We can see from phylogenetic tree that cluster separation depends neither on geography of virus isolation (Ukrainian isolate are similar to American) nor on plant cultivar as Ukraine isolate was extracted from *Sum and substance* hosta cultivar whereas similar American isolate was selected from hosta *Striptease*.

Our data is in agreement with literuture data according to with analisys of CP i TGB1 aminoacid sequenses of all known HVX strains confirmed their monophyletic origin. Never the less the relationships between different isolates of HVX are still unclear [8].

It was also shown that the HVX-CP gene is less variable than TGB1, which suggests that CP is possibly under more stringent selection pressure than TGB1. The substitutions observed among the isolates in their respective sequences of the two genes were irregularly distributed. The 3'-proximal part of CP was the least variable region. This is probably due to its critical role in mediating essential functions such as interaction with the genomic RNA, movement and encapsidation [9,10].

Observed HVX genetic variability possibly has biological value. There are many instances where it has been shown that a single amino acid changes in the CP of a plant virus has a significant impact on virus/host interactions. Hence, additional investigations are required to determine the biological significance of the observed amino acid sequence diversity in CP and TGB1 of HVX.

Reference

1. Currier S, Lockhart BEL. Characterization of a potexvirus infecting Hosta spp // Plant Dis. – 1996. – Vol. 80 – P. 1040–1043.
2. Koláčková A., Holková L. First report of Hosta virus Xinfecting Hostain the Czech Republic // New Disease Reports – 2011 – Vol. 24 – P:9.
3. Lockhart B., Currier S. Viruses occurring in Hosta spp. in the USA // Acta Hort – 1996 – Vol.19 – P. 62–67.
4. Park M.H., Ryu K.H. Molecular evidence supporting the classification of Hosta virus X as a distinct species of the genus Potexvirus // Arch Virol – 2003 – Vol.148 – P:2039–2045.
5. Adedire O.L., Wen R.H., Windham A., Windham M., Hajimorad M.R. Hosta virus X in Hostaidentified in Tennessee, USA // Plant Pathology – 2009 – Vol.58 – P:405.
6. Koenig R., Lesemann DE., Potexvirus group. Descriptions of Plant Viruses // CMI/AAB. – England – 1978 – No. 200].
7. Tamura K., Peterson D., Peterson N. et al. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods//Mol. Biol. Evol. – 2011. – V. 28, № 10. – P. 2731–2739.
8. Fajolu O., Wen R.-H., Windham S., Windham M. T., Moulton J. K., Hajimorad M.R. Genetic variability and phylogenetic analysis of hosta virus X // Arch Virol – 2009 – Vol.154 – P.1909–1916.
9. Lewandowski D. J. Hosta Virus X // Agriculture and Natural Resources – 2008 – HYG-3069-08.
10. Tang J., Hardy C., Lebas B.S.M., Ward L.I. Presence of Hosta virus X in New Zealand // Australasian Plant Dis. – 2012 – Vol.7 – P:39–40.

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ФІЛОГЕНЕТИЧНИЙ АНАЛІЗ Х ВІРУСУ ХОСТИ ДЕТЕКТОВАНОГО НА ТЕРИТОРІЇ УКРАЇНИ

Х-вірус хости (Potexvirus, HVX) був ідентифікований методом ІФА в різних сортах хоста з колекції Національного ботанічного садаНАН України ім. М.М. Гришка. Отримано сиквенс гена білка оболонки українського ізоляту HVX. Отримана послідовність порівнювалася з відомими штамами з Генбанка. Филогенетические дерева були побудовані за допомогою методу об'єднання сусідів і максимальної правдоподібності. Ген капсидного білка українського ізоляту на 99-100% подібні за нуклеотидним і амінокислотним послідовностей з американськими изолятами.

Ключові слова: х-вірус хости, імуноферментний аналіз, генбанк.

