Activation of AtMPK9 through autophosphorylation

PhD Theses

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I. Introduction

Reversible protein phosphorylation at serine, threonine, and tyrosine residues is a ubiquitous mechanism of signal transduction in all eukaryotes. The mitogen-activated protein kinase (MAPK or MPK according to the plant nomenclature) family of protein kinases provide prevalent mechanisms for transducing extracellular mitogen and stress stimuli into cellular responses.

The majority of the MAPKs, the so-called conventional MAPKs, are activated within a three-tiered, canonical kinase cascade. Upon the appropriate stimuli, MAPK kinase kinases activate dual-specificity MAPK kinases, which in turn phosphorylate both the tyrosine and the threonine residue of the conserved TXY (threonine-X-tyrosine) motif of MAPKs. The conventional MAPKs are all characterized by the presence of this TXY motif in their activation loop of the catalytic domain and require phosphorylation of both phosphoacceptor amino acids to become fully active.

Another group of MAPKs, referred to as atypical MAPKs, are activated through a distinct MAPKK-independent activation mechanism. In animal cells, ERK3/4 and ERK7/8 belong to the atypical MAPKs. These ERKs show a low level of homology, but similar structural organization, possessing a unique C-terminal extension with largely unknown functions. The activation of these kinases is also ensured by different mechanism. Phosphorylation of a single serine residue within the activation motif of ERK3/4 is sufficient for their activation. Whereas, ERK7/ERK8 does not require an upstream kinase for activation, rather both residues are autophosphorylated involving an autocatalytic mechanism.

Plants feature the most extended MAPK family of all eukaryotes implying the versatility of these signalling mechanisms to regulate cellular functions. About 5% of the genomes of green plants encode protein kinases and roughly 10% of plant kinases are involved in MAPK pathways. The plant MPKs are classified into four phylogenetic groups designated A–D. Comparing the sequences at the activation loops, MPKs fall into two types: those carrying the amino acid motif TEY (A–C group) and those with a plant-specific TDY phosphorylation motif (D group). The TEY-type MPKs possess the evolutionarily conserved common docking (CD) domain, a characteristic feature of classical MAPKs. In accordance, the A–C group member kinases function in canonical MAPK pathways i.e., they are activated by pertinent MAPK kinases. Beside their TDY type of activation loop, the D group MAPKs the differ from the classical MAPKs in several other aspects too. Similar to atypical mammalian MAPKs, they have a long C-terminal extension and a diverged CD domain, wherein certain acidic residues are replaced with basic amino acids. Neither the interacting partners nor the activation mechanisms of the D-type MPKs have been described in detail previously.

In accordance with their versatile physiological functions, the hundreds of MAPK substrates identified range from transcription factors to cytoskeletal proteins and they are involved in cytoskeletal rearrangement. In plants, knowledge of MAPK substrates, specifically the phosphorylation of cytoskeletal proteins is limited.

According to the protein association studies, EB1a, EB1c, γ-tubulin and GCP4 can act as a putative substrate of AtMPK6. γ-tubulin is a subunit of microtubules; EB1 (end binding protein) is a microtubule associated protein, which is localised at the positive end of microtubules

during interphase; while the GCP4 (γ-tubulin complex protein 4) is a component of the γ-tubulin complex.

Despite the various improvements of prokaryotic cell-based protein synthesis and the use of several eukaryotic cell types for routine protein production, cell-free *in vitro* translation is a rational choice of method for alleviating the paucity of functional recombinant kinases. The significance of *in vitro* translation could be hardly overestimated since this approach opened the door for studies of molecular mechanisms of protein biosynthesis and the latter developments made it suitable for preparative scale, directed protein synthesis. Various sources of translation machinery have been explored for construction of *in vitro* translation systems, but due to its low cost and capacity for producing properly folded, multidomain eukaryotic proteins wheat germ protein extracts appear particularly suitable for cost-effective production of eukaryotic proteins. The wheat germ-based translation systems are ideal for production of milligram quantities of proteins and parallel synthesis of a panel of proteins. Furthermore, simple and rapid generation of required DNA templates for *in vitro* transcription is ensured by the availability of optimized vectors and PCR protocols; thus, transition from a gene to a purified protein can be accomplished in a few days.

