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Regulation of cell polarity during morpho- and pathogenesis in plants: the role of kinases

FINAL REPORT

The main questions of our proposal were:

- How ROP-GTPases are linked to upstream receptors and downstream kinases in plants?
- What are the roles of these kinases in the establishment of cell polarity, morphogenesis and pathogenesis?
- What are the similarities and differences in cell polarity establishment during pollen tube growth and fungal haustorium formation considering the role of these kinases?
- What are the similarities and differences among monocotyledonous (barley) and dicotyledonous (Arabidopsis) plants in the regulation of fungal susceptibility by ROP-related kinases?

Due to our research efforts, we can give answers for all these questions, however, due to unexpected changes and new findings the questions became more focused during the project:

- **How ROPs are linked to their downstream receptor kinases belonging to the RLCK VI_A class?**
- **What are the biological function of the ROP-activated RLCK VI_A kinases (are they involved in polarity regulation and/or pathogenesis in barley/Arabidopsis)?**
- **What can be the substrates of these ROP-activated kinases?**
- **Are ROP GTPases regulated by upstream kinases (are ROPs themselves kinase substrates)?**

Background

Plants have a specific family of Rho-type small G-proteins (Rho-of-plants or ROPs) which have primary roles in the regulation of plant morphogenesis through the determination polar cell growth and cell expansion. In addition, they are involved in the plant's responses to fungal pathogens. Our knowledge

on the downstream events of ROP-dependent signaling are rather scarce. In the frame of a previous OTKA-supported project we identified a plant-specific kinase subfamily (RLCKVI_A) the members of which are activated by ROP GTPases. These kinases are structurally unrelated to their functional animal/yeast counterparts (PAKs) and have no any known RHO(ROP)-binding motif and the way of their activation by ROPs is unknown. The RLCK Class VI family of Arabidopsis protein kinases has 14 members falling into two groups based on the similarity of their kinase domains (Jurca et al., 2008). A targeted yeast-two-hybrid interaction matrix showed that groupA but not of groupB interact with ROP G-proteins (Dorjgotov et al., 2009). In our laboratory, several RLCK VI kinaseses falling to groupA have been isolated from various species (Medicago, Arabidopsis, Hordeum) and their in vitro protein phosphorylation activity was found to be dependent on the presence of GTP-bound ROP proteins (Dorjgotov et al., 2009; Huesmann et al., 2012; Reiner et al., 2014).

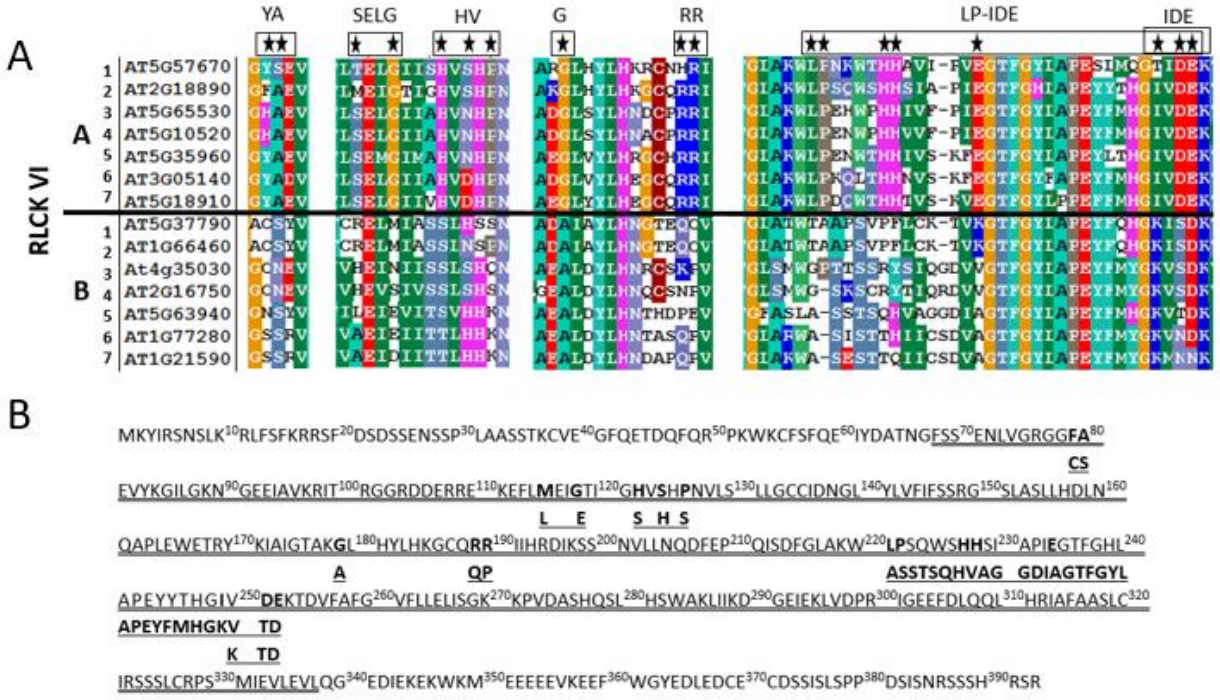

Figure 1. Sequence differences between the RLCK VI_A and VI_B group kinases (A). Asterisks indicate the most characteristic amino acids which were mutated in the RLCK VI_A2 kinase as shown (B). Abbreviations of these amino acids were used to name the various motifs.

Plant-specific RopGEFs (ROP Guanine Nucleotide Exchange Factors) appear to be responsible for ROP activation during tip growth in plants. Using a phosphomimetic ROP mutant we could demonstrate that overexpression of this mutant in pollen tubes prevents RopGEF-mediated ROP activation as well as downstream signaling. These previous findings provided the basis of the present proposal that aimed to reveal the biological role of the ROP-RLCKVI_A interaction and the kinase potentially phosphorylating the ROPs themselves.

Results

How ROPs are linked to their downstream receptor kinases belonging to the RLCK VI_A class?

