

An Immunochemical Testing of Pathophysiological Reactions of Several PSTV-Infected Tomato (*Lycopersicon esculentum* L.) Cultivars

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Abstract. Several tomato cultivars were infected with a severe strain of PSTV and a pathophysiological reaction was characterized by means of enzyme-linked immunosorbent assay with serum containing IgGs to disease-associated host-specific leaf proteins. A strong expression of disease-associated symptoms (stunting, epinasty, leaf blade malformation and rugosity) and strong immunochemical reaction was found for cultivars Bizon, Linia, Revermun and Rutgers. The immunochemical assay revealed appearance of a major antigenic protein having a molecular mass of about 70 kD in these cultivars. The immunochemical reaction with disease-associated proteins reached a maximum four weeks after inoculation of PSTV. A weak immunochemical reaction was observed, if proteins from cvs. Sonato, Harzfeuer and Karlik were analyzed. Cvs. Sonato and Harzfeuer did not show characteristic symptoms such as stunting, leaf blade malformation and rugosity. Except for stunting, the same was true for cv. Karlik. On the other hand, no significant difference in PSTV accumulation was observed among the cultivars analyzed.

Reciprocal hybrids obtained by crossing cvs. Revermun and Karlik showed strong symptoms of the disease (Revermun-type) and increased activity of nuclease due to PSTV infection. On the contrary, an immunochemical analysis revealed a low level of the disease-associated antigens in these hybrid tomatoes, suggesting rather recessive genetic background determining their expression during PSTV-caused pathogenesis.

Viroids are known to be pathogenic RNAs, which do not code for proteins (Sänger 1982). On the other hand, it has been shown, for example, that the potato spindle tuber viroid (PSTV) and the citrus exocortis viroid (CEV) caused in tomatoes the pathogenesis resulting in an accumulation of disease-associated host protein(s) of high antigenicity (Camacho-Henriquez and Sänger 1982, Galindo *et al.* 1984, Granell *et al.* 1987, Matoušek *et al.* 1988, 1990).

The production of disease-associated antigen(s) in certain plants infected with viroids could probably be used for an immunochemical quantification and characterization of the pathophysiological reaction. Such approach could also be useful for studying pathogenesis in relation to viroid and plant genotypes.

In the present work, we have compared viroid-induced pathophysiological reactions of several tomato cultivars by means of immunochemical detection of disease-associated antigens.

MATERIAL AND METHODS

Plant Material

The following cultivars of tomato (*Lycopersicon esculentum* L.) were used: Revermun, Sonato (Netherlands); Harzfeuer (FRG); Bizon, Karlik (USSR); Rutgers (USA) and Linia (Czechoslovakia). Three week old tomato seedlings were infected exclusively with a severe strain of PSTV originally isolated from *Solanum commersonii* in Scotland (Cammack and Harris 1973). PSTV inoculation was made with crushed infectious foliage as described by deBokx and Chaffari (1969). The plants were grown in air conditioned boxes with a 16 h photoperiod supplied by fluorescent lamps with irradiance of $150 \mu\text{mol}^{-2} \text{s}^{-1}$ and a temperature of 26–27 °C. The third leaves (counting from stem apex) were collected (from eight inoculated and eight healthy plants of each cultivar) for analysis succesively two week after inoculation.

Hybrid tomatoes were obtained by reciprocal crossing of cvs. Revermun and Karlik. The hybrids and their parental cultivars were grown in a greenhouse under natural illumination. The third fully developed leaves (as described above) were collected for analysis three weeks after PSTV inoculation. Cv. Revermun and both reciprocal hybrids showed the syndrome characteristic for the disease (stunting, epinasty, leaf blade malformation and rugosity) at that time. Strong stunting was observed for cv. Karlik as a main response to PSTV infection. Twenty plants of each variant were analyzed.

Detection and Quantification

PSTV was detected and quantified using "dot-blot" procedure with ^{32}P labelled cDNA of PSTV (Palukaitis *et al.* 1985).

Preparation of Leaf Extracts

The extraction buffer contained PBS (0.1 M sodium phosphate, pH 7.2, 0.15 M sodium chloride), 10 mM 2-mercaptoethanol, 5 % polyvinylpyrrolidone

Abbreviations used: PSTV, potato spindle tuber viroid; PBS, 0.1 M phosphate buffered saline (0.15 M NaCl, pH 7.2).