II. Objectives

Although reversible protein phosphorylation is a ubiquitous mechanism of signal transduction in plants, many details of its regulation and function have not been described, yet. MPKs have central role in numerous signal transduction pathways from cell division to apoptosis. According to the amino acid composition of TXY phosphorylation motif, plant MAPKs could be divided into two subtypes: the TEY motif possessing MPKs (members of A-C group) and the plant specific TDY phosphorylation site holding MPKs (representatives of D group). While the activity regulation of A-C group member MPKs is relatively well described, studies scarcely have provided insights into the activation mechanism of D-type MPKs. We aimed to reveal the molecular details of activity regulation of AtMPK9, a D group member MPK. We planned to apply wheat germ protein extract based *in vitro* translation and transient protoplast protein overexpression as primary tools of investigations. We intended to identify the phosphorylation pattern of AtMPK9 via collaboration with protein mass spectrometry (MS) experts of the Biological Research Centre of Hungarian Academy of Sciences.

Our second objective was the determination of the putative substrates of AtMPK6 by using our previously published kinase substrate identification method.

We aimed to answer the following questions with our studies:

- Could we produce functional AtMPK9 by bacterial expression or *in vitro* translation system?
- Could we identify the phosphorylation of the TDY activation loop *in vivo* and *in vitro*?

- Which amino acids of AtMPK9 are phosphorylated?

- What mechanisms activate AtMPK9?

- Is there any MAPK kinase, which can activate the AtMPK9 in *in vivo* expression system?

- Which microtubule-associated proteins are phosphorylated by AtMPK6 *in vitro*?

III. Materials and methods

Gateway and ligation independent cloning

Construct of AtMKK4-GOF was generated by Gateway technique, which relies on the DNA recombination enzymes of the λ-bacteriophage. The *in vitro* translation vector constructs were created by ligation independent cloning, a straightforward method, which is not restricted by the occurrence of recognition sites of restriction endonucleases in the DNA sequence of fragments to be cloned.

***In vitro* mutagenesis**

TDY and LOF (loss-of-function, kinase inactive) mutants of AtMPK9 were created by the PCR-based *in vitro* mutagenesis of Stratagene with the usage of appropriate mutation containing primers.

Protoplast transient expression

Recombinant proteins of interest can be produced by cell culture derive protoplast. The cells are treated with the mixture of cellulase, hemicellulase, and pectinase to remove the cell wall. The obtained protoplasts can be transfected by the protein coding plasmid DNA and the transiently expressed proteins can be studied following an overnight incubation.

***In vitro* translational system**

Wheat germ-based, cell-free translational mixture was applied to produce preparative amount of recombinant proteins in a quick, simple and highly efficient way. The translation mixture contains the endogenous ribosomes, translational factors (initiation, elongation and termination), aminoacyl tRNA synthetases, the mRNA template, creatine phosphate, creatine kinase and amino acids. The reagent contains ATP and GTP, which are required for the activation of amino acids and the elongation of proteins, respectively. The regeneration of the ATP is provided by the creatine phosphate – creatine kinase system. The AtMPK9, AtMPK6 protein constructs and the putative substrates were produced by *in vitro* translation.

***In vitro* kinase assay**

In vitro kinase assays were performed with *in vitro* translated, affinity purified or transiently expressed, immunoaffinity purified AtMPK9 and AtMPK6. Either the *in vitro* translated, bead-bound proteins or myelin basic protein (MBP) were used as substrate in the presence of [γ-^{32}P]ATP. The phosphorylated substrates were separated on SDS-PAGE and analysed by Typhoon Phosphorimager.

