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SHORT COMMUNICATION

Early trans-plasma membrane responses to Tobacco mosaic virus infection

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Abstract Early trans-plasma membrane behavior after in vivo mechanical inoculation of *Nicotiana tabacum* with Tobacco mosaic virus (TMV) was investigated and compared to virus quantification in leaf tissues. To identify early events related to virus/host interaction, the systemic virus TMV was used to infect lower leaves and tests were carried out on upper leaves which were not directly infected. Non-invasive microelectrodes were used to estimate trans-plasma membrane electron transport and membrane potential after artificial inoculum of virus, monitoring the plant for the following 15 days. Virus infection was assessed by ELISA and quantified by quantitative RT-PCR. Collected data showed that after 2-day post-inoculation (dpi), TMV was able to modify membrane parameters: transient hyperpolarization of trans-membrane potential was observed until 10 dpi, while redox activity in infected samples was higher compared to control until end of tests. Conversely, ELISA diagnostic test was not able to reveal the virus presence in tobacco leaves until 6 dpi, while leaf symptoms were manifested after 13 dpi.

Keywords t-PMEP · t-PMET · TMV

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Introduction

Plasma membrane behavior may reflect health status of animal cells (Ly and Lawen 2003) and viral diseases cause alterations to membrane potential (Akeson et al. 1992; Helenius et al. 1985; Wiley and Skedel 1987). Similar consequences were also observed throughout early stages of recognition between microorganisms and plants (Elmore and Coaker 2011), whereas effects caused by viruses to membrane potential of the host are little investigated. The infection of Tobacco ringspot virus caused alterations in trans-membrane potentials of cells of *Vigna sinensis*, altering their capability to restore their constitutive status after a treatment with the metabolic inhibitor sodium azide in comparison to control cells (Stack and Tattar 1978). Variations in ion fluxes that characterized the initial step in the signal transduction pathway caused by Tobacco mosaic virus or Papaya mosaic virus infections were also found in in vitro treated protoplasts of *Gomphrena globosa* (Schwarzstein 1997). A significant step forward was achieved by Shabala et al. (2010); they demonstrated that changes in K^+ fluxes occurs within minutes since mesophyll portions without cuticle are incubated with Potato virus X suspension. This approach represent an useful method to recognize virus–plant matching during first stages of infections, but no correlation with virus inoculum can be estimated due to the infection techniques. Electrophysiological trials performed on leaves of naturally diseased grapevines showed how virus infection may cause differences in the

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trans-plasma membrane potential (t-PMEP) and how this response is quite constant in plant with settle infection (Rinaldelli et al. 2014; Panattoni et al. 2015). Experiments carried out applying chemicals on *Nicotiana tabacum* infected by Cucumber mosaic virus showed as the pathogen can change the t-PMEP of the host (Rinaldelli et al. 2012). The higher activity of trans-plasma membrane electron transport (t-PMET) observed in ampelovirus- or nepovirus-infected grapes suggests that viral pathogens can also modify the redox homeostasis (Rinaldelli et al. 2014). However, these findings indicate interactions between viral pathogen and the trans-plasma membrane activities of hosts during settle virus infections.

The objective of this work is to look for the t-PMEP and t-PMET reactions during early stage of virus infection in in vivo plants, in which health status was monitored by diagnostic tests before symptoms appearance. Tobacco mosaic virus (TMV) was selected as viral pathogen to test because it is a common plant virus which management is notoriously hard (Panattoni et al. 2013a; Luvisi et al. 2012a, 2015).

Materials and methods

TMV and plant materials

An isolate of TMV was maintained in tobacco plants (*N. tabacum* L. cv. Turkish) in insect-free greenhouse environment following common cultural practices. The tobacco plants, employed as the inoculum source of pathogen, were infected by a single isolate of TMV type-strain identified by molecular test (Luvisi et al. 2011). Leaves of diseased or healthy tobacco plants were grinded using 0.03 M phosphate buffer at pH 7.2 (1:4 w/v, g/mL) to obtain crude sap. In vivo infections were carried out via mechanical inoculation of crude sap from infected plants or healthy one (mock inoculation) to healthy tobacco plants in growth stage 1104 (Cooperation Centre for Scientific Research Relative to Tobacco 2009), characterized by development of four leaves. One leaf of each lower node was inoculated (inoculated leaf). Tests were carried out on leaves of upper node (systemic leaves), where first half of the leaf was used for diagnostic assay (ELISA) and second half of the leaf for quantitative RT-PCR (qRT-PCR), while the remaining one was used for electrophysiological test. Five plants inoculated with infected crude sap and five control plants (mock inoculation) were sampled every 24 h for 15 days after infection.

