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Effects of modulation of potassium channels in tobacco mosaic virus elimination

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ABSTRACT

To evaluate the effects on modulation of K^+ channel in antiviral treatments, we report the application of potassium channel blockers (PCBs) tetraethylammonium chloride (TEA) and cesium chloride (Cs) in *in vitro* plantlets or callus cultures infected by Tobacco mosaic virus (TMV). The effect of PCBs were evaluated in: a) *in vitro* TMV-infection trials, where PCB-treatments were applied during infection process of healthy plantlet; b) chemotherapy trials, where PCB-treatments were applied in combination to antiviral drugs on TMV-infected plantlets; c) in antiviral trials using callus culture and sieving technique. Both PCBs were able to reduce relative concentration of virus [calculated as Fold Changes (FC) relative to control samples] during infection process, with major effect observed at 7 dpi and in medium or upper portions of plantlet. Positive effects of PCBs were also observed following chemotherapy trials, where TEA significantly improves antiviral effectiveness of the antiviral drugs, causing higher FC compared to that obtained with just chemotherapy treatment since 3th subculture. Nevertheless, plantlets were positive to TMV up to 6th subculture, thus virus elimination was not achieved). Finally, the callus culture and sieving technique lead to 11.1 ± 3.8% of TMV-negative plantlets, while TEA-treated mother-calli lead to 22.2 ± 3.8% of negative plantlets. Concluding, findings suggest that modulation of K^+, previously indicated as essential in early stages of viral infection, could be also involved in antiviral techniques.

1. Introduction

In animal cells, ion channels are emerging as key factors required during virus replicative cycles, and have been assigned critical roles in virus entry, survival, and release [1,2,3]. The selective transport of K^+ is involved in many physiological functions, including homeostasis of the membrane potential and the repolarization of the action potential in excitable cells [4]. Studies in *Chlorella* lead also to suggest that a viral-encoded K^+ protein might be the evolutionary ancestor of all K^+ channel proteins [5]. The importance of K^+ channel activity during virus lifecycle was confirmed in virus families such as *Bunyaviridae* [6] and its role was also investigated in plants. In protoplast of *Gomphrena globosa* artificially infected by Papaya mosaic virus and Tobacco mosaic virus changes in average ion currents were observed, identifying an early event in the signal transduction pathway related to virus/host interaction [7]. The central role of K^+ channel activity during early stage of infection was observed by [8]. Excised mesophyll segments with removed epidermis were placed peeled side down directly on drop of purified Potato virus X suspension and changes in K^+ fluxes occurred within minutes from viral incubation. Unfortunately, the use of potassium channel blocker (PCB) to the leaf alongside virus did not ameliorate infection symptoms after artificial foliar inoculation [8] but no data about effect of PCB on virus movement and replication in tissues next to the infection sites was reported. The phenomenon of rapid K^+ release from host cells during the early phase of viral infection was also reported in infection of *Chlorella* cells by Paramecium bursari chlorella virus [9].

In order to evaluate the modulation of K^+ channels in antiviral treatments, we report the effects of PCBs such as tetraethylammonium chloride (TEA) or cesium chloride (Cs) in *in vitro* systemic infection of Tobacco mosaic virus (TMV), one of most studied viruses which pathosystem still serves, as reported by [10]; as an excellent model for dissecting various aspects of plant–virus interactions. TEA [11,12] and Cs [13,14] are blockers of ionic channels and their application decreased plant K^+ uptake compared with the control treatment [15]. The effect of PCBs were evaluated in: a) *in vitro* TMV-infection trials, where

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PCB-treatments were applied during infection process of healthy plantlet; b) chemotherapy trials, where PCB-treatments were applied in combination to antiviral drugs on TMV-infected plantlets; c) in antiviral trials using callus culture and sieving technique.

2. Materials and methods

2.1. TMV production, purification and assay

A collection of virus isolates of TMV was maintained in tobacco plants (*Nicotiana tabacum* cv. Turkish) under greenhouse conditions (18-24 °C), insect free. The plants, infected by an isolate of virus type-strain identified by RT-PCR [16], were used as the inoculum source of viruses. Viral particles were purified from diseased *N. tabacum* plants form leaves (50 g of tissue) [17]. According to [18]; polyethylene glycol (PEG) precipitation method was performed as reported by [17] omitting the sucrose cushion centrifugation to limit losses in TMV amount [19]. 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. TMV quantity in plantlets or callus culture was esteemed by qRT-PCR employing a CFX96 Real-Time thermocycler (Bio-rad, USA), following [20]. TMV primers designed by [20] were used to amplify 173 bp segment. Quantitative PCR assays were carried out on 70 ng of tobacco total RNA. The PCR was set in order 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) was set at 35 to discriminate between healthy or TMV-infected samples. Prior to use purified TMV as inoculum for *in vitro* plantlets or callus culture, the virus was diluted in sterile 0.01 M sodium phosphate buffer.

