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STABILITY OF PLANT VIRUS GENOTYPE AND RISK ASSESSMENT FOR ARISING OF NOVEL VIRUS VARIANTS WITH ELEVATED EPIDEMIC POTENTIAL IN ANTHROPOGENICALLY TRANSFORMED ENVIRONMENT

Данная работа посвящена моделированию продолжительного хронического воздействия тяжелых металлов, внесенных в грунт в нетоксичных концентрациях, на развитие вирусной инфекции системно пораженных растений. Нами продемонстрировано отсутствие статистически достоверных изменений в геноме ВТМ и подтверждена стабильность генома РНК-содержащих вирусов растений под хроническим воздействием абиотических факторов антропогенно трансформированной окружающей среды.

This work has been focused on modeling long-term chronic influence of heavy metals applied into soil in subtoxic concentrations on virus infection development in systemically infected plants. Here we have demonstrated the absence of statistically significant changes in TMV genome and confirmed high stability of the genome of RNA plant viruses under chronic effect of abiotic factors of anthropogenically transformed environment.

Introduction. Chronic interplay among abiotic environmental factors and higher multicellular organisms and microorganisms including viruses is insufficiently explored. Following the examples of viruses, bacteria and plants it is known that intense short-lived effects of radioactive irradiation, UV irradiation, various chemical substances, temperature, etc. may induce genetic changes of mentioned organisms. However, the outcomes of prolonged chronic influence of abiotic environmental factors on plants and viruses are virtually unknown, – first of all, due to the difficulties in modeling such experiments. Meanwhile, the chronic type of interactions is the most important aspect in evolutionary terms, as it represents a powerful factor for organisms' variability [1].

In short, it has been established that heavy metal contamination of ecosystems favours plant virus spread [2, 3], more intense accumulation of viruses by systemically infected plants and delay in the onset of virus-specific symptoms [4]. We have also demonstrated positive correlation between the heavy metal content in soil and virus concentration in tissues of plants grown in such soil [5]. It has been revealed that bivalent cations of heavy metals may induce the appearance of the various aggregates of virus particles *in vi*tro [6]. Long-term virus passaging in heavy metal-stressed plants has been shown to affect neither virus infectivity nor the appearance of local virus-specific symptoms [5, 6].

According to the proposed hypothesis (partially confirmed by the outcomes of laboratory and small-scale field experiments), chronic effect of abiotic environmental stress factors leading to intensification of plant virus infection development may also contribute to genetic changes of RNA viruses of plants invoking further alterations of their biologic characteristics (in terms of the host range, symptoms' severity, pathogen's virulence). This, in turn, may be reflected at the population level in the form of more wide spread of virus infections in the ecosystems.

This work has been focused on modeling long-term chronic influence of heavy metals applied into soil in subtoxic concentrations on virus infection development in systemically infected plants for further assessment of probability of appearance/generation/prevalence of novel/mutated virus variants. Such variants may possess different biological characteristics posing certain threat in terms of elevated virulence or more severe symptoms, efficient spread, etc.

Materials and methods. In this work we have employed a well-studied model system "*Tobacco mosaic virus* – *Nicotianatabacum* cv. Samsun plants". Tobacco plants inoculation was done mechanically at the stage of four true leaves [7]. The following generations of experimental plants were inoculated using homogenate obtained from previous generations of plants of the respective group. Such procedure was repeated every time when inoculating plants. In such a way we have passed the virus three times (four generations of plants were used in total), and the duration of the experiment has totaled to 16 months. Plant samples from each generation were checked for virus presence and relative content via indirect ELISA [8] using specific anti-TMV polyclonal rabbit antisera. ELISA data (not shown here for the lack of space) confirmed stable virus transmission to the subsequent plant generations.

Further, we have analyzed possible molecular and evolutionary changes in virus genotype and the preservation of its genetic information to study the adaptation of the organisms to changing environmental conditions, as well as co-evolution of the parasite and its host using the 'plant-virus' model system under effect of anthropogenic stress factors.

Ten plants from the each experimental group were used for total RNA extraction using RNeasyPlantMinikit (Qiagen, UK) following the manufacturer's recommendations. RNA samples were further used for the RT-PCR amplification of cDNA (using specific primers and One-step-RT-PCR kit (Qiagen, UK) [9]) corresponding to the part of coat protein gene of *Tobacco mosaic virus* (TMV). Obtained RNA and cDNA preparations were checked using agarose gel electrophoresis [10]. Obtained cDNA preps have been sequenced and analyzed with bioinformatics packages (MEGA5 plus BioEdit) to see their mutual homology and reveal tentative differences in the sequence of CP gene of TMV induced by chronic influence of the heavy metals exerted on tobacco plants. The scheme of this experiment was generally adopted from Kearney et al. (1999) [11] and showed on Fig.1.

