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In silico identification and experimental validation of amino acid motifs required for the Rho-of-plants GTPase-mediated activation of receptor-like cytoplasmic kinases

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Key message

Several amino acid motifs required for Rop-dependent activity were found to form a common surface on RLCKVI_A kinases. This indicates a unique mechanism for Rho-type GTPase-mediated kinase activation in plants.

Abstract

Rho-of-plants (Rop) G-proteins are implicated in the regulation of various cellular processes, including cell growth, cell polarity, hormonal and pathogen responses. Our knowledge about the signalling pathways downstream of Rops is continuously increasing. However, there are still substantial gaps in this knowledge. One reason for this is that these pathways are considerably different from those described for yeast and/or animal Rho-type GTPases. Among others, plants lack all Rho/Rac/Cdc42-activated kinase families. Only a small group of plant-specific receptor-like cytoplasmic kinases (RLCK VI_A) has been shown to exhibit Rop-binding-dependent *in vitro* activity. These kinases do not carry any known GTPase-binding motifs. Based on the sequence comparison of the Rop-activated RLCK VI_A and the closely related but constitutively active RLCK VI_B kinases, several distinguishing amino acid residues/motifs were identified. All but one of these was found to be required for the Rop-mediated regulation of the *in vitro* activity of two RLCK VI_A kinases. Structural modelling indicated that these motifs might form a common Rop-binding surface. Based on *in silico* data mining, kinases that have the identified Rop-binding motifs are present in Embryophyta but not in unicellular green algae. It can, therefore, be supposed that Rops recruited these plant-specific kinases for signalling at an early stage of land plant evolution.

Keywords: *Arabidopsis thaliana*; cellular signalling, kinase activity; protein-protein interaction; RLCK; Rop-binding motifs

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Introduction

Rho-type small GTP-binding proteins are molecular switches that regulate cellular signalling pathways: they are "ON" in the GTP-bound state and "OFF" in the GDP-bound state. When in the GTP-bound "ON" conformation, they can interact with and regulate a plethora of effector proteins. These proteins control the organization of the cytoskeleton, the pattern of gene expression and the activity of the plasma membrane NADPH oxidase (Jaffe and Hall 2005, Nagawa et al. 2010). The family of eukaryotic Rho-type G-proteins are subdivided into several subfamilies. The Rho and Cdc42 subfamilies are shared by yeasts and animals, while the Rac GTPases are present only in animals (Brembu et al. 2006). Plants have one unique subfamily of Rho-type GTPases, the Rop ("Rho-of-plants") G-proteins (Brembu et al. 2006). The overall structure of all Rho-type proteins was conserved during evolution. Nevertheless, all subfamilies evolved to interact with a specific set of effectors regulating well-defined cellular processes. For example, Rops have specific regulators, as well as effectors, that are implicated in plant-specific processes. Among others, these include cell wall remodelling, hormonal signalling and plant-pathogen interactions (Nagawa et al. 2010). Likewise, Rops have characteristic amino acid residues in the regions involved in the protein-protein interactions of Rho-type GTPases (Berken and Wittinghofer 2008).

Rops, like their yeast and animal counterparts, link receptors to intracellular signalling pathways. Plant membrane receptors that fall within the class of receptor-like serine/threonine kinases (RLKs), can indirectly promote the GDP-to-GTP exchange of Rops via the plant-specific Rop guanine nucleotide exchange factors (RopGEFs) (Miyawaki and Yang 2014, Fehér and Lajkó 2015). The negative Rop regulators and the GTPase-activating proteins

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(RopGAPs), as well as the guanine nucleotide dissociation inhibitors (GDIs), can also convey various extra- or intracellular signals towards the G-proteins (Fehér and Lajkó 2015).

Rops can mediate these signals towards diverse sets of downstream effectors in a cell type- and signal-specific manner. The Rop effectors include plant-specific small scaffold proteins such as the Rop-interacting CRIB-motif containing proteins (RICs), the interactor of constitutively active Rop/Rop-interacting proteins (ICR/RIPs), and the receptor for activated C-Kinase 1 (RACK1) (Nagawa et al. 2010). These proteins have been implicated in having roles in the regulation of cell polarity (Yang and Fu 2007, Murphy and Peer 2012) and in the formation of a multi-subunit protein complex involved in pathogen defence (the so-called rice "defensome";Kawano et al. 2010). However, our current knowledge about the signalling cascades further downstream of these scaffold proteins is rather limited (Hong et al. 2016). Plant-specific cell wall-modifying enzymes, the Cinnamoyl-CoA Reductase 1 (CCR1) and the UDP-glucose transferase 1 (UGT1), are also Rop effectors (Nagawa et al. 2010). Furthermore, Rops interact with the respiratory burst oxidase homolog (Rboh) enzymes, generating reactive oxygen species (Wong et al. 2007). Rbohs affect cell growth, plant development and plant responses to abiotic and biotic environmental constraints (Kaur et al. 2014).

Yeast and animal Rho-type GTPases are linked to several signalling pathways via specific effector kinases. The activity of these kinases is directly regulated by the G-proteins (Zhao and Manser 2005) and these kinases are involved in the regulation of rather basic cellular processes, including cell morphology, cell division, vesicular trafficking, etc. (Schwartz 2004). Surprisingly, none of these kinase families is present in plants. Therefore, the linking of plant Rho-type G-proteins to downstream kinase signalling has, until recently, been undetected.

Yeast two-hybrid screening experiments resulted in the identification of plant kinases specifically binding the constitutively active ("GTP-bound") Rop form (in *Arabidopsis*:

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Molendijk et al. 2008, in *Medicago*: Dorjgotov et al. 2009, in *Hordeum*: Huesmann et al. 2012). These kinases belong to plant-specific receptor-like cytoplasmic kinases (RLCKs). Their kinase domain shows strong similarity to that of the receptor-like serine/threonine kinases (RLKs). However, RLCKs have no receptor configuration: they have neither extracellular ligand-binding nor transmembrane domains (Lin et al. 2013). Although RLCKs belong to ten different classes, the members of the RLCK Class VI have mostly been found to bind Rops and have Rop-dependent *in vitro* kinase activity (for a review, see Fehér and Lajkó 2015). The only exception at present is the *Arabidopsis* Novel Cystein Rich Kinase or NCRK, belonging to RLCK Class VIII (Molendijk et al. 2008). The function of plant Rop effector kinases is mostly unknown but certain members have been implicated in the regulation of plant-pathogen interactions and morphogenesis (Huesmann et al. 2012, Reiner et al. 2014, Enders et al. 2017).

Rop-binding kinases have none of the sequence motifs that play a role in the binding and activation of yeast and animal kinases by Rho-type G-proteins (Dorjgotov et al. 2009, Fehér and Lajkó 2015). The mechanism of the activation of these potential Rop effector plant kinases is unidentified at present.

Proteins belonging to the same protein family share evolutionarily conserved amino acid residues in given positions. These residues are present in all members and are important for their common general function. As such, they are devoid of mutations during evolution. Multiple sequence alignments may also highlight subgroup-dependent conservation patterns. These "specificity-determining positions" (SDPs) suggest that the members of the subgroup acquired specific function(s) in addition to the global function of the whole family (Chakraborty and Chakrabarti 2015). Within the *Arabidopsis* RLCK VI class, only half of the 14 proteins (forming the group RLCK VI_A) were found to be able to bind Rops (Dorjgotov et al. 2009). Multiple sequence alignment allowed us to identify amino acid motifs and potential SDPs that

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differentiate between the Rop-binding (group A) and the non-binding (group B) RLCK VI members. Here, we report on the role of these motifs in the Rop-binding and protein phosphorylation activities of two *Arabidopsis* RLCK VI_A kinases.

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Materials and methods

Sequence analyses

Classification of the *Arabidopsis* RLCK Class VI kinases into A and B groups has been described elsewhere (Jurca et al. 2008). The *Arabidopsis* RLCK VI_A-homologous kinases of *Chlamydomonas reinhardtii*, *Physcomitrella patens*, *Marchantia polymorpha*, *Selaginella moellendorffii*, and *Oryza sativa* were identified using the Pattern Hit Initiated Basic Local Alignment Search Tool (PHI-BLAST) of the National Center for Biotechnology Information (NCBI) at https://blast.ncbi.nlm.nih.gov/Blast.cgi. The kinase domain of the RLCK VI_A3 (At5g65530) kinase was used as a query sequence, together with the GXXXHXXH amino acid pattern (X=any amino acid) characteristic for all *Arabidopsis* RLCK VI_A members with the Expected-value of 0.01 and the PHI-BLAST threshold of 0.005. The search results were narrowed down for the above-listed species that have fully sequenced genomes and represent unicellular green algae, liverworts (Marchantiophyta), mosses (Bryophyta), Lycopodiophyta and Angiosperms (Eudicots and Monocots), respectively. Sequence alignments were made using the ClustalW algorithm (Thompson et al. 1994). A specificity-determining site (SDP) analysis was conducted using the JDet software package (Muth et al. 2012) downloaded from http://csbg.cnb.csic.es/JDet/ (last accessed on 27.11.2017).

The three-dimensional (3D) model of the RLCK VI_A2 kinase was built using the SWISS-MODEL SERVER (http://swissmodel.expasy.org) (Biasini et al. 2014). The kinase domain of the BRI1-associated receptor kinase 1 (BAK1; the Protein Data Bank Identity [PDBID] of the crystallographic structure is 3ulz) was used as a template. The "DeepView" Swiss-PdbViewer software was used (http://spdbv.vital-it.ch/) for visualization.

