The Transcriptional Response of *Arabidopsis thaliana* L. Genes *AtKu70*, *AtRAD51* and *AtRad1* to X-Ray Radiation

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Abstract: The study of influence of the fractionated and acute ionizing radiation on plants revealed that it is able to induce genomic instability. The hypothesis that transcription rate of several evolutionary conserved DNA repair genes *AtKu70*, *AtRAD51* and *AtRad1*, which keeps genome stability in cells of model plant *Arabidopsis thaliana*, changes differently depending on dose and mode of ionizing radiation exposure had been tested. Gel electrophoresis-based reverse transcription polymerase chain reaction (RT-PCR) method was used for quantifying mRNA transcription levels. The data demonstrated that mode and dose of irradiation affect transcription rate of the genes *AtKu70*, *AtRAD51* and *AtRad1*. The fractionated and acute X-ray irradiation may result in adaptive response through the induction of key DNA double-strand break (DSB) repair genes *AtKu70* and *AtRAD51*, as well as in genome instability through transcriptional activation of error-prone *AtRad1*-mediated DNA DSB repair combined with decreased expression of *AtRAD51*. In plants at doses within the range of 3-9 Gy, an adaptive influence is prevailed, but at doses of 12-21 Gy an error-prone repair of double-strand DNA damage is activated. Fractionation of dose has a significant effect on the transcription of the genes *AtKu70*, *AtRAD51* and *AtRad1* only at doses of 15 Gy and 21 Gy. Acute dose of 15 Gy activates error-prone *AtRad1*-mediated DSB repair and repressed both *AtRAD51*-dependent and *AtKu70*-dependent repair pathways, while fractionated irradiation at the same total dose induces more accurate homologous recombination and canonical non-homologous end joining of the DNA strands. In case of *A. thaliana* exposed to X-rays at dose 21 Gy, the situation is going reversed because of strong induction of the all three genome caretaker genes in leaves of acutely irradiated plants in contrast to the plants under fractionated exposure.

Key words: X-ray exposure, DNA repair, gene expression, transcription rate, genome instability.

1. Introduction

Preserving the integrity and self-identity of the genetic apparatus is tremendously important for both developing and mature organisms. It is acquiring special significance for the higher plant [1]. The sedentary plants are more susceptible to the action of the adverse genotoxic factors in comparison with actively moving animals. Restricted ability of the cells of post-embryonic tissues to repopulation and limited mitotic potential of the meristematic cells taken together probably resulted in progressive evolution of the effective repair of the lethal DNA damages in plants [1, 2]. Perhaps this explains radioresistance of seeds of the most of the higher plants. However, the

sensitivity of plants to the small doses of ionizing radiation, especially fractionated or chronic, is little different from the radiosensitivity of animal organisms [3]. The reason for these similarities and differences in the biological action of radiation on plant and animal objects remains unclear, but presumably is associated with DNA repair effectiveness [4].

According to modern concepts, basic proteins and enzymatic pathways for repair of the main radiation-induced lesions—DNA breaks are evolutionary conserved among eukaryotes. Therefore, it can be assumed that the particular modification of radiosensitivity of plant cells under the influence of radiation might be caused by the change in the transcriptional activity of genome caretaker genes in a manner resembling those found in mammalian cells [5-7].

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The main pathways for genome recovery after exogenous and endogenous DNA damage include (directly or as the one of the intermediate stages of processing of damaged DNA) repair of double-stranded and single-stranded DNA breaks with non-ligatable ends, gap filling and DNA interstrand crosslink resolution. As known, three enzymes play a key role in mentioned above repair pathways for maintaining the structural integrity of plant DNA, i.e., RAD51, a homologue of bacterial recombinase RecA; Ku70, a component of DNA-protein kinase complex and Rad1 (UVH1), endonuclease/exonuclease involved in the removal of damaged nucleotides and “nicked” ssDNA ends by Rad1/Rad10 (XPF/ERCC1) complex. Genes coding these proteins belong to the important group of caretaker genes. The effect of the acute and fractionated X-ray ionizing irradiation of the mature *Arabidopsis thaliana* plants at doses significantly smaller than median lethal dose (~150 Gy) on the expression of the genes *AtKu70*, *AtRAD51* and *AtRad1* can touch not only repair of induced single-strand DNA breaks (SSBs) and double-strand DNA breaks (DSBs), but also effectiveness of preventing of endogenous DNA damage.

