Summary of the Ph.D. thesis

Characterization of ROP GTPase-activated Arabidopsis receptor-like cytoplasmic kinases (RLCK class VI_A)

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INTRODUCTION

Higher plants, being rooted, are bombarded by a wealth of external stimuli. Their cells are also subject to many endogenous signals, such as hormones and developmental cues, some of which comes from neighboring cells and involve intercellular communication. All these signals must be recognized, integrated, and translated into responses at the cell or organ level. The mechanisms of signal perception and many components of signaling pathways are highly conserved among eukaryotes, although plants have evolved along different lines from animals and show some unique features and novel combinations of ancient themes.

Ample research on experimental models such as *Drosophila, Caenorhabditis, Xenopus* oocytes, mammalian cell lines, and yeast, lead to the identification of basics elements involved in signaling that are remarkably conserved. An extracellular signal binds to or stimulates a plasma membrane-based receptor, which activates a GTP-binding protein (G-protein). The G-protein alternates between two conformational states GDP (inactive form) or GTP (active form) -bound states and thus acts as a molecular switch. GTP is hydrolyzed to GDP by an intrinsic GTPase activity in the protein. When activated, the G-protein either regulates a cascade of protein kinases or modulates the activity of effector molecules and second messengers, which, in turn, regulate the activities of protein kinases. Protein kinases regulate the activities of other proteins and/or enzymes, which mediate gene expression or metabolic pathways. Protein phosphatases are also recognized as important components of signaling. The signal perception/transduction pathways, thus, involve at least four elements: receptors, G-proteins, effector molecules or second messengers, and protein kinases or phosphatases. Two classes of signaling G proteins are known: heterotrimeric G proteins and monomeric RAS superfamily of small GTPases.

In plants, the intracellular signaling pathways linking cell surface receptors to nuclear or metabolic responses are still poorly understood. The *Arabidopsis* genome sequence reveals that, among others, plants lack many of the signaling G proteins used by animals and yeast. Plants, however, contain a unique family of small GTPases,

termed ROP (Rho-of-plants). Rho-type GTPases belong to the RAS superfamily of small GTP-binding proteins which serve as two-state molecular switches depending on their GDP- or GTP-bound conformation (Wennerberg et al. 2005; Lundquist 2006). Rho GTPases are implicated in diverse cellular processes through the regulation of cytoskeletal organization and dynamics, NADPH oxidase activity and gene expression (Bokoch 2000; Bustelo et al. 2007). The GTP-binding and hydrolyzing activity of these multifunctional proteins is tightly regulated by a dedicated group of protein factors (Narumiya 1996). The effector proteins downstream of Rho GTPases are also numerous and further increase the specificity of Rho GTPase signaling (Cotteret and Chernoff 2002; Karnoub et al. 2004). Rho-type small GTPases are ancient proteins present in most eukaryotes with considerable structural conservation. However, the early split of viridiplantae form the animal-fungal-amobeozoa lineage led to the separated evolution of plant ROP GTPases (Brembu et al. 2006). This separation resulted in the accumulation of unique features as the primary structure, regulation and signaling properties of ROP GTPases are considered (Brembu et al. 2006; Berken and Wittinghofer 2008). Among others, no Cdc42/Rac-interactive-binding (CRIB) motif-containing kinases (p21-activated kinases or PAKs), characteristic for other eukaryotes (Hofmann 2004; Cotteret and Chernoff 2002), could be identified in plants. PAKs play key roles in fundamental and general cellular processes, such as cytoskeletal rearrangements and the stimulation of mitogen-activated protein kinase (MAPK) cascades, in animal and yeast cells (Hofmann et al. 2004; Bokoch 2003). Therefore it is striking that similar pathways have not been identified in plants up to now. Moreover, plants do not have cognate Ras GTPases although these proteins are also key elements of MAPK-mediated mitogen signaling in other eukaryotes (Dunn et al. 2005). It is generally believed therefore that ROP GTPases, as the only signaling type small GTPases in plants with combined Rho and Ras functions, should be linked to kinase cascades in a presently unknown way (Berken 2006; Yang 2007). In our laboratory a previous research showed that ROPs interact with and activates RLCKs in Medicago

(Dorjgotov et al. 2009), and the aim of this work was to characterize the function of the corresponding RLCK group in Arabidopsis.

RESEARCH OBJECTIVES

Our general aim was to characterize the members of the Arabidopsis thaliana RLCK Class VI family of protein kinases. This protein family was selected based on the previous observations that these plant-specific proteins may serve as ROP GTPase effector kinases. Despite of their potential significance in ROP GTPase mediated signaling, hardly any functional information is available about the fourteen Arabidopsis RLCK Class VI members.