Mutagenesis

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The cDNA clones of the RLCK VI_A2 (At2G18890) and VI_A3 (At5g65530) kinases and AtRop1 (AT3G51300) were obtained from the *Arabidopsis* Biological Resource Center (ABRC; http://www.*Arabidopsis*.org/). The cDNAs were amplified by polymerase chain reaction (PCR) using Phusion high-fidelity polymerase (Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences are listed in Supplementary Table 1. The PCR fragments were inserted into the pEntry2b vector at the EcoRI/XhoI sites. This vector was previously modified, removing the SalI site to change the reading frame. The sequences of the kinases and Rop1 were altered using the overlap extension polymerase chain reaction approach combined with Gateway cloning (Atanassov et al. 2009). The RLCK VI_A group-specific amino acids were changed to those present in B group kinase(s), as indicated in Fig. 1. In the case of the LP-IDE motif, the whole region between the RLCK VI_A2/3 and the RLCK VI_B5 kinases was exchanged (Fig. 1). The mutated kinase cDNAs were cloned by LR recombination (Life Technologies, Carlsbad, CA, USA) into the Gateway version of the yeast two-hybrid vector pGADT7 (pDest-GADT7) obtained from ABRC. The mutated kinase cDNA clones were PCR amplified and cloned into the EcoRI/XhoI sites of the pET28a vector for bacterial protein production and purification, as described in detail elsewhere (Dorjgotov et al. 2009). The plasmid constructs (pDest-GBKT7; pET26b) carrying the constitutively active (G15V) mutant forms of the *Arabidopsis* Rop1 GTPase were developed using similar procedures. The mutagenic primer pairs are listed in Supplementary Table 1.

Protein-protein interactions

Yeast two-hybrid system-based protein-protein interaction studies were conducted according to the Yeast Protocols Handbook of Clontech, available online at http://www.clontech.com/ (last accessed 08.07.2016). The *Saccharomyces cerevisiae* yeast strain, AH109, was used as the host. Yeast transformation, with the pDest-GADT7 (kinase) and pDest-GBKT7 (Rop)

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constructs, described above, was carried out using the lithium acetate (LiAc)-mediated method. The transformants were grown on appropriate drop out media to monitor transformation efficiency as well as the activation of the *his3* (in the presence of 0-10 mM 3-aminotriazole dependent on the required selection stringency) or the *ade* reporter genes, as indicated. Production of the appropriate fusion proteins in the yeast cells was verified by protein purification and Western blotting with antibodies against the yeast GAL4 activation domain and plant Rop GTPases (anti-*Arabidopsis* RAC3 polyclonal antibody), respectively, that were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Bimolecular fluorescence complementation (BiFC) tests were conducted based on the double ORF expression (pDOE) vector system developed by Gookin and Assmann (2014) and made available by the ABRC. The pDOE vectors allow for the parallel expression of two proteins linked to the N- and C-terminal regions of the mVenus yellow fluorescent protein, respectively. The vectors also carry a separate fluorescent protein (mTurquoise) for transformation control. In order to facilitate the monitoring of protein-protein interaction in pollen tubes, the mTurquoise marker of the pDOE11 vector, controlled by the agrobacterial mannopine synthase (mas) promoter, was exchanged for the mCherry red fluorescent protein, controlled by the pollen-specific tomato Lat52 promoter (Twell et al. 1990). The mCherry-coding DNA sequence was PCR amplified as an XhoI/SacI fragment (by Phusion polymerase) and cloned into the corresponding sites of the pLat52:YFP-carrying vector, pWEN240 (Klahre et al. 2006), resulting in pLat52:mCherry. The pLat52:mCherry chimeric gene could be transferred as an EcoRI/KpnI fragment into the pDOE11 vector, replacing the pmas: mTurquoise marker. The vector backbone of this pDOE11-mCherry plasmid was decreased by cloning its HindIII/KpnI fragment into the corresponding sites of pWEN240, resulting in the pDOE11mCTr vector, which, in parallel with BiFC in pollen tubes, is suitable for monitoring transfection efficiency.

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The PCR-amplified cDNA coding for the constitutively active form of the Rop1 G-protein was cloned into the NcoI/SpeI site of the pDOE11mCTr vector. In this way, Rop1 was fused to the N-terminal part of the mVenus protein. The mutant kinase cDNAs were inserted into the Rop1 CA containing vector at the PmlI site using the SLICE (seamless ligation cloning extract) method (Zhang et al. 2012). Primers are listed in Supplementary Table 1. The ligation extract was made from *Escherichia coli* JM109 cells, according to Motohashi (2015). The constructs were tested by sequencing for the correct fusion of the proteins. Particle bombardment of the modified pDOE11mCTr constructs into tobacco (*Nicotiana tabacum* SR1) pollen was achieved via a helium-driven PDS-1000/He particle delivery system (Bio-Rad, Hercules, California, USA), as described in (Kost et al. 1998).

Microscopy

Cells expressing Lat52:mCherry were tracked by using a spinning disk confocal microscope (Visitron, Germany). This system integrates the Yokogawa CSU-WD confocal scan unit (Yokogawa, Japan), an Olympus IX-83 microscope base (Olympus, Germany) and a Zyla 4.2 Plus sCMOS (Andor, Northern Ireland) dual camera system. The microscope objectives were LUCPlan FL 20x (dry, 0.45NA) and 40x (dry, 0.6NA). For mCherry detection, a 561 nm laser, an LP565 nm dichroic mirror and a 582-636 nm emission filter were used. The laser intensity and the camera exposure settings were kept constant during image capturing.

Protein purification and in vitro kinase assays

The protein expression in *Escherichia coli* cells, the metal affinity chromatography-based purification of 6xhistidine-tagged (6xHIS) kinase and the GTPase proteins, as well as the *in vitro* protein kinase activity tests, were conducted exactly as described in (Dorjgotov et al. 2009).

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Results

Identification of amino acid sequence motifs potentially involved in the binding of RLCKs to Rops

The RLCK Class VI family of *Arabidopsis* protein kinases has 14 members that fall into two groups, based on the similarity of their kinase domains (Jurca et al. 2008). A targeted yeast two-hybrid interaction matrix showed that members of group A, but not those of group B, can interact with Rop G-proteins (Dorjgotov et al. 2009). Several RLCK VI kinases that fall into group A have been isolated from various species (*Medicago*, *Arabidopsis*, *Hordeum*) (Dorjgotov et al. 2009, Huesmann et al. 2012, Reiner et al. 2014). The *in vitro* protein phosphorylation activity of these kinases was found to be increased by the presence of GTP-bound Rops (Dorjgotov et al. 2009, Huesmann et al. 2012, Reiner et al. 2014).

The primary amino acid sequences of the seven Rop-activated *Arabidopsis* RLCK VI group A members were compared with those of the seven Rop-independent group B members. This resulted in the identification of several short amino acid motifs/regions that characteristically different between the two groups (Fig. 1A). The motifs have been named after their representative amino acids. These were found to be dispersed across the whole kinase domain (Fig. 1B). Using the JDet software package (Muth et al. 2012), we could verify that the motifs we selected for detailed characterization overlap with the potential "specificity determining positions" SDPs (Supplementary Fig. 1). The subgroup-specific conservation of amino acids in SDPs indicates the functional significance of those residues (Chakraborty and Chakrabarti 2014).

Mutational analysis of the potential Rop-binding motifs characterizing RLCK VI group A

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To prove the role of the identified sequence motifs of the RLCK VI_A2 kinase in Rop binding, they were mutated one at a time. The amino acid motifs characteristic of group A kinases (shown in Fig. 1A) were changed to amino acids present in the same position in group B kinases. Since group B kinases are constitutively active kinases (Dorjgotov et al. 2009), it was supposed that such exchanges would directly relate to Rop-binding. The cDNA clones' coding for the mutant kinases were linked to the Gal4 activation domain. These were co-expressed in yeast cells with the constitutively active *Arabidopsis* Rop1 G-protein (Rop1 CA) linked to the Gal4 DNA-binding domain. A pairwise combination of the kinase mutants with the Rop1 CA protein was tested in the yeast two-hybrid system for protein-protein interaction (Fig. 2A). Interaction stability was evaluated using different selection strengths (0, 1, 3, 10 mM 3-amino-1,2,4-triazole, 3-AT). In the presence of 10 mM 3AT, all mutations, except one (designated as YA), prevented colony growth, indicating weak or no RLCK-to-Rop binding. Omitting 3AT from the medium resulted in growing yeast colonies in the case of the G (strong growth) and the LP-IDE (weak growth) mutants. Therefore, these mutations weakened but did not prevent the interaction of the investigated proteins, unlike the mutations HV, LS, RR and IDE.

To further support the above observations, *in vitro* kinase activity assays were carried out. The 6xHIS-tagged mutant forms of the RLCK VI_A2 kinase were expressed in, and purified from, *Escherichia coli* cells. Fig. 2B shows the auto-phosphorylation of RLCK VI_A2 kinase in the presence or absence, respectively, of the similarly purified *Arabidopsis* Rop6 CA protein. The G mutant of RLCK VI_A2 behaved in a manner that was similar to the wild type. Three of the six other mutant kinases lost their Rop-dependent auto-phosphorylation activity (YA, HV, IDE), while the remaining kinase mutants (LS, RR, LP-IDE) showed at least certain level of activity both with and without the G-protein (Fig. 2B). These data confirmed that all but one of the identified motifs play a role in Rop-binding and some are important for the Rop-dependent regulation of the kinase activity.

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The presence of Rop-binding motifs affects the substrate phosphorylating activity of RLCK VI_A3

The RLCKVI_A2 kinase has a low affinity for the artificial kinase substrate, myelin basic protein. Therefore, some of the above described mutations have been replicated in the RLCK VI_A3 kinase, which has previously been demonstrated to efficiently phosphorylate this substrate (Reiner et al. 2014). These mutations altered the conserved HV, LS and RR amino acid motifs in the same way as in the case of the A2 mutant (Fig. 1). These mutations were selected because they resulted in the loss of the Rop-binding of RLCK VI_A2 but with different consequences for its activity (Fig. 2B). Fig. 3 shows that all three mutations prevented the RLCK VI_A3 kinase from binding to the active Rop G-protein (Fig. 3A). The RLCK VI_A3 kinase has a basal myelin basic protein phosphorylation activity that is considerably augmented in the presence of Rop1 CA (Fig. 3B). The myelin basic protein-phosphorylation activity of the RLCK VI_A3 kinase was lost due to the HV mutation, whereas Rop-independent activities could be observed in the cases of the LS and RR mutations (Fig. 3B). These results were in accordance with the auto-phosphorylation pattern of the corresponding RLCK VI_A2 kinase mutants. All RLCK VI_A3 forms exhibited a strong auto-phosphorylation activity that masked any Rop-dependent differences in autophosphorylation.