Trans-plasma membrane assays

Carbon fiber microelectrodes (CFME) (point diameter 5 µm) (Carbostar-1, Kation Scientific, USA) were employed as indicated by Rinaldelli et al. (2012) for the detection of ions in solution. The microelectrodes were firstly calibrated to evaluate their linear response to redox chemicals. The microelectrodes were plunged into recording solution (RS) composed by of 5×10^{-4} M potassium ferricyanide ($K_3[Fe(CN)_6]$) (PF) set to pH 5.6 with MES [2-(*N*-Morpholino) ethanesulfonic acid], whereas the amount of potassium ferrocyanide ($K_4[Fe(CN)_6]$) was increased from 0 to 20, 50, and 100 µM. With every shift of potassium ferrocyanide amount, the potential of the CFME was set at +400 mV to observe the oxidation current in the solution. To evaluate the cell reductase activity, which was defined as concentration of ferrous ($[Fe^{2+}]$), calibration curves were employed (Rinaldelli et al. 2012). The microelectrode was set at 5 µm above leaf exposed tissues (Taylor and Chow 2001). The setting up of tissues and the detection methods for redox measurements were carried out as indicated in literature (Rinaldelli et al. 2012). To measure steady t-PMET values, the tobacco samples were kept in RS solution for at least 5 min of constant oxidation current before recording the value.

To collect t-PMEP data, leaf portions were set up as reported by Luvisi et al. (2012b). The samples were preincubated for 1 h in basal solution (BS) (Panattoni et al. 2013b) set to pH 5.6 using TRIS (2-amino-2-hydroxymethyl-propane-1,3-diol). BS was constantly aerated permitting to flood the test chamber at a constant flux speed of 10.0×10^{-3} L min^{-1}. Micropipettes (point diameter <1 µm) were fabricated from single-barreled borosilicate capillaries (World Precision Instruments, Sarasota, USA) and employed as measuring electrodes (Rinaldelli et al. 2012). The penetration of the micropipettes was done in the median area of the mesophyll using a micromanipulator to accomplish the procedure. The adequate penetration of micropipette was achieved after 5 min of stabilization of membrane potential (Ober and Sharp 2003). Experimental trials were implemented at 22 °C (±0.5), in the light (30 W m^{-2}). Measurements were performed under Faraday cage.

TMV assay

Immunoenzymatic assays (ELISA) were performed employing commercial detection kits for TMV (Loewe Biochemica, Germany). Leaf portions excised from diseased or pathogen-free tobacco plants were collected and employed in tests as positive and negative reference samples, respectively. Absorbance values were normalized as

R values (OD-infected explant/OD-healthy control). The value of 2.0 of *R* was set as limit-value to discern a positive versus a negative sample (Luvisi et al. 2012a).

TMV quantity, defined as TMV genome equivalents per tobacco μg RNA, was evaluated by qRT-PCR employing a CFX96 Real-Time thermocycler (Bio-rad, USA), according to Edelbaum et al. (2009). In the assay, we employed the fluorescent dye SYBR Green I and tobacco β-actin genomic DNA as reference gene. Specific primers by Edelbaum et al. (2009) were used to amplify 173 bp segment. Quantitative molecular tests were carried out on 70 ng of tobacco total RNA. The PCR was set to achieve 15 min activation at 95 °C, 45 cycles of 20 s at 95 °C, 30 s at 60 °C, 30 s at 72 °C, and 20 s at 82 °C (melting: ramp from 60 to 99 °C with 1 °C of temperature increase every 5 s). Threshold cycle value (Ct) lower than 35 were used to identify infected samples.

