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ULTRASTRUCTURAL CHARACTERISTICS OF MESOPHYLL CELLS OF TRANSGENIC TOBACCO PLANTS WITH HUMAN INTERFERON ALPHA 2B GENE INFECTED BY TOBACCO MOSAIC VIRUS

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Summary: Tobacco plants resistant to the antibiotic kanamycin were obtained using the Agrobacterium-mediated transformation method. The transgenic nature of these plants was confirmed by PCR analysis using primers specific to genes *ifn-α2b* and *nptII*. RT-PCR analysis demonstrated that the human *ifn-α2b* gene was transcribed in the plants obtained after transformation. Extracts from transgenic plants possessed interferon-like antiviral activity in EPT cells infected by vesicular stomatitis virus (3.274-6312 IU/g of fresh weight, or $1.553 * 10^{-3}$ - $3.009 * 10^{-3}$ IU/mg of total soluble protein). Transgenic plants were infected by tobacco mosaic virus. Electron microscopic analysis of cells of transgenic and control tobacco plants showed no significant differences in their ultrastructure.

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Keywords: Transgenic plants; *Nicotiana tabacum*; interferon; tobacco mosaic virus.

Abbreviations: CP – capsid protein; TMV – tobacco mosaic virus; EPT – established piglets testicular cells; VSV – vesicular stomatitis virus.

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INTRODUCTION

Plants viral diseases are one of the most important factors that lead to a significant reduction in crop yield. Therefore, the creation of resistant plants remains a relevant area of genetic research. These plants can be created by conventional breeding or by genetic engineering methods and can be resistant to one or more viruses (Bruening, 2006).

However, resistant plants are mainly produced using genetic engineering methods. Viral genes such as capsid protein (CP) genes or virus transport proteins can be used for construction of virus resistant transgenic plants. For example, tobacco plants with expressed CP of tobacco mosaic virus (TMV) were resistant to tobacco Potyviridae (Abel et al., 1986; Savenkov and Valkonen, 2001; Prins et al., 2008). Also, sweet potato plants with *hpt* gene resistant to sweet potato feathery mottle virus and clover plants resistant to the alfalfa mosaic virus (Panter et al., 2012) were created using genetic engineering methods.

There is another possible approach to create plants resistant to viruses using a non-viral gene. In the 90's of the 20th century, it was shown that the transfer of the human interferon gene into the genome of plants may increase plant resistance to plant virus infection (Rivkin et al., 1995). This approach suggested the possibility of presence in plants of interferon response, similar to that in mammals (Silverman, 2007). But today there is evidence that the transfer into plants of only one gene encoding interferon is not sufficient for acquisition of resistance to viruses (Mitra et al., 1996). RNase L and protein kinase activity are necessary for efficient operation of interferon protection system in plants (Lim et al., 2002; Silverman, 2007). The basic work related to constriction of transgenic plants resistant to viruses which synthesize human interferon was carried out in the late 90's. Nevertheless, the question about the possibility of the use of interferon to protect plants from plant viruses and the impact of viruses on transgenic cells still remains open.

Typically, the effect of viral infection is determined by the degree of symptomatic lesion of the plant. However, ultrastructural changes in the cells of virus-infected plants are also observed. For example, in tobacco mesophyll cells TMV virions are localized in the cytoplasm and the vacuole, while plastids and the nucleus were virus free. Infected TMV plants have thickened cell wall, increased quantity of starch grains and mitochondria (Xu and Feng, 1998). It was shown that TMV affected plasmodesma, expanding them to improve transport of viral RNA (Reichler and Beachy, 1998). An increase of lysosomes, dictiosomes and multivesicular bodies was observed as plants responded to viral infection in TMV-infected plants (Reunov et al., 2006). In this way plant viral infection can be regarded as biotic stress and the ultrastructural changes in the cells of infected plants can be regarded as a response to this stress. It also should be noted that viral infected transformed plants are subjected to double stress including the contact with Agrobacteria in the transformation process and the contact with the virus. Therefore, the ultrastructure of these plants may have singularities. In addition, the discussion about the possible negative influence of genetic transformation on the plant, including the effect on the ultrastructure

of the transformed cells, still goes on. The influence of the viral infection on the ultrastructure of the cells of transgenic plants is still not understood.

The aim of this work was to study the ultrastructure of tobacco cells transformed with the human *ifn-α2b* gene as well as the ultrastructure of transgenic plants infected with the tobacco mosaic virus.