The identified motifs may form a binding surface for Rops

Although the three-dimensional (3D) structure of RLCK VI kinases is, at present, unknown, a theoretical model was generated based on the crystal structure of the kinase domain of the Brassinosteroid Insensitive 1-associated Receptor Kinase 1 (BAK1; protein data bank id.: 3ULZ). Fig. 4 shows the distribution of the mutated amino acids using this *in silico* 3D model

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of the RLCK VI_A2 kinase. Most of the amino acids implicated in Rop-binding are parts of a more or less continuous surface above the substrate-binding cleft of the kinase model (Fig. 4). The only RLCK VI_A-specific motif (YA) that was found to affect the kinase activity but not Rop-binding (Fig. 2) is not part of this surface but resides in the ATP-binding pocket (Fig. 4).

The part of the RLCKL VI_A2 kinase domain carrying the motifs required for Rop-binding was then used to replace the corresponding region of RLCK VI_B5 (Fig. 5a). The chimeric kinase could not bind Rop1 CA in the yeast two-hybrid system (Fig. 5b). Either the chimeric kinase could not properly fold or the investigated motifs are required but are not sufficient for Rop-binding.

In planta **protein interactions**

The bimolecular fluorescence complementation (BiFC) assay was used to determine the *in vivo* interaction of the wild type and mutant RLCK VI_A kinases with the constitutively active Rop1 form. The cDNAs coding for the kinases and the Rop1 CA GTPase were fused to the cDNA fragments coding for the C- and N-terminal regions, respectively, of the yellow fluorescence protein (mVenus). Both chimeric genes were placed under the control of *Arabidopsis* Ubiquitin-10 promoter in the same vector molecule, ensuring a similar level of co-expression. The vector molecule also carried a red fluorescent protein (mCherry) gene as a visible marker for transformation success. This marker was under the control of the pollen-specific tomato Lat52 promoter. Fig. 6 shows that, in agreement with the yeast-two-hybrid and kinase activity data, the wild type but not the binding-mutant kinases could interact with the active Rop1 protein in tobacco pollen tubes.

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Rop-binding motif-containing kinases are evolutionarily conserved in the Embryophyta

The presence of Rop-activated RLCK VI_A kinases has been experimentally proved in *Medicago* (Dorjgotov et al. 2009), *Arabidopsis* (Molendijk et al. 2008, Dorjgotov et al. 2009, Reiner et al. 2014) and *Hordeum* (Hoefle et al. 2011) species. In order to determine the evolutionary conservation of this kinase type in the plant kingdom, we performed an *in silico* analysis. A Pattern Hit Initiated BLAST search was performed in the representative, fully sequenced, plant genomes of *Chlamydomonas reinhardtii*, *Physcomitrella patens*, *Marchantia polymorpha*, *Selaginella moellendorffii* and *Oryza sativa* using the *Arabidopsis* RLCKVI_A3 sequence and the characteristic amino acids of the HV Rop-binding motif as a pattern. Even though only the HV motif was used for screening, the identified *Physcomitrella*, *Marchantia*, *Selaginella* and *Oryza* kinases contained all Rop-binding motifs that are characteristic of the *Arabidopsis* RLCK VI_A kinase group (Fig. 7). However, no such kinase sequences were found in the *Chlamydomonas* genome, indicating that this kinase family might have appeared during the early evolution of land plants (Embryophyta).

Discussion

Rho-type small GTP-binding proteins are linked to a variety of signal transduction pathways in eukaryotic cells. They are known to recruit various downstream effector proteins to regulate cellular processes that are principally associated with the (re)organization of the cytoskeleton (Schwartz 2004). Among these effectors, kinases occupy a central role in yeast, and especially, in animal cells. These kinases are numerous and fall into several classes, such as the p21-activated kinases (PAKs), the Rho-kinases (ROKs), the mixed-lineage kinases (MLKs), the myotonin-related Cdc42-binding kinases (MRCKs), the citron kinases (CRIKs) and the novel protein kinase (PKN) (Zhao and Manser 2005). This number of GTPase-associated kinases

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underlines the complexity of the phosphorylation cascades that are downstream of the Rho-type (Rho, Cdc42, Rac) G-proteins in animals. Although they have different domain structures, the kinases listed above are all able to bind one or several types of GTP-bound Rho proteins. Their GTPase-binding ability is due to the presence of defined structural elements (amino acid motifs) outside of their kinase domains (Zhao and Manser 2005).

The mechanism of G-protein-mediated kinase activation is best known in the case of p21-activated or PAK kinases (Bokoch 2003, Rane and Minden 2014). These kinases can interact with GTP-bound Cdc42 and with Rac, but not with Rho GTPases, and this interaction dramatically increases their *in vitro* kinase activity (Manser et al. 1994). The crystal structure of the human PAK1 protein reveals that the kinase is present as a homodimer in solution. The kinase activity is autoinhibited in trans due to its N-terminal kinase inhibitor domain (Parrini et al. 2002). This kinase inhibitor domain overlaps with the binding site for the Cdc42/Rac GTPases (p21-binding domain or PBD) (Rane and Minden 2014). The core of this binding domain is the 16-amino acid-long Cdc42/Rac-interactive-binding or CRIB motif (Burbelo 1995). The binding of the GTP-bound G-proteins to the PBD results in a major change in PAK1 conformation. This results in the release of the catalytic domain from autoinhibition (Rane and Minden 2014). PAKs have an ancient origin, for example, the Cdc42-regulated Ste20 and Cla4 protein kinases of *Saccharomyces cerevisiae* belong to the same group (Zhao and Manser 2005). However, genes that code for either PAK kinases carrying the CRIB motif or for any other Rho-associated kinases of animals could not be identified in the plant genomes. Bearing in mind the central role of these kinases in basic cellular processes, such as cell growth and division, this was a rather unexpected finding.

Motifs, like the CRIB motif, are, however, present in several plant Rho (Rop)-associated proteins, such as the upstream regulator Rop GTPase-activator proteins (RopGAPs) (Wu et al.

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2000) and the downstream effector Rop-interacting CRIB-motif-containing (RIC) scaffold proteins (Wu et al. 2001). Theoretically, Rops can be linked to kinase signalling either indirectly through the CRIB-containing RIC scaffold proteins or in a CRIB-independent way through plant-specific Rop-binding motifs. The discovery of the Rop-binding plant receptor-like cytoplasmic kinases strengthened the latter possibility (Molendijk et al. 2008, Dorjgotov et al. 2009, Huesmann et al. 2012, Reiner et al. 2014). These kinases have neither p21-binding nor autoinhibitory nor any other conserved domains outside of their catalytic regions (Jurca et al. 2008). Therefore, the models explaining the regulation of animal Rho-associated kinases (Zhao and Manser 2005) cannot be applied to their plant counterparts.

All but one (NCRK; Molendijk et al. 2008) of the known *Arabidopsis* Rop-binding kinases belong to the family designated as RLCK Class VI (Fehér and Lajkó 2015). This class of plant RLCKs can be subdivided into two groups (Jurca et al. 2008) that differ in their ability to bind Rops (Dorjgotov et al. 2009). Multiple sequence alignment of all RLCK VI proteins highlighted a conservation pattern characteristic only of RLCK VI group A (Fig. 1). Seven of these conserved motifs/regions were investigated in detail. During evolution, certain members of a protein family may acquire specific function(s) in addition to the global function of the whole family. This is indicated by the preserved residues in the "specificity-determining positions" (SDPs) of their structure (Chakraborty and Chakrabarti 2014). While the mutations of fully conserved amino acids of a protein family result in non-functional proteins, mutations of SDPs only alter specific functional aspects, such as the regulation or substrate-specificity of subfamily members. These residues tend to appear on protein surfaces and influence the interaction of the proteins with other molecules. The identification of SDPs is of utmost importance to the characterization or manipulation of specific protein functions. Therefore, several *in silico* SDP detection methods have been developed (Chagoyen et al. 2016). Using the JDet software package (Muth et al. 2012), we were able to verify that the motifs we had selected for detailed

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characterization contain potential SDPs (Supplementary Fig. 1). Furthermore, it was experimentally demonstrated that these residues/motifs contribute to Rop-binding and to the regulation of kinase activity (Figs. 3, 5 and 6), in keeping with the functional significance of SDPs.

All the investigated residues/motifs reside within the kinase domain. The modelled three-dimensional structure of the RLCK VI_A2 kinase indicated that all but one of the investigated residues form a common surface (Fig. 4). It is, therefore, hypothesized that this surface may serve for docking GTP-bound Rops. This surface is just above the ATP- and substrate-binding cleft of the kinase (Fig. 4). One can suppose that Rop-binding may directly affect the conformation of the catalytic domain, rendering it able to bind and/or phosphorylate its substrates.

Arginines are abundant residues at protein-protein interfaces due to the versatility of the intermolecular interactions of their side chain (Crowley and Golovin 2005). The two arginines, $R_{189}R_{190}$, (numbering is based on the RLCKVI_A2 sequence shown in Fig. 1) are positioned just before the highly conserved HRD motif of the activation loop of the RLCK VI_A kinases. These residues seem to be responsible for the Rop-inducibility of kinase activity since exchanging these residues for those present in the constitutively active B group kinases rendered the activity of the RLCKVI_A2/3 kinases Rop independent (Figs. 2 and 3). These amino acids, therefore, may contribute to maintaining an inactive conformation that is released upon the binding of Rops.