III. Results

We demonstrated that the *in vitro* translated AtMPK9 possessed high activity towards MBP without addition of any MAPKK and the high *in vitro* kinase activity was accompanied by the double phosphorylation of the TDY amino acid triplet. Furthermore, *in vitro* mutagenesis of either of the phosphoacceptor amino acids resulted in loss of kinase activity implying inevitable bisphosphorylation of the TDY motif in activation AtMPK9. We also confirmed by MS analysis the phosphorylation requirement of TDY motif and demonstrated that the phosphoacceptor amino acids could be phosphorylated independently.

We failed to identify any AtMPK9 interacting endogenous protein of wheat-germ extract by MS analysis suggesting an upstream kinase independent phosphorylation of AtMPK9.

Our protoplast transient protein expression based experiments demonstrated NaCl treatment induced activation of AtMPK9. In contrast to the salt treatment, elevation of the kinase activity of AtMPK9 was negligible with any of the co-expressed constitutively active AtMAPKKs, which hints an MAPKK-independent, but autophosphorylation-dependent activation mechanism of AtMPK9. We transformed protoplasts to express wild-type and loss-of-function (WT and LOF) AtMPK9. Western blot analysis of salt treated samples with anti-p-ERK antibody demonstrated that the TDY motif of WT AtMPK9 became phosphorylated under stress conditions. In contrast, the treated and non-treated LOF AtMPK9 samples were indistinguishable; none of them were detectable with the anti-p-ERK antibody. These results

provided an additional line of evidence for autophosphorylation relying activation mechanism of AtMPK9.

Next, we treated protoplasts expressing WT AtMPK9 or LOF AtMPK9 with the phosphatase inhibitor okadaic acid. Analysis of TDY phosphorylation state with anti-p-ERK antibody showed that okadaic acid treatment led to a dramatic increase in TDY phosphorylation of WT, whereas phosphatase inhibitor treatments did not result in TDY loop phosphorylation of the kinase inactive LOF AtMPK9 mutant.

We implemented *in vitro* kinase reactions with the purified, λ phosphatase-treated and -untreated WT AtMPK9 where LOF AtMPK9 was used as substrate. According to the obtained results, the LOF AtMPK9 was not phosphorylated by the WT AtMPK9 indicating intramolecular autophosphorylation of AtMPK9.

The LC–MS/MS data showed that active and inactive forms of AtMPK9 hold different phosphorylation patterns in their C-terminal extensions. Besides the TDY motif, Ser401, Ser443 and Ser464 were also phosphorylated in the WT AtMPK9 but none of these residues were detected to be phosphorylated with the catalytically inactive LOF. Interestingly, one serine is followed by an alanine residue instead of a proline, implying that AtMPK9 is capable of phosphorylating serine residues that do not fulfil the S/T-P definition of MAPK phosphorylation sites.

Finally, we *in vitro* translated the components of cytoskeleton associated AtMPK6 protein complex to identify the putative substrates of the kinase. The *in vitro* kinase assay demonstrated that AtMPK6 exclusively phosphorylated the plant specific EB1c.

IV. Conclusions

Regulation of AtMPK9, as a representative of D group of *Arabidopsis thaliana* MPKs, possessing TDY activation loop, was previously unknown. According to our results, the *in vitro* translated AtMPK9 showed surprisingly high MBP kinase activity in radioactive kinase assay and this high kinase activity was accompanied by dual phosphorylation of the TDY motif of the activation loop as was demonstrated by immunoblotting using anti-p-ERK antibody and MS/MS analysis.

Phosphorylation of both phosphoacceptor amino acids within the TDY motif is essential for kinase activity since *in vitro* mutagenesis of either the threonine or the tyrosine residue (T185A, Y187F, T185A/Y187F) drastically reduced the activity of the *in vitro* translated AtMPK9 mutants. Non-mutated phosphoacceptor residues in the TDY mutants were phosphorylated according to MS/MS analysis in single mutants, but no phosphorylation was identified in the T185A/Y187F double mutant and the LOF kinase. These results indicate that phosphorylation of both phosphoacceptor amino acids in the TDY motif are essential for the activation of AtMPK9 by an autophosphorylation mechanism.

