of proteins involved in immune signaling is regulated by ubiquitin-mediated proteolysis (Zeng *et al.*, 2006, Dreher and Callis, 2007, Delauré *et al.*, 2008, Dielen *et al.*, 2010, Marino *et al.*, 2012, Duplan and Rivas, 2014). SKP1 proteins participate in the formation of SCF E3 ubiquitin ligase complexes but little is known about their individual function (Vierstra, 2009, Dezfulian *et al.*, 2012). As HvSKP1-like interacts with HvRBK1 a possible function of HvSKP1-like in the barley-*Bgh* interaction was assumed (Reiner *et al.*, 2015a). Transcript abundance of *HvSKP1-like* in barley plants slightly increased 12 hours after inoculation with *Bgh*. This result might indicate that *HvSKP1-like* is transcriptionally regulated when the fungus forms its appressorium. So far only for the pepper *SKP1* (*CaSKP1*) gene transcriptional regulation in response to incompatible pathogen challenge or salicylic acid treatment was reported (Chung *et al.*, 2006).

Furthermore TIGS of *HvSKP1-like* in barley epidermal cells resulted in enhanced fungal penetration success (Reiner *et al.*, 2015a). Since TIGS of the HvSKP1-like-interacting protein *HvRBK1* in barley epidermal cells supported fungal growth this result is in accordance with similar functions of HvRBK1 and HvSKP1-like (Huesmann and Reiner, *et al.*, 2012, Reiner *et al.*, 2015a).

Previously, a feedback mechanism which negatively regulates HvRACB signaling was discussed in order to explain the opposite functions of HvRACB and HvRBK1 in the barley-*Bgh* interaction (Huesmann and Reiner, *et al.* 2012, Dörmann *et al,* 2014). The observed regulation of HvRACB abundance via a mechanism involving HvRBK1 and HvSKP1-like would represent such a negative feedback mechanism (Reiner *et al.*, 2015a). Silencing of *HvRBK1* or *HvSKP1-like* would then hinder proteasomal degradation of CA HvRACB and result in the accumulation of the susceptibility factor. This in turn would possibly support downstream signaling of HvRACB, hence leading to increased colonization success of *Bgh*. Controlling signaling output of active HvRACB in the barley-*Bgh* interaction via proteasomal degradation of HvRACB would describe an additional regulatory mechanism to its negative regulation by HvMAGAP1 (Hoefle *et al.*, 2011). In animals, functional regulation of RHOs or their regulatory proteins via the ubiquitination pathway is a well described mechanism (Nethe and Hordijk, 2010, Visvikis *et al.*, 2010). The recent observation that the E3 ubiquitin ligase SPL11 degrades the ROPGAP SPL11-interacting protein 6 (SPIN6) that negatively modulates OsRAC1-mediated immune signaling in rice showed for the first time that this also occurs in plants (Liu *et al.*, 2015).

Taken together, similar to HvRBK1, HvSKP1-like has a function in basal resistance against *Bgh*. The assumption that HvSKP1-like controls in concert with HvRBK1 abundance of the susceptibility factor HvRACB might explain the contradicting functions that HvRACB acts in susceptibility whereas HvRBK1 and HvSKP1-like are involved in resistance against *Bgh*. However it cannot be excluded that susceptibility factors other than HvRACB are targeted by this complex.

3.7. Hypothetical models of RAC/ROP signaling in plant-powdery mildew interactions

During the last years quite a few studies contributed to the understanding of RAC/ROP signaling in plant-powdery mildew interactions. Based on the most recent findings and the results from this study the following working models should highlight similarities and differences in RAC/ROP signaling of barley and *Arabidopsis* in the interaction with their respective powdery mildew fungi.

First a model of HvRACB-signaling in the interaction of barley with the barley powdery mildew fungus *Bgh* was discussed. This model summarizes the current knowledge of HvRACB which supports fungal accommodation and is involved in cytoskeleton rearrangement (Fig.3). Upon fungal contact HvRACB gets activated in a not yet understood manner. Active HvRACB is supposed to loosen microtubules thereby promoting fungal accommodation (Schultheiss *et al.*, 2002, Schultheiss *et al.*, 2003, Hückelhoven *et al.*, 2013a). Furthermore it was observed that active HvRACB, which might represent a fungal effector target, limits polar F-actin reorganization when under attack from *Bgh* (Opalski *et al.*, 2005, Hückelhoven *et al.*, 2013b). HvRIC171, an effector of active HvRACB supports fungal accommodation by a so far not understood mechanism (Schultheiss *et al.*, 2008). By contrast, HvRBK1 which also interacts with the active form of HvRACB restricts colonization success of *Bgh* through direct or indirect stabilization of microtubules (Huesmann and Reiner *et al.*, 2012). The identified HvRBK1-interacting HvSKP1-like protein is likewise involved in basal resistance against *Bgh*. In an HvRBK1-involving pathway HvSKP1-like controls abundance of active HvRACB. This negative feedback might occur through ubiquitin-dependent proteasomal degradation of active HvRACB after its interaction with and/or phosphorylation by HvRBK1 (Reiner *et al.*, 2015a). Another negative regulator of active HvRACB is HvMAGAP1 (Hoefle *et al.*, 2011). HvMAGAP1 promotes polar organization of microtubules at sites of fungal defense and limits susceptibility to fungal penetration. Function of HvMAGAP1 in turn is possibly fine-tuned by HvELMOD_C (Hoefle and Hückelhoven, 2014).

HvRAC1 which also interacted and activates HvRBK1 was excluded from this model because its function in susceptibility or basal resistance to *Bgh* is not well understood and its influence on CA HvRACB abundance was not investigated.

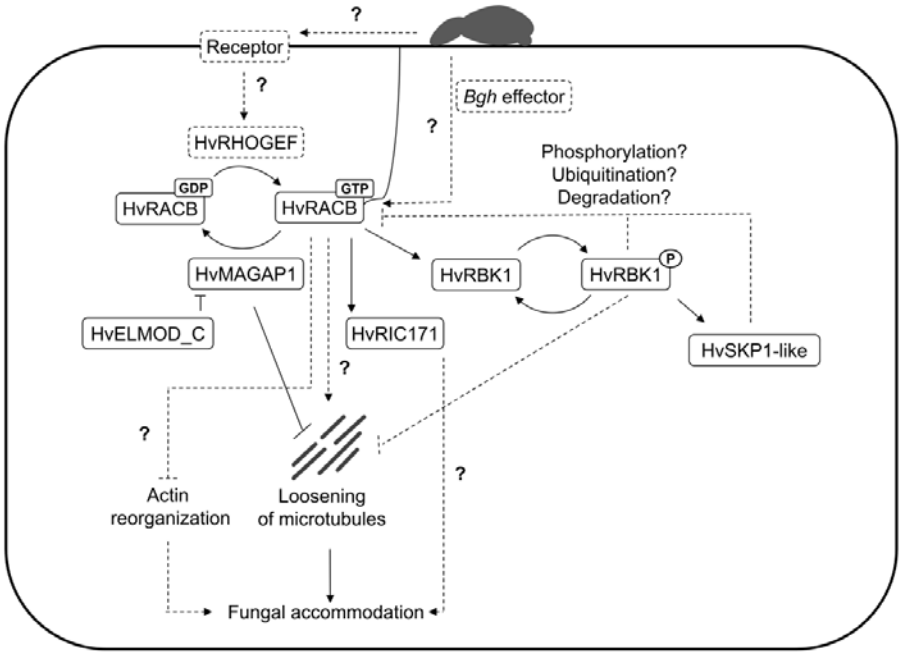

Fig 3. Proposed working model of HvRACB-signaling in the barley-*Bgh* interaction
The powdery mildew fungus *Bgh* is recognized by a so far unknown receptor which activates a not yet identified HvROPGEF. This HvROPGEF in turn mediates conversion of the inactive GDP-bound HvRACB into its active GTP-bound form. The active HvRACB which might be a *Bgh* effector target, is assumed to loosen the microtubule network thereby enabling fungal accommodation. In addition, CA HvRACB limits polar F-actin reorganization during fungal attack. HvRIC171 which interacts with active HvRACB supports fungal accommodation in a not yet understood manner. HvRBK1 which also interacts with the active form of HvRACB stabilizes microtubules in order to prevent fungal colonization success. Together with its interacting HvSKP1-like protein, HvRBK1 is assumed to negatively control abundance of active HvRACB by post-translational modifications. Fine-tuning of HvRACB-signaling via HvRBK1- and HvSKP1-like involving ubiquitin-dependent proteasomal degradation of active HvRACB would represent an additional mechanism to control signaling output of active HvRACB. HvMAGAP1 is a negative regulator of active HvRACB and counteracts the function of active HvRACB. Function of HvMAGAP1 in turn is fine-tuned by HvELMOD_C

In the interaction of *Arabidopsis* and *Arabidopsis*-powdery mildew fungi knowledge about AtRAC/ROP signaling is limited (Fig.4). Besides having an influence on cytoskeleton reorganization, AtROP6 is suggested to play a role in susceptibility of *Arabidopsis* to powdery mildew (Poraty-Gavra *et al.*, 2013). AtROP6 interacts and activates AtRLCK VI_A3 which is most similar to HvRBK1 in barley (Reiner *et al.*, 2015b). Like HvRBK1, AtRLCK VI_A3 is involved in basal resistance and restricts proliferation of *E. cruciferarum*. Unlike what was reported for HvRBK1, AtRLCK VI_A3 has no proven influence on microtubule stability. However a role of AtRLCK VI_A3 in trichome morphogenesis was observed. Cytoskeleton reorganization might provide a mechanistic link between trichome morphogenesis and fungal colonization. AtROPGAP1 and AtROPGAP4 which are most similar to HvMAGAP1 act as negative regulators of active AtROP6 and restrict fungal accommodation (Hoefle *et al.*, 2011, Huesmann *et al.*, 2011). In comparison to HvRACB-signaling in the barley-*Bgh* interaction, AtRAC/ROP-signaling in the *Arabidopsis-E. cruciferarum* interaction is less well understood. Downstream interacting

partners of AtRLCK VI_A3 are so far unknown and therefore, one can only speculate on an AtROP6-antagonizing function of AtRLCK VI_A3 in the *Arabidposis-E. cruciferarum* interaction.

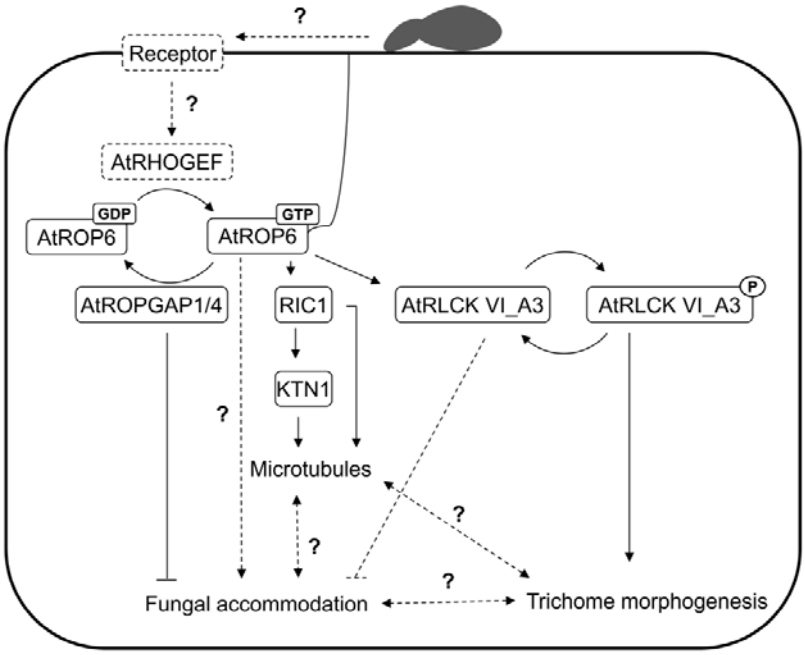

Fig 4. Proposed working model of AtROP6-signaling in the *Arabidopsis*-powdery mildew interaction
Active AtROP6 is supposed to support establishment of *Arabidopsis* powdery mildew fungi and has an influence on microtubule organization. AtROP6 also interacts with AtRLCK VI_A3 which influences trichome branching and restricts fungal colonization success. As fungal invasion as well as trichome branching is linked to cytoskeleton rearrangement there might be a link between both observations. AtROPGAP1 and AtROPGAP4 are negative regulators of AtRAC/ROP activity and restrict fungal reproductive success.

Taken together RAC/ROP-signaling in plant-powdery mildew interactions is partially conserved. RLCKs of the subfamily VI_A3 are likely RAC/ROP-effectors in both *Arabidopsis* and barley. Their function in resistance or limiting susceptibility against powdery mildew fungi is conserved but the respective associated mechanisms seem to be different in *Arabidopsis* and barley. While in barley microtubule organization could be directly linked to pathogen invasion, involvement of the cytoskeleton in fungal establishment in *Arabidopsis* is indirectly suggested. For that reason it would be helpful to identify possible downstream interacting partner of AtRLCK VI_A3 to better understand its mode of action during fungal colonization. In barley it would be challenging to verify possible post-translational modifications of HvRACB. Verification of proposed ubiquitination and degradation of HvRACB would add a second level to HvRAC/ROP-regulation in barley and would contribute to further understanding of HvRAC/ROP signaling towards susceptibility of *Bgh*.

However for HvRBK1 as well as AtRLCK VI_A3, it would be interesting to further reveal their function in the complex interlinked RAC/ROP signaling network. Due to the observation that both RLCKs interacted with several RAC/ROP proteins they might represent an interesting branching point at which several possibly antagonistic RAC/ROP signaling pathways converge.

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5. Appendix

5.1. Huesmann and Reiner *et al.*, 2012

Barley ROP Binding Kinase1 Is Involved in Microtubule Organization and in Basal Penetration Resistance to the Barley Powdery Mildew Fungus[1][C][W]

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Certain plant receptor-like cytoplasmic kinases were reported to interact with small monomeric G-proteins of the RHO of plant (ROP; also called RAC) family in planta and to be activated by this interaction in vitro. We identified a barley (*Hordeum vulgare*) partial cDNA of a ROP binding protein kinase (HvRBK1) in yeast (*Saccharomyces cerevisiae*) two-hybrid screenings with barley HvROP bait proteins. Protein interaction of the constitutively activated (CA) barley HvROPs CA HvRACB and CA HvRAC1 with full-length HvRBK1 was verified in yeast and in planta. Green fluorescent protein-tagged HvRBK1 appears in the cytoplasm and nucleoplasm, but CA HvRACB or CA HvRAC1 can recruit green fluorescent protein-HvRBK1 to the cell periphery. Barley HvRBK1 is an active kinase in vitro, and activity is enhanced by CA HvRACB or GTP-loaded HvRAC1. Hence, HvRBK1 might act downstream of active HvROPs. Transient-induced gene silencing of barley HvRBK1 supported penetration by the parasitic fungus *Blumeria graminis* f. sp. *hordei*, suggesting a function of the protein in basal disease resistance. Transient knockdown of HvRBK1 also influenced the stability of cortical microtubules in barley epidermal cells. Hence, HvRBK1 might function in basal resistance to powdery mildew by influencing microtubule organization.

ROP (RHO, RAT SARCOMA HOMOLOG, of plants, also called RAC, RAT SARCOMA-related C3 botulinum toxin substrate) small GTP-binding proteins are signal transduction proteins that are considered as molecular switches for signaling toward cell development, hormone responses, and the cytoskeleton (Nibau et al., 2006). Additionally, ROPs are involved in resistance and susceptibility to plant diseases (Chen et al., 2010a, 2010b; Trusov et al., 2010). ROPs exist in a GTP-bound active form for targeting of downstream effectors and in a GDP-bound inactive form. The C terminus of ROPs is posttranslationally lipid modified for membrane association. Depending on their lipid modification motifs, ROPs are categorized in two phylogenetic subgroups: type I ROP proteins possess a CAAX prenylation motif, whereas those of type II lack a typical CAAX box but are palmitoylated. Activated type I ROPs can be further acylated at a conserved Cys residue, and these lipid modifications influence localization and function of ROPs (Sorek et al., 2007, 2011; Berken and Wittinghofer, 2008). In metazoans, RHO proteins physically interact with a plethora of proteins that regulate many processes in a cell type-specific manner (Bustelo et al., 2007). In plants, several proteins have been identified that bind to and signal upstream or downstream from ROPs (Berken and Wittinghofer, 2008; Yalovsky et al., 2008; Mucha et al., 2011). However, our knowledge of protein interaction partners of ROPs and their physiological relevance is still incomplete. In metazoans, RHO signaling involves upstream and downstream protein kinases (Denhardt, 1996). Similarly, plant ROPs can be activated via receptor-like kinases that activate plant-specific ROP nucleotide exchangers for exchange of ROP-bound GDP for GTP (Zhang and McCormick, 2007; Löcke et al., 2010; Humphries et al., 2011). Active ROP-GTP interacts with downstream factors (also called ROP effectors) to trigger cellular responses. ROP-GTP binds putative downstream protein kinases in yeast (*Saccharomyces cerevisiae*) and in planta. One type of a protein kinase that interacts with active ROPs is a Cys-rich receptor kinase, named AtNCRK (Molendijk et al., 2008). A second type of Arabidopsis (*Arabidopsis thaliana*) ROP binding kinases (AtRBKs) is otherwise known as receptor-like cytoplasmic kinase of the VIA subfamily (Arabidopsis AtRLCK VIA) or ROP-interacting receptor-like kinases (*Medicago truncatula* MtRRKs; Molendijk et al., 2008; Dorjgotov et al.,

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2009). These RLCKs are activated in vitro by ROPs (Dorjgotov et al., 2009).

Plant receptor-like kinases and downstream kinases are key to plant immunity (Tena et al., 2011). However, the picture of plant kinases involved in pathogen signal transduction or in modulation of the immune response is complex, and no function has yet been described for ROP binding kinases. However, transcripts of Arabidopsis AtRLCK VIAs accumulate in response to stress and hormones and, in the case of AtRBK1, in response to the pathogens *Botrytis cinerea* and *Phytopthora infestans* (Jurca et al., 2008; Molendijk et al., 2008).

The barley (*Hordeum vulgare*) ROP protein HvRACB is required for full susceptibility to powdery mildew disease caused by the biotrophic fungal pathogen *Blumeria graminis* f. sp. *hordei* (Schultheiss et al., 2002; Hoefle et al., 2011). Additionally, the barley ROPs HvRACB, HvRAC3, and HvRAC1 have the potential to support susceptibility to fungal penetration when expressed as constitutively activated (CA) mutant variants (Schultheiss et al., 2003; Pathuri et al., 2008). Barley CA HvRAC1, however, also supports callose deposition as well as the oxidative burst and the hypersensitive reaction in those cells of barley, which successfully defend penetration by *B. graminis* f. sp. *hordei* (Pathuri et al., 2008). This is similar to the function of rice (*Oryza sativa*) OsRAC1 in resistance to *Magnaporthe grisea* (Ono et al., 2001; Chen et al., 2010b). HvRACB further influences polar growth processes in barley leaf epidermis and root hairs (Pathuri et al., 2008; Hoefle et al., 2011). HvRACB regulates polarity of filamentous F-actin in barley epidermal cells during interaction with *B. graminis* f. sp. *hordei* and modulates the number of cells to which fungal infection structures, haustoria, get access (Schultheiss et al., 2002; Opalski et al., 2005). Barley HvRIC171 interacts with HvRACB in yeast and in planta (Schultheiss et al., 2008). HvRIC171 is a member of the so-called ROP-interactive CRIB (Cdc42/Rac-interactive binding) motif-containing protein family (RIC). RICs contain the conserved RHO-binding CRIB motif, which is also found in nonplant RHO effectors. Otherwise, RICs are structurally diverse (Wu et al., 2001). Barley HvRIC171 is located at the cell periphery where it further accumulates upon coexpression of CA HvRACB and upon attack from *B. graminis* f. sp. *hordei*. This may indicate activation of HvROPs and recruitment of downstream effectors at the site of fungal attack in barley (Schultheiss et al., 2008; Hückelhoven and Panstruga, 2011). Accordingly, HvRIC171 enhances susceptibility to haustorium invasion when overexpressed (Schultheiss et al., 2008). Another barley HvRACB-binding protein is HvMAGAP1, a microtubule (MT)-associated ROP GTPase-activating protein. ROPGAPs inactivate ROPs by the stimulation of GTP hydrolysis (Wu et al., 2000). HvMAGAP1 antagonizes HvRACB in susceptibility to penetration by *B. graminis* f. sp. *hordei* and supports polar organization of cortical MTs during fungal attack (Hoefle et al., 2011). Similarly, Arabidopsis AtROPGAP1 and AtROPGAP4 limit susceptibility to the powdery mildew fungus *Erysiphe cruciferarum* (Hoefle et al., 2011; Huesmann et al., 2011). However, little is known about other ROP-binding proteins of barley that are potentially involved in signaling toward pathophysiological or cell developmental responses.

Here, we describe identification of HvRBK1, a potential ROP effector kinase from barley. CA HvRACB and HvRAC1-GTP enhance kinase activity of HvRBK1 in vitro. CA HvRACB and CA HvRAC1 interact with HvRBK1 in planta. Transient knockdown of HvRBK1 destabilizes MTs and enhances susceptibility to penetration by *B. graminis* f. sp. *hordei*. Data suggest a function of barley ROP binding kinase1 in basal resistance to powdery mildew.

RESULTS

Identification and Characterization of Barley HvRBK1

To identify potential ROP binding proteins of barley, we carried out yeast two-hybrid (YTH) screenings of a cDNA library from a pool of near-isogenic barley lines resistant or susceptible to powdery mildew infected with *B. graminis* f. sp. *hordei*. We used barley HvRACB, CA HvRACB, and CA HvRAC1 as bait proteins (for more details, see Hoefle et al., 2011). One partial cDNA was repeatedly fished with all three ROP baits out of the cDNA library. A corresponding full-length cDNA was generated by 5′ RACE. We identified a matching genomic contig sequence of 7,372 bp in the draft sequence of the barley genome (contig_6719 at http://webblast.ipk-gatersleben.de/barley/index.php) and generated a corresponding gene model. The gene model identified a corresponding TATA box and was consistent with the full-length character of our cDNA. The gene is on the long arm of barley chromosome 7H and contains seven introns and eight exons (Supplemental Fig. S1A).

The cDNA encodes a protein of 543 amino acids with similarity to ROP binding kinases of Arabidopsis (AtRLCK VIA2, AtRLCK VIA3, AtRLCK VIA-4 [AtRBK1], and AtRLCK VIA6 [AtRBK2]), and *M. truncatula* (MtRRK1 and MtRRK2). Therefore, we named the predicted protein barley ROP binding kinase1 (HvRBK1; Supplemental Fig. S1B).