Another highly conserved region of the Rop-binding RLCK VI_A kinases is the one that carries the investigated LS and HV motifs (Fig. 1). This short amino acid sequence resembles the CRIB motif of plant and animal Rho-type GTPase-binding proteins (Fig. 8). Mutations in this region prevented Rop-binding with (LS) or without (HV) restoring kinase activity indicating that it

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might indeed be a functional Rop-binding motif (Figs. 2 and 3). This CRIB-like motif is also close to the activation loop in the 3D model and may contribute to the Rop-dependent kinase activation mechanism (Fig. 4).

A domain replacement experiment, in which the kinase domain of the B group kinase RLCK VI_B5 was exchanged for that of the RLCKVI_A2 kinase, indicated that the investigated motifs, although required, are not sufficient for the interaction/activation (Fig. 5). This agrees with previous observations that N- or C-terminal truncation of RLCK VI_A kinases outside of the kinase domain could also prevent Rop-binding (Molendijk et al. 2008, Dorjgotov et al. 2009). It can be hypothesized that the whole protein sequence contributes to the proper conformation of the kinase correctly positioning the Rop-binding motifs on the surface. The crystallization of a GTP-Rop-RLCK VI_A complex could provide a clue to clarify the role of the above or of any other residues in the plant-specific Rop-dependent kinase activation mechanism.

RLCK VI_A type kinases seem to be present in all land plants but are missing from the unicellular alga, *Chlamydomonas* (Fig. 7). Plants and opisthokonta (fungi and animals) diverged early during evolution and plants and animals independently acquired multicellularity. Likewise, Rho-type GTPases and related signalling cascades also developed independently in these taxa, resulting in striking differences (Brembu et al. 2006). Rops themselves form a structurally distinct class of eukaryotic Rho-type G-proteins (Berken and Wittinghofer 2008). Their structural characteristics allow them to recognize and bind a plethora of plant-specific regulators and effectors (Berken and Wittinghofer 2008, Fricke and Berken 2009, Nagawa et al. 2010, Schaefer et al. 2011, Fehér and Lajkó 2015). The recruitment of RLCK kinases by the Rop G-proteins and the associated plant-specific kinase-activation mechanism are further examples of the unique nature of small GTPase-mediated signalling in plants.

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Author contribution statement

DBL, IV, MD, DM carried out most of the experimental work; GF contributed by planning and synthesizing the mutagenic oligonucleotide primers; FA was responsible for microscopy; AF ensured the financial support, planned and supervised the work, took part in experiments and wrote the manuscript.

Conflict of interest statement

The authors declare that they have no conflict of interest.

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Figure legends

Fig. 1. Identification and mutagenesis of amino acid motifs potentially responsible for the Rop GTPase-binding ability of RLCK VI_A kinases. (a) Protein sequence alignment of RLCK Class VI group A and group B kinases resulted in the identification of four regions exhibiting characteristic amino acid differences. The characteristic amino acids, which are conserved in group A but not in group B, are indicated by asterisks. These amino acids of the RLCK VI_A2 and A3 kinases were mutated to test their role in Rop-binding. The amino acids that were mutated in parallel are boxed. The motifs were named after their characteristic amino acids, as indicated above them. The colours highlight the amino acid similarities of the aligned sequences. (b) The amino acid sequence of the RLCK VI_A2 kinase with the double-underlined kinase domain and the positions of the mutations. The amino acids of RLCK VI_A2, highlighted in bold, were exchanged with those that are present in the same positions in RLCK VI_B sequences, as indicated under the sequence. The whole LP-IDE region was exchanged for the corresponding region of RLCK VI_B5, as shown (the IDE motif was also modified on its own, as indicated).

Fig. 2. Interaction and activation of RLCK VI_A2 kinase mutants with the constitutively active Rop1^{CA} G-protein. a). Yeast two-hybrid protein-protein interaction assay. The kinase mutants (see Fig. 1 for details) and the wild type (WT) were fused to the Gal4 activation while the Rop1^{CA} GTPase was fused to the Gal4 DNA-binding domains and expressed in yeast. Colony growth on a -leu -trp plate is shown to confirm transformation; growth at increasing selection pressure (-leu -trp -his; -leu -trp -his +5mM 3AT; -leu -trp -his -ade) demonstrates interaction strength. Empty pGADT7 and pGBKT7 vectors were used as controls. (b) Auto-phosphorylation activity of the RLCK VI_A2 kinase mutants in the presence (+) and absence (-) of the active Rop GTPase. Autoradiogram of kinase activity (upper row, P^{32}) and Coomassie Brilliant Blue (CBB) stained proteins in the polyacrylamide gel (lower row) are shown.

Fig. 3. Interaction and activation of RLCK VI_A3 kinase mutants with the constitutively active Rop1^{CA} G-protein. (a) Yeast two-hybrid protein-protein interaction assay. The kinase

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mutants (LS, HV, RR) and the wild type (WT) were fused to the Gal4 activation while the Rop1^{CA} GTPase was fused to the Gal4 DNA-binding domains and expressed in yeast. Colony growth on a -leu -trp plate is shown to prove transformation; growth on *-leu -trp -his* plates indicates interaction with the active G-protein. Empty pGADT7 and pGBKT7 vectors were used as controls. (b) Myelin basic protein (MyBP) phosphorylation activity of the RLCK VI_A3 kinase mutants in the presence (+) and absence (-) of the active Rop GTPase. Autoradiogram of kinase activity (upper part, P^{32}) and stained proteins in the polyacrylamide gel (lower part, Coomassie Brilliant Blue [CBB]) are shown (M – Molecular weight marker; H2O – no kinase control).

Fig. 4. Predicted 3D model of RLCK VI_A2 structure with highlighted characteristic amino acids (arrows) discriminating RLCK VI_A and VI_B group kinases (see Fig. 1). The various motifs are labelled using different colours (YA-magenta, LS-red, HV-green, RR-white, G-blue and LP-IDE yellow).

Fig. 5. The region of the RLCK VI_A2 kinase carrying the investigated motifs is not sufficient to convey Rop1^{CA}-binding to RLCK VI_B5. The part of the VI_A2 kinase with the investigated Rop-binding motifs was used to replace the corresponding region of the B5 kinase. (a) The sequence alignment of the full length RLCK VI_B5 kinase with the chimeric RLCK VI_B5_A2 protein is shown. The positions of the investigated Rop-binding motifs are indicated above the sequences by the one-letter-codes of the characteristic amino acids. (b) Result of a yeast two-hybrid assay testing the capability of the chimeric RLCK VI_B5_A2 protein shown in panel (a) to interact with Rop1$^{CA.}$ The full length wild type RLCK VI_A2 and VI_B5 kinases were used as positive and negative controls, respectively. The kinases were fused to the Gal4 activation while the Rop1^{CA} was fused to the Gal4 DNA-binding domains, respectively. Colony growth on a *-leu -trp* plate is shown to confirm successful yeast transformation by the two constructs; growth on *-leu -trp -his* plates indicates protein-protein interaction via the reconstituted Gal4-mediated complementation of histidine auxotrophy. Empty pGADT7 and pGBKT7 vectors (-) were also used as controls.

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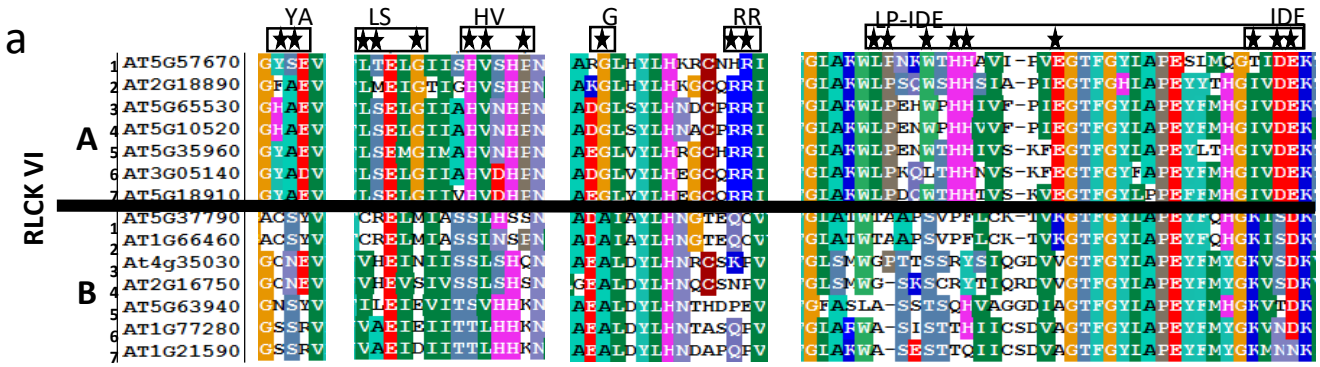
Fig. 6. Testing the interaction of RLCK VI_A3 kinase variants with the constitutively active Rop1^{CA} G-protein in pollen tubes. Confocal laser scanning images of transfected pollen tubes are shown. The constructs used for transfection carried a Lat52:mCherry chimeric gene to visualize transfected pollen tubes due to their red fluorescence. The same construct was used to express the Rop1^{CA} protein fused to the N-terminal fragment of the mVenus fluorescent protein, and the wild type (WT) or mutant (HV, LS, RR) RLCK VI_A3 kinase forms fused to the C-terminal mVenus fragment. Interaction of Rop1^{CA} to the WT kinase resulted in bimolecular fluorescence complementation as indicated by the fluorescence of the reconstituted mVenus protein (green). mVenus fluorescence could not be detected after the transfection of pollen tubes with constructs expressing Rop1^{CA} together with the HV, LS, or RR kinase mutants. The same vector without a kinase gene (-) was used as a control. White arrows show pollen tube tips. Scale bar is 30µm.