The aim of this investigation was to examine dose-effect dependence of transcriptional response of caretaker genes *AtKu70*, *AtRAD51* and *AtRad1* under acute and fractionated X-ray irradiation at non-lethal doses. As a model for repeated action of radiation, a fractionation of total dose into three equal fractions had been served, with one fraction per 24 h (the sum of portions was equal to the each acute dose), at which mode the cells have repaired the majority of reparable primary-induced DNA damage before exposure to the next fraction of irradiation. As a reference gene, *AtEfla* have been used, one of the housekeeping genes, which encodes an isoform of the alpha subunit of the elongation factor-1 complex, which is responsible for the enzymatic delivery of aminoacyl tRNAs to the ribosome. Strong correlation between transcription rate of this gene and general

transcription level is common to the viable plant cells.

2. Materials and Methods

2.1 X-Ray Exposure

The relative concentration of the pre-mRNAs of genes *AtKu70*, *AtRAD51* and *AtRad1* in rosette leaves of *A. thaliana* Col-0 plants was examined after acute and fractionated irradiation in a RUM-17 X-ray equipment (National Cancer Institute, Kiev, Ukraine, 2015). Photon energies of 180 keV had been used in the experiments as a model for low linear energy transfer (LET) ionizing radiation exposure. Dose rate was 89 cGy/min and the summarizing dose ranged from 3 Gy to 21 Gy. When dose had been fractionated, the time interval between fractions was 24 h. Dosage and exposure modes were as follows: 3 Gy applied as acute versus three fractions 1 Gy + 1 Gy + 1 Gy as fractionated dose with interval 24 h; similarly, 6 Gy vs. 2 Gy + 2 Gy + 2 Gy, 9 Gy vs. 3 Gy + 3 Gy + 3 Gy, 12 Gy vs. 4 Gy + 4 Gy + 4 Gy, 15 Gy vs. 5 Gy + 5 Gy + 5 Gy and 21 Gy vs. 7 Gy + 7 Gy + 7 Gy. The doses had chosen much lower than the median lethal dose LD₅₀, but nevertheless effective in induction of phenotypic effects, such as inhibition/stimulation of growth processes and flowering. Plants were germinated on soil in open growth-chambers at temperature 20-24 °C under standard conditions of 16/8 h light/dark per day, where the light mode of illumination was on 80 µM/m²·s. Plants were exposed to X-rays at 35 d of growth before beginning of flowering. Experiments had been repeated for three times.

2.2 RNA Extraction and Reverse Transcription

Reverse transcription polymerase chain reaction (RT-PCR) was performed for detection of differences in gene's transcription rate [8]. On the template of mRNA extracted from fresh leaves 2 h after irradiation (standard time for early transcriptional response detection), a reverse transcription reaction

was carried out and then PCR has been performed. Total RNA was isolated from 500 mg tissue of 2-3 rosette leaves from each of 25 plants per dose value variant using the RIBO-Sorb kit from AmpliSens (Russian Federation). The procedure involved disruption and mechanical homogenization of fresh tissue material frozen with liquid nitrogen just before RNA isolation. Reverse transcription of total RNA for the preparation of cDNA was done using REVERTA-L kit from AmpliSens (Russian Federation) containing M-MLV reverse transcriptase and random hexamers as polymerization primers. All manipulations were carried out according to the manufacturer's protocol.

2.3 PCR Conditions

PCR (GeneAmp PCR System 2400, Applied Biosystems, USA) was performed in 20 μ L total volume, using 5 μ L cDNA as a template, 9.8 μ L ddH₂O, 2 μ L of 10 mM dNTP mix (Thermo Fisher Scientific, USA Fermentas), 0.2 μ L Taq DNA polymerase (Thermo Fisher Scientific, USA), 2 μ L of Taq DNA polymerase buffer, 0.5 μ L of 10 μ g/L each of two primers, forward and reverse. The PCR conditions were as follows: heating to 95 °C for 4 min; 35 cycles of 95 °C for 45 s, 63 °C for 45 s and 72 °C for 60 s, followed by final extension for 10 min. Target cDNA sequences were amplified with oligonucleotide primers: Fw 5'-TCCGTCCGGCTATTCCTGGC-3', Rev 5'-TGCGTCACCGGCTTTTCGCT-3' for *At1g16970* (*AtKu70*); Fw 5'-ACCCAGCACGGACCTTTCCC-3', Rev 5'-ATCGAGCTTCCGCTTCTGGC-3' for *At5g20850* (*AtRAD51*); Fw 5'-AAGCCAATGCTGTTCGCCCC-3', Rev 5'-TCTTCCGCCTGCTTTTCGGG-3' for *At5g41150* (*AtRad1*); Fw 5'-AGCCCCTTCGTCTCCCACTT-3', Rev 5'-GCCAAGTACCTCCGCCACCT-3' for *At1g07940* (*AtEfla*). The PCR with the product of reverse transcription without M-MLV reverse transcriptase as negative control was used, for

disabling false positives due to the occasional genomic DNA contamination.

