

THE ROLE OF *ARABIDOPSIS* GENES INVOLVED IN ABIOTIC (OSMOTIC, OXIDATIVE AND GRAVITROPIC) STRESS RESPONSE REGULATIONS

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SUMMARY. The warming of overall climate requires to breed plant cultivars tolerant to extreme osmotic tolerance, e.g. to high salt concentration in order to improve their chance to survive deleterious effects of abiotic stress conditions. Our initial aim is to isolate and characterize abiotic stress response regulatory genes arisen from *Arabidopsis thaliana* which is known as a model species for such investigations in higher plant. For this reason, a Ser/Thr protein kinase, the CRK5 was chosen. The CRK5 protein kinase is partly functionally characterized exhibiting a role in the regulation of gravitropic responses of *Arabidopsis thaliana* roots (Rigó *et al.*, 2013). The CRK5 is a plasma membrane associated kinase that forms U-shaped patterns facing outer lateral walls of root epidermis cells. The CRK5 phosphorylates the hydrophilic loop of the auxin efflux transporter PIN2 *in vitro*. Thus, delayed gravitropic response of *crk5* mutant reflects defective phosphorylation of PIN2 and deceleration of its brefeldin sensitive membrane recycling. Recently, we have been investigating the regulatory role of CRK5 protein kinase under osmotic (salt) and oxidative (hydrogen peroxide) stresses. The aim was to gather additional information regarding its role in the regulation of responses to either high salinity or oxidative stress, and consequently, regarding the impact on the auxin biosynthesis, transport and signaling. CRK protein kinase is hypothesized to be involved in the regulation of the effect of reactive oxygen species (ROS, e.g. hydrogen peroxide).

Keywords: abiotic stress response regulation, *Arabidopsis thaliana*, CRK5 protein kinase, gravitropism, reactive oxygen species

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Connection of gravitropic response and the plant hormone auxin

Plant roots are involved in many metabolic processes including water and nutrient acquisition, anchorage, propagation, storage functions and secondary metabolite synthesis. All these vital functions are controlled by a number of regulatory genes including transcription factors, protein kinases and transporters controlling local hormone content (Scheres *et al.*, 2002; Petricka *et al.*, 2012). Development of roots is regulated by different plant hormones like auxin, cytokinin, brassinosteroids, abscisic acid and gibberellin. These hormones participate in root development by integrating their signals with auxin biosynthesis, transport, and signaling (Petricka *et al.*, 2012). Among these hormones the auxin is considered to be the master regulator (Benkova *et al.*, 2003; Quint and Gray, 2006; Saini *et al.*, 2013). Auxin is involved in every aspect of plant growth and development such as embryogenesis, organogenesis and tissue patterning. It controls various stages of root development, root elongation, system architecture and tropisms (Sauer *et al.*, 2013).

During evolution, the adaptation to circumstances of Earth gravitation was essential for the developmental processes, for the formation and location of organs and organ systems. Due to the gravitational force, both terrestrial and water plants have growth axis parallel with the gravitation vector. In higher plants the direction of main roots is identical with the gravity vector (positive gravitropism); while the photosynthesizing shoots develop into an opposite direction showing the so called negative gravitropism. The first person who described the root and shoot gravitropism was Charles Darwin (Darwin, 1880). Since his basic discovery, it became clear that the sensors for the gravitational direction in plants are specialized gravity sensing cells called statocytes containing special amyloplasts (starch containing plastids) named statoliths. These specific statocyte cells are found in the root columella and stem endodermis. Mutants impaired in starch synthesis are unable to change their direction of growth according to the direction of gravitation. It was also shown that the movement of statoliths along the gravitational vector alters their interactions with actin filaments which are responsible for statoliths sensing and movement. During the movement of the statoliths, several plasma membrane localized mechanoreceptors are activated which immediately trigger several signaling and secondary messenger molecules. Among these, the release of the Ca^{2+} ions, the Ca^{2+} and calmodulin (CaM) sensing and the consequent phosphorylation cascade are the most important processes. N. Cholodny and F.W. Went described in 1926 that the gravitation sensing signal transduction process results in the asymmetric distribution of plant hormone auxin (Went, 1974). The molecular mechanisms of the amyloplast dependent gravity response and its connection with auxin signaling are largely unknown (Morita, 2010; Hashiguchi *et al.*, 2013). During vertical to horizontal rotation, auxin is transported basipetally, from the upper to the lower side of the plant roots, where elevated auxin concentration inhibits cell elongation. The cells at the upper part of the root - due to the

