CREATING OF THE PLASMID CONSTRUCTION PROTEIN KINASE AtKIN10, CONJOINT WITH RFP TO STUDY THE CELLULAR LOCALIZATION OF THIS ENZYME.

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This work describes the process of cloning the gene AtKIN10 and the new plasmid construct pART7--*AtKIN10-RFP* creation, which produces the chimeric protein KIN10-RFP, for protoplasts transformation. This construction was used to study the subcellular localization of protein kinase AtKIN10, and its possible links with elements of the cytoskeleton.

Plants use a wide range of adaptive mechanisms in response to different types of stress. They live in an environment that is constantly changing. Unlike animals, plants cannot move and choose a location with optimal conditions for them, so they must constantly adjust and adapt to environmental changes. Understanding of these adaptation mechanisms can help significantly in creating plants, resistant to different types of stress. That's why, the study of mechanisms of plant resistance to abiotic and biotic stress is very topical.

Green plants are the principal solar energy converter sustaining life on Earth. Unfortunately, little is known about plants adaption to darkness, or how they adapt to unpredictable abiotic environmental stresses. Plants use a wide variety of adaptive mechanisms for the stress perception and the formation of appropriate responses.[1,2] Plants react to stressors through the formation of appropriate adaptive responses and general stress responses. With the rapid development of transkryptomik and metabolomic methods, it became clear that the formation of stress response is the result of a complex network of many signaling cascades. [3] Although some of the stress-signalling cascades have been dissected in detail, the intersection points between different types of stress as well as the identity of the signalling intermediates and key regulators remain largely unknown.[4]

It is known that protein kinase KIN10 (also known as AKIN10/At3g0190) with Arabidopsis thaliana, is one of the main regulators of transcription in response to several stress factors of different nature, such as dark, nutrient deficiency, depressed photosynthesis and others.[5-6] It was also shown that KIN10 plays the main role in the regulation of anabolic and catabolic pathways that take part in the formation of cellular response to stress factors under conditions of normal growth and development.[7] Therefore, further study of protein kinase KIN10 mechanisms is an important research area for a better understanding of the molecular nature of the formation of cellular response to the effect of various factors.

Bioinformational researches of the catalytic domain of kinases, that phosphorylate cytoskeletal elements, suggest that KIN10, may be is involved in

phosphorylation of microtubules.

For further investigation of cellular localization of protein kinase KIN10 and clarification of its possible function in phosphorylation cytoskeletal elements, AtKIN10 gene was cloned and construction pART7-KIN10-RFP was created, which produces the chimeric fusion protein KIN10-RFP, to transform protoplasts.

Materials and methods

Arabidopsis thaliana plants were germinated from seed and grew in a culture room at 22 ° C and the light length period of 14 hours a day under artificial lighted. Plants aged 6 - 8 weeks were used for further protoplasts selection from young leaves.

Isolation and transformation of protoplasts was performed as follows:

Young leaves of Arabidopsis were placed on a Petri dish with plasmolysis buffer (500 мM manitol, 10 мM CaCl2, 10 мM MES-KOH (pH 5,6)). The plant material was cut by blade into thin strips. Sliced plant material was moved to a new Petri dish with the enzyme solution for Arabidopsis (1% Cellulysin, 0,3% Macerase, 0,4 M manitol, 20 мM MES-KOH (pH 5,6), 20 mM KCl, 10 мM CaCl2, 0,1% BSA) for 3-4 hours in the dark. After that, the enzyme solution with plant material was filtered through a nylon mesh and collected in a new centrifuge tube of 50 ml volume. To 1 volume of protoplast suspension was added 0.5 volume of 200 mM CaCl2 and gently stirred. Protoplast suspension was precipitated by centrifugation at 500 - 700 r / min for 2 min and resuspended in 3 ml of chilled medium W5 (125мM $CaCl_2$, 154 мM NaCl, 2 мM MES-KOH (pH 5,6), 5 мM KCl). Protoplasts were left to incubate in an ice bath in the dark in the horizontal tubes for 30 min. Then protoplasts were precipitated by centrifugation at 500-700 rev / min. 1 minute. and resuspended in the same volume of solute MaMg (0,4 M manitol, 15 мM MgCl2, 5 мM MES-KOH (pH 5,6)). In separate tubes 10 mkl of plasmid DNA were added (10-20 mg), then 100mkl of the protoplast suspension was added to MaMg solute and gently stirred. At the last turn, to a mixture of DNA from protoplasts was added 110 ml 110 μL solute of PEG-CMS (40% polyethylene glycol (PEG 4000, Fluka), 1 0,28 M manitol, 0,14 M $Ca(NO_3)_2$). The mixture was well but gently stirred and was left to incubate in the dark at 23 ° C for 20 min. After incubation stage to the transformation mixture was slowly added incubation medium (0,6M manitol, 4мM MES-KOH (Ph 5,7), 4мM KCl, 3мM $CaCl_2$) by the following algorithm: 0.5ml, 1ml, 2ml and 3ml in the end, and was left to incubate in the dark for about 10 - 15 hours. The next day, the efficiency of transformation of protoplasts was assessed by using epifluorescent microscope.

