

Abstract of Ph.D. thesis

**MOLECULAR CHARACTERISTIC OF ROP
(RHO OF PLANTS) GTPASE ACTIVATED
RECEPTOR-LIKE CYTOPLASMIC KINASES
IN ARABIDOPSIS**

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Introduction

The Rho-type GTPases have central roles in cellular processes associated with cytoskeletal dynamics (e.g. cell movement, cell division, cell shape, and cell polarity). These proteins operate as molecular switches: they activate signal transduction pathways when they are in GTP-bound conformation, but their signalling activity ceases when they are GDP-bound. If the Rho GTPase is in the GTP-bound form, it can further activate a diverse set of downstream signalling effector proteins. There are some upstream regulator proteins which regulate the activity of ROPs. One of these proteins are the guanine nucleotide exchange factor or GEF proteins which catalyse the GDP to GTP exchange activating ROPs. In contrast, the GAPs (GTPase accelerator proteins) inactivate ROPs via the promotion of GTP hydrolysis, while the GDIs (guanine nucleotide dissociation inhibitors) stabilize the inactive state of ROPs.

Plants have a specific group of Rho-type GTPases, the „Rho of plants” (ROP) family. Our knowledge about the signalling pathways associated with ROPs is yet incomplete. ROPs differ from other Rho-type GTPases in the regions which are responsible for effector binding, suggesting that ROP GTPases have specific effectors. Indeed, plants lack the Rho GTPase-activated PAK kinases, which are

very important mediators of Rho GTPase signalling in yeast as well as in animals. Therefore our question was: are there any ROP GTPase-activated kinases, which may have PAK-like functions in plants? Due to a yeast two-hybrid screening approach two ROP-interacting kinases could be identified. These kinases interacted with the GTP- but not with the GDP-bound ROP form what is typical for ROP effectors. Furthermore, the *in vitro* activity of these kinases was dependent on the presence of GTP-bound ROP. These ROP-activated kinases belong to the subfamily VI of receptor-like cytoplasmic kinases (RLCKs) of *Arabidopsis*. They have a receptor kinase-like catalytic domain, but they don't have extracellular or transmembrane regions and that's why they can found in the cytoplasm. Based on their primary structure, the 14 Arabidopsis RLCK VI kinases can be classified into two groups (A and B). Only the members of group A have ROP GTPase-binding ability, but it was not observed in the case of group B kinases, their activity is ROP GTPase independent.

Research objectives

Based on the results of our laboratory, we have been looking for answers to the following questions during my phd work:

1. What kind of amino acid motifs have the *Arabidopsis* receptor-like cytoplasmic kinases (subgroup VI class A) that can bind to ROP GTPases?
2. What is the role of the identified amino acid motifs in the activation of kinases?
3. How common are these kinase motifs in plants?
4. What is the effect of mutation of identified amino acids on the *in planta* function of kinases?

Materials and methods

The cDNA clones of the RLCK VI_A2 and VI_A3 kinases and AtRop1 were obtained from the *Arabidopsis* Biological Resource Center. The cDNAs were amplified by polymerase chain reaction (PCR). The RLCK VI_A group-specific amino acids were changed to those present in B group kinase(s). The mutated kinase cDNAs were cloned by LR recombination into the Gateway version of the yeast two-hybrid vector. The mutant kinases were produced in and purified from a bacterial protein expression system. The plasmid constructs (pDest-GBKT7; pET26b) carrying the constitutively active (G15V) mutant forms of the *Arabidopsis* Rop1 GTPase were developed using similar procedures. The prepared mutant constructs were used for our experimental work:

- Yeast two-hybrid screening
- Protein production and purification from bacteria
- Western blot analysis on purified proteins
- *In vitro* kinase assay on purified proteins

Protein–protein interactions *in planta*:

