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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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*The final publication is available at Springer via http://dx.doi.org/*doi: 10.1007/s00299-014-1725-1

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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 thalina* 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 resulted in the recruitment of green fluorescent protein (GFP)-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 *Arapidopsis*, *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.

Key words:

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 transmenbrane RLK protein family. Together over 600 RLKs and RLCKs exist in *Arabidopsis* and over 1000 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-incombatibility (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; Fu et al. 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 up-regulation of gene expression under abiotic stress or hormone treatments as well as in response to the pathogens *Botrytis cinerea* and *Phytophtora 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.

MATERIAL AND METHODS

*The final publication is available at Springer via http://dx.doi.org/*doi: 10.1007/s00299-014-1725-1

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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 it was 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 10h 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 10h 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 5-15 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'AACCCGGGCTATGGCTGTTGAAGAGATG3' 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* were amplified using primer pairs

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Rop4BamHI5´cDNAp 5'GAATTTGCTGGATCCATGAGTGCTTCGAG3' and M13rev 5'AACAGCTATGACCATGA3'or Rop6BamHIfwd 5'AGGATCCATGAGTGCTTCAAGGTTTATC3' and Rop6SalIrev 5'CCGCGGGATGTCGACTCAGAGTATAGAAC3' 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`TTTGAATTCATGGCTGTTGAAGAGATGGAG3` and At5g65530_Bam_rev 5`AAAGGATCCTTACTCCATTAAGAGCTGTCTATG3` and the restriction sites *Eco*RI and *Bam*HI. Generation of the different *Arabidopsis* and barley *RAC/ROP* constructs based on the vector pGBKT7 were 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

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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 hours after bombardment using confocal laser scanning microscopy (Leica TCS SP5; Leica Microsystems). GFP fluorescence of tagged AtRLCK VI_A3 was excited with a 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, 1mM of DTT, 10 µM of ATP, 0.2 MBq [□-^{32}P] of ATP and 0.25 µg/µl of myelin basic protein. After incubation for at least 30 minutes at room temperature, the reactions were stopped by adding 5 µl of 5x 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). 1µg 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

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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 sec at 98°C followed by 28-40 cycles of 10 sec at 98°C, 15 sec at 55°C and 50 sec 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 strenght, genomic DNA (gDNA) was isolated from 5 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 sec at 95°C, 30 sec at 54°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. 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

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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 dominant negative (DN) forms of barley RAC/ROPs in yeast and *in planta*. HvRBK1 is closely related to the VI_A subfamily of *Arabidopsis* receptor-like cytoplasmic kinases and shows highest similarity to AtRLCK VI_A3 (Huesmann et al. 2012). In order 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 dominant negative (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. Similarly to HvRBK1, AtRLCK VI_A3 shows no exclusive specificity for type I or type II RAC/ROPs.

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 hours after bombardment (hab) by confocal laser scanning microscopy. This was obvious

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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). In order 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 quantitative real-time PCR (qPCR)

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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 summary, increased susceptibility of *Atrlck VI_A3* mutant lines to *E. cruciferarum* suggest a function of AtRLCK VI_A3 in basal resistance to powdery mildew. In order 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 suggest 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 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

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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

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RAC/ROP proteins (Dorjgotov et al. 2009; Huesmann et al. 2012). Data provides evidence that AtRLCK VI_A3 physically interacts with AtROPs and could hence be involved in AtROP-dependent downstream signaling in *Arabidopsis*. 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 regulate 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; Pathuri et al. 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; Schultheiss et al. 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 by a powdery mildew fungus (Hoefle and Hückelhoven 2008). Gene expression studies of DN AtROP6 plants showed that they over-express 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

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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; Fu et al. 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

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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 require 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. Over-expression 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

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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 authors read and approved the final manuscript.

ACKNOWLEDGMENTS

This work was supported by grants from the German Research Foundation (HU886/3 and SFB924 to R.H.) and the Hungarian Scientific Research Fund (OTKA K101112).
The authors gratefully acknowledge the support by the Faculty Graduate Center Weihenstephan of TUM Graduate School at Technische Universität München, Germany.