Among similar kinases, a rice protein annotated as OsPERK1 was the most similar protein found in monocots (Supplemental Fig. S1B, Fig. 1). Barley EST libraries and the draft genome contain HvRBK1-similar sequences, hinting at a possible small gene family of barley ROP binding kinases similar to the AtRLCK VIA family in Arabidopsis. Among the Arabidopsis kinases, AtRLCK VIA3 was most similar to the barley HvRBK1 (55% identical between amino acids 149 and 542) followed by AtRLCK VIA4 (synonymous to AtRBK1, 52% identical between amino acids 142 and 542; Supplemental Fig. S1B). Arabi-

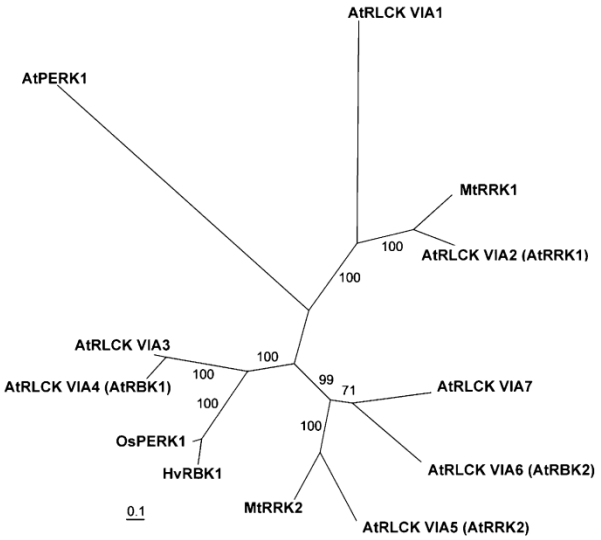

Figure 1. Unrooted phylogenetic tree (best scoring tree after the bootstraps) of predicted RLCKs of the Arabidopsis RLCKVIA family (At5G57670 = AtRLCK VIA1, At2G188900 = AtRLCK VIA2, At5G65530 = AtRLCK VIA3, At5G10520 = AtRLCK VIA4, At5G35960 = AtRLCK VIA5, At3G05140 = AtRLCK VIA6, At5G18910 = AtRLCK VIA7), *M. truncatula* MtRRKs (MtRRK1, MtRRK2), barley HvRBK1, rice OsPERK1, and the predicted transmembrane receptor-like kinase AtPERK1 (AT3G24550) taken as an outgroup. Numbers indicate support values after bootstraps.

dopsis *AtRLCK VIA3* (At5g65530) and *AtRLCK VIA4* (*AtRBK1*, AT5G10520) genes also have an *HvRBK1*-like gene structure with seven introns and eight exons, which differs from that of the other *RLCK VIA* genes. We carried out a phylogenetic analysis based on protein sequences using protein alignments created by custalw2 (Thompson et al., 1994) and analyzed by RaXML program (Stamatakis et al., 2008). This further supported that HvRBK1 might be the ortholog of AtRLCK VIA3 or of its paralog AtRBK1 (Fig. 1). *Bos taurus* interleukin-1 receptor-associated kinase4 is the most similar kinase from metazoan species that is represented in GenBank.

The kinase domain of plant ROP binding kinases is highly conserved in mono- and dicots. It is positioned at the C-terminal part of the protein. By contrast, the N terminus of HvRBK1 is not strongly conserved in rice OsPERK1 and highly dissimilar to that of the related Arabidopsis ROP binding kinases AtRLCK VIA3 or AtRBK1 (Supplemental Fig. S1B). The N terminus of these ROP binding kinases is enriched with Ser residues in all three species. In HvRBK1, however, this is less pronounced when compared with the other three RLCKs from rice and Arabidopsis. The predicted open reading frames of Arabidopsis AtRBK1 and AtRLCK VIA3 code proteins shorter than barley HvRBK1, which possesses an N-terminal extension (Supplemental Fig. S1B).

In independent YTH assays, the interaction of the full-length HvRBK1 with barley HvROPs was confirmed and auto-activation was excluded (Fig. 2). Further YTH assays with the partial HvRBK1 from the original screening showed that amino acids 210 to 543 of the barley HvRBK1 C terminus were sufficient for the interaction (not shown). Hence, the kinase domain of HvRBK1 may directly interact with barley ROPs. In all YTH assays, HvRBK1 preferentially interacted with CA HvROPs when compared with corresponding dominant negative (DN) HvROPs (Fig. 2, and see below).

Together, barley HvRBK1 is similar to Arabidopsis ROP binding AtRLCK VIAs in sequence and in the capacity to bind ROPs in the YTH assay.

Activated Barley HvRACB and HvRAC1 Increase the Activity of HvRBK1 in Vitro

It was previously described that the in vitro activity of Arabidopsis and *M. truncatula* kinases belonging to the RLCK class VIA is dependent on the presence of active ROPs (Dorjgotov et al., 2009). Therefore, we tested whether the barley RLCK VIA-like kinase HvRBK1 has similar biochemical properties. We investigated the ability of the recombinant HvRBK1 protein to phosphorylate the myelin basic protein in vitro in the presence and absence of HvROP GTPases (HvRACB and HvRAC1, respectively) in various conformations or concentrations (Fig. 3). Activity of HvRBK1 was considerably increased in the presence of the CA HvRACB mutant in a concentration-dependent way. Under the same conditions, the wild-type or DN forms of HvRACB had no significant effect on the kinase activity (Fig. 3A). The effect of GTP-loaded HvRAC1 on the in vitro activity of HvRBK1 was also tested, and a ROP concentration-dependent increase in activity was evident in these assays (Fig. 3B).

HvROPs Recruit HvRBK1 to the Cell Periphery for Protein-Protein Interaction

To see where HvRBK1 might interact with barley HvROPs in the cell, we fused the GFP to the N terminus of the protein. Confocal laser scanning microscopy (CLSM) localized GFP-HvRBK1 in the cytoplasm and nucleoplasm when transiently expressed in barley epidermal cells after biolistic transformation (Fig. 4A). The subcellular localization of GFP-HvRBK1 changed upon coexpression of the CA HvRACB or CA HvRAC1 but not upon coexpression of DN HvRACB taken as a control that does not bind HvRBK1 (Figs. 2 and 4A). Both barley CA HvROPs led to the accumulation of GFP-HvRBK1 at the cell periphery. Translocation to the cell periphery was partial when CA HvRACB was coexpressed and complete when CA HvRAC1 was coexpressed (Fig. 4A, Supplemental Fig. S2). Quantitative measurement of fluorescence intensities of GFP-HvRBK1 supported that CA HvRACB and CA HvRAC1 but not DN versions recruit HvRBK1 to the cell periphery (Figs. 4, B and C). Strength of translocation largely reflected the subcellular localization of the

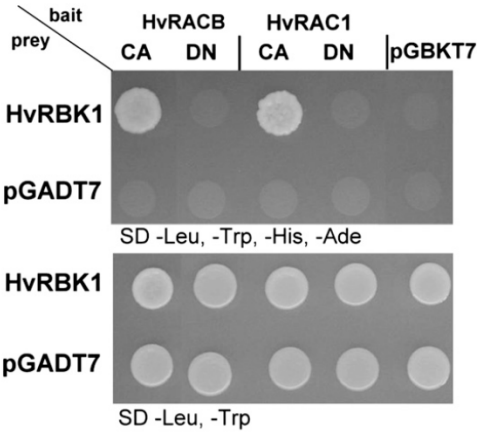

Figure 2. Targeted YTH assay of HvRBK1 and barley HvROPs in yeast. Targeted YTH assay in yeast strain AH109 cotransformed with *HvRBK1* in the pGADT7 prey vector and barley *HvROP*s in pGBKT7 bait vector. Bait and prey interaction allows growth on selective synthetic dextrose media without −Leu, −Trp, −His, and adenine (−Ade). No growth was observed on selective media when yeast was cotransformed with expression constructs of *HvROP*s and the empty vector pGADT7 or with *HvRBK1* and empty vector pGBKT7. As control for successful cotransformation, yeast cells were dropped on selective synthetic dextrose media −Leu, −Trp (bottom picture).

respective CA HvROP proteins at the cell periphery as indicated by imaging GFP-CA HvRACB or GFP-CA HvRAC1, respectively (Supplemental Fig. S3; see also Schultheiss et al., 2003).

The measurement of fluorescence resonance energy transfer (FRET) further supported interaction of HvRBK1 and HvRACB in planta. We generated fusion protein constructs with cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) for acceptor photobleaching. We coexpressed HvRBK1-YFP with either CFP-CA HvRACB or CFP-DN HvRACB and bleached the acceptor HvRBK1-YFP at the periclinal cell periphery facing the surface of the leaf epidermis. This led to significant fluorescent enhancement of CFP-CA HvRACB but not of CFP-DN HvRACB used as donors (Table I). This corroborated results from the YTH and the in planta recruitment assays. Activated HvRACB may thus recruit HvRBK1 by direct protein interaction at the periphery of barley epidermal cells.

Barley HvRBK1 Is Expressed in Pathogen Response

To test whether barley *HvRBK1* is expressed in response to *B. graminis* f. sp. *hordei*, we extracted total RNA from barley first leaves densely inoculated with conidia of *B. graminis* f. sp. *hordei*. Reverse transcription-PCR showed that transcripts of *HvRBK1* slightly accumulated from 8 h after inoculation with *B. graminis* f. sp. *hordei* onward when compared with non-inoculated controls from the same batch of plants (Supplemental Fig. S4). These data were confirmed by checking pathogen-responsive expression of *HvRBK1* in publically available transcriptome data at the database PLEXDB (Wise et al., 2007). We identified the Genechip probe set contig14061_at as the one that represents the sequence of *HvRBK1*. Expression profiles of the microarray dataset BB10, which contains transcription profiling of barley plants differing at *MLA1* and *MLA6* powdery mildew resistance loci (Meng et al., 2009), revealed a slightly increased expression of *HvRBK1* in all investigated plant genotypes inoculated with powdery mildew, from 8 h after inoculation onward. According to that, expression of *HvRBK1* increases when barley is challenged by both virulent and avirulent *B. graminis* f. sp. *hordei*.

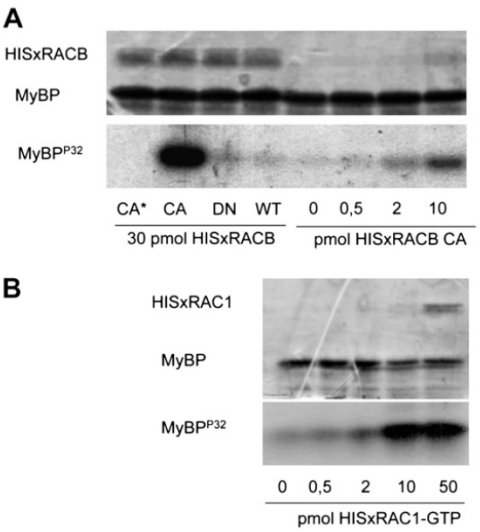

Figure 3. Active HvRACB/HvRAC1 GTPases increase the activity of HvRBK1 in vitro. The in vitro myelin basic protein (MyBP) phosphorylating activity of HvRBK1 is shown in the presence of purified HIS-tagged barley HvRACB (A) and HvRAC1 (B) GTP-binding proteins. A, HISxHvRACB was added to the kinase reaction in CA, DN, and wild-type (WT) forms (30 pmol each). As a negative control (CA*), the same amount of the CA HvRACB GTPase was also added to a reaction mixture not containing the kinase protein (CA*). Moreover, kinase reactions were carried out in the presence of various CA HvRACB GTPase amounts (0–10 pmol). Protein loading is shown by Coomassie Brilliant Blue staining of the proteins in the kinase reaction after their separation in a polyacrylamide gel (top image), whereas kinase activity is demonstrated by the autoradiographic detection of radioactive MyBPP32 in the same gel (bottom image). B, HISxHvRAC1 was added to the kinase reaction at the indicated concentrations after loading the wild-type protein with GTP. The top image shows the result of the CBB staining and the bottom one the autoradiography of the gel used for the separation of proteins in the kinase reactions.

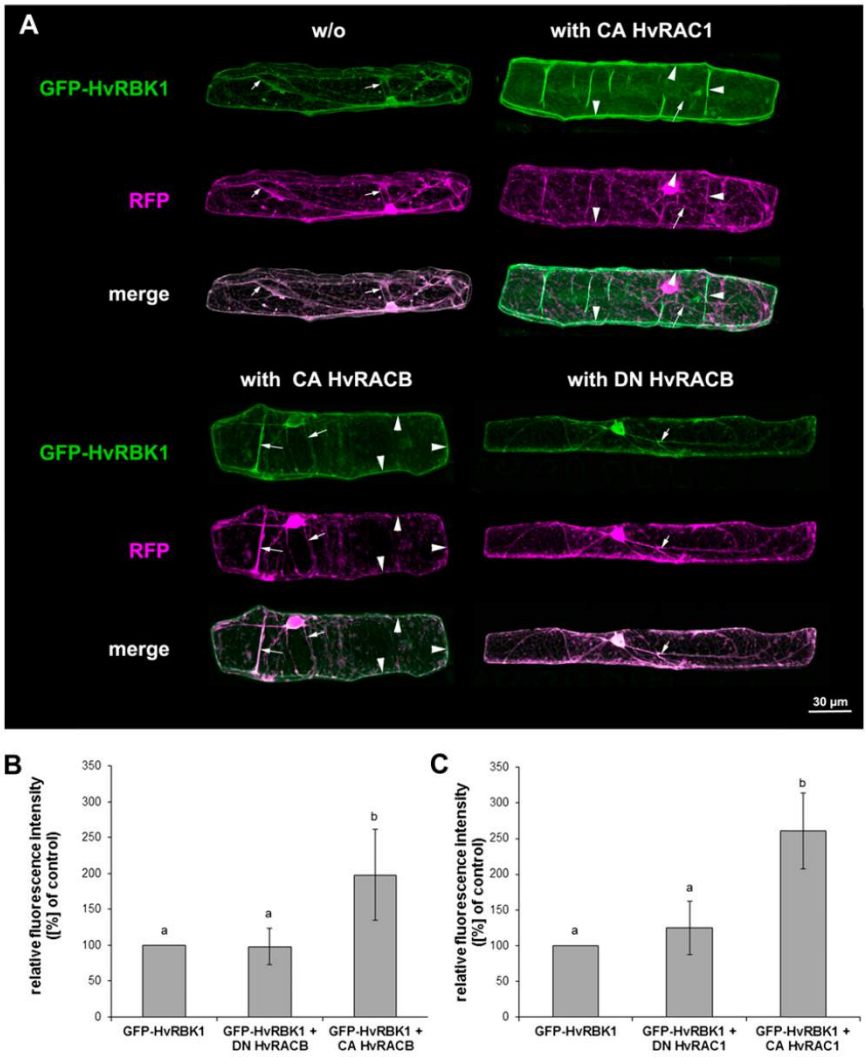

Figure 4. Recruitment of GFP-HvRBK1 by HvROPs in barley epidermal cells. A, Subcellular localization of GFP-HvRBK1 in epidermal cells of barley. A, Confocal laser-scanning micrographs of barley epidermal cell expressing GFP-HvRBK1 (green) and RFP (magenta). Soluble RFP was cotransformed as marker for cytoplasmic and nuclear localization. White color in the merged channels demonstrates similar localization of GFP-HvRBK1 and RFP in cytoplasmic strands (arrows). GFP-HvRBK1 was expressed alone (−/−) or together with the unlabeled HvROPs CA HvRACB, CA HvRAC1, or DN HvRACB. Coexpression of GFP-HvRBK1 and CA HvRACB or CA HvRAC1 results in recruitment of GFP-HvRBK1 to the cell periphery/plasma membrane (arrowheads) as demonstrated by color separation and the green cell periphery in the fluorescent merged pictures. DN HvRACB does not alter cytoplasmic localization of GFP-HvRBK1. Pictures are maximum projections of 20 to 30 optical sections at 2-μm increments. B and C, Fluorescence intensity of GFP-HvRBK1 at the cell periphery is greatly modified upon coexpression of different HvROPs. To quantify recruitment of GFP-HvRBK1 by CA HvRACB and CA HvRAC1, both proteins were coexpressed with GFP-HvRBK1 and mCherry as a marker for cytoplasmic and nuclear localization. As negative control, CA HvROP variants were replaced by DN HvRACB or DN HvRAC1. Additionally, the GFP-HvRBK1 was expressed with mCherry and empty vector as control. Mean pixel intensity was measured at the cell periphery and normalized against mCherry pixel intensity in the nucleus. Columns show means of three independent experiments with 95% confidence intervals as error bars. Intensities were significantly different between the CA HvRACB or CA HvRAC1 expressing cells versus the respective controls after ANOVA (Tukey test, $P < 0.05$, as indicated by different letters in the figure).

Table I. *Efficiency of FRET between HvRBK1-YFP and variants of CFP-HvRACB*

Acceptor	FRET Efficiency[a]	
	CFP-DN HvRACB	CFP-CA HvRACB
	%	
HvRBK1-YFP	3.1 ± 2.5	16.2 ± 6.9***

[a]Quantitative analysis of acceptor photobleaching FRET measurements. Data show the means of three independent experiments. Seven to 10 cells were analyzed in each experiment. FRET efficiency was significantly elevated in cells transformed with HvRBK1-YFP and CFP-CA HvRACB compared with cells cotransformed with HvRBK1-YFP and CFP-DN HvRACB (two-tailed Student's *t* test; ***, $P > 0.001$). Errors represent the SDs.

Barley HvRBK1 Influences MT Stability and Fungal Penetration

The MT cytoskeleton can be regulated via ROP signaling and has key functions in plant defense against invading pathogens. To test whether HvRBK1 influences the cytoskeleton, the MT arrangement was investigated upon transient-induced gene silencing (TIGS) (Douchkov et al., 2005) of *HvRBK1*. For this, the TIGS construct pIPKTA30N-*HvRBK1* was generated and cotransformed with pGY-1-*RFP-HvMAGAP-Cter* as a reliable red fluorescent protein (RFP) MT marker in barley (Hoefle et al., 2011). Off-target analysis using the Si-Fi program (http://labtools.ipk-gatersleben.de/; Nowara et al., 2010) indicated that *HvRBK1* may be the only target for this TIGS construct in the barley transcriptome. Microscopic evaluation was performed by confocal laser-scanning microscopy 48 h after bombardment. Three types of MT arrangement were distinguished: well-ordered parallel arrangement (Fig. 5A), disordered/randomized arrangement (Fig. 5B), and fragmented MTs (Fig. 5C). In repeated independent experiments, TIGS of *HvRBK1* significantly enhanced the frequency of cells with fragmented MTs in comparison to the empty vector control (Fig. 5D). The marker RFP-HvMAGAP1-Cter represents the MT-associating domain of HvMAGAP1 fused to RFP. It was selected as a MT marker because it lacks any ROP interaction domain and thus may not interfere with ROP signaling in the assays (Hoefle et al., 2011). We alternatively used full-length RFP-HvMAGAP1 or DsRED-MBD, a DsRED fusion of the MT-associated protein4 MT-binding domain (Marc et al., 1998). With both alternative MT markers, silencing of HvRBK1 caused similar effects, leading to more cells with fragmented MTs (Supplemental Fig. S5). We then used the same TIGS construct to assess a potential function of HvRBK1 in interaction with *B. graminis* f. sp. *hordei*. TIGS of *HvRBK1* rendered barley more susceptible to penetration by virulent *B. graminis* f. sp. *hordei* in single epidermal cells after biolistic transformation. The penetration success of *B. graminis* f. sp. *hordei* increased from 35% in control cells to 55% in cells bombarded with the TIGS construct of *HvRBK1* (Fig. 5E).

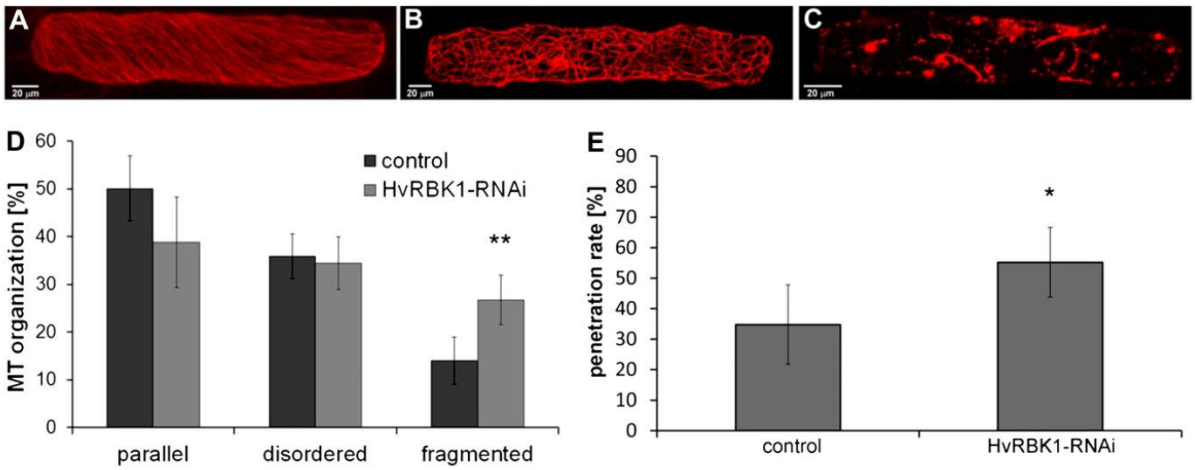

Figure 5. Influences of TIGS of *HvRBK1* on fungal penetration success and MT organization in epidermal cells of barley. Epidermal cells were transiently transformed with the MT marker RFP-HvMAGAP1 to image typical MT arrays in epidermal cells. A, Parallel arrangement of MTs. B, Disordered/randomized arrangement of MTs. C, Fragmented MTs. D, Frequencies of cells falling into the categories of different MT arrays (A–C). Columns represent means of four independent experiments (50 cells for each plasmid combination were investigated per experiment), with significantly more fragmented MTs after TIGS of *HvRBK1*. We counted MT arrays after projections of 20 to 25 optical sections at 2-μm increments with the MT marker RFP-HvMAGAP1-Cter. Similar results were obtained with two alternative MT markers (Supplemental Fig. S5). E, TIGS of *HvRBK1*, when compared with empty TIGS vector control, led to enhanced fungal penetration rate as indicated by more haustoria-containing cells per total cells attacked by *B. graminis* f. sp. *hordei*. Columns show the mean of five independent biological experiments. Error bars show SD of the mean (two-sided Student's *t* test; *, $P < 0.05$ and **, $P < 0.01$). [See online article for color version of this figure.]