The comparison of the primary amino acid sequences of the seven ROP-activated Arabidopsis RLCK VI groupA members with that of the seven Rop-independent groupB members allowed the identification of several short aminoacid motifs/regions that are characteristically different between the two groups (Fig. 1). The motifs are not grouped together, but are dispersed in the whole kinase domain. To prove the role of the identified sequence motifs in ROP binding, they were mutated one-by-one in the case of the RLCK VI_A2 kinase. The amino acid motifs characteristic for groupA kinases have been changed to amino acids present in the same position in groupB kinases. A yeast two-hybrid system-based protein-protein interaction matrix was established via the pairwise combination of the kinase mutants with the ROP G-protein. All mutations, except one, prevented or weakened the RLCK-to-ROP binding, indicating that indeed these motifs have role in the interaction (Fig. 2). To further support the above observations, in vitro kinase activity assays were carried out. Four out of the six investigated kinases lost their ROP-dependent autophosphorylation activity as compared to the wild-type, while the remaining two kinase mutants (G; RR) kept some of their activity even in the absence of the G-protein. The RLCKVI_A2 kinase turned to be to have low affinity for the artificial kinase substrate myelin basic protein. Therefore, some of the mutations have been replicated in the RLCK VI_A3 kinase (Fig. 3), which previously have been demonstrated to strongly phosphorylate this substrate. In accordance with the autophosphorylation results of RLCK VI_A2 kinase, the myelin basic protein-phosphorylation activity of the RLCK VI_A3 kinase was lost due to the HV mutation, while ROP-independent activities could be observed in the cases of the LS and RR mutations. These data confirmed that the

identified motifs play role not only in ROP-binding but at least some of them is important for the ROP-dependent activity of the kinase. The aminoacids implicated in ROP-binding share a more-or-less common surface of the proteins above the substrate-binding cleft. The only RLCK VI_A-specific motif (YA) that was found not to affect ROP-binding but the kinase activity, is not part of this surface, strengthening the suitability of the models (Fig. 4).

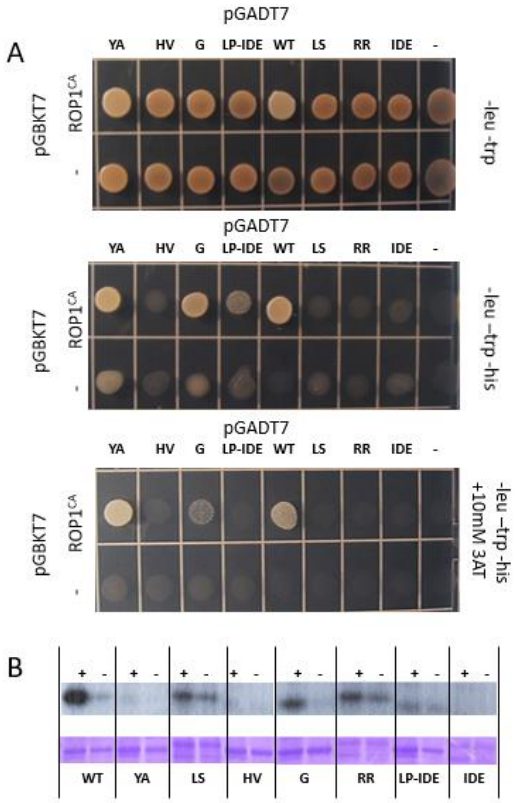

Figure 2. Interaction and ROP1^{CA}-dependent activity of RLCK VI_A2 mutants.

"A" shows yeast two hybrid interaction of ROP1 CA with the various kinase mutants (see Fig. 1) and the wild type (WT). Two selection levels are shown to demonstrate interaction strength differences. "B" shows the in vitro autophosphorylation activity of the kinase variants in the presence and absence of ROP1^{CA}.

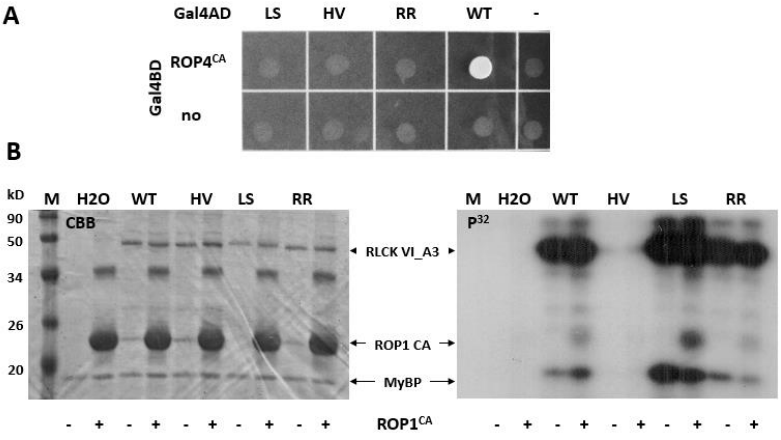

Figure 3. The experiment shown in Fig. 2 has been repeated with some RLCK VI_A3 mutants. In this case the phosphorylation of the myelin basic protein substrate was used to demonstrate kinase activity.

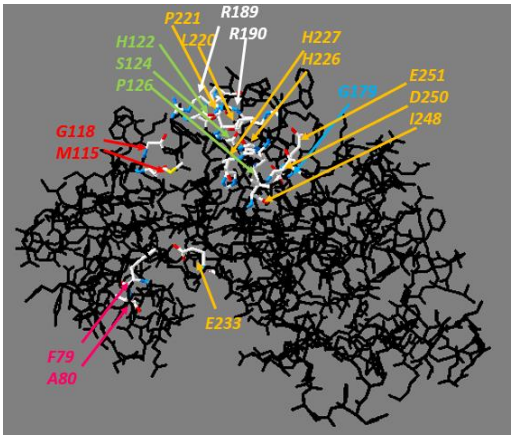

Figure 4. 3D model of the RLCK VI_A2 kinase with the mutated amino acids highlighted. Different motifs are indicated by different colors. The motif not affecting ROP-binding is shown in magenta.