Serial dilutions (1:10) of purified TMV were used to build up standard curves employed to appraise the quantity of TMV in the diseased tobaccos. Viral particles were purified from diseased *N. tabacum* plants in which leaves (50 g of tissue) were sampled 3 weeks after artificial infection (Uhde-Holzem et al. 2010). According to Rettcher et al. (2015), polyethylene glycol (PEG) precipitation method was performed as reported by Uhde-Holzem et al. (2010) omitting the sucrose cushion centrifugation to avoid losses in pathogen amount. Following the sucrose gradient step, selected fractions were pooled, followed by centrifugation at 248,000×*g* for 3 h. The TMV amount in the final sample was assessed by reading at wavelength of 260 nm (OD_{260}) applying the viral extinction coefficient of 3.0. The viral presence was measured in three different hosts repeating the measurements three times for each plant (three biological and three technical repeats).

Statistical analysis

Absorbance, [Fe^{2+}] and membrane potential were elaborated using Sigma-Plot software (version 11; Systat Software, San Jose, CA, USA). The software was used to perform *t* test. $P < 0.05$ was considered to be significant.

Results

t-PMEP values are reported in Fig. 1a. Stress conditions may lead to increase impalement attempts (Rawyler et al. 2002), but no significant increase was observed in infected samples. At 2-day post-inoculation (dpi) systemic leaves of infected plants showed significant increment in hyperpolarization compared to control (up to 17.7% of increase at 5 dpi compared to control) but no changes in t-PMEP measures were detected in comparison to healthy leaves after 10 dpi, confirming the similar behavior observed in virus-infected grapevines when infection was settled (Rinaldelli et al. 2014). Because the membrane potential gradient is consequence of H^+-ATPase work (Sondergaard et al. 2004), diseased cells could be regarded as more energized of control cells at first stage of infection. Concerning the membrane electron transport (Fig. 1b), the redox values measured by the microelectrode were linear in relation to the increase of potassium ferrocyanide amount (while a steady concentration of 500 μM ferricyanide was maintained), observing biggest activity at 5 μm (data not shown), in accordance to the data showed by Taylor and Chow (2001) for *Zea mais* roots. In virus-infected tobacco the [Fe^{2+}] produced by tissues of systemic leaves of infected plants increase significantly after 2 dpi, and difference were maintained during the whole monitored period, showing higher t-PMET activity compared to control (33.3–94.7% of increment).

qRT-PCR showed higher analytical sensitivity compared to ELISA test (Fig. 2). Even if some samples from infected plants showed a different behavior compared to control plants since 8 dpi, ELISA reading of samples collected from inoculated plants overtake the positive threshold at 9 dpi ($R = 2.1 \pm 0.4$) (Fig. 2a). Conversely, using qRT-PCR, Ct lower than 35 (31.1 ± 2.1) were observed at 6 dpi (Fig. 2b). The quantity of TMV at 6 dpi, esteemed as virus genome equivalents per tobacco μg RNA, was 1.841. However, at 6 or 9 dpi, plants were symptomless regardless positive diagnostic tests. Following inoculation with TMV, the tobacco plants presented symptoms at 13 dpi in inoculated plants, with ELISA readings of 2.8 ± 0.4, Ct value of 23.9 ± 2.7 and TMV genome equivalents of 17.845.

Discussion

Throughout initial stages of the host infection caused by TMV, the coat protein of TMV can behave as an elicitor for the plant who responds with alteration in membrane ion flow rates: a signal transduction cascade observed in various virus–host combinations (Schvarzstein 1997). In the case of TMV, a disease that affects the host as a whole, the virus moves from diseased cells towards cells not yet infected, and the further systemic transfer is regulated by various physiological responses that involve several pathogen and host factors (Sholthof 2005). Furthermore, as reported by Atkinson et al. (1996), interactions with the plant cell membrane and with proteins associated to cytoskeleton can cause alterations in ion flow rates and in transduction pathway signals. With regard to membrane electron transport, the redox homeostasis of cells is deeply bound to the amount of NAD^+ and NADH (Del Principe