MATERIALS AND METHODS

Nicotiana tabacum L. cv Petit Havana plants were used for genetic transformation by *Agrobacterium tumefacience* GV3101 strain, which carried the pCB124 vector with the human *ifn-α2b* gene and the selective *nptII* gene. Explants were co-cultivated with the bacterial suspension for 30 min, then they were transferred to MS medium (Murashige and Skoog, 1962) supplemented with 2.5 mg/dm^3 BAP, 0.05 mg/dm^3 NAA, 500 mg/dm^3 cefotaxime and 25 mg/dm^3 kanamycin for plant regeneration under selective conditions. Green plants that were obtained on a selective medium were transferred to 1/2MS (MS medium with half-reduced macrosalt content) supplemented with 25 mg / dm^3 kanamycin and 500 mg/ dm^3 cefotaxime.

Plant leaves were weighed and used for extracts preparation in phosphate buffer (pH 7.4, 1% dodecyl sulfate and 1 mM PMSF).

The antiviral activity of plant extracts was carried out by inhibition of cytopathic effect of the test virus in EPT culture obtained in the virology laboratory of the Kolomiychenko Institute of Otolaryngology Academy of Medical Sciences of Ukraine. Indiana strain vesicular stomatitis virus from the Museum of the Virology Department of Shupik NMAPGE was used. Recombinant human interferon alpha-2b with 100 000 IU/ml antiviral activity (Biopharma, Ukraine) was used as the reference product. The study was conducted using a standard micromethod in Sarstedt (Germany) 96-well culture plates and RPMI-1640 culture medium. Intact cell monolayers (K-) and 100 infectious doses of the test virus with full manifestation of the cytopathic effect of test virus (K +) were used as the control.

Wild-type TMV that was obtained from the Department of Virology of the SAC "Institute of Biology" Taras Shevchenko Kyiv National University was used to infect the plants. Infection was carried by virus mechanical inoculation in second and third tiers tobacco leaves. Cuttings from the middle part of the upper leaves (diameter 2 mm) of plants were fixed in a 3% glutaraldehyde solution (Merck, Germany) and the standard method of Klymchuk et al. (2001) for electron microscopic examination was used. Transverse sections of silver-gold (60 ± 10 nm thick) on leaf plates received ultramicrotom LKB 8800, painted lead citrate (Reynolds, 1963) and examined under a transmission electron microscope JEM-I230 (JEOL, Tokyo, Japan) at accelerating voltage of 100 kV. Morphometric analysis was performed using the UTHSCSA Image Tool 3.0, using an extensive range of electron microscopic images.

RESULTS AND DISCUSSION

Tobacco plants resistant to the antibiotic kanamycin were obtained using the Agrobacterium-mediated transformation method. The transgenic

nature of these plants was confirmed by PCR analyses (using primers specific to genes *ifn-α2b* and *nptII*). The transcription of human *ifn-α2b* gene was confirmed by RT-PCR analyses. Analysis of antiviral interferon-like activity of plant extracts was performed using EPT cell culture infected by VSV. It was found that the extracts demonstrated interferon-like activity up to 3.274-6312 IU/g fresh weight or $1.553*10^{-3}$ – $3.009*10^{-3}$ IU/mg total soluble protein (Table 1). Thus, extracts from transgenic tobacco plants with human *ifn-α2b* gene obtained using the Agrobacterium-mediated transformation method demonstrated antiviral interferon-like activity on EPT cells infected by VSV.

These plants were infected by TMV. Morphological changes such as deformation of leaves and chlorotic areas were detected in the transgenic and control plants 9-10 days after the virus inoculation. RT-PCR analyses (Fig. 1) confirmed the presence of viral RNA in the transgenic and control plants, which were infected by TMV. Thus, despite the fact that the extracts of transgenic plants possessed antiviral activity on cells EPT infected by VSV, these plants were infected by TMV.

The electron microscopic study, which included analysis of ultrastructural organization of mesophyll cells was carried out on leaf cross sections. A morphometric analysis of cells and organelles was carried out at their diametric sections. The following groups of plants were studied: wild-type plants (control); control plants infected by TMV; transgenic plants with the human *ifn-α2b* gene infected by TMV, and transgenic plants with the human *ifn-α2b* gene that were not infected.

Ultrastructural differences between cells of wild-type and transgenic plants with *ifn- α2b* were not found. (Fig 2A, C). 12-13 chloroplasts were observed in diametrically cut cells. They were located mainly at the periphery, closely to the cytoplasmic membrane. Chloroplast stroma was characterized by a poorly developed internal membrane system with stroma thylakoids, grana and great-sized starch granules. Mitochondria in cells of wild-type and transgenic plants were mostly round, oval, rarely elongated. Free ribosomes, polyribosomes, dictiosomes and a small number of vesicles were found in the hyaloplasm of mesophyll cells. The cell walls of adjacent mesophyll cells were punctured by plasmodesmata. The average thickness of cell walls in the areas of localization of plasmodesmata increased from 220-240 to 300-320 nm without any significant changes in the structural organization of plasmodesmata.

