Functional analysis of the CDPK-Related Kinase (CRK) family in *Arabidopsis thaliana*

PhD thesis

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Szeged 2019

Introduction

In Plants the Calcium-Dependent Protein Kinase (CDPK) is a Serine/Threonine type of protein kinase that plays vital roles in their growth, development and responses to various stresses. CDPKs have been identified throughout the plant kingdom as well as in several protists but are absent in animals (Harper and Harmon, 2005). CDPKs constitute one of the largest subfamilies of plant protein kinase family. This kinase family has a characteristic structure with an N-terminal Ser/Thr protein kinase domain fused to a carboxy-terminal calmodulin-like domain containing EF-hand calcium-binding sites (Harmon et al., 2000; Cheng et al., 2002). Most of the CDPKs have been predicted to have myristoylation sites at their N-terminal site assuming that these proteins are membrane localized and they also possess a nearby Cysteine residue that may serve as a site for palmitoylation (Hrabak et al., 2003; Harper et al., 2004; Hegemann et al., 2006).

The CDPK-Related Kinase (CRK) family is closely related to CDPKs and consists of eight members in *Arabidopsis* plant. Unlike the CDPKs that are more explored, limited information is available about the functional role of plant CRKs *in vivo* in *Arabidopsis thaliana*. For a decade only predictions of their membrane localization have been reported (Podell and Gribskov, 2004). Most of the AtCRKs, including the AtCRK5 posses a conserved C-terminal calmodulin (CaM) binding domain, which overlaps with the kinase auto inhibitory domain (Zhang et al., 2002). The N-terminal region of most AtCRKs is predicted to be myristoylated and palmitoylated (Renna et al., 2013; Rigó et al., 2013). An analysis of the CRK protein kinase family previously in the plant tomato was reported to consist of six members and the first tomato CRK gene (Sl*CRK6*) was shown to carry disease resistance against plant pathogen (Wang et al., 2016). Previously partially characterized T-DNA insertion mutant of the AtCRK5 protein kinase was reported to show the asymmetric distribution of auxin causing a disturbed gradient leading to delayed gravitropic bending of At*crk5-1* mutant roots. This delay in the gravitropic bending capacity of At*crk5-1* mutant was found to be a consequence of improper phosphorylation of auxin efflux transporter PIN2 protein (Rigó et al., 2013). Recently we also reported it to be an important kinase involved in the hypocotyl hook development during skotomorphogenesis (Baba et al.,2019). In the current study we

characterized functionally the AtCRK protein kinase family members as until now there was only very limited information available about their localization and stress related activity during *Arabidopsis thaliana* growth and development. The main focus was on their subcelluar localization and general characterization involving their root gravitropic features, with further special emphasis on two of its highly expressed individual members AtCRK1and AtCRK5 during continuous light stress involving redox homeostasis and embryo development respectively.

Aims

(1) Identification and acquisition of At*crk* T-DNA insertion mutants from public mutant collection and characterization of these mutant lines to generate homozygous lines and test the expression of mutated genes (genotyping process).

(2) Phenotyping of the selected homozygous At*crk* mutants and performing the germination and root growth assays, also testing their root/hypocotyl gravitropic bending capability under gravistimulation.

(3) To report the subcellular localization of cDNA cloned version of 35S-AtCRK-eGFP tagged proteins initially by the protoplast transient expression assay and finally in the roots of transgenic transformed seedlings.

(4) Characterization of the potential role of AtCRK1 protein kinase in response to its sensitivity to continuous light stress involving the regulation of $^1O_2$-triggered cell death and maintenance of cellular redox homeostasis.

(5) Characterization of the role of AtCRK5 protein kinase during embryogenesis by the network of auxin-gibberellic acid hormonal crosstalk through a cooperative role of auxin efflux carriers PIN1, PIN4, PIN7 and influx carrier AUX1.

Techniques and methods applied:

- Genotyping
- Phenotyping
- Gene cloning and plant transformation
- Protoplast transformation assay
- PSII photochemical activity measurements by Imaging-PAM
- Detection of $^{1}O_2$ by Singlet Oxygen Sensor Green
- Determination of cell death by Evan's blue staining
- Differential centrifugation
- Western blot/ Immunolocalization
- RNA isolation, CDNA synthesis
- PCR and qRT-PCR
- Confocal Laser Scanning Microscopy/ Cell-R Microscopy
- Competitive GA ELISA assay
- *In vitro* kinase assay/Mass spectrometry
- Bioinformatics analysis and tools