Cloning of the gene AtKIN10 was performed with Arabidopsis thaliana cDNA. For the synthesis of cDNA from Arabidopsis thaliana seedlings was isolated total RNA. RNA isolation was performed using TRIsol-reagent (Invirogen) according to the protocol recommended by the company - the manufacturer. The quality and integrity of the isolated RNA was determined spectrophotometry and by agarose gel electrophoresis. Synthesis of cDNA was

performed in a total volume of 20 ml. For cDNA synthesis reaction 3 ml general RNA was used , 2 ml (5X First-Strand Buffer), 1ml DTT (20 mM), 1ml dNTP Mix (10 mM), 1ml (dT25), 11ml H2O. The reaction mixture was stirred on a vortex and rapidly precipitated on microcentrifuge. Microtubes with a mixture was incubated for 5 minutes at 70 ° C, then rapidly cooled on ice and added by 1 ml of RevertAid M-MuLV Reverse Transcriptase (Fermentas). Microtubes with the reaction mixture was incubated at 42 ° C for 1.5 h. After this, enzymatic reaction was inactivated by incubation at 72 ° C for 10 minutes.

The coding sequence without the stop codon AtKIN10 was amplified from synthesized cDNA by PCR by using appropriate primers containing XhoI and SmaI restriction sites (KIN10XhoI_for 5'-GCCTCGAGATGGATGGATCAGGCACAGG-3' and KIN10SmaI_rev 5'-CGCCCGGGGAGGACTCGGAGCTGAGCAA-3'). Amplification profile with Pfu DNA polymerase (Fermentas) was as follows:

initial denaturation 95 ° C for 3 min, next 35 cycles of the following amplification were performed as follows: 30 sec at 95 ° C, 30 sec at 56 ° C and 1 min at 72 ° C, final elongation - at 72 ° C for 3 min.

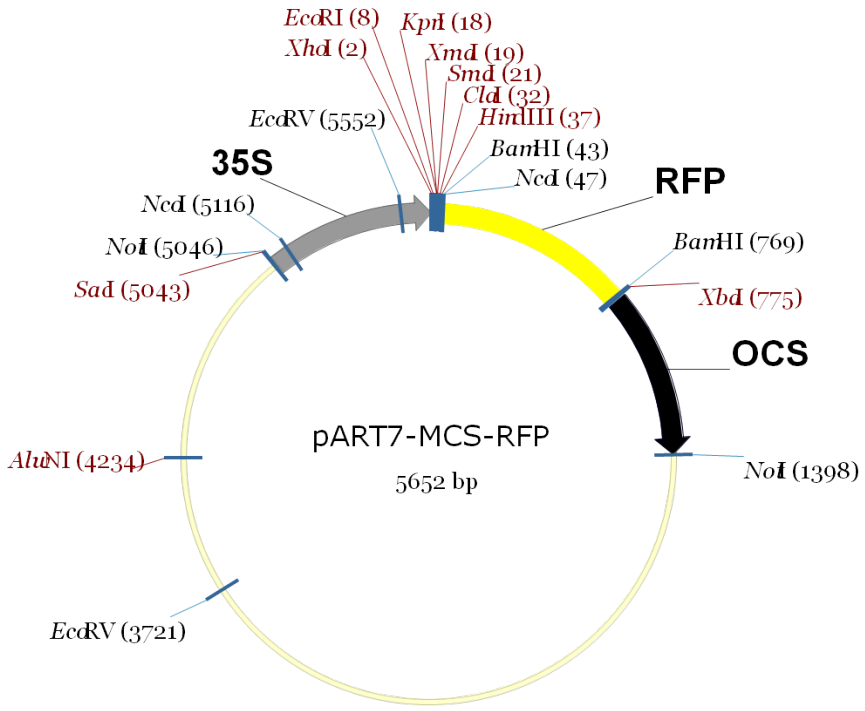

Fig.1. Binary plasmid vector pART7-RFP

After treatment with appropriate restriction enzymes and alkaline phosphatase, DNA fragments of binary vector pART7-RFP (Fig. 1.) and PCR fragment AtKIN10 were separated by electrophoresis on 1% agarose gel (Fig. 2.) and subsequently excised and excreted from the gel.