The authors declare no conflict of interest

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FIGURES

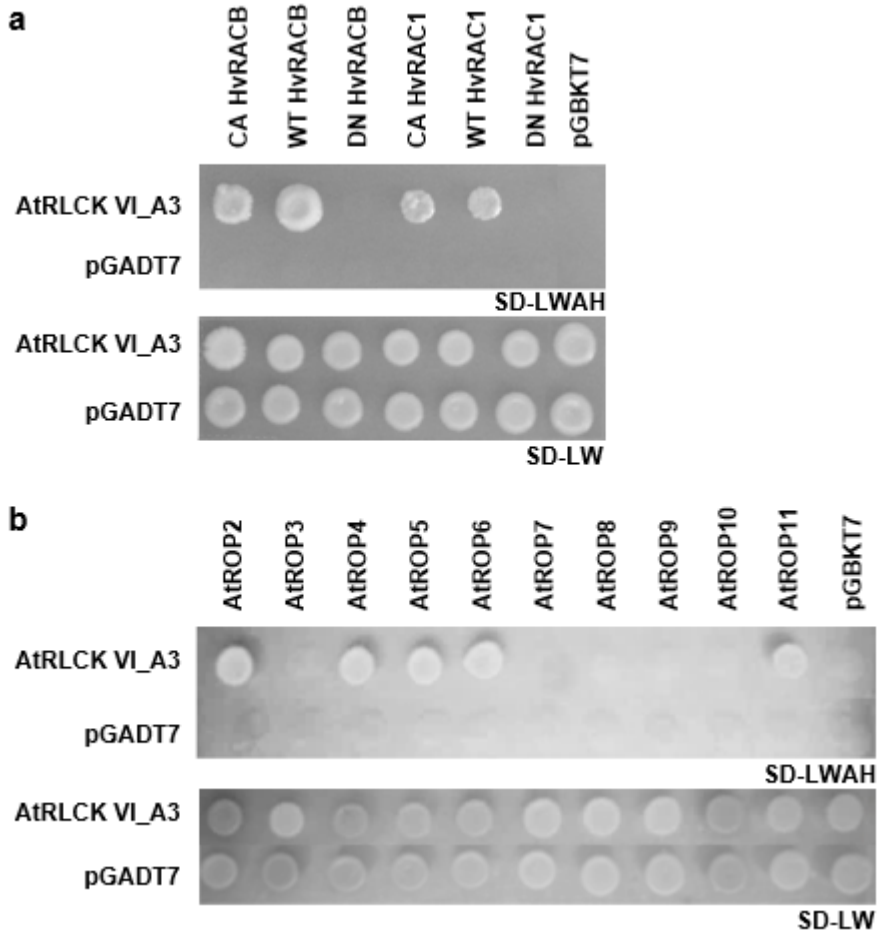

Fig. 1 Targeted yeast two hybrid assay of AtRLCK VI_A3 with different barley and *Arabidopsis* RAC/ROPs. **a** AtRLCK VI_A3 interacts with the constitutively active (CA) and wild-type forms (WT) of barley RACB (HvRACB) and RAC1 (HvRAC1) but not with their dominant negative (DN) forms. **b** AtRLCK VI_A3 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.