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ФИЛОГЕНЕТИЧЕСКИЙ АНАЛИЗ Х ВИРУСА ХОСТЫ ДЕТЕКТИРУЕМОГО НА ТЕРРИТОРИИ УКРАИНЫ

Х-вирус хосты (Potexvirus, HVX) был идентифицирован методом ИФА в различных сортах хосты из коллекции Национального ботанического сада НАН Украины им. Н.Н. Гришка. Получен сиквенс гена белка оболочки украинского изолята HVX. Полученная последовательность сравнивалась с известными штаммами из генбанка. Филогенетические деревья были построены с помощью метода объединения соседей и максимального правдоподобия. Ген капсидного белка украинского изолята на 99-100% подобен по нуклеотидным и аминокислотным последовательностям с американскими изолятами.

Ключовые слова: х-вирус хосты, иммуноферментный анализ, генбанк.

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COMPLEMENTARY RESULTS OF LUMINESCENT AND TRANSMISSION ELECTRON MICROSCOPY PROVIDE STRIKING EVIDENCE OF HEAVY METAL IONS' EFFECT ON THE FORMATION OF AGGREGATES OF *TOBACCO MOSAIC VIRUS* VIRIONS BOTH *IN VITRO* AND *IN VIVO*

In vitro electron microscopy studies showed that virus particles treated with heavy metals aggregate creating either 'typical' lateral (side-to-side) aggregates of virions or star-like ones not reported previously. Luminescent light microscopy of epidermal cells of virus-infected tobacco plants demonstrated that metal treatment has led to the appearance of mostly amorphous and noncompact inclusion bodies, which were not typical for cells of plants not stressed with a heavy metal. Finally, electron microscopy of thin sections of tissues of virus-infected tobacco plants showed that metal-affected cells contained higher numbers of larger crystalline multilayered inclusions consisting of virus particles.

Key words: virus-infected tobacco, metal-affected cells.

Introduction. Previously we have shown that heavy metal contamination of ecosystems favours plant virus spread [1, 2], more intense accumulation of viruses by systemically infected plants and delay in the onset of virus-specific symptoms [3]. We have also demonstrated positive correlation between the heavy metal content in soil and virus concentration in tissues of plants grown in such soil [4]. 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 [4, 5].

According to the proposed hypothesis (partially confirmed by the outcomes of laboratory and small-scale field

experiments), chronic effect of abiotic environmental stress factors may lead to intensification of plant virus infection development. Many plausible reasons for this may be suggested including: (i) more efficient intercellular and/or systemic virus transport; (ii) more efficient virus replication/accumulation at the cell level (for instance, due to the plant defenses' failure); (iii) formation of novel virus variants tolerant to the stress exerted by the metals.

This work has been focused on the second option, as we have studied the *in vitro* and *in vivo* effects of heavy metals on *Tobacco mosaic virus* (TMV) virions and formation of virus-induced inclusion bodies in infected cells.

Materials and methods. In this work we have used a well-studied model system "*Tobacco mosaic virus* – *Nicotiana tabacum* cv. Samsun plants".

Tobacco plants were virus-inoculated mechanically in two upper leaves at the stage of four true leaves using carborundum powder [6]. The concentration of inoculum was 150 μg/ml. The development of systemic viral infection was monitored visually by symptoms, and using indirect ELISA [7] to measure virus content in the plants (not shown here for the lack of space).

Heavy metals Zn and Pb in the form of water-soluble salts (ZnSO4x7H2O and Pb(NO3)2 (Alfarus, Ukraine) have been used to simulate a soil contamination. The compounds were dissolved in sterile distilled water and added to soil separately (monometal contamination) at the 5X maximum permissible concentrations (MPC). Values of 1X MPC for the metals under study were as follows: Zn – 300 mg/kg, and Pb – 100 mg/kg [8]. The heavy metals were applied to soil 5 days prior to plant inoculation with TMV.