2.2. In vitro plantlets

In vitro tobacco plantlets were obtained following [21]. Nodes from healthy or TMV-infected plants were collected and surface sterilized before transfer to aseptic culture tubes with [22] growth medium. Plantlets were maintained in a controlled environment chamber that assured maintenance of health condition, with a temperature regime of 22 ± 1 °C, 16 h photoperiod, 50 $\mu Em^{-2} s^{-1}$; plantlets were transferred to fresh growth medium at 14-day intervals. After an acclimatization period of two months, the health condition of each plantlet was confirmed by qRT-PCR. Plantlets with three nodes were used to evaluate the effect of PCBs in *in vitro* TMV-infection trials, where PCB-treatments were applied during infection process of healthy plantlet and in chemotherapy trials, where PCB-treatments were applied in combination to antiviral drugs on TMV-infected plantlets.

2.3. Effect of PCB in in vitro TMV-infection trials

Effects of PCBs in *in vitro* TMV-infection trials were evaluated using the following growth media: TMV medium (MS medium with 4.5 $\mu g\ mL^{-1}$ of TMV); TMV-TEA 20/40 (MS medium with 4.5 $\mu g\ mL^{-1}$ of TMV and 20 or 40 mM of TEA); TMV-Cs 10/20 (MS medium with 4.5 $\mu g\ mL^{-1}$ of TMV and 10 or 20 mM of Cs); TEA 20/40 (MS medium with 20 or 40 mM of TEA); Cs 10/20 (MS medium with 10 or 20 mM of Cs); MS medium. Healthy explants were cultivated in a 2-step process. In the first step (inoculation), the plantlet were transferred on TMV-PCB (Fig. 1A) or TMV-medium (Fig. 1B), and cultured for 24 h. After infection treatment (second step of *in vitro* culture), plantlets were transferred to PCB medium or MS medium for 14 days of growth. All experiments were performed in triplicate; each experiment consisted of 15 *in vitro* plantlets.

2.4. Effect of PCB in chemotherapy trials

For chemotherapy trials, *in vitro* tobacco explants were obtained from artificially TMV-infected *Nicotiana tabacum* L. cv. Turkish. Antiviral chemicals such as ribavirin (RB) [23,24] and mycophenolic acid (MPA) [25–28] (Sigma–Aldrich, St. Louis, MO) were tested individually against TMV in TEA-addicted (20 mM) growth medium (Fig. 1C). Growth media with antiviral chemicals without TEA or with TEA only were used as control (Fig. 1C). Drugs were hydrated in stock solution and, immediately before use, ultra-filtered and added to proliferation medium after sterilization (0.10 mM for RB and 0.40 mM for MPA) [29]. The experimental design involved growth of infected plantlets for six consecutive subcultures (each one 14-days long). After each subculture, the apical portion (1 cm) of each plantlet was transferred to fresh growth medium and the residue was assayed by qRT-PCR [30]. All experiments were performed in triplicate; each experiment consisted of 15 *in vitro* plantlets.

2.5. Effect of PCB in callus culture and sieving technique

Following [31]; callus cultures were initiated from virus-infected leaves and grown on control medium containing MS salts, Gamborg's B5 vitamins [32], 30 g/l sucrose, 1 mg/l benzylaminopurine, 0.1 mg/l naphthaleneacetic acid, and 8 g/l phytoagar. The treatment medium differs from control medium by adding TEA (20-40 mM). Calli were grown for 8 weeks in a growth chamber under the conditions described above for plant growth until they reached a size > 1 cm. TMV-infected callus (mother callus) was divided in small callus particles (micro-calli) using the Cell Dissociation Sieve (Sigma-Aldrich, Inc. St. Louis, MO) under sterile conditions (Fig. 1D). As reported by [31] the micro-calli (250 μm in diameter) were transferred for callus proliferation to semi-solid media (3 g/l phytoagar). After 6 weeks, both control calli than TEA-treated calli were transferred solid MS media for further growth. Shoot induction occurred within 3–4 weeks of culture and health status was checked. All experiments were performed in triplicate; each experiment consisted of 15 micro-calli.

2.6. Virus assay

Sampling was carried out during the following 7 and 14 days after transplanting, considering tissues (stem and leaves) of the lower, medium or upper node. For the lower node analysis, portion of stem tissues in contact with medium was removed prior analysis. TMV extraction and quantification was achieved following [20]. Virus amounts were determined by qRT-PCR using a CFX96 Real-Time thermocycler (Bio-rad, USA), coupled with the DNA-minor-groove binding fluorescent dye SYBR Green I. Relative quantification was calculated using the comparative cycle threshold (Ct) method in which the change in the amount of the target viral RNA was normalized in relation to the level of infection of control plantlets, chosen as a baseline. Thus, the relative level of infection of the treated plantlet was calculated as Fold Changes (FC) relative to control samples. Specific primers by [20] were used to amplify 173 bp segment. qRT-PCR was performed using 70 ng of plant total RNA. The cycling conditions were 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, rising by 1 °C each step, waiting 5 s for each step thereafter).

2.7. Statistical analysis

Data were elaborated using Sigma-Plot software (version 11; Systat Software, San Jose, CA). The software was used to perform analysis of variance (ANOVA). To evaluate differences in virus concentration, T-test was used to compare fold changes between treated or untreated plantlets. Data expressed in percent were converted in arcsin values. $P \leq 0.05$ was considered to be significant.