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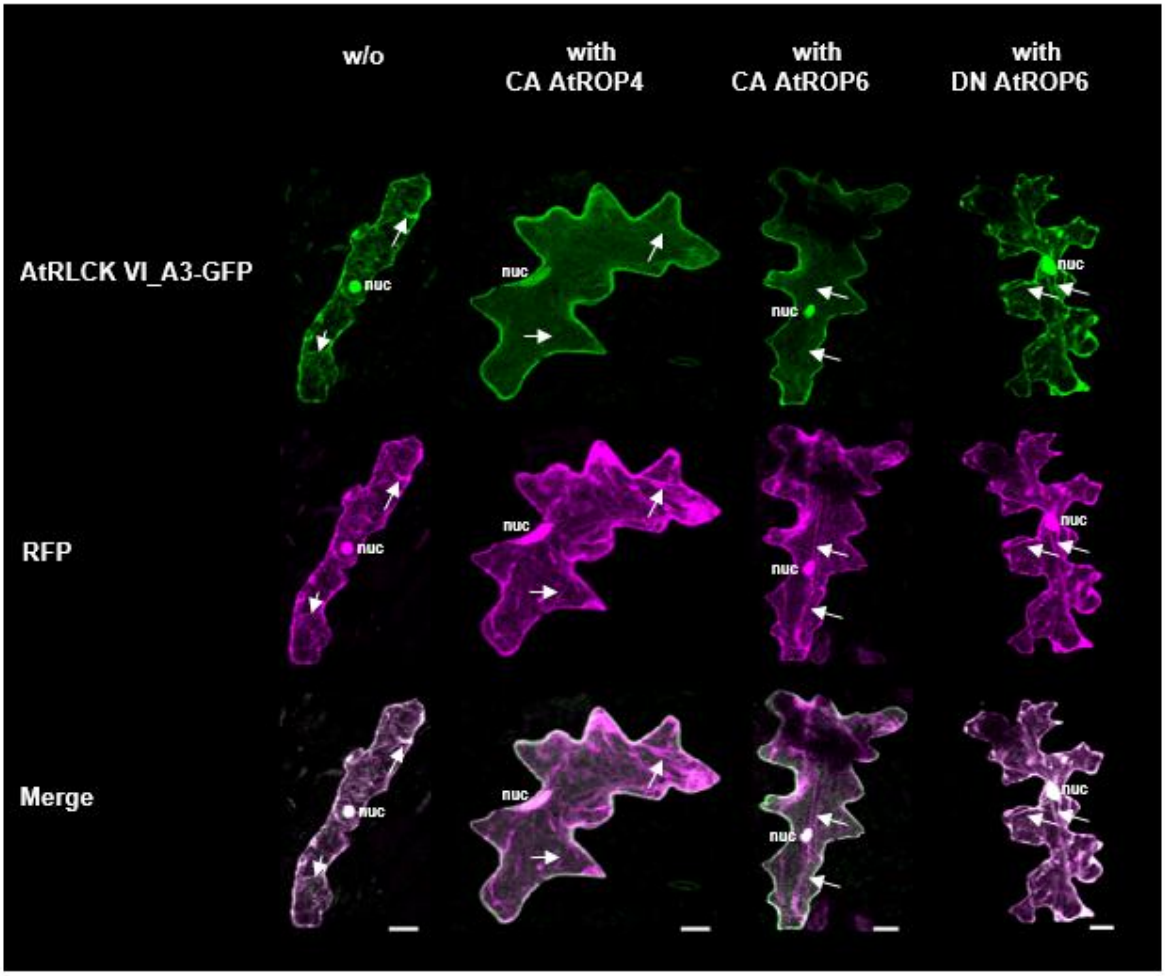

Fig. 2 Subcellular localization of AtRLCK VI_A3 in transiently transformed *Arabidopsis* epidermal cells 24 hours after bombardment (hab). AtRLCK VI_A3-GFP co-localizes with soluble RFP in cytoplasmic strands (arrows) and nucleoplasm. Upon co-expression of either CA AtROP4 or CA AtROP6, AtRLCK VI_A3-GFP 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. Nucleus (nuc).

