PROJECT FINAL REPORT

Function of CRK5 in regulation of auxin signaling and gravitropic responses in Arabidopsis

NKFI number: PD-128055

Supervisor: Gábor Rigó

Aims:

By sensing the Earth's gravity, higher plants adjust the growth of their shoots and roots with opposite polarity, which is controlled by the direction of gravity vector and the asymmetric distribution of the plant hormone auxin. Our previous study revealed that crk5-1 mutant displays delayed geotropic responses and reduced elongation of primary roots with more lateral roots. This phenotype is the result of altered auxin distribution caused by abnormal localization of PIN2 auxin efflux protein, which is phosphorylated by CRK5. The aim of our proposed research was to reveal the role played by CRK5 in geotropism and root development. To achieve this goal we attempted to identify and characterize proteins, which were phosphorylation substrates of CRK5 kinase and/or interacted with it. We intended to identify the CRK5-phosphorylated amino acid residues of PIN transporters and other target proteins by proteomic methods. To study the function of such modifications, point mutations were aimed to be generated which could block or mimic the phosphorylation. Biological significance of CRK5- mediated protein phosphorylation was meant to be studied by the expression of these mutant proteins in transgenic plants. We aimed to identify CRK5-interacting proteins by biochemical/proteomic methods, i.e. immunoprecipitation (pull-down) technique followed by mass spectrometry analysis. Protein interactions were planned to be characterized by bimolecular fluorescence complementation (BiFC) assays in plant cells. The results of the proposed research were expected to contribute to the better understanding of signal transduction pathways controlled by CRK5.

3. Hypothesis, aim of the project and the proposed studies

In the proposed project we have proposed to investigate the molecular mechanisms by which CRK5 regulates growth and geotropic responses of *Arabidopsis thaliana*. We have intended to decipher the function of CRK5-mediated protein phosphorylation, identify interacting proteins and characterize their role in root and shoot geotropism. In order to achieve these goals, the following particular aims have been pursued:

-Identification and confirmation of novel CRK5 phosphorylation targets.
-Studying phosphorylation targets of CRK5, identification of phosphorylation sites in PIN2 and other CRK5 substrates.
-Studying the function of PIN2 and other phosphorylation targets by altering their CRK5-dependent phosphorylation sites using in vitro mutagenesis.

-Analysis of the importance of PIN2 and PIN3 phosphorylation sites in gravitropic responses and their developmental effects.
-Identification of CRK5-interacting proteins and protein complexes. Confirmation of CRK5 protein-protein interactions in plant cell.

Results:

According to the workplan, our first task was to generate the Analog kinase (AS) variant of the CRK5 kinase. In order to do so we created two version of the AS variant kinase. The so-called gatekeeper methionine amino acid - which determines the size of ATP molecules to be accepted - was modified to Alanin (His_6-ASCRK5A) or Glycin (His_6-ASCRK5G) amino acids via PCR based mutagenesis. This task was completed and we confirmed the successful modification by sequencing. After cloning them into protein overexpression vector (pET28c), they were transformed into protein expression bacterial strain (BL21De3Rosetta). The modified recombinant proteins were purified by Ni-NTA affinity chromatography, and the purity, quality and quantity of proteins were confirmed by denaturing SDS gel electrophoresis (Figure 1.).

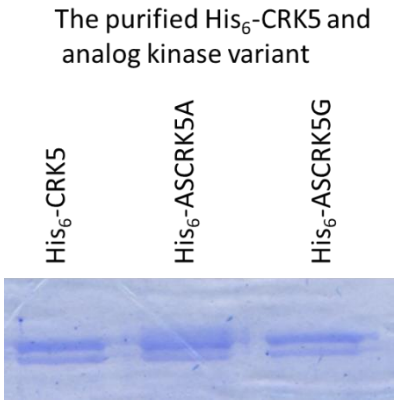


Figure 1. The purified His_6-CRK5 and analog kinase variant

The modified kinases were used for in vitro kinase assay and PNBM alkylation reactions, as recommended by the manufacturer (ABCAM). In order to get the best results, reaction conditions had to be optimized, which was followed by Western blot testing. At this stage, we could verify that the modified kinases were active, and they could use the altered ATPγS analogues: the N6-benzyl-and the isopentenyl-ATPγS. As the analog-kinase variant of the CRK5 kinase was found not working properly in plant cells (see later), we performed a test again under *in vitro* conditions with the His_6-ASCRK5 kinase variant purified from E. coli. We carefully re-analysed our previous results and concluded that the positive signal was overly faint, and the band considered as a good signal was not acceptable. The red circle represents the thiophosphorylation event on His_6-PIN2HL. As it is visible on Figure 2, we could detect the thiophosphorylation event in the case of His_6-CRK5 kinase, and at a lower level in the case of His_6-ASCRK5A, but phosphorylation of His_6-PIN2HL could not be detected in the case of His_6-ASCRK5G.