DISCUSSION

ROP binding kinases of class RLCK VIA are candidates for downstream effector proteins in plant ROP signaling (Molendijk et al., 2008; Dorjgotov et al., 2009). This hypothesis is supported by our data showing that ROPs bind an RLCK VIA-like barley protein HvRBK1 in yeast and in planta and are capable of increasing its activity in vitro. TIGS of barley *HvRBK1* led to enhanced susceptibility to powdery mildew, suggesting a function of this kinase in interaction with the biotrophic fungal leaf pathogen. Data establish a function of the barley ROP binding kinase HvRBK1 in basal resistance to powdery mildew and further support an important function of ROP signaling in plant-microbe interactions.

We identified HvRBK1 in nonbiased YTH screenings using HvRACB or HvRAC1 proteins as baits. YTH assays with full-length HvRBK1 supported that it can interact with HvRAC1 and the powdery mildew susceptibility factor HvRACB (Schultheiss et al., 2002; Hoefle et al., 2011). CA forms of HvRACB and HvRAC1 were able to change subcellular localization of HvRBK1. CA HvRAC1 completely and CA HvRACB partially recruited HvRBK1 to the cell periphery. Differences in the strength of recruitment may be explained by the fact that CA HvRAC1 is more exclusively associated with the cell periphery than CA HvRACB, of which a portion is often visible in the cytoplasm and nucleoplasm when fused to GFP (Schultheiss et al., 2003; Supplemental Fig. S3). Hence, subcellular localization of HvRBK1 seems to mirror that of the coexpressed activated barley ROP. The recruitment experiments support that HvRBK1 is bound by ROPs in planta. FRET experiments further added direct evidence for the in planta interaction of activated HvRACB and HvRBK1 (Table I).

The capability for the phosphorylation of an artificial substrate, myelin basic protein, by purified HvRBK1 confirmed that HvRBK1 is an active kinase. The constitutively active but not the DN mutant or the wild-type form of HvRACB significantly enhanced kinase activity. HvRAC1-GTP also enhanced the kinase activity in vitro in a concentration-dependent way. Similarly, *Medicago* and Arabidopsis RLCK VIA2 kinases exhibit active ROP-dependent activity in vitro (Dorjgotov et al., 2009). Therefore, the regulation of the activity of RLCK VIA kinases by active ROPs might be a general feature in plants.

Based on the YTH assay and in planta and in vitro experiments, HvRBK1 can be considered as a potential ROP-effector kinase that may be involved in HvRACB/HvRAC1-dependent downstream signaling in barley. The fact that CA HvRACB but not DN HvRACB activated the kinase in vitro and recruited GFP-HvRBK1 to the cell periphery supports the idea that active ROPs signal through HvRBK1 for downstream events. Possibly HvRBK1 resides in the cytoplasm when ROPs are inactive. Activation of ROPs might then recruit HvRBK1 to the cell periphery where downstream substrates of HvRBK1 might be phosphorylated for local regulation. The physiological substrates of plant RLCK VIA kinases are actually unknown. Phosphomimetic mutations in ROPs alter their function and interaction with upstream plant-specific ROP nucleotide exchanger proteins (Fodor-Dunai et al., 2011). However, thus far, there is no evidence that ROPs themselves are substrates of RLCK VIA kinases.

Considering the involvement of the barley ROPs in plant-fungal pathogen interactions (Schultheiss et al., 2002; Pathuri et al., 2008; Hoefle et al., 2011), as well as the elevated expression of the HvRBK1 gene in infected leaves, it was reasonable to suppose a function of HvRBK1 in pathogenesis. It was unexpected, however, that barley HvRBK1 appeared to be required for basal resistance rather than susceptibility to powdery mildew because of strong evidence that barley ROP signaling is involved in susceptibility to penetration by the barley powdery mildew fungus (Schultheiss et al., 2002, 2008; Pathuri et al., 2008; Hoefle et al., 2011). The fact that ROP signaling proteins are involved in both susceptibility and basal resistance in plants would make it an attractive target for a fungal virulence strategy. A function of HvRBK1 in basal resistance could be explained by a negative feedback regulation of the susceptibility factor HvRACB by HvRBK1. Absence of the kinase then would lead to enhanced activation of downstream events, which are independent of HvRBK1. Alternatively, HvRBK1 might be activated by ROPs that function in pathogen defense rather than in susceptibility. HvRBK1-interacting HvRAC1 has a potential role in defense. Expression of CA HvRAC1 enhances callose deposition and oxidative defense at the sites of attack from *B. graminis* f. sp. *hordei.* However, this is not sufficient to cause resistance, and the expression of CA HvRAC1 in barley even weakens basal penetration resistance to *B. graminis* f. sp. *hordei.* By contrast, the CA HvRAC1 genotypes show enhanced basal penetration resistance to the rice blast fungus *Magnaporthe oryza* (Pathuri et al., 2008). Barley HvRAC1 is highly similar to rice OsRAC1 (Schultheiss et al., 2003), which is a key factor of plant immunity, including resistance to rice blast, and of pathogen-triggered cell death (Ono et al., 2001; Chen et al., 2010a, 2010b; Trusov et al., 2010). OsRAC1 is involved in immunity triggered by nonspecific pathogen-associated molecular patterns and by race-specific virulence effectors (Chen et al., 2010a; Kawano et al., 2010). Together, plant ROPs and their interactors are pivotal factors in both susceptibility and immunity to diverse fungal pathogens.

MTs reorganize upon attack from *B. graminis* f. sp. *hordei* in barley epidermal cells (Baluska et al., 1995; Hoefle et al., 2011), and MT depolymerizing drugs weaken penetration resistance of barley coleoptiles to nonadapted powdery mildew fungi (Kobayashi et al., 1997). A polarized reorganization of the MTs correlates with basal resistance to fungal penetration. This is partially regulated by HvMAGAP1 that supports

polarization of MTs under fungal attack (Hoefle et al., 2011). The fact that HvMAGAP1 fulfills a ROP-antagonistic function in association with MTs supports the hypothesis that MTs themselves are targets of ROP signaling (Hoefle et al., 2011; Mucha et al., 2011). Transient silencing of HvRBK1 weakened stability of MTs and supported fungal penetration (Fig. 5). This is consistent with a function of MTs in building penetration barriers against *B. graminis* f. sp. *hordei*. In Arabidopsis, ROP-mediated MT organization involves different ROPs that have partially antagonistic functions in the development of epidermal pavement cells (Fu et al., 2005, 2009; Xu et al., 2010). One may therefore speculate that different barley ROP pathways can also have MT destabilizing or organizing functions to which HvRBK1 contributes. Taken together, barley HvROPs, HvMAGAP1, and HvRBK1 may function in MT dynamics in the interaction with invading fungal pathogens. Parasitic *B. graminis* f. sp. *hordei*, however, might take advantage of this by manipulating ROP signaling in barley for either suppression of basal penetration defense or for getting support from the host when forming the haustorial complex within an intact barley cell (Hoefle et al., 2011). In such a scenario, HvRBK1 might play a role in penetration defense by supporting MT stability. However, our data do not exclude that a virulent fungus could also make use of HvRBK1, depending on the spatiotemporal pattern of ROP activity during fungal invasion.

MATERIALS AND METHODS

Plant Growth, Pathogens, and Inoculation Conditions

Barley (*Hordeum vulgare*) plants of the cultivar Golden Promise were grown in a growth chamber at 18°C with 60% relative humidity and a photoperiod of 16 h with 150 μmol m^{-2} s^{-1}. *Blumeria graminis* f. sp. *hordei*, race A6 was maintained on Golden Promise under the above-described conditions.

In transient transformation experiments, detached primary leaf segments of barley were placed on 0.5% (w/v) H_2O-agar 7 d after germination and inoculated with >100 conidia mm^{-2}.

Isolation of Hv*RBK1*

The complete coding sequence of *HvRBK1* was amplified by 5′-RACE from a barley cDNA pool with the 5′/3′-RACE Kit, 2nd Generation (Roche). From total RNA, 1.5 μg were transcribed into first-strand cDNA via the gene-specific primer SP1 5′-GCGACTCCAAGCGCGATATTG-3′. The isolated single-strand cDNA-containing mix was purified with the High Pure PCR Product Purification Kit (Roche). Purified single-strand cDNA was used for poly(A) tailing of the 3′-end by a terminal transferase and subsequent amplification of the *HvRBK1* coding sequence according to the manufacturer's instructions. The *HvRBK1* sequence was amplified on the dA-tailed cDNA with an oligo(dT)-anchor primer and a second gene-specific primer, SP2 5′-CATTTGGGTGGTTTACGTGC-3′. In a second nested PCR with a PCR-anchor primer and a third gene-specific primer, SP3 5′-GAACTGAACCTGTCAGTGGC-3′, the specific *HvRBK1* 5′-RACE product was obtained and ligated into the pGEM-T-vector (Qiagen) and sequenced.

YTH Screening and Targeted YTH Assay

YTH screening and transformation of yeast (*Saccharomyces cerevisiae*) was performed according to the yeast protocols handbook and the Matchmaker library construction and screening kits manual (Clontech). The YTH screening for HvRACB and HvRAC1 interaction partners is described in detail in Hoefle et al. (2011). For the targeted YTH assay, HvRBK1 was fused in the YTH vector pGADT7 with the GAL4 activation domain. Transformed yeast cells were selected on synthetic dextrose medium lacking Leu and Trp. Selection of yeast expressing interacting proteins was performed on synthetic dextrose medium lacking Leu, Trp, His, and adenine.

TIGS

Transient transformation of 7-d-old barley leaves of cultivar Golden Promise was performed as described earlier (Douchkov et al., 2005; Eichmann et al., 2010) with the PDS-1000/He System (Bio-Rad Laboratories) plant transformation gun with hepta-adapter. The RNAi construct for TIGS pIPKTA30N-*HvRBK1* was produced by blunt end insertion of a 370-bp (bp 636–1,006 of the coding sequence) fragment of *HvRBK1* in antisense orientation into the Gateway compatible entry vector pIPKTA38. Then the cDNA fragment was transferred as inverted repeats into the pIPKTA30N destination vector by a Gateway clonase reaction (Douchkov et al., 2005). Seven micrograms per shot of the RNAi construct pIPKTA30N-*HvRBK1*, the empty vector pIPKTA30N, and 3.5 μg of the reporter plasmid pGY-1-*GFP* were used in TIGS experiments.

Protein Localization and Protein-Protein Interaction in Planta

For localization studies, leaves of Golden Promise were transiently transformed with *GFP-HvRBK1* fusion constructs under control of the 35S promoter via particle bombardment. N-terminal fusion constructs of *HvRBK1* with *GFP* were achieved by insertion of the coding sequence in frame with *GFP* lacking the stop codon into the expression vector pGY-1 by the *Bam*HI and *Sal*I restriction sites. Soluble RFP, or mCherry in pGY-1, under the control of the P35S promoter was cotransformed as transformation marker. Each shot delivered 1 μg of the fusion construct and 0.5 μg of the transformation marker. In transient coexpression experiments, each shot delivered 1 μg of *GFP-HvRBK1* fusion construct together with 1.1 μg of pGY-1-CA*HvRACB* or 1.1 μg of pGY1-CA*HvRAC1*. Leaves were inspected 24 h after bombardment by CLSM. Pictures were generated by sequential scanning to avoid channel crosstalk. GFP was excited by a 488-nm laser line and detected at 500 to 550 nm, whereas RFP was excited by a 561-nm laser line and detected at 571 to 610 nm.

Interaction of CFP-HvRACB and HvRBK1-YFP in planta was verified by FRET analysis. A C-terminal fusion of HvRBK1 with YFP was achieved by amplification of *HvRBK1* with the primer pair HvKinFW 5′-AACCCGGGATGAAACTAAGGGAGTATTTCC-3′ and HvKinREV 5′-AACCCGGGGACACTGCTCCAACGC-3′ introducing *Sma*I restriction sites and insertion into the *Sma*I restriction sites of the pGY-1 vector containing *YFP*. The construction of CFP-CA HvRACB and CFP-DN HvRACB was described in Hoefle et al. (2011). Leaves of 7-d-old barley plants were cotransformed by particle bombardment with 1.2 μg of pGY-1-*CFP*-CA*HvRACB* or pGY-1-*CFP*-DN*HvRACB* together with 1.2 μg of pGY-1-*HvRBK1-YFP*. FRET analysis was performed 24 h after bombardment by the acceptor photobleaching method using the Leica Application Suite, Advanced Fluorescence 1.8.0 software (Leica Microsystems). For description of the FRET analysis, see Hoefle et al. (2011).

For subcellular fluorescence intensity measurements, leaves of barley were transiently transformed via particle bombardment with soluble mCherry (pGY1-*mCherry*) as cytoplasmic and nucleoplasmic marker, the *GFP-HvRBK1* fusion construct, and respective constructs with the CA or DN variants of HvRACB and HvRAC1 under control of the 35S promoter. As control, *mCherry* and the *GFP-HvRBK1* construct were cotransformed. Leaves were analyzed by CLSM 24 h after bombardment. All cells within an experiment were scanned with the same microscope settings. Mean pixel intensities were measured in the red channel (mCherry) in the nucleus and in the green channel (GFP-HvRBK1) at the cell periphery. The values of the peripheral intensities were normalized against the values of the nucleus and relative intensities compared with the control were calculated and plotted.

Analysis of MT Organization

To evaluate MT arrays in cells, leaves of Golden Promise were transiently transformed via particle bombardment with the *HvRBK1* RNAi construct

pIPKTA30N-*HvRBK1* and a *GFP* expression construct as transformation marker (as described above). To observe the MT dynamics, the cells were also cotransformed with pGY1-*RFP-HvMAGAP1* expressing the MT-associating barley *HvMAGAP1* as an RFP-fusion protein. Alternatively, cells were cotransformed with the MT marker *DsRED-MBD* (Marc et al., 1998) or with the RFP fused C-terminal MT-associating part of *HvMAGAP1* (Hoefle et al., 2011). The leaves were evaluated 48 h after bombardment by CSLM as described above. Each cell was distributed in one of the three categories of MT organization: parallel, disordered, or fragmented.

Gene Expression Analysis

Total RNA from leaves of 3-week-old barley plants was extracted from frozen plant material using TRIzol reagent (Invitrogen). From each sample, 1 μg of total RNA was reverse transcribed into cDNA using the QuantiTect reverse transcription kit (Qiagen). For semiquantitative two-step reverse transcription-PCR reactions, 1 μg of cDNA from each sample was used. To monitor differences in the initial template amounts, PCR reactions were stopped in the exponential phase. The *UBIQUITIN* transcript of barley was amplified in parallel as quality and quantity control with specific primers Ubi-5′ 5′-ACCCTCGCCGACTACAACAT-3′ and Ubi-3′ 5′-CAGTAGTGGCGGTC-GAAGTG-3′. To amplify the *HvRBK1* transcript, the primer pair Kinasefisch 5′-GCCATGAAACTAAGGGAGTAT-3′ and wt26SP3 5′-GAACTGAAC-CTGTCAGTGGC-3′ was used. As control for normal proliferation of the powdery mildew fungus, transcripts of the β-subunit of MTs were amplified with fungus-specific primers Bgh_beta-tub_F 5′-TCTGCCATTTTC-CGCGGTAA-3′ and Bgh_beta-tub_R 5′-CGTTGCTTACTTCCTCTGGA-3′.

Kinase Activity Measurements

The cDNA clones of the barley proteins have been inserted into bacterial expression vectors in order to allow the purification of 6x-His-tagged (6xHIS) proteins from bacterial cultures. The vectors used were pET28a (for HvRBK1), pET28b (for HvRacB) from Novagen (Merck KGaA) and pQE-70 (for HvRac1) from Qiagen. For recombinant protein production, the ArcticExpress (DE3) RIL competent cells were used according to the supplier's advice (Agilent Technologies). Protein purification was achieved using HisSelect Sepharose (Sigma-Aldrich) as described elsewhere (Dorjgotov et al., 2009, Fodor-Dunai et al., 2011) and the removal of contaminating chaperons derived from the ArcticExpress(DE3)RIL competent cells according to Joseph and Andreotti (2008). For the in vitro kinase assays, the reaction mix was set up as 1 pmol of purified 6xHIS kinase, 0,5-50 pmol of purified 6xHIS GTPase, 20 mM of Tris-HCl, pH = 7.6, 5 mM of MgCl2, 50 mM of NaCl, 1 mM of DTT, 10 μM of ATP, 0.2 MBq [γ-^{32}P] of ATP, and 0.25 μg/μL of myelin basic protein. The reactions were stopped by 5 μL of 5× SDS loading buffer after 60 min at room temperature. Proteins were separated on SDS-polyacrylamide gels that were stained by Coomassie Brilliant Blue, dried, and exposed to x-ray films using standard methods.

The HvRAC1 protein was loaded by GTP after using EDTA for nucleotide removal as described by Rodriguez-Viciana and McCormick (2006).

Sequence data from this article can be found in the GenBank data libraries under the following accession numbers: barley, *HvRBK1* (HE611049), *HvRACB* (AJ344223), *HvRACD* (AJ439334), *HvRAC1* (AJ518933), *HvRAC3* (AJ518932), *HvROP6* (AJ439333), *HvMAGAP1* (AK371854), and *HvUBIQUITIN* (M60175); Arabidopsis (*Arabidopsis thaliana*), *AtROP6* (At4g35020, NP_829654), *AtPERK1* (NP_189098, At3g24550), *AtRLCKVIA1* (NP_001078762, At5g57670), *AtRLCK-VIA2* (AtRRK1, AAT99800, At2g188900), *AtRLCKVIA3* (AAO63452, At5g65530), *AtRLCKVIA4* (AtRBK1, AED91559, At5g10520), *AtRLCKVIA5* (AtRRK2, AED94033, At5g35960), *AtRLCKVIA6* (AtRBK2, NP_187165, At3g05140), and *AtRLCKVIA7* (AAO64835, At5g18910); rice (*Oryza sativa*), *OsPERK1* (BAD45880) and *Medicago truncatula*: *MtRRK1* (FM886833) and *MtRRK2* (FM886834); and *Medicago sativa*, *MsROP6* (CAI84892).

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Figure S1. Genomic structure of the *HvRBK1* gene and the comparison of the deduced amino acid sequence of HvRBK1 to related proteins.

Supplemental Figure S2. Quantification of GFP-HvRBK1 signal intensity in cells coexpressing different versions of HvROPs.

Supplemental Figure S3. Subcellular localization of GFP-CA HvRACB and GFP-CA HvRAC1.

Supplemental Figure S4. *HvRBK1* expression pattern in the interaction of barley with *B. graminis* f. sp. *hordei*.

Supplemental Figure S5. Influences of TIGS of *HvRBK1* on MT organization in epidermal cells of barley.

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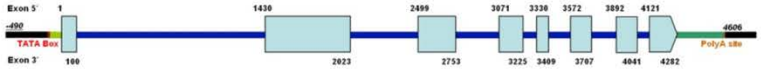
Supplemental Data Huesmann and Reiner *et al.*, 2012

Supplemental Data. Huesmann et al. Plant Physiology (subm)

A



B

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AtRLCKVIA3      --------------------------MAVEEMEK-KIKGSQETELHKNDLSIEDPS-PRC 32
AtRBK1          --------------------------MAVEDNKNSESKNHQEVELHRNDLGLEDSSSPRG 34
HvRBK           -MKLREYFLLEPAGGCGGGGAERAEDSLPASGEEVQGVNQEEGRRSYGEPADEDCGSIGS 59
OsPERK1         MTKLKEYLVIESAEVGNEDG--RQEDLLVKEG---PGEHQENTIRDSDEAKNEDCSNSGS 55
                                          .        ::     .:   ** .

AtRLCKVIA3      VLEIPV-----------------------------------MSSDSD-------------- 44
AtRBK1          VLG-------------------------------------MVSDSD-------------- 43
HvRBK           VTDAAVPSEDPSEGQSND------------------SSDSQCAGSDGGCKEVPEVYSKSG 101
OsPERK1         VTEAVVSSEEPSDRSSTEEPSDRSSTEEPSDSSSGGDSSSQSADSDGGSKEAPKMDSKAG 115
                *                                          :.**

AtRLCKVIA3      --NSSS-CSSCSPD----KSSSPLSTTPPNVSSFH------------QWNKMIESIK--- 82
AtRBK1          --NSSSSCSSCSSDDKSSSTSSPFSNTTKTVSSSHH---------GLQWNKMIESIK--- 89
HvRBK           GNDENSECTDQSSPRAVLDISVSGSVDSDESSSVEQPAVPSRSVQPLQWRNLISGLILSR 161
OsPERK1         -NDDSSECTDQSSPRAVLDISVSGSVDSDESSSVEQPAESN---HNTQWRNLISGLILRR 171
                  :..* *:. *.     . * . *  .   ** .           **.::*..:

AtRLCKVIA3      KKSIRRFSVIPLLASYELTRK---KKQPKLSPCSENDFD---CDQILVAKPSWRNFTFDE 136
AtRBK1          KKSMRRFSVIPLLASYELTRKNLRRKQPKLTP-SESAFT---CEAFFMAKPSWRNFTYEE 145
HvRBK           KRLMARAVTFPQRSKNTGLKRYLGRMRSGKNQMDCSAIAPEIFPEIEKWRPSWRSFDYDE 221
OsPERK1         RKSMARAGTFPQRTKTTGLKRYLERMRSGKNQIDCGAIAPEILPEISKWRPSWRSFDYSE 231
                :: : *  .:*  :.    ::   : :.  .  . . :       :   :****.* :.*

AtRLCKVIA3      LVAATDNFNPENMIGKGGHAEVYKGVLPDGETVAIKKLTRHAKEVEERVSDFLSELGIIA 196
AtRBK1          LAVATDYFNPENMIGKGGHAEVYKGVLINGETVAIKKLMSHAKEEEERVSDFLSELGIIA 205
HvRBK           LCAATDRFSSDNLIGKGGHAEVYKGQLADGQFVAVKRLTKGGNK-EDRISDFLSELGIIA 280
OsPERK1         LCAATDKFSSENLIGKGGHAEVYKGHLADGQFVAVKRLTKGGNK-EDRISDFLSELGIIA 290
                * .*** *..:*:************ * .:*: **:*:*   .:: *:*:************

AtRLCKVIA3      HVNHPNAARLRGFSCDRGLHFVLEYSSHGSLASLLFGSEECLDWKKRYKVAMGIADGLSY 256
AtRBK1          HVNHPNAARLRGFSSDRGLHFVLEYAPYGSLASMLFGSEECLEWKIRYKVALGIADGLSY 265
HvRBK           HVNHPNAAQLLGFSVEGGLHLVLQFSPHGSLASLLHGAKGALRWKARFNIALGVAEGLFY 340
OsPERK1         HVNHPNAAQLLGFSVEGGLHLVLQFSPHGSLASVLHGTKGALKWKARFNIALGIAEGLLY 350
                ********:* *** : ***:**:::.:******:*.*:: .* ** *:::*:*:*:** *

AtRLCKVIA3      LHNDCPRRIIHRDIKASNILLSQDYEAQISDFGLAKWLPEHWPHHIVFPIEGTFGYLAPE 316
AtRBK1          LHNACPRRIIHRDIKASNILLNHDYEAQISDFGLAKWLPENWPHHVVFPIEGTFGYLAPE 325
HvRBK           LHEGCHRHIIHRDIKASNILLTEDYQPQISDFGLAKWLPDKCTHQVVFPIEGTFGYMAPE 400
OsPERK1         LHEGCHRRIIHRDIKASNILLTEDYQPQISDFGLAKWLPDKWTHHVVFPIEGTFGYMSPE 410
                **: * *:************..**:.**************:: .*::**********::**

AtRLCKVIA3      YFMHGIVDEKTDVFAFGVLLLEIITGRRAVDTDSRQSIVMWAKPLLEKNNMEEIVDPQLG 376
AtRBK1          YFMHGIVDEKIDVFAFGVLLLEIITSRRAVDTASRQSIVAWAKPFLEKNSMEDIVDPRLG 385
HvRBK           YFMHGIINEKTDVFAYGVLLLELVTGRKAVDS-SRQSLVIWAKPLLESNNMKGLVDPSLD 459
OsPERK1         YFMHGIINEKTDVFAYGVLLLELVTGRKAVDS-SRQSLVIWAKPLLDSNNMKELVDPSLD 469
                ******::** ****:******::*.*:***: ****:* ****:*:.*.*: :*** *.