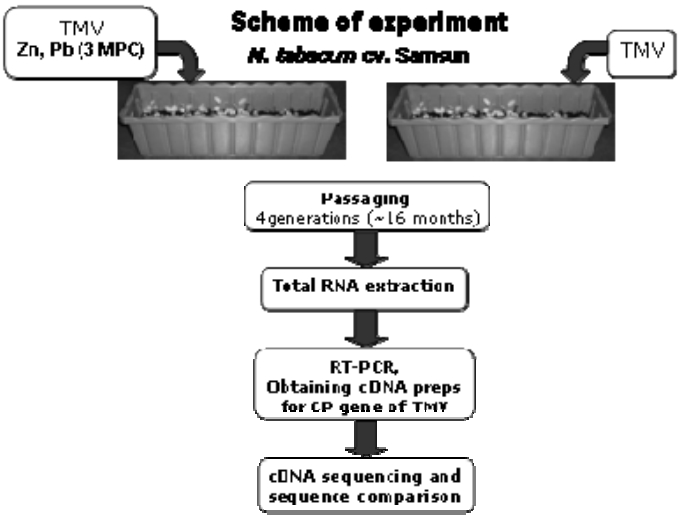

Figure 1. The experiment algorithm for long-term passaging of TMV in tobacco plants subjected to chronic effect of heavy metals

Results and discussion. Ten tissue samples of tobacco plants from each experimental group (two groups were continuously stressed with heavy metals for several generations, the other consisted of untreated virus-infected plants) were then subjected for total RNA extraction. Electrophoretic studies confirmed successful total RNA isolation from all groups of plants (Figure 2).

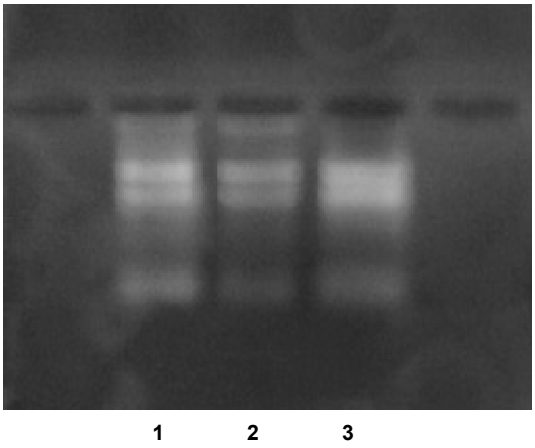

Figure 2. Electrophoretic studies of total RNA preparations isolated using RNeasyPlantMinikit (Qiagen, UK) from:
1 – TMV-infected tobacco plants not subjected to heavy metal stress ("control" plants);
2 – TMV-infected tobacco plants grown in Zn-amended soil;
3 – TMV-infected tobacco plants grown in Pb-amended soil

As can be seen from Figure 2, all obtained RNA preparations were comparable in yield and quality.

Further, total RNA preparations were used for the synthesis of corresponding cDNAs via RT-PCR using primers specific to the part of TMV U1 strain (known as reference or common strain and kindly provided by MSU, Russia). The analysis of obtained amplification products (Figure 3) confirmed that all total RNA samples (see Figure 2) (and hence, all respective plants) indeed contained recognizable (nucleotide sequence of) TMV. Obtained cDNAs were characterized with visually analogical and expected size in the gel (700 bp).

Although the technique in which the RT-PCR has been conducted cannot be considered as of quantitative nature, all measures were taken to ensure the uniformity of the samples' preparation and PCR itself. However, as can be seen from Figure 3, total RNA samples from metal-treated plants yielded more intense product band (tracks 2 and 3). The speculative nature of this argument nevertheless, has been directly confirmed by the outcomes of semiquantitative ELISA (Figure 4) demonstrating higher virus content in systemically invaded tissues of tobaccos subjected to heavy metal stress. This is in agreement with our previous results as for the same model system, as for TMV-infected tomatoes or PVX-infected potato plants [4, 12]. Hence, higher virus content in systemically infected host plants seems to be a general phenomenon when stressed with subtoxic concentrations of heavy metals.