(insoluble form), 0.2 mM phenylmethylsulfonylfluoride (PMSF) and 10 % sucrose. Leaf cuttings obtained either from positive (infected) or from healthy plants were extracted using a Pollähne roller press, with 1 ml of the buffer per 1 g of leaf blades (fresh mass). Leaf homogenates were sedimented in a refrigerated centrifuge for 10 min at 19 000 *g*. Samples were stored at -20°C until used. Just before analysis they were re-sedimented once more under the same conditions.

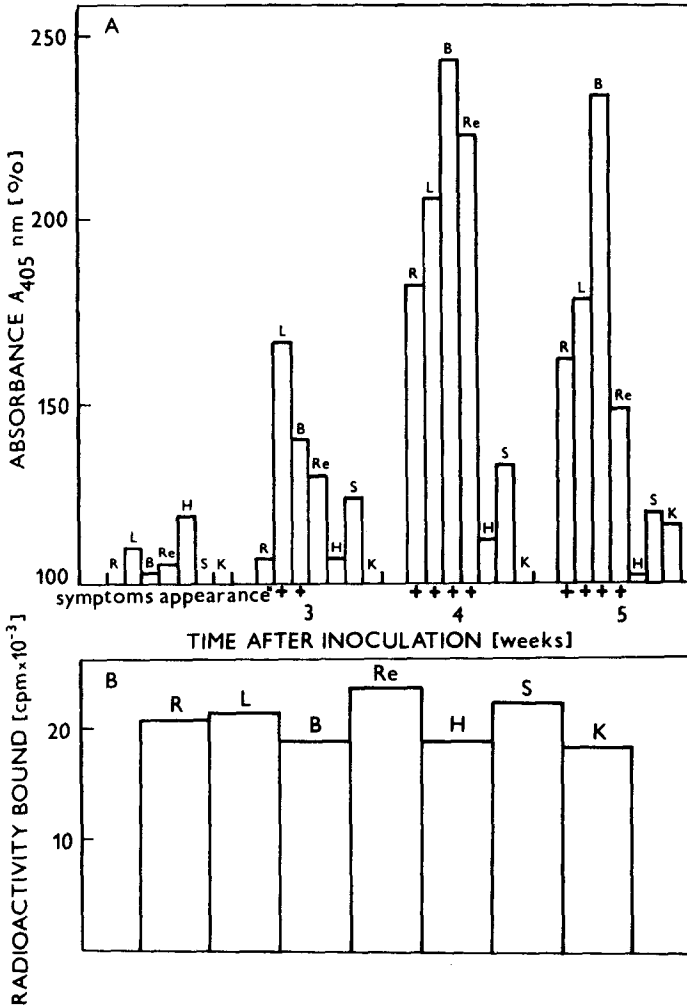


Fig. 1. Analysis of immunochemical reaction (A) and level of PSTV (B) in different tomato cultivars. A – reaction was measured by ELISA and expressed in percents relative to healthy plants. R- cv. Rutgers; L- cv. Linia; Re- cv. Revermun; H- cv. Harzfeuer; S- cv. Sonato; K- cv. Karlik, B- cv. Bizon. B – Amount of nucleic acids applied to nitrocellulose corresponds to 6.5 mg of fresh mass. Specific radioactivity was 1.6×10^7 cpm μg^{-1} of cDNA PSTV insert.

Enzyme-linked Immunosorbent Assay

The rabbit IgGs were raised against PBS-soluble proteins from PSTV-infected tomato cv. Rutgers and purified by affinity chromatography on bead macroporous cellulose with immobilized "healthy" antigens as described by Matoušek *et al.* (1990). 0.1, 1 and 5 µg of protein of each variant were absorbed on polystyrene microtiter plates overnight at 4 °C in 0.2 M sodium bicarbonate buffer, pH 9.6 containing 5 mM 2-mercaptoethanol and 0.2 mM PMSF. Under such conditions of protein coating a preferential reaction with antigens from PSTV-infected leaves is reached in ELISA-test (Matoušek *et al.* 1990). Incubation of the immobilized antigens with specific IgGs was performed for 2 h at 37 °C. Detection was achieved using goat anti-rabbit IgGs labelled with alkaline phosphatase. p-Nitrophenyl phosphate served as a substrate. Hydrolyzed enzyme substrate was determined by measuring the extinction at 405 nm. The ELISA tests were performed three times for each variant. The mean value of absorbance, relative to the healthy control-variants is given in per cents.

Protein Transfer and Enzyme Immunoassay

Proteins were separated by polyacrylamide gel electrophoresis (Laemmli 1970) and transferred to nitrocellulose membranes under SDS-denaturing conditions (Towbin *et al.* 1979). Enzyme immunoassays were performed using pig's anti-rabbit IgGs labelled with horse radish peroxidase.

