Final Report

NN-111085

The primary goal of research proposal was studying involvement of γ-tubulin in cell cycle regulation and stress responses by analysing its phosphorylation pattern and identifying its protein interacting partners. The international collaboration resulted in realization of these original aims furthermore provided previously unknown information about regulation of a D-type Arabidopsis mitogen activated kinase (AtMPK9), the mechanism of plant DNA damage response, auxin signalling pathways, and a novel approach for protein kinase substrate identification. The obtained results are discussed according to the indicated topics instead of their chronological order.

Posphorylation studies on γ-tubulin complex

The collaborating laboratory performed immunopurification from extracts of Arabidopsis cultured cells using anti-AtMPK6 antibody, identified proteins of eluted complex by LC-MALDI-TOF mass spectrometry and reproducibly found γ-tubulin as a member of the AtMPK6 complex. Since they found activated AtMPK6 in the complex, we set out to test whether γ-tubulin can be phosphorylated by MPK6. γ-tubulin was translated in vitro and an in vitro kinase assay was carried out with AtMKK4-activated AtMPK6. The results indicated that γ-tubulin is not phosphorylated by AtMPK6. The finding that γ-tubulin is not a substrate of AtMPK6 suggested that other proteins of γ-tubulin complexes might be phosphorylated by this protein kinase. A database search indicated AtGCP4 to be a plausible candidate for MAP kinase phosphorylation with a docking motif for MAPKs as well as a MAPK phosphorylation site at its N-terminus (http://elm.eu.org). We cloned and translated in vitro GCP4 protein and performed an in vitro kinase assay with activated MPK6. Similarly to γ-tubulin, phosphorylation of GCP4 protein could not be detected. The Czech collaborating laboratory demonstrated that microtubule plus ends proteins, the EB1s, were associated with microtubules polymerized from cell extracts of Arabidopsis. Furthermore, they found that AtMPK6 interacted with EB1 proteins immunopurified with anti-EB1 antibody that recognised multiple EB1 family members. To test whether plant EB1proteins were substrates for AtMPK6, we cloned and translated in vitro EB1a and EB1c and carried out a protein kinase assay with activated MPK6. We found that EB1a, similarly to γ-tubulin and GCP4, was not phosphorylated, while EB1c protein was phosphorylated by AtMPK6 under the same in vitro kinase assay conditions. These finding are in concert with computer predictions, since EB1c but not EB1a and EB1b has predicted MAP kinase phosphorylation sites and docking motif on C-terminal of the molecule (http://elm.eu.org, http://gps.biocuckoo.org). These results are essential part of the below publication which demonstrates that AtMPK6 interacts with γ-tubulin on specific subsets of mitotic microtubules during late mitosis and has a role in maintaining regular planes of cell division under stress conditions.

NEW PHYTOLOGIST 207:(4) pp. 1061-1074. (2015)

γ-tubulin protein interaction studies

On of the primary goals of project was to reveal possible regulation of the E2F/DP dimer via interaction with γ-tubulin. To this end, we created vector construct for in vitro translation of E2FA/B/C, DPA/B. We had many difficulties with the production of γ-tubulin by in vitro translation since it is prone to polymer formation. Finally, we managed to develop an optimized protocol for γ-tubulin synthesis and purification; thus, we could address the question if E2F/DP complexes interact with the tubulin. The accomplished experiments demonstrated that all three E2Fs (A/B/C) directly interact with γ-tubulin while DP1 and DP2 could bind to it via E2Fs. Furthermore, we also showed

that γ-tubulin and DPs compete for the same binding site of E2FA/B since addition of DPA and B decreased γ-tubulin binding capacity of the two activator E2F transcription factors. The collaborating partners confirmed these results in planta by immunoprecipitating the E2F- γ-tubulin complexes from GFP-E2F expressing plants by using GFP antibody. Chromatin immunoprecipitation by γ-tubulin selective antibody and sequence analysis were also implemented by the collaborating partners. These experiments demonstrated that γ-tubulin could play a role in cell cycle regulation since the sequence analysis of immunoprecipitated complex identified promoters of E2FB binding sites such as CDKB1.1, CycD3.1, and PCNA. Finally, the E2FA/B- γ-tubulin interactions were also confirmed by electron microscopy in the Czech research laboratory. These results form a manuscript in preparation.