For thin-sectioning studies, leaf tissue was processed, thin sectioned and analyzed with a transmission electron microscope according to generally-accepted protocols [9]. For microscopy, copper grids or blends (Sigma, USA) were coated with chloroform-dissolved 0.2% polyvinyl formaldehyde (Serva, Germany), dried overnight on filter paper at room temperature, and then strengthened with carbon coating. The samples deposited onto grids were stained with 2.5% uranyl acetate and 0.02 N lead citrate (Serva, Germany), and examined using JEM-1200 ex or JEM 1400 (JEOL, Japan) transmission electron microscopes. The sections were photographed at a magnification of 5,000-60,000x.

For luminescent microscopy studies we used fresh leaf tissue of tobacco plants, acridine orange dye and green light filter following generally-accepted protocol [10]. Photographs were made under UV-light at an instrumental magnification of 630x.

For *in vitro* studies, water-soluble salts of heavy metals (ZnSO4x7H2O and Pb(NO3)2 (Alfarus, Ukraine) have been used at the range of concentrations (5-22 mM, calculated for metal). TMV was used in the distilled water suspension of 0.165 mg/ml. Salt and virus suspensions were mixed 1:1 on a glass slide and incubated at room temperature for 30 min [11]. The resulting suspension was used for transmission electron microscopy studies following the generally-accepted procedure at a magnification of 5,000-30,000x.

Results and discussion. *In vitro* studies showed that TMV particles treated with heavy metals aggregate in two ways creating either 'typical' lateral (side-to-side) aggregates of virions known from literature or star-like ones not reported previously (Figure 1, 2). Together with available data [12], the importance of bivalent metal ions for the formation of TMV-specific inclusion bodies in infected plant cells has been suggested.

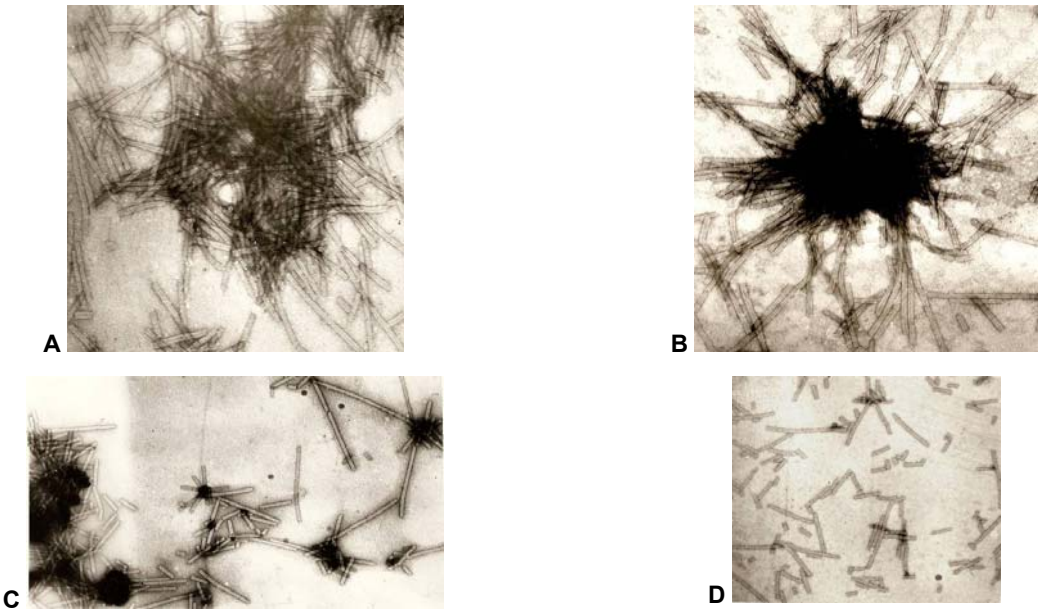

Figure 1. Electron micrograph of TMV suspension:
(A, B) incubated with Zn salt (instrumental magnification 40000x); (C) incubated with Pb salt (instrumental magnification 30000x); (D) not treated with metal (instrumental magnification 30000x)

Indeed, this has also been confirmed in part by *in vivo* luminescent analysis of epidermal cells of TMV-infected tobacco plants. Normally, TMV U1 strain induced the development of quasi-crystal intracellular inclusion bodies characterized by compactness, visual homogeneity and geometrically 'proper' shape. Zinc treatment has led to the appearance of the inclusion bodies, most of which being amorphous and noncompact, with visible 'fissures' (Figure 2).