Summary

In this study we studied the subcellular localization studies of the AtCRK family members in roots using the 35S promoter driven AtCRK-eGFP fusion proteins analysed by confocal laser scanning microscopy (CLSM). We reported that in analogy to the previously characterized one of its member AtCRK5, the majority of the overexpressing AtCRK-eGFP fusion proteins indicated plasma membrane localization in transgenic plants, except AtCRK1-eGFP which additionally to its plasma membrane localization, displayed mostly the peculiar prominent endomembrane GFP signal too. Further the T-DNA insertional mutants for the various At*CRK* family members were selected and the homozygous mutants of most of them were initially genotyped. These At*crk* mutants were later phenotyped by performing the germination and root growth assays, and testing their root/hypocotyl gravitropic bending capability under gravistimulation. We reported that the members of the AtCRK family mutants displayed the delayed germination ability and reduced root growth rate comparing to the wild type *Arabidopsis* Col-0. Moreover, the root geotropic bending capacity was found to be reduced in most of the At*crk* mutants which was accompanied by altered localization of the auxin efflux PIN2-GFP protein. The hypocotyl bending capacities of the At*crk* mutants depicted also a delay in the hook bending. This observation suggests that auxin transport could be impaired in the AtCRK family mutants in roots/hypocotyl. Previously it was determined that AtCRK5 is able to phosphorylate some of the PIN proteins *in vitro*, therefore we speculate that in analogous to AtCRK5 function - the other AtCRK family members also depicted a prominent role in regulation of the root gravitropic response of *Arabidopsis thaliana* by involvement of the phosphorylation event of specific auxin transporters.

Furthermore we also reported a novel, continuous light dependent phenotype of the AtCRK1 family member mutant At*crk1-1*. In this mutant the enhanced $^1O_2$ content was accompanied with low carotenoid and chlorophyll content, also the decreased functional PSII parameters like effective PSII quantum yield (ΦPSII) lower electron transport rate (ETR) and non-photochemical quenching (NPQ), suggesting that the fitness of the photosynthesis in this mutant is seriously impaired by its susceptibility to photoxidative stress. It is already known that $^1O_2$ hyperaccumulation can cause directly cell death. So in the case of

At*crk1-1,* necrotic lesions and cell death could also be observed during continuous light conditions, which resembles the reaction of *flu* mutant from dark to light transition or *ch1* mutant to high light stress. $^1O_2$ is not only toxic but it can also operate as a stress signal, leading to extensive changes in gene expression, promoting programmed cell death (PCD) or acclimation. Based on At*crk1-1* overproduction of the $^1O_2$ under continuous light condition, we may say that AtCRK1 is a suppressor of $^1O_2$ production, and can function as a regulator of $^1O_2$ triggered cell death displaying its pivotal role in regulation and responses to continuous light and cellular redox homeostasis.

We also reported other functional role of AtCRK5 protein kinase which is related to its regulation in embryogenesis of *Arabidopsis thaliana.* We found that the At*crk5-1* mutant has a delayed embryogenesis accompanied with decreased levels of gibberellic acid (GA) during early embryogenesis. The At*crk5-1* mutant was also deficient in the maintenance of the local auxin concentration during embryogenesis as demonstrated by decreased expression of the auxin sensor DR5::GFP. The abundance of the polar auxin transport (PAT) proteins PIN1-GFP, PIN4-GFP, PIN7-GFP and AUX1::YFP in the At*crk5-1* mutant embryos was also found decreased. It is already known that AtCRK5 is able to phosphorylate the PIN1, PIN2 and PIN3 proteins *in vitro.* Here we also report that AtCRK5 protein kinase can phosphorylate *in vitro* the hydrophilic loops of some additional PIN proteins like PIN4 and PIN7 which are involved in auxin efflux transport during embryogenesis. Therefore, we propose that AtCRK5 protein kinase – additionally its regulatory role in root gravitropic responses and hypocotyl bending – can also govern the embryo development in *Arabidopsis thaliana* through the fine tuning of auxin-GA level by phosphorylation of targeted polar auxin transport (PAT) proteins. We conclude that AtCRK5 also exhibits a prominent role in the regulation of embryogenesis by mediating the auxin homeostasis through a cooperative approach of auxin efflux carriers PIN1, PIN4, PIN7 and influx carrier AUX1. Based on these novel results a manuscript is currently in preparation.