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 constitutive active ROP1 form. The “gene gun” purchased from the project budget was used for these experiments. In agreement with the yeast-two-hybrid and kinase activity data, only the wild type but not the binding-mutant kinases can interact with the active Rop1 protein in tobacco pollen tubes. The effects of the ectopically expressed wild type kinase and its mutant forms were evaluated on pollen tube growth and polarity in the presence and absence of the constitutive active ROP1 G-protein. Sub-apical pollen tube diameter and total pollen tube length were measured in the transformed pollen tubes expressing mCherry as a marker. The expression of the kinases alone affected both pollen tube length (growth) and tip widening (polarity) dependent on their ROP-binding and ROP-dependent/independent activities (Fig. 5). These observations support the view that these kinases are involved in pollen tube morphogenesis downstream of ROPs.

In silico sequence analysis indicated that Pyscomitrella, Marchantia, Selaginella and Oryza possess kinases that share all the Rop-binding motifs characteristic for the Arabidopsis RLCK VI_A kinases (Fig. 6). No such kinase sequences were found, however, in the Chalamydomonas genome indicating that this kinase family might appeared during the early evolution of land plants (Embryophyta).

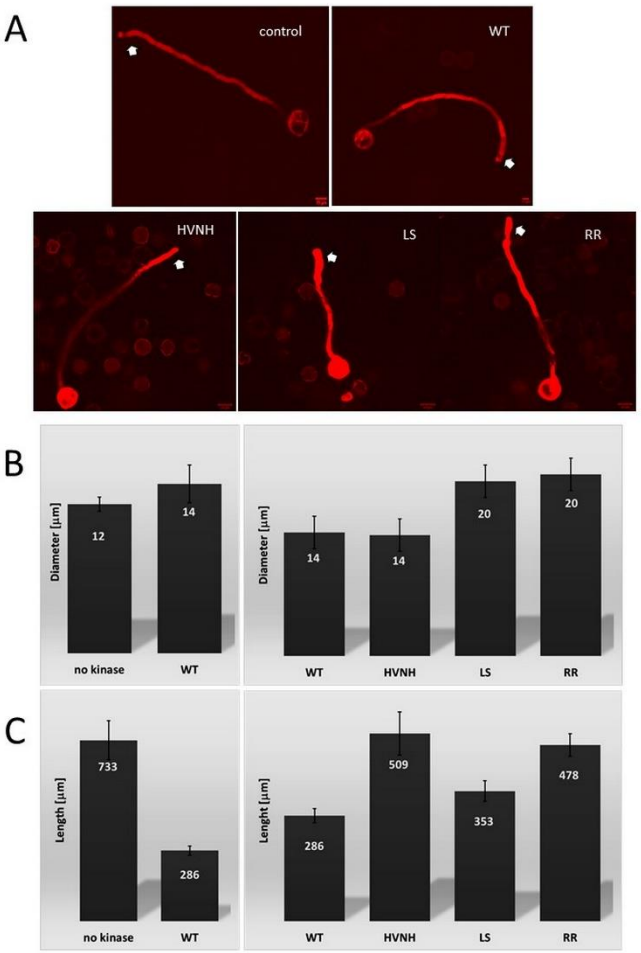

Figure 5. Transient expression of the mutant RLCK VI_A3 kinase forms in tobacco pollen tubes affect pollen tube polarity (A, B) and growth (A, C). Red fluorescence indicates successful transfection (Lat52-mCherry marker of the expression vector).

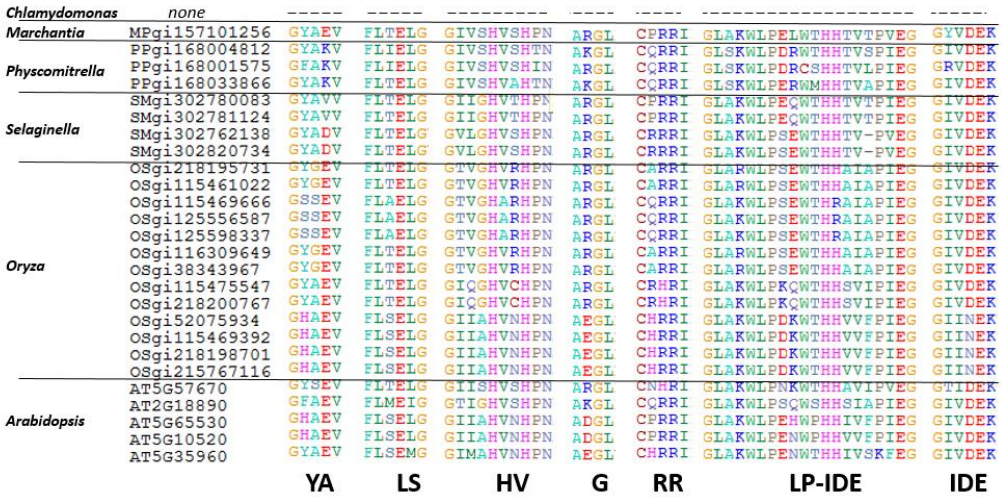

Figure 6. Representation of RLCK VI_A-like kinases in Embryophyta.

Altogether, in this part of the project, we could identify the characteristic amino acid motifs of plant ROP-activated kinases. We demonstrated that these motifs not only regulate ROP-binding but are also involved in the regulation of kinase activity. Furthermore, our data indicated that these ROP-effector kinases are involved in the regulation of pollen tube growth and polarity.

These results have been summarized in a manuscript that is about to be submitted to the Plant Journal (project participants are underlined):

Dézi Bianka Lajkó, Ildikó Valkai, Mónika Domoki, Dalma Ménesi, Györgyi Ferenc, Ferhan Ayaydin, Attila Fehér (2017) Identification of amino acid motifs affecting the Rho-of-plants (ROP) GTPase-mediated activation of plant RLCK VI_A kinases. Plant Journal (under submission)

The manuscript to be submitted in few days is available at this link to support the review process:

https://app.box.com/s/8s2d9f5ypiajgbewcgeztxa2mhnxu8p5

What are the biological function of the ROP-activated RLCK VI_A kinases (are they involved in polarity regulation and/or pathogenesis in barley/Arabidopsis)?