decreasing auxin concentration – will elongate further leading to root bending into the direction of the gravitational vector. Direction of the gravitropic responses are controlled by the asymmetric distribution of auxin, resulting in the downward and upward bending of the horizontally placed roots and shoots, respectively (Blancaflor, 2013). In the last decade, the details of the auxin transport mechanism were mostly clarified by the discovery of the PINFORMED (PIN) proteins which are the main plasma membrane located auxin transporters (Palme and Gailweiler, 1999; Feraru and Friml, 2008; Friml, 2010). However, the question how the secondary Ca^{2+} /CaM signal regulates the asymmetric auxin transport, cell elongation and finally the positive gravitropic root growth response has remained unanswered. Last year Rigó *et al.* (2013) described the functional characterization of *Arabidopsis thaliana* CRK5 protein kinase. This protein kinase is a member of the Ca^{2+} /CaM-dependent protein kinase (CDPK) related CRK family which consists of eight members with unknown functions (Harmon *et al.*, 2000; Harmon, 2003; Harper *et al.*, 2004).

The members of the CDPK superfamily

The Ser/Thr protein kinase CDPK superfamily including the CRK (CDPK-related) and the SnRK (sucrose nonfermenting1-related) protein kinases is found only in the plant kingdom. The most characterized members of this superfamily are the CDPKs and the SnRKs. The *Arabidopsis thaliana* genome analysis revealed 34 CDPKs, 8 CRKs and 38 SnRKs (The Arabidopsis Genome Initiative, 2000; Hrabak *et al.*, 2003). The three plant SnRKs subfamilies (SnRK1, SnRK2 and SnRK3) have different functions involving regulation of energy metabolism (SnRK1, Hardie *et al.*, 1998) or environmental stress reactions (SnRK2 and SnRK3, Gong *et al.*, 2002). Many SnRK2 members from several plant species are activated by hyperosmotic and salinity stresses (Umezava *et al.*, 2004) Unlike SnRKs, there is only very few information about the function of plant CRKs *in vivo*. Data concerning mainly their biochemical characterization (e.g. predicting plasma membrane localization of these proteins) has been published (Podell and Gribskov, 2004; Rigó *et al.*, 2008). Information about physiological substrates of individual CRK isoforms is scarce (Harper and Harmon, 2005) and available only for AtCRK3 for which the glutamine synthetase enzyme (AtGLN1;1) participating in leaf senescence was found to act as a substrate (Li *et al.*, 2006).

The role of the CRK5 protein kinase in gravitropic regulations

Rigó *et al.* (2013) described the functional analysis of the *Arabidopsis* CRK5 protein kinase. This protein kinase is a member of the CDPK-related CRK family which consists of eight members with unknown functions (Hrabak *et al.*, 2003). They described that the CRK5 protein kinase phosphorylates the PIN2 auxin efflux protein

which is a key transporter of auxin during *Arabidopsis* root gravitropic response. It was also shown that the CRK protein kinase has an important role in the regulation of root and shoots gravitropisms in *Arabidopsis*. At 24 hours after reorientation by 135° , the wild type (wt) roots completely bended to the direction of gravitational vector, while the roots of the T-DNA mutant allele of CRK5 protein kinase (*crk5-1*) rotated only at 90° (Fig. 1). The inflorescence of wild type (wt) *Arabidopsis* has shown normal 90° rotation, while inflorescence of the *crk5-1* mutant plants could rotate only at 45° as it was demonstrated by Rigó *et al.* (2013). As a conclusion, the inactivation of CRK5 protein kinase inhibits the normal gravitropic responses of roots and shoots in *Arabidopsis*, respectively.