Table 1. Antiviral activity of extracts of tobacco plants with human *inf α2b* gene tested on ETP cells infected by VSV.

Plant	Vector	Sample	IU/mg total soluble protein	IU/g fresh weight
N. tabacum	pCB 124	Leaves	$1.553*10^{-3}$	3274
N. tabacum	pCB 124	Leaves	$3.009*10^{-3}$	6312
N. tabacum	pCB 124	Leaves	$1.614*10^{-3}$	5000
N. tabacum control	-	Leaves	0	0

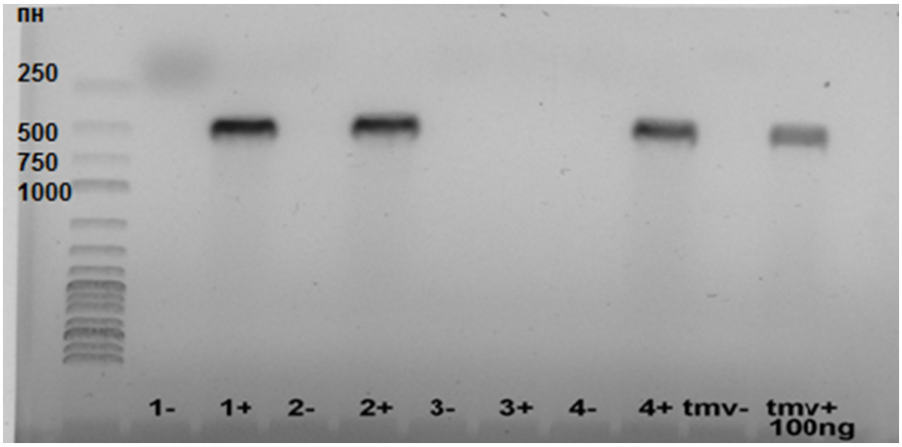

Figure 1. Products of RT-PCR analysis of total RNA from plants with the gene *ifn-α2b* and infected by tobacco mosaic virus in terms of the presence of viral RNA; 1, 2 - transformed plants; 3 - not infected control plants; 4 - infected control plants; tmv - native virus preparation (- without RT, with RT +).

Thus, it could be argued that transgenic plants were not new organisms, their cell structures were consistent with the cell structures of wild-type plants and displayed no significant differences.

Also, compared with cell ultrastructure of control plants, no significant differences were found in the

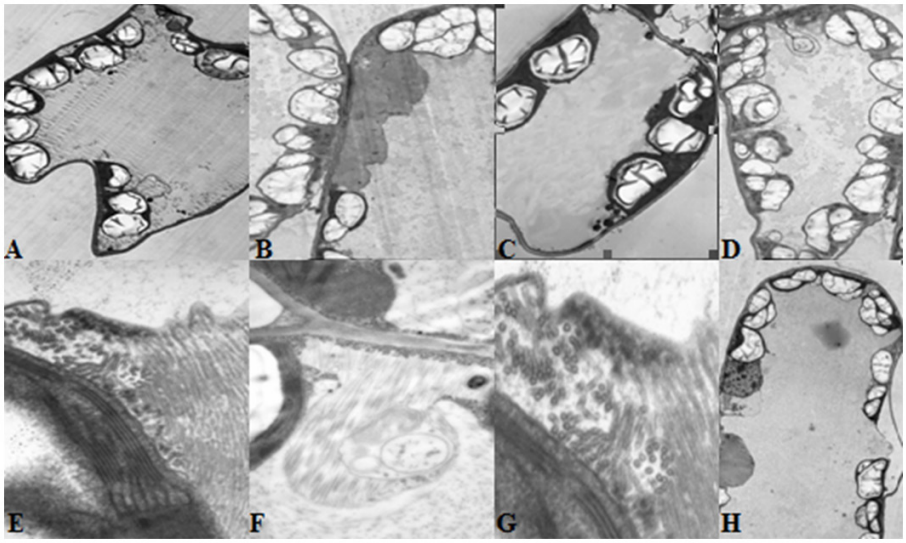

Figure 2. Ultrastructure of tobacco cells. A - ultrastructure of control cells without TMV (magnification 2K); B - ultrastructure of control cells infected with TMV (2K); C - ultrastructure of cells of transgenic plants with human interferon alpha-2b gene without TMV (2k); D - ultrastructure of cells of transgenic plants with human interferon alpha-2b gene infected by TMV (2K); E - viral unit in cells of control plants (30K); F - formation of vesicular bodies in infected cells of control plants (20K); G - viral aggregates in cells of transgenic plants with human interferon alpha-2b gene (80 K); H - cell wall plasmolysis in cells of control plants (2K).