Bimolecular fluorescence complementation tests were used to detect protein-protein interactions *in planta*. BiFC tests were conducted based on the pDOE vector system. 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. In our case, the tested proteins were the constitutively active ROP1 and wild type and mutated RLCK VI_A3 kinases. The PCR-amplified cDNA coding for the constitutively active form of the Rop1 G-protein was cloned into the appropriate site of the vector. In this way, Rop1 was fused to the N-terminal part of the mVenus protein. The mutant kinase cDNAs were inserted into the vector using SLICE (seamless ligation cloning extract) method. The vector also carry a separate fluorescent protein for transformation control. The constructs were bombarded into *Nicotiana tabacum* pollens, then pollens were germinated under *in vitro* conditions. Cells expressing Lat52:mCherry were tracked by using a spinning disk confocal microscope. The laser

intensity and the camera exposure settings were kept constant during image capturing. Subapical pollen tube diameter and total pollen tube length were measured in the mCherry-marked transformed pollen tubes. The Evaluation was made by ImageJ software.

Plant experiments:

For our experiments we used the *rlck vi_a2* T-DNA insertion mutant line. Seeds were surface sterilized and then were incubated in cold sterile water overnight at 4 °C and then spread on sterile medium. The seedlings were grown under sterile conditions *in vitro*; the plants were grown in short-day (8h) or continuous light at 22 °C constant temperature. The medium was ½ MS with 0.8% agar and 0.5% sucrose. Later we planted our plants into the greenhouse in short-day conditions, and when reaching the right size, we used long-day conditions until the *Arabidopsis* inflorescence was harvested. Subsequently, the cDNAs encoding the different kinase mutants, regulated by the promoter of the RLCK VI_A2 gene, were introduced into the *rlck vi_a2* mutant *Arabidopsis* line by *Agrobacterium*-mediated genetic transformation. The transformed plants were selected by seed germination in the presence of 20 mg/l Hygromycin antibiotic. The segregation tests using the progeny of the transformed plants were also carried out in the presence of 20 mg/l Hygromycin. Lines

showing appropriate segregation rates were selected and hypocotyl growth experiments were performed.

Results

Production of RLCK VI_A3 kinase mutants affected in ROP binding:

Based on *in silico* comparison, several positions were identified where the amino acids are characteristically different in the sequences of group A and B kinases. It was supposed that these residues might be responsible for the difference in ROP GTPase-binding. Although these amino acid motifs are dispersed along the kinase sequences, on 3D kinase models most of them form a common surface. We replaced these amino acids one by one in the ROP-activated RLCK VI_A2 kinase with typical amino acids of B-type kinases, using site-specific mutagenesis. Changing the motifs prevented ROP GTPase binding in yeast two hybrid system.

The *in vitro* auto-phosphorylation activity of the kinase was completely eliminated in the case of certain mutations, while in other instances the activity become independent of the presence of the ROP GTPase. These results were confirmed with the RLCK VI_A3 kinase. As this kinase (in contrast to VI_A2) well phosphorylates the myelin basic protein substrate, we could demonstrate that the mutations affect

substrate phosphorylation and auto-phosphorylation in the same way.

The evolutionary conservation of the ROP-binding motifs of RLCK VI_A3 kinases:

We were interested in how ROP-activated kinases are widespread in the plant kingdom. Therefore the evolutionary conservation of the position of ROP-binding amino acids was investigated in kinase sequences of different plant taxa with available whole genome sequence. We could conclude that RLCK VI A-type kinases exist in multicellular plants but not in unicellular algae, indicating that this kinase family might have appeared during the early evolution of land plants (Embryophyta).

The interaction of RLCK VI_A3 kinase and ROP1 GTPase *in planta* and the effect of RLCK VI_A3 kinase on pollen tube growth:

The bimolecular fluorescence complementation (BiFC) assay was used to determine the *in vivo* interaction of the wild type and mutant RLCK VI_A3 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). Bimolecular fluorescence complementation confirmed

that only the wild type kinase but not of the LS, HV and RR mutant forms could interact with the ROP GTPase since only this combination resulted in green mVENUS fluorescence. Observing pollen tube morphology it could be observed the transient expression of Rop1 CA resulted in very short pollen tubes with balloon-like widened tips, since ectopic expression of active ROPs results in the loss of cell polarity and blocks cell growth. Co-expression of A3 kinase or its mutants with Rop1 CA did not affect the pollen tube phenotype in a significant way, indicating that the kinases are not rate limiting for this ROP1 overexpression phenotype.

