The role of Arabidopsis CRK5 protein kinase in the regulation of root gravitropic response

Ph.D. Thesis Resume

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INTRODUCTION

By sensing the Earth's gravity, plants adjust the growth of their shoots and roots with opposite polarity along the direction of gravity vector. Both positive and negative gravitropic responses, directing downward and upward bending of horizontally placed roots and shoots, respectively, are controlled by asymmetric distribution of the plant hormone auxin. Cellular transport of auxin is controlled by the AUX/LAX influx and PIN-FORMED (PIN) efflux carriers. Gravity is perceived by specific starch-containing statocyte cells in the root columella and stem endodermis. Mutations impairing starch biosynthesis, biogenesis, and sedimentation of starch-containing plastids (i.e., statoliths) and their interactions with actin filaments, endoplasmic reticulum, and plasma membrane highlight the importance of mechanosensitive ion channels and components of calcium/calmodulin and inositol-phosphate signaling pathways that connect gravisensing with the regulation of polar localization of auxin transporters. While our knowledge about factors controlling polar localization, activity and stability of auxin transporters is steadily increasing, little is known about how sensing of the gravitation stimulus leads to specific changes in auxin transport through factors participating in the regulation of Ca^{2+}/calmodulin signalling.

The results described in this Ph.D. thesis uncover the function of CRK5, a member of the Ca^{2+}/calmodulin-dependent kinase-related family, in the control of root gravitropic response. CRK5 has been originally identified in yeast two-hybrid screens as an interacting partner of nuclear PRL1 subunit of the spliceosome-activating NTC (nineteen) complex. Similarly to other seven closely related members of the CRK family, the CRK5 kinase (At3g50530) carries an N-terminal MGxC myristylation/palmitoylation motive, which is conserved in all plasma membrane-associated CRK proteins examined so far in other plant species. The N-terminal domain of CRK5 also harbours a nuclear localisation signal, which is followed by a conserved serine/thronine kinase domain, an autoinhibitory domain and four degenerated EF-hand motives. In all currently known members of the CRK kinase family, the EF-hand motives are not capable to bind Ca^{2+} but interact with calmodulin, which increases the activating T-loop phosphorylation of CRK5-related tomato CRK1 kinase but results only in minimal enhancement of its substrate phosphorylation activity. Except for CRK5 characterized first during the course of our work, regulatory functions and cellular substrates of other members of CRK family are currently unknown.

GOALS

As a major goal, the present work aimed at characterizing the regulatory functions of CRK5 protein kinase that belongs to the family of CDPK Related Kinases (CRKs) in *Arabidopsis thaliana*. In this study, the most important goals were

1. To isolate a null mutation in the *CRK5* gene and identify developmental abnormalities caused by the *crk5* mutation.

2. As we found that the *crk5-1* T-DNA insertion mutation causes a delay of shoot and root gravitropic responses, in addition to conferring inhibition of root elongation and enhancement of lateral root differentiation, our further goal was to clarify how inactivation of CRK5 alters the gravitropic growth responses regulated by the plant hormone auxin.

3. In order to characterize the regulation of *CRK5* gene and subcellular localisation of CRK5 kinase, we aimed at generating gene constructs that maintain natural regulatory properties of the *CRK5* gene and permit *in vivo* localization of the kinase in fusion with either the green fluorescent protein (GFP) or the β-glucuronidase (GUS) reporter enzyme. Furthermore, using these constructs, we wished to perform genetic complementation of the *crk5-1* mutation, in order to verify that the observed gravitropic defects indeed resulted from inactivation of the *CRK5* gene.

4. As the AUX1 auxin influx and PIN (chiefly PIN3 and PIN2) efflux carriers play a critical role in the regulation of auxin-dependent gravitropic response of roots, we wished to determine how inactivation of the *CRK5* kinase affects the function, membrane localization and stability of AUX1 and PIN proteins. In addition, using an auxin-induced DR5-GFP reporter, we wished to follow how the *crk5-1* mutation compromises the establishment of asymmetric auxin distribution during gravistimulation through affecting the function of auxin transport proteins.

