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# The occurrence of a soluble protein ($E_1$) in cucumber cotyledons infected with plant viruses

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**Abstract**

Analysis on polyacrylamide gels of the soluble protein fraction of cucumber cotyledons infected with tomato spotted wilt virus (TSWV) revealed a stimulated protein component, designated as protein $E_1$. The amount of protein $E_1$ was related to the size and number of local lesions produced on the inoculated cotyledons. Protein $E_1$ stained positive for carbohydrate and could be partly purified by gel filtration on Sephadex G 100. The molecular weight was estimated to be approximately 22000 d.

Protein $E_1$ seems to be serologically unrelated to any of the structural proteins of TSWV. Following inoculation of cucumber cotyledons with the yellow strain of cucumber mosaic virus (CMV), a protein with identical mobility on 7.5 and 10% polycrylamide gels to protein $E_1$ was detected. These proteins are probably identical and their accumulation is a result of the infection process. There was a close correspondence between the amount of protein $E_1$ and the severity of symptom expression.

A protein with a mobility differing slightly from protein $E_1$ occurred in the soluble protein fraction of *Nicotiana rustica* after infection with either TSWV or tobacco mosaic virus.

## Introduction

Symptoms of tomato spotted wilt virus (TSWV) infection of cucumber cotyledons developed three or four days after inoculation. These symptoms consisted of gradually expanding yellow spots with small necrotic centres. Electron microscopic examination of cells from these lesions revealed large amorphous areas of high electron density, described as dark diffuse masses (Ie, 1971). In later stages of infection mature virus particles were detected, often present in clusters in the cisternae of the endoplasmic reticulum. The development of macroscopic symptoms and definite subcellular structures after inoculation with TSWV may be associated with alterations in the protein composition of the soluble fraction. During our investigations of possible alterations in the protein composition a protein component, designated $E_1$, was detected in crude extracts of diseased cotyledons, but it was not found in similar extracts from healthy cotyledons. To study the origin of this component the soluble protein fraction of cucumber cotyledons infected with either cucumber mosaic virus (CMV) or tobacco mosaic virus (TMV) were also analysed. The soluble protein fractions of *N. rustica*, infected with TSWV and TMV, respectively, were also investigated for the presence of protein components comparable or identical with protein $E_1$. The results obtained are discussed in relation to observations made by other workers on the occurrence of proteins in soluble fractions.

## Materials and methods

*Viruses, plants and growth conditions.* TSWV was obtained from Mr T. S. Ie and maintained in *Tropaeolum majus*. The yellow strain of CMV was kindly supplied by Dr. J. Dijkstra and TMV 'Wageningen' by Dr. D. Noordam.

Cucumber cotyledons were inoculated 10 and 15 days after sowing, just before the first primary leaf began to develop. *N. rustica* was cultivated as described earlier (Tas et al., 1976).

*Isolation of the soluble protein fraction.* The yellow lesions resulting from TSWV and CMV infection of cucumber cotyledons were cut out using a razor blade. In the case of *N. rustica*, infected by TSWV and TMV, which cause chlorosis and mosaic symptoms, respectively, systemically infected leaves were used.

For isolation of the soluble protein fraction, 4 g of leaf material was homogenized in 2 ml ice cold extraction buffer (0.1 *M* Tris-HCl, 0.5 *M* sucrose, 0.1 % cysteine-HCl and 0.1 % ascorbic acid, pH 8.0) in a precooled mortar at 2–4°C (Van Loon and Van Kammen, 1970). The homoganete was pressed through 2 layers of gauze and centrifuged for 15 min at 15000 *g*. The pellet was discarded and the supernatant was centrifuged for 30 min at 30000 *g*. The resulting supernatant was centrifuged for 2.5 h at 105000 *g*. The supernatant from the last centrifugation (soluble protein fraction) was used either immediately or after storage at –20°C.

*Polyacrylamide gel electrophoresis.* The soluble protein fractions were analyzed by electrophoresis on polyacrylamide gels according to Van Loon and Van Kammen (1970). Electrophoresis was for 20 min at 2 mA/tube followed by 4.5 mA/tube until the tracker dye was a few mm from the bottom of the gel (approximately 45 min).