Other Methods

Nuclease extracts were prepared as described by Matoušek and Dědič (1988). Nuclease activity was measured by means of a radial diffusion test on agarose (Matoušek *et al.* 1987). One unit of nuclease activity is defined as the amount of enzyme that releases 0.001 A_{260} unit of acid soluble products in 1 min at 37 °C. Proteins were quantified by the method according to Bradford (1976).

RESULTS

The first characteristic morphological symptoms of infection (especially leaf blade malformation and rugosity) were clear-cut for cvs. Linia and Bison 21 d after inoculation. Significant immunochemical reaction exceeding the control level was observed especially for extracts from cv. Linia at the same time (Fig. 1A). The first symptoms of infection for cvs. Revermun and Rutgers were observed 23–24 d after inoculation, while cvs. Harzfeuer and Sonato did not show characteristic morphological symptoms (Fig. 1A). Nevertheless, a very weak immunochemical reaction was found, if proteins from the two cultivars were analyzed (Fig. 1A). Except for stunting, we did not observe characteristic

symptoms like epinasty, leaf malformation and rugosity for cv. Karlik. We observed no immunochemical reaction exceeding the control level for this cultivar (Fig. 1A). The strongest relative immunochemical reaction was observed for the sensitive cultivars Linia, Bison, Revermun and Rutgers four weeks after PSTV-inoculation, while later (five weeks after inoculation) the reaction was somewhat weaker (Fig. 1A).

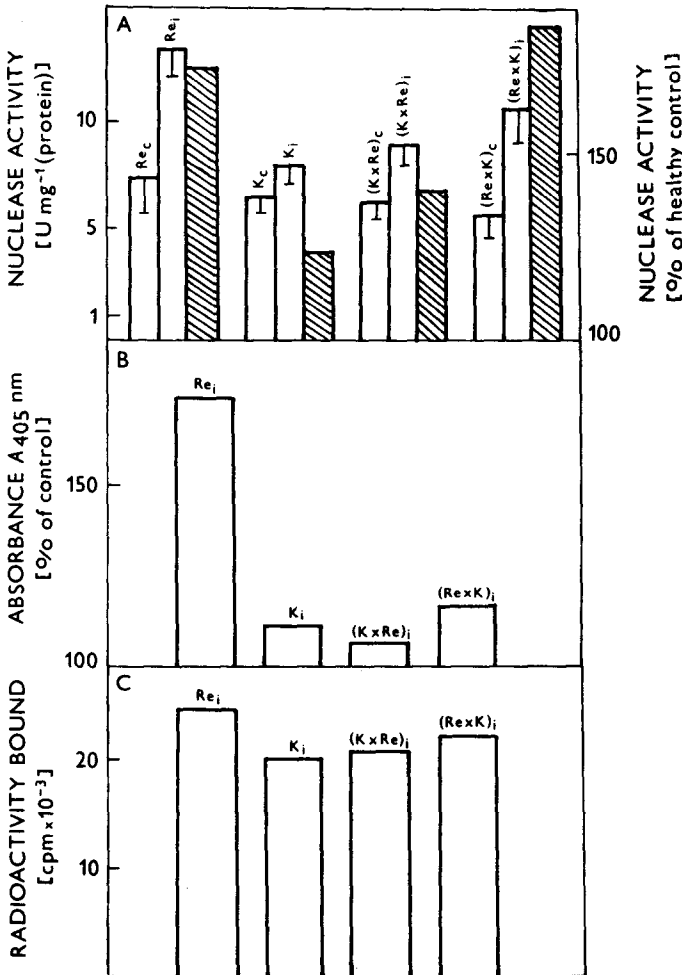


Fig. 3. Analysis of activity of acid nuclease (A), immunochemical reaction (B) and level of PSTV (C) in "sensitive" and "tolerant" cultivars and their reciprocal hybrids.

A, B - "sensitive" cultivar Revermun, (Re); "tolerant" cv. Karlik, (K); Karlik x Revermun, (K x Re); Revermun x Karlik, (Re x K). Infected and healthy genotypes, respectively, are designated by indexes "i" and "c".

C - amount of nucleic acids applied to nitrocellulose corresponds to 6.5 mg of fresh mass. Specific radioactivity was 1.9×10^7 cpm μg^{-1} of cDNA PSTV insert.

Confidence intervals are given at a 5 % level.

The plants were analyzed for the presence of PSTV four weeks after inoculation by means of hybridization technique using ^{32}P -labelled rcDNA of PSTV. All tomato cultivars were infected successfully under our experimental conditions. Moreover, levels of radioactivity detected in individual cultivars (Fig. 1B) did not differ too significantly, suggesting the similar rates of PSTV accumulation in cultivars analyzed.