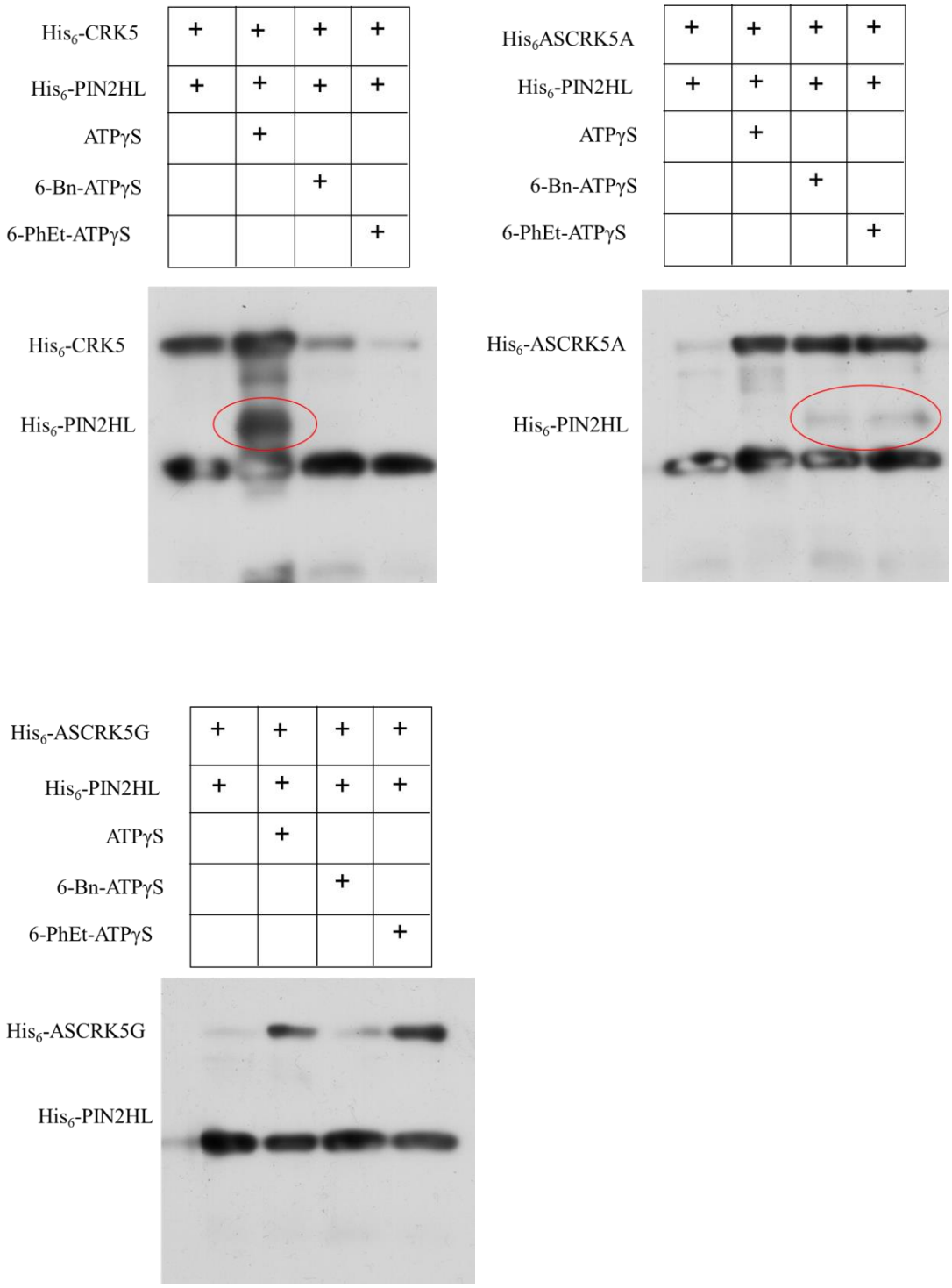


Figure 2. Western blot detection of thiophosphorylation event with specific antibody. Red circle represents the thiophosphorylation event.

We repeated the kinase activity measurement under *in vitro* conditions in another classical way, using radioactive γ-ATP and HiS_6-PIN2 hydrophilic loop, plus MBP (Myelin Basic Protein) as a substrate. Compared to the wild type His_6-CRK5 kinase no phosphorylation event on HiS_6-PIN2 hydrophilic loop could be detected neither in the case of A nor G version of analog-

sensitive CRK5 kinase, only in the case of MBP, where the signal was very low, but still detectable. The red circle represents the phosphorylation event, on His_6-PIN2HL and MBP (Figure 3.).

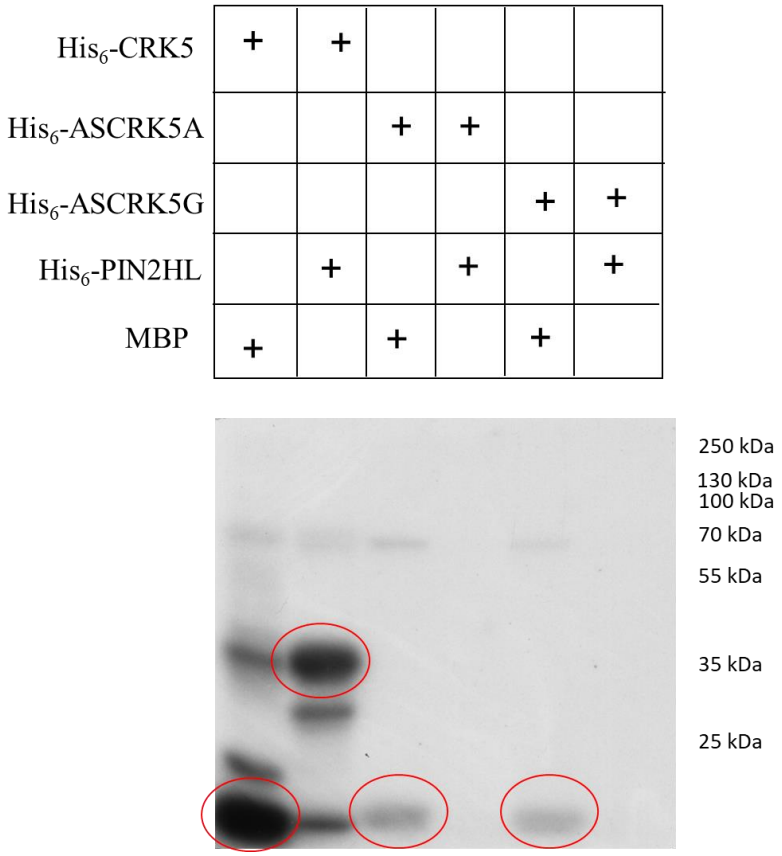
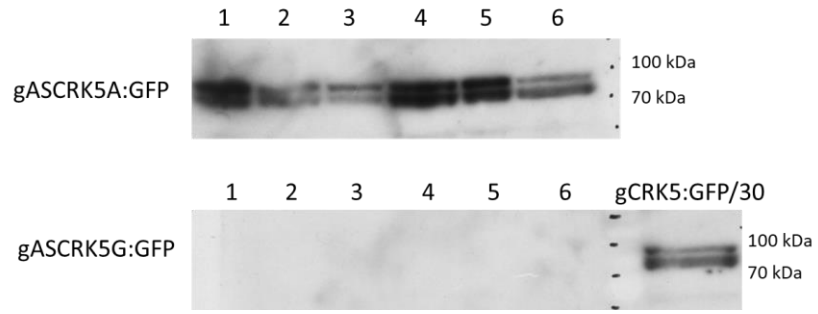