AtRLCKVIA3      NDFDETEMKRVMQTASMCIHHVSTMRPDMNRLVQLLRGDDQLAE-QKPGGARTVS---LD 432
AtRBK1          NMFNPTEMQRVMLTASMCVHHIAAMRPDMTRLVQLLRGEDGPAELQQKAGERTMS---VN 442
HvRBK           AGYDLEEMALTLAVASMCIHHSANLRPSMKSVVRFLKGDRESLELMGK--PKPTKPPMFD 517
OsPERK1         VGYDPEEMAHILAVASMCIHHSSSSRPSMKSVVRFLKGDRESLEMMQMQRPKLMKPLMFD 529
                  ::  **   : .****:** :  **.*. :*::*:*:    *       :  .   .:

AtRLCKVIA3      DCDL-DHTSSSYLNDLTRHRQLSME- 456
AtRBK1          ACDLQDHTSSSYLNELRRHRQLLME- 467
HvRBK           SCDSEDYTRTSYLNDLDKHKQLALEQ 543
OsPERK1         SGDSEDYTRSSYLNDLDRHKKLALEQ 555
                  *  *:* :****:* :*::* :*
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Figure S1. Genomic structure of the HvRBK1-coding gene and the comparison of the deduced amino acid sequence of HvRBK1 to related proteins. A, Structure of the genomic sequence (> contig_6719 length=7372 rbca=7HL at http://webblast.ipk-gatersleben.de/barley/index.php) of HvRBK1. TATA Box and poly-A sites are indicated in red. Transcribed untranslated regions are indicated in green. Light blue boxes indicate exons. Blue lines indicate introns. ATG starts at indicated nucleotide position 1 and stop codon TGA ends at indicated position 4282. 5`cis region is limited by -490 base pairs. 3´ region beyond the poly-A signal is neglected. B, Alignment of full-length HvRBK1, AtRLCK

VIA3, AtRBK1 (AtRLCK VIA4) and rice OsPERK1 protein sequences (accession numbers are given before the references). Sequence alignment revealed high similarity of kinases at the C-terminus (kinase domain) and limited similarity at the N-terminus. The latter one is, however, serine-rich in all four proteins and in particular in OsPERK1, AtRLCK VIA3, and AtRBK1. Grey shaded letters show amino acids identical in OsPERK1 and HvRBK1 only. Asterisks indicate amino acids identical in all four proteins. Blue underlined letters mark the highly conserved protein kinase ATP-binding region signature and red underlined letters mark the serine/threonine protein kinases active-site signature. Alignment was made using the ClustalW program (Thompson et al., 1994).

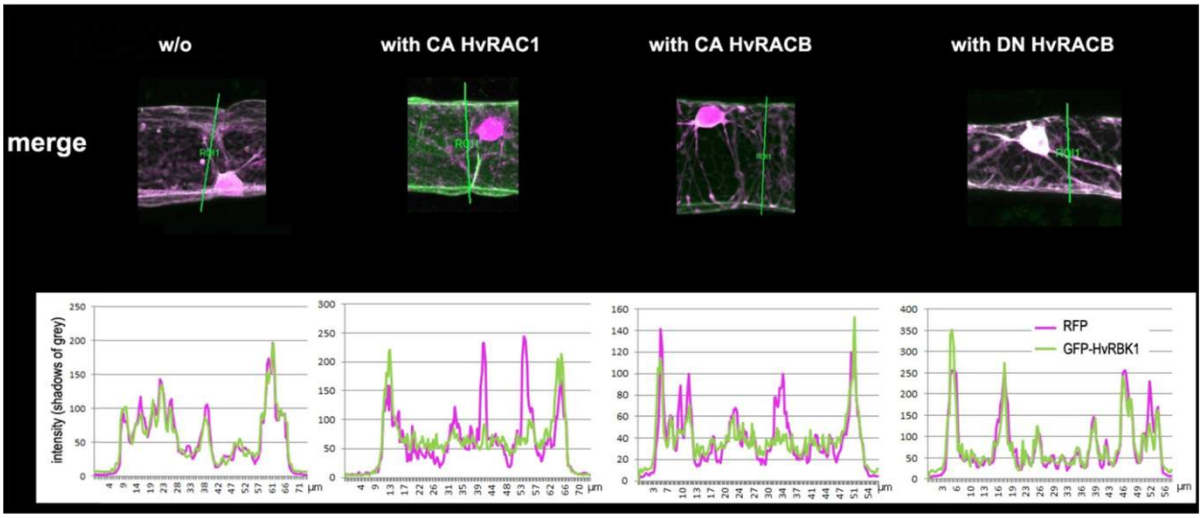

Figure S2. Quantification of GFP-HvRBK1 signal intensity in cells co-expressing different versions of HvROPs. Merged channel pictures represent magnifications of the same cells as in Figure 4. Pixel intensity was measured along the green line in a maximum projection of 25 optical sections at 2 µm increments. GFP-HvRBK1 signal is high in the cell periphery but weak in cytoplasmic strands when CA HvRACB or CA HvRAC1 are coexpressed. By contrast GFP-HvRBK1 signal is higher in the cytoplasm and weaker in the cell periphery without co-expression of a CA HvROP or with co-expression of DN HvRACB. RFP signal is always high in the cytoplasm.

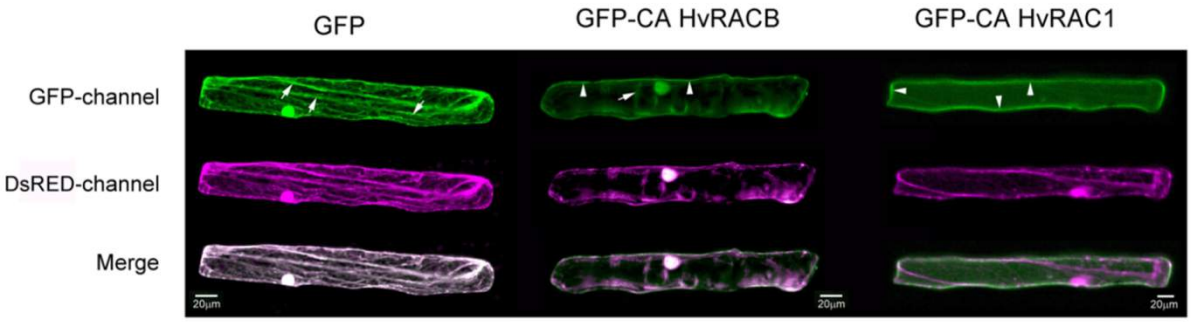

Figure S3. Subcellular localization of GFP-CA HvRACB and GFP-CA HvRAC1. To demonstrate subcellular localization of CA HvRACB and CA HvRAC1, both proteins were fused to GFP and co-expressed with DsRED as a marker for cytoplasmic and nuclear localization. Pictures represent whole cell maximum projection of 25 optical sections at 2 µm increments. As indicated in GFP and merged channels, GFP-CA RACB has a more peripheral localization (arrow heads) as compared to free GFP (left) that is detectable in cytoplasmic strands (arrows) and nucleoplasm. By contrast, GFP-CA HvRAC1 is more exclusively associated with the cell periphery (arrow heads), when compared to GFP-CA HvRACB.

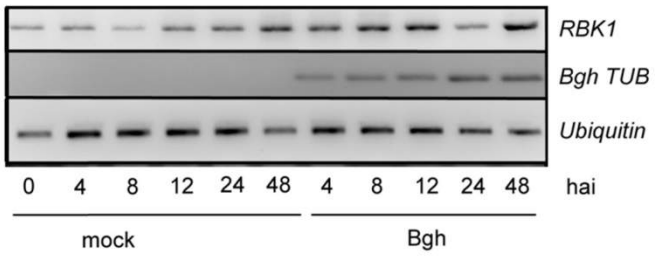

Figure S4. *HvRBK1* expression pattern in the interaction of barley with *B. graminis* f.sp. *hordei*. RNA was isolated from *B. graminis* f.sp. *hordei* or mock inoculated barley leaves harvested at 4, 8, 12, 24, 48 hpi, and reverse transcribed to cDNA, which was used as template in PCR reactions. Constitutively expressed *HvUBIQUITIN* served as quality control of the cDNA synthesis. Fungal *Bgh TUBULIN* (*TUB*) served as a positive control for successful inoculation.

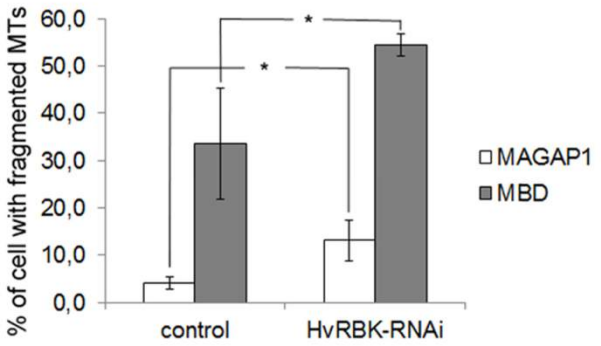

Figure S5. Influences of TIGS of HvRBK1 on microtubule organization in epidermal cells of barley. Epidermal cells were transiently transformed with the MT-markers RFP-HvMAGAP1 (full length) (MAGAP1) or DsRED-MBD (MBD) and the HvRBK1 RNAi construct or the empty RNAi vector control. Columns represent means ± standard deviations of at least five independent experiments (50 cells for each plasmid combination were investigated per experiment), with significantly more fragmented MTs after TIGS of HvRBK1 when compared to controls (two-sided student´s t-test $p<0.05$ for both MT markers*). Noteworthy, when experiments were performed with the MT marker DsRED-MBD (MAP4), we observed a high basal level of fragmented MT in control cells.

5.2. Reiner *et al.*, 2015a

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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Running title: SCF-complex function in mildew interaction

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

Accesion numbers: *HvRACB* (AJ344223), *HvRBK1* (HE611049), *HvSKP1-like* (LN714776)

Number of words of the manuscript: 6988

SUMMARY

In an increasing number of plant-microbe interactions it became 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 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. Now, we identified a barley type II S-phase kinase 1 associated (SKP1)-like protein (HvSKP1-like) as molecular interactor of HvRBK1. SKP1 proteins are subunits of the SKP1-cullin 1-F-box (SCF)-E3 ubiquitin ligase complex that acts in 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 but not of dominant negative HvRACB in barley epidermal cells. In addition, silencing of *HvSKP1-like* enhanced susceptibility of barley to haustorium establishment by *Bgh*. In summary, our results suggest that HvSKP1-like together with HvRBK1 controls abundance of HvRACB at the same time modulating the outcome of the barley-*Bgh* interaction. A possible feedback mechanism from RAC/ROP-activated HvRBK1 on the susceptibility factor HvRACB is discussed.

INTRODUCTION

The ubiquitin-26S proteasome system is a major regulator for protein stability. Being responsible for controlled protein degradation, the system is involved in almost all processes of plant biology, including hormone signalling, cell division, plant development and responses to abiotic and biotic stress (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). While E1 and E2 enzymes are little specific, E3 ubiquitin ligases confer substrate specificity to the ubiquitination pathway. One type of E3 ubiquitin ligases, the CRL (cullin-RING) ligases, are subdivided into several groups of which the S-phase kinase associated protein 1-cullin 1-F-box (SCF) E3 ubiquitin ligases represent 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 on *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). Type II protein structure is similar to that of type I proteins except that they show a carboxy-terminal elongation. Additionally, the type II-encoding genes contain several introns (Kong *et al.*, 2007). The function of *SKP1-like* genes in plants is hardly characterized. For ASK1 and ASK2 proteins it was shown that they are involved in flower development, male sterility, embryogenesis and seedling development (Liu *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 derepression of jasmonate-, auxin and ethylene signalling, thereby linking regulation of defence gene expression via hormone signalling to ubiquitin-mediated proteolysis (Potuschak *et al.*, 2003, Trujillo and Shirasu, 2010). It is also 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 accumulation of the intracellular plant resistance (R) protein homolog SUPPRESSOR OF *npr1-1,* CONSTITUTIVE 1 (SNC1) (Duplan and Rivas, 2014, Gou *et al.*, 2012, Gou *et al.*, 2009). Within the last years it became further 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 (Delauré *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) 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 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 accommodation of haustoria in intact epidermal cells (Hoefle *et al.*, 2011, Pathuri *et al.*, 2008, Schultheiss *et al.*, 2002, Schultheiss *et al.*, 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 infection success of *Bgh*. By contrast, TIGS of *HvRACB* limits fungal infection and expression of *CA HvRACB* supports it (Schultheiss et al. 2003). Hence, a mechanism was 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 protein abundance of CA HvRACB but not of inactive, dominant negative (DN) HvRACB ($HvRACB^{T20N}$). TIGS of *HvSKP1-like* supported fungal infection success. These findings suggest a role of the E3 ubiquitin ligase component HvSKP1-like in controlling 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 8 introns and 9 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 most similar proteins in *Arabidopsis* and rice (Fig 1., Supplementary Fig. S1). The barley SKP1-like protein was classified into type II of SKP1 proteins 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. Due to 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) as well as the 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 (Schultheiss et al. 2008, Hoefle et al. 2011, Huesmann et al. 2012). Because HvSKP1-like interacted with HvRBK1 in yeast, we wanted to know whether these proteins can physically interact *in planta*. Subcellular co-localization

of proteins can give a first hint towards protein-protein interaction. We hence transiently co-expressed green fluorescent protein (GFP)-tagged HvSKP1-like with red fluorescing mcherry-tagged HvRBK1 in barley epidermal cells. Both proteins similarly localized in the cytoplasm and nucleoplasm 24 hours after bombardment (hab) with plasmid-coated gold particles (Fig. 3a). Single optical sections supported 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 (Supplementary Fig. S2a). In addition, GFP-HvSKP1-like largely co-localized with the cytoplasmic and nucleoplasmic marker mcherry upon transient co-expression in barley epidermal cells (Fig. 3b; Supplementary Fig. 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 that area. We measured 15.6 % FRET efficiency by 24 hours 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 RNAi of *HvRBK1* enhance protein abundance of CA HvRACB in protoplasts

Recent findings in the animal research field revealed that SCF-E3 ubiquitin ligase complexes target HvRACB-related GTPases for their proteasomal degradation (Wei *et al.*, 2013, Zhao *et al.*, 2013). Since 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. 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 DMSO solvent control-treated protoplasts was set to 100%. 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 with an *HvRBK1*-RNAi construct (Huesmann *et al.*, 2012) or the empty RNAi-vector pIPKTA30N. Usage of cytosolic GFP-CA HvRACBΔCSIL 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 HvRACBΔCSIL 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 of GFP-CA HvRACBΔCSIL signal on Western-blots (data not shown). To address the question whether *HvRBK1*-RNAi limits specifically protein abundance of GFP-CA HvRACBΔCSIL, we repeated the experiment with free GFP. In contrast to GFP-CA HvRACBΔCSIL, 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 specific degradation of HvRACB.

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

Because *HvRBK1*-RNAi had an effect on protein abundance of GFP-CA HvRACBΔCSIL 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. Fluorescence intensity of CFP-CA HvRACB was measured at the plasma membrane 48 hours after bombardment and normalized to mcherry in the nucleus. 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* compared to 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. Fluorescence intensity of CFP was unaffected by *HvRBK1*- or *HvSKP1-like*-RNAi constructs, indicating specific influence of both RNAi-constructs on the protein stability of HvRACB (Fig. 5b). To investigate whether the increase of CA HvRACB abundance is associated with its activation state *CFP-WT* (wild type) *HvRACB* or *CFP-DN HvRACB* were transiently transformed into barley epidermal cells together with the respective RNAi construct for TIGS and *mcherry* for normalization. Fluorescence intensity of CFP-WT HvRACB increased upon silencing of either *HvRBK1* or *HvSKP1-like* by more than two or three fold, respectively, compared to cells transformed with *CFP-WT HvRACB* alone (Supplementary Fig. S3). The increase in fluorescence intensity of CFP-WT HvRACB was less pronounced when compared to that of CFP-CA HvRACB (Fig. 5a). Co-transformation of *CFP-DN HvRACB* with silencing constructs of either *HvRBK1* or *HvSKP1-like* increased fluorescence intensity of inactive CFP-DN HvRACB by around 40% or 50%, respectively,

compared to the controls (Fig. 5c). Although this increase was statistically significant, it was weak when compared to that of wild type or activated HvRACB. This suggested similar functions of HvSKP1-like and HvRBK1 in preferentially regulating abundance of the active HvRACB protein.

HvSKP1-like modulates fungal infection success.

To investigate a possible function of the HvRBK1-interacting HvSKP1-like in the barley-*Bgh* interaction, we analyzed transcript abundance by reverse transcription quantitative real-time PCR. A time course analysis of gene expression during fungal development showed a slight but statistically non-significant accumulation of transcripts 12 hours after inoculation (hai) with *Bgh* when compared to non-inoculated controls (Fig. 6a). 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*. 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 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 upon 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 to the empty RNAi-vector pIPKTA30N control (Fig. 6c). Together, this suggests a function of HvSKP1-like in limiting 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 abundance of HvRACB through proteasomal degradation, thereby influencing the outcome of the interaction of barley with *Bgh*. HvSKP1-like seems not to directly interact 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 SCF complex indirectly. *In silico* analysis classified HvSKP1-like into 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) upon transient co-expression. HvRACB appears as a possible target of an HvSKP1-like-containing SCF complex, because CA HvRACB abundance increased upon treatment with the proteasome inhibitor MG132 in protoplasts and upon silencing of either *HvSKP1-like* or *HvRBK1* in barley leaf epidermal cells. In leaf mesophyll protoplasts, an increase of CA HvRACB protein was detected upon silencing of *HvRBK1,* too*,* but was inconsistent upon silencing of *HvSKP1-like*. This might be due to 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). Besides their F-box domain, F-box

proteins contain in most cases a recognition domain composed of kelch repeats, leucine-rich repeats (LRR) or other motives 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 were yet 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, Schultheiss *et al.*, 2003). Although HvRBK1 can be activated by CA HvRACB, TIGS of *HvRBK1* enhances infection success of *Bgh* (Huesmann *et al.*, 2012). Huesmann and co-workers (2012) hence hypothesized on 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 abundance of active HvRACB in concert with HvRBK1 and that 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 that was observed in SCF^{FBXL19}-mediated degradation of human RAC1 (HsRAC1), in which phosphorylation of HsRAC1 by AKT kinase acts as signal for association of the F-box protein FBXL19 with phosphorylated HsRAC1 (Zhao *et al.*, 2013). Potential phosphorylation of HvRACB by HvRBK1 hence needs to be investigated in future. Interestingly, phosphomimetic mutations of RAC/ROP

GTPases also influence their activity via accessibility for ROPGEFs (Fodor-Dunai *et al.*, 2011).

In contrast to human SCF^{FBXL19} that mediates 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). Since HvRBK1 interacts with and is activated by CA HvRACB but does not interact with DN HvRACB (Huesmann *et al.*, 2012), we speculate that preferentially the active form of HvRACB is targeted for degradation. This is supported because abundance of CFP-DN HvRACB only slightly increased upon silencing of *HvRBK1* or *HvSKP1-like*. DN HvRACB might be less sensitive to HvRBK1- and SCF-mediated degradation, because it cannot activate HvRBK1 and subsequent negative feedback on its own abundance. However, we cannot exclude 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 silencing of these factors.

Inactivation of HvRACB signalling via proteasome-dependent degradation of HvRACB would describe an additional mechanism to regulation of HvRACB signalling by the barley MICROTUBULE-ASSOCIATED ROP-GTPASE ACTIVATING PROTEIN 1 (HvMAGAP1) (Hoefle *et al.*, 2011). HvMAGAP1 interacts with HvRACB and limits susceptibility of barley to *Bgh*, hence reflecting the regulating function of ROPGAPs in controlling signalling output of ROP GTPases. Similar to what is described for barley, T-DNA insertion lines of *Arabidopsis AtROPGAP1* and *AtROPGAP4* and of the ortholog *AtRLCK VI_A3* have 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

accommodation of fungal haustoria similar to CA HvRACB (Schultheiss *et al.*, 2008). HvMAGAP1, HvRBK1 and the E3 ligase subunit HvSKP1-like thus 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 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 fulfill redundant, distinct and antagonistic functions in plant-microbe interactions (Chen *et al.*, 2010) and in plant development (Yang, 2008), better understanding is needed of posttranslational regulation of RAC/ROPs in these processes. In this regard it is interesting that most recently a connection was 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 signalling output of OsRAC1 in rice. This reminds of results from the animal research field, in which regulation of RHO GTPases and their regulatory proteins by ubiquitin-mediated proteasomal degradation is widely discussed as an additional mechanisms to the control of RHO GTPases by RHOGEFs and RHOGAPs (Liu *et al.*, 2015, Nethe and Hordijk, 2010, Visvikis *et al.*, 2010).