Fig.2. PCR fragment of protein kinase KIN10 on the agarose gel.

After isolation of DNA fragments from agarose gel by using specialized Kits, plasmid vector pART7-RFP, which was treated with the appropriate restriction enzymes and phosphatase, was used for the subsequent ligation reaction (Fermentas T4 DNA Ligase) with the DNA fragment-KIN10 at a ratio of 1:6.

Results

During the cloning AtKIN10 in pART7-RFP (Fig. 3). the transformation of competent cells of E. coli (dH5a) was conducted. Competent cells were transformed by ligation reaction mixture. By creating antibiotic resistance in transformed bacterial plasmid, which carries the gene for resistance to ampicillin, the initial selection of transformants was made.

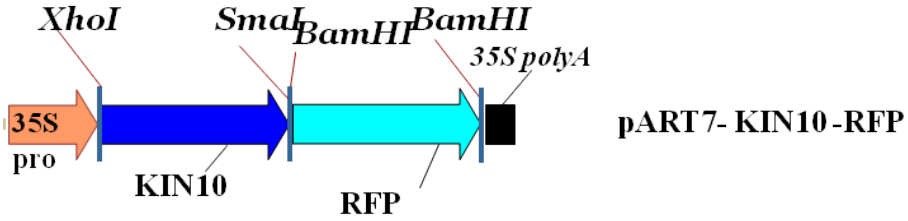

Fig.3. Plasmid construction pART7-KIN10-RFP

With the transformed colonies plasmid DNAwas excreted . Analysis and screening of plasmid DNA for the presence cDNA AtKIN10 in them was conducted . Positive clones were selected for further amplification and transformation of plant protoplasts. During the transformation of protoplasts, was featured the functionality of created plasmid construction. About 5% of the population of transformed protoplasts had fluorescence Fig.4. This approach shows that the chimeric protein kinase AtKIN10 of the RFP can be input into plant cells by transient expression of the chimeric gene.

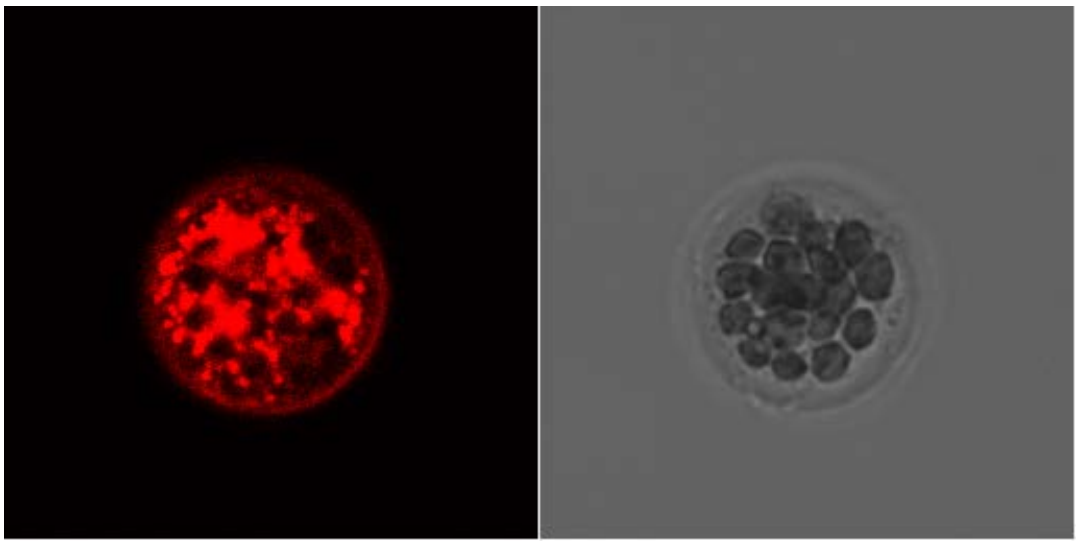

Fig.4. KIN10-RFP expression in Arabidopsis protoplasts after transformation vector in pART7-AtKIN10-RFP.

Conclusion

As a result of the research intracellular localization AtKIN10 was showed. It was found that AtKIN10 is uniformly distributed throughout the volume of the cytoplasm. Using of the created AtKIN10-RFP construction can be a useful tool for the further study of cellular signaling and of the role of this kinase in the regulation of cytoskeleton elements. The created vector construction can be used for future creation of binary vector for stable transformation of plants.

References

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