5. Finally, following the purification and biochemical characterization of the CRK5 kinase, we wanted to examine whether CRK5 modulates the functions of those PIN auxin efflux carriers that act in the regulation of root gravitropic response by recognizing them as direct phosphorylation substrates.

METHODS

By PCR-based screening, T-DNA insertion mutations were isolated and their positions determined using DNA sequencing. Transcription of *crk5* alleles was examined by real-time quantitative PCR (qRT-PCR) measurements. Upon inspection of phenotypic traits of the *crk5-1* mutant, developmental defects caused by the mutation were defined and characterized by a delay of shoot and root gravitropic responses and elevated number of lateral root primordia. To verify that these altered traits were indeed caused by the *crk5-1* mutation, genetic complementation of the mutation was performed. In order to accomplish this, by replacing the stop codon of *CRK5* we generated modified gene constructs that allowed the expression of CRK5 in fusion with either green fluorescent protein (GFP) or β-glucuronidase reporters in plants. Using qRT-PCR, we measured *CRK5* mRNA levels, and parallel monitored the activity of CRK5-GUS protein in various plant tissues and organs by histochemical staining. By confocal laser microscopy (CLM) studies, we observed that the CRK5-GFP protein is localized in the plasma membranes of protoplasts, whereas in specific cell types of plants it shows polar U-shape membrane localization. Membrane-association of CRK5-GFP was further verified by western blotting of purified cell fractions. By CLM monitoring the accumulation of auxin-induced DR5-GFP reporter, we found that the *crk5-1* mutation elevates auxin export from the root tip towards the elongation zone, and delays the establishment of asymmetric auxin distribution between upper and lower sections of gravistimulated roots. qRT-PCR measurements indicated that the decrease of auxin and DR5-GFP levels in the root tips was not due to transcription inhibition of key genes controlling auxin biosynthesis and transport. Using heat maps constructed by confocal imaging, we found that the *crk5-1* mutation did not alter the localization of fluorescent reporter-labelled AUX1, PIN1, PIN3, PIN4 and PIN7 auxin transporters, but led to characteristic changes in the amount and polar localization of PIN2 in the epidermis and cortex cells of *crk5-1* mutant compared to wild type. Using the exocytosis inhibitor brefeldin (BFA) and membrane specific dye FM4-64, we observed that the *crk5-1* mutation delays exocytosis and alters the amount and proper polar membrane localisation of PIN2. A His_6-tagged form of CRK5 kinase was purified by Ni^{2+}-affinity chromatography and subsequent kinases assays showed that CRK5 is activated by autophosphorylation in a Ca^{2+}-independent fashion. Finally, by mass spectrometry studies we found that CRK5 phosphorylates the cytoplasmic hydrophilic loop domain of PIN2 at six different positions.

RESULTS

In order to determine the function of CRK5 kinase, we have first identified two T-DNA insertion mutations in the *CRK5* gene using a PCR-based screening approach. By qRT-PCR measurement of transcription of mutant alleles, we verified that one of these, *crk5-1*, corresponded to a null mutation. Examination of altered developmental traits indicated that negative and positive gravitropic responses of shoots and roots, respectively, suffer a considerable delay in the *crk5-1* mutant compared to wild type. In addition, elongation of main root showed about 30% inhibition, whereas the number of lateral roots was increased by 30%, indicating a likely alteration of auxin homeostasis, in the *crk5-1* mutant compared to wild type. By Lugol-staining, we observed that *crk5-1* and wild type roots accumulated comparable amounts of starch. Thus, the delay of gravitropic response did not reflect a change in starch accumulation required for gravisensing in statocyte cells of columella layer of root tips. To confirm that the observed delay of gravitropic response was indeed caused by the *crk5-1* mutation, a genetic complementation test was performed. To accomplish this and further characterization of CRK5, we created gene constructs for expression of CRK5 kinase in fusion with either green fluorescent protein (GFP) or β-glucuronidase reporters by maintaining the gene's original native regulation in plants. Monitoring the activity of CRK5:GUS reporter indicated that CRK5 is expressed ubiquitously in all plant organs. The highest CRK5-GUS levels were observed in root tips, along the vascular bundles, and in flowers. These results were corroborated by qRT-PCR measurement of *CRK5* mRNA levels in different organs. With the gCRK5:GFP construct designed for studying *in vivo* localization of CRK5, all phenotypic alterations caused by the *crk5-1* mutation, including the inhibition of root elongation, enhancement of lateral root differentiation and delayed gravitropic responses of shoots and roots, could be successfully complemented and restored to wild type.