EXPERIMENTAL PROCEDURES

Plants, pathogen and inoculation

Barley (*Hordeum vulgare* L.) cultivar Golden Promise and Pallas were grown in a growth chamber at 18°C, 60% relative humidity and a 16 h photopheriod with 150 µmol m^{-2} s^{-1}. *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 it is 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 primers HvK2_fw 5'GCATTGGCAAGAATGTCAGAAAGTGAG3' and K2potStopp 5'CTGTATGTGCACAAATTTCCTAGGC3' and ligated into the pGEM-T easy vector (Promega) and sequenced. All overexpression constructs for microscopic analysis and protoplast transformation were derived on the pUC18-based plant expression vector pGY1 that contains the 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 primers HvK2fw_SmaI AACCCGGGATGTCAGAAAGTGAG and M13rev AACAGCTATGACCATGA and introduced into pGY1 via *Sma*I and *Sal*I restriction sites. The fluorescence tag containing constructs pGY1-*GFP* and pGY1-*mcherry* and pGY1-*CFP* have been described elsewhere (Hoefle *et al.*, 2011, Huesmann *et al.*, 2012). For fluorescence resonance energy transfer (FRET) measurements *CFP* was amplified using primers CFP *Sma*I fw + 1 5'ACCCGGGTATGGTGAGCAAGGGCGAGG3`and CFP *Sma*I rev -1 5'ACCCGGGGTACAGCTCGTCCATGCCGA3' and inserted into the *Sma*I restriction site of pGY1-*HvSKP1-like* to obtain an N-terminal fusion of *HvSKP1-like* with *CFP*. Generation of pGY1-*HvRBK1-YFP* was described earlier (Huesmann *et al.*, 2012). pGY1-*GFP-HvSKP1-*

like, was obtained in the same way as pGY1-*CFP-HvSKP1-like*, only that *GFP* instead of *CFP* was amplified. pGY1-*mcherry-HvRBK1* was obtained by amplification of *mcherry* with primers GFP5'*BamH*I 5'GGATCCATGGTGAGCAAGGGCGAG3' and GFP3'noStop+2 5'CCTTGTACAGCTCGTCCAT3' and subsequent blunt-end ligation of *mcherry* into *Sma*I digested pGY1-*HvRBK1*. Via *BamH*I restriction sites, *mcherry* was cloned into *BamH*I digested pGY1-*CA HvRACB* (Schultheiss *et al.*, 2003) to obtain an N-terminal fusion of mcherry with CA HvRACB. Construction of pGY1-*CFP CA HvRACB* and pGY1-*CFP DN HvRACB* was described in Hoefle et al. 2012. Constructs for RNAi-mediated transient induced gene silencing (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. TIGS construct of *HvRBK1* was described elsewhere (Huesmann *et al.*, 2012). Construction of pGY1-*GFP CA HvRACBΔCSIL* was described elsewhere (Schultheiss *et al.*, 2003). Constructs for the yeast-two hybrid assays were based on the vectors pGBKT7 and pGADT7 (Clontech). The *HvSKP1-like* gene was amplified using the primers HvK2 *Nde*I fw 5`ACATATGATGTCAGAAAGTGAGTTATC3` and M13fwd (-40) 5`GTTTTCCCAGTCACGAC3`. Via the restriction sites *Nde*I and *Sal*I (*Xho*I for pGADT7), open reading frame of *HvSKP1-like* was cloned into pGADT7. Generation of the different barley *RAC/ROP* constructs based on the vector pGBKT7 as well as generation of the construct pGADT7-*HvRBK1* was previously described (Hoefle *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 the MEGA6 software (Tamura *et al.*, 2007). Phylogeny was tested with a bootstrap of 500 (Poisson's correction model). Protein sequences of the 21 ASKs were retrieved from TAIR (http://www.arabidopsis.org/). According to the criteria and nomenclature of Kahloul et al., (2013) 30 OSKs were selected for the phylogenetic analysis. 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 (Hoefle *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, selection of yeast transformants was conducted on selective media lacking leucine and tryptophan (SD-LW). Selection for protein interaction was performed on selective media lacking leucine, tryptophan, adenine and histidine (SD-LWAH). Several transformants of each combination were resuspended in water, diluted to OD_{600}=1 and 10-fold serial dilutions were spotted in parallel on SD-LW and SD-LWAH medium.

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

For subcellular localization, protein-protein interaction studies as well as subcellular fluorescence intensity measurements, barley epidermal cells were transiently transformed using microprojectile bombardment (Schweizer *et al.*, 1999). Leaves of 7-days-old barley plants were bombarded with DNA-coated gold particles (25 mg/ml, Ø 1.0 µm) using the PDS-1000/He biolistic delivery system equipped with the single adapter (Bio-Rad, Hercules, USA). Bombardment was performed under 26 Hg vacuum and 900 pounds per square inch (psi) gas pressure. 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 pGY1-*mcherry* into barley epidermal cells. Protein localization was analyzed 24 hours after bombardment (hab) using confocal laser scanning microscopy (CLSM, Leica TCS SP5; Leica Microsystems). 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 the LAS AF 1.8.0 software (Leica Microsystems) and processed using PhotoImpact X3 (Ulead). For FRET measurements, each shot delivered 1.5 µg of pGY1-*CFP-HvSKP1-like* or 0.8 µg pGY1-*CFP* respectively and 1.3 µg of pGY1-*HvRBK1-YFP* into barley epidermal cells. FRET efficiency between CFP-HvSKP1-like or CFP respectively and HvRBK1-YFP was measured by the acceptor photobleaching method 24 hab and analyzed by CLSM using the LAS AF 1.8.0 software (Leica Microsystems). For bleaching, a region of interest was selected at the cell periphery with little cytoplasmic streaming, where 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 picture with the identical settings and sample position was taken. FRET efficiency

(FRETeff) in the bleached area was calculated from the donor fluorescence intensity before (Dpre) and after bleaching (Dpost) according to the following equation: FRETeff = (Dpost-Dpre)/Dpost. In total 10 cells per experiment and variant were analyzed. 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 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. Mean pixel intensity of the CFP-tagged proteins was measured at the plasma membrane and normalized against mean pixel intensity of soluble mcherry in the nucleus 48 hours after transformation. Fluorescence intensity was plotted relative to either CFP-CA HvRACB, CFP-WT HvRACB, CFP or CFP-DN HvRACB. In each experiment, the microscopic settings remained constant and 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 a HyD.

Transient induced gene silencing (TIGS) and functionality testing of the *HvSKP1-like* RNAi-construct

Transient induced gene silencing was performed as it is described earlier (Huesmann *et al.*, 2012). 7.0 µg per shot of the empty vector pIPTKA30N or pIPKTA30N-*HvSKP1-like* and 3.5 µg of the transformation marker pGY1-*GFP* were used. Barley leaf segments were bombarded 48 hours prior to inoculation with *Bgh*. Another 48 hours later, fungal structures were stained with 0.3% calcofluor (wt/vol in water) for 30 s and haustoria frequency of *Bgh* on GFP-expressing epidermal cells was defined as 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 it is previously described (Hoefle *et al.*, 2011). Per shot, 1.0 µg of the pGY1-*GFP-HvSKP1-like* together with 1.0 µg pIPKTA30N-*HvSKP1-like* or 1.0 µg of the empty vector pIPKTA30N respectively and 0.5 µg of pGY1-*mcherry* were transiently transformed into barley epidermal cells.

Protoplast transformation

The lower epidermis of primary leaves of 7-days-old barley plants was removed and the leaves were floated up to 1.5 hours on enzyme solution (0.45 M Mannitol, 10 mM MES pH 5.7, 10 mM $CaCl_2$, 0.3 % (wt/vol) Gamborg B5, 0.5% (wt/vol) Cellulase R10, 0.5% (wt/vol) Macerozyme R10, 0.5% (wt/vol) 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 $CaCl_2$, 5 mM KCl, 2 mM MES pH 5.7) and filtered through an 40 µM nylon mesh. Protoplasts were collected by centrifugation at 1 000 rpm for 3 min at room temperature and washed once with W5 solution. After resuspension in 1-2 ml W5 solution, protoplasts were incubated for 30 min. on ice. The supernatant was discarded and the protoplasts were re-suspended with a density of 2 x 10^6 protoplasts/ml in MMG solution (0.4 M Mannitol, 15 mM $MgCl_2$, 2mM MES pH5.7). For each transformation, 20 µg of plasmid DNA was mixed with 200 µl of protoplasts and 220 µl of PEG solution (40% (wt/vol) PEG 4000, 0.2 M Mannitol, 0.1 M $CaCl_2$). After incubation for 10 minutes at room temperature in the dark, 880 µl of W5 solution was added and the protoplasts were collected by centrifugation at 1 000 rpm for 3 min at room temperature. Transformed protoplasts were re-suspended in 2 ml WI solution (0.5 M Mannitol, 20 mM KCl, 4 mM MES pH 5.7) in each

well of a 6-well tissue culture plate and incubated under very dim light at room temperature for 16-20 hours prior to analysis.

Protein extraction, Immunoblotting and Antibodies

Protoplasts were harvested 16-20 hours after transformation, by centrifugation at 3 000 rpm for 3 min. Protoplasts were lysed with 50 µl IP Buffer (50 mM Tris HCl pH 7.5, 150 mM NaCl, 10 mM EDTA, 0.1 % Nonidet P-40, 50 µM MG132, 1 mM PMSF, 1% protease inhibitor cocktail, Sigma-Aldrich) 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 a Bradford assay (Bradford, 1976) using the Bio-Rad protein assay reagent (Bio-Rad). Afterwards, the supernatant was diluted with 6 x Laemmli Buffer, heated at 95°C for 10 min and about 40 µg of total protein per sample was subjected to immunoblot analysis. SDS-Page, immunoblotting and Ponceau S staining were performed according to standard methods. Antibodies used for immunoblotting were anti-GFP [3H9] (Chromotek) and horseradish peroxidase-conjugated anti-rat (Sigma-Aldrich). Signals were detected by chemiluminescence detection using SuperSignal West Femto Chemiluminescent substrate (Thermo Scientific).

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 polyethylene glycol-mediated transformation method. 17 hours after transformation, protoplasts were treated for 3 hours with 10 µM MG132 solved in DMSO or DMSO respectively. Afterwards 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. Results were plotted relative to the DMSO-treated

protoplasts which served as control. In each experiment 10 protoplasts per treatment were analyzed.

Gene expression analysis

Total RNA was extracted from inoculated and mock-inoculated barley leaves of the cultivar Pallas at 0, 12, 24 and 48 hours after inoculation. Five individual leaves per time-point were pooled and RNA was extracted from frozen plant material using Ribozol^{Tm} RNA Extraction Reagent (Amresco) according to the manufacturer's protocol. 1µg of total RNA was reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. Quantitative real-time PCR was performed to analyze expression of *HvSKP1-like* in inoculated and mock-inoculated barley leaves using Maxima SYBR Green/ROX qPCR Master Mix (2x) (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 sec at 95°C, 30 sec at 55°C and 1 min at 72°C. A dissociation curve analysis was finally conducted as follows: 1 min at 95°C, 30 sec at 55°C and 30 sec 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'TCTCGTCCCTGAGATTGCCCACAT3' and Ubc2 rev 5'TTTCTCGGGACAGCAACACAATCTTCT3'. HvSKP1-like was amplified with the primers qPCR-HvK2-fw 5'GGAGCGCCAGAAACTAAAGG3' and qPCR-HvK2-rev 5'CCAGAACCTCCATCCCCATT3'. In addition, HvPR-1b was amplified using primers HvPR1b 5'TGGTATAGAGCAGGCCCATAGAA3' and HvPR1b 5'GCTAGGGCGAGCAAGATGAC3'. All Primers were tested for similar amplification efficiency and expression of HvSKP1-like and data were evaluated using MxPro QPCR software (Stratagene).

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FIGURE LEGENDS

Fig. 1 Phylogenetic relationships of plant SKP1-like proteins. The evolutionary history was inferred by 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 Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with 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).

Fig. 2 Targeted yeast-two hybrid assay of HvSKP1-like with 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 media lacking leucine, tryptophan, adenine and histidine (SD-LWAH). Yeast growth on selective media lacking leucine and tryptophan (SD-LW) indicates successful co-transformation. Yeasts in figure a and b were dropped in parallel on the same plate but figure was split for better labelling. Expression of proteins was driven by the ADH1 promotor. CA, constitutively active, DN, dominant negative, WT, wild-type.

Fig. 3 Subcellular localization of HvSKP1-like and *in planta* interaction of HvSKP1-like with HvRBK1. (a) GFP-HvSKP1-like largely co-localizes with mcherry-HvRBK1 in cytoplasmic strands in transiently transformed barley epidermal cells 24 hours after bombardment (hab). (b) GFP-HvSKP1-like showed cytoplasmic localization similar to soluble mcherry that was co-transformed as cytoplasmic and nucleoplasmic marker by 24 hab. Expression of fusion proteins was driven by the CaMV 35S promotor. Confocal images show maximum projection of 23-26 optical sections at 2 µm increments. The scale bar is 20 µm. (c) Quantitative analysis of fluorescence resonance energy transfer (FRET) by acceptor photobleaching in transiently transformed barley epidermal cells 24 hab confirmed interaction of CFP-HvSKP1-like and HvRBK1-YFP *in planta*. All constructs are under the control of the CaMV 35S promotor. Data show mean ± standard error of the mean (SEM) of three independent experiments. In each experiment 10 cells were analyzed. $^{**}P \leq 0.01$ according to an unpaired Student's *t* test.

Fig. 4 Effect of the proteasome inhibitor MG132 and *HvRBK1*-RNAi on protein abundance of CA HvRACB in barley protoplasts 17 hours after transformation. (a) mcherry-CA HvRACB and GFP expressing barley protoplasts were incubated for 3 hours with 10 µM MG132 or DMSO as a control. To quantify fluorescence intensity, mean pixel intensity of mcherry-CA HvRACB was measured at the plasma membrane and normalized against mean pixel intensity of GFP in the nucleus. Fluorescence intensity of MG132 treated protoplasts is significantly increased compared to fluorescence intensity of control DMSO-treated protoplasts. All constructs are under the control of the CaMV 35S promotor. Data show mean ± SEM of four independent experiments. In each experiment 10 protoplasts were analyzed. $^{*}P \leq 0.05$ 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 HvRACBΔCSIL or GFP and either the empty RNAi-vector pIPKTA30N or *HvRBK1*-RNAi respectively. Protein

abundance of GFP-CA HvRACBΔCSIL is increased in protoplasts co-expressing *HvRBK1*-RNAi compared to protoplasts co-expressing the empty RNAi-vector. Protein abundance of GFP is not changed in protoplasts co-expressing pIPKTA30N or *HvRBK*-RNAi respectively. All constructs are under the control of the CaMV 35S promotor. Ponceau S staining of the western blot served as loading control.

Fig. 5 Effect of *HvRBK1*- and *HvSKP1-like*-RNAi on fluorescence intensity of CFP-CA HvRACB, CFP-DN HvRACB or CFP in transiently transformed barley epidermal cells 48 hours after bombardment. (a) Fluorescence intensity of CFP-CA HvRACB is significantly increased upon co-expression with *HvRBK1*-RNAi or *HvSKP1-like*-RNAi but not upon co-expression with the empty RNAi-vector pIPKTA30N. (b) Fluorescence intensity of CFP remains similar upon co-expression with the empty RNAi vector pIPKTA30N, *HvRBK1*-RNAi or *HvSKP1-like*-RNAi respectively. (c) Fluorescence intensity of CFP-DN HvRACB is significantly increased upon co-expression with *HvRBK1*-RNAi or *HvSKP1-like*-RNAi but increase of fluorescence intensity is lower than what was observed for CFP-CA HvRACB. In all experiments, confocal laser scanning microscopy was used to measure mean pixel intensity of CFP, CFP-CA HvRACB or CFP-DN HvRACB at the plasma membrane and mean pixel intensity of the co-expressed soluble mcherry in the nucleus. CFP, CFP-CA HvRACB or DN-HvRACB fluorescence was normalized against mcherry. Data represents mean ± SEM of 3-4 independent experiments. In each experiment 10 cells per combination were analyzed. Different letters indicate significant difference (ANOVA Duncan test, $P \leq 0.05$). All constructs contain the CaMV35S promoter.

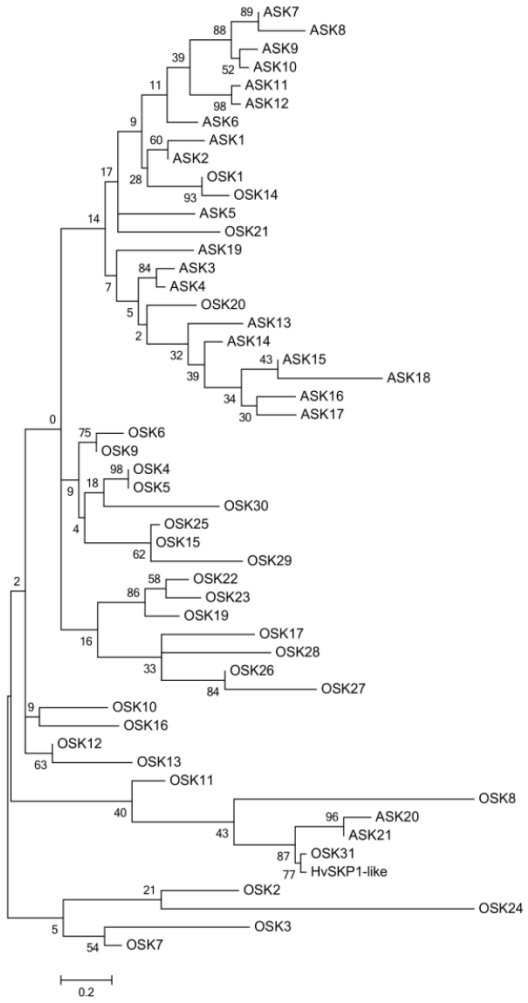
Fig. 6 Functional analysis of HvSKP1-like in the barley-*Blumeria graminis* f. sp. *hordei* (*Bgh*) interaction. (a) Reverse transcription quantitative real-time PCR analysis of *HvSKP1-like* and *HvPR-1b* gene expression in response to *Bgh* infection 12, 24 and 48 hours after inoculation. Gene expression was normalized to the housekeeping gene *HvUbc2* and relative expression is compared to the non-inoculated samples at the same time-point. Data show mean ± SEM of three independent experiments. Gene expression 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 to cells expressing the empty RNAi-vector pIPKTA30N. Barley leaves were inoculated 48 hours after bombardment with *Bgh* and a minimum of 50 interaction sites per experiment were analyzed 48 hours after inoculation with the fungus. Data show mean ± SEM of six independent experiments. $^{*}P \leq 0.05$ according to an unpaired Student's *t* test. All constructs contain the CaMV 35S promoter. (c) Efficiency of *HvSKP1-like*-RNAi directed against the *GFP-HvSKP1-like* expression construct in transiently transformed barley epidermal cells 48 hours after bombardment. In three independent experiments a minimum of 50 mcherry fluorescence displaying cells were analyzed for accumulation of GFP-HvSKP1-like. The number of cells expressing both mcherry and GFP-HvSKP1-like was significantly reduced when we co-transformed with *HvSKP1-like*-RNAi compared to cells co-transformed with the empty RNAi vector *pIPKTA30N*. All constructs are under the control of the CaMV 35S promotor. Data show mean ± SEM. $^{**}P \leq 0.01$ according to an unpaired Student's *t* test.

SUPPORTING INFORMATION LEGENDS

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 hours 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. Expression of fusion proteins was driven by the CaMV 35S promotor. Confocal image shows selected single optical sections. The scale bar is 20 µm.

Fig. S3 Effect of *HvRBK1*- and *HvSKP1-like*-RNAi on fluorescence intensity of CFP-WT HvRACB in transiently transformed barley epidermal cells 48 hours after bombardment. Fluorescence intensity of CFP-WT HvRACB is significantly increased upon transformation of *CFP-WT HvRACB* with *HvRBK1*-RNAi or *HvSK*P1-*like*-RNAi. Data represents mean ± SEM of 4 independent experiments. In each experiment 10 cells per combination were analyzed. Different letters indicate significant difference (ANOVA Duncan test, $P<0.05$).