Figure 2. Luminescent microscopy of TMV-induced inclusion bodies in epidermal hairs of leaves of infected tobacco plants (UV light, acridine orange dye, green light filter, instrumental magnification 630x):
(A) compact virus inclusion (orange) typical for plants not treated with heavy metals; (B) noncompact virus inclusion (orange) often found in metal-stressed plants

Further, we were primarily concerned with observing the progress of TMV infection in cells of systemically infected tobacco plants subjected to heavy metal stress. The point was to use electron microscopy as a proxy measure to see whether virus replicates more efficiently in a single cell, and to elucidate the consequences of dual stress at the cell level.

Intact palisade parenchyma cells of tobacco plants had a typical morphology and properly shaped nuclei, chloroplasts and mitochondria. The nucleus with nucleoli normally was located close to the centre of the cell, whereas large chloroplasts were oval in shape, had dense stroma, fully-formed thylacoids and lamellas, and typically resided at the cell periphery. Cells of tobacco plants systemically infected with TMV showed typical mild pathologies mainly involving a moderate vacuolization of the cytoplasm and deformation of chloroplasts. The nuclei of such cells were larger than those of intact cells, but the cell wall, mitochondria and cell membrane did not demonstrate significant alterations. Virus-specific crystalline inclusion bodies have been found in the cytoplasm close to the cell periphery (Figure 3).

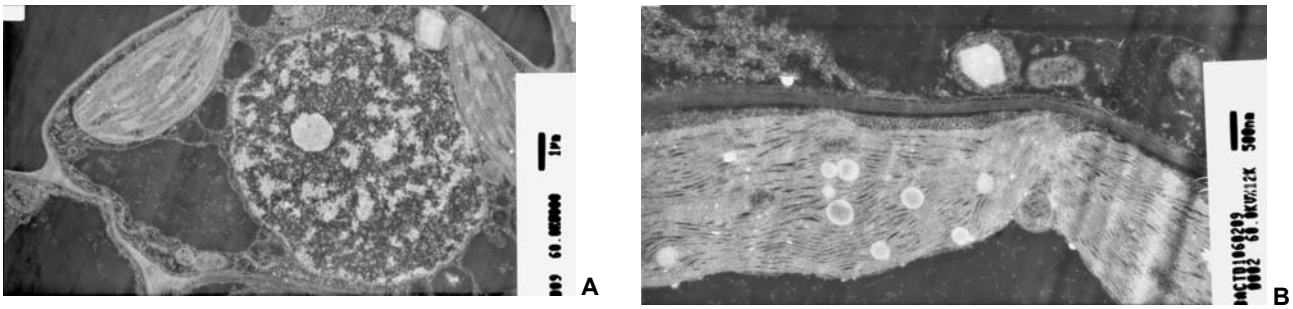

Figure 3. Electron micrographs of intact (A) and TMV-infected (B) cells of tobacco plants

Mesophyll cells of Zn-treated TMV-infected tobacco plants demonstrated higher degree of vacuolization. Their organelles (mainly, the nucleus and chloroplasts) were often significantly distorted and usually displaced close to the cell membrane. Virus particles formed numerous prominent crystalline inclusion bodies located close to the cell periphery forming layered structures composed of TMV virions. In Zn-treated cells TMV particles have been repeatedly observed in nuclei and chloroplasts.