Fig. 7. Comparison of the investigated kinase motifs in various plant species having complete sequenced genomes and belonging to different taxa. The Pattern Hit Initiated Basic Local Alignment Search Tool (PHI-BLAST; NCBI) was used to identify the homologs of the *Arabidopsis thaliana* RLCK VI_A3 (At5g65530) kinase, having the GXXXHXXH amino acid pattern (X=any amino acid). The expected value was set to 0.01 and the PHI-BLAST threshold to 0.005. The sequence alignment was made using the ClustalW algorithm (Thompson et al. 1994). Only the regions investigated in the present work are shown.

Fig. 8. Comparison of animal, yeast and plant CDC42/RAC1-interactive binding (CRIB) motifs with the LS-HV Rop-binding motifs of RLCK VI_A kinases. AtGAP, *Arabidopsis thaliana* Rop GTPase activating protein; AtRIC *Arabidopsis thaliana* Rop-interacting CRIB-motif-containing protein; AtRLCK VI_A, *Arabidopsis thaliana* receptor-like cytoplasmic kinase class VI group A; HsPAK1, *Homo sapiens* P21-activated kinase 1; ScSte20, *Saccharomyces cerevisiae* 'Sterile 20' protein kinase; the CRIB consensus sequence is based on yeast and animal protein sequences (Burbelo et al. 1995).



b

MKYIRSNSLKRLFSFKRRSFDSDSSENSSPLAASSTKCVEGFQETDQFQRPKWKCFSFQEIYDATNGFSSENLVGRGGFAEVYK

GILGKNGEEIAVKITRGGRDDERREKEFLMEIGTIGHVSHPNVLSLLGCCIDNGLYLVFIFSSRGSLASLLHDLNQAPLEWETR

YKIAIGTAKGLHYLHKGCQRRIIHRDIKSSNVLLNQDFEPQISDFGLAKWLPSQWSHHSIAPIEGTFGHLAPEYYTHGIVDEKT

DVFAFGVFLLELISGKKPVDASHQSLHSWAKLIIKDGEIEKLVDPRIGEEFDLQQLHRIAFAASLCIRSSSLCRPSMIEVLEVL

QGEDIEKEKWKMEEEEEVKEEFWGYEDLEDCECDSSISLSPPDSISNRSSSHRSR

Fig. 1.

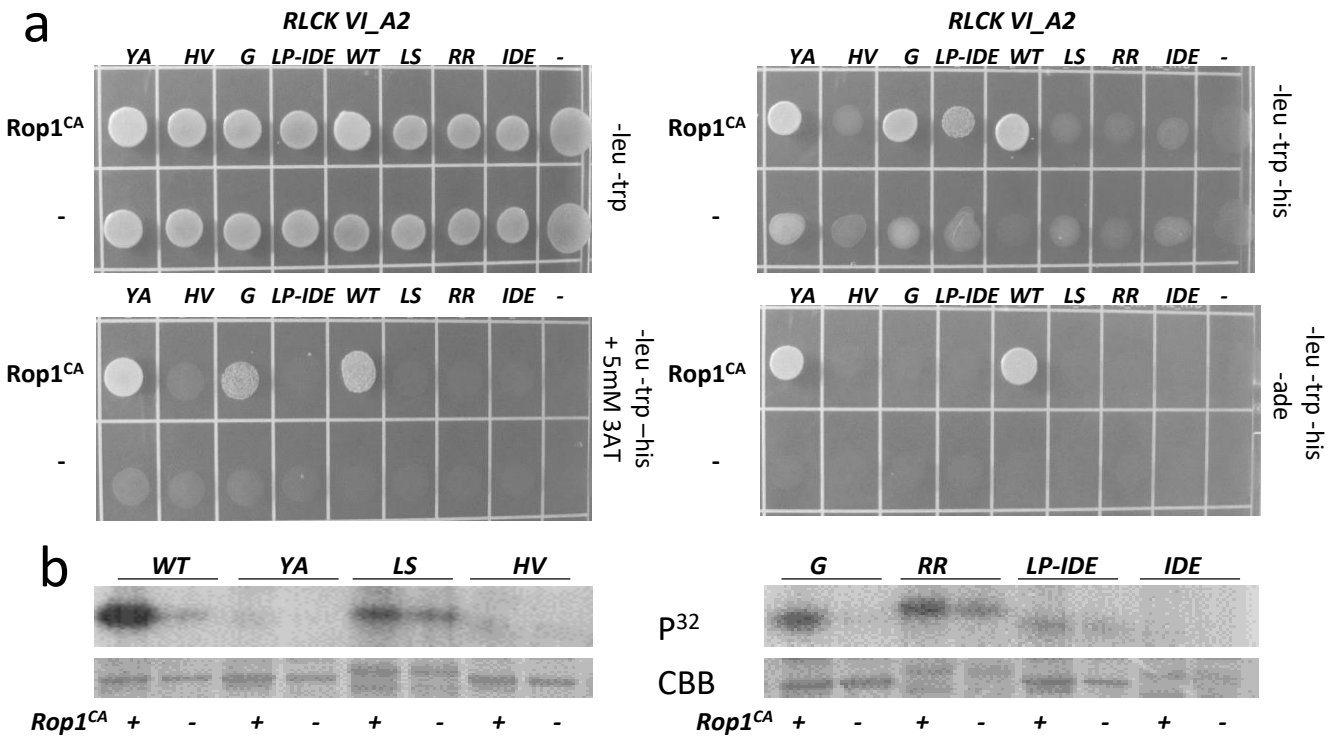
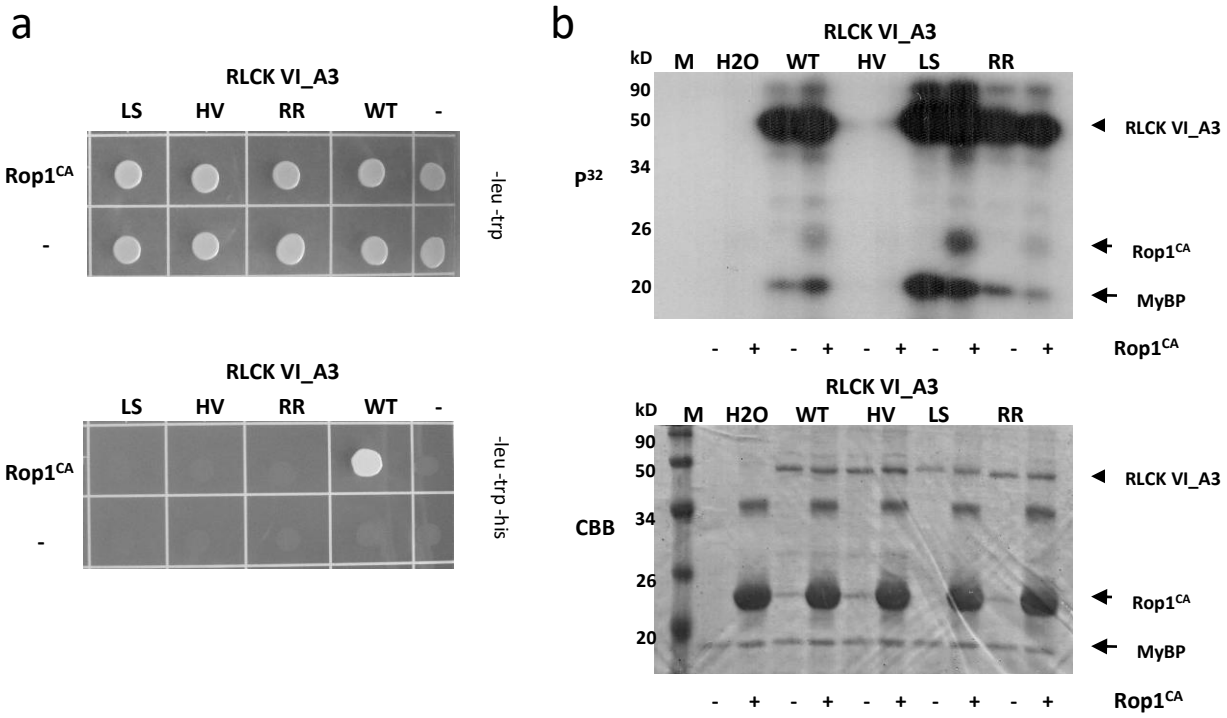

a
RLCK VI_A2
YA HV G LP-IDE WT LS RR IDE -
Rop1CA
-
-leu -trp
-leu -trp -his
-leu -trp –his + 5mM 3AT
-leu -trp -his -ade
b
WT YA LS HV
G RR LP-IDE IDE
P32
CBB
Rop1CA + - + - + - + -

Fig. 2.


a
RLCK VI_A3
LS
HV
RR
WT
-
Rop1CA
-
-leu -trp
RLCK VI_A3
LS
HV
RR
WT
-
Rop1CA
-
-leu -trp -his
b
RLCK VI_A3
kD
M
H2O
WT
HV
LS
RR
90
50
34
26
20
P32
RLCK VI_A3
Rop1CA
MyBP
- + - + - + - + - +
Rop1CA
RLCK VI_A3
kD
M
H2O
WT
HV
LS
RR
90
50
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CBB
RLCK VI_A3
Rop1CA
MyBP
- + - + - + - + - +
Rop1CA

Fig. 3.