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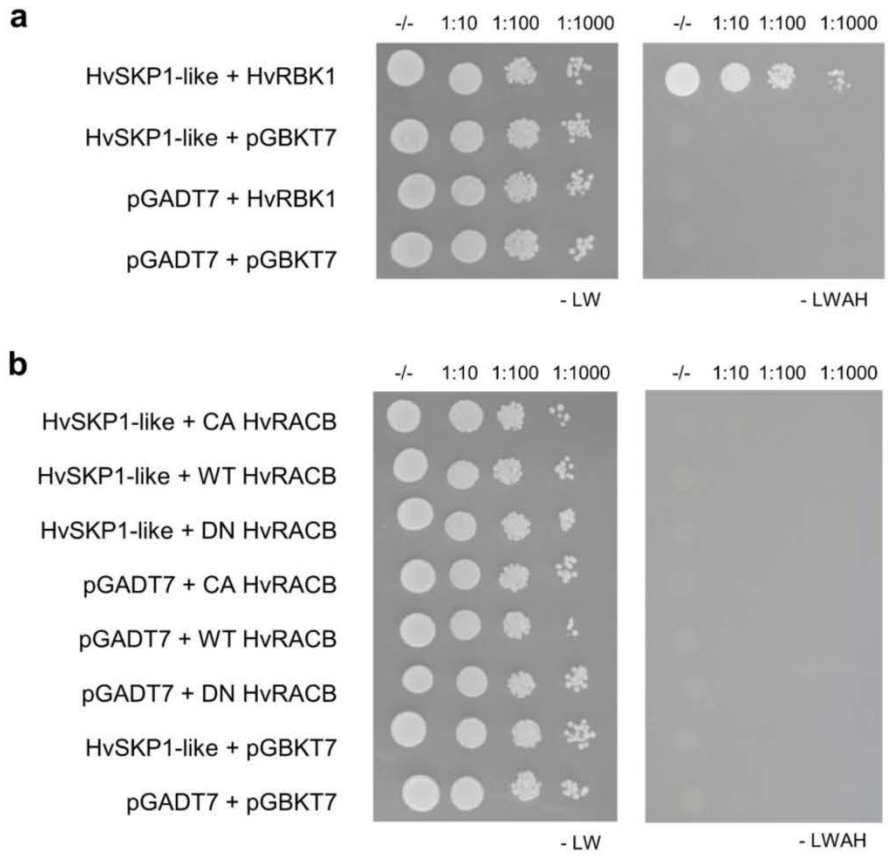

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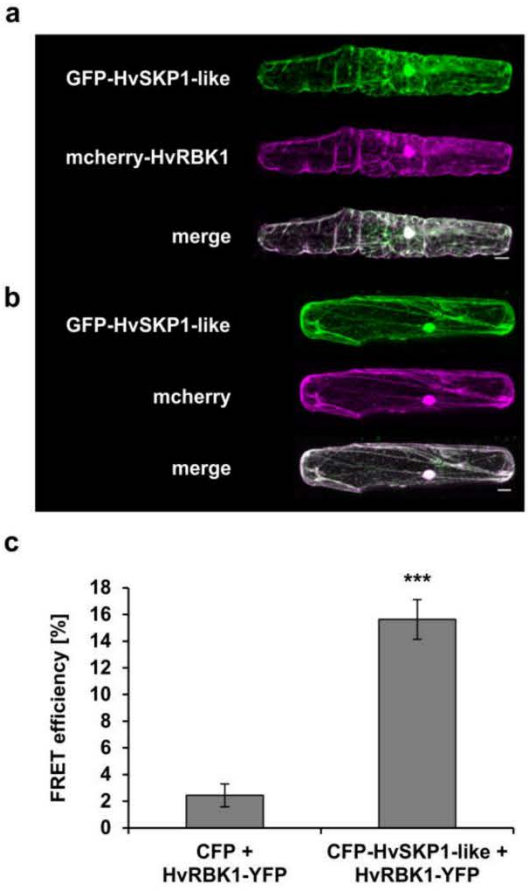

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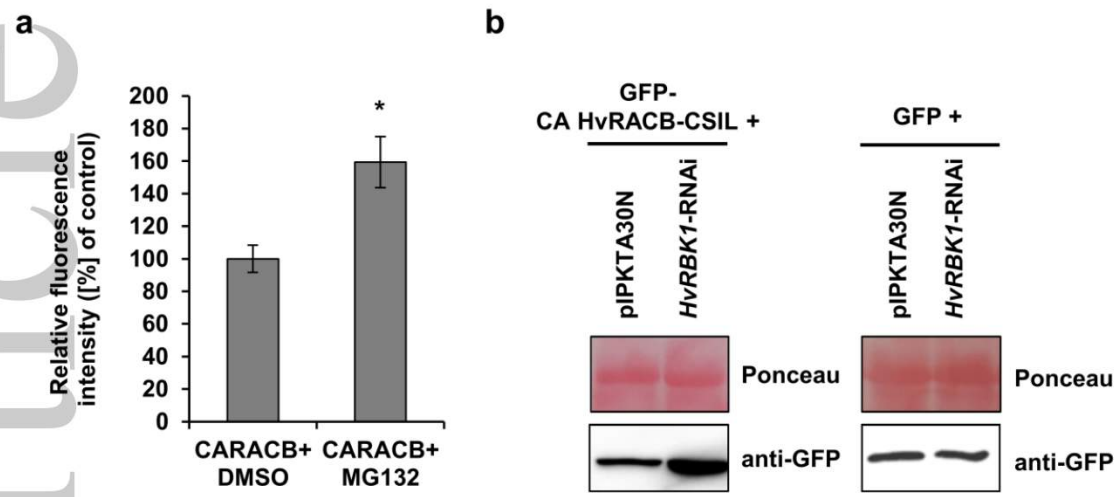

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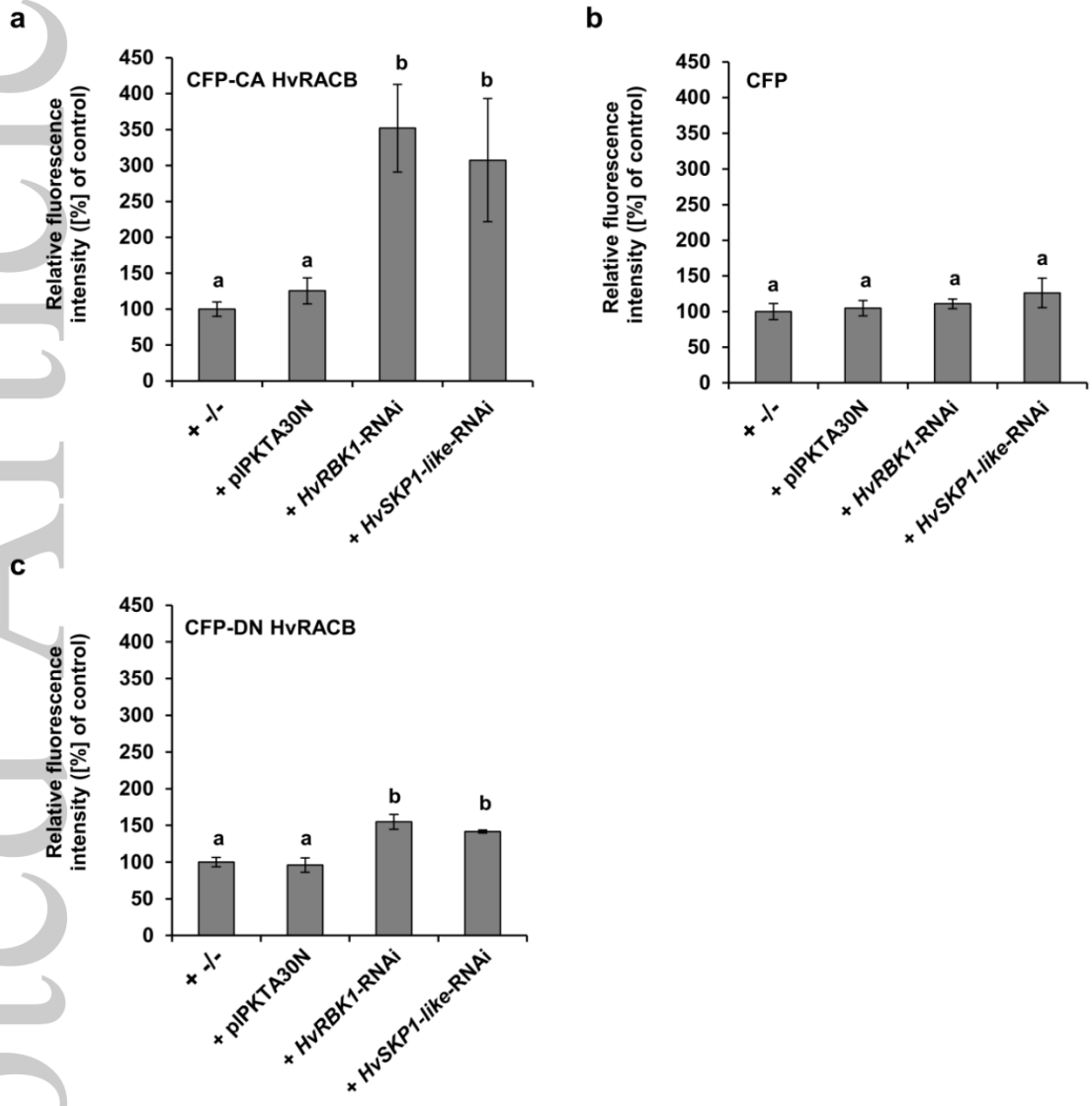

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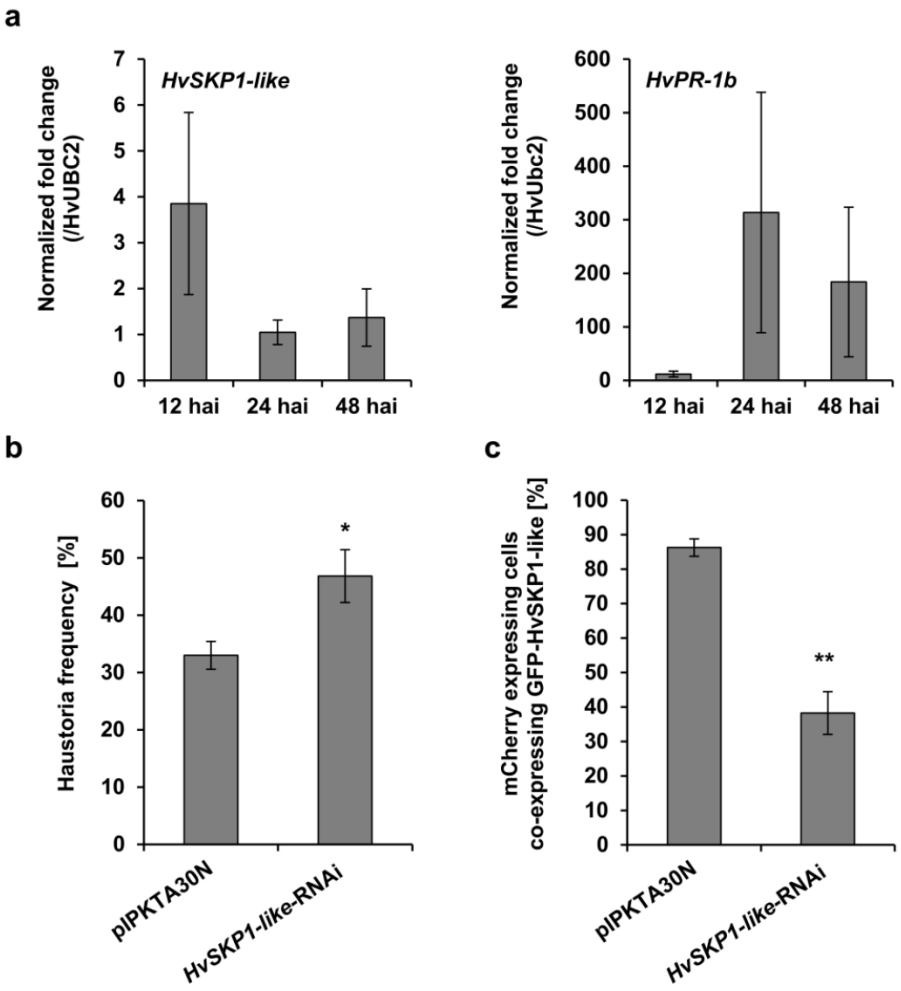

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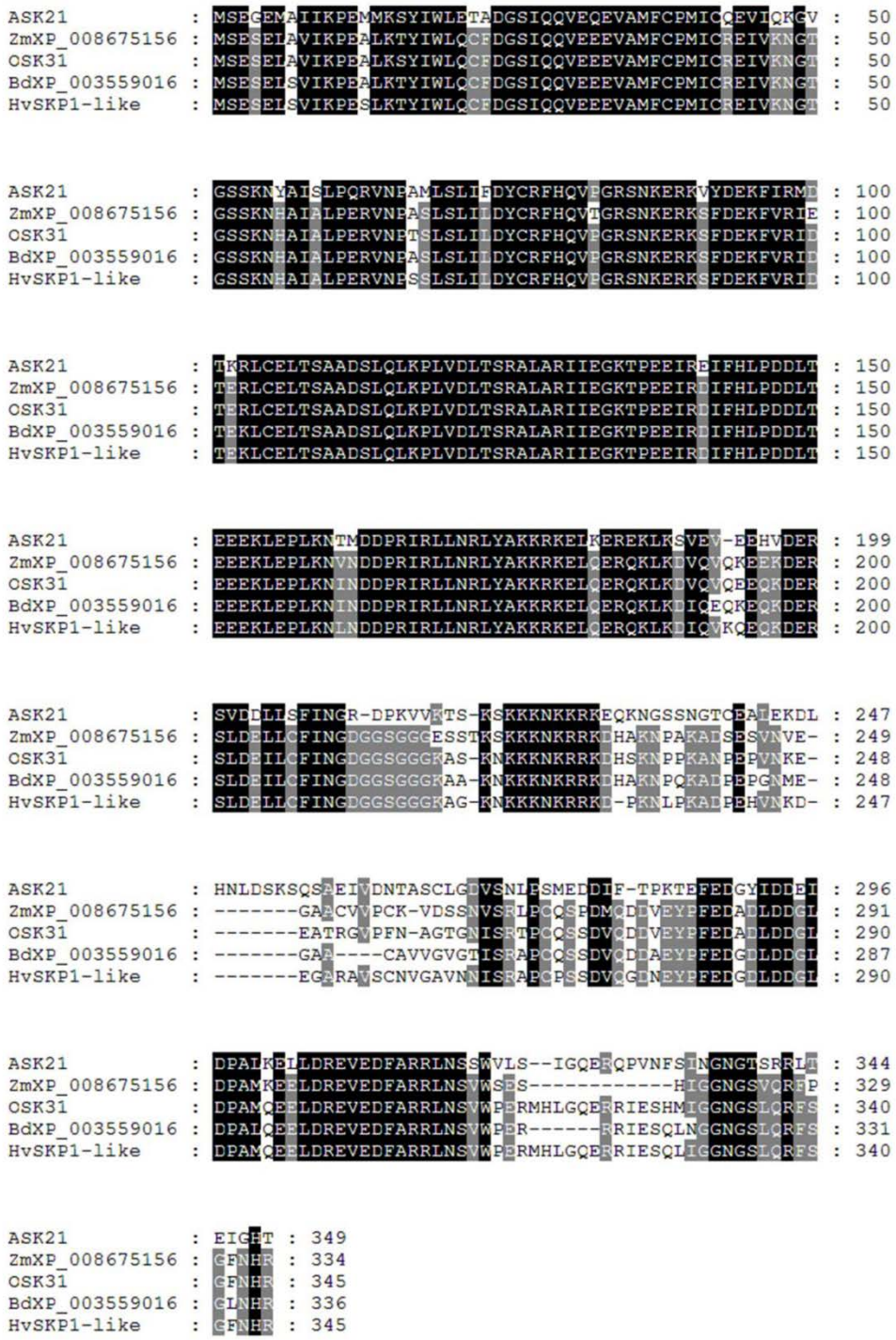
Supplemental Data Reiner *et al.*, 2015a



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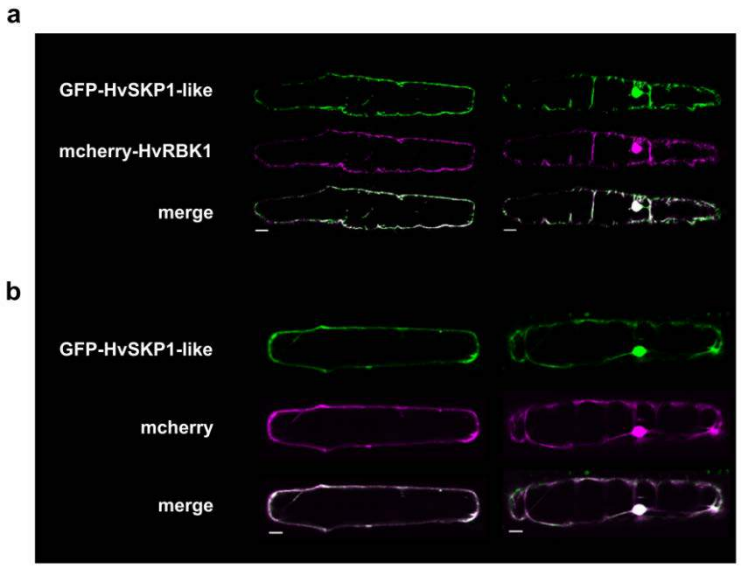

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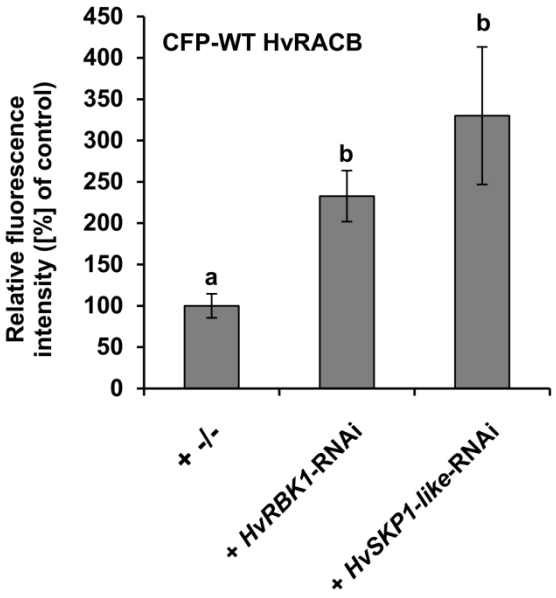

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5.3. Reiner *et al.*, 2015b

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ORIGINAL PAPER

The *Arabidopsis* ROP-activated receptor-like cytoplasmic kinase RLCK VI_A3 is involved in control of basal resistance to powdery mildew and trichome branching

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Abstract
Key message **The *Arabidopsis* receptor-like cytoplasmic kinase AtRLCK VI_A3 is activated by AtROPs and is involved in trichome branching and pathogen interaction.**
Abstract Receptor-like cytoplasmic kinases (RLCKs) belong to the large superfamily of receptor-like kinases, which are involved in a variety of cellular processes like plant growth, development and immune responses. Recent studies suggest that RLCKs of the VI_A subfamily are possible downstream effectors of the small monomeric G proteins of the plant-specific Rho family, called 'Rho of plants' (RAC/ROPs). Here, we describe *Arabidopsis thaliana* AtRLCK VI_A3 as a molecular interactor of AtROPs. In *Arabidopsis* epidermal cells, transient co-expression of plasma membrane located constitutively activated (CA) AtROP4 or CA AtROP6 resulting in the recruitment of green fluorescent protein-tagged AtRLCK VI_A3 to the cell periphery. Intrinsic kinase activity of AtRLCK VI_A3 was enhanced in the presence of CA AtROP6 in vitro and further suggested a functional interaction between the proteins. In the interaction of the biotrophic powdery mildew fungus *Erysiphe cruciferarum* (*E. cruciferarum*) and its host plant *Arabidopsis*, *Atrlck VI_A3* mutant lines supported enhanced fungal reproduction. Furthermore *Atrlck VI_A3* mutant lines showed slightly reduced size and an increase in trichome branch number compared to wild-type plants. In summary, our data suggest a role of the AtROP-regulated AtRLCK VI_A3 in basal resistance to *E. cruciferarum* as well as in plant growth and cellular differentiation during trichome morphogenesis. Results are discussed in the context of literature suggesting a function of RAC/ROPs in both resistance and susceptibility to pathogen infection.

Keywords Receptor-like cytoplasmic kinase (RLCK) · RAC/ROP GTPase · *Arabidopsis thaliana* · *Erysiphe cruciferarum* · Trichome

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Introduction

Receptor-like cytoplasmic kinases (RLCKs) belong to the receptor-like kinase (RLK) superfamily that is involved in a variety of biological processes like plant growth, development and immune responses (Afzal et al. 2008). RLCKs share a conserved serine/threonine (Ser/Thr) kinase domain (Afzal et al. 2008; Gish and Clark 2011) together with the large and diverse transmembrane RLK protein family. Together over 600 RLKs and RLCKs exist in *Arabidopsis* and over 1,000 in rice (Shiu et al. 2004). In contrast to RLKs, RLCKs do not possess an extracellular and transmembrane domain resulting in their cytoplasmic localization. However, some RLCKs are anchored to the plasma membrane through myristoylation motifs (Murase et al. 2004; Tang et al. 2008; Veronese et al. 2006). As protein kinases, RLCKs transmit intracellular signals through phosphorylation of target proteins or through RLK

complex-mediated transphosphorylation events. Although little is known about their precise biological functions, several RLCKs were reported to be, either alone or in concert with RLKs, involved in plant development and immunity (Lin et al. 2013). In this regard, RLCKs play a role in the embryonic patterning process (Bayer et al. 2009), self-incompatibility (Murase et al. 2004), organ separation (Burr et al. 2011), ethylene (Laluk et al. 2011) and brassinosteroid signaling (Sreeramulu et al. 2013; Tang et al. 2008). In plant immunity, RLCKs are involved in both pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI) (Lu et al. 2010; Swiderski and Innes 2001; Zhang et al. 2010). RLCKs are divided into 13 subfamilies (RLCK I-XIII) (Shiu et al. 2004). *Arabidopsis* RLCKs of the subfamily VI_A interact with the plant-specific Rho family of small monomeric G proteins called 'Rho of plants' (RAC/ROPs) (Jurca et al. 2008; Molendijk et al. 2008). A function of the VI subfamily of RLCKs as RAC/ROP downstream signaling effectors in plants was supported by RAC/ROP GTPase-dependent activation of RLCKs in *Arabidopsis, Medicago trunculata* (*M. trunculata*) and barley (*Hordeum vulgare* L) (Dorjgotov et al. 2009; Huesmann et al. 2012). RAC/ROP proteins regulate processes like cell development, hormone signaling, cytoskeleton rearrangement and plant disease resistance or susceptibility via various downstream effector proteins including RLCKs (Berken 2006; Nibau et al. 2006). RAC/ROPs act as molecular switches, transducing extracellular signals into intracellular responses by shuttling between an inactive GDP-bound and an activated GTP-bound state. Several regulatory molecules including guanine nucleotide exchange factors (ROPGEFs), GTPase activating proteins (ROPGAPs) and guanine nucleotide dissociation inhibitors (GDIs) adjust the balance between these two forms (Nibau et al. 2006). Furthermore, RAC/ROPs are divided into two phylogenetic subgroups (type I and type II) depending on their posttranslational lipid modifications, which anchor the active proteins in the plasma membrane (Winge et al. 2000). Besides the eleven characterized RAC/ROP proteins in *Arabidopsis* (Li et al. 2001), six and seven RAC/ROPs are described in barley (Schultheiss et al. 2003) and rice (Chen et al. 2010), respectively. In *Arabidopsis*, AtROP4 and AtROP6 are involved in the auxin binding protein 1 (ABP1)-mediated formation of lobed pavement cells through organization of the actin and microtubule cytoskeleton as well as in pathogen response (Fu et al. 2005, 2009; Poraty-Gavra et al. 2013; Xu et al. 2010). In addition, AtROP4 and AtROP6 are described as activators of AtRLCKs. Nevertheless, the functional knowledge about RAC/ROP-regulated RLCK signaling in plants is limited. Recently, the barley RLCK ROP binding kinase1 (HvRBK1), which is closely related to AtRLCK VI_A3, was shown to serve as RAC/ROP effector in the barley–barley powdery mildew interaction (Huesmann et al. 2012). HvRBK1 interacted with the susceptibility factor HvRACB, which is required for successful invasion of intact barley epidermal cells by the biotrophic fungus *Blumeria graminis* f. sp. *hordei*, the causal agent of powdery mildew disease (Hoefle et al. 2011; Schultheiss et al. 2002). All RLCKs described as RAC/ROP interactors are members of the RLCK VI subfamily that is divided into group A and B based on their domain structure (Jurca et al. 2008; Molendijk et al. 2008). In *Arabidopsis*, the RLCK VI subfamily shows upregulation of gene expression under abiotic stress or hormone treatments as well as in response to the pathogens *Botrytis cinerea* and *Phytophthora infestans* (Jurca et al. 2008; Molendijk et al. 2008). Here, we describe the HvRBK1-related *Arabidopsis* AtRLCK VI_A3 as direct molecular interactor of *Arabidopsis* RAC/ROPs. AtRLCK VI_A3 interacts with AtROPs in yeast and shows increased kinase activity in the presence of constitutively activated (CA) AtROP6 in vitro. Furthermore, *AtRLCK VI_A3* mutant lines show a reduced growth phenotype and an increased number in trichome branching. Finally, a slight increase in susceptibility towards the powdery mildew fungus *Erysiphe cruciferarum* (*E. cruciferarum*) was observed in *AtRLCK VI_A3* mutant lines suggesting a function of AtRLCK VI_A3 in the *Arabidopsis*-powdery mildew pathosystem.