Acknowledgement
This research was supported by OTKA project No. PD115502 and No. PD128055, NKFI project No. NN118089, OTKA grant No. 124828 and GINOP-2.3.2-15-2016-00001. AIB was supported by the C1 Ministerial PhD Fellowship from Tempus Public Foundation, and the Doctoral School in Biology, University of Szeged, Hungary.

PUBLICATION LIST (MTMT ID: 10053163)

Mandatory peer-reviewed international publications for the fulfilment of doctoral process and on which this thesis is based:

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: Functional Analysis of the *Arabidopsis thaliana* CDPK-Related Kinase Family: At*CRK1* Regulates Responses to Continuous Light. *Int. J. Mol. Sci.*,19, 1282(2018) **(I.F: 4.183).**

Baba AI, Valkai I, Labhane N, Andrási N, Szabados L, Fehér A, Rigó G and Cséplő A: AtCRK5 protein kinase exhibits an essential role in embryogenesis of *Arabidopsis thaliana*. ***(Manuscript in preparation).***

Baba AI, Andrási N, Valkai I, Gorcsa T, Koczka L, Darula Z, Medzihradszky KF, Szabados L, Fehér A, Rigó G, and Cséplő Á: AtCRK5 Protein Kinase Exhibits a Regulatory Role in Hypocotyl Hook Development during Skotomorphogenesis. *Int. J. Mol. Sci.*, 20, 3432(2019) **(I.F: 4.183).**

Other peer-reviewed international publications

Andrási N, Rigó G, Zsigmond L, Pérez-Salamó I, Papdi C, Klement E, Pettkó-Szandtner A, **Baba AI**, Ayaydin F, Dasari R, Cséplő A, and Szabados L: The *Arabidopsis* Heat Shock Factor A4A is target of MPK4 and regulates responses to combined stresses. *Journal of Experimental Botany, erz217*(2019) **(I.F: 5.360).**

Kovács H, Aleksza D, **Baba AI**, Hajdu A, Király A, Zsigmond L, Toth SZ, Kozma Bognar L, Szabados L: Light control of salt-induced proline accumulation is mediated by ELONGATED HYPOCOTYL 5 in *Arabidopsis*. *Frontiers in Plant Science* (2019) ***(Under revision).***

Book chapter:

Baba AI, Rigó G, Andrási N, Tietz O, Palme K, Szabados L, Cséplő A: Striving Towards Abiotic Stresses: Role of the Plant CDPK Superfamily Members. In: Palocz-Andresen M., Szalay D., Gosztom A., Sípos L., Taligás T. (eds) *International Climate Protection. Springer, Cham.*, 99-105(2019).

Conference lectures, poster presentations and scientific trainings:

1. Hungarian Molecular Life Sciences conference (March 29-31, 2019, Eger, HUNGARY) **(Poster)**. CRK5 protein kinase exhibits an essential role in embryogenesis of *Arabidopsis thaliana*. **Abu Imran Baba,** Ildikó Valkai, Nitin Labhane, Norbert Andrási, Laura Zsigmond, László Szabados, Attila Fehér, Gábor Rigó and Ágnes Cséplő.

2. HUMBOLDT-KOLLEG 2018 "Sustainable development and climate change: connecting research, education, policy and practice" (September 19-22, 2018, Belgrade, SERBIA). Functional Characterization of CDPK Related Kinase (CRK) Family in *Arabidopsis thaliana*. **Abu Imran Baba**, Gábor Rigó, Ferhan Ayaydin, Ateeq Ur Rehman, Norbert Andrasi, László Szabados and Ágnes Cséplő.

3. EPSO/FESPB 2018 Congress, (18-21 June, 2018, Copenhagen, DENMARK) **(Poster)** Functional analysis of CDPK Related Kinase (CRK) family in *Arabidopsis thaliana* plant. **Abu Imran Baba,** Gábor Rigó, Ferhan Ayaydin, Norbert Andrási, Ateeq Ur Rehman, János Urbancsok, Laura Zsigmond, Ildikó Valkai, Imre Vass, Taras Pasternak, Klaus Palme, László Szabados, Ágnes Cséplő.

4. STRAUB DAYS, Biological Research Centre, HAS (10-11 May, 2018, Szeged, HUNGARY) **(Poster).** Functional analysis of the *Arabidopsis thaliana* CDPK Related Kinase (CRK) family. **Abu Imran Baba,** Gábor Rigó, Ferhan Ayaydin, Norbert Andrási, Ateeq Ur Rehman, János Urbancsok, Laura Zsigmond, Ildikó Valkai, Imre Vass, Taras Pasternak, Klaus Palme, László Szabados, Ágnes Cséplő.