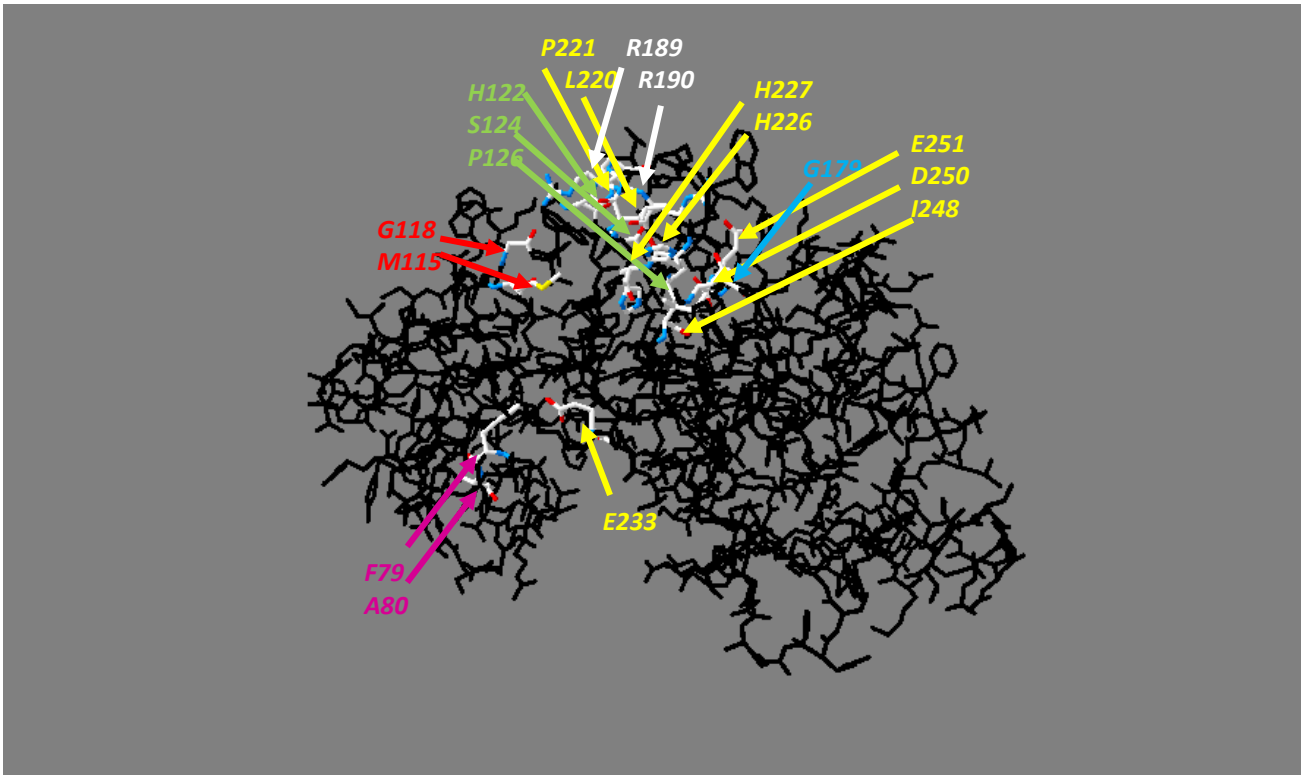
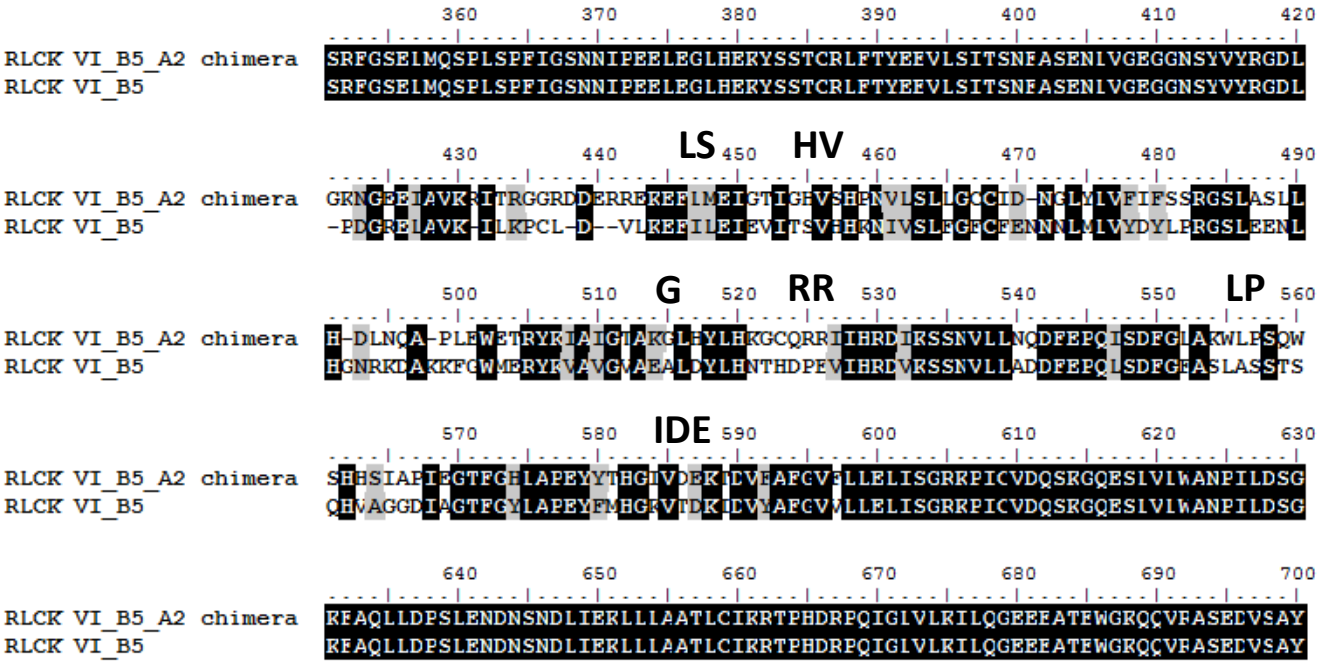

P221
R189
L220
R190
H122
S124
P126
H227
H226
E251
D250
I248
G118
M115
E233
F79
A80

Fig. 4.

a


RLCK VI_B5_A2 chimera
RLCK VI_B5
LS
HV
G
RR
LP
IDE

b

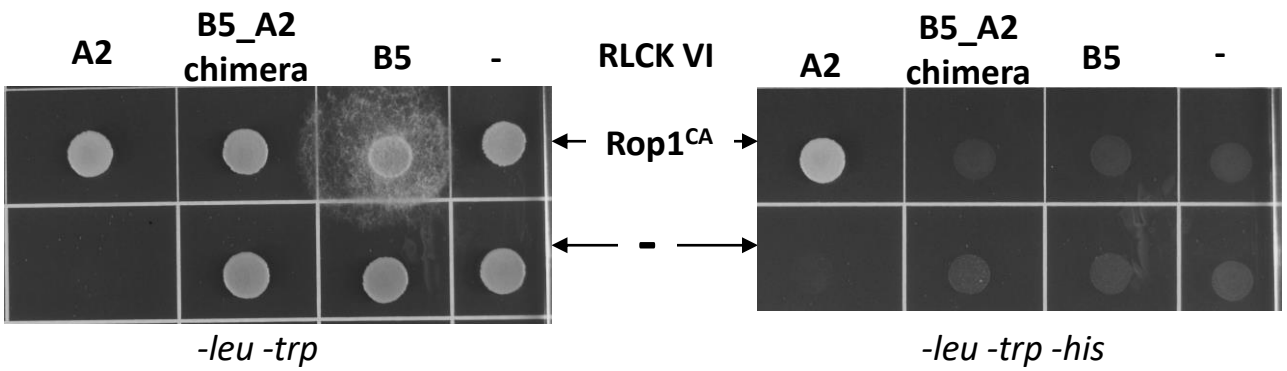

A2
B5_A2 chimera
B5
-
RLCK VI
Rop1CA
-leu -trp
-leu -trp -his

Fig. 5.

