

Isolation and characterization of plant RHO (ROP) GTPase activated kinases

Ph.D thesis

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Introduction

The lifestyles of plants and animals differ at the most basic level. While animals are capable of changing their location, plants's lifestyles must accomodate to their unchanging location. As such, plants' do not have a determined developmental program. This means that environmental factors have a primary influence on the developmental program. Small environmental changes cause variation in the functions of hundreds of plant genes, thus allowing the organism to adapt its metabolic pathways and continual development to the given situation.

When G-proteins bind a GTP molecule and when they release GDP and inorganic phosphate, their structure changes. This structural change is associated with the change in the protein's signaling activity as well [1,2]. In their GTP-bound form, the small GTPases are capable of interacting with the end-products, or effector proteins, in the signaling pathway. This interaction results in a change in the functioning of the effector proteins, thus the conclusion that the GTP-bound form is the "switched on" form of the G-proteins. In contrast, the GDP-bound form has no interaction with the end-products and does not pass on signals, thus is considered the "switched off" form. Thus the ROP GTPases, like other members of the RAS superfamily, function as 2-state molecular switches, which, depending on their form, switch on or off signaling pathways. ROP GTPase regulating proteins can be placed in three role-based groups: proteins that speed up GTP degradation (GAPs; GTPase Activating Proteins), nucleotide-exchanging proteins (GEFs; Guanine nucleotide Exchange Factors) and nucleotide-anchoring proteins (GDIs; Guanine nucleotide Dissociation Inhibitors) [3]. GAP and GDI proteins typically interact with GTPases, resulting in or restricting to the GDP-bound, deactivated form, thus inhibiting signaling. In contrast, GEFs assist in releasing GDP, stimulating GTP binding and the activation of the GTPases. On the whole, GTPase signaling activity is determined by the ratio of GDP- and GTP-bound proteins.

Significant variations between plants and animals exist with respect to small GTPases (RAS/RHO) too, which play a role in signaling. The cell division controlling RAS GTPases are completely lacking in plants and only group of RAC-related GTPases, the ROP (RHO proteins of plants) GTPase group exists, but it distinctly differs from them [4,5] ROP GTPases play a key role in the regulation of plant cell growth as they determine the

direction apical growth [6-12]. Among the known ROP GTPase effector proteins, there are numerous proteins which play a role in the reorganization of the cytoskeleton (for example, RIC, ARP, WAVE, formin, RBOH, ICR/RIP, CCR, UGT) [6,8,13-20]

Plants have fewer G-proteins and associated receptors, but more receptor-like kinases (RLK) [21,22]. The wall cress genome contains more than six hundred different receptor kinase genes, which is at least six times more than the human genome. In total, the wall cress genome encodes more than a thousand kinase proteins, while the human genome has only 500-600 similar proteins. Comparable differences can be seen when considering the genes responsible for protein degradation as well. The large number of receptors and signaling proteins guarantees the plant cell a rapid, flexible response to environmental change. In spite of the fact that plants code for an extremely large number of kinase genes, the ROP GTPase-activated plant kinase effectors have not been identified.

Objectives

Regulation of plant development is extremely variable. In our laboratory, we are studying those processes, at the cell and molecular level, which allow for the regulation of rapid and environmentally-adaptive plant cell division, shape and differentiation. In association with this, our laboratory has been studying the role of RHO-type (ROP) GTPases in plant cell signaling pathways. When we began working with plant signaling pathways, and specifically the key role of GTPase interactions (end-products and regulators), there was little information available in the literature. Thus it was relatively simple to state our objectives: to discover new elements in the primarily unknown plant ROP GTPase-activated signaling pathways. Our goal was to use independent methods to reveal and confirm interactions between newly identified elements and ROP GTPases, both *in vitro* and *in vivo*. Primarily, we were interested in whether these small proteins, functioning as molecular switches, were taking part in the activation of kinase reaction chains, as in yeasts and animals. Finally, we aimed to determine the biological role of the newly identified elements. In this way, we hoped to expand upon the presently limited known information about signaling pathways in plant cells.

Results and discussion

The first part of my PhD work was to find proteins (regulators and end-products) that interacted with ROP GTPases using the yeast-dihybrid system. Initially we identified three ROP GTPase cDNA in alfalfa and fully characterized them [23]. We then chose to further study the cDNA for which no data was available in the literature. We labeled it as MsROP6 GTPase and using a 2-step PCR, we produced the following: deactivated (DN-Dominant Negative), continually activated (CA-Constitutively Active) and non-isoprenilated (incorrect intracellular localization, L).