Figure 3. Results of the radioactive kinase assay. Red circle represents the phosphorylation event.

In order to isolate possible phosphorylation targets of CRK5 protein kinase from *Arabidopsis thaliana* using the Analog –kinase system, GFP-tagged version of analog sensitive kinase variants of CRK5 gene were generated. To achieve this goal, the bacterial recombineering system was adopted as described (Bitrian et al., 2011) and the experimental conditions were optimized in our laboratory. In this case, we modified the gatekeeper Methionin residue of the CRK5 kinase to Alanine (A) or to Glycine (G): gASCRK5A:GFP or gASCRK5G:GFP. The constructed gene fusions were tested by sequencing and were introduced into the genome of wild type and *crk5-1* mutant plants by *Agrobacterium*-mediated plant transformation. After transformation, we propagated independent lines. In T3 generation homozygous lines were achieved, and these lines were used for further analysis. The localization of ASCRK5A:GFP and ASCRK5G:GFP proteins in the cells were checked by LSM microscopy, followed by Western blot detection to obtain the CRK5-GFP protein, finally the gravitropic stress response test was performed.

We found, that the modified version of the gASCRK5-GFP construct had the same localization pattern in all of the tested *Arabidopsis* transformed lines with Alanine (A) modified version of the gASCRK5A-GFP. While in the case of the Glycine (G) modification we could not detect the fusion gASCRK5G-GFP signal by LSM microscopy. We tested several independent lines in each cases. We performed a Western blot to verify the existence of the fusion protein as well. The fusion protein could be detected just in the case of the Alanine (A) modified version, while in the case of the Glycine (G) version no Western blot signal was visible in the samples of the several tested independent transformed lines. This result correlated with the previous LSM microscopy results (Figure4.).

A,



B,

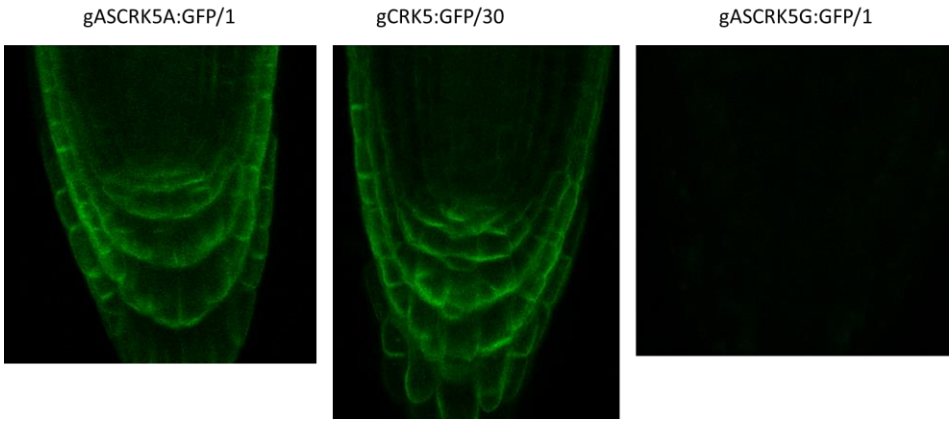


Figure 4. A, Results of the Western blot detection. B, Results of LSM microscopy.

We implemented the gravitropic stress response test, (Rigo et.al. 2013), but regrettably, pursuant to our observations the modified version of the gASCRK5G-GFP was not able to complement the *crk5-1* mutant (Figure5.). Furthermore, the modification of the gatekeeper Methionine residue to Glycine dramatically decreased the ASCRK5G-GFP stability in plant cells. According to (Zang et.al. 2005), in case the analog-sensitive kinase loses its activity, a second mutation could restore the kinase activity. There was not enough time to complete the process of second mutagenesis, as this was the third/last year of this project.

