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Biosynthesis of tobacco mosaic virus RNA in tobacco protoplasts

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Abstract

Changes in the number of protoplasts, viability, protein and chlorophyll contents and ribonucleases activity were studied in tobacco mesophyll protoplasts *in vitro* inoculated with tobacco mosaic virus (TMV). The number of protoplasts slowly increased during the cultivation period and the viability decreased from 95 to 67 % in the control noninoculated protoplasts, and to 55 % in the infected protoplasts. 30 h after inoculation the protein and chlorophyll contents strongly decreased to 25 - 30 % and 17 - 19 %, respectively, in comparison with contents 3 h after inoculation. The chlorophyll *a/b* ratio decreased from 2.11 and 2.02 to 0.79 and 0.60 in healthy and infected protoplasts, respectively. The activities of ribonucleases in protoplasts quickly decreased during experiment but they were higher in infected than in noninfected protoplasts (between 20 to 30 h after inoculation they were 132 to 146 % higher than that in healthy controls). These activities corresponded to the multiplication curve of TMV.

Key words: chlorophyll, *Nicotiana tabacum*, protein, ribonuclease activity, viability

Introduction

Virus RNA can be synthesized in an infected host cell from intermediates of the reductive pentose phosphate pathway during photosynthesis (Hampton *et al.* 1966, Jensen 1968, Tu *et al.* 1968) or from intermediates of the oxidative pentose phosphate pathway (Tien and Tang 1963, Merrett and Sunderland 1967, Huth 1973, Šindelář 1975, 1986, Makovcová and Šindelář 1981), or from nucleotides released from degraded host RNA. These three metabolic pathways are involved in virus RNA biosynthesis, but they usually depend on the type of virus, host, and environmental conditions.

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The pathway of host RNA degradation by ribonucleases, in which the nucleotides necessary for virus RNA biosynthesis are released, is a universal pathway. However, it seems that this pathway is used only when the sources of nucleotides formed *de novo* by the reductive and oxidative pentose phosphate pathways are insufficient for the synthesis of a virus RNA. This is true for the tobacco mosaic virus (Reddi 1963, Cheo 1971, Šindelářová *et al.* 1988), or for fast virus RNA synthesis (potato virus Y, Šindelář *et al.* 1990).

The biosynthesis of virus RNA is a complex process still not entirely obvious, therefore we focused on study of the sources of intermediates for the biosynthesis of virus RNA from the host RNA degradation pathway using the model of tobacco mesophyll protoplasts infected *in vitro* with tobacco mosaic virus.

Materials and methods

Two month old tobacco (*Nicotiana tabacum* L. cv. Samsun) plants were grown under constant conditions in soil, at an irradiance of 60 μmol m^{-2} s^{-1} (photoperiod 12/12 h) and average temperature of 25 °C, were used for protoplast preparation.

Preparation and inoculation of protoplasts: Protoplasts were prepared according to Šindelářová and Šindelář (1994) with exception the leaves were sterilized for 30 min in 2 % commercial bleach (SAVO). Protoplasts were inoculated with freshly purified TMV (Gooding and Hebert 1967) by the method described previously (Šindelář and Šindelářová 1994). Protoplasts were incubated under continuous light, at temperature of 25 °C, in CPW medium (Šindelářová and Šindelář 1994) or MS medium (Murashige and Skoog 1962) without organic supplements, carbon source and plant growth regulators.

TMV content assay: The TMV content was determined by the DAS-ELISA method (Clark and Adams 1977) with rabbit anti-TMV antibodies and alkaline phosphatase (Boehringer) labelled antibodies prepared from our isolate of TMV-*vulgare*. Virus content was estimated from calibration curve of purified TMV with use of computer software described in Manfal (1987).