ultrastructure of transgenic plant cells infected by TMV, despite that extracts from these plants demonstrated interferon-like antiviral activity (Fig 2B, D). A large number of virions accumulated in the infected mesophyll cells. These viral particles aggregated with dense packing of virions. In the central vacuole scattered virus particles were observed. In sections of virus aggregates longitudinal profiles of closely spaced together virions and their transverse profiles were observed (Fig. 2E, G). The length of individual virions was 300±50 nm, with a diameter in cross sections 16±1 nm and a 4 nm diameter of the central cavity (lumen). It should be noted that cytoplasmic aggregates of TMV were defined in the areas where cell membranes of adjacent cells directly contacted and also in areas distant from cell walls. These features were characteristic for infected plants, both transgenic and wild-type. However, areas of cytoplasm with densely spaced aggregates of virions and multivesicular bodies as well as individual virions were detected in the vacuoles of wild-type cells. The nuclei of the mesophyll cells in all infected plants were rounded with relatively smooth shell contours, preferably with condensed chromatin, and these nuclei were similar to nuclei of wild-type plant cells. However, variations in chloroplast ultrastructure were detected in TMV infected plant cells. Two-three starch grains were detected in the stroma of chloroplasts in cells of both infected control and transgenic plants instead of one large starch grain detected in cells of non-infected plants. Chloroplasts of infected cells were smaller compared to chloroplasts of control plants. In chloroplasts of infected cells the size of plastoglobuli was 170±25 nm compared to 200±30 nm in the control plants. Mitochondria in the infected cells were characterized by a tendency to increase the number of cristae compared with mitochondria structure of wild-type, non-infected plants. Also, TMV infected cells of non-transgenic plants showed relatively higher frequency of multivesicular bodies, which were located in both hyaloplasm and cell vacuoles (Fig. 2F). The appearance of multivesicular bodies likely reflected an increased level of autolytic processes. The phenomenon of plasmolysis was detected in infected non-transgenic control plants (Fig. 2H).

Earlier Rivkin et al. (1995) showed that interferon can be synthesized in the cells of transgenic plants and this influenced the development of plant virus infection. However, in transgenic plants some components of 2.5A system, which are need for effective activity of the interferon system are absent (Lim et al., 2002). Thus, interferon does not ensure complete protection of plants from plant virus, but it can be active against mammalian viruses. Our results indicated that transgenic plants were not fully protected from the TMV infection. This result is not entirely consistent with the experiments of Rivkin et al. (1995). These studies (Rivkin et al., 1995) do not indicate full resistance of transgenic plants with the interferon gene. They noted only slight slowing of the manifestation of the disease. Our work was primarily aimed at studying the processes of plant virus infection at a cellular level. Our results indicated that both transgenic and non-transgenic plants were infected by TMV, but there were some differences in the ultrastructure of infected cells of transformed and non-transformed plants.

Thus, although resistance of plants to the viral infection was claimed previously (Rivkin et al., 1995), we can not confirm that transgenic plants with infα2b gene are resistant to TMV infection. According to our results we can conclude that phenotype and ultrastructure of transgenic plants did not differ from wild-type plants. The ultrastructure of infected transgenic and control plant cells revealed no fundamental differences and the responses of transgenic and control plants to the viral infection were similar. The overall response of plants to TMV was similar to previously described (Xu and Feng, 1998; Reunov et al., 2006). However, extracts of transgenic plants showed antiviral interferon like activity on ETP cells infected by VSV. This activity indicated that an interferon-like product was synthesized. The ultrastructure of the cells of the transgenic plants infected by TMV was not different from the ultrastructure of the infected wild-type plants. This indicated that interferon that was synthesized in transgenic plants did not protect these plants against TMV. This phenomenon can be explained by the lack of necessary components of the 2-5A interferon system in plants. (Lim et al., 2002; Silverman, 2007).

CONCLUSION

Tobacco plants resistant to the antibiotic kanamycin were obtained using the Agrobacterium-mediated transformation method. The transgenic nature of these plants was confirmed by PCR analysis using primers specific to genes *ifn-α2b* and *nptII*. RT-PCR analysis demonstrated that the human *ifn-α2b* gene was transcribed in the plants obtained after transformation. Extracts from transgenic plants showed interferon-like antiviral activity in EPT cells infected by VSV (3.274-6312 IU/g of fresh weight, or 1.553 * 10^{-3} - 3.009 * 10^{-3} IU/mg of total soluble protein). Transgenic plants were infected by TMV. Electron microscopic analysis of cells of transgenic and control tobacco plants showed no significant differences in their ultrastructure.

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