Materials and methods

Plants, pathogen and inoculation

All experiments were performed in the *Arabidopsis thaliana* ecotype Columbia (Col-0). *AtRLCK VI_A3-1* (SALK_148741.46.10.x) and *AtRLCK VI_A3-2* (SALK_010841C) were obtained from the Nottingham Arabidopsis Stock Centre (NASC) and selected for homozygosity by genotyping. The *AtRLCK VI_A3-2* allele was complemented by the transgenic construct 35S:AtRLCK VI_A3. Plant transformation was performed using the floral dip method (Clough and Bent 1998) as described previously (Weis et al. 2013). For uniform germination, seeds were kept in 0.05 % Agarose at 4 °C in the dark for 2 days before sowing them on soil. Plant growth took place in a growth chamber at 22 °C and a 10 h photoperiod with 120 µmol m^{-2} s^{-1} and 65 % relative humidity.

Erysiphe cruciferarum (*E. cruciferarum*) was grown on Col-0 and on *phytoalexin deficient4 (pad4)* mutants in a growth chamber at 22 °C and a 10 h photoperiod with 120 µmol m^{-2} s^{-1} and 65 % relative humidity. For microscopic analysis of disease progression, 5–7-week-old *Arabidopsis* plants were inoculated with *E. cruciferarum* in

a density of 3–5 spores mm^{-2}. Inoculation density for quantitative real-time PCR (qPCR)-based quantification of *E. cruciferarum* infection ranged between 5 and 15 spores mm^{-2} on 3-week-old *Arabidopsis* seedlings.

Cloning procedures

The AtRLCK VI_A3 cDNA fragment was amplified from an *Arabidopsis* cDNA pool. All constructs for the microscopic subcellular localization studies were based on the pUC18-based plant expression vector pGY1 (Schweizer et al. 1999) that contains the CaMV 35S promoter and CaMV 35S terminator site. The fluorescence tag containing constructs pGY1-*GFP* and pGY1-*RFP* have been described elsewhere (Hoefle et al. 2011). For fusion of the AtRLCK VI_A3 open reading frame (ORF) to GFP, the AtRLCK VI_A3 coding sequence was PCR amplified using primers At5g65530SmaI fwd 5′AACCCGGG CTATGGCTGTTGAAGAGATG3′ and At5g65530SmaI rev 5′GGCCCGGGACTCCATTAAGAGCTGTCTATG3′. The resulting fragment was cloned into the *Sma*I site of pGY1-*GFP* o. stop to obtain a C-terminal fusion construct of AtRLCK VI_A3 with GFP. The constitutively active (CA, G15V) and dominant negative (DN, T20N) AtROP mutants were obtained by site-directed mutagenesis using overlap extension PCR. ORF of CA *AtROP4* and CA *AtROP6* was amplified using primer pairs Rop4BamHI5′cDNAp 5′GAATTTGCTGGATCCATGAGTGCTTC GAG3′ and M13rev 5′AACAGCTATGACCATGA3′or Rop6BamHIfwd 5′AGGATCCATGAGTGCTTCAAGGT TTATC3′ and Rop6SalIrev 5′CCGCGGGATGTCGACTC AGAGTATAGAAC3′, respectively, and cloned into pGY1 via *Bam*HI and *Sal*I restriction sites. pGY1-DN *AtROP6* was obtained by cutting out *CFP* from pGY1-*CFP* DN *AtROP6* via *Bam*HI restriction site followed by subsequent religation of the vector. The binary vector pLH6000 (GenBank accession number AY234328) was used for the generation of *AtRLCK VI_A3* mutant complementation lines. *AtRLCK VI_A3* was cut out via *Kpn*I restriction site from pGY1-*AtRLCK VI_A3* and ligated into pLH6000 containing 35S promoter. The construct was transferred into the *Agrobacterium tumefaciens* (*A. tumefaciens*) strain AGL-1 as it is described by Weis et al. (2013). Constructs for the yeast-two hybrid assays were based on the vectors pGBKT7 and pGADT7 (Clontech). The *AtRLCK VI_A3* gene was cloned into pGADT7 using the primers At5g65530_Eco_for 5′TTTGAATTCATGGCTGTTGAA GAGATGGAG3′ and At5g65530_Bam_rev 5′AAAGGA TCCTTACTCCATTAAGAGCTGTCTATG3′ and the restriction sites *Eco*RI and *Bam*HI. Generation of the different *Arabidopsis* and barley *RAC/ROP* constructs based on the vector pGBKT7 was previously described (Hoefle et al. 2011). To allow the purification of 6x-His-tagged proteins from bacterial cultures, *AtRLCK VI_A3* was inserted into pET28a (Novagen) while *AtROP6* and its mutant forms were cloned into pET26b (Novagen), respectively, (Dorjgotov et al. 2009).

Yeast-two hybrid assays

The *Saccharomyces cerevisiae* (*S. cerevisiae*) strain AH109 (Clontech) was used for targeted yeast-two hybrid interaction studies. Yeast transformation was carried out following the yeast protocols handbook (Clontech). Selection of yeast transformants was conducted on selective media lacking leucine and tryptophan (SD-LW), selection for protein interaction was performed on selective media lacking leucine, tryptophan, adenine and histidine (SD-LWAH).

Protein localization *in planta*

For subcellular localization studies, *Arabidopsis* epidermal cells were transiently transformed using microprojectile bombardment (Schweizer et al. 1999). Leaves of 4-week-old *Arabidopsis* plants were bombarded with DNA-coated tungsten particles (50 mg/ml, Ø 1.1 µm) using the particle inflow gun (Finer et al. 1992). Bombardment was performed under 0.85 bar vacuum and 8.5 bar helium gas pressure. For each shot, 0.9 µg DNA of the construct of interest or 0.5 µg of the transformation marker RFP is delivered into *Arabidopsis* epidermal cells. Protein localization is analyzed 24 h after bombardment using confocal laser scanning microscopy (Leica TCS SP5; Leica Microsystems). GFP fluorescence of tagged AtRLCK VI_A3 was excited with 488 nm laser. Emission was detected at 500–550 nm. RFP fluorescence was excited at 561 nm and detected at 571–610 nm. Images were obtained using the LAS AF software (Leica) and processed using PhotoImpact X3 (Ulead).

Kinase activity measurements

For recombinant protein production either the ArcticExpress (DE3) RIL competent cells (Agilent Technologies) or the *Escherichia coli* strain Rosetta™ [BL21 (DE3)/(pLysS)] (Novagen) were used as described elsewhere (Dorjgotov et al. 2009, Huesmann et al. 2012). Subsequent protein purification was carried out using Ni-IDA-Agarose (Biontex) according to the manufacturer's protocol. Contaminating chaperons derived from the ArcticExpress (DE3) RIL competent cells were removed as described elsewhere (Joseph and Andreotti 2008). For the in vitro kinase activity measurements, the reaction mix was set up as 4 pmol of purified AtRLCK VI_A3, 1–100 pmol of purified AtROP6, 20 mM Tris–HCL, pH 7.6, 5 mM of

$MgCl_2$, 50 mM of NaCl, 1 mM of DTT, 10 µM of ATP, 0.2 MBq [$_{\gamma}$-^{32}P] of ATP and 0.25 µg/µl of myelin basic protein. After incubation for at least 30 min at room temperature, the reactions were stopped by adding 5 µl of 5× SDS loading buffer. Proteins were separated on SDS–polyacrylamide gels that were subsequently stained by Coomassie Brilliant Blue. After drying, the gels were exposed to X-ray films using standard methods. Immunoblotting was performed using standard protocols as describe earlier (Dorjgotov et al. 2009).

Semi-quantitative RT-PCR

Total RNA was extracted from leaves of 7-week-old *Arabidopsis* plants. Leaves of three individual plants were pooled and RNA was extracted with the Spectrum™ Plant Total RNA Kit (Sigma-Aldrich). 1 µg of total RNA was reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen) following the manufacturer's instructions. Ubiquitin 5 (UBQ5, At3g62250) was used as constitutively expressed reference gene and amplified with the specific primers AtUBQ5fwd 5′CCAAGCCGAAGAAGATCAAG3′ and AtUBQ5rev 5′ACTCCTTCCTCAAACGCTGA3′. To amplify the *AtRLCK VI_A3* transcript of the complete coding sequence, the primers At5g65530_Eco_for 5′TTTGAATTCATGGCTGTTGAAGAGATGGAG3′ and At5g65530_Bam_rev 5′AAAGGATCCTTACTCCATTAAGAGCTGTCTATG3′ were used. The PCR program was as follows: 30 s at 98 °C followed by 28–40 cycles of 10 s at 98 °C, 15 s at 55 °C and 50 s at 72 °C. Final extension was performed for 5 min at 72 °C.

Analysis of powdery mildew development

For microscopic analysis of disease progression, *E. cruciferarum* infected leaves of 7–8-week-old *Arabidopsis* plants were harvested 5 days after inoculation and cleared in ethanol acetic acid solution [6:1(v/v)]. Fungal structures were stained with acetic ink [10 % blue ink (v/v) in 25 % acetic acid] and in 4–5 independent experiments, 10 colonies per leaf of in total ≥13 leaves of each genotype were evaluated using bright-field microscopy. Statistical analysis (ANOVA Duncan) was performed using the SPSS version 21 software (IBM). For qPCR-based quantification of *E. cruciferarum* infection strength, genomic DNA (gDNA) was isolated from five pooled seedlings per genotype 5 days after infection with the fungus. gDNA was isolated as previously described (Fraaije et al. 1999) except that the extraction buffer additionally included 5 mM 1,10 phenanthroline monohydrate and 2 % (w/v) polyvinilpyrrolidone. qPCR was performed in 10 µl volume using Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific) according to the manufacturers protocol. In each triplicate reaction, 100 ng of DNA and a final primer concentration of 10 µM were used. qPCR was performed on the Mx3005P Real-Time PCR system (Stratagene) and cycling conditions were as follows: 10 min at 95 °C, followed by 40 cycles of 30 s at 95 °C, 30 s at 54 °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. For the quantitation of gDNA, primers for a β-tubulin gene of *E. cruciferarum* and primers for the RbcS gene of *Arabidopsis* were used as it is described elsewhere (Engelsdorf et al. 2013). Data were analyzed using MxPro QPCR software (Stratagene).

Analysis of trichome branching

Trichome branching was analyzed under the bright-field microscope on 7–8-week-old discolored *Arabidopsis* leaves. A minimum of 800 trichomes from 10 different plants per genotype were analyzed. ANOVA followed by Duncan test using SPSS version 21 software (IBM) was performed to show significant differences.

Results

AtRLCK VI_A3 interacts with barley and *Arabidopsis* RAC/ROPs in yeast

In a previous study, we showed that the barley HvRBK1 interacts with the CA but not the DN forms of barley RAC/ROPs in yeast and *in planta*. HvRBK1 is closely related to the VI_A subfamily of *Arabidopsis* RLCKs and shows highest similarity to AtRLCK VI_A3 (Huesmann et al. 2012). To test, whether AtRLCK VI_A3 might show a similar specificity of interaction with RAC/ROPs as HvRBK1, we performed a heterologous targeted yeast-two hybrid assay. AtRLCK VI_A3 interacted with the CA and wild-type (WT) forms of HvRACB and HvRAC1 but not with their DN forms (Fig. 1a). These results reflect the interaction patterns of HvRBK1 and barley RAC/ROPs in yeast and *in planta* (Huesmann et al. 2012) and point towards a possible functional conservation of these RLCKs as interaction partners of RAC/ROPs in *Arabidopsis* and barley. The interaction of AtRLCK VI_A3 with AtROPs was tested in a targeted yeast-two hybrid assay (Fig. 1b). In addition to those AtROPs used by Dorjgotov et al. (2009), we included AtROP3, AtROP5 and AtROP7 in our yeast-two hybrid matrix. Among the 10 tested wild-type forms of *Arabidopsis* ROPs, AtRLCK VI_A3 interacted with type I AtROP2, AtROP4, AtROP5, AtROP6 as well as with type II AtROP11. Similar to HvRBK1, AtRLCK VI_A3 shows no exclusive specificity for type I or type II RAC/ROPs.

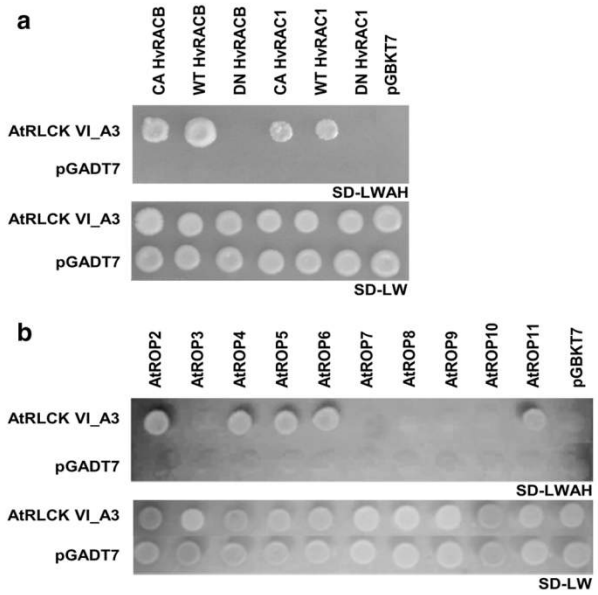

Fig. 1 Targeted yeast-two hybrid assay of AtRLCK VI_A3 with different barley and *Arabidopsis* RAC/ROPs. **a** AtRLCK VI_A3 interacts with the constitutively active (CA) and wild-type forms (WT) of barley RACB (HvRACB) and RAC1 (HvRAC1) but not with their dominant negative (DN) forms. **b** AtRLCK VI_A3 interacts with AtROP2, AtROP4, AtROP5, AtROP6 and AtROP11 in yeast. Yeast growth on selective media lacking leucine, tryptophan, adenine, histidine (SD-LWAH) shows positive interaction. Selective media lacking leucine and tryptophan (SD-LW) indicates successful co-transformation

Constitutively active AtROPs recruit AtRLCK VI_A3 to the cell periphery

AtRLCK VI_A3 does not possess any obvious protein targeting or myristoylation motifs. To examine the subcellular localization of AtRLCK VI_A3 in vivo, we fused a green fluorescence (GFP) tag to the C-terminal part of AtRLCK VI_A3. The construct, together with the red fluorescent protein (RFP) as cytoplasmic and nucleoplasmic marker, was transiently transformed into *Arabidopsis* epidermal cells using microprojectile bombardment. AtRLCK VI_A3 fluorescence was detected in the nucleoplasm and cytoplasmic strands 24 h after bombardment (hab) by confocal laser scanning microscopy. This was obvious from the co-localization of AtRLCK VI_A3-GFP with RFP in the nucleus and cytoplasmic strands, which manifested as an extensive whitish signal in the merged confocal channels (Fig. 2). Co-expression of either CA AtROP4 or CA AtROP6 strongly diminished labeling of cytoplasmic strands by AtRLCK VI_A3-GFP. Instead fluorescent AtRLCK VI_A3-GFP was enriched at the cell periphery enveloping the cytoplasm. Nuclear fraction of AtRLCK VI_A3-GFP signals was not strongly affected by co-expression of CA AtROP4 or CA AtROP6. Thus, AtRLCK VI_A3 was partially recruited to the cell periphery by the activated plasma membrane-associated AtROPs. This suggests that protein interaction of CA AtROPs with AtRLCK VI_A3 sequestered AtRLCK VI_A3-GFP at the plasma membrane. By contrast, co-expression of DN AtROP6 did not alter cytoplasmic localization of AtRLCK VI_A3-GFP.

AtROP6 enhances AtRLCK VI_A3 activity in vitro

It was previously shown, that CA or GTP-loaded RAC/ROPs can activate *Arabidopsis, M. trunculata* or *H. vulgare* kinases which fall into the RLCK clade VI_A (Dorjgotov et al. 2009; Huesmann et al. 2012). To test the ability of AtROP6 to activate AtRLCK VI_A3, in vitro kinase assays were performed. AtRLCK VI_A3 was able to phosphorylate the myelin basic protein (MyBP) in vitro in the absence of AtROP6 indicating a basal phosphorylation activity of the kinase. However, in the presence of WT or CA AtROP6, this basal activity of AtRLCK VI_A3 increased while the presence of DN AtROP6 had no significant effect on kinase activity. In addition, MyBP-phosphorylating activity of AtRLCK VI_A3 increased in the presence of CA AtROP6 in a dose-dependent manner (Fig. 3a, b). These findings support the hypothesis that AtRLCK VI_A3 is a downstream signaling effector of AtROPs.

Atrlck VI_A3 mutants are more susceptible to the biotrophic fungus *E. cruciferarum*

We next investigated whether AtRLCK VI_A3 has similar function in the plant–fungal pathogen interaction as it was described for HvRACB and HvRBK1 in the barley–barley powdery mildew interaction (Huesmann et al. 2012). For this purpose, two homozygous AtRLCK VI_A3 T-DNA insertion lines, *Atrlck VI_A3-1* and *Atrlck VI_A3-2,* which contain the T-DNA insertion in the fourth and second exon, respectively, were genotyped (see below Fig. 5a) and analyzed for altered infection phenotypes. *Atrlck VI_A3* mutant seedlings were inoculated with conidia of the biotrophic fungus *E. cruciferarum* and 5 days after inoculation (dai) genomic DNA (gDNA) was isolated for subsequent qPCR (qPCR) analysis. The amount of fungal gDNA relative to plant gDNA was determined and compared to the wild type. Both *Atrlck VI_A3* mutant lines showed a slight but significant increase in fungal DNA compared to the wild-type plants (Fig. 4a). Furthermore, the obtained qPCR data correlated well with the microscopic analysis of disease progression. The number of conidiophores per colony on *Atrlck VI_A3* mutant lines was higher at 5 dai in comparison to that on the wild-type plants (Fig. 4b). In

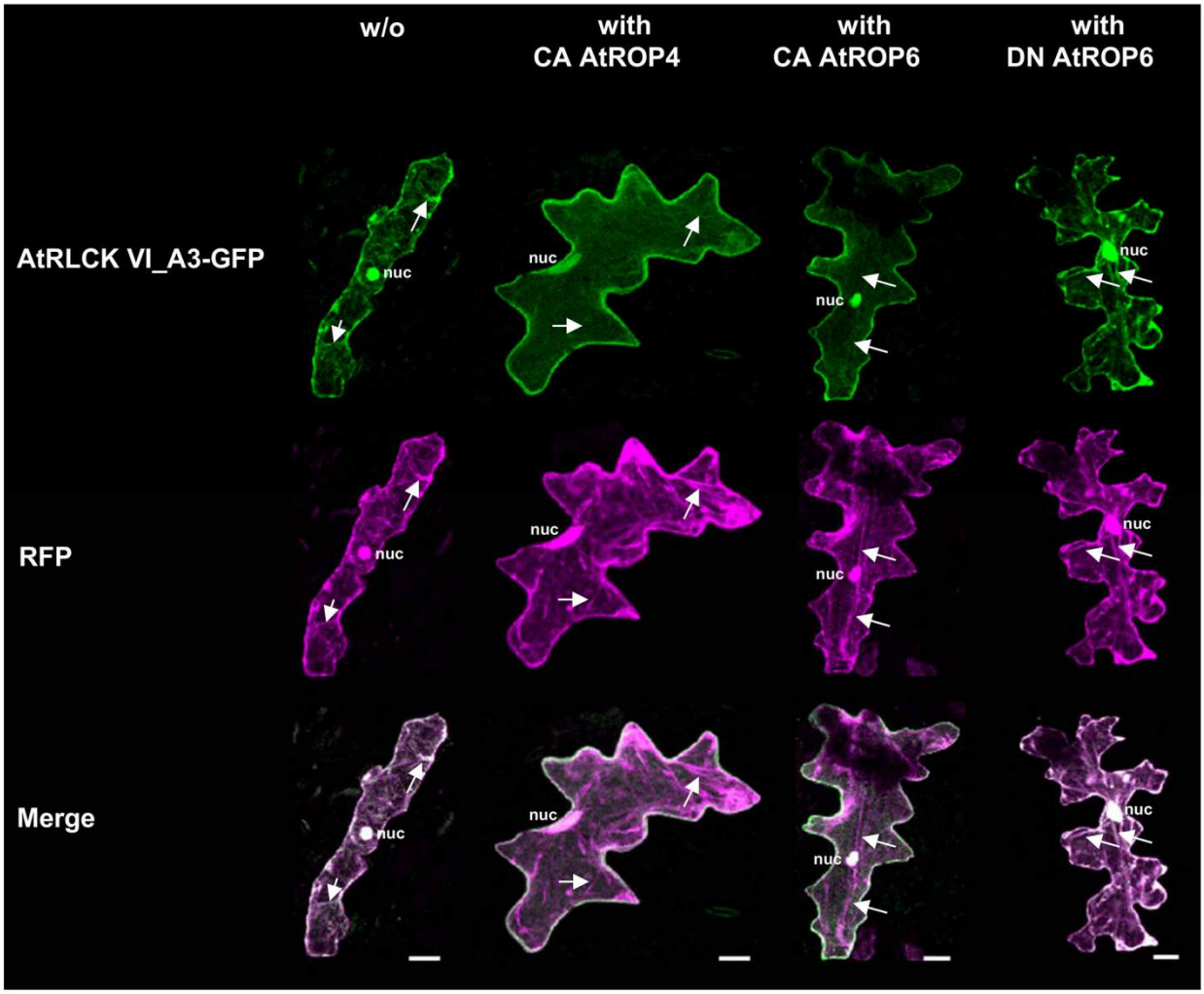

Fig. 2 Subcellular localization of AtRLCK VI_A3 in transiently transformed *Arabidopsis* epidermal cells 24 h after bombardment (hab). AtRLCK VI_A3-GFP co-localizes with soluble RFP in cytoplasmic strands (*arrows*) and nucleoplasm. Upon co-expression of either CA AtROP4 or CA AtROP6, AtRLCK VI_A3-GFP is recruited to the cell periphery/plasma membrane. Co-expression of DN AtROP6 does not alter cytoplasmic localization of AtRLCK VI_A3-GFP. Confocal images are maximum projections of 20–30 optical sections at 2 µm increments. The *scale bar* is 20 µm in all pictures. *nuc* nucleus

summary, increased susceptibility of *Atrlck VI_A3* mutant lines to *E. cruciferarum* suggests a function of AtRLCK VI_A3 in basal resistance to powdery mildew. To confirm the role of AtRLCK VI_A3 in the *Arabidopsis–E. cruciferarum* interaction, the ectopic expression construct CaMV35S::AtRLCK VI_A3 was introduced in the *Atrlck VI_A3-2* mutant background for complementation. Expression of the transgene in 7-week-old plants was analyzed by semi-quantitative RT-PCR of the complete coding sequence (Fig. 5a). Abundance of AtRLCK VI_A3 transcript was clearly increased in both complemented *Atrlck VI_A3* mutant lines (*Atrlck VI_A3-2-C1* and *Atrlck VI_A3-2-C2*) compared to the wild type. In contrast, no corresponding transcript could be amplified from the *Atrlck VI_A3* mutant lines. Microscopic analysis of disease progression showed that the development of conidiophores per colony on the complemented *AtRLCK VI_A3-2* mutant line resembles that on the wild-type plants (Fig. 5b). This suggests that overexpression of AtRLCK VI_A3 in the *Atrlck VI_A3-2* mutant background rescued the pathogenesis phenotype.