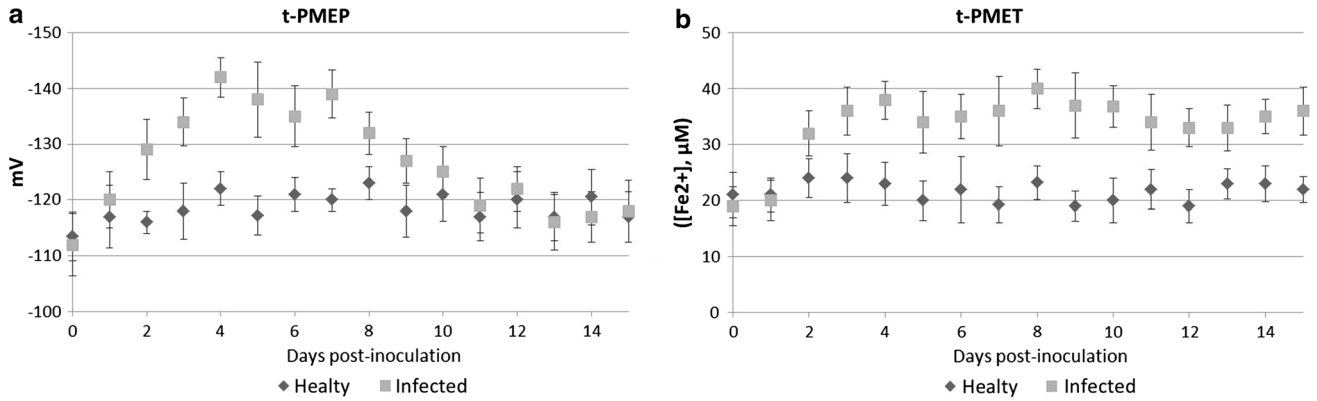

Fig. 1 **a** Trans-plasma membrane potential (t-PMEP, mV) and **b** electron transport (t-PMET, $[Fe^{2+}]$, µM) of systemic leaves of *Nicotiana tabacum* infected by TMV

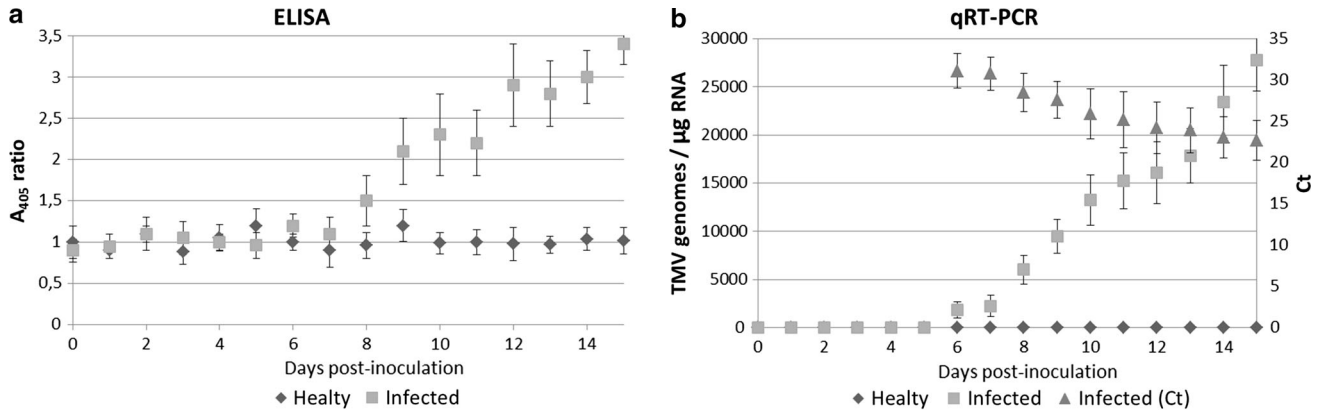

Fig. 2 Health status of systemic leaves of TMV infected *Nicotiana tabacum* plants compared to untreated one, assayed with **a** ELISA test and **b** qRT-PCR. *Ct* cycle threshold