MANUSCRIPT IN PREPARATION

Describing activity regulation of AtMPK9

During the time frame of proposal, we continued our on-going research with School of Biological Sciences, Royal Holloway University of London, which aimed at description of activity regulation of a D-type Arabidopsis mitogen activated kinase (MAPK), AtMPK9. We demonstrated that in contrary to the canonical MAPKs, the in vitro translated AtMPK9 showed high kinase activity without the addition of upstream activator MKKs. This high kinase activity was accompanied by dual phosphorylation of the TDY motif of the activation loop as it was demonstrated by immunoblot using p-ERK antibody. Phosphorylation of both phosphoacceptor amino acids within the TDY motif is essential for kinase activity since in vitro mutagenesis of either the threonine or tyrosine residue drastically reduced the activity of in vitro translated AtMPK9 mutants. The MKK independent activation mechanism has been corroborated by in vivo studies using transfected Arabidopsis protoplasts. AtMPK9 was activated upon salt treatment when expressed in protoplasts, while none of the constitutively active mutant MKKs were able to activate AtMPK9. Furthermore, contrary to wild-type AtMPK9, the protoplast-expressed kinase inactive mutant version of AtMPK9 was not recognized by p-ERK antibody following salt treatment, again indicating that the T-loop phosphorylation is an autocatalytic process. Finally, mass spectrometry analysis of in vitro translated and affinity-purified AtMPK9 complex failed to identify any interacting, endogenous protein kinases of wheat germ extract. According to our in vitro kinase activity assays, this autophosphorylation is intramolecular rather than intermolecular since the inactive LOFAtMPK9 mutant was not phosphorylated when mixed with an active AtMPK9. LC/MS/MS analysis of the tryptic digests demonstrated that the C- terminus of the kinase is also autophosphorylated: 3 serines at this unstructured domain were phosphorylated, but only in the wild-type AtMPK9. These results have been published in the “Biochemical Journal”.

BIOCHEMICAL JOURNAL 467:(1) pp. 167-175. (2015)

Functional analysis of Retinoblastoma Related in DNA damage response

In collaboration with five laboratories, we aimed at revealing the function of retinoblastoma related (RBR) in DNA damage response. The cooperating partners previously generated many needed, Arabidopsis mutant lines and using these tools implemented confocal microscopy, chromatin immunoprecipitation, and protein mass spectrometry analysis. The confocal microscopy results showed that RBR and AtBRCA1 are recruited to and co-localise with a subset of γH2AX labelled foci at DNA damage sites in an ATM and ATR dependent manner. The task of our laboratory was to test the direct interaction of RBR and AtBRCA1. To this end, we created vectors for in vitro translation of RBR, AtBRCA1, E2FA and E2FB. Using the in vitro translated proteins, we demonstrated the previously described interaction of RBR with E2F proteins. Importantly, the RBR also formed

complex with AtBRCA1 implying direct interaction of these proteins. The paper which presents these results describes that RBR -mainly known as a regulator of cell cycle and asymmetric cell division in plant meristems- is also involved in maintaining genome integrity in these growth zones through two functions, (i) protection and sensing of DNA integrity and (ii) transcriptional regulation of important DDR genes. Unfortunately, the project grant number is not indicated on the publication, even though our contribution is closely related to the original proposal.

EMBO JOURNAL 36:(9) pp. 1261-1278. (2017)

Studying histone ubiquitination in DNA damage response

The above indicated publication was followed up by studying the putative role of histone ubiquitination in plant DNA damage response (DDR). It has been described that phosphorylation of H2AX is preceded by monoubiquitination by a RING type ubiquitin ligases in mammalian cells, and we confirmed that DNA stress recruits RBR and BRCA1 to γH2AX-labelled heterochromatic foci. Therefore, we started analysing the putative ubiquitination of Arabidopsis H2AX (AT1G54690). A publication representing a coexpression network analysis from 963 microarray chips indicated that AtBRCA1, RUB1 conjugating enzyme 2 (AT2G18600) and C3H4-type RING finger protein (AT5G60250) form the same regulon hinting their involvement in DDR. In order to reveal the existence of a putative H2AX ubiquitination cascade, we inserted the coding regions of ubiquitin activating enzyme (AT2G30110), H2AX, and the above described two proteins into GST-, 6xHIS-, and biotin-tagging pEU vectors. All four proteins were successfully in vitro translated by using wheat-germ protein extract system. Ubiquitin activating enzyme activity of AT2G30110 was demonstrated by complementing the in vitro translation with biotin labelled ubiquitin and detection of ubiquitinated of AT2G30110 by western blotting with using streptavidine coupled peroxidase. We also managed to show the interaction of the in vitro translated conjugating enzyme and ligase. Since we could demonstrate neither interaction nor ubiquitination of H2AX with the applied RING finger protein, we in vitro translated H2B (AT3G09480.1), another histone which is known to be monoubiquitinylated. The protein interaction studies demonstrated that in contrary to H2AX, H2B interacts with AT5G60250. Presently, we study ubíquitination of H2B by AT5G60250.