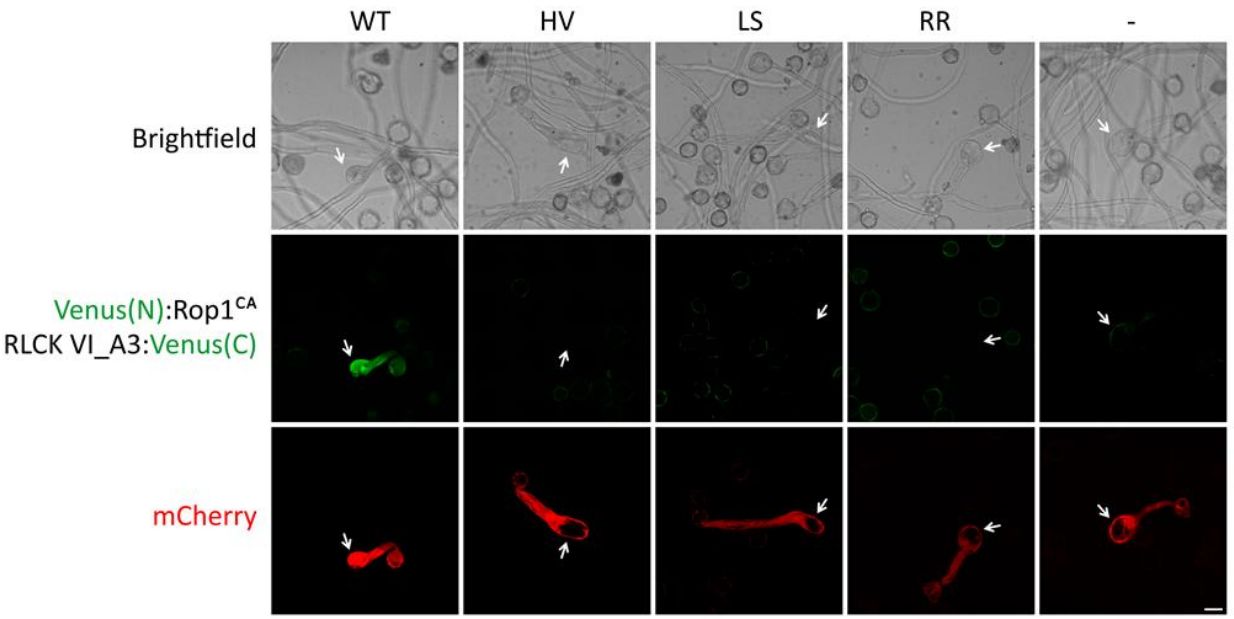
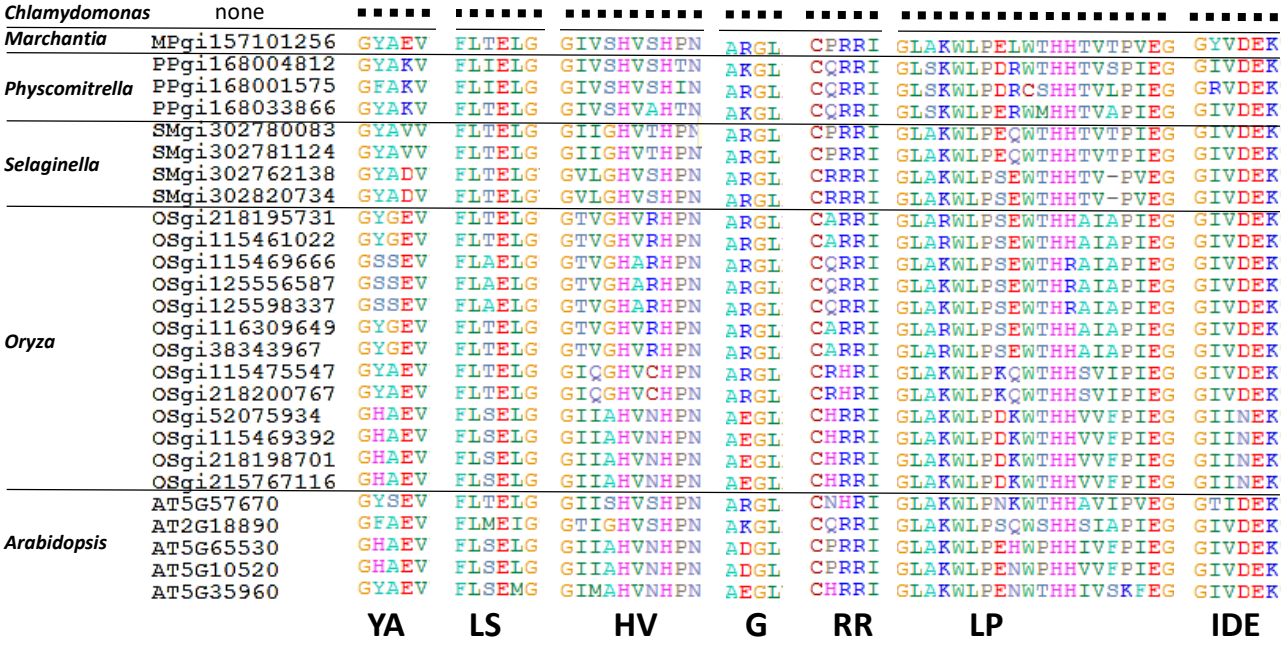

WT
HV
LS
RR
-
Brightfield
Venus(N):Rop1CA
RLCK VI_A3:Venus(C)
mCherry

Fig. 6.


Chlamydomonas none
Marchantia MPgi157101256 GYAEV FLTELG GIVSHVSHPN ARGL CPRRI GLAKWLPELWTHHTVTPVEG GYVDEK
Physcomitrella PPgi168004812 GYAKV FLIELG GIVSHVSHTN AKGL CQRRI GLSKWLPDRWTHHTVSPIEG GIVDEK
PPgi168001575 GFAKV FLIELG GIVSHVSHIN ARGL CQRRI GLSKWLPDRCSHHTVLPIEG GRVDEK
PPgi168033866 GYAKV FLTELG GIVSHVAHTN AKGL CQRRI GLSKWLPERWMHHTVAPIEG GIVDEK
Selaginella SMgi302780083 GYAVV FLTELG GIIGHVTHPN ARGL CPRRI GLAKWLPEQWTHHTVTPIEG GIVDEK
SMgi302781124 GYAVV FLTELG GIIGHVTHPN ARGL CPRRI GLAKWLPEQWTHHTVTPIEG GIVDEK
SMgi302762138 GYADV FLTELG GVLGHVSHPN ARGL CRRRI GLAKWLPSEWTHHTV-PVEG GIVDEK
SMgi302820734 GYADV FLTELG GVLGHVSHPN ARGL CRRRI GLAKWLPSEWTHHTV-PVEG GIVDEK
Oryza OSgi218195731 GYGEV FLTELG GTVGHVRHPN ARGL CARRI GLARWLPSEWTHHAIAPIEG GIVDEK
OSgi115461022 GYGEV FLTELG GTVGHVRHPN ARGL CARRI GLARWLPSEWTHHAIAPIEG GIVDEK
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OSgi125556587 GSSEV FLAELG GTVGHARHPN ARGL CQRRI GLAKWLPSEWTHRAIAPIEG GIVDEK
OSgi125598337 GSSEV FLAELG GTVGHARHPN ARGL CQRRI GLAKWLPSEWTHRAIAPIEG GIVDEK
OSgi116309649 GYGEV FLTELG GTVGHVRHPN ARGL CARRI GLARWLPSEWTHHAIAPIEG GIVDEK
OSgi38343967 GYGEV FLTELG GTVGHVRHPN ARGL CARRI GLARWLPSEWTHHAIAPIEG GIVDEK
OSgi115475547 GYAEV FLTELG GIQGHVCHPN ARGL CRHRI GLAKWLPKQWTHHSVIPIEG GIVDEK
OSgi218200767 GYAEV FLTELG GIQGHVCHPN ARGL CRHRI GLAKWLPKQWTHHSVIPIEG GIVDEK
OSgi52075934 GHAEV FLSELG GIIAHVNHPN AEGL CHRRI GLAKWLPDKWTHHVVFPIEG GIINEK
OSgi115469392 GHAEV FLSELG GIIAHVNHPN AEGL CHRRI GLAKWLPDKWTHHVVFPIEG GIINEK
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OSgi215767116 GHAEV FLSELG GIIAHVNHPN AEGL CHRRI GLAKWLPDKWTHHVVFPIEG GIINEK
Arabidopsis AT5G57670 GYSEV FLTELG GIISHVSHPN ARGL CNHRI GLAKWLPNKWTHHAVIPVEG GTIDEK
AT2G18890 GFAEV FLMEIG GTIGHVSHPN AKGL CQRRI GLAKWLPSQWSHHSIAPIEG GIVDEK
AT5G65530 GHAEV FLSELG GIIAHVNHPN ADGL CPRRI GLAKWLPEHWPHHIVFPIEG GIVDEK
AT5G10520 GHAEV FLSELG GIIAHVNHPN ADGL CPRRI GLAKWLPENWPHHVVFPIEG GIVDEK
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YA LS HV G RR LP IDE

Fig. 7.

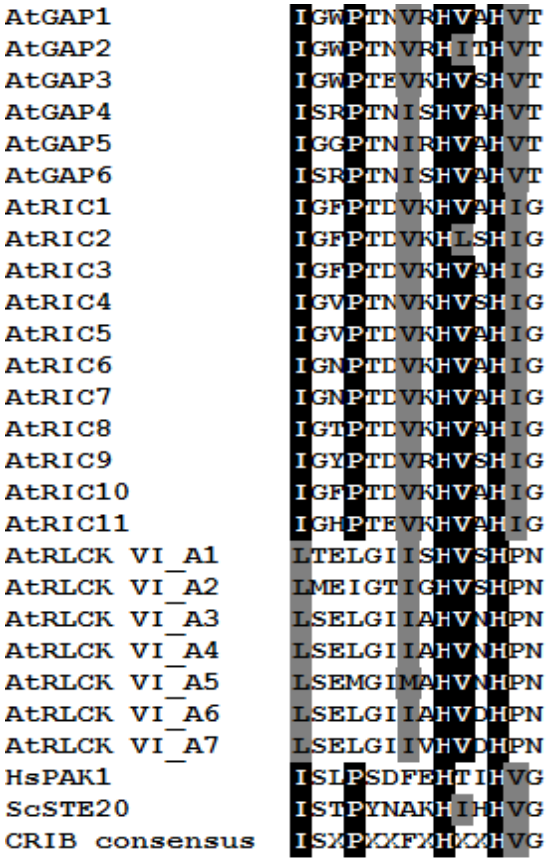
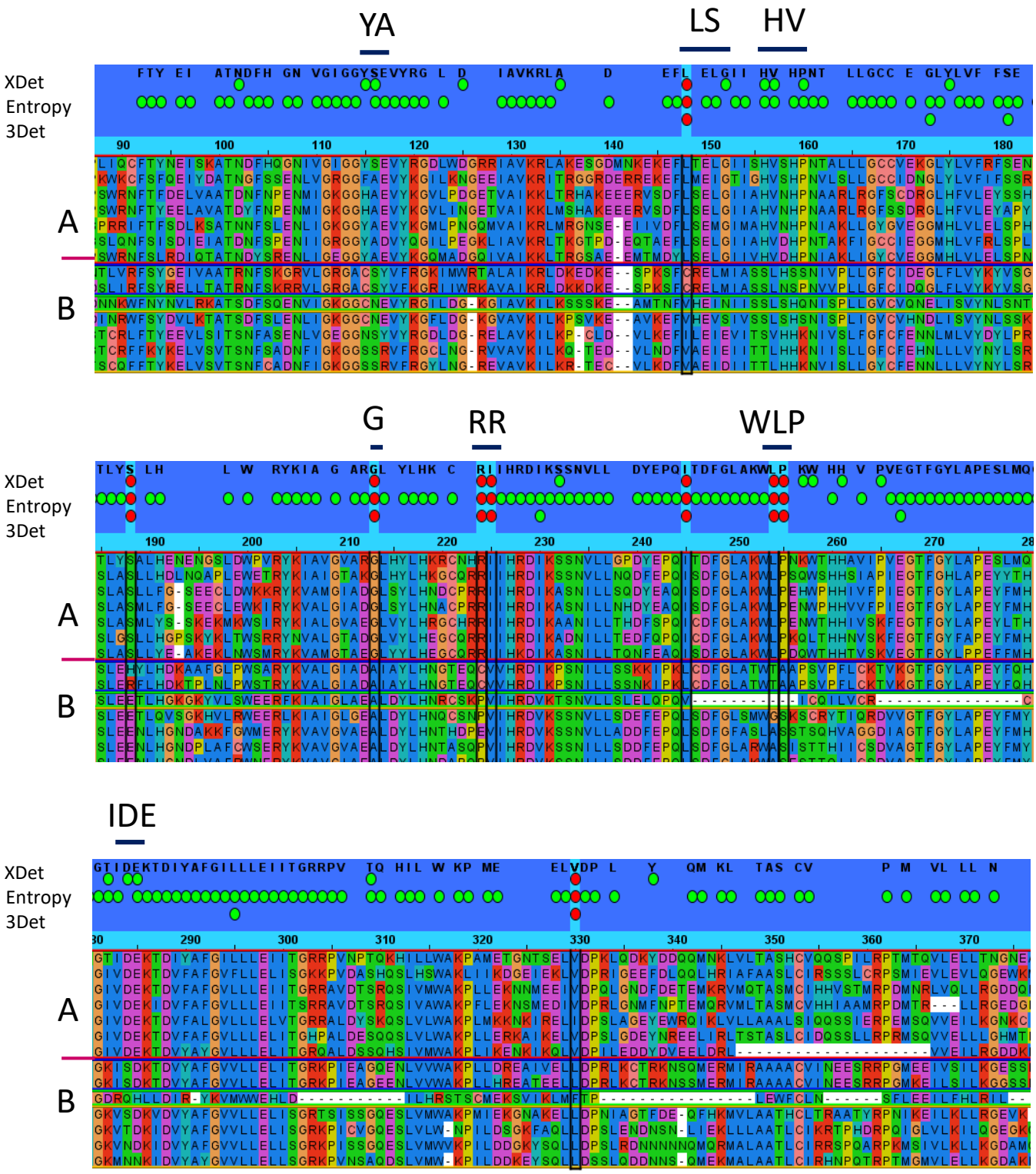