et al. 2011; Gray et al. 2011), which ratio is determined by movement of electrons from cellular NADH throughout plasma membrane to an external electron acceptor. In human trials, Herst and Berridge (2006) reported that a trans-membrane changes can act as signaling system capable to regulate the metabolism of host cells and it can change various cell functions such as cell redox homeostasis and protection against diseases. Our data suggest an interaction between TMV and trans-plasma membrane behavior, not only once the viral infection was settle, but since first stages after inoculum. Trans-plasma membrane seems to be very sensitive to virus accumulation and presence and TMV-effects were revealed before positive responses of health assays. The occurrence of fast changes of ion flow rates in plant cells at the initial stage of viral infection was described for green algae (Neupartl et al. 2008), tobacco, tomato, potato, periwinkle, and sugar beet (Shabala et al. 2010). In addition, Shabala et al. (2010) point out the relevance of membrane behavior in initial events of virus infection process and, particularly, in host reactions to the pathogen attack. In these tests, perceptible amounts of virus were present during interaction with host cell which respond after minutes since incubation. In tests reported in this paper, we simulate a natural infection process, in which just a part of plant (the infected leaf) was inoculated. TMV needs time to systematically invade the whole plant (as well as the systemic leaves) and for some days post inoculation (in our test condition the number was 5 dpi) non-inoculated leaves were negative to ELISA and qRT-PCR, generating a temporary false negative test result, while membranes of host cells seem to react earlier to virus present within the plants, transiently (t-PMEP) or constantly (t-PMET).

Conclusion

This work should be complemented by future studies that will examine if such effects in the distal part of the plant (where the virus is not detected) are due to the virus itself (perhaps already present but under detection limit), or to the virus replication in the inoculated leaves, or eventually

to the plant defense mechanisms. As reviewed by Liu and Nelson (2013), the virus replication and its diffusion among cells is dependent to complexes composed by pathogen and plant proteins and viral RNA. The role of various plant membranes is also crucial in virus amass, whereas virus transport is helped by membranes, microfilaments or microtubules. However, changes that imply alterations of the ions concentrations, increases of reactive oxygen species (ROS), trigger of kinase cascades, alteration of the plant hormones cannot be excluded as factors responsible for such membrane alterations.

Author contribution statement Conceived and designed the experiments: AL, ER, and AP. Performed the electrophysiological experiments: ER and AP. Performed the inoculation experiments and diagnostic assay: AL, AM, ES, AA and DR. Analyzed the data: ES. Contributed reagents/materials/analysis tools: AA, ES. Wrote the paper: AL and LDB. Edited the paper: ER and AM.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

References

Akeson M, Scharff J, Sharp CM, Neville DM (1992) Evidence that plasma membrane electrical potential is required for vesicular stomatitis virus infection of MDCK cells: a study using fluorescence measurements through polycarbonate supports. J Membr Biol 125:81–91

Atkinson MM, Midland SL, Keen NT (1996) Syringolide 1 triggers ca2+ influx, K^+ efflux, and extracellular alkalization in soybean cells carrying the disease-resistance gene Rpg4. Plant Physiol 112:297–302

Cooperation Centre for Scientific Research Relative to Tobacco (2009) A scale for coding growth stages in tobacco crops. https://www.coresta.org/sites/default/files/technical_documents/main/Guide-No07-Growth-Stages_Feb09_0.pdf. Accessed 31 Aug 2017

Del Principe D, Avigliano L, Savini I, Catani MV (2011) Trans-plasma membrane electron transport in mammalian: functional significance in healthy and disease. Antioxid Redox Sign 14:2289–2317

Edelbaum D, Gorovits R, Sasaki S, Ikegami M, Czosnek H (2009) Expressing a whitefly GroEL protein in *Nicotiana benthamiana* plants confers tolerance to tomato yellow leaf curl virus and cucumber mosaic virus, but not to grapevine virus A or tobacco mosaic virus. Arch Virol 154:399–407

Elmore JM, Coaker G (2011) The role of the plasma membrane H^+-ATPase in plant-microbe interactions. Mol Plant 4:416–427

Gray JP, Eisen T, Cline GW, Smith PJS, Heart E (2011) Plasma membrane electron transport in pancreatic β-cells is mediated in part by NQO1. Am J Physiol Endovasc 301:113–121

Helenius A, Kielien M, Wellsteed J, Mellman L, Rudnick G (1985) Effect of monovalent cations on Semliki Forest virus entry into BHK-21 cells. J Biol Chem 260:5691–5697

Herst PM, Berridge MV (2006) Plasma membrane electron transport: a new target for cancer drug development. Curr Mol Med 6:895–904