5. LIMITS OF KNOWLEDGE. INTERNATIONAL SCIENTIFIC CONFERENCE HUMBOLDT-KOLLEG. (22-25 June 2017, Krakow, POLAND) **(Lecture and Poster).** Functional characterization of the CDPK-Related Kinase (CRK) family members in higher plant *Arabidopsis thaliana.* **Abu Imran Baba**, Gábor Rigó, Taras Pasternak, Klaus Palme, Csaba Koncz, László Szabados, Ágnes Cséplő.

6. PLANT BIOLOGY EUROPE FESPB/EPSO Congress (26-30 June, 2016, Prague, CZECH REPUBLIC) **(Poster)** Regulation of differential growth in apical hook of *Arabidopsis*: role of hormonal fine tuning. **Abu Imran Baba**, Norbert Andrási, Teréz Gorcsa, Gábor Rigó, László Szabados, Ágnes Cséplő.

7**.** STRAUB DAYS, Biological Research Centre, HAS (25-26 May, 2016, Szeged, HUNGARY) **(Poster)**. Auxin-Ethylene Interaction in the *Arabidopsis crk5-1* mutant in Dark. Localization of the Polar Auxin Transport Proteins. **Abu Imran Baba**, Norbert Andrási, Teréz Gorcsa, Gábor Rigó, László Szabados and Ágnes Cséplő.

8. International Biological Conference at BABES BOLYAI University, (April, 2016, Kolozsvár ROMANIA) **(Lecture).** Involvement of the calcium-dependent protein kinase (CDPK)-related kinase CRK5 in regulation of gravitropic responses under de-etiolated conditions. **Abu Imran Baba**, Norbert Andrási, Gábor Rigó, László Szabados and Ágnes Cséplő.

9. FIBOK Biotechnological Conference, (21-22 March 2016 Gödöllő, HUNGARY) **(Lecture).** Regulation of plant gravitropic stress response by CRK5 protein kinase involving polar auxin transport (PAT) protein localization in etiolated *Arabidopsis thaliana.***Abu Imran Baba**, Norbert Andrási, Gábor Rigó, László Szabados and Ágnes Cséplő.

10. HUMBOLDT-KOLLEG "Symposium on International Climate Protection" (13-14 November, 2015, Budapest, HUNGARY) **(Poster)** CRK5 Protein Kinase is a Regulator of Abiotic Stress Responses in Higher Plant *Arabidopsis Thaliana*. Ágnes Cséplő, **Abu Imran Baba**, Norbert Andrási, Teréz Gorcsa , Gabor Rigó, Laszlo Szabados.

11. 17th EMBL PhD Symposium titled "Just by Chance? Randomness and variability shaping biology" (22-24 October, 2015, Heidelberg, GERMANY) **(Poster)** AUXIN-ETHYLENE INTERACTIONS IN THE *ARABIDOPSIS crk5-1* MUTANT IN DARK. **Abu Imran Baba**, Norbert Andrási, Teréz Gorcsa, Gábor Rigó, László Szabados and Ágnes Cséplő

12. **Training Program at Les Houches Winter School** organized by University of Grenoble in Chamonix (13-24 March, 2017, Les Houches, FRANCE) titled "Biology at different scales: interplay between physics and integrative biology".

Fellowships:

1. Full PhD C1 ministerial Scholarship from "***Hungarian Ministerial Scholarship Board***" (**Tempus Public Foundation)** (2015-2018).

2. Young researcher travel and fee waiver grant from ***Alexander von Humboldt Scientific society*** Krakow to attend "International Scientific Conference Humboldt-kolleg" in Krakow, Poland (2017).

3. Fellowship from ***University of Grenoble***, to attend two weeks "Les Houches Winter Training School" in Chamonix, France (2017).

4. EMBL/EMBO grant for attending International "***17th EMBL PhD Symposium***" at EMBL Heidelberg, Germany (2015).

5. Young Researcher Fellowship from ***Hungarian Academy of Sciences*** (2019).

Conflict of interest

We as the corresponding and/or contributing first authors of the below mentioned peer reviewed publication and the unpublished part of this thesis declare that the authors have no conflict of interest related to this study. We also declare that the PhD candidate Abu Imran Baba worked under our supervision and had a great contribution to the overall results evidenced in this thesis.

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: Functional Analysis of the *Arabidopsis thaliana* CDPK-Related Kinase Family: AtCRK1 Regulates Responses to Continuous Light. *Int. J. Mol. Sci.*,19,1282(2018).

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29 August, 2019, Szeged

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