In order to identify the antigens responsible for immunochemical differences among infected cultivars, the immunoblot technique was performed (Fig. 2). Several antigens were detected in crude extracts. However, accumulation of at least one major antigenic protein with a molecular mass of about 70 kD seemed to be responsible for specific differences in immunochemical reactions obtained for crude protein extract from infected tomato cultivars. A low level of this antigen was also observed in infected plants of the cvs. Sonato and Harzfeuer. Moreover, traces of the antigen were found in the cv. Karlik as well as in uninfected controls (results not given).

Reciprocal hybrids obtained by crossing cvs. Karlik (low antigen accumulation during pathogenesis) and Revermun (high level of disease-associated antigen in infected plants) showed strong symptoms of disease characteristic for infected cv. Revermun. For comparison of pathophysiological reactions in these genotypes we analyzed levels of acid nuclease, which was found earlier to be increased in PSTV-infected tomato leaves (Matoušek *et al.* 1988). Increased level of the nuclease activity was observed for all PSTV-infected genotypes (Fig. 2A). The highest specific activity of the nuclease was observed in infected hybrid of combination (Re \times K) and in PSTV-infected cv. Revermun, whereas lower specific activity was measured in PSTV-infected hybrid plants (K \times Re) and in infected cv. Karlik. However, analysis of protein extracts from infected hybrid tomatoes by ELISA revealed, very low immunochemical reactions relative to the uninfected samples (Fig. 2B) and low accumulation of 70 K antigen (results not shown). These results corresponded to Karlik-type of pathogenesis (Fig. 2B).

Data described in Fig. 2C indicate no strong differences in PSTV accumulation among the individual genotypes.

DISCUSSION

It follows from our results that the occurrence of immunochemically detectable antigens was in good correlation with the expression of symptoms related to PSTV-infection in certain tomato cultivars. Although no apparent characteristic symptoms were observed for the infected cvs. Sonato and Harzfeuer, the weak accumulation of disease-associated proteins as revealed by immunochemical reaction suggests that some pathophysiological process occurred, which could not be clearly determined by a "visual" method.

The plants of cvs. Bizon, Linia, Rutgers, and Revermun were very sensitive to infection, while cvs. Harzfeuer, Sonato and to a certain extent cv. Karlik were more "tolerant" to PSTV infection. At least one major antigen with a molecular mass of about 70 kD, as estimated under denaturing conditions, was found to be responsible for the immunochemical differences detected among the "sensitive" and "tolerant" cultivars. It has been shown that the investigated protein has accumulated essentially in viroid-infected tomato leaves, but not in tomato leaves infected with potato viruses X, Y, and M (Matoušek *et al.* 1990). Probably, the detected antigen corresponds to the 70K-subunit of disease-associated tomato leaf protein described also by Galindo *et al.* (1984). The protein had a molecular mass of about 140 kD as estimated under native conditions and its accumulation was restricted to viroid and satellite RNA infection (Galindo *et al.* 1984).

On the one hand, it was shown in this study that appearance of the 70K antigen correlate with symptom expression in some genotypes. On the other hand, the antigen did not accumulate significantly in the reciprocal hybrids between cvs. Karlik and Revermun when infected with PSTV, although the symptoms of infection were well developed in these plants. These results suggest a rather recessive genetic background, determining its expression and its relative independence on morphological symptoms developed during viroid caused pathogenesis. These observations are in good accordance with previous finding (Matoušek *et al.* 1990) that the antigen appearance did not correlate with development of a viroid-like syndrome after treating the plants with AgNO₃. Granell *et al.* (1987) used treatments with silver ions for induction of viroid-like symptoms and showed similarity of this phenomenon to viroid caused pathogenesis. It was demonstrated that ethylene is involved in the mechanism of the symptom production and accumulation of at least ten pathogenesis-related proteins (Granell *et al.* 1987). Possibly, the tomato cultivars examined in our experiments differed also in other (minor) antigens, which accumulated in infected leaves, but these proteins were not clearly detected and identified by the method used. The genotypes also differed at least in the level of nuclease activity increased during PSTV infection. The results described above support the suggestion that different processes occur simultaneously during viroid-caused pathogenesis and are conditional on the host genotype. For instance, the low level of 70K antigen in the hybrid tomatoes (recessive inheritance) could be explained provided that its accumulation is due to posttranslational modification of some (pre-synthesized) host protein. The process of posttranslational modification could be regulated in the "tolerant" genotypes by some dominant suppressor gene(s) and could be thus in these genotypes less intensive.

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