Gels were stained with a saturated solution of Amidoblack 10 B in 5 % trichloracetic acid and destained with 7 % acetic acid (Van Loon, 1973) Carbohydrate was stained with Schiff's reagent according to Zacharius et al. (1969).

*Estimation of molecular weight.* The molecular weight of protein $E_1$ was estimated by the method of Zwaan (1967) using the equation $\log M = a(r) + b$ in which r = retardation quotient and a and b are constants. The retardation quotient, r, can be defined as $r = m_1/m_2$ in which $m_1$ is the absolute mobility in the gel of high concentration and $m_2$ that in the gel of lower concentration.

Electrophoresis was carried out on gels containing 7.5 and 10 % acrylamide. Ovalbumin (43000 d) and bovine serum albumin (68000 d) were co-electrophoresed as standard proteins.

*Filtration on Sephadex G-100.* Ten to twenty ml of soluble protein fraction were applied onto a Sephadex G-100 column (75 × 5 cm) equilibrated with 0.5 *M* Tris-HCl, pH 7.5, and eluted with the same buffer at a speed of approximately 20 ml/h. Fractions were collected every 20 min. Suitable fractions were pooled and concentrated by lyophilization. The powder was dissolved in distilled water and dialyzed against 0.005 *M* Tris-glycine buffer pH 8.6.

*Differential precipitation with ammonium sulphate.* Solid ammonium sulphate was

added to the soluble protein fractions to a final concentration of 35%, 50%, 75% and 100%. Precipitates were collected by centrifugation for 10 min at 30000 *g*. dissolved in 0.005 *M* Tris-glycine buffer pH 8.6, and dialysed overnight against the same buffer.

## Results

Cucumber cotyledons were inoculated with TSWV or with water (control). Eight days after inoculation, when the yellow lesions were fully developed, the soluble protein fractions of diseased and healthy cotyledons were prepared and analysed

Fig. 1. A. Polyacrylamide gel electrophoresis on 7.5% gels of the soluble protein fraction of cucumber cotyledons after infection with TSWV (1), with the yellow strain of CMV(2) and with water (3). B. Polyacrylamide gel electrophoresis on 10% gels of precipitates obtained by ammonium sulphate saturation between 50 and 75% of the soluble protein fraction. The cucumber cotyledons were inoculated with the yellow strain of CMV (1) and with water (2). C. Polyacrylamide gel electrophoresis of the soluble protein fractions of *N. rustica* after inoculation with TSWV (1), with TMV (2) and with water (3) on 7.5% gels. In A and B the position of $E_1$ is indicated by an arrow. The electrophoretic front in C is indicated with f, and the position of the newly occurring protein in *N. rustica* by arrows.