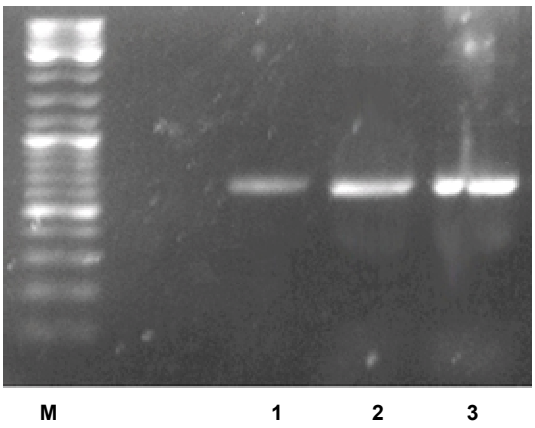

Figure 3. Electrophoretic studies of cDNA preparations obtained (One-step-RT-PCR kit (Qiagen, UK)) with total RNA preparations isolated from:
1 – TMV-infected tobacco plants not subjected to heavy metal stress ("control" plants);
2 – TMV-infected tobacco plants grown in Zn-amended soil; 3 – TMV-infected tobacco plants grown in Pb-amended soil

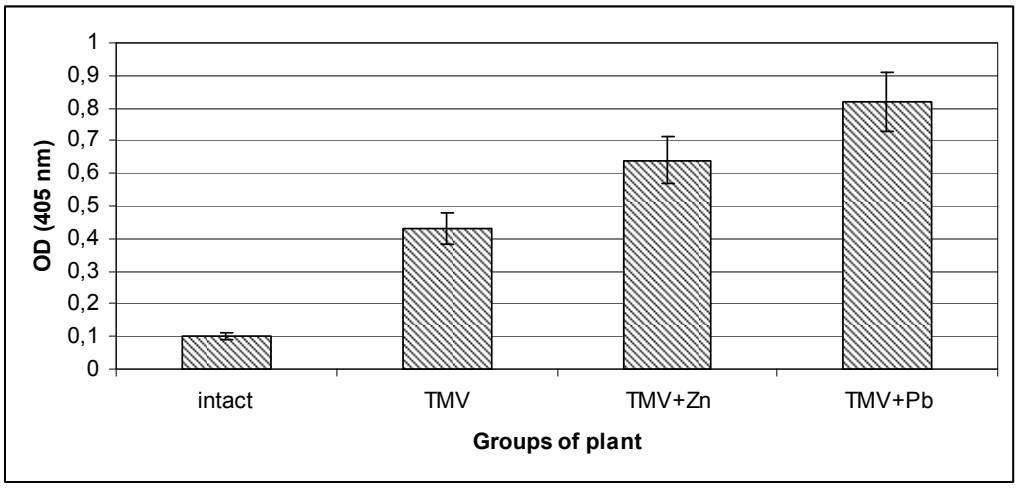

Figure 4. Semiquantitative ELISA outcomes confirming efficient TMV replication in every plant group and showing higher virus content in tobacco plants grown in meal-enriched soil

Further on we have carried out sequencing of the amplicons (see Figure 3) and their comparison. The outcomes have clearly shown nearly complete (99,9%) identity of cDNAs corresponding to the part of coat protein gene of TMV for all groups of experimental plants, including that subjected to long-term passaging under heavy metal stress (Figure 5).

Here we demonstrated the absence of statistically significant changes in the nucleotide sequence of CP gene of TMV under long-term chronic influence of heavy metals. Therefore, we have not confirmed the mutagenic effect of heavy metal in relation to TMV replicating in tobacco plants and have demonstrated high degree of evolutionary stability of RNA genome of the plant virus. In this regard we may suppose that heavy metals (at least zinc and lead, which are the most common metal pollutants found everywhere where human activity is conducted on a regular basis [1-4], and at least in these concentrations in soil) do not pose an appreciable threat in the context of sudden appearance of novel (possibly, more dangerous) isolates of plant viruses.

Of course, in this work we had several limitations among which: (1) single model system; but taking into account its wide use for various experiments, extremely wide spread (and significance) of TMV and that our previous works were mostly based on this system, we believe this choice is justified; (2) choice of heavy metals (discussed above); (3) duration of virus passaging in plants; this is the most speculative part of the story, as there is virtually no data to build upon; we deem that several generations of plants should have been enough for genetic changes (in the genome of TMV) to occur, should they appear. Considering the quasi-species nature of RNA viruses described for many of them including TMV, it is highly probable that novel virus variants may appear even under 'normal' conditions by that time, i.e. without any effects induced by additional stressors. In such case we would presumably see slight differences in one of the less conservative coat protein gene of TMV. As this is not the case, another reason may lay with the primers designed to detect conservative part of the CP gene of TMV. Should the mutations appear in a different part of the gene, it would pass unnoticed. This is taken into consideration and will be the subject for further work.