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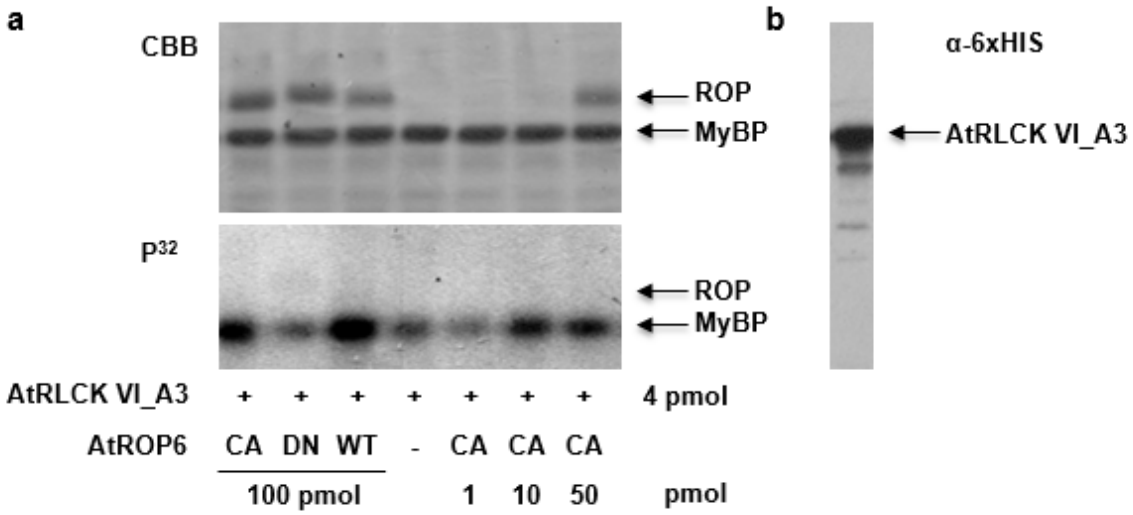

Fig. 3 *In vitro* activity of AtRLCK VI_A3 in the presence and absence of AtROP6. **a** The in vitro myelin basic protein (MyBP) phosphorylating activity of AtRLCK VI_A3 is increased in the presence of WT and CA AtROP6. Moreover, MyBP phosphorylating activity of AtRLCK VI_A3 is increased in a dose-dependent 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.

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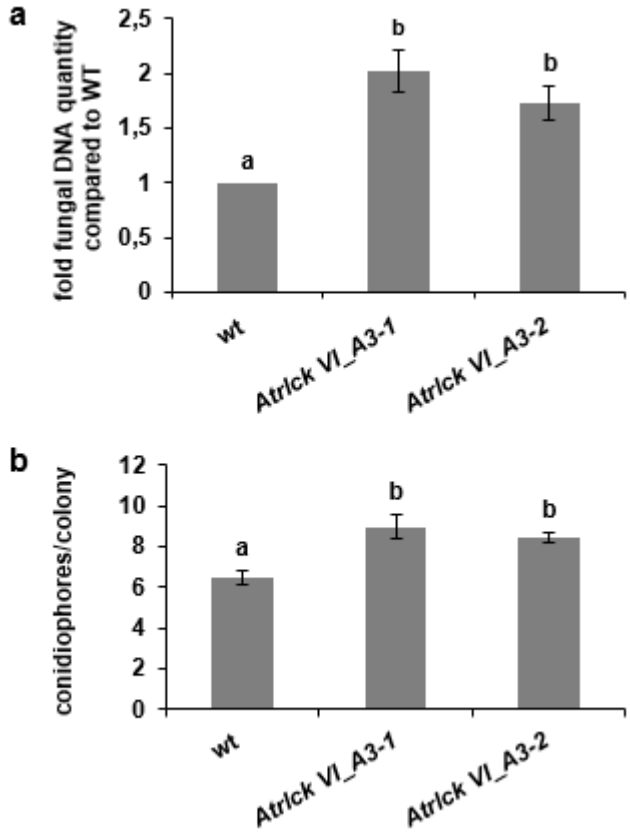

Fig. **4** *Erysiphe cruciferarum* infection phenotypes of *Atrlck VI_A3* mutant lines. **a** qPCR analsysis of powdery mildew infection of *Atrlck VI_A3* mutant lines 5 days after inoculation (dai). Ratios of *E. cruciferarum* to *Arabidopsis* gDNA were determined and subsequently normalized to the 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 5 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 were 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).