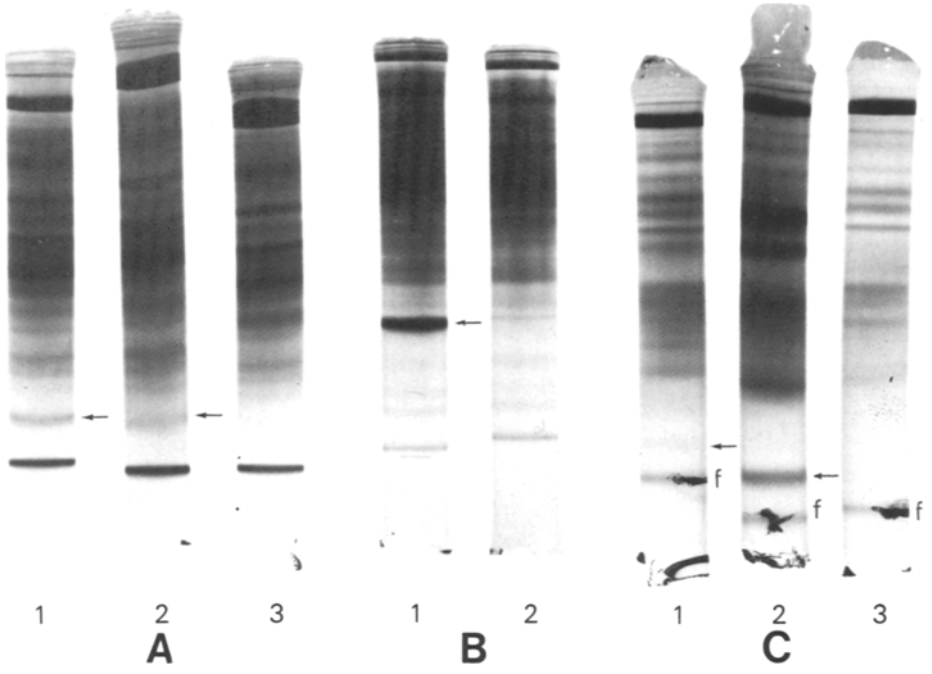


*Fig.* **1**. *A. Analyse van de fractie oplosbare eiwitten uit de cotylen van komkommerplanten, die met TSWV (1), met de gele stam van CMV (2) en met water (3) waren geïnoculeerd op polyacrylamidegels (7.5%). B. Elektroforese van het neerslag dat in de oplosbare eiwitfractie bij verzadiging met ammoniumsulfaat tussen 50 en 75% ontstaat. De komkommercotylen waren met CMV (1) en water (2) geïnoculeerd. C. Analyse op polyacrylamidegels van de oplosbare eiwitfractie uit N. rustica na inoculatie met TSWV (1), TMV (2) en water (3). Het front wordt op de gels van dit experiment met de letter f aangeduid. Het eiwit $E_1$ en de nieuw gevormde eiwitten in N. rustica met pijlen.*

on polyacrylamide gels. The protein band patterns obtained were highly reproducible. Soluble protein fractions from TSWV-infected cotyledons contained a protein band, Rf 0.89 on 7.5% gels, which was not discernible in fractions from healthy cotyledons (Fig. 1, A1 and A3; Fig. 3A and B). This component was designated protein $E_1$. In addition, two other 'new' bands were observed with Rf values of 0.58 and 0.10 on 7.5% gels. The intensity of these bands was much less than that of protein $E_1$. These proteins were not studied further.

Comparison of the soluble protein fractions prepared at different times after infection, revealed that component $E_1$ was first detectable when the yellow lesions appeared, approximately 3–4 days after infection. Subsequently, the intensity of the band increased with the severity of symptoms. Protein $E_1$ was not detected in the areas between lesions and in uninoculated leaves.

The appearance of protein $E_1$ was not due to the yellowing of the cotyledons. Examination of soluble protein fraction obtained from cotyledons artificially yellowed by cultivation in the dark with short periods of illumination failed to reveal protein $E_1$.

Protein $E_1$ could be partly purified from TSWV infected cotyledons by Sephadex G-100 gel filtration (Fig. 2 and 3). Sample IV (fractions 57–64) contained protein $E_1$ as a major component (Fig. 3F). About 70% of the amount present in the soluble fractions could be precipitated with ammonium sulphate in the range of 55–70% saturation, and 20 and 10% in the range of 45–55 and 70–99%, respectively. Upon analysis of the 55–70% saturation fraction from healthy cotyledons, a faint band, with the same Rf values as protein $E_1$, was observed on 10% polyacrylamide gels (Fig. 1, B2). Using standard proteins as markers the molecular weight of protein $E_1$ was estimated to be about 22000 d. Staining of the gels for carbohydrate gave a positive reaction for protein $E_1$ (Fig. 3F).

Antiserum against TSWV failed to react with the soluble protein fraction from infected cotyledons, with the pooled fractions (57–64) obtained after Sephadex filtration and with protein $E_1$ concentrated by ammonium sulphate precipitation. An antiserum produced against protein $E_1$, having a titre of 1/4 with homologous antigen, also failed to react with either intact TSWV in double diffusion tests or with TSWV proteins resolved on SDS-polyacrylamide gels (Tas et al., 1976). Protein $E_1$ seems therefore to be unrelated to any of the structural proteins of TSWV.

To test the hypothesis that protein $E_1$ is a result of virus infection, cucumber

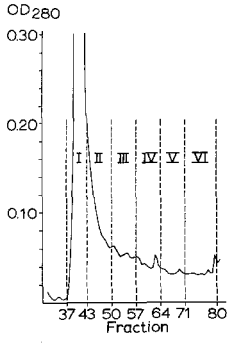


Fig. 2. Elution pattern from Sephadex G 100 of the soluble protein fraction isolated from cucumber cotyledons infected with TSWV. Fractions were pooled as indicated to give samples I to VI.