AtGAP1 IGWPTNVRHVAHVT
AtGAP2 IGWPTNVRHITHVT
AtGAP3 IGWPTEVKHVSHVT
AtGAP4 ISRPTNISHVAHVT
AtGAP5 IGGPTNIRHVAHVT
AtGAP6 ISRPTNISHVAHVT
AtRIC1 IGFPTDVKHVAHIG
AtRIC2 IGFPTDVKHLSHIG
AtRIC3 IGFPTDVKHVAHIG
AtRIC4 IGVPTNVKHVSHIG
AtRIC5 IGVPTDVKHVAHIG
AtRIC6 IGNPTDVKHVAHIG
AtRIC7 IGNPTDVKHVAHIG
AtRIC8 IGTPTDVKHVAHIG
AtRIC9 IGYPTDVRHVSHIG
AtRIC10 IGFPTDVKHVAHIG
AtRIC11 IGHPTEVKHVAHIG
AtRLCK VI_A1 LTELGIISHVSHPN
AtRLCK VI_A2 LMEIGTIGHVSHPN
AtRLCK VI_A3 LSELGIIAHVNHPN
AtRLCK VI_A4 LSELGIIAHVNHPN
AtRLCK VI_A5 LSEMGIMAHVNHPN
AtRLCK VI_A6 LSELGIIAHVDHPN
AtRLCK VI_A7 LSELGIIVHVDHPN
HsPAK1 ISLPSDFEHTIHVG
ScSTE20 ISTPYNAKHIHHVG
CRIB consensus ISXPXXFXHXXHVG

Fig. 8.



Supplementary Fig. 1. Analysis of the multiple sequence alignment of the Arabidopsis RLCK_VI kinase family members by the Jdet software package (Muth et al. 2012). The package applies three different approaches (Xdet, Entropy-based prediction, and 3Det) to predict SDPs and subfamilies. Green spots sign SDPs predicted by the given program. Red spots indicate positions that were predicted as SDPs by all three methods. The motifs investigated in the manuscript are indicated by lines and names above the analysis result. The RLCKVI_A group was predicted as a single subfamily while the other RLCK VI kinases (we designated together as RLCK VI_B group) fall into three subfamilies (separated by lines) based on the prediction.

Title: Identification of amino acid motifs required for the Rho-of-plants (ROP) GTPase-mediated activation of receptor-like cytoplasmic kinases Submitted to: Planta

Dézi Bianka Lajkó, Ildikó Valkai, Mónika Domoki, Dalma Ménesi, Györgyi Ferenc, Ferhan Ayaydin, Attila Fehér

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RLCK VI_A2 mutagenesis

VI_A2 EcoRI Full Fw	GCAGAATTCATGAAGTACATTCGAAGCAA	5' end, cloning to pEntry2b (SalI site removed) at EcoRI site
VI_A2 XhoI Full Rev	GCACTCGAGCTAGCGACTACGATGCGAAGAAC	3' end, cloning to pEntry2b (SalI site removed) at XhoI site
VI_A2 IDE Fw	CTATACACATGGGAAAGTGACTGACAAGACTGATGTGTTTG	mutagenic
VI_A2 IDE Rev	CAAACACATCAGTCTTGTCAGTCACTTTCCCATGTGTATAG	mutagenic
VI_B5A2 chimera Fw1	TGGATTGGCAAAATGGGCTTCAAGTACCTCA	mutagenic
VI_B5A2 chimera Rev1	TGAGGTACTTGAAGCCCATTTTGCCAATCCA	mutagenic
VI_B5A2 chimera Fw2	GGTAAAGTAACCGACGAGAAGACTGATGTG	mutagenic
VI_B5A2 chimera Rev2	CACATCAGTCTTCTCGTCGGTTACTTTACC	mutagenic
VI_A2 G Fw	GGACAGCAAAAGCGCTTCATTATTTACAC	mutagenic
VI_A2 G Rev	GTGTAAATAATGAAGCGCTTTTGCTGTCC	mutagenic
VI_A2 RR Fw	CAAAGGTTGTCAGCAACCGATCATACACAG	mutagenic
VI_A2 RR Rev	CTGTGTATGATCGGTTGCTGACAACCTTTG	mutagenic
VI_A2 YA Fw	AGGTAGAGGTGGATGTACAGAGGTGTATAA	mutagenic
VI_A2 YA Rev	TTATACACCTCTGTACATCCACCTCTACCT	mutagenic
VI_A2 LS Fw	AGAGTTTTTACTGGAGATTGAAACAATAGGAC	mutagenic
VI_A2 LS Rev	GTCCTATTGTTTCAATCTCCAGTAAAAACTCT	mutagenic
VI_A2 HV Fw	ACAATAAGATCTGTCCAACATTCTAATGTC	mutagenic
VI_A2 HV Rev	GACATTAGAATGTTGGACAGATCTTATTGT	mutagenic

RLCK VI_A3 mutagenesis

VI_A3 EcoRI Full Fw	GCAGAATTCATGGCTGTTGAAGAGATGGA	5' end, cloning to pEntry2b (SalI site removed) at EcoRI site
VI_A3 XhoI Full Rev	GCACTCGAGCTCCATTAAGAGCTGTCTAT	3' end, cloning to pEntry2b (SalI site removed) at XhoI site
VI_A3 HV Fw	GCTGCGTTTCGATGGCTAAGACTTGCGATTATCCCAAGC	mutagenic
VI_A3 HV Rev	AATCGCAAGTCTTAGCCATCGAAACGCAGCTAGGCTTCGTG	mutagenic
VI_A3 LS Fw	GATTTCTTAGCGGAGCTCGACATAATCGCACATGTTAACC	mutagenic
VI_A3 LS Rev	GTGCGATTATGTCGAGCTCCGCTAAGAAATCACTGAC	mutagenic
VI_A3 RR Fw	CATAACGATTGCCCTCAGCCGATTATTCACCGAGAC	mutagenic
VI_A3 RR Rev	GTCTCGGTGAATAATCGGCTGAGGGCAATCGTTATG	mutagenic

Rop1 mutagenesis

AtRop1 EcoRI Full Fw	GCAGAATTCATGAGCGCTTCGAGGTTCGTAA	5' end, cloning to pEntry2b (SalI site removed) at EcoRI site
AtRop1 SalI Full Rev	TCTGTCGACATAGAATGGAGCATGCCTTCTGCGC	3' end, cloning to pEntry2b (SalI site removed) at XhoI site
AtRop1 CA Fw (BtgZI)	GTTGGCGATGTCGTCGTCGGAAAAACTTGTTTGTTGA	mutagenic
AtRop1 CA Rev (BtgZI)	TCAACAAAACAAGTTTTTCCGACAGCGACATCGCCAC	mutagenic

pENTRY primers

pE2B-Seq	GCGTTTCTACAAACTCTTCC	to amplify GATEWAY-ready PCR fragments in extension overlap PCR
SeqLB	GTAACATCAGAGATTTTGAGACAC	to amplify GATEWAY-ready PCR fragments in extension overlap PCR

BIFC constructs

pDOEPmlA3SLICEFw	GGGGTCCCTACGTAGTCACGCTGTTGAAGAGATGGAGAAGAAG	PCR cloning into the PmlI site of the pDOE11 vector by SLICE
pDOEPmlA3SLICERev	CAGAACCTCCGGACGTCACCTCCATTAAGAGCTGTCTATGTCG	PCR cloning into the PmlI site of the pDOE11 vector by SLICE
Rop1 NcoI Full Fw	CCATGGGGATGAGCGCTTCGAGGTTCG	Cloning into the pDOE11 vector
Rop1 SpeI Full Rev	TGACTAGTTCATAGAATGGAGCATGCCTTC	Cloning into the pDOE11 vector