ABSTRACT

of the dissertation for the Philosophy Doctor degree (PhD) in the specialty 6D060700 - "Biology"

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Study of the roles of *Arabidopsis thaliana* Poly (ADP-ribose) polymerases in covalent modifications of DNA strand breaks termini *in vitro* and *in vivo*

General description of work. This thesis is devoted to studies of PARP-dependent covalent poly(ADP-ribosyl)ation of DNA substrates *in vitro* and *in vivo*.

The relevance of research. Plants cannot change their position in the soil, and therefore are constantly exposed to environmental and genotoxic agents, including ultraviolet and ionizing radiation. In addition, plants continuously generate oxygen radicals (ROS) as by-products of metabolic reactions, which are synthesized in relatively large quantities in mitochondria, chloroplasts, peroxisomes, and on plasma membranes. All this, first of all, acts on cellular DNA, causing its damage at the level of changes in nitrogenous bases, sugar-phosphate backbone and DNA breaks. If cells are unable to detect and repair DNA strand breaks, then this can lead to harmful consequences such as chromosomal aberrations, genomic instability and cell death. Maintaining the integrity of the genome by repairing DNA damage is important in both embryonic and somatic cells.

Poly (ADP-ribose) polymerase (PARP) catalyzes the synthesis of ADP-ribose polymers covalently attached to acceptor proteins. In this case, NAD^+ acts as a donor of ADP-ribose residues.

The genome of *Arabidopsis thaliana*, a widely used model plant organism, encodes at least three putative PARP enzymes: AtPARP1 (At4g02390), AtPARP2 (At2g31320), and AtPARP3 (At5g22470). It has been shown that plant PARPs are structurally homologous to mammalian PARP proteins. The high degree of conservatism at the amino acid sequence level between arabidopsis and mammalian enzymes suggests that PARP performs similar functions in plants as well as in animal systems. In addition to structural similarities, PARP has enzymatic activity and is functionally homologous to PARP enzymes in mammals. Both AtPARP1 and AtPARP2 are localized in the nucleus and in the presence of DNA breaks, attaches the ADP-ribose residues from NAD^+ to itself (self-modification) and to acceptor proteins *in vitro* and *in vivo*.

In contrast to mammals, little is known about PARylation in plants. It is practically unknown about poly(ADP-ribose) acceptor proteins and proteins that interact with ADP-ribose. In plants, no PARylated proteins were found, except for histones and PARP. The identification of new acceptor proteins will help to understand the regulatory function of PARylation in plant development under stress responses.

One of the best-known roles for PARPs is as a sensor for DNA damage. PARP1 binds in a PARylated form with SSB and DSB and attracts the DNA repair protein machine to the sites of DNA damage.

Talhaoui and colleagues discovered a previously unknown phenomenon of post-replicative DNA modification by PARylating the ends of DNA breaks. This reaction is catalyzed by PARP1 and PARP2 mammalian enzymes *in vitro.* It has been found that mammalian PARP enzymes can directly ADP-ribosylate the 5' and 3' ends of DNA oligonucleotides. Currently, there is no direct evidence for the presence of PARylated DNA adducts *in vivo*. However, the efficient *in vitro* PARylation of DNA breaks by purified recombinant PARP proteins indicates that this type of post-replicative DNA modification can also occur in living cells.

Given the high degree of homology between PARP of *A. thaliana* and mammals, it can be assumed that PARP of plants, as well as animal homologues, exhibit catalytic activity with respect to DNA substrates. In addition, the availability of genetic mutants of arabidopsis deficient in the PARP1 and PARP2 genes makes it possible to study PARP1 and PARP2-dependent PARylation of genomic DNA *in vivo.*

In the presented dissertation work, PARP1 and PARP2 dependent covalent poly(ADP-ribosyl)ation of DNA substrates were studied for the first time *in vitro* and *in vivo* in *Arabidopsis thaliana*.

Purpose of the study. Isolation and characterization of cDNA genes for *Arabidopsis thaliana* poly (ADP-ribose) polymerases and a detailed study of the substrate specificity and the role in the covalent modification of DNA strand breaks of recombinant enzymes *in vitro* and *in vivo*.

Research objectives:

1. Isolation and functional expression of cDNA genes encoding *AtPARP1*, *AtPARP2* and *AtPARP3 Arabidopsis thaliana* in *E. coli*.

2. Characterization of the substrate specificity of *A. thaliana* AtPARP1, AtPARP2, and AtPARP3 for oligonucleotide substrates with different configurations and structures of the 5' and 3' ends. Study of NAD^+ dependence and sensitivity of high molecular weight PAR–DNA adducts to protease and DNase treatment.