Therefore we decided to carry out the following studies:

- in silico analysis of their primary structure
- gene expression profiling based on a gene-specific real-time quantitative PCR approach,
- co-expression study of RLCKs and other ROP GTPase effectors and regulators based on in silico data analysis
- determination of RLCK-ROP GTPase interaction specificity in a yeast two-hybrid interaction matrix experiment
- investigation of *in vitro* phosphorylating activity and its dependence on the presence of ROP GTPases in active/inactive conformation
- generation of transgenic plants overexpressing or silencing RLCK VI members and their molecular and phenotypic characterization

METHODS

- Bionformatic methods
- Plant maintenance, stress induction, estradiol induction
- Transgenic plant generation
- Genetic engineering methods, GATEWAY technology
- PCR mutagenesis

- RNA/DNA isolation, cDNA synthesis
- Real-time quantitative PCR, semi-quantitative PCR
- Protein-protein interaction tests: yeast two hybrid assay, pull down assay
- Bacterial protein expression and purification, Western blot analysis
- *In vitro* kinase assay
- Microscopy

RESULTS AND DISCUSSION

1. In silico analysis of the primary structure of AtRLCK VI family members

The RLCK VI subfamily contains 14 proteins in Arabidopsis. The phylogenetic comparison of the Arabidopsis RLCK VI sequences revealed that the 14 proteins form two groups and within the groups there is a pair-wise similarity of certain sequences, indicating that gene duplication played a significant role in the formation of this protein family as well. The proteins are highly homologous to each other, especially in the kinase domain, but are divergent from the related kinase families. The group A of Arabidopsis RLCK VI kinases is characterized by the presence of an N-terminal serine-rich region (four out of the seven proteins) while several (four) group B members carry an N-terminal UspA domain (Kerk et al. 2003).

2. Gene expression profiling of AtRLCKs VI kinases based on a gene-specific real-time quantitative PCR approach

AtRLCK VI genes exhibited diverse expression pattern in the various plant organs as well as in response to stress/hormone treatments. In certain cases, the paralogous genes retained a similar relative expression pattern. The presented data indicate that the activity of several members of the RLCK VI kinase subfamily is regulated at the transcriptional level during plant development as well as in response to environmental stresses. Their tightly regulated and generally low expression may indicate that these proteins are involved in specific rather than general cellular processes.

3. Co-expression study of RLCKs and other ROP GTPase effectors and regulators based on in silico data analysis

In order to strengthen the view that RLCK VI members may indeed serve as ROP effectors *in planta*, the co-expression of ROPs and RLCK VI_A kinases was analysed *in silico* based on microarray data sets. Moreover, to have a full picture on potential partners involved in given ROP-dependent signaling pathways all known ROP regulators and effectors were included into the study.

The hierarchical clustering analysis of microarray expression data showed clear correlation of the relative expression pattern of several of the investigated proteins with organ/tissue specific expression. Five main expression clusters (A-E) could be established. These clusters include genes with preferential or more abundant expression in the pollen and flower; stem, node and hypocotyl; root; shoot and root tip; flower and silique; and all over the plant.

4. RLCK-ROP GTPase interaction specificity

In order to test RLCK-ROP interaction specificity a yeast two hybrid interaction matrix was established using 10 RLCK VI kinases (5-5 from groups A and B, respectively) Iand eight Arabidopsis ROP GTPases. The interaction was found to be specific for the RLCKVI group A kinases only. However, in an in vitro pull down assay a randomly selected kinase from the group B showed also interaction with a ROP GTPase. This difference in the results obtained by the various protein-protein interaction approaches may be related to the differing conditions stabilizing or weakening the interaction. However, based on the in vitro kinase activity assays (see further), we suppose that the interaction pattern obtained in the yeast assay is more specific and can better simulate the in planta conditions.

Primary sequence analysis revealed several characteristic differences in the sequences of the kinases belonging to group A that showed interaction with ROPs in yeast two hybrid assays. Several residues/motives could be identified that may have a role in the RLCK-ROP binding.