To determine the function of RLCK VI_A kinases transgenic and mutant plants have been generated and transient gene expression assays have been used. In our laboratory, we have selected the widely-expressed RLCK VI_A2 kinase for detailed characterization. In the frame of a research collaboration (prof. Ralph Huckelhoven; Technical University of Munich), the Arabidopsis RLCK VI_A3 kinase and its barley homolog, named HvRBK1, were characterized.

Characterization of the Arabidopsis RLCK VI_A2 kinase

At the start of the project, there were no T-DNA insertion mutants available for the RLCK VI_A2 kinase, however, we already had some lines expressing a construct with estradiol-inducible RNA-interference. This system allowed the characterization of germination and seedling growth (estradiol induction did not work well at the whole plant level). It could be observed that the hypocotyls and cotyledons of the transgenic seedlings were smaller in the presence of estradiol. This observation could be strengthened with a T-DNA insertion mutant we found in the GABIKAT collection. Although the insertion was in an intron, gene expression experiments verified that the

line is a RLCK VI_A2 knock-out (or null) mutant. Expression of the RLCK VI_A2 cDNA in the mutant background, using 35S promoter, complemented the mutant phonotypes described below verifying the mutation specificity. Using the *rlck vi_a2* mutant as well as 35S:RLCK VI_A2 overexpressing lines, a detailed phenotypic analysis was carried out (Fig-s 7-8). It was proved by scanning electron and confocal laser scanning microscopy that the shorter hypocotyl, cotyledon, and root of mutant seedlings is a result of decreased cell elongation. In contrast, the overexpressor had larger, more elongated cells. Greenhouse plants of the mutant exhibited smaller that of the overexpressor larger rosette leaves. Scanning electron microscopy followed by software-mediated cell size analysis proved that not only the size but the circularity of epidermal cells was smaller in the mutant. In addition, the mutant as well as the overexpressor both had disturbed phyllotaxis. The mutant also showed somewhat distorted flower morphology with smaller petals and sepals, and around 10% seed (embryo) abortion. Moreover, the mutant had a smaller the overexpressor a larger root meristem as well as altered root elongation. These phenotypes closely resemble those obtained by overexpressing the dominant negative form of the ROP2 GTPase in Arabidopsis (Li et al., 2001).

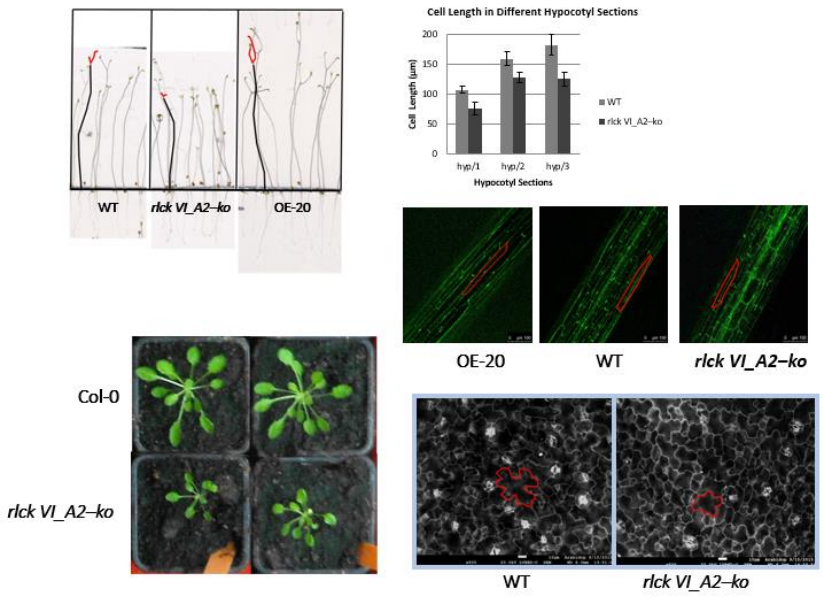

Figure 7. Effect of the RLCK VI_A2 kinase on cell size and plant growth (examples).

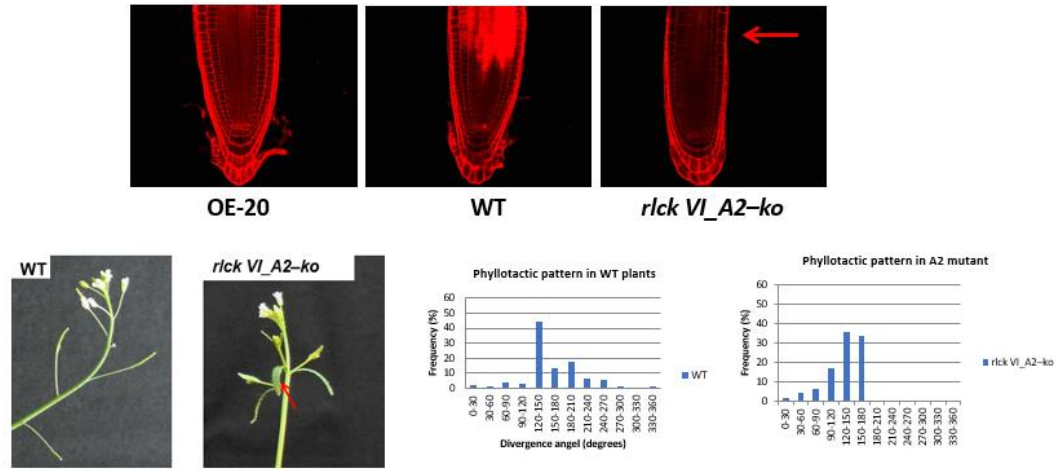

Figure 8. Effect of the RLCK VI_A2 kinase level on meristem function (examples).

Our collaborator Prof. Ralph Hückelhoven is an expert of plant-pathogen interactions and he is especially interested in the role of ROP GTPase signaling in this process. Therefore, we sent them the mutant and transgenic lines for analysis considering pathogen responses. None of the lines exhibited altered sensitivity against Erysiphe cruciferarum infection.