Atrlck VI_A3 mutant lines show growth retardation and increased trichome branch number

When we characterized the *Atrlck VI_A3* mutant lines they showed a retarded growth phenotype in comparison to wild-type plants (Fig. 6a). This growth retardation could be

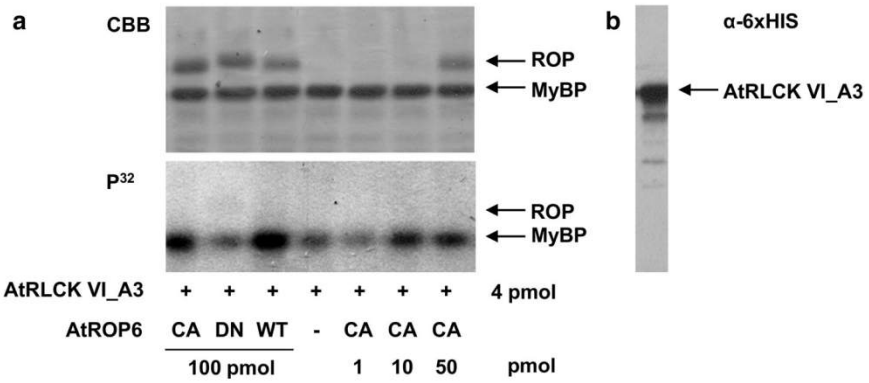

Fig. 3 In vitro activity of AtRLCK VI_A3 in the presence and absence of AtROP6. **a** The in vitro myelin basic protein (MyBP) phosphorylating activity of AtRLCK VI_A3 is increased in the presence of WT and CA AtROP6. Moreover, MyBP-phosphorylating activity of AtRLCK VI_A3 is increased in a dose-dependent manner of CA AtROP6. Protein loading is shown by Coomassie Brilliant Blue (CBB) staining. Autoradiographic detection of phosphorylated MyBP is shown in the *lower part*. **b** An aliquot from the kinase assay mixture (before adding AtROPs) was used for Western blot to show the presence of AtRLCK VI_A3

also complemented by overexpression of AtRLCK VI_A3 in the *Atrlck VI_A3-2* mutant background. Trichomes are easily accessible model to investigate changes in cellular differentiation. To further explore the function of AtRLCK VI_A3 in *Arabidopsis* polar cell growth, we analyzed the trichome branch number of the different genotypes (Fig. 6b). Usually leaf trichomes have three branches and only a small percentage of trichomes possess four branches. *Atrlck VI_A3* mutant lines showed an increase in the frequency of trichomes with four branches compared to wild-type plants. Complementation of *Atrlck VI_A3* via *AtRLCK VI_A3* overexpression restored the frequency of trichomes with three branches to wild-type level. Introduction and rescue of the trichome branch phenotype suggest a role of AtRLCK VI_A3 in cellular differentiation during trichome morphogenesis.

Discussion

Within the last years, it became evident that RLCKs of the VI_A subfamily are possible downstream signaling effectors of RAC/ROP proteins in plants (Dorjgotov et al. 2009; Huesmann et al. 2012; Molendijk et al. 2008). In this study, we identified AtRLCK VI_A3 as a molecular interactor of AtROPs and suggest a function for this kinase in trichome morphogenesis and basal resistance to the powdery mildew fungus *E. cruciferarum*. Besides the C-terminal kinase domain, AtRLCK VI_A3 possesses a serine-rich region of unknown function in its N-terminus (Jurca et al. 2008). Based on phylogenetic analysis, AtRLCK VI_A3 might be the ortholog of the barley RLCK HvRBK1 which interacts with the powdery mildew susceptibility factor HvRACB in yeast and *in planta* (Huesmann et al. 2012). A heterologous targeted yeast-two hybrid assay showed that AtRLCK VI_A3 can interact with the same barley RAC/ROPs as HvRBK1, supporting a possible functional conservation of AtRLCK VI_A3 and HvRBK1. We further confirmed the interaction of AtRLCK VI_A3 with AtROPs in a yeast-two hybrid assay as it was described by Dorjgotov et al. (2009). In general, AtRLCK VI_A3 did not show a clear preference for interaction with either type I or type II AtROPs in yeast. By contrast to what was reported for other RLCKs (Murase et al. 2004; Tang et al. 2008; Veronese et al. 2006), AtRLCK VI_A3 did not show any motif for membrane localization and hence AtRLCK VI_A3 was predicted to localize in the cytoplasm. Transient expression of GFP-tagged AtRLCK VI_A3 in *Arabidopsis* epidermal cells supported this. However, co-expression of plasma membrane-anchored CA AtROP4 or CA AtROP6, respectively, resulted in recruitment of AtRLCK VI_A3 to the cell periphery suggesting direct protein interaction of AtRLCK VI_A3 and CA AtROPs at the plasma membrane. Recruitment of RLCKs by CA RAC/ROP proteins to the cell periphery was previously reported in similar experiments by Molendijk et al. (2008) and Huesmann et al. (2012). We showed that AtRLCK VI_A3 is an active kinase with low intrinsic kinase activity. Both the CA and the WT form of AtROP6 but not DN AtROP6 were able to enhance the kinase activity of AtRLCK VI_A3 in vitro. Concentration-dependent enhancement of AtRLCK VI_A3 kinase activity by CA AtROP6 further confirmed that this RLCK may serve as a Rho-GTPase effector similarly to other RLCK VI_A proteins that can be activated by RAC/ROP proteins (Dorjgotov et al. 2009; Huesmann et al. 2012). Data provide evidence that AtRLCK VI_A3 physically interacts with AtROPs and could hence be involved in AtROP-dependent downstream signaling in *Arabidopsis*.

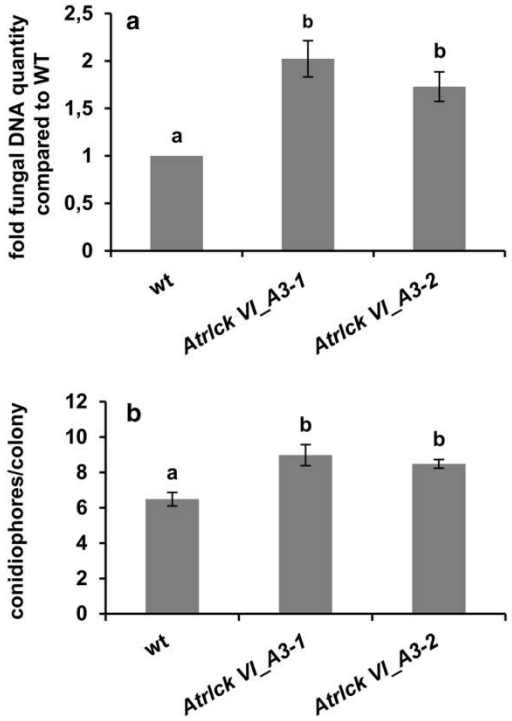

Fig. 4 *Erysiphe cruciferarum* infection phenotypes of *Atrlck VI_A3* mutant lines. **a** qPCR analysis of powdery mildew infection of *Atrlck VI_A3* mutant lines 5 days after inoculation (dai). Ratios of *E. cruciferarum* to *Arabidopsis* gDNA were determined and subsequently normalized to the wild-type. Data show mean ± standard error of the mean (SEM) of three independent inoculation events. In each inoculation event at least two different samples per genotype (each derived from five pooled seedlings) were evaluated. Different *letters* indicate significant difference (ANOVA Duncan test, $P > 0.05$). **b** Quantification of *E. cruciferarum* growth on *Atrlck VI_A3* mutant lines. The number of conidiophores per colony was counted 5 days after inoculation. In 4 independent experiments, 10 colonies per leaf of in total ≥13 leaves of each genotype were evaluated. Average mean ± SEM is shown. Different *letters* indicate significant difference (ANOVA Duncan test, $P > 0.05$)

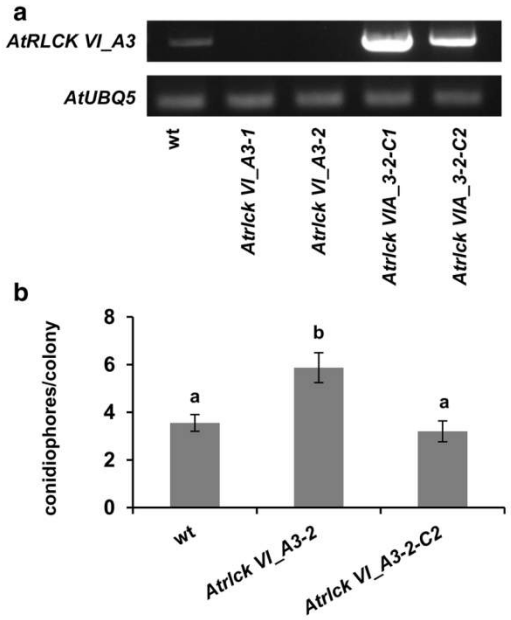

Fig. 5 Complementation of *Atrlck VI_A3-2* by overexpressing *35S:AtRLCK VI_A3* (*Atrlck VI_A3-2-C1* and *Atrlck VI_A3-2-C2*). **a** Expression of AtRLCK VI_A3 was determined by semi-quantitative RT-PCR in 7-week-old *Arabidopsis* genotypes. AtUBQ5 (At3g62250) was used as internal control. **b** Quantification of *E. cruciferarum* growth on *Arabidopsis* plants. The number of conidiophores per colony was counted 5 days after inoculation. In 5 independent experiments, 10 colonies per leaf of in total ≥13 leaves of each genotype were evaluated. Average mean ± SEM over all experiments is shown. Different *letters* indicate significant difference (ANOVA Duncan test, $P > 0.05$)

AtRLCK VI_A3 shows stronger binding preference for activated AtROPs. This is consistent with previous findings in which RAC/ROP effector molecules preferably bind to the CA forms of RAC/ROP proteins (Berken 2006). RAC/ROP proteins as well as RLCKs are involved in plant immunity. RLCK of subclade VII regulates PTI signaling like, for example, BIK1 or PBS1, respectively (Lu et al. 2010; Swiderski and Innes 2001). RAC/ROP proteins either positively or negatively regulate resistance to fungal and bacterial pathogens (Chen et al. 2010; Ono et al. 2001; Pathuri et al. 2008, 2009; Poraty-Gavra et al. 2013). In barley, CA HvRACB supports accommodation of infection structures of *Blumeria graminis* f. sp. *hordei* in intact epidermal cells whereas RNAi-mediated knock-down of HvRACB rendered cells less accessible for fungal ingrowth (Schultheiss et al. 2002, 2003; Hoefle et al. 2011). Because CA HvRACB was identified as an interaction partner and activator of HvRBK1, which is the barley RLCK most similar to AtRLCK VI_A3, we tested the function of AtRLCK VI_A3 in the interaction of *Arabidopsis* with the adapted powdery mildew fungus *E. cruciferarum*. Similar to the related HvRBK1 in the barley–barley powdery mildew pathosystem, AtRLCK VI_A3 appears to have a function in basal resistance because reproductive success of the fungus was increased on *Atrlck VI_A3* mutant lines. The observation that RAC/ROP-activated AtRLCK VI_A3 and barley HvRBK1 act in limiting susceptibility is not intuitive because there is evidence that RAC/ROP activity in *Arabidopsis* and barley contributes to susceptibility to powdery mildew fungi (Dörmann et al. 2014; Hoefle et al. 2011; Huesmann et al. 2011; Pathuri et al. 2008; Poraty-Gavra et al. 2013). Recently, it was shown that DN AtROP6, restricted reproductive success of the powdery mildew fungus *Golovinomyces orontii* on *Arabidopsis*, perhaps suggesting a function of wild-type AtROP6 in susceptibility (Poraty-Gavra et al. 2013). Indeed, CA AtROP6 was observed to accumulate at the site of invasion

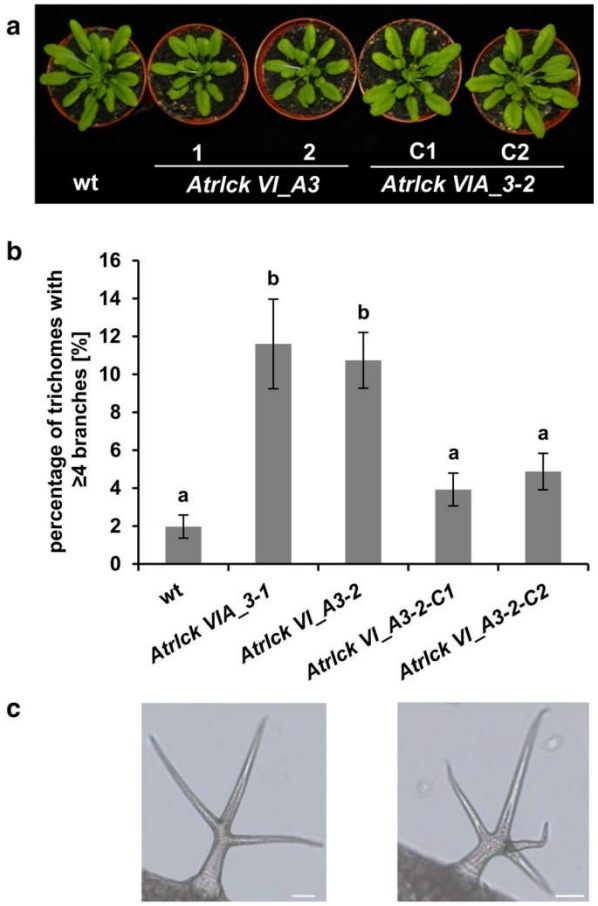

Fig. 6 Developmental phenotypes of *A. thaliana* genotypes. **a** Retarded growth phenotype of 7-week-old *AtRLCK VI_A3* mutant plants compared to the wild-type. Complementation of *AtRLCK VI_A3-2* by overexpressing *35S:AtRLCK VI_A3 (AtRLCK VI_A3-2-C1* and *AtRLCK VI_A3-2-C2)* restores plant size. **b** Quantitative analysis of trichome branch number of 7–8-week-old wild type, *AtRLCK VI_A3* mutant and mutant complementation lines. At least 800 trichomes of 10 leaves from different plants were analyzed for each genotype. *Bars* represent mean values ± SEM. Mean values with different *letters* are significantly different (ANOVA Duncan test, $P > 0.05$). **c** Light micrographs of mature leaf trichomes of *Arabidopsis*. The *scale bar* represents 50 µM in each picture

by a powdery mildew fungus (Hoefle and Hückelhoven 2008). Gene expression studies of DN AtROP6 plants showed that they overexpress salicylic acid response genes. However, genetic interaction studies showed that the salicylic acid response pathway was not responsible for the restriction of powdery mildew disease on DN AtROP6 plants (Poraty-Gavra et al. 2013). *Arabidopsis Atropgap1* and *Atropgap4* T_DNA insertion lines show enhanced susceptibility to *E. cruciferarum* (Huesmann et al. 2011). ROPGAPs negatively control RAC/ROP downstream effects by supporting hydrolysis of RAC/ROP-bound GTP and hence *ropgap* mutants may have enhanced RAC/ROP activity. AtRLCK VI_A3, as well as other AtRLCK kinases of clade VI_A, AtROPGAP1 and AtROPGAP4 interact with a similar set of AtROPs in yeast-two hybrid assays including AtROP6 (Dorjgotov et al. 2009; Hoefle et al. 2011; Huesmann et al. 2011). However, it is not known, which AtROPs are activated during fungal attack in *Arabidopsis* and whether and how interaction of activated AtROPs with AtRLCK VI_A proteins is specified *in planta*. Therefore, the function of AtRLCK VI_A3 is difficult to explain as an AtROP downstream effector in a linear signal transduction model. Instead, a negative feedback regulation of AtROPs by AtRLCK VI_A3 might explain these findings like it was previously discussed for HvRACB and HvRBK1 (Huesmann et al., 2012). In such a scenario, AtRLCK VI_A3 could be activated by an AtROP that functions in susceptibility but activated AtRLCK VI_A3 would trigger a negative feedback control of the AtROP or of downstream effectors. Alternatively, AtRLCK VI_A3 could act as a RAC/ROP downstream effector at the interface of antagonistic RAC/ROP signaling events or in parallel supporting immune responses downstream of another type of AtROP. Interestingly, there are both resistance-promoting and disease-promoting RAC/ROPs in rice (Chen et al. 2010). However, because we did not observe an obvious deregulation of plant immune responses in *Atrlck VI_A3* mutants, we currently favor the model that AtRLCK VI_A3 interferes with susceptibility. Mutualistic inhibition of distinct AtROP signaling events is postulated to operate in development of jigsaw puzzle-shaped pavement cells in *Arabidopsis.* The antagonistic AtROP2/4-RIC4 and AtROP6-RIC1 pathways organize fine actin microfilaments at sites of lobe outgrowth and well-ordered microtubules at neck regions. While the AtROP2/4-RIC4 pathway promotes accumulation of F-actin and lobe formation, the AtROP6-RIC1 pathway is responsible for microtubule stabilization, constraining radial cell expansion (Fu et al. 2005, 2009). Interestingly, the predicted barley ortholog of AtRLCK VI_A3, HvRBK1, supports microtubule stability (Huesmann et al. 2012). Possibly, crosstalk of antagonistic ROP pathways might converge at RLCK VI_As. Potentially contrasting functions of individual RAC/ROPs and RLCK VI_As in plant-pathogen interactions represent thus a basis for further studies on the RAC/ROP signaling network.

Trichomes are a well-studied model for cellular differentiation in *Arabidopsis* (Ishida et al. 2008). RAC/ROP proteins play a role in cell development and trichome morphogenesis was previously linked to RAC/ROP signaling (Fu et al. 2002; Szymanski 2005). We thus wondered whether AtROP-activated AtRLCK VI_A3 has also a function in trichome morphogenesis. Indeed, *AtRLCK VI_A3* mutant plants showed an increase in trichome

branch number in comparison to wild-type plants. Trichomes are specialized epidermal cells that are evenly distributed on *Arabidopsis* leaves and stems (Hülskamp et al. 1994). Usually mature leaf trichomes have three branches and only a small number of trichomes possess four branches (Schellmann and Hülskamp 2005). Interestingly, trichome development and branching is regulated through the microtubule and actin cytoskeleton (Mathur 2006). Reorganization and stabilization of microtubules plays also a crucial role in the initiation of trichome branches as indicated by pharmacological and mutant analyses (Mathur and Chua 2000; Sambade et al. 2014). In addition, a recent study showed that bacterial toxin-mediated changes in RAC/ROP activity cause failure of trichome branching (Singh et al. 2013). Taken together, the development of leaf pavement cells and trichome morphogenesis requires similar processes of cytoskeleton organization. This suggests that RAC/ROP proteins operate in trichome morphogenesis, which is further supported because AtRLCK VI_A3 regulates trichome branching as a possible AtROP signaling component. It would be interesting to learn whether altered cytoskeleton organization explains both enhanced trichome outgrowth and enhanced success of the epidermis-invading powdery mildew fungus. In barley, there is evidence that cytoskeleton organization by HvRACB and associated proteins regulates fungal entry and root hair development (Hoefle et al. 2011; Dörmann et al. 2014). It seems astonishing, that reduced leaf rosette size of *Atrlck VI_A3* mutants is linked with enhanced susceptibility to powdery mildew since in most cases dwarf plants display reduced susceptibility to biotrophic pathogens. However, we did not observe spontaneous cell death or defense gene expression in non-inoculated *Atrlck VI_A3* mutants. This suggests that reduced rosette growth does not result from constitutive expression of defense responses. Overexpression of *AtRLCK VI_A3* in the mutant background rescued all phenotypes studied but did not result in opposite phenotypes. This suggests that AtRLCK VI_A3 is not the rate-limiting factor in regulating growth, trichome branching and powdery mildew development. It appears likely, that upstream RAC/ROP activity rather than abundance of AtRLCK VI_A3 would regulate activity and thus output of AtRLCK VI_A3 signaling. Together, RAC/ROP proteins and RLCKs of clade VI_A can directly interact with each other. Active RAC/ROP supports RLCKs VI_A activity and both types of signaling proteins regulate similar processes in cell development and interaction with powdery mildew fungi. This supports that RLCKs of clade VI_A are indeed RAC/ROP signaling kinases. Identification of further interactors and substrates of these kinases is needed for future understanding of RAC/ROP signaling pathways and crosstalk.

Author contribution statement RH initiated the project. RH, CH, TR and AF designed experiments. TR, ChH, CH, DM and AF performed experiments. TR and ChH analyzed data. TR, RH and CH interpreted results. TR wrote the manuscript and CH and RH critically revised the manuscript. All the authors read and approved the final manuscript.

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