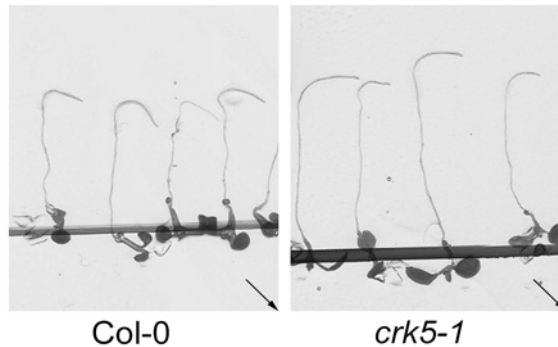


Figure 1. Gravitropic defects caused by the *crk-1* mutation. Root assays: comparison of gravitropic responses of wild type (wt) and the mutant (*crk5-1*) 7 days old seedlings. The direction of gravity is indicated by black arrow.

The reason for the impaired gravitropic responses in *crk5-1* mutant is the alteration of the auxin distribution as compared to those of the wild type. Upon gravistimulation by 135° rotation of the wild type roots, the asymmetric activation of the auxin sensor *DR5::GFP* signal in the columella root cap and epidermis cells was in four hours (Ottenschlager *et al.*, 2003; Rigó *et al.*, 2013). On contrary, in the *crk5-1* mutant, the maximum of the asymmetric localization of *DR5::GFP* expression in the root cap and epidermis was observed only at 9 h after application, thus a five hours delay in the root gravitropic response in *crk5-1*. It was also shown that *in vitro* the CRK5 protein kinase phosphorylates the PIN2 auxin efflux protein which is a key factor of the basipetal auxin transport. The results of immunolocalization of the PIN2 protein - which is the most important basipetal auxin efflux transporter – demonstrated an altered PIN2 pattern in the *crk5-1* mutant when compared to the wild type one (Rigó *et al.*, 2013). The intracellular localization of PIN2 is normally apical in epidermal and basal in cortex cells in *Arabidopsis* wild type roots and this localization is determined by a dynamic phosphorylation/dephosphorylation processes of the PIN2 protein.

Improper phosphorylation of PIN2 in the case of *crk5-1* mutant roots leads to improper auxin transport, and consequently, to a delay of the auxin transport. So, it was found a direct functional correlation between the asymmetric auxin distribution, improper PIN2 phosphorylation and gravitropic response delay in *crk5-1* roots (Rigó *et al.*, 2013).

Additional interesting information is that the intracellular localization of the green fluorescence protein (GFP) tagged CRK5 shows a U-shape facing outer lateral walls of root epidermis cells plasma membrane distribution (Fig. 2 a). Such localization pattern was only observed for boron transporters in *Arabidopsis* (Miwa *et al.*, 2007; Takanoa *et al.*, 2010). This U-shape like pattern may indicate a role for CRK5 protein kinase in the microelement and water transport regulations, too.

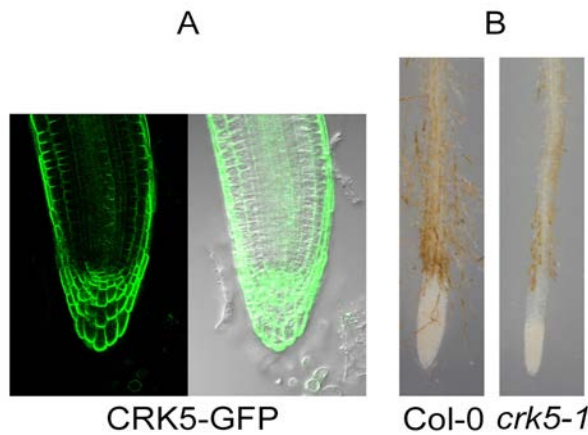


Figure 2. (A) Localization of the *CRK5::GFP* fusion protein in *Arabidopsis* root cap cells. Note U-shape like localization pattern in root tips. (B) Demonstration of hydrogen peroxide content in wild type (Col-0) and mutant (*crk5-1*) roots of 6 days old seedlings by 3, 3-diaminobenzidine (DAB) staining. The detection of hydrogen peroxide was performed according to Ren *et al.*, 2002