*Fig. 2. Elutie van de oplosbare eiwitfractie uit cotylen van komkommerplanten, die met TSWV waren geïnfecteerd, over een Sephadex G-100-kolom. De opgevangen fracties werden tot 6 monsters samengevoegd en geanalyseerd (Fig. 3).*

Fig. 3. Densitometer traces on 7.5% polyacrylamide gels of the soluble protein fractions of healthy cucumber cotyledons (A), TSWV infected cotyledons (B) and fractions I (C), II (D), III (E), IV (F), V (G) and VI (H) obtained after elution of the soluble protein fraction of TSWV infected cotyledons from Sephadex G 100. The fractions were concentrated and compared by equal volume. The gels with fraction IV (F) were stained with amidoblack (——) and with Schiff's reagent (- - - -).

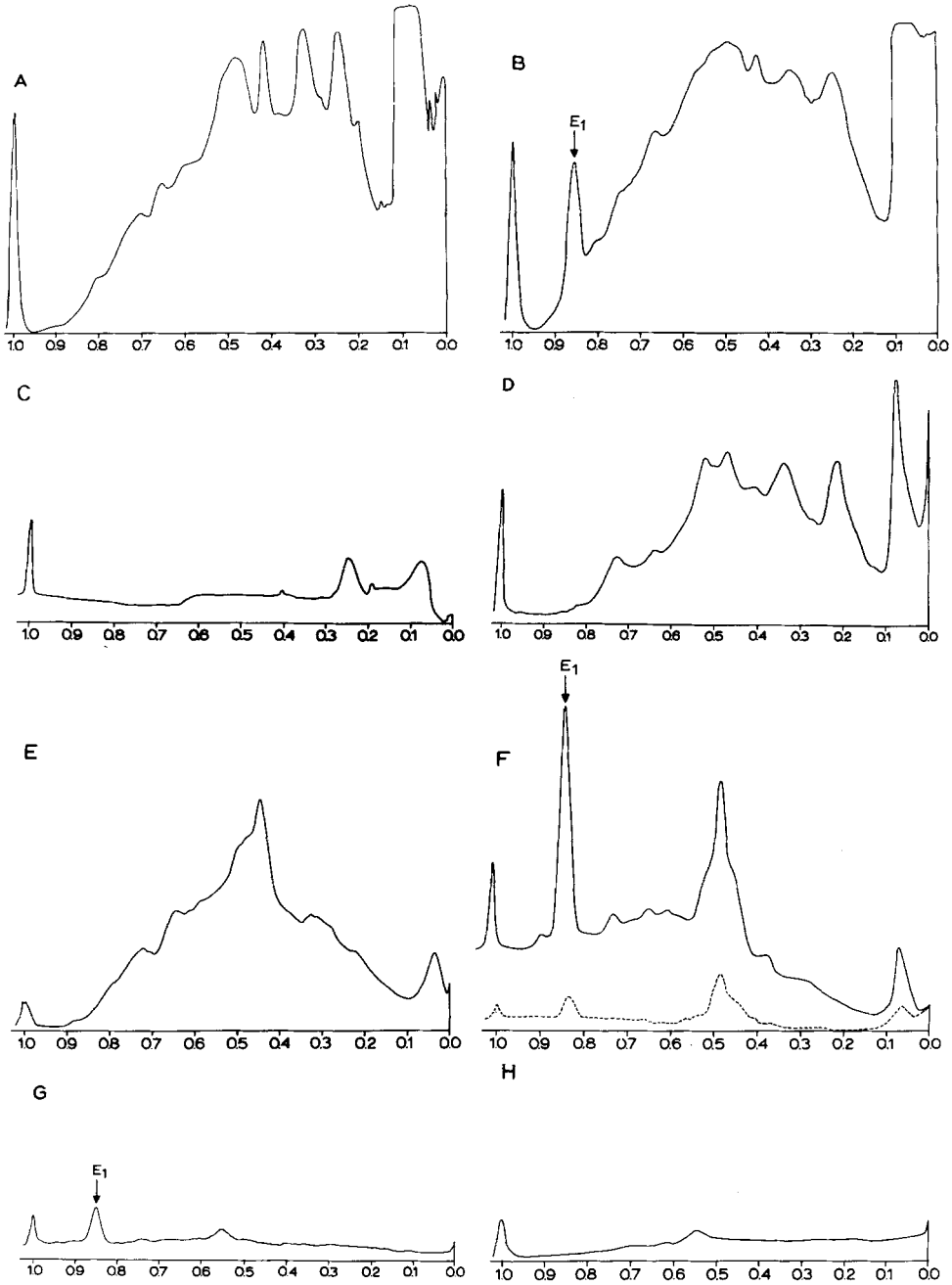


*Fig. 3. Dichtheidspatroon op 7.5% polyacrylamidegels van de oplosbare eiwitfracties uit gezonde (A) en met TSWV (B) besmette komkommercotylen en de 6 monsters I-VI (C-H), die bij de elutie van een oplosbare eiwitfractie over een Sephadex-kolom waren verkregen. De monsters waren voor de analyse geconcentreerd en op gelijk volume gebracht. Gel F was gekleurd met amino-zwart (———) en met Schiff's reagens (- - - -).*

Fig. 4. Densitometer traces of the soluble protein fraction of cucumber cotyledons eight days after inoculation with water (A) and with TMV (B). Electrophoresis was on 7.5% polyacrylamide gels. The protein zone corresponding to protein $E_1$ is indicated.

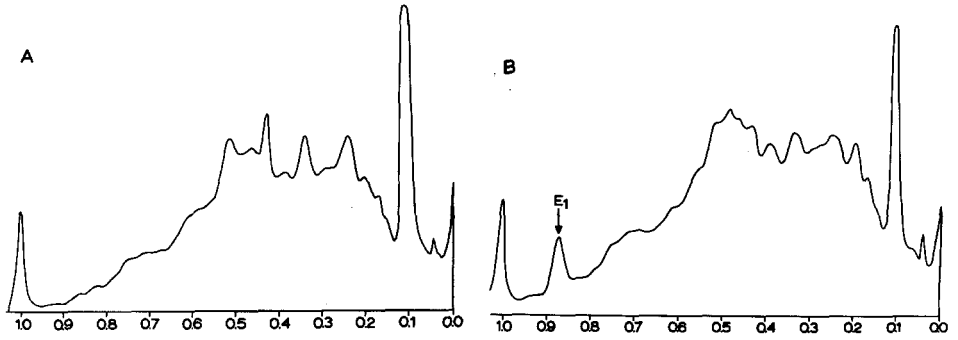


*Fig. 4. Dichtheidspatronen van de oplosbare eiwitfractie van komkommercotylen, die acht dagen tevoren met water (A) en TMV (B) waren geïnoculeerd. De fracties waren op polyacrylamidegels (7.5%) geëlektroforeerd. De eiwitcomponent die correspondeert met eiwit $E_1$ is met $E_1$ aangeduid.*