3. Study of the role of the conserved triad "histidine-tyrosine-glutamic acid (H-Y-E)" in the catalytic domain of AtPARP in the ADP-ribosylating activity of the enzyme.

4. Study of the auto (ADP)-ribosylating activity of AtPARP2.

5. Analysis of the structure and composition of PAR–DNA adducts by treatment with various enzymes and identification of their nature using MALDI-TOF MS

6. Study of Poly-(ADP-ribosyl)ation of plant genomic DNA under the action of a genotoxic agent - bleomycin.

Object of study. AtPARP, *Arabidopsis thaliana*.

Subject of study. Study of covalent poly (ADP-ribosyl)ation of the ends of DNA strand breaks catalyzed by PARP proteins of *Arabidopsis thaliana in vitro* and *in vivo*.

Research methods. Isolation of total nucleic acids from *Arabidopsis*; Agarose gel electrophoresis of nucleic acids; Isolation of mRNA; Generation of cDNA of a gene using reverse transcription reaction (ROT) and polymerase chain reaction

(PCR); Development of oligonucleotide primers using the OLYGO program; Restriction of the accumulated DNA and vector; Protein electrophoresis under denaturing conditions in the presence of sodium dodecyl sulfate; Immunoblotting with polyclonal antibodies; Affinity chromatography; Western blotting; Treatment of plants with genotoxic agents; Southern blotting.

Scientific novelty of the research.

The cDNA of the *AtPARP1*, *AtPARP2*, and *AtPARP3* genes has been isolated and characterized. Functional expression of AtPARP1, AtPARP2, and AtPARP3 with a histidine terminus in *E. coli* and purification of the recombinant protein were carried out. It has been shown for the first time that purified recombinant *A. thaliana* AtPARP1 and AtPARP2 convert DNA oligonucleotide duplexes into high molecular weight products in the presence of NAD^+ via ADP-ribosyl transferase activity. It has been shown that AtPARP2 has a high DNA ADP-ribosylation activity compared to AtPARP1, but forms shorter chains containing up to 20 ADP-ribose units. It has been shown for the first time that AtPARP3 does not exhibit the ADP-ribosylating activity typical of PARP enzymes, despite its structural similarity to mammalian PARP3 and PARP1. It has been shown for the first time that AtPARP1 preferentially modifies overhanging strand duplexes, to a lesser extent, DNA nick and gap duplexes, while AtPARP2 prefers nick and gap duplexes over overhanging strand DNA substrate. It has been established that a highly conserved glutamic acid residue in the catalytic triad AtPARP1 and AtPARP2 is required for the manifestation of poly (ADP-ribosyl)ation DNA activity. Biochemical analysis of the structure and composition of PAR-DNA adducts generated by AtPARP1 and AtPARP2 showed that, like their mammalian counterparts, AtPARP enzymes use the 5'-terminal DNA phosphates as an acceptor residue for the covalent attachment of an ADP-ribose unit for the synthesis of poly (ADP-ribose). The molecular mechanism of ADP-ribosylation of DNA catalyzed by AtPARP was revealed by identifying the degradation products of PAR-DNA using Nudix, a human NUDT16 hydrolase. The putative molecular structure of the ADP-ribose-p-DNA adduct was further confirmed by MALDI-TOF MS analysis of ADP-ribosylated DNA fragments.

The theoretical and practical significance of the work

Isolation and characterization of cDNA genes of *Arabidopsis thaliana* poly(ADP-ribose) polymerases and study of the role of *A. thaliana* poly(ADP-ribose) polymerases in the covalent modification of DNA strand breaks *in vitro* and *in vivo* has the great theoretical importance for understanding the mechanisms of repair and post-replicative modification of plant genomic DNA.

The results obtained are fundamental and can serve as a basis for the development of molecular technology to improve plant resistance to various abiotic and biotic stresses. The results can be used in testing seeds of important agricultural crops for resistance to environmental adverse factors.

The main provisions for the defense:

- *Arabidopsis thaliana* AtPARP1 and AtPARP2 poly(ADP-ribose) polymerases carry out ADP-ribosylation of terminal phosphate residues of DNA strand breaks.

- Poly(ADP-ribose) polymerase 1 preferentially PARylates the recessed DNA duplex with a preference for Rec>Nick>Gap duplexes, while Poly(ADP-ribose) polymerase 2 more efficiently PARylates nick-gap duplexes with a preference for Nick>Gap>Rec duplexes .