The genomic clone of the RLCK VI_A2 kinase was cloned by PCR from an artificial bacterial chromosome library of the Arabidopsis genome. The app. 2kb long promoter and the first intron of the coding sequence was isolated and linked to the β-glucuronidase (GUS) marker gene. The construct was transformed into Arabidopsis and second generation homozygous transgenic plants were characterized for GUS activity. In agreement with the phenotypes, the RLCK VI_A2 promoter was highly active in whole seedlings (Fig. 9), and in the elongating tissues of adult plant stems and roots, along the vasculature, and in young flowers. This pattern largely overlaps with that of the ROP2 GTPase.

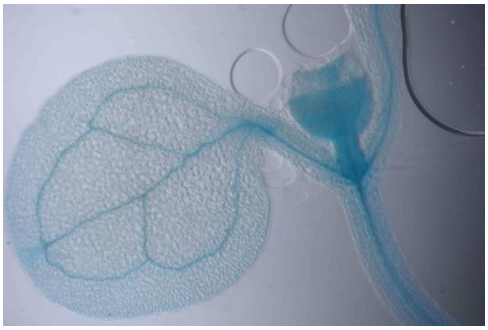

Figure 9. The activity of the RLCK VI_A2 promoter in 12-day-old seedlings.

The promoter sequence of the RLCK VI_A2 GTPase was analyzed for transcription factor binding sites. It was established that the BELLRINGER (BLR1) transcription factor (TF) has five binding sites in the proximal promoter region. This TF is known to regulate meristem function and among others controls phyllotaxis and flower development. The *bellringer* null phenotype resembles to that of the *rlck vi_a2* mutations. To verify the link, we expressed the RLCK VI_A2 promoter:GUS construct in the *bellringer* null mutant background and analyzed the GUS activity in second generation transformants. The promoter activity was almost totally compromised in the mutant background indicating most likely a direct regulation. Several of the ROP GTPases also have BLR1 binding sites in their promoters.

Based on the similarities between RLCK VI_A2 and ROP2 phenotypes and expression patterns, it is supposed that these two proteins may from a signaling unit. We decided to follow two strategies to verify this interaction in planta. The RLCK VI_A2 promoter was linked to the constitutively active form of the ROP2 GTPase and this construct was transformed into wild type as well as *rlck vi_a2* mutant background. Due to the presence of the unregulated active GTPase, the plants have severe phenotypes. The comparison of the phenotypes in the wild type and the mutant background can provide information about those active-GTPase-dependent processes where the kinase is involved. These detailed phenotypic analyses are still under way.

The other strategy is to verify the ROP2-RLCK VI_A2 interaction via co-immunoprecipitation. For this purpose, a tandem affinity purification tag (TAP-tag) was linked to the N-terminal of the kinase creating a gene expression construct that was expressed in transgenic plants under the control of 35S promoter. The TAP-tagged kinase was shown to be functional as it complemented the *rlck vi_a2* mutant phenotypes. Expression and purification of the kinase in/from plant extracts was verified using TAP-tag antibodies. However, the protease recognition site of the construct was not working and the protein could not be removed from the immunoglobulin beads used for purification. This caused problems in further downstream experiments (western, MALDI sequencing). To overcome the problem, a polyclonal antibody was ordered against a specific peptide of the RLCK VI_A2 kinase. The antibody works well with purified kinase proteins. However, despite its affinity purification, the antibody gave strong background with total plant

proteins that hampered its direct application. To further increase our chances, a published ROP2 GTPase-specific polyclonal antibody was purchased. We will attempt to immunoprecipitate ROP2 as well as RLCK VI_A2 containing protein complexes from wild type and *rop2* as well as *rlck vi_a2* mutant plants and determine their composition by MALDI sequencing. This approach is still also under way. We have reserved MALDI capacity for March.

During our phenotypic studies we investigated the growth of kinase mutant and overexpressor seedlings in the presence of various growth regulators (auxin, brassinosteroid, abscisic acid, gibberellic acid). Interestingly and unexpectedly, we found, that gibberellic acid treatment can complement the *rlck vi_a2* mutation (Fig. 10). This suggest that the kinase might affect gibberellic synthesis. We carried out gene expression studies that support the possibility that the kinase has lower gibberellic acid level. At present samples are collected to send to the laboratory of Miroslav Strnad (Olomouc, Czech Republic) for the analysis of gibberellic acid content and metabolism in the mutant.

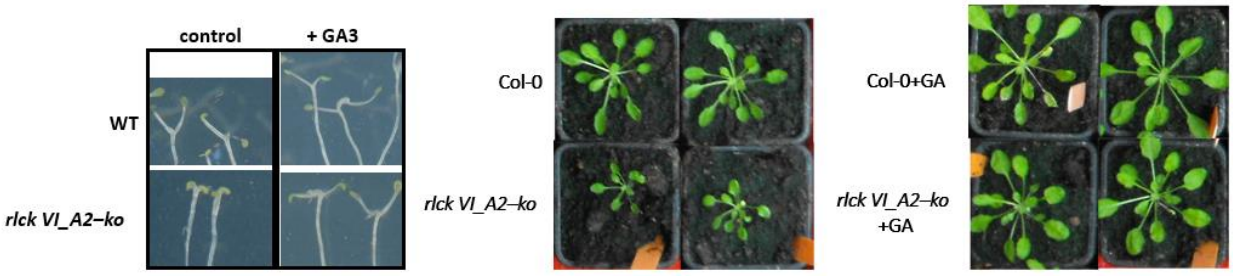

Figure 10. Complementation of the hypocotyl/cotyledon elongation and plant growth phenotypes of the RLCK VI_A2 mutant by exogenous gibberellic acid (GA).

These experiments will serve the basis of a manuscript on RLCK VI_A2 function in cell elongation and plant morphogenesis (Ildiko Valkai, Dézi Lajkó, Katalin Pichehrerné Gémes, Dalma Ménesi and Attila Fehér will be among the authors from the lab). Although the presently available data would allow publication, we think that proving the RLCK VI_A2-ROP2 link and/or the effect on gibberellic acid synthesis would increase the impact considerably. Therefore, we delay the publication for few months to see the outcomes of the above approaches.