-We carried out yeast-dihybrid screening on the cDNA library generated from alfalfa root. After the screening, the gene sequences that were "fished out" using the MsROP6 GTPase CA+L mutants (CA-Constitutively Active + non-isoprenilated-incorrect intracellular localization, L) were identified in the GenBank data (Blastx: <http://www.ncbi.nih.gov/BLAST>). The results indicated that the cDNAs code for the following proteins: two receptor-like cytoplasmic kinases (RLCK, Receptor-like Cytosolic Kinase), two p65 microtubule-bound proteins, one protein homologous to NEDD1, one MtN24 (234as) root-specific protein, one DUF1620 (domain unknown function) domain carrying protein and one part of a completely unknown protein

-We concentrated on two receptor-like cytoplasmic kinases, since there was no available information about plant ROP GTPase interactions with kinases, yet proteins with similar characteristics play basic roles in the cell function of other eukaryotes, such as the GTP-bound CDC42/RAC GTPase interaction with the PAK1 (p21 protein activated kinase) kinase family [24,25]. All PAK1 kinases have a CRIB (CDC42/Rac-interactive binding) motif, which is responsible for the interaction with GTPases. We named the two receptor-like cytoplasmic kinases ROP-interacting Receptor-like Kinases (RRK for short). The two alfalfa-RRK, as previously stated, belong to a previously unknown plant kinase family, which is called receptor-like cytoplasmic kinase VI family (RLCK VI) [26].

-From the yeast two hybrid experiment, we came to the conclusion that the ***two RRK kinases interact with the MsROP6 CA+L forms*** and not with the DN form. Thus the two MtRRK kinases do not interact with the GDP-bound ROP GTPase, similar to the animal and yeast PAK1 kinases.

-In animal and yeast systems the kinases that bind RHO/RAC/CDC42 GTPases usually have a CRIB motif (for example, PAK1 kinases [24]), yet in the complete genome

sequence of wall cress, no kinases were found to contain the CRIB motif. If the CRIB motif isn't present, then which part of the kinase is responsible for the interaction? Is there a distinct region or amino acid sequence which is responsible for binding to the activated ROP GTPase? To answer this question, we broke the MtRRK1 kinase into various pieces. We tested the interaction of the segments with ROP GTPase using the yeast-dihybrid system. We came to the conclusion that **there is no identifiable part or region of the kinase** which is, by itself, responsible for the interaction with activated ROP GTPase. It is probable that there is not one, single peptide pattern that is responsible, but rather amino acid side chains found on various parts of the kinase take part in the interaction.

-In the literature there are examples of the small GTPases (such as RHOA, RAB, RIC1) as well as regulatory proteins (GEF, GDI) which are regulated by direct phosphorylation [27,28]. Since the MtRRK kinases do not possess CRIB motifs, a logical question developed as to whether MsROP6 is actually the substrate for the MtRRK kinases. To test this, we planned a kinase reaction using radioactively labeled [γ -32P] ATP, for which we overexpressed proteins in bacteria and purified them. Using the MALDI technique, we the MsROP6 protein CA form was phosphorylated with non-radioactive ATP by the MtRRK1 kinase. The results of the investigation in the MTA SzBK Proteomic Laboratory and the radioactive kinase reactions both showed that the **MsROP6 GTPase is not the substrate of the MtRRK kinases.**

We examined whether the kinase activity of MtRRK kinases is dependent on MsROP. We examined the phosphorylation of myelin basic protein (a universal substrate for serine threonine kinases) by RRK1 kinase in the presence of activated GTPase. The MtRRK1 kinase is only able to significantly phosphorylate the myelin basic protein if the MsROP6 CA mutant or MsROP6 WT wild type and 1mM GTP are present. In the absence of GTPase, MyBP phosphorylation was barely observable. Likewise, phosphorylation was very weak with MsROP6WT wild type and 1mM GDP. **GTP-bound MsROP6 GTPase activates MtRRK.**

-If MtRRKs follow MsROP6 in the signaling pathway, then is MsROP6 specify for MtRRK1/2, or will other GTP-bound ROP GTPases also be able to activate their kinase activity? To answer this question, we carried experiments similar to those above in the presence of some wall cress ROP GTPases as well. The experiments showed that

AtROP1 and AtROP6 also activated the MtRRK1 kinase. Thus it is not a specific reaction, other ROPs can activate the kinase *in vitro*.

We also examined whether GTPases in non-plant RHO families are capable of activating it. We chose a non-plant RHO-type GTPase (RX). Unfortunately, we are not sure of which organism the GTPase is from. Dr. Attila Szűcs and his colleagues found it in the cDNA library of *Medicago sativa* root, but it is not a plant sequence. They believe that it originated from a symbiotic or parasitic fungus. It is a small GTP-bound protein belonging to the CDC42 GTPase sub-family [23]. We studied its interaction with both MtRRKs using the yeast-dihybrid system. The alfalfa RRK kinases did not interact with the non-plant RX GTPases in the yeast-dihybrid system. **The MtRRK kinases only interact with plant ROP GTPases and are only activated by ROP GTPases. In other words, they are plant-specific effectors of ROP GTPases.**