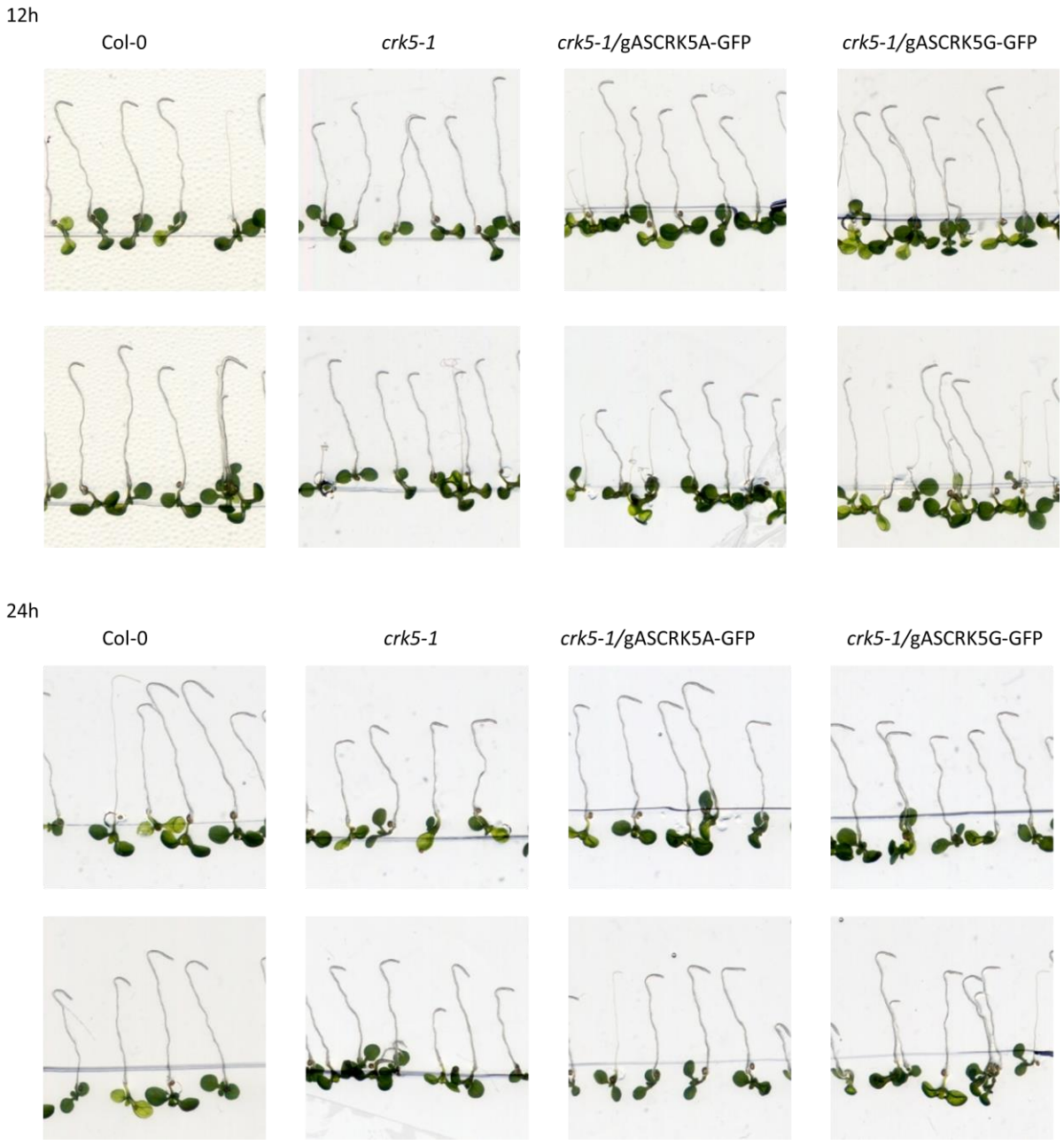


Figure 5. Representative image of the gravitropic stress response. Two time points: 12h and 24h, and after the plates were scanned.

For identification of CRK5 phosphorylation targets, the analog-kinase containing transgenic lines were planned to use, but sadly the modifications seemed to be able to inactivate the kinase activity and disable the biological function of the CRK5 kinase. The generated transgenic plant lines could not be used to isolate the possible phosphorylation target genes of the CRK5 kinase. In the future, we would like to attempt the second suppressor-mutagenesis to generate an active, analog- sensitive CRK5 kinase.

PIN phosphorylation:

During the first year of the PD OTKA Grant, I was the supervisor of a MsC diploma student, Lilla Koczka from University of Szeged. Her experiments based on one of the parts of my project. She performed the cloning of the full-length hydrophilic loop of various pin proteins, PIN1, PIN2 and PIN3 hydrophilic loop region. The PIN1 hydrophilic loop was not part of this project, but we executed the cloning and analysis. This protein function is not related to the gravitropic response, rather the embryonic development of *Arabidopsis thaliana*. Additionally, parts of the experiments were performed with the participation of an MsC biologist student, and as a result, a successfully defended thesis came into existence.
Lilla Koczka:
Determination of the Arabidopsis thaliana CRK5 protein kinase phosphorylation sites on the PIN1, PIN2 and PIN3 hydrophilic loop region.

We performed the cloning of the cDNA fragments encoding the hydrophilic loop region of PIN, PIN2 and PIN3 proteins into various bacterial protein expression vector. The recombinant proteins were successfully produced in E. coli, and later purified to homogeneity by Ni-NTA, or GST-sepharose chromatography. Purity was checked by Coomassie staining or Western blot techniques. The red rectangle represents the purified proteins. (Figure 6.)