ONGOING RESEARCH ACTIVITY

Analysing interaction of RBR with various E2F mutants

Laboratory of Molecular Regulators of Plant Growth of Biological Research Center of Hungarian Academy of Sciences has a long standing expertise in E2F dependent regulation of plant growth. They study various E2F mutant Arabidopsis lines to determine functionality of diverse protein domains of E2Fs. To identify the minimal protein domain requirements of RBR-E2F interactions, we created in vitro translation vectors possessing wild-type, transactivation and dimerization truncated mutant E2FA/B/C coding regions. All mutant variants of E2F proteins and RBR were successfully produced by in vitro translation, and the pull-down assays showed that wild-type E2Fs interact with RBR. Presently, we characterize the RBR-E2F interactions by using the mutant E2F proteins.

ONGOING RESEARCH ACTIVITY

Due to our established in vitro translation method and expertise in protein interaction studies, we had collaborative projects, which were not closely related to the aims of original research project. Most of these research activities resulted in publication. We give a short description of our responsibility in those publications whose acknowledgment indicated the OTKA support.

Method development for plant protein kinase substrate screening

The paper describes a novel approach for screening of putative substrates of plant protein kinases. The developed method relies on transient protoplast expression of putative substrate-kinase partners and detection of phosphorylated substrates by capillary isoelectric focusing (cIEF) coupled nanofluidic immunoassay. Our research group confirmed the data obtained by the presented novel method by producing activated AtMPK3 and wild-type and phosphorylation minus mutant WUS proteins with in vitro translation. The implemented in vitro kinase assay provided results were in harmony with the data of transient protoplast experiments thus demonstrated applicability of the novel assay.

BMC PLANT BIOLOGY 16:(1) Paper 204. 13 p. (2016)

Characterization of auxin transporter PIN6

This research collaboration involved 7 research group to use genetic, molecular and pharmacological approaches for characterizing the molecular mechanism(s) controlling the subcellular localization of PIN-FORMED (PIN) 6 PIN6. Depending on tissue type, PIN6 either localizes to endomembrane or plasma membrane. The paper provides evidence that this dual localization is controlled by PIN6 phosphorylation and demonstrates that PIN6 is phosphorylated by mitogen-activated protein kinases (MAPKs) MPK4 and MPK6. These phosphorylations were shown by our laboratory by in vitro kinase assays following production of protein of interest by in vitro translation. The publication proposes that PIN6 subcellular localization is under the control of developmental signals acting on tissue-specific determinants controlling PIN6-expression levels and PIN6 phosphorylation.

NEW PHYTOLOGIST 217:(4) pp. 1610-1624. (2017)

Analysis of PIN1 phosphorylation

The publication reports that the PIN auxin efflux carrier family possesses three highly conserved putative mitogen-activated protein kinase (MAPK) sites adjacent to the phosphorylation sites of the well-characterised AGC kinase PINOID. To test whether PIN1 is phosphorylated by AtMPK4/6, in vitro kinase assay was performed using in vitro translated proteins. As indicated by radiolabelled phosphate incorporation the hydrophilic loop (HL) of wild-type PIN1 was phosphorylated by MPK6. In contrast, substitution of the three MAPK phosphorylation residues with alanines (T227A/T248A/T286A) resulted in a marked diminution of phosphorylation. These results indicate that T227, T248 and T286 are MAPK phosphorylation sites on PIN1. To verify MPK6-mediated phosphorylation of these residues, we also performed Liquid Chromatograph-Mass Spectrometer/Mass Spectrometer (LC-MS/MS) analysis. Phosphorylation of T227, T248, T286 and the previously reported S337 was confirmed by the obtained data. The paper describes that these newly identified phosphorylation sites are conserved in plant PIN proteins and their phosphorylation by MAPKs leads to a partial loss of the plasma membrane localisation of PIN1. Consequently, MAPK-mediated modulation of PIN trafficking may participate in environmental adjustment of plant growth.

FEBS LETTERS 592:(1) pp. 89-102. (2018)