Characterization of the Arabidopsis RLCK VI_A3 kinase

Parallel with our research on the RLCK VI_A2 kinase, in the laboratory of Ralph Hückelhoven the phenotype of the *rlck vi_a3* T-DNA insertion mutant was investigated and we joined to this approach. We could observe together that the mutant plants are smaller, and their leaf trichomes are more branched. Our German colleagues have determined meanwhile the fungal sensitivity of the mutant and found that the mutation resulted in a higher frequency of infections. Therefore, this kinase is a fungal resistance factor. To complete the study, we carried out the *in vitro* characterization of the RLCK VI_A3 activity and demonstrated its ROP GTPase dependence.

These experiments resulted in a joint publication:

Reiner, T., Hoefle, C., Huesmann, C., Ménesi, D., Fehér, A., & Hückelhoven, R. (2014). The Arabidopsis ROP-activated receptor-like cytoplasmic kinase RLCK VI_A3 is involved in control of basal resistance to powdery mildew and trichome branching. Plant Cell Reports, 34, 457–468. https://doi.org/10.1007/s00299-014-1725-1

Production of triple knock-out lines in Arabidopsis

To overcome potential cross-complementation by the various RLCK VI_A kinases, we decided to knock down the VI_A1, VI_A2 and VI_A3 kinases in the same plant line. We used the RLCK VI_A3 T-DNA insertion mutant as starting material and we introduced a construct into it. This construct contained gene-specific fragments of the A2 and A1 kinase cDNAs linked to a sequence motif resulting in trans-acting microRNA activation. This approach called microRNA-mediate gene silencing (MIGS) resulted in decreased VI_A1, and VI_A2 expression in VI_A3 mutant background. We verified that the expression of the other four RLCK VI_A kinase gene was not affected. The phenotype of the triple mutant was similar that of the RLCK VI_A2 mutant but more sever, especially as phyllotaxis and flower morphology was concerned (Fig. 11). More interestingly, we frequently observed bifurcating pollen tubes culturing the pollens of the mutant (Fig. 11). This implicates the kinases again in pollen tube polarity.

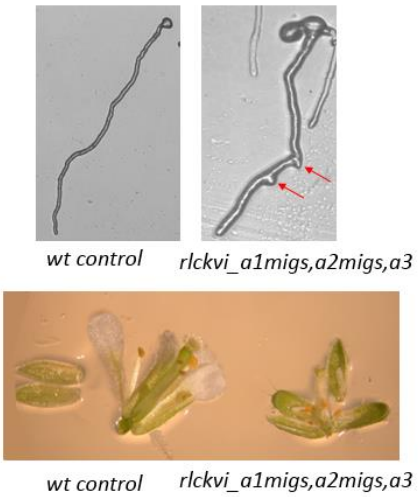

Figure. 11. Examples of the phenotype of a triple knock-out line. Pollen tube polarity (upper part) and flower organ morphology (lower part).

Characterization of the barley RLCK VI_A3-like RBK1 kinase

In Ralph Hückelhoven's laboratory the main model plant is barley (Hordeum vulgare). During their search for ROP-interacting proteins having role in fungal infection/resistance they found a barley RLCK VI_A3-like kinase designated RBK1 (ROP-BINDING KINASE1). They showed that this kinase is a fungal resistance factor since its silencing decreased the frequency of fungal penetration and infection. This was due to the disruption of the cortical microtubule network. We contributed to this work by proving the barley ROP G-protein, RACB and RAC1, dependent activity of the RBK1 kinase providing the first evidence for ROP-activated kinases in monocots. This work was carried out after the submission of the present proposal but got published before the official start of it (due to administrative reasons our project could start almost a year later than originally planned in the proposal). Therefore, this publication does not have this grant mentioned in the Acknowledgements in agreement with the rules.

Huesmann, C., Reiner, T., Hoefle, C., Preuss, J., Jurca, M. E., Domoki, M., Fehér, A., and Hückelhoven, R. (2012). Barley ROP binding kinase1 is involved in microtubule organization and in basal penetration resistance to the barley powdery mildew fungus. Plant Physiology, 159(1), 311–320. https://doi.org/10.1104/pp.111.191940

What can be the substrates of these ROP-activated kinases?

Determination of kinase substrates is still not an easy approach. We followed three possible ways in our project as was proposed in our grant application.

The chemical genomic approach

This modern approach was considered at the beginning as the most straightforward. The method is based on a so called "analog-sensitive" mutation introduced into the ATP-binding region of the kinase (Allen et al., 2007). Due to this mutation, the kinase can accept and use N6-substituted ATP analogues during substrate phosphorylation. If this analog is ATP-γS, then the mutant kinase, and only the mutant kinase, will be able to thiophosphorylate its substrates in a complex mixture such as in a plant extract. The thiophosphorylated substrates can be identified using a specific antibody and sequenced.

First, the analog sensitive mutation had to be introduced into the kinase. Since we learned during our studies that the alfalfa RLCK VI_A2 kinase has much higher in vitro activity than its Arabidopsis homolog, we used for this approach the alfalfa kinase. The mutant kinase proved to be inactive in an in vitro kinase assay. According to the literature, this mutation frequently results in the loss of phosphorylation activity. The solution is to introduce a further, so called "suppressor mutation" into the protein to restore activity. We followed this strategy, however, the recommended mutation still did not restore the activity. Based on the comparison of analog mutation tolerant and non-tolerant kinases we determined a further site as a potential suppressor mutation site. We mutated this site alone or in combination with the first suppressor mutation and found that the triple mutant has restored activity and can also accept the N6-substituted ATP analog. Therefore, the commercially available kit for analog-sensitive kinase substrate screening was purchased. In control experiments, we could prove that the system is working (Fig. 12), however using plant extracts we always got a very strong background even if we did not add the kinase to the reaction. After several trials, we contacted the manufacturer of the kit but they could not advise us. We attempted to contact the inventors of the kit but they

never responded. We carried out further trials trying to improve the protocol but we failed. After using up the antibody we had to give up this approach.