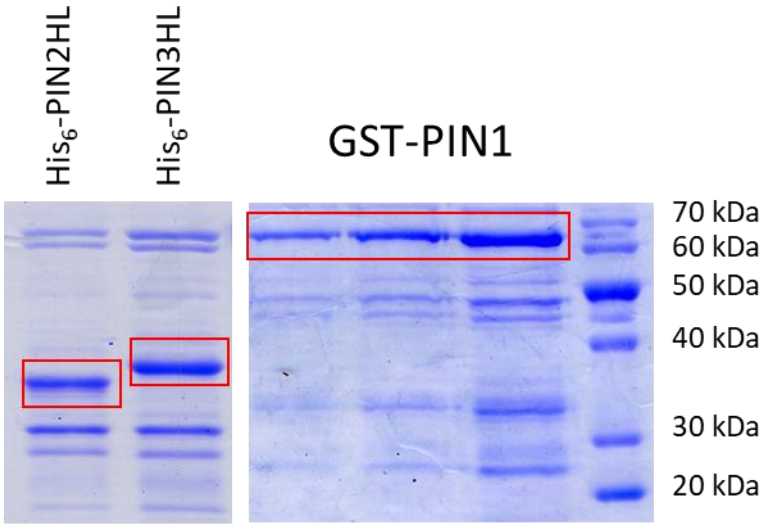


Figure 6. Coomassie staining of the various purified PIN hydrophilic loop proteins.

Radioactive kinase assay was performed to confirm that the CRK5 protein kinase was indeed able to phosphorylate the hydrophilic loop region of all three PIN proteins (PIN1, PIN2 and PIN3). The red circle represents the phosphorylation event on GST-PIN1HL, His_6-PIN2HL, His_6-PIN3HL and MBP purified proteins (Figure 7).

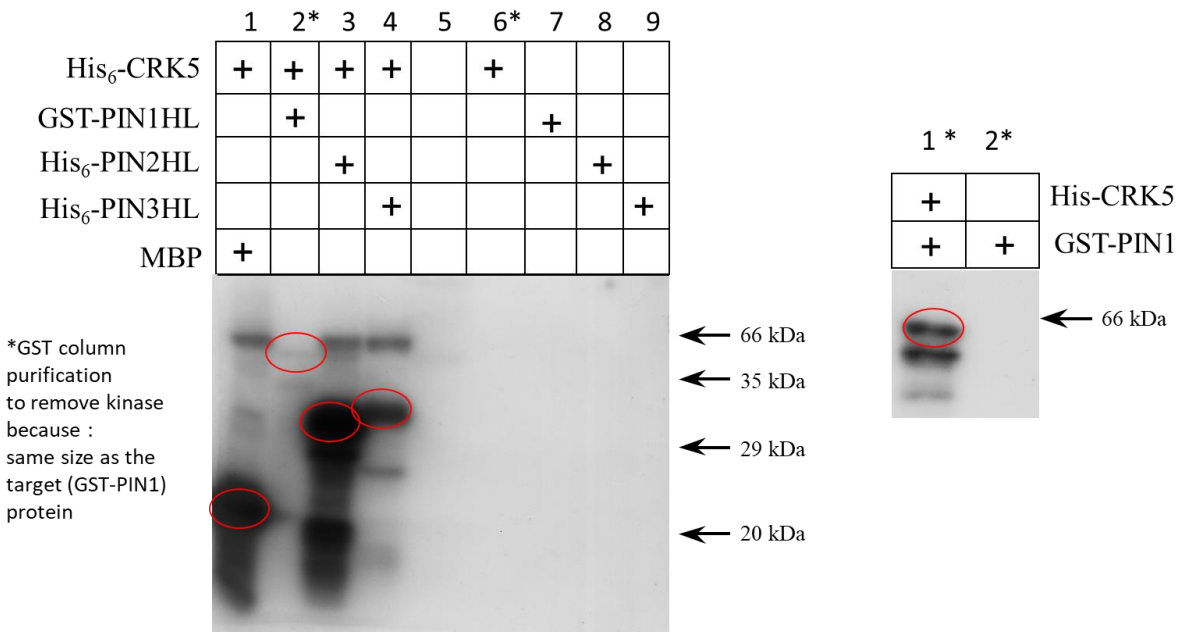


Figure 7. Results of the radioactive kinase assay. Red circle represents the phosphorylation event on GST-PIN1HL, His_6-PIN2HL, His_6-PIN3HL and MBP purified proteins. On the right panel, we increased the amount of the GST-PIN1Hl protein.

The experiment was performed in the presence of non-radioactive ATP as well, and after SDS PAGE and Coomassie staining the PIN protein fragments were isolated from gel. In the Laboratory of Proteomics Research (operating in BRC HAS, Leader: Katalin Medzihradszky) more phosphorylation sites were identified on all PIN proteins: 2 in PIN1HL, approx. 15 in PIN2HL, and approx 12 in PIN3HL proteins. We analysed the identified phosphorylation sites, and compared to the already identified sites with other kinases (Figure 8. Figure 9.).

GST-PIN1:

At1g73590

red: starting point of the HL

MSPILGYWKIKGLVQPTRLLLEYLEEKYEEHLYERDEGDKWRNKKFELGLEFPNLPYYIDGDVKLTQSMAIIRYIADKHN
MLGGCPKERAEISMLEGAVLDIRYGVSRIAYSKDFETLKVDFLSKLPEMLKMFEDRLCHKTYLNGDHVTHPDFMLYDALD
VVLYMDPMCLDAFPKLVCFKKRIEAIPQIDKYLKSSKYIAWPLQGWQATFGGGDHPPKSDLVPRGSYRGAKLLISEQFPD
TAGSIVSIHVDSDIMSLDGRQPLETEAEIKEDGKLHVTVRRSNASRSDIYSRRSQGLSATPRPSNLTNAEIYSLQSSRNP
TPRGSSFNHTDFYSMMASGGGRNSNFGPGEAVFGSKGPTPRPSNYEEDGGPAKPTAAGTAAGAGRFHYQSGGSGGGGGAH
YPAPNPGMFSPNTGGGGGTAAKGNAPVVGGKRQDGNGRDLHMFVWSSSASPVSDVFGGGGGNHHADYSTATNDHQKDVKI
SVPQGNSNDNQYVEREEFSFGNKDDDSKVLATDGGNNISNKTTQAKVMPPTSVMTRLILIMVWRKLIRNPNSYSS.