2.4 Gel Densitometry and Transcription Rate Evaluation

Differences in the relative level of transcription were examined using densitometric quantification of 1% agarose and 0.005% of 10 mg/L EtBr containing gel electrophoresis images. Images were captured with a Cannon PowerShot G5 camera and then ImageJ program (National Institutes of Health, USA) was used to measure band intensities. The relative intensities of specific bands were calculated by reference to the 500-bp band from the molecular weight marker (Thermo Fisher Scientific, USA). Results were normalized by the concentration of mRNA of housekeeping gene *AtEfla*. An average value of gel images from two repeated PCR reactions per every dose point was analyzed. As a measure of the relative transcription rate of the gene, a normalized concentration of RT-PCR amplification of pre-mRNA (under identical conditions) isolated from the equivalent samples of leaves tissue was taken. The differences in the mature mRNA degradation rate did not affect this estimate.

2.5 Statistical Analysis

Obtained data then were averaged using a log-exponential transformation and analyzed using standard statistical methods. Differences between groups were tested for statistical significance using two-tailed Mann-Whitney U test and Kruskal-Wallis test (Monte Carlo method, 10,000 iterations) based on a confidence probability level $P > 0.95$. Data presented in graphs and diagrams are averages with sample errors, similar to standard deviations in the case of Gaussian-based statistics, but non-symmetric because of log-exponential transformation of the raw values. The relationship between transcription rates of studied genes was characterized by Spearman's rank correlation and Pearson partial correlation coefficients.

3. Results and Discussion

3.1 The Contrary Effects of Different Doses of X-Rays on the Activity of DNA Repair Genes

Dose and transcription rate curves reflect contrary effect of different doses of X-rays on activity of DNA repair genes. As shown in Fig. 1, acute and fractionated irradiation at dose 3 Gy induce increased expression of the genes *AtKu70* and *AtRAD51*, but transcriptional activity of *AtRad1* is not changed. However, transcription rate of a reference gene *AtEfla* after acute exposure decreased three folds. Acute irradiation within doses 9-12 Gy has lower stimulative effect on the concentration of *AtRAD51* and *AtKu70* transcripts up to the absence of stimulation of *AtRAD51* or even suppression of transcription of *AtKu70* at 12 Gy and 15 Gy. Under condition of acute exposure at dose of 21 Gy, the second maximum of expression level of genes *AtKu70*, *AtRAD51* and *AtRad1* is observed. In the case of fractionated exposure, an effect of X-rays is similar at doses lower than 9 Gy, and very differs from acute irradiation influence at doses 12-21 Gy. Fractionated irradiation generally less efficiently induces expression of repair genes *AtKu70* and *AtRAD51*, compared with acute irradiation at stimulating doses 3 Gy and 21 Gy, but more efficiently induces expression of mentioned genes at inhibitory dose 15 Gy (Fig. 1).

The most intrigued finding is silencing of the gene *Ku70*, the main factor of non-homologous end-joining during DSB repair in eukaryotic somatic cells, when plants were exposed to X-rays at acute dose 12 Gy and also to acute irradiation at 15 Gy. This combines with the activation of *Rad1* expression, which involved in highly error-prone single-strand annealing (SSA) and microhomology-mediated end joining (MMEJ) [9-11]. Also, the total expression of *AtKu70*, *AtRAD51* and *AtRad1* raised the minimum under conditions of acute radiation exposure at 15 Gy.

Analysis of obtained data confirmed that the most reliable (but in some cases insufficient) marker of

radiation-induced transcriptional response is an increased level of the *AtRAD51* mRNA. Transcriptional activation of *AtRAD51* was observed at doses from 3 Gy to 9 Gy, and absence of this effect observed at higher doses, except acute dose of 21 Gy (Fig. 1c and Fig. 2a). It should be mentioned that *RAD51* protein takes part not only in DNA repair, but also in meiotic recombination. Therefore, activation of *AtRAD51* in floral meristem cells is critical at the reproductive stage of ontogenesis [12].