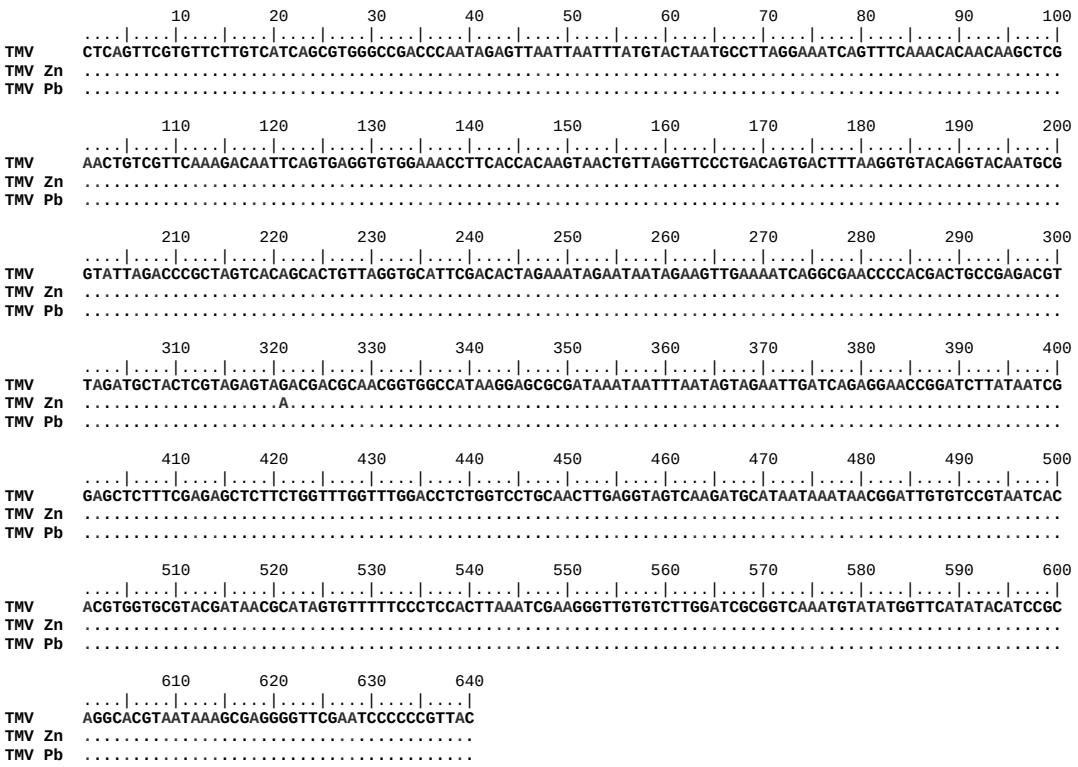

Figure 5. Comparison of sequences of cDNAs corresponding to the part of coat protein gene of TMV (aligned using MEGA 5 package and processed using BioEdit package) for different plant groups (TMV – TMV-infected plants not treated with heavy metals; TMVZn – TMV-infected plants growni nZn-amended soil; TMVPb – TMV-infected plants grownin Pb-amended soil)

Conclusions. In this work we have demonstrated the absence of statistically significant changes in the part of CP gene of TMV after long-term passaging in systemically infected host plants, *Nicotianatabacum*cv. Samsun, and have not demonstrated the mutagenic effect of heavy metals towards TMV.

Using TMV as a model virus we have confirmed high stability of the genome of RNA viruses under chronic effect of abiotic factors of anthropogenically transformed environment.

Considering the obtained experimental outcomes it is deemed highly unlikely that there is a real danger for the random and unexpected appearance of novel plant virus strains in anthropogenically stressed environment (taking into account the limitations above).

Acknowledgments. The authors are indebted to Dr. Tetyana Shevchenko (Department of Virology, Taras Shevchenko' Kyiv National University) for providing specific anti-TMV polyclonal rabbit antiserum and to Dr. Ivan Boubriak (Biochemistry Department, Oxford University) for his invaluable help with sequencing.

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Надійшла до редколегії 18.04.12