6xHIS-PIN2:

At5g57090

red: starting point of the HL

? maybe presence in the control

MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSQFPETAGSITSFRVDSDVISLNGREPLQTDAEIGDDGKLHVVVRRS
SAASS?MISSFNKSHGGGLNSSMITPRAS?NLTGVEIYSVQSSREPTPRASS?FNQTDFYAMFNASKAPSPRHGYT?NS?
YGGAGAGPGGDVYSLQSSKGVTPRTSNFDEEVMKTAKKAGRGGRSMSGELYNNNSVPSYPPPNPMFTGSTSGASGVKKKE
SGGGGSGGGVGVGGQNKEMNMFVWSSSASPVSEANAKNAMTRGSSTDVSTDPKVSIPPHDNLATKAMQNLIENMSPGRKG
HVEMDQDGNNGGKSPYMGKKGSDVEDGGPGPRKQQMPP.

6xHIS-PIN3:

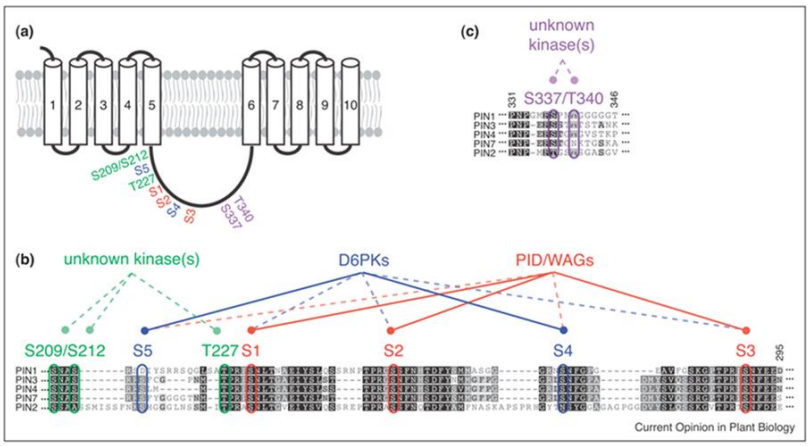
At1g70940

red: starting point of the HL

MGSSHHHHHHSSGLVPRGSHMASMTGGQQMGRGSQFPETAASIVSFKVESDVVSLDGHDFLETDAEIGDDGKLHVTVRKS
NASRRSFCGPNMTPRPSNLTGAEIYSLSTTPRGSNFNHSDFYNMMGFPGGRLSNFGPADMYSVQSSRGPTPRPSNFEENC
AMASSPRFGYYPGGGAGSYPAPNPEFSSTTTSTANKSVNKNPKDVNTNQQTTLPTGGKS?NS?HDAKELHMFVWSSNGSP
VSDRAGLNVFGGAPDNDQGGRSDQGAKEIRMLVPDQSHNGETKAVAHPASGDFGGEQQFSFAGKEEEAERPKDAENGLNK
LAPNSTAALQS?KTGLGGAEASQRKNMPP.

?"maybe presence in the control": it was surly detectable in the kinase treated sample, and also presence in the non-treated control sample but smaller extent. These sites are maybe not specific for the CRK5 kinase. Other experiments are necessary to confirm them.

Figure 8. The identified possible phosphorylation sites of the different PIN proteins hydrophilic loop.



Topology and annotated partial sequence alignment of the 'long' PINs. **(a)** Schematic representation of the 'long' PINs with the predicted transmembrane-spanning domains and the cytoplasmic hydrophilic loop (CL) with identified phosphosites. **(b)** Sequence alignment of the N-terminal part of the CL with the five D6PK and PID/WAG phosphosites and their respective phosphosite preferences indicated by the blue/red color code and continuous or dashed lines [19••,29••,30••]. Additional phosphosites that are unlikely targets of D6PK or PID are shown in green [19••,21,22]. **(c)** Alignment of the C-terminal part of the CL with two potential phosphosites that are likely not phosphorylated by PID or by D6PK [19••,36••]. Amino acid references are for PIN1.

The identified phosphorylation sites on different PIN proteins by other kinases
Same as CRK5:
S1: only case of PIN2
S2: in case of PIN1 and PIN2
All others phosphorylation sites are different.

Figure 9. Summary of phosphorylation site results.

In order to get the function of this phosphorylation site in plants, we planned to make two phosphor-mimicking mutant version (change the possible phosphorylation site to alanine or aspartate,) in all analysed PIN protein. For this study, we ordered the BAC clones from TAIR (https://www.arabidopsis.org) which contained the various PIN gene genomic fragments. Due to the unexpected complexity of PIN2 and PIN3 phosphorylation - 15 and 12 phosphorylation sites have been identified in PIN2 and PIN3, respectively, - the multistep in vitro mutagenesis turned out to be a complicated and time-consuming task, which took much longer time than initially predicted. In our hands, the efficiency of the bacterial recombineering system was too low or unfunctional in these cases. We attempted to optimize the system under our conditions, contacted the authors of the original article, but after several round of sequencing reaction, positive good clones could not be detected. Two other methods were tested to generate the modified version of selected PIN genes: one of them was the SLICE method based on Motohashi, K. et.al. (2015)., and the other was a method previously used in our laboratory, and published by Szekely et. al. (2008). So far we do not have a positive construct. We ordered and propagated various types of PIN mutant plant lines; PIN1 mutant: pin1(GK_051A10), PIN2 mutant: agr1-1(N268), agr1-2 (N269), ethylene insensitive root(N8058), agr1(SALK_122916), agr1(SALK_144447), PIN3 mutant: pin3-4(N9363) pin3-5 (N9364), pin3(SALK_113246), for testing the complementation effect of the various modified PIN gene constructs.