There was no evidence for the interaction of AtMPK9 with MAPKKs and our studies confirmed the previous results. The MAPKK-independent activation mechanism has been corroborated by *in vivo* studies using transformed *Arabidopsis* protoplasts. AtMPK9 was activated upon salt, H_2O_2 and flagellin treatment when expressed in protoplasts, whereas none of the constitutively active mutant MAPKKs were able to activate AtMPK9.

Furthermore, in contrast with WT AtMPK9, the protoplast-expressed inactive mutant version of AtMPK9 was not recognized by anti-p-ERK antibody following salt treatment, again indicating that the T-loop phosphorylation is an autocatalytic process.

It is well known that MAPKs are negatively regulated by multiple phosphatases. Okadaic acid inhibits protein phosphatase 2A (PP2A), PP2B and, to a lesser extent, PP1. We treated protoplasts expressing WT AtMPK9 with the phosphatase inhibitor okadaic acid. Treatment of WT AtMPK9 expressing cells resulted in dramatically increased phosphorylation of TDY loop and activation of WT AtMPK9, whereas the phosphorylation of TDY motif in the kinase-inactive LOF AtMPK9 was insignificant following okadaic acid treatment. Application of phosphatase inhibitors provided an additional line of evidence for autophosphorylation of AtMPK9 and its regulation by okadaic acid-sensitive phosphatases *in vivo*.

These results collectively suggest that activation of AtMPK9 does not rely on upstream kinase, but involves an autophosphorylation mechanism and signalling inputs by protein phosphatases. According to our *in vitro* kinase activity assays, this autophosphorylation is intramolecular rather than intermolecular, since the inactive LOF AtMPK9 mutant was not phosphorylated when mixed with an active AtMPK9.

The role of the disordered C-terminus of AtMPK9 is unknown and the D-type MPKs are divergent in their C-termini. LC–MS/MS analysis of the tryptic digests demonstrated that the C-terminus of the kinase is also autophosphorylated: three serines at this unstructured domain were phosphorylated, but only with the WT AtMPK9. Interestingly, one of these residues does not fulfil the minimal requirement of the MAPK

phosphorylation motif since the modified serine is not followed by a proline. Considering this putative function of the C-terminus, phosphorylation of serines in this domain of AtMPK9 is also expected to have a physiological function in regulating the kinase activity, but further work is necessary to determine the biochemical mode of its regulation.

V. List of publications

Publications directly related to the subject of the dissertation:

- **Nagy, S. K.**, Darula, Z., Kállai, B. M., Bögre, L., Bánhegyi, G., Medzihradszky, K. F., Horváth, G. V. and Mészáros, T. (2015) Activation of AtMPK9 through autophosphorylation that makes it independent of the canonical MAPK cascades. Biochem. J. **467**, 167–175. IF: 4.396
- Kohoutová, L., Kourová, H., **Nagy, S. K.**, Halada, P., Mészáros, T., Irute, M., Bögre, L. and Binarová, P. (2015) The Arabidopsis mitogen-activated protein kinase 6 is associated with γ-tubulin on microtubules, phosphorylates EB1c and maintains spindle orientation under nitrosative stress. New Phytol. in press. IF: 7.672
- **Nagy, S. K.** and Mészáros, T. (2014) In vitro translation-based protein kinase substrate identification. In Cell-Free Protein Synthesis, Methods in Molecular Biology, pp 231–243, Humana Press, New York.

Other publications:

- Szeitner, Z., Lautner, G., **Nagy, S. K.,** Gyurcsányi, R. E. and Mészáros, T. (2014) A rational approach for generating cardiac troponin I selective Spiegelmers. Chem. Commun. (Camb). **50**, 6801–4. IF: 6.834
- Kálmán, F. S., Lizák, B., **Nagy, S. K.**, Mészáros, T., Zámbó, V., Mandl, J., Csala, M. and Kereszturi, É. (2013) Natural mutations lead to enhanced proteasomal degradation of human Ncb5or, a novel flavoheme reductase. Biochimie **95**, 1403–1410. IF: 3.123