The yeast three-hybrid approach

Although the yeast two-hybrid approach is not very specific, it may indicate potential kinase substrates that can be further tested. In our case, the system had to be modified since the kinase

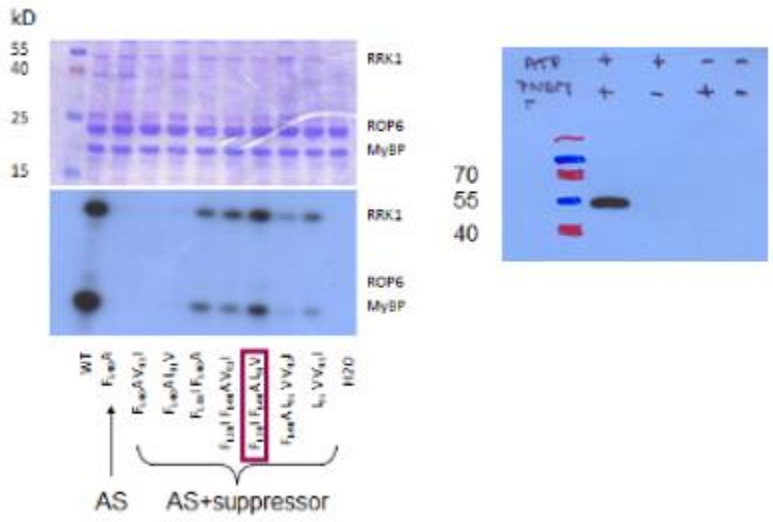

Figure 12. The analog sensitive (AS) version of the Medicago RLCK VI_A2 kinase got active due to suppressor mutations (left) and accepted N6-substituted ATP-γS (right).

is active only in the presence of active ROP GTPase. Therefore, the three hybrid system of Clontech was adapted. First, the pBridge vector was improved changing the week ADH1 promoter for a stronger one. Then, the cDNA coding for the constitutively active mutant form of the ROP1 GTPase was inserted between a methionine-repressed promoter and the RLCK VI_A2 kinase was expressed from the vector as a bait. In this system, the kinase-prey interaction could be tested in the presence and absence of the active ROP GTPase on methionine-containing and methionine-free media, respectively. Working of the system was tested using the active ROP1 GTPase as prey: on methionine-containing medium only the Gal4-activator domain-linked ROP1 GTPase was produced and the interaction resulted in yeast growth; on the methionine-free medium the ROP1 gene of the pBRIDGE vector was also active and competed out the Gal4-AD-containing ROP1 from the interaction preventing yeast growth. Despite this positive control experiment, the screening approaches failed. No real yeast colony growth could be observed on methionine-free media after screening an Arabidopsis flower cDNA library. Repeating the screening in the presence of methionine resulted in the identification of one potential RLCK VI_A2 interacting protein: KIP1.

KIP1 stands for “kinase-interacting protein 1”. These protein is a huge scaffold protein that was shown to be the partner of plasma membrane receptor kinases, RLKs. It was implicated in pollen tube growth and therefore might indeed be linked to ROP GTPase signaling. We obtained only a fragment of the cDNA as a result of the screening. We purified the corresponding protein fragment and found that this fragment could not be in vitro phosphorylated by RLCK VI_A2. This is in agreement with the other observation that the kinase-KIP1 interaction was independent from the presence of active ROP1 in the three-hybrid system. Therefore, we postulated that this protein might not be an RLCK VI_A2 substrate but can provide a surface for its interaction with other proteins (if the interaction is valid in planta). Neither the full length cDNA of the *KIP1* gene nor T-DNA insertion mutants are available in public collections and therefore further characterization of the interaction was not feasible.

The targeted approach

We supposed that the RLCK VI_A2 kinase might feed back to GTPase signaling and therefore we tested whether it can phosphorylate ROP regulator or other effector proteins. We cloned several of these cDNAs into bacterial expression vectors, purified the proteins and tested in in vitro assays but none of them proved to be a RLCK VI_A2 substrate.

These approaches, despite our efforts, did not led to publishable results.

Are ROP GTPases regulated by upstream kinases (are ROPs themselves kinase substrates)?

Results of our previous grant resulted in the finding that ROPs have an evolutionary conserved phosphorylation site that can regulate their activation. In this project, we aimed to identify the kinase that potentially phosphorylates this site. We had two candidate kinase groups based on their phosphorylation consensus: the AGC kinases and the CDK kinases. Since in animal cells one of the AGC-type kinases, the Akt kinase, phosphorylates this site, we started our project with investigation of this possibility. We cloned the Arabidopsis AGC1-7 kinase that was shown to be involved among others in pollen tube polarity, a ROP-regulated process. The kinase cDNA was expressed in bacteria, the protein was purified and used in in vitro kinase assays. Using the

artificial substrate myelin basic protein, the kinase was characterized as active. Unfortunately, the kinase proved to be not phosphorylating the ROP GTPase in vitro in any form (GDP-, GTP-bound and empty GTPases were all tested; Fig. 13). We performed a control experiment with an animal PKA kinase belonging to the same class. This kinase although could phosphorylate the ROP GTPase but not at the investigated S74 residue (the S74A mutant ROP was phosphorylated at the same efficiency as the wild type; Fig. 13). The phosphorylation site of the plant and animal GTPases differs in one amino acid: in plants there is an asparagine instead of a proline characteristic for animal RHO GTpases. This might cause the difference. This asparagine, however, fits into the phosphorylation consensus of the plant-specific calcium-dependent kinases (CDKs). Since CDKs are also implicated in many ROP-regulated processes, verifying this direct link would have a great impact.