Interestingly, on the border range of stimulating doses of acute irradiation (9 Gy), an additional amplicon of *AtKu70* have been detected, the respective mature mRNA ~ 845 bp in length (Fig. 1b, 9 Gy, fractionated irradiation) that probably reflect the stimulation of pre-mRNA processing. Also when 15 Gy dose was fractionated on three equal fractions with the 24 h timeout interval of exposure (the given mode of irradiation stimulates *AtKu70* and *AtRAD51* expression, but represses *AtRad1*), an additional unknown *AtEfla* amplicon length of ~420 bp has been detected (Fig. 1a). Further search in the databases of annotated nucleotide sequences gives reason to speculate on RNA interference between the *AtEfla* pre-mRNA and complementary siRNA of *Ath_wt_00191* (GenBank accession number DQ950747.1). These data indicate possible involvement of mechanisms of mRNA maturation and RNA interference in the regulation of transcriptional response to ionizing radiation.

It was found that the obtained dose and gene transcription rate curves (Figs. 2a and 2b) are in good consistent with the authors' previous observation on radiation-induced modification of growth and development of *A. thaliana*. Thus, X-ray irradiation at doses 3 Gy and 21 Gy stimulates plant stem growth, earlier flowering and pods ripening. Irradiation at dose of 12 Gy has contrarily inhibitory influence on growth and development processes of seedlings. Furthermore, inhibition of stem growth, decrease of the pods size and the average number of pods per plant, increased level

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Fig. 1 Transcription rate of the genes *AtKu70*, *AtRAD51*, *AtRad1* under acute and fractionated X-rays exposure.

Ac: acute X-ray irradiation, main amplicon; Ac*: fractionated X-ray irradiation, additional amplicon; Fr: fractionated X-ray irradiation, main amplicon; Fr*: fractionated X-ray irradiation, additional amplicon.

Arbitrary unit is equal to the transcription rate of *AtEfla* in leaves of unirradiated plants. The absence of amplicon is an evidence for gene activity, lower than threshold of the densitometric detection of specific mRNA.

Group differences at significance level: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

of sterility, decreased seed germination, phenotypic abnormalities were observed in *A. thaliana* grown from seeds of plants, which had been irradiated at dose of 12 Gy. These markers indicate genome instability in meristematic cells and gametes, as well as in embryo tissues cells in F_1 generation progeny of

irradiated plants.

Significantly, the doses of X-ray, which led to stimulation of vegetative and generative sphere of plant vital activity, coincide with doses inducing or increasing transcription rate of genes *AtRAD51* and *AtKu70*. Contrariwise, exposure to ionizing radiation

Fig. 2 Relative transcription rate of genes *AtKu70*, *AtRAD51*, *AtRad1* under different doses of X-rays exposure.

Arbitrary unit is equal to the transcription rate of *AtE1a* in leaves of unirradiated plants. The absence of amplicon is an evidence for gene activity, lower than threshold of the densitometric detection of specific mRNA.

Tables at the top right corners contain values of Spearman's rank correlation coefficient. Correlation coefficients significance level P values marked as: * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$.

at inhibitory doses reduces transcription rate of mentioned genes and simultaneously increases transcriptional activity of *AtRad1*.

It is crucial that the stimulation or inhibition of growth, growth delay, appearance of phenotypic deviations (anomalies of habitus and development) in irradiated plants and in their progeny significantly correlate with the transcription level of the genes which have been studied. In particular, the survival

and growth of irradiated plants are negatively correlated with the activity of gene *AtRad1*. Radiation-induced expression of *AtKu70* and *AtRAD51* has a stimulating effect on the growth and development of *A. thaliana* after fractionated radiation exposure. A suppression of *AtKu70* combined with low transcriptional activity of *AtRAD51* vice versa leads to decreased survival, inhibition of growth and development, formation of abnormal phenotype of

plants in post-radiation period. Therefore, relative transcription rates of the genes *AtRAD51*, *AtKu70* and *AtRad1* affect growth and development of plants, exposed to X-ray radiation, as well as appearance of abnormal phenotype of some plants in F₁ generation. Transcriptional repression of *AtKu70* and simultaneously up-regulation of *AtRad1*, involved in the error-prone DSB DNA repair pathways MMEJ and SSA, seem to be associated with radiation-induced genome instability resulting in phenotype deviations, stem growth retardation and reduced number of flowers per plant. Additionally, the data show that radiation-induced delay of flowering phenotype apparently is determined by another molecular pathway than repair of the plant genome.