Immunoprecipitation, identification of interaction partners:

We found, that 1% of Triton X-100 in the presence of 150mM NaCl could well solubilize the CRK5-GFP protein. We used 35S-GFP expressing plant lines as a control, plant growing, sample collection and extraction procedure were performed as in the case of the gCRK-GFP expressing plant lines. We collected various source of plant materials for isolation of CRK5-GFP protein complexes: 1, whole green flowering plants from greenhouse, 2, in vitro root culture 3, etiolated plants. We used highly specific, GFP-TRAP-MA magnetic agarose beads from Chromotec (https://www.chromotek.com/). We sent the isolated protein complexes into the Laboratory of Proteomics Research (operating in BRC HAS, Leader: Katalin Medzihradszky) for complete analysis. We gathered the information into the following table:

Root:		
T9_Q8H190	At1g73260	Putative trypsin inhibitor
T1_Q9ZVJ6	At2g38750	Annexin D4
M2_Q9LYG3	At5g11670	NADP-dependent malic enzyme 2
Etiolated:		
D3_Q9LQ55	At1g59610	Dynamin-2B
T1_Q9ZVJ6	At2g38750	Annexin D4
T8_Q1H555	At3g11510	Ribosomal protein S11 family
T2_P43297	At1g47128	Cysteine proteinase RD21a
Green plants:		
T7_Q41932	At4g05180	Oxygen-evolving enhancer protein 3-2, chloroplastic
T6_Q9C5C2	At5g25980	Myrosinase 2
T2_P43297	At1g47128	Cysteine proteinase RD21a
T10_P50883	At2g37190	60S ribosomal protein L12-1
T5_Q9LLR6	At5G59310	Non-specific lipid-transfer protein 4

Figure 9. This table represents the most prominent identified CRK5-GFP interaction partners.

The identified possible interaction partners were cloned from various source of cDNA library. The sequenced clones were cloned into the following BiFC vectors using LR Clonase techniques: pUBC-cYFP-DEST, and pUBC-nYFP-DEST binary vector system. We tested the construct in *Arabidopsis* cell suspension using *Agrobacterium*-mediated co-transformation, and LSM technics to confirm the interaction with CRK5 kinase.

In this case, we used the shot name for e.g.: T9_T1 etc. for the easiest labelling of LSM picture.

We could detect the interaction in all cases. Depending on the co-transformation efficiency, we had few or high amount of positive cells. As we used cell suspension, the cells formed small aggregates, or sometimes had a non-round phenotype. From our previous experiments using

yeast two hybrid system, CRK5 was known to be able to form a dimer. Interestingly we could confirm this dimer formation by using BiFC technics. In the following picture, we present the typical LSM microscopy images (Figure 10).

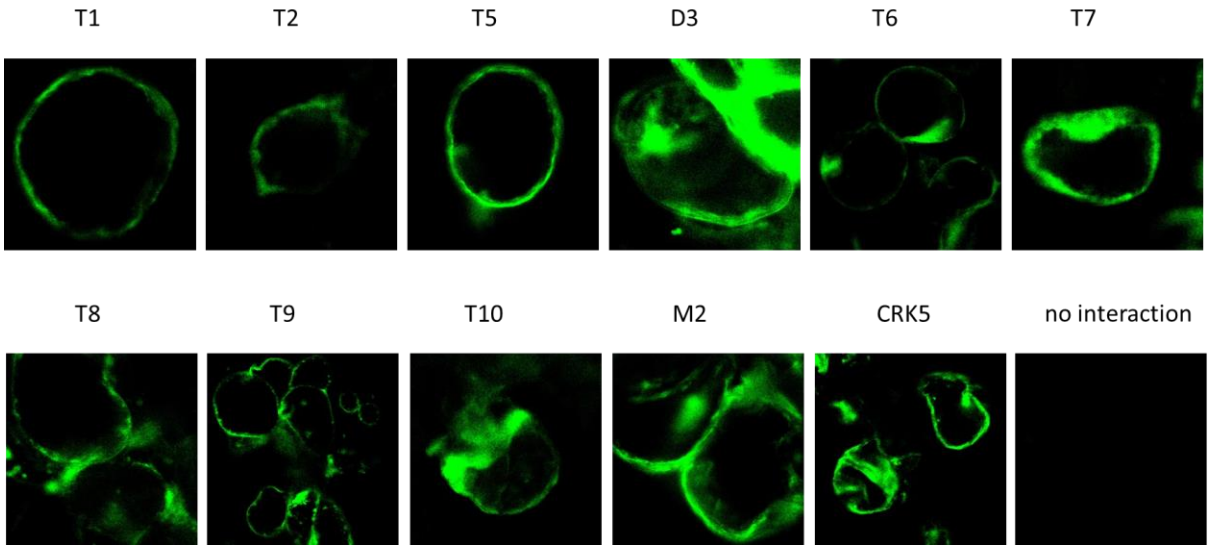


Figure 10. Green signal represents the interaction of the various proteins from the MALDI results with the CRK5 protein kinase.