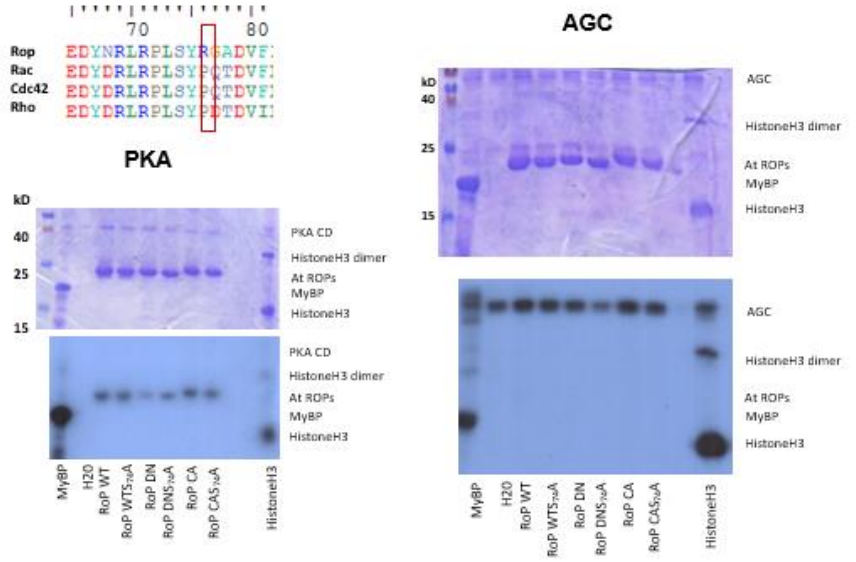

Figure 13. The consensus S74 phosphorylation site and the phosphorylation assays using animal PKA and plant AGC kinases with the Arabidopsis ROP1 substrate.

Three cDNAs coding for CDKs expressed in the pollen (CDK17, 32, 34) have been cloned into bacterial expression vectors and purified. The kinases were active as they could phosphorylate the myelin basic protein in vitro. In vitro phosphorylation experiments using the ROP1 GTPase as substrate proved to be successful: CDK17 and 34 phosphorylated the ROP G-protein strongly (Fig. 14), while CDK32 exhibited a much lower efficiency. The phosphorylation strength was dependent on ROP1 conformation: the active, GTP-bound form was a much weeker substrate as compared to the GDP-bound form. Unfortunately, however, the S74 mutant of ROP1 could also be phosphorylated by the CDKs at a similar level then the wild type. To determine the

phosphorylation site, the in vitro phosphorylated ROP1 GTPase was subjected to trypsin digestion and MALDI sequencing. Sequencing revealed five sites that were phosphorylated and an app. 30-amino-acid-long region that cannot be seen by the MALDI at al. The sequencing also verified the S74 was not phosphorylated. We selected the site that was represented by the most of phosphorylated peptides, S97. We mutated the site into alanine (S97A) and glutamic acid (S97E). The mutant ROP1 GTPase cDNAs were inserted into yeast two hybrid vectors to test the effect of the mutation on protein-protein interactions. The interaction of ROP1 mutants with the positive regulator RopGEF2, the negative regulator ropGDI1, and the effectors RIC2 and RLCK VI_A3 were tested. None of the interaction showed a clear dependence of the phosphorylation-mimic mutation (S97E). Rather the S97A mutation was effective; it prevented the interaction in most cases. In a parallel approach, the ROPS97A and S97E mutants were expressed in growing pollen tubes. No mutation-dependent pollen tube growth phenotypes could be observed indicating that the mutant ROPs can be activated and support polarity in the pollen tubes.

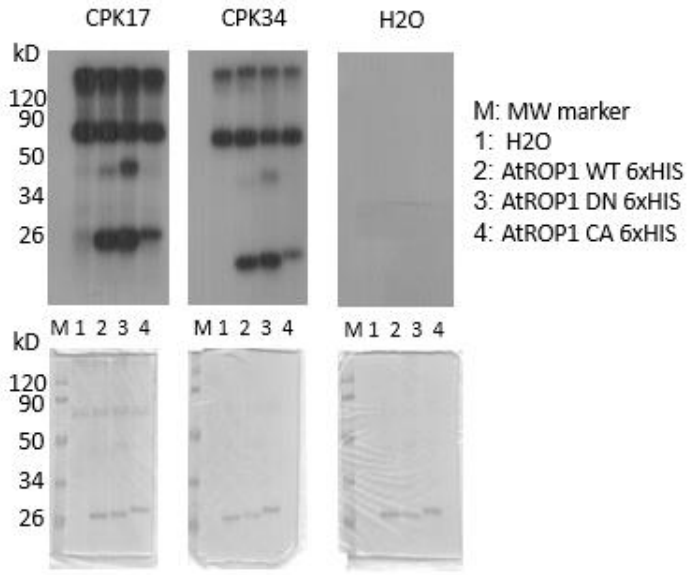

Figure 14. CPK 17 and 34 phosphorylates ROP1 in vitro dependent on its conformation.

This project part on the one hand did not resulted in a kinase phosphorylating the ROP1 at the S74 residue that has a phenotype (S74E prevents ROP activation and polarity establishment), on the other hand we identified a kinase capable for phosphorylating ROPs but in this case the site and the caused phenotype could not be determined yet. The identification of this latter would require considerable work efforts for which we do not have financial support at present.

The answers for the questions we raised in the project proposal:

Based on the above experiments we can answer the original questions although with different details:

- **How ROP-GTPases are linked to upstream receptors and downstream kinases in plants?**
  ROPs are linked to receptor kinases via their positive activator nucleotide exchange factors. How receptors activate these factors is not known yet. We showed that ROPs can directly link to and activate downstream RLCK VI_A kinases. This requires the presence of several defined amino acid motifs on the kinase that form a ROP-binding surface. Some of these motifs is involved only in binding while others are also important for activation.
- **What are the roles of these kinases in the establishment of cell polarity, morphogenesis and pathogenesis?**
  The RLCK VI_A kinases are involved in a variety of processes where ROPs also have regulatory roles. These include cell elongation and plant growth, meristem functioning, polarity establishment during pollen tube growth, and fungal pathogen resistance.
- **What are the similarities and differences in cell polarity establishment during pollen tube growth and fungal haustorium formation considering the role of these kinases?**
  These kinases seem to produce pollen tube growth if overexpressed as well as prevent ffungal penetration and haustorium formation. Therefore, they may act in a similar way during the two processes.
- **What are the similarities and differences among monocotyledonous (barley) and dicotyledonous (Arabidopsis) plants in the regulation of fungal susceptibility by ROP-related kinases?**
  In the case of the Arabidopsis RLCK VI_A3 and its barley homologue RBK1 we could demonstrate that they behaved similarly in vitro as well as in vivo under fungal infection.

Szeged, 2017.02.02.

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