5. In vitro activation of RLCK_VI kinases by ROP GTPases

Inspite of the potential interactions of ROPs with various RLCK kinases *in vitro*, the kinase activity assays clearly indicated specificity towards a functional interaction of ROPs only with the RLCK VI_A kinases. One kinase which belongs to the group B (AtRLCK VI_B3) and other from RLCK class VII that were tested also, could not be activated by an AtROP GTPase indicating specificity towards RLCK VI_A kinases. The presence of the GTPases in the GTP-bound conformation is the most efficient in increasing the phosphorylation activity of the kinase. Based on the fact that RLCK VI_A kinases are preferentially and strongly activated by the GTPase in active conformation, it could be hypothesize that these kinases are potential downstream ROP GTPase effectors. This possibility was disapproved by Molendijk et al. (2008) first reporting ROP-RLCK interactions. They could not observe ROP GTPase-dependent *in vitro* autophosphorylation or AtROP4 substrate phosphorylation with ROP-interacting RLCK kinases immunoprecipitated from insect cells where they were co-expressed.

Although it is likely that RLCK VI_A kinases serve as ROP effectors, it is not clear how the GTPases can activate the kinases. We know a bit more as the region of the ROP GTPase involved in kinase activation is considered. A specific region of RhoGTPases, the so called Rho-insert region, is implicated in effector binding and activation (Karnoub et al. 2004). Plant ROP GTPases have an insert region with characteristic differences as compared to human or yeast Rac, Rho ad Cdc42 proteins that is an indication that plants have evolved specific ROP GTPase effectors (Berken, 2008). In our laboratory it was identified that this region is important for the activation of the MtRRK1 (MtRLCK VI_A2) kinase (Dorjgotov et al. 2009).

6. Transgenic plants characterization

Cell polarization is intimately linked to plant development, growth, and responses to the environment. It is well established that plant cells use conserved mechanisms such as Rho family GTPases (ROPs) to integrate both plant-specific and conserved polarity cues and to coordinate the cytoskeketon dynamics/reorganization and

vesicular trafficking required for polarity establishment and maintenance. Our data showed that kinases belonging to the RLCK Class VI *Arabidopsis thaliana* can be specifically activated by GTP-bound ROP GTPases *in vitro* further supporting the view that plant ROP GTPases may directly regulate downstream kinase signaling. In this direction we proceeded with analysis of transgenic plants over-expressing or silencing AtRLCK VI_A2. We suspect that AtRLCK VI_A2 as a potential ROP effector may have a role in polarity establishment as well. Directional cell expansion (e.g. trichomes) and tip growth (e.g. pollen tubes) are two basic processes underlying the morphogenesis of polar cell types in plants.

Our preliminary data that need further confirmation indicated that both over-expressing as well as silencing of the gene disturb polar growth of the pollen tube and might affect trichome branching. Over-expression of AtRLCK VI_A2 increase the frequency of trichomes with fewer branches while silencing of the gene may lead to the apparition of trichomes with more branches, although only with a low frequency. Over-expressing AtRLCK VI_A2 in the pollen tube may result in the formation of bifurcated pollen tube tip. Silencing of AtRLCK VI_A2 could lead to the formation of ballooned pollen tube tips. Further more detailed investigations will be carried out to confirm the potential role of RLCK VI_A kinases in these and further ROP GTPase-regulated processes (e.g. pathogen response, ROS production) to support the view that RLCKs are indeed plant specific ROP GTPase effector kinases.

In summary, we established that AtRLCK VI_A kinases interact and are activated *in vitro* by AtROP GTPases. Furthermore, the characterization of expression patterns and transgenic plants allowed us to hypothesize that these kinases may serve as ROP GTPase effectors in plants. This signaling step has several plant-specific aspects. Further studies will allow interesting insights into the evolution of cellular signaling mechanisms and will provide valuable new information about the regulation of plant development.

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LIST OF PUBLICATIONS

Publications related to the thesis

- **Jurca M.,** Bottka S., Fehér A., 2008: *Characterization of a family of Arabidopsis receptor-like cytoplasmic kinases (RLCK class VI).* Plant Cell Reports. 27(4):739-48. IF:2,301

- Fehér A., **Jurca M.,** Fodor-Dunaine C., Dorjgotov D., 2008: *Regulation of ROP GTPase activity at the gene expression level.* The Open Plant Science Journal. 2: 21-30

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- Fehér A., Dorjgotov D., **Jurca M.,** Fodor C., Fricke I., Berken A., 2010: *Links between Rho GTPases and kinase signaling in plants* – Poster, July 2. SEB Main Meeting, Prague

- Dunainé-Fodor C., Dorjgotov D., Szűcs A., Ötvös K., **Jurca M.**, Fehér A., 2008: *Investigation of the elements of Rho-GTPase-dependent signalling in plants.*

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- **Jurca M.,** Feher A., 2007: *Expression profiling of receptor-like cytoplasmic protein kinases (class VI) of Arabidopsis.* – poster presentation - 3rd International qPCR Symposium, München - Germany, Proceedings ISBN-13: 978-3-00-020385-5

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