Now I would like to write a few words about the possible functions of the identified interaction partners:

T-1 AnnexinD4:

It is involved in membrane-related processes, such as intracellular vesicular trafficking, endo- and exocytosis, phagocytosis and autophagy, due to their inherent ability to bind and position the membrane structures in relation to each other in a calcium-dependent manner.
(Konopka-Postupolska and Clark, 2017).

D-3 Dynamin-2B: dynamin-like proteins (DLPs) implicated in membrane remodelling, chlatrin/GTP/microtubule binding (dynamin and chlatrin-mediated endocytosis)
(Ferguson, S.M. and De Camilli, P., 2012).

T-5 Non-specific lipid-transfer protein 4:

Lipid transport, response to abscisic acid, response to salt stress, response to water deprivation. AtLTPI-4 is associated with the plasma membrane, can transfer long-chain as well as very long-chain fatty acids into the extracellular space, involved in suberin formation of *Arabidopsis thaliana* crown galls (Deeken, R. , et. al. 2016). Suberin consists of a polymer matrix, which contains polyaromatics and polyaliphatics (Graça and Santos, 2007).

M-2 NADPH-dependent malyc enzyme 2:

It is involved in plant defense responses and lipid biosynthesis, cytosolic isoforms role in plant defense responses and lignin biosynthesis (Wheeler MC, et. al. 2005). Control the cytosolic pH by balancing the synthesis and degradation of malate (Martinoia, E. and Rentsch, D. 1994).

T-6 Myrosinase2:

A family of enzymes involved in plant defense against herbivores. Its known biological function is to catalyze the hydrolysis of a class of compounds called glucosinolates.
When the plant is attacked by pathogens, insects, or other herbivore, the plant uses myrosinase to convert glucosinolates,-which are otherwise benign- into toxic products like isothiocyanates, thiocyanates, and nitriles, (Barth, C. and Jander, G. 2006).

T-2 Cysteine proteinase RD21A:

Cysteine protease that plays a role in immunity, senescence, and biotic and abiotic stresses involved in elicitor-stimulated programmed cell death (PCD). Involved in water stress-induced cell death through its protease activity that is released to the cytoplasm after vacuolar collapse. Function in plants defense response, (Pogorelko, G.V. et al. 2019).

T-9 Putative trypsin inhibitor:

Involved in modulating programmed cell death in plant-pathogen interactions.
www.arabidopsis.org

T-8 Ribosomal protein S11 family,
T-10 60S ribosomal protein L12-1:

Response to cold, ribosomal large subunit assembly, ribosome biogenesis, translation.
www.arabidopsis.org

T-7 Oxygen-evolving enhancer protein 3-2:

“Encodes the PsbQ subunit of the oxygen evolving complex of photosystem II.”
This interaction partner is interesting, because this protein localize in the chloroplast. Maybe this interaction is unspecific.
www.arabidopsis.org

More characterization is necessary to make a better conclusion of these interactions. Some proteins are involved in plasma membrane trafficking, while others have structural or defense related action. Interestingly we could isolate such interaction partners, that are not related to gravitropic stress responses. For more precise analysis more time is needed.

As we found that the CRK5 kinase could phosphorylate the PIN1 and PIN3 HL loop, we extended the CRK5 studies and started the functional analysis of CRK5 kinase during embryogenesis (where a possible function of PIN1 has), and during the germination, mainly in scotomorphogenesis (where a possible function of PIN1 and PIN3 have). Together with my colleagues we expect to get severeal interesting results regarding the CRK5 function in embryogenesis and germination.
These results are planned to be published this year in two independent articles:

Abu Imran Baba, Ildikó Valkai, Nitin Labhan, Lilla Koczka,Norbert Andrási, László Szabados, Attila Fehér, Gábor Rigó and Ágnes Cséplő
CRK5 protein kinase exhibits pivotal role in embryogenesis of Arabidopsis thaliana. (manuscript in preparation)

Abu Imran Baba, Ildikó Valkai, Nitin Labhan, Lilla Koczka,Norbert Andrási, László Szabados, Attila Fehér, Gábor Rigó and Ágnes Cséplő
Role of CRK5 kinase in and skotomorphogenesis of Arabidopsis thaliana.
(manuscript in preparation)

In *Arabidopsis* plants there are 8 members of CRK gene family. In this PD OTKA grant we are just focusing on the CRK5 kinase function, but previously we started the analysis of the *Arabidopsis thaliana* CDPK-Related kinase family members. We could partially complete this latter task, and the results were published in the following journal:

International Journals of Molecular Sciences as:
Functional Analysis of the Arabidopsis thaliana CDPK-Related Kinase Family: AtCRK1 Regulates Responses to Continuous Light.
Baba AI, **Rigó G**, Ayaydin F, Rehman AU, Andrási N, Zsigmond L, Valkai I, Urbancsok J, Vass I, Pasternak T, Palme K, Szabados L, Cséplő Á
Int J Mol Sci. 2018 Apr 25;19(5). pii: E1282. doi: 10.3390/ijms19051282.

Based on this work, my PhD student (share with Ágnes Cséplő 50-50%) Abu Imran Baba write his PhD thesis

Title:
Functional analysis of CDPK Related Kinase (CRK) family members in *Arabidopsis thaliana*

Home defence: 05.04.2019.

Reference:

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The MPK4-phosphorylated Heat Shock Factor A4A regulates responses to combined salt and heat stresses (accepted manuscript in Journal of Experimental Botany)