Viability of protoplasts, protein and chlorophyll content and ribonucleases activities assay: The 0.1 cm^3 aliquot of protoplasts was used for determination of the number of living protoplasts by means of Methylene Blue staining with a haemocytometer (Hooley and McCarthy 1980). The remain content of flask were centrifuged at 2 000 *g* for 10 min, the sedimented protoplasts were resuspended in 2 cm^3 20 mM Tris-HCl buffer, pH 7.0, containing 1 mM EDTA, 2.5 mM $MgCl_2$, 30 mM 2-mercaptoethanol and then they were disrupted by ten passes through a syringe with thin needle (the disruption was monitored by light microscope). The chlorophyll content in this suspension was determined by the method of Arnon (1949), protein content by the method of Bradford (1977) using BSA as a standard, glucose-6-phosphate dehydrogenase activity by the method described by Šindelář (1986), and the ribonucleases (RNases) activity according to Cheo (1971).

Statistical treatment: The results are presented as arithmetical means of 3 - 5 determinations. The statistical significance of differences was evaluate by the *t*-test.

Percent of inoculated protoplasts was determined according to Šindelář and Šindelářová (1994).

Chemicals: Protoplast releasing enzymes were obtained from *Serva Feinbiochemica GmbH (*Heidelberg, Germany*)*. Before use, both *Cellulase R-10* and *Macerozyme R-10* were dissolved in the incubation medium, centrifuged for 10 min at 22 000 *g*, and cold sterilized through a 0.45 μm filter. The solutions of TMV and poly-L-ornithin (PLO) were sterilized in the same way. All other biochemicals were obtained from *Sigma Chemical Co.* (St. Louis, USA).

Results and discussion

The number of protoplasts infected by tobacco mosaic virus always exceeded 68 % (in average 77.8 % ± 8.6 %). The number of protoplasts in 1 cm^3 incubating medium slowly increased from 5.3 × 10^4 to 6.3 × 10^4, viability decreased in control and infected protoplasts from 95 to 67 and 55 %, respectively (Fig. 1).

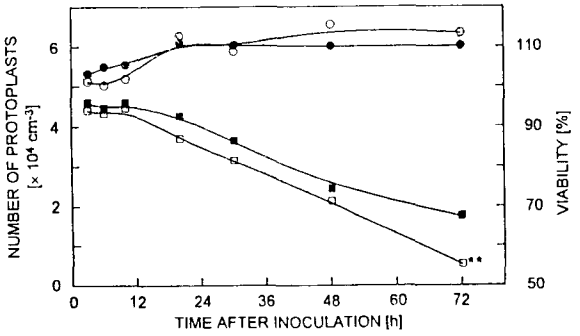

Fig. 1. Number (*circles*) and viability (*squares*) of healthy (*closed symbols*) and tobacco mosaic virus inoculated (*open symbols*) tobacco mesophyll protoplasts in cultivation medium. ** - the difference is statistically significant at $P < 0.01$.

The protein content 3 h after inoculation was 242 μg per 10^6 living control protoplasts and 180 μg per 10^6 living infected protoplasts. During the first 24 h this content drastically decreased in control and infected protoplasts to 60 and 54 μg respectively, which represent 25 % and 30 % of the initial values. The decrease was faster in infected than in noninfected protoplasts (Fig. 2).

The chlorophyll content also decreased during the first 24 h to 19 % in control and to 17 % in infected protoplasts in comparison with content 3 h after inoculation (Fig. 3). The photosynthetic apparatus was gradually degraded, which was manifested by decreases in chlorophyll *a* and *b* contents and especially chlorophyll *a*/*b* ratio (from 2.11 to 0.79 in healthy and from 2.02 to 0.60 in infected protoplasts 30 h after inoculation).

Activities of glucose-6-phosphate dehydrogenase were low, their values being within the limits of experimental errors. Therefore the results are not presented.

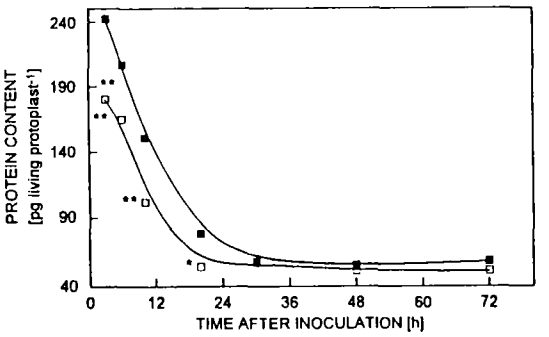

Fig. 2. Protein content in healthy (*closed squares*) and tobacco mosaic virus inoculated (*open squares*) tobacco mesophyll protoplasts. **,* - the differences are statistically significant at $P < 0.01$ and $P < 0.05$, respectively.