The role of ROP-binding ability of RLCK VI_A2 kinase in the biological function of kinase:

Characterization of the *rlck vi_a2* T-DNA insertion mutant revealed that hypocotyls of mutant seedlings were 25% shorter in 40 μ E red light compared to the hypocotyl length of Columbia-0 (Col) wild-type plants, and a reduction in the size of the cotyledons was also observed. We were able to restore the hypocotyl length and cotyledon size of the *rlck vi_a2* mutant plants by expressing the wild type (WT) RLCK VI_A2 cDNA, moreover, in these lines, significantly higher hypocotyl lengths were measured compared to Col plants. RLCK VI_A2_HV, LS and RR mutant kinases were also expressed in the *rlck*

vi_a2 mutant background to determine whether the ROP-binding ability of kinase plays a role in this process. We also performed hypocotyl length measurements in high intensity continuous red light. Our results showed similarity with the results obtained in experiments with wild type A2 kinase. In case of length of the hypocotyl, all mutant kinase variants have been restored the phenotypic changes observed in the absence of RLCK VI_A2, as well as cotyledons were larger than Col-0 plants.

Conclusions:

Multiple sequence alignment of all RLCK VI proteins highlighted a conservation pattern characteristic only of RLCK VI group A. 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. 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. We have also demonstrated that the characteristic amino acid differences between the two groups of the *Arabidopsis* RLCK VI kinase family also function as SDPs and are consistent with the different ROP GTPase binding ability and regulation of kinases. Furthermore, it was experimentally demonstrated that these residues/motifs contribute to ROP-binding and to the regulation of kinase activity, in keeping with the functional significance of SDPs. The modelled three-dimensional structure of the RLCK VI_A2 kinase indicated that all but one of the investigated residues form a common surface. The exception is the motif (YA) that is not involved in the ROP GTPase binding. 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. 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.

The polar growth of pollen tubes is one of the best studied processes among the processes regulated by plant ROP GTPases. Overexpression of ROP1 CA GTPase causes loss of polarity of pollen tubes and widening of pollen tube peaks. This widening phenotype was not affected by RLCK VI_A3 kinases expressed in parallel with ROP1 CA GTPase protein.

Furthermore, the overexpression of constitutively active RLCK VI_A3 kinases did not alter the diameter values at the peak of the pollen tubes. Our observations suggest that the function of the RLCK VI_A3 kinase is not necessary for the polarity of the pollen tube, and that the kinase activity does not influence these regulatory processes.

In complementation experiments with *rlck vi_a2* kinase mutant plants, the reduced hypocotyl extension phenotype of mutant seedlings was able to complement both wild-type and ROP GTPase-binding mutant kinase forms. It can be assumed that the activity of kinases is only modulated by ROP GTPase binding but is not essentially required. It cannot be excluded that RLCK VI_A kinases have ROP GTPase dependent and independent functions also. Similar results of a mutant seedling with no kinase activity suggest that the phosphorylation activity of the kinase is not necessary for its biological function. Kinases often also perform functions that are independent of their activity through the cohesion / positioning of protein complexes.

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Publications

Article related to this Ph.D thesis:

Lajkó D.B., Valkai I., Domoki M., Ménesi D., Ferenc G., Ayaydin F., Fehér A. (2018).

In silico identification and experimental validation of amino acid motifs required for the Rho-of-plants GTPase-mediated activation of receptor-like cytoplasmic kinases. Plant Cell Rep. Apr;37(4):627-639. doi: 10.1007/s00299-018-2256-y. Epub 2018 Jan 16. PMID: 29340786 IF₂₀₁₈: 2.989

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Signals fly when kinases meet Rho-of-plants (ROP) small G-proteins. Plant Sci. Aug;237:93-107. doi: 10.1016/j.plantsci.2015.05.007. Epub 2015 May 18. PMID: 26089155 IF₂₀₁₅: 3.362

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