- Enzymes AtPARP1 and AtPARP2 use the 5'-terminal phosphates of DNA as an acceptor residue for covalent attachment of an ADP-ribose unit to synthesize the PAR polymer with the formation of a phosphodiester bond between the 5'P of DNA and C1' of ADP-ribose.

- *Arabidopsis thaliana* AtPARP proteins are structurally similar to other members of the PARP family and contain a highly conserved "H-Y-E" catalytic triad in their ART domains, in which a glutamic acid residue is required for DNA PARylation.

- AtPARP3 does not exhibit ADP-ribosylating activity.

Main results and conclusions:

1. It was found that the product of *AtPARP1*, *AtPARP2* and *AtPARP3* gene expression are globular proteins with a molecular weight of 111.2 kDa, 70.2 kDa, and 91.5 kDa, respectively.

2. It was shown for the first time that purified recombinant *A. thaliana* AtPARP1 and AtPARP2 convert DNA oligonucleotide duplexes into high molecular weight products in the presence of NAD^+ due to ADP-ribosyl transferase activity; AtPARP2 has higher ADP-ribosylation activity than AtPARP1; the dependence of the products of ADP-ribosylation of DNA on the presence and concentration of NAD^+ has been established.

3. It has been shown for the first time that AtPARP3 does not exhibit the ADP-ribosylating activity typical of PARP enzymes, despite its structural similarity to mammalian PARP3 and PARP1. Removal of the autoinhibitory and N-terminal BRCT domains for constitutive AtPARP3 hyperactivation did not result in the restoration of ADP-ribosyl transferase activity, suggesting that AtPARP3 in plants may have evolved to perform a different function.

4. It has been shown for the first time that the efficiency of the formation of PAR-DNA products catalyzed by AtPARP1 and AtPARP2 strongly depends on the structure of the DNA duplex. AtPARP1 preferentially modifies recessed strand duplexes, to a lesser extent, DNA nick and gap duplexes, while AtPARP2 prefers nick and gap duplexes over recessed DNA substrate.

5. It has been established that human and plant PARPs share a conserved H-Y-E catalytic triad: the catalytic triad of human PARP1 (H862-Y896-E988) corresponds to that of AtPARP1 (H833-Y867-E960) and AtPARP2 (H486-Y520-E614) of *Arabidopsis thaliana*. The mutant forms of the $AtPARP1^{E960K,\ E960Q,}$ and $AtPARP2^{E614K}$ enzymes exhibited DNA MARylation activity, which indicates the need for a highly conserved glutamic acid residue in the AtPARP1 and AtPARP2 catalytic triad for poly(ADP)-ribosylating DNA activity.

6. Biochemical analysis of the structure and composition of PAR-DNA adducts generated by AtPARP1 and AtPARP2 showed that, like their mammalian counterparts, AtPARP enzymes use the 5'-terminal DNA phosphates as an acceptor residue for the covalent attachment of an ADP-ribose unit for synthesis poly(ADP-

ribose). The molecular mechanism of AtPARP catalyzed ADP-ribosylation of DNA was revealed by identifying the degradation products of PAR-DNA using Nudix, human NUDT16 hydrolase. The putative molecular structure of the ADP-ribose-p-DNA adduct was further confirmed by MALDI-TOF MS analysis of ADP-ribosylated DNA fragments.

Connection with the plan of the main scientific works. The dissertation work was carried out within the framework of the scientific project AP05131478 "Study of the role of *Arabidopsis thaliana* poly (ADP-ribose) polymerases in the covalent modification of break ends in the DNA chain *in vitro* and *in vivo*" of the Ministry of Education and Science of the Republic of Kazakhstan.

Approbation of work. Materials of the dissertation work were reported: at international scientific conferences of students and young scientists "Farabi Alemi", al-Farabi KazNU, Almaty, 2018-2020; at the international conference "European Biotechnology Congress" (2018, Athens, Greece); at the international virtual conference «THE PARP FAMILY & ADP-RIBOSYLATION (Cold Spring Harbor Laboratory)» (2020 New York, USA).

Publications. The main content of the dissertation is reflected in 10 printed works, including 1 article and 2 theses in journals with a nonzero impact factor included in the Web of Science or Scopus database, 4 articles in republican scientific publications recommended by the CCSES MES RK, and 3 theses in materials of international conferences.

The structure of the thesis. The thesis is presented on 141 pages and consists of indications and abbreviations, an introduction, a literature review, materials and methods, results and discussion, a conclusion, a list of used literature sources from 464 titles of which 464 are in English; contains 5 tables, 37 figures.