The role of CRK5 protein kinase in abiotic stress responses

The plant ecosystems are significantly influenced by continuous fluctuation of the climate. As a realistic tendency, a serious decrease in plant productivity is forecasted in the near future within considerable parts of Europe. The most critical environmental factors affecting plant productivity are drought, extreme cold and heat and the increase in the salt content of the soil. Therefore, the aim to increase abiotic stress adaptability of plants has become of great interest. For this reason, we continued to further investigate the functional characterization of the CRK5 addressing the study of abiotic (osmotic and oxidative) stress response features of this protein kinase. We

found that one mutant allele of the CRK5 protein kinase develops longer roots than the wild type allele under high saline concentration (Rigó *et al.*, 2011). This means that the CRK5 protein kinase may also influence the osmotic stress regulation. We also found that the hydrogen peroxide level in *crk5* mutant roots was altered compared to wild type roots (Fig. 2 b). It is very intriguing to understand how CRK5 might be a novel player of ROS signal transduction in roots. The hydrogen peroxide is a multifunctional molecule which participates in regulation of several signaling pathways including within the oxidative stress responses of plants (Petrov and Van Breusegem, 2012). We hypothesize that the CRK5 protein kinase may regulate auxin signaling through alteration of redox homeostasis of reactive oxygen species (ROS). Although the functional characterization of the CRK protein kinase is still under examination, the detailed investigations of the loss-of- function mutant of the CRK5 protein kinase revealed that this Ser/Thr type kinase may participate not only in gravitropic response but also in the regulation of the response to abiotic stress.

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REFERENCES

- Benková, E., Michniewicz, M., Sauer, M., Teichmann, T., Seifertová, D., Jürgens, G., Friml, J. (2003) Local, efflux-dependent auxin gradients as a common module for plant organ formation, *Cell*, **115**, 591-602
- Blancaflor, E.B. (2013) Regulation of plant gravity sensing and signaling by the actin cytoskeleton, *Am. J. Bot.*, **100**, 143-152
- Darwin, C. (1880) *The power of movement in plants*, London, John Murray
- Feraru, E., Friml, J. (2008) PIN Polar Targeting, *Plant Physiology*, **147**, 1553-1559
- Friml, J. (2010) Subcellular trafficking of PIN auxin efflux carriers in auxin transport, *Eur. J. Cell Biol.*, **89**, 231-235
- Gong, D.M., Guo, Y., Jagendorf, A.T., Zhu, J.K. (2002) Biochemical characterization of the *Arabidopsis* protein kinase SOS2 that functions in salt tolerance, *Plant Physiology*, **130**, 256-264
- Hardie, D.G., Carling, D., Carlson, M. (1998) The AMP-activated/SNF1 protein kinase subfamily: metabolic sensors of the eukaryotic cells?, *Annual Review Biochemistry*, **67**, 821-855
- Harmon, A.C., Gribskov, M., Harper, J.F. (2000) CDPKs - a kinase for every Ca²⁺ signal?, *Trends Plant Sci.*, **5**, 154-159
- Harmon, A.C. (2003) Calcium-regulated protein kinases of plants, *Gravity Space Biol. Bull.*, **16**, 83-90