Pb-treated plant cells also reacted on viral infection rather similarly (Fig.7). To our opinion, lead ions had more dramatic effect on the progress of TMV infection in tobacco parenchyma cells. As such, organelles (especially the nucleus and chloroplasts) were more damaged. Cells of virus-infected tobacco plants grown in Pb-enriched substrate also showed higher degree of vacuolization, distinct abnormalities of the cell wall structure (visual 'fragility') and expansion of the intercellular space. These virus-infected cells, as well as Zn-effected, also contained numerous abnormally large starch grains in their chloroplasts. It worth to note that according to microscopy data the cytoplasm of such cells contained more virions and fully-formed virus-specific crystalline inclusions as compared to the cells of Zn-treated virus-infected plants (Figure 4).

In this work we were primarily concerned with observing the progress of TMV infection in cells of systemically infected tobacco plants subjected to heavy metal stress. The point was to use electron microscopy as a proxy measure to see whether virus replicates more efficiently in a single cell, and to elucidate the consequences of dual stress at the cell level.

Figure 4. Electron micrographs of TMV-infected cells of tobacco plants grown in (A) Zn-amended or (B) Pb-amended soil

Visual estimates of the course of viral infection clearly show that heavy metals Zn or Pb potentiate virus accumulation in cells. This is in accordance with the quantitative ELISA results, when Zn and Pb induced respectively 2-times and 4,5-times increase in TMV content as compared to that in virus-infected plants not treated with heavy metals (i.e., representing virus load at the plant level). In addition, the microscopy provided evidence that metal-affected cells contained higher numbers of larger crystalline multilayered inclusions consisting of TMV particles.

In TMV-infected parenchyma cells of tobacco plants stressed by another stressor, heavy metal (Zn or Pb), similar pathological changes were observed: vacuolization, some distortion/damage of major organelles (nucleus and chloroplasts) more evident in Pb-treated cells, occurrence of large starch grains in the chloroplasts and trans-/malformation of the cell wall (Pb-specific effect). One can say that the overall cell degradation is much more severe when cells are affected by both TMV and a heavy metal.

Conclusions. In this work we have demonstrated plausible coherence of results obtained using different kinds of microscopy for TMV virions *in vitro* and viral infection *in vivo*. Presented data show that heavy metals may have direct effects on virions' aggregation and also can (indirectly) influence the formation of viral inclusions in the cell. Combined effect of viral infection and heavy metals has more serious consequences for cells of tobacco plants and may be indicative of more efficient virus replication in chronically stressed cells.

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References

1. Polischuk V.P. 2005. Effects of radioactive and chemical pollution on plant virus frequency / V.P. Polischuk, T.P. Shevchenko, I.G. Budzanivska [et al]. NATO Security through Science Series, Environmental Security: Vol.2., Equidosimetry (F. Brechignac and G. Desmet, eds.). – P.87-92.

2. Polischuk V.P., Budzanivska I.G., Shevchenko T.P. Abiotic environmental factors: effects on epidemiology of plant virus infections Cooper J.I. et al. (eds.). 2006. Virus Diseases and Crop Biosecurity. NATO Security through Science Series, Springer. – P.121-132.

3. Shevchenko O., Kamzel O., Budzanivska I., Shevchenko T., Spaar D. and Polischuk V. 2006. Heavy metal soil contamination delays the appearance of virus-induced symptoms on potato but favours virus accumulation. Archives of Phytopathology and Plant Protection, 40(6): 406-413.

4. Kamzel O.A., Petrenko S.M., Budzanivska I.G., Shevchenko O.V. 2007. Correlation between the zinc content and TMV replication efficiency in tobacco plants. The Bulletin of Taras Shevchenko' Kyiv National University. Biology Series, 49-50: 100-101.

5. Iutynska O.A., Bysov A.S., Shevchenko O.V. Reproduction and systemic transport of TMV in plants under modeled stress // Agroecology Journal. – Spec. issue. – 2011. – P.85-88.

6. Hill S.A. Methods in plant virology. Oxford: Alden Press, 1984. 167 p.

7. Crowther J.R. ELISA. Theory and practice. N.Y.: Humana Press, 1995. – 96 p.

8. Kabbata-Pendias, A. & Pendias. H. (1986) Trace Elements in Soils and Plants, Boca Raton, Florida: CRC press, Inc., 349 p.