cotyledons were inoculated with the yellow strain of CMV. The soluble protein fraction from these cotyledons contained a component with a mobility, identical (Rf 0.89) to that of protein $E_1$ on 7.5% polyacrylamide gels (Fig. 1, A2). On 10.0% polyacrylamide gels, the protein from cotyledons infected with TSWV and CMV appeared also to have the same mobility (Rf 0.70).

Inoculation of cucumber cotyledons with TMV resulted in the development of very small necrotic lesions occupying a small proportion of the leaf area. The soluble protein fraction profile of such cotyledons was almost identical to the profile of the corresponding fraction from healthy cotyledons (Fig. 4). A week band was found in the area where protein $E_1$ occurred. As in the case of TSWV and CMV-infected cucumber plants, the amount of $E_1$ appeared to be related to the leaf area afflicted with symptoms.

The soluble protein fractions isolated from *N. rustica* leaves following infection with with either TSWV or TMV also exhibited a 'new' protein band of Rf 0.90 on 7.5% polyacrylamide gels. The mobility of this protein differed slightly from that of protein $E_1$ on 7.5% gels (Fig. 1C). The protein from infected *N. rustica* also appeared to possess a carbohydrate moiety as demonstrated by staining with Schiff's reagent. The nature of this protein, its relationship to protein $E_1$, were not investigated further.

## Discussion

A protein component ($E_1$) occurring in the soluble protein fraction of cucumber cotyledons infected with TSWV has been detected. A protein occurring in the soluble protein fraction of cucumber cotyledons infected with CMV is probably the same as protein $E_1$ in view of their identical mobility on both 7.5 and 10% polyacrylamide gels.