- Harper, J.F., Breton, G., Harmon, A. (2004) Decoding Ca (2+) signals through plant protein kinases, *Annu. Rev. Plant Biol.*, **55**, 263-288
- Harper, J.F., Harmon, A.C. (2005) Plants, symbiosis and parasites: a calcium signaling connection, *Nature Reviews Molecular Cell Biology*, **6**, 555-566
- Hashiguchi, Y., Tasaka, M., Morita, M.T. (2013) Mechanism of higher plant gravity sensing, *Am. J. Bot.*, **100**, 91-100
- Hrabak, E.M., Chan, C.W., Gribskov, M., Harper, J.F., Choi, J.H., Halford, N., Kudla, J., Luan, S., Nimmo, H.G., Sussman, M.R., Thomas, M., Walker-Simmons, K., Zhu, J.K., Harmon, A.C. (2003) The *Arabidopsis* CDPK-SnRK superfamily of protein kinases, *Plant Physiology*, **132**, 666-680
- Li, R.J., Hua, W., Lu, Y.T. (2006) *Arabidopsis* cytosolic glutamine synthetase AtGLN1; 1 is a potential substrate of AtCRK3 involved in leaf senescence, *Biochemical and Biophysical Research Communication*, **342**, 119-126
- Miwa, K., Takano, J., Omori, H., Seki, M., Shinozaki, K., Fujiwara, T. (2007) Plants tolerant of high boron levels, *Science*, **318**, 1417
- Morita, M.T. (2010) Directional gravity sensing in gravitropism, *Annu. Rev. Plant Biol.*, **61**, 705-20
- Ottenschläger, I., Wolff, P., Wolverton, C., Bhalerao, R.P., Sandberg, G., Ishikawa, H., Evans, M., Palme, K. (2003) Gravity-regulated differential auxin transport from columella to lateral root cap cells, *Proc. Nat. Acad. Sci. U.S.A.*, **100**, 2987-2991
- Palme, K., Gälweiler, L. (1999) PIN-pointing the molecular basis of auxin transport, *Curr. Opin. Plant Biol.*, **2**, 375-381
- Petricka, J.J., Winter, C.M., Benfey, P.N. (2012) Control of *Arabidopsis* root development, *Ann. Rev. Plant Biol.*, **63**, 563-90
- Petrov, V.D., van Breusegem, F. (2012) Hydrogen peroxide a central hub for information flow in plant cells, *AoB PLANTS*, pls014
- Podell, S., Gribskov, M. (2004) Predicting N-terminal myristoylation sites in plant proteins, *BMC Genomics*, **5**, 37-52
- Quint, M., Gray, W.M. (2006) Auxin signaling, *Curr Opin Plant Biol*, **9**, 448-453
- Ren, D., Yang, H., Zhang, S. (2002) Cell death mediated by MAPK is associated with hydrogen peroxide production in *Arabidopsis*, *J. Biol. Chem.*, **277**, 559-565
- Rigó, G., Ayaydin F., Szabados L., Koncz C., Cséplő A. (2008) Suspension protoplasts as useful experimental tool to study localization of GFP-tagged proteins in *Arabidopsis thaliana*, *Acta Biol. Szeged.*, **52**, 59-61
- Rigó, G., Ayaydin, F., Kovács, H., Szabados, L., Cseplo, A. (2011) AtCRK5, a CDPK-related serine/threonine protein kinase may participate in regulation of salt tolerance in *Arabidopsis thaliana*, In: *Proceedings of the Conference "Protection of the Environment and the Climate": TÁMOP-Humboldt College for Environment and Climate Protection*, Palocz-Andresen, M., Nemeth, R., Szalay, D. (ed), Sopron, University of West Hungary, 70-74

- Rigó, G., Ayaydin, F., Tietz, O., Zsigmond, L., Kovacs, H., Pay, A., Salchert, K., Darula, Z., Medzihradsky, K. F., Szabados, L., Palme, K., Koncz, C., Cseplo, A. (2013) Inactivation of plasma membrane-localized CDPK-RELATED KINASE5 decelerates PIN2 exocytosis and root gravitropic response in *Arabidopsis*, *Plant Cell*, **25**,1592-1608
- Saini, S., Sharma, I., Kaur, N., Pati, P.K. (2013) Auxin: a master regulator in plant root development, *Plant Cell Rep.*, **32**, 741-757
- Sauer, M., Robert, S., Kleine-Vehn, J. (2013) Auxin: simply complicated, *J. Exp. Bot.*, **64**, 2565-2577
- Scheres, B., Benfey, P., Dolan, L. (2002) Root development, *Arabidopsis Book*, **1**, e0101
- Takano, J., Tanaka, M., Toyoda, A., Miwa, K., Kasai, K., Fujii, K., Onouchi, H., Naito, S., Fujiwara, T. (2010) Polar localization and degradation of *Arabidopsis* boron transporters through distinct trafficking pathways, *Proc. Natl. Acad. Sci.*, **107**, 5220-5225
- The *Arabidopsis* Genome Initiative (2000) Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*, *Nature*, **408**, 796-815
- Umezawa, T., Yoshida, R., Maruyama, K., Yamaguchi-Shinozaki, K., Shinozaki, K. (2004) SNRK2C, a SNF1-related protein kinase 2, improve drought tolerance by controlling stress-responsive gene expression in *Arabidopsis thaliana*, *Proc. Natl. Acad. Sci., USA*, **101**,17306-17311
- Went, F. W. (1974) Reflections and speculations, *Annu. Rev. Plant Physiol.*, **25**, 1-26