Identification of an N-terminal myristoylation signal in CRK5 suggested potential membrane localisation of the protein. Indeed, confocal imaging of CRK5:GFP protein expressed either transiently in protoplasts or stably in transgenic plants confirmed that CRK5 is a plasma membrane-associated kinase. This result was further confirmed by co-localization with the membrane specific dye FM4-64 and western immunoblot detection of CRK5-GFP in purified cell fractions. In the root epidermis, cortex and columella cells, CRK5-GFP was detected in U-shape patterns oriented towards the root surface. In epidermal cells treated with the exocytosis

inhibitor fungal toxin brefeldin (BFA), CRK5 showed initial internalization into BFA bodies, but then ultimately accumulated in cell nuclei upon prolonged treatment. Although the function of nuclear CRK5 is yet unclear, its nuclear import suggests a functional significance of its previously observed interaction with the PRL1 subunit of the spliceosome-activating NTC complex.

The delay of root gravitropic response indicated a change in the regulation of auxin homeostasis in the *crk5-1* mutant. In fact, we found that the activity of auxin-induced DR5-GFP reporter was considerably lower in *crk5-1* root tips compared to wild type, which could plausibly explain why the elongation of primary root showed about 30% inhibition in the mutant. At the same time, our control qRT-PCR measurements indicated that transcription of key genes involved in the regulation of auxin biosynthesis, degradation and transport did not show remarkable difference between wild type and *crk5-1* mutant. Consequently, the decrease of auxin levels in *crk5-1* root tips did not result from altered regulation of genes that modulate the accumulation and transport of auxin.

By monitoring the formation of asymmetric auxin gradient in gravistimulated roots using an auxin-induced DR5-GFP reporter, we observed that the intensity of DR5-GFP5 signal has increased within 1 to 2 hours in the lower sections of wild type roots facing the gravity vector, while this could only be detected only 9 hours after gravistimulation in *crk5-1* roots. In response to treatment with the auxin efflux inhibitor the intensity of DR5-GFP signal was further reduced, whereas treatment with the auxin influx inhibitor NOA restored the levels of DR5-GFP in *crk5-1* root tips to those detected in wild type. This indicated that the *crk5-1* mutation enhanced the auxin flux from the root tip through the cortex toward the shoot with the assistance of AUX1 influx carrier and plausibly explained why the *crk5-1* mutation elevated by 30% the number of auxin-induced lateral roots above the elongation zone.

The threshold and polarity of auxin transport is determined by the amount and polar localization of auxin transporters. Our confocal microscopic studies indicated that the PIN1-GFP, PIN3-GFP, PIN4-GFP and PIN7-GFP auxin efflux and AUX1-YFP influx carriers show similar cellular localization in wild type and *crk5-1* roots, which are grown either vertically or subjected to gravistimulation by 135° rotation. In conclusion, polar localization of these auxin carriers remained unaffected by the *crk5-1* mutation. By contrast, the PIN2 auxin export carrier