9. Mendgen K. Electron Microscopy of Plant Pathogens. Edited by D.E. Lesemann. – Springer, 1991. – 336 p.

10. Konstantinova-Shlesinger, M.A., (Ed.) (1961) Luminescent analysis, Moscow: State Publisher of physical-mathematical literature, 405 p.

11. Nedoluzhko, A. & Douglas, T., (2001) Ordered association of tobacco mosaic virus in the presence of divalent metal ions. J. of Inorganic Chem. 84, pp.233-240.

12. Durham, A.C.H. & Hendry, D.A., (1977) Cation binding by tobacco mosaic virus. Virology 77, pp.510-519.

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ВЗАЄМОДОПОВНЮЮЧІ РЕЗУЛЬТАТИ ЛЮМІНЕСЦЕНТНОЇ ТА ТРАНСМІСІЙНОЇ ЕЛЕКТРОННОЇ МІКРОСКОПІЇ СВІДЧАТЬ ПРО ВПЛИВ ІОНІВ ВАЖКИХ МЕТАЛІВ НА УТВОРЕННЯ АГРЕГАТІВ ВІРІОНІВ ВІРУСУ ТЮТЮНОВОЇ МОЗАЇКИ ЯК В УМОВАХ *IN VITRO*, ТАК І *IN VIVO*

Електронномікроскопічні дослідження в умовах in vitro показали, що оброблені важкими металами вірусні частки агрегують у "типові" структури, в яких віріони з'єднані латерально, та утворюють зіркоподібні структури, неописані раніше. Результати світлової люмінесцентної мікроскопії продемонстрували, що під впливом важких металів в епідермальних клітинах вірус-інфікованих рослин тютюну утворюються головним чином аморфні некомпактні вірусні включення, нетипові для клітин рослин, які не підлягали впливу стресу, спричиненого важкими металами. Електронна мікроскопія ультратонких зрізів тканин вірус-інфікованих рослин тютюну показала, що важкі метали викликали появу більшої кількості великих багатошарових включень, які складалися з вірусних часток.

Ключові слова: люмінесцентна мікроскопія, мікроскопія ультратонких зрізів, важкі метали, вірус-інфіковані рослини тютюну, вірусні включення.

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ВЗАИМОДОПОЛНЯЮЩИЕ РЕЗУЛЬТАТЫ ЛЮМИНЕСЦЕНТНОЙ И ТРАНСМИССИВНОЙ ЭЛЕКТРОННОЙ МИКРОСКОПИИ СВИДЕТЕЛЬСТВУЮТ О ВЛИЯНИИ ИОНОВ ТЯЖЕЛЫХ МЕТАЛЛОВ НА ОБРАЗОВАНИЕ АГРЕГАТОВ ВИРИОНОВ ВИРУСА ТАБАЧНОЙ МОЗАКИ КАК В УСЛОВИЯХ *IN VITRO*, ТАК И *IN VIVO*

Электронномикроскопические исследования в условиях in vitro показали, что обработанные тяжелыми металлами вирусные частицы агрегируют в "типичные" структуры, в которых вирионы соединены латерально, и образуют звездоподобные структуры, описаные ранее. Результаты световой люминесцентной микроскопии показали, что под влиянием тяжелых металлов в эпидермальных клетках вирус-инфицированных растений табака образуются главным образом аморфные некомпактные вирусные включения, нетипичные для клеток растений, которые не подлежали воздействию стресса, вызванного тяжелыми металлами. Электронная микроскопия ультратонких срезов тканей вирус-инфицированных растений табака показала, что тяжелые металлы привели большему количеству многослойных включений, которые состояли из вирусных частиц.

Ключевые слова: люминесцентная микроскопия, микроскопия ультратонких срезов, тяжелые металлы, вирус-инфицированные растения табака, вирусные включения.