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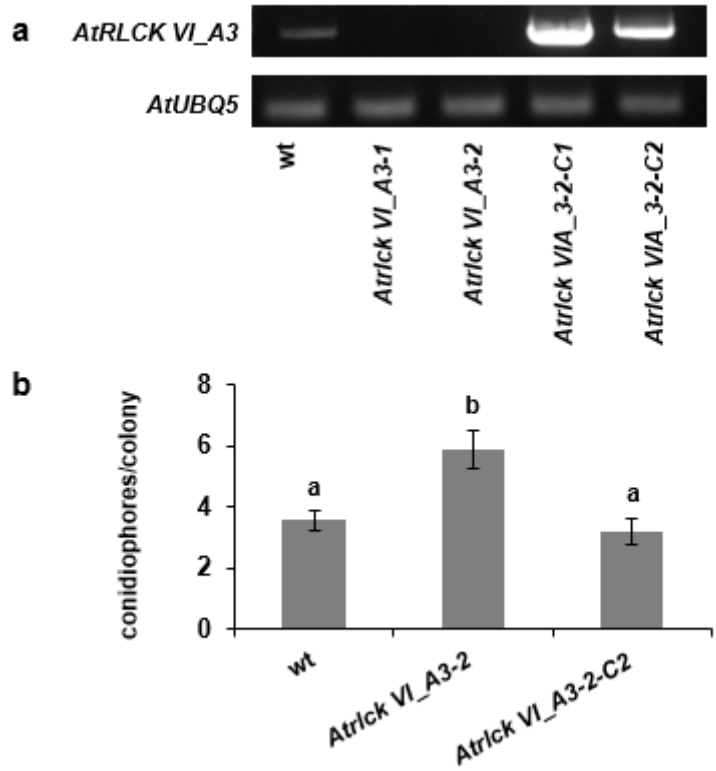

Fig. 5 Complementation of *Atrlck VI_A3-2* by overexpressing *35S:AtRLCK VI_A3* (*Atrlck VI_A3-2-C1* and *Atrlck VI_A3-2-C2*). **a** Expression of AtRLCK VI_A3 was determined by semi-quantitative RT-PCR in 7-week old *Arabidopsis* genotypes. AtUBQ5 (At3g62250) was used as internal control. **b** Quantification 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).

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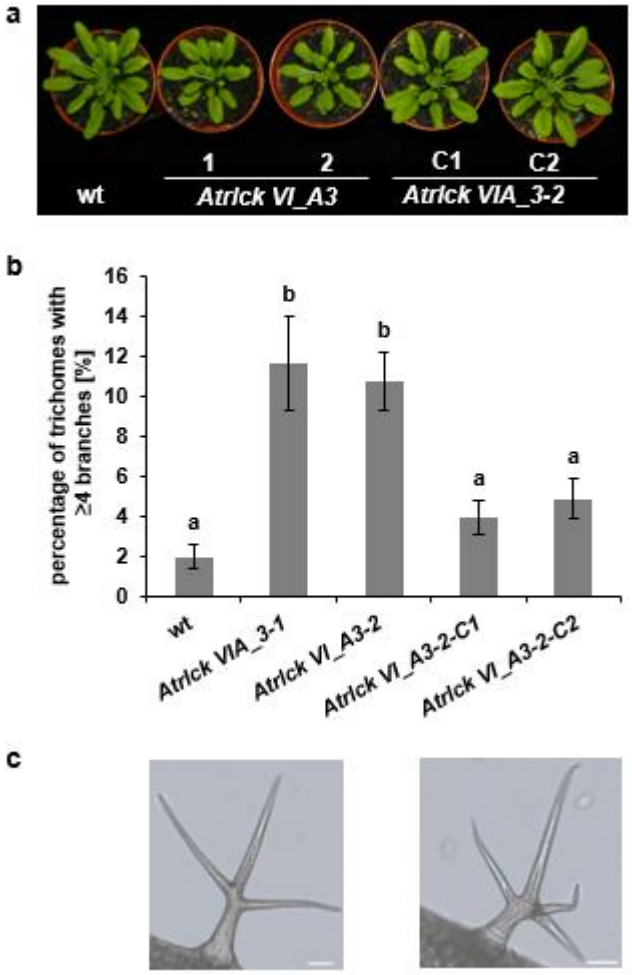

Fig. 6 Developmental phenotypes of *A. thaliana* genotypes. **a** Retarded growth phenotype of 7-week-old *AtRLCK VI_A3* mutant plants compared to the wild-type. Complementation of *AtRLCK VI_A3-2* by overexpressing *35S:AtRLCK VI_A3 (AtRLCK VI_A3-2-C1* and *AtRLCK VI_A3-2-C2)* restores plant size. **b** Quantitative analysis of trichome branche 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.

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