Liu C, Nelson RS (2013) The cell biology of Tobacco mosaic virus replication and movement. Front Plant Sci 4:12

Luvisi A, Panattoni A, Triolo E (2011) Thiopurine prodrugs for plant chemotherapy purposes. J Phytopathol 159(5):390–392

Luvisi A, Panattoni A, Triolo E (2012a) Eradication trials of tobacco mosaic virus using chemical drugs. Acta Virol 56:155–158

Luvisi A, Rinaldelli E, Panattoni A, Triolo E (2012b) Membrane transport of antiviral drugs in plants: an electrophysiological study in grapevine explants infected by Grapevine leafroll associated virus 1. Acta Physiol Plant 34:2115–2123

Luvisi A, Panattoni A, Materazzi A (2015) Heat treatments for sustainable control of soil viruses. Agron Sustain Dev 35:657–666

Ly JD, Lawen A (2003) Transplasma membrane electron transport: enzymes involved and biological function. Redox Rep 8:3–21

Neupartl M, Meyer C, Woll I, Frohns F, Kang M, Van Etten JL, Kramer D, Hertel B, Moroni A, Thiel G (2008) Chlorella viruses evoke a rapid release of K^+ from host cells during the early phase of infection. Virology 372:340–348

Ober ES, Sharp RE (2003) Electrophysiological responses of maize roots to low water potential: relationship to growth and ABA accumulation. J Exp Bot 54:813–824

Panattoni A, Luvisi A, Triolo E (2013a) Elimination of viruses in plants: twenty years of progress. Span J Agric Res 11(1):173–188

Panattoni A, Rinaldelli E, Triolo E, Luvisi A (2013b) In vivo inhibition of trans-plasma membrane electron transport by antiviral drugs in grapevine. J Membr Biol 246:513–518

Panattoni A, Rinaldelli E, Materazzi A, Luvisi A (2015) Modulation of viral infection in plants by exogenous guanosine. Acta Physiol Plant 37(11):226

Rawyler A, Arpagaus S, Braendle R (2002) Impact of oxygen stress and energy availability on membrane stability of plant cells. Ann Bot 90:499–507

Rettcher S, Jungk F, Kuhn C, Krause HJ, Nolke G, Commandeur U, Fischer R, Schillberg S, Schroper F (2015) Simple and portable magnetic immunoassay for rapid detection and sensitive quantification of plant viruses. Appl Environ Microb 81:3039–3048

Rinaldelli E, Panattoni A, Luvisi A, Triolo E (2012) Effect of mycophenolic acid on trans-plasma membrane electron transport and electric potential in virus-infected plant tissue. Plant Physiol Biochem 60:137–140

Rinaldelli E, Luvisi A, Panattoni A (2014) Virus interference with trans-plasma membrane activity in infected grapevine leaves. Acta Physiol Plant 36(12):3345–3349

Schvarzstein M (1997) Changes in host plasma membrane ion fluxes during the *Gomphrena globosa* Papaya Mosaic Virus interaction. MSc Thesis, Department of Botany, University of Toronto, Canada

Shabala S, Babourina O, Rengel Z, Nemchinov LG (2010) Non-invasive microelectrode potassium flux measurements as a potential tool for early recognition of virus-host compatibility in plants. Planta 232:807–815

Sholthof H (2005) Plant virus transport: motions of functional equivalence. Trends Plant Sci 10:376–382

Sondergaard TE, Schulz A, Palmgreen MG (2004) Energization of transport processes in plants. Role of the plasma membrane H^+-ATPase. Plant Physiol 136:2475–2482

Stack JP, Tattar TA (1978) Measurement of transmembrane electropotentials of *Vigna sinensis* leaf cells infected with tobacco ringspot virus. Physiol Plant Pathol 12:173–178

Taylor AR, Chow RH (2001) A microelectrochemical technique to measure trans-plasma membrane electron transport in plant tissue and cells in vivo. Plant Cell Environ 24:749–754

Uhde-Holzem K, Schloesser V, Viazov S, Fischer R, Commandeur U (2010) Immunogenic properties of chimeric potato virus X particles displaying the hepatitis C virus hypervariable region I peptide R9. J Virol Methods 166:12–20

Wiley DC, Skedel JJ (1987) The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annu Rev Biochem 56:365–394