Thus, it is suggested that at least some of the radiation-induced effects, such as temporal stimulation of development, radioadaptation and genome instability could be partly explained by change of transcription rate and co-expression of caretaker genes *AtKu70*, *AtRAD51* and *AtRad1* (Fig. 3). Specific transcription factors, such as suppressor of gamma response 1 (SOG1) [2, 5], and RNA-interference can be considered as potential regulators of transcription

of these genes.

3.2 Co-regulation of Transcriptional Response of Genes, Participating in Maintenance of the *A. thaliana* Genome Integrity

Obtained results demonstrate that mode and dose of irradiation significantly affect concentration of transcripts of caretaker genes *AtKu70*, *AtRAD51* and *AtRad1* in cells of *A. thaliana* plants. The dependence of the transcription rate on dose is not linear. Moreover, there is reason to describe changes in transcription of genes *AtRAD51* and *AtKu70* under influence of X-ray radiation rather as regulation and modulation of expression than simply induction or suppression. It was found that cells in tissues of rosette leaves of 5-week-old *Arabidopsis* plants express gene *AtRad1* constitutively and that this gene does not belong to the class of radiation-induced loci, such as *AtRAD51*. At the same time, gene *AtKu70* is regulated in a complex constitutive-inducible manner. It was proposed that observed changes in transcription rate are based on two different, but complementary mechanisms: (1) “switch on/switch off” regulation and (2) “turn up/turn down” gene regulation. The first

Fig. 3 Scheme of transcriptional co-regulation of *AtKu70*, *AtRAD51* and *AtRad1* under conditions of acute (a) and fractionated (b) X-ray irradiation.

one is responsible for the general activation or repression of the gene, the second for a more fine-tuning or “quantitative” setting of its expression.

The wave-like character of the transcription activity-dose dependence curves might be explained by the model of negative feedback (Fig. 3). The solid bold arrows revealed significant Pearson partial correlation coefficients between the transcriptional activity of genes, which is a marker of possible co-regulation. The dotted line shows probably spurious correlation between *AtRAD51* and *AtRad1*, caused by *AtRad1-AtKu70* and *AtRAD51-AtKu70* pair wise covariance (negative pair correlation coefficient is high, but partial correlation between transcription rates of genes *AtRAD51* and *AtRad1* is not statistically significant). The arc form arrow indicates the negative feedback.

Analysis of covariance of transcription level of mentioned repair genes under conditions of acute and fractionated radiation reveals to suggest down-regulation of *AtRad1* by *AtKu70* expression. It is likely that only mature *Ku70* protein has a suppressive activity as a negative regulator of *AtRad1*, observed only under conditions of fractionated exposure. This can be explained by assuming that “turn down” of *AtRad1* transcription takes more than 2 h after exposure for mRNA processing and translation of *AtKu70*. Negative regulation of *AtRad1* by *Ku70* protein is consistent with another data obtained for budding yeast and gives evidence of the conservativeness of this mechanism [13].

It should be emphasized that *AtRAD51* and *AtKu70* are obviously positively co-regulated. Under conditions of acute X-ray irradiation, the gene *AtRad1* is regulated independently of *AtKu70* and *AtRAD51*. Thus, it can be concluded that expression of inducible gene *AtRAD51* and partly inducible gene *AtKu70* is governed by the same transcriptional factor SOG1 [2, 5]. Regarding *AtRad1*, investigation confirms the regulation of its expression by SOG1-independent mechanism, as indicated by the observation that

transcription activity of *AtRad1* under X-ray exposure do not match by activity of the genes *AtKu70* and *AtRAD51*.

4. Conclusions

The effect of X-ray radiation at different doses, significantly lower than median lethal dose for survival of *A. thaliana* plant, is mediated by the modification of expression of caretaker genes, participating in maintenance of the structural integrity of the cell’s chromosomal apparatus. The positive or negative sign of the effect is determined by the ratio of activity of two groups of genes: those involved in the correct recovery of the structure of DNA and genes responsible for error-prone repair pathways.

X-ray radiation can lead to both radio adaptation response through the induction of transcription of *AtRAD51* and *AtKu70* genes, and genome instability through the transcriptional activation of error-prone *AtRad1*-mediated DNA DSB repair combined with decreased activity of high fidelity *AtRAD51*-depended repair. Overall, the transcriptional response of caretaker genes might play the main role in plant adaptation to the repeated exposure of genotoxic factors.

The obtained data allow to link phenotype deviations observed when doses of 3-21 Gy were used with a modification of the expression of key caretaker genes. Thus, an evidence in favor of the hypothesis about the impact of relatively small doses of radiation on the plant by a mechanism other than the direct induction of DNA damage has been received.

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