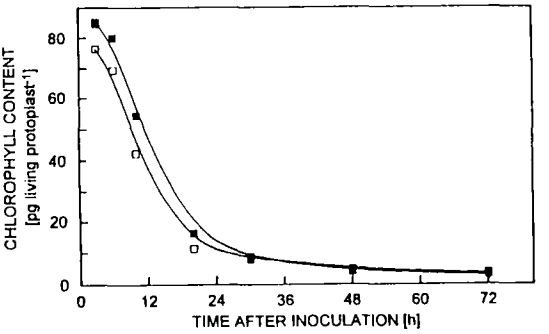

Fig. 3. Chlorophyll content in healthy (*closed squares*) and tobacco mosaic virus inoculated (*open squares*) tobacco mesophyll protoplasts.

Activities of ribonucleases in protoplasts quickly decreased, but their decrease was slower in infected protoplasts (Fig. 4) than in healthy ones. Ribonucleases activity in infected protoplasts increased to 132 - 146 % from the noninfected 20 to 30 h after inoculation (Fig. 5).

We obtained the same results for viability, activity of ribonucleases and protein, chlorophyll and TMV contents in the case that MS medium was used instead of the CPW medium.

With respect to low activity of glucose-6-phosphate dehydrogenase (a key enzyme of oxidative pentose phosphate pathway) and with regards to strong decrease of chlorophyll content, there is no evidence for involvement of oxidative or reductive pentose phosphate pathways in virus RNA synthesis.

On the contrary, the pathway of the host rRNA degradation by ribonucleases was enhanced. In tobacco mesophyll protoplasts infected with TMV, the ribonuclease activity during the whole cultivation period was increased, reaching 132 - 146 % of

the value of the noninfected protoplasts 20 to 30 h after inoculation. The curve of ribonucleases, degrading host rRNA to free nucleotides necessary for intense biosynthesis of TMV-RNA, well corresponds to the multiplication curve of TMV.

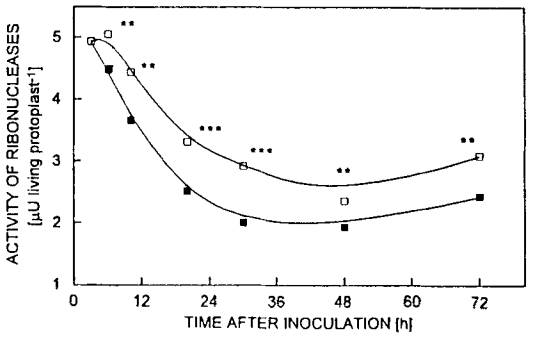

Fig. 4. Activities of ribonucleases in healthy (*closed squares*) and tobacco mosaic virus inoculated (*open squares*) tobacco mesophyll protoplasts. ***,** - the differences are statistically significant at $P < 0.001$ and $P < 0.01$, respectively.

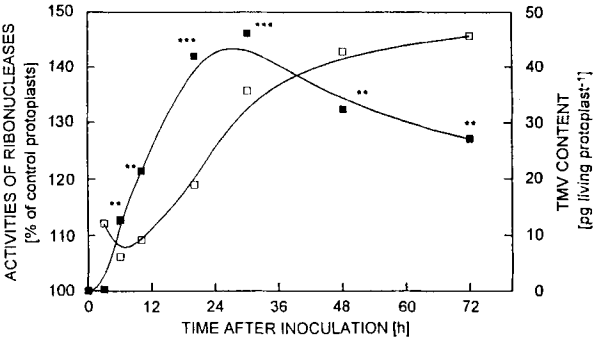

Fig. 5. Activities of ribonucleases (% of healthy protoplasts, *closed squares*) and TMV multiplication curve (*open squares*). ***,** - the differences are statistically significant at $P < 0.001$ and $P < 0.01$, respectively.

Thus, we assume that TMV-RNA in tobacco mesophyll protoplasts is preferentially synthesized from the nucleotides of degraded host RNA.

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