-The results of the above experiments lead to the conclusion that plant ROP GTPases contain one (or more) patterns that are responsible for the interactions with RRK kinases and kinase activation, and that this pattern is missing in other, non-plant RHO GTPases. We know from the literature that animal RHO/RAC/CDC42 GTPases have a characteristic peptide loop (RHO insertion region) which has an important role in activation [29,30]. Nonetheless, variation of this region does not affect the interaction between the proteins [31-33]. Plant ROP GTPases also have a characteristic RHO-insertion region and its amino acid sequence hardly varies within the plant ROP GTPase sub-family, yet this region is extremely different from the peptide loop found on GTPases in other RHO families. This can't be considered a functional insert region in RAS GTPases, as it is only characteristic of RHO-type GTPases. According to the literature, this change doesn't affect the GTPase proteins' structure, but it does affect its ability to activate the effector protein [31,33]. We also carried out a similar exchange on the MsROP6 GTPase and we named the produced mutant MsROP6 insertion region mutant, or MsROP6 Δ ^{IRAS}. We examined the interactions between the Δ ^{IRAS} MsROP6 proteins and both RRKs using the yeast-dihybrid system and found that the change in the ROP-insert region did not affect the interaction between the kinases and the GTPases. Nonetheless, the reaction with MsROP6 Δ ^{IRAS}+CA mutant protein did show that it was not capable of activating the MtRRK1 kinase. **The ROP insert region responsible for activation of RRK, but not binding of RRK kinases.**

-We cloned the MtRRK kinases into the appropriate reading frame behind a 35S promoted GFP (green fluorescent protein) gen. The plasmids containing 35S-MtRRK1-GFP and 35S-MtRRK2-GFP gene fusions were transformed into wall cress protoplasts using polyethylene glycol (PEG) and after one day of growth, the cells were observed under a confocal microscope. **We determined that both kinases are localized within the cell's cytoplasm.**

-We transformed both YFP-MtRRK1 and CFP-MsROP6+CA vector constructions into wall cress protoplast cells (YFP, yellow fluorescent protein; CFP, cyan fluorescent protein). Microscopic examination showed that **MsROP6 CA mutant and MtRRK1 kinase have common localization in the cell.** Thus there is the possibility of interactions within the cell, since they are in the same vicinity.

-The SecA gene has normal expression in sample of mRNA (cDNA) from different parts of alfalfa. The expression of different genes were not compared to one another, but rather in relation to the given genes normalized, average expression in all samples. We were able to determine that **MtRRK1 is more highly expressed in the roots and tubers** than in other plant parts, while MtRRK2 shows very low relative expression in cell cultures and flowers. **Like MsROP6 GTPase and MtRRK2 kinase, it expresses in relatively constant amounts everywhere,** but has rather high relative expression in the flower.

- We identified other potential interacting proteins for MtRRK2 in the yeast-dihybrid screening. After testing interaction, the amino acid-based identification of partners produced the following:

Proteins interacting with the Golgi apparatus, vesicular trafficking and cell wall synthesis: Dynamin, RGP1 (Reversibly glycosylated protein), SRP (Signal recognition particle), Golgi associated protein, Fasciclin-like protein, Kinesin-like.

Proteins associated with transcription (transcription factors): bHLH transcription factor protein, Myb-like transcription protein, Zinc-finger transcription protein, Makorin zinc-finger type RNA-binding protein, Mi-2 chromatin remodeling protein

Proteins associated with signaling pathways: POZ/BTB domain-carrying protein, PIL (Photoreceptor Interacting-like Protein), DUF315 (Domain of unknown function 315) domain carrying protein, which was later discovered to have ROP-GEF function.

Summary

-We identified strongly interacting potential partners for MsROP6 GTPase using the yeast-dihybrid screening. Amongst these, we chose 2 receptor-like cytoplasmic kinases for further study. We named the two receptor-like cytoplasmic kinases MtRRK1 and MtRRK2 (RRK=ROP interacting Receptor-like Kinase).

-These kinases are only found in plants and are members of the plant receptor-like cytoplasmic kinases VI. family (RLCK VI).

-A domain or motif responsible for ROP GTPase binding in the short amino and carboxy groups outside the kinase domain was not found. The whole kinase protein (with the exception of the short terminal regions) is necessary for interaction.

-The MsROP6 GTPase is not a substrate for these kinases, but in GTP-bound form an activator, which means that it is able to activate the MtRRK kinases kinase activity. While they don't show specificity within the plant ROP GTPases, a non-plant CDC42-like GTPase was not able to induce activity. Thus we consider the signaling connection to be plant-specific.

-The insertion region of the MsROP6 GTPase is responsible for activating the MtRRK kinases, but an altered insertion region doesn't inhibit interaction.

-Both RRK kinases are found in the cytoplasm.

-The cellular localization of MsROP6 and MtRRK1 overlaps.

In summary, we were among the first to identify plant kinase molecules which taken part in RHO (ROP) GTPase-dependent signaling. This signaling step has several plant-specific aspects, of which further studies will provide interesting insight into the evolution of the signaling pathway, as well as providing valuable information about the regulation of plant development.

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Publication list

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Dorjgotov D, Jurca E.M, Dunaine-Fodor Cs, Szucs A, Otvos K, Klement E, Biro J, Fehér A
Plant Rho-type (Rop) GTPase-dependent activation of receptor-like cytoplasmic kinases
in vitro
FEBS letters (2009) (in press)
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Other publications

Attila Feher, Manuela E. Jurca, Csilla Fodor-Dunai and Dulguun Dorjgotov
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The Open Plant Science Journal, 2008, 2, 21-30

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CELL BIOL INT, 27, 191-192 (2003) Impact factor: 1.092