that in addition to PIN3 and AUX1 plays a pivotal role in the regulation of root gravitropic response, showed characteristic change in its accumulation and polar localization in the *crk5-1* mutant. In apical (shootward-oriented) membranes of root epidermal cells the level of PIN2 was remarkably lower compared to wild type. Whereas PIN2 was localized in the basal membrane (i.e., facing the root tip) of wild type cortex cells, PIN2 showed apical membrane localization in the majority of cortex cells and appeared in the inner lateral membranes of endodermis cells facing the vascular tissues in *crk5-1* mutant roots. This indicated that relocalization of PIN2 altered the direction of auxin export from the root tip toward the elongation and differentiation zones, and vascular dividing tissues in the *crk5-1* mutant. Establishment of asymmetric PIN2 distribution between the lower and upper sections of gravistimulated roots, similarly to asymmetric distribution of auxin monitored by the DR5-GFP reporter, and consequently bending of the roots toward the gravity vector showed a considerable delay in the *crk5-1* mutant compared to wild type. Thus, due to the change in polar localization of PIN2, normal recycling of auxin though the cortex toward the root tip is disturbed, while the transport of auxin from the root tip to the elongation zone is enhanced in the *crk5-1* mutant. Altered localization pattern of PIN2 in the *crk5-1* mutant is very similar to that observed in wild type roots treated with low concentration of BFA, which shifts the localization of PIN2 from the basal to apical membranes of cortex cells, and thereby inhibits gravitropic response of roots. Subsequently, we indeed found that not only the levels of PIN2 were reduced in the apical membranes of epidermal cells of root transient zone, but BFA-dependent internalization of PIN2 was also accelerated in these cells by the *crk5-1* mutation.

Membrane recycling, polar localization and degradation of PIN proteins is regulated by phosphorylation of their cytoplasmic hydrophilic loop domains. Using purified His_6-tagged CRK5 kinase and PIN2loop substrate proteins in kinase assays *in vitro*, we found that CRK5 phophorylates the central hydrophilic loop of PIN2. From the six different CRK5 phosphorylation sites determined by mass spectrometry analysis, three occurs only in PIN2 and not in any other PIN protein. A lack of phosphorylation of these three unique PIN2 loop residues may therefore specifically alter the function and localization of PIN2. Further confirmation of this by site-directed mutagenesis is an important goal of our future studies.

SUMMARY

CRK5 is a member of the Arabidopsis thaliana Ca^{2+}/calmodulin-dependent kinase-related kinase family. Here, we show that inactivation of CRK5 inhibits primary root elongation and delays gravitropic bending of shoots and roots. Reduced activity of the auxin-induced DR5-green fluorescent protein reporter suggests that auxin is depleted from *crk5* root tips. However, no tip collapse is observed and the transcription of genes for auxin biosynthesis, AUXIN TRANSPORTER/AUXIN TRANSPORTER-LIKE PROTEIN (AUX/LAX) auxin influx, and PIN-FORMED (PIN) efflux carriers is unaffected by the *crk5* mutation. Whereas AUX1, PIN1, PIN3, PIN4, and PIN7 display normal localization, PIN2 is depleted from apical membranes of epidermal cells and shows basal to apical relocalization in the cortex of the *crk5* root transition zone. This, together with an increase in the number of *crk5* lateral root primordia, suggests facilitated auxin efflux through the cortex toward the elongation zone. CRK5 is a plasma membrane-associated kinase that forms U-shaped patterns facing outer lateral walls of epidermis and cortex cells. Brefeldin inhibition of exocytosis stimulates CRK5 internalization into brefeldin bodies. CRK5 phosphorylates the hydrophilic loop of PIN2 *in vitro*, and PIN2 shows accelerated accumulation in brefeldin bodies in the *crk5* mutant. Delayed gravitropic response of the *crk5* mutant thus likely reflects defective phosphorylation of PIN2 and deceleration of its brefeldin-sensitive membrane recycling.

PUBLICATION LIST

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