The absence of any reaction between the soluble protein fraction from TSWV-infected cotyledons and TSWV antiserum, and the occurrence of the component in

cotyledons infected with either TSWV or CMV indicated that this protein is not related to a virus structural or a virus-coded protein.

The occurrence of a component, with an Rf-value of about 0.90 on 7.5% polyacrylamide gels in the soluble protein fraction of virus infected plants seems to be a general phenomenon. Besides the protein band detected by us in soluble protein fractions from TSWV, CMV and TMV infected cucumber cotyledons and *N. rustica* leaves, a new protein band with an Rf value of 0.94 on 7.5% gels was found by Van Loon and Van Kammen (1970) in *N. glutinosa* following infection with TMV. Ziemiecki and Wood (1976) studying the soluble protein fraction of cucumber cotyledons following infection with two strains of CMV, reported the occurrence of a protein with an Rf value of 0.89–0.90 after electrophoresis on 7% gels. The latter authors could not demonstrate a reaction between this protein and antiserum against purified CMV. This protein seems therefore also unrelated to a virus structural protein. The Rf values of the protein bands measured by the different authors varied somewhat; this may reflect small differences in the conditions of electrophoresis and perhaps also in the size of the carbohydrate moieties, which can differ from plant species to plant species. Kassanis et al. (1974) have analyzed the soluble protein fraction of *N. tabacum* cv Xanthi-nc infected with TMV, CMV and potato virus X. Several new bands were found on 10% polyacrylamide gels; one of these (b2) may be identical to the one mentioned above. The bands found by Kassanis et al. (1974) were also observed after injection of polyacrylic acid into plants.

Since no increase of protein $E_1$ occurred following inoculation of cucumber cotyledons with water, it can be discounted that $E_1$ results from inoculation injury. The possibility that $E_1$ is of viral origin can also be excluded because it occurs after infection with either TSWV or CMV. Protein $E_1$ must, therefore, be coded for by the host genome. Its occurrence in infected leaves can be explained either by an induction or by a stimulation of its synthesis during virus infection. Thus, this protein may be either absent from or may occur in very small amounts in healthy leaves. The presence of a faint band on 10% polyacrylamide gels after analysis of a soluble protein fraction from healthy tissue concentrated with ammonium sulphate supports the latter possibility. Ziemiecki and Wood (1976) also concluded that the synthesis of the new components was stimulated by the virus infection. Van Loon and Van Kammen (1970), and Kassanis et al. (1974), however, believe that the new proteins are induced during the virus infection.

Protein $E_1$ does not appear to have a function in the localization of TSWV since it also occurs following systemic infection with CMV causing a mosaic pattern on the leaves. The biological function of $E_1$ remains to be investigated.

**Samenvatting**

*Het optreden van een oplosbaar eiwit ($E_1$) in komkommercotylen na infectie met het tomatebronsvlekkenvirus en het komkommermozaïekvirus*

In een oplosbare eiwitfractie uit cotylen van komkommer die met bronsvlekkenvirus van de tomaat (TSWV) waren geïnfecteerd werd na analyse op polyacrylamide gels een nieuwe eiwitcomponent ($E_1$) gevonden (Fig. 1). De hoeveelheid $E_1$-eiwit, die op de gels gevonden werd, bleek sterk afhankelijk te zijn van het aantal lesies en hun

afmeting. Door filtratie van de oplosbare fractie over een Sephadex-kolom kon het eiwit gedeeltelijk gezuiverd worden (Fig. 2 en 3). Eiwit $E_1$ bleek een glycoproteïne te zijn en was niet serologisch verwant met een van de structurele eiwitten van TSWV. Na inoculatie met komkommermozaïekvirus (CMV) werd eenzelfde eiwitcomponent gevonden (Fig. 1). Na inoculatie van *N. rustica* met TSWV en tabaksmozaïekvirus (TMV) werd een eiwit gevonden waarvan de beweeglijkheid iets van het eiwit $E_1$ verschilde (Fig. 4). Uit de resultaten kon geconcludeerd worden dat door de virusinfectie in een plant de productie van een niet virusspecifiek eiwit wordt gestimuleerd of geïnduceerd.

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