

# Plant Cell Death Caused by Fungal, Bacterial, and Viral Elicitors: Protective Effect of Mitochondria-Targeted Quinones

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**Abstract**—Chitosan (partially deacetylated chitin), a component of fungal cell walls, caused epidermal cell (EC) death in the leaves of pea (*Pisum sativum* L.) and tobacco *Nicotiana tabacum* or *Nicotiana benthamiana* detected by destruction of cell nuclei. The mitochondria-targeted quinone SkQ1 prevented the destruction of EC nuclei induced by chitosan. Chitosan increased and SkQ1 suppressed the activity of protein kinases in *N. benthamiana* and *P. sativum* and eliminated the effect of chitosan. Chitosan induced the generation of reactive oxygen species (ROS) in the guard cells (GC) of pea plants. Treatment with chitosan or H<sub>2</sub>O<sub>2</sub> did not cause destruction of GC nuclei; however, it resulted in disruption of the permeability barrier of the plasma membrane detected by propidium iodide fluorescence. Treatment with bacterial lipopolysaccharide but not peptidoglycan caused destruction of pea EC nuclei, which was prevented by SkQ1. Leaves of tobacco plants containing the *N* gene responsible for resistance to tobacco mosaic virus (TMV) were infiltrated with *Agrobacterium tumefaciens* cells. These cells contained a genetic construct with the gene of the helicase domain of TMV replicase (*p50*); its protein product *p50* is a target for the *N*-gene product. As a result, the hypersensitive response (HR) was initiated. The HR manifested itself in the death of leaves and was suppressed by SkQ3. Treatment of tobacco epidermal peels with the *A. tumefaciens* cells for the *p50* gene expression stimulated the destruction of EC nuclei, which was inhibited by SkQ1 or SkQ3. The *p50*-lacking *A. tumefaciens* cells did not induce the destruction of EC nuclei. The protective effect of mitochondria-targeted antioxidants SkQ1 and SkQ3 demonstrates the involvement of mitochondria and their ROS in programmed cell death caused by pathogen elicitors.

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**Key words:** programmed cell death, mitochondria-targeted quinones, chitosan, lipopolysaccharide, peptidoglycan, tobacco mosaic virus, guard cells, epidermal cells

The strategy of innate immunity in humans and animals is as follows: cell receptors recognize the conservative molecular structures of large groups of pathogens, i.e. pathogen-associated molecular patterns (PAMPs) [1, 2]. Plant innate immunity has three branches: 1) basal (horizontal) resistance induced by PAMPs (PAMP-triggered

immunity, PTI) or DAMPs (damage-associated molecular patterns); 2) vertical resistance induced by pathogen effectors (effector-triggered immunity, ETI), and 3) RNA interference or gene silencing, i.e. suppression of the expression of viral and nonviral pathogen-related genes [3-7].

**Abbreviations:** DCF, 2',7'-dichlorofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; EC, epidermal cells; ETI, effector-triggered immunity; GC, guard cells; HR, hypersensitive response; LPS, lipopolysaccharide; PAMP, pathogen-associated molecular patterns; PCD, programmed cell death; PG, peptidoglycan; PI, propidium iodide; PTI, PAMP-triggered immunity; ROS, reactive oxygen species; SkQ1, 10-(6'-plastoquinonyl)decyl triphenylphosphonium; SkQ3, 10-(6'-methylplastoquinonyl)decyl triphenylphosphonium; TMV, tobacco mosaic virus.

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PAMPs include external molecular structures of fungi and bacteria located on the surface of their cells: fungal chitin, lipopolysaccharide (LPS) of the outer membrane of Gram-negative bacteria, peptidoglycan (PG) of bacterial cell walls, and the protein of bacterial flagella (flagellin). DAMPs are the compounds released from a plant under the influence of hydrolytic enzymes of the pathogen. Pathogen effectors are the agents that disturb the PTI-activating system of signal transduction from plant cell receptors interacting with PAMPs or realization of immune response in PTI. Their recognition in ETI is ensured by *R* (resistance)-genes [3, 5, 7, 8].

An example of ETI is the interaction between tobacco mosaic virus (TMV) and a tobacco plant. The resistance gene in TMV-resistant types of tobacco is the *N*-gene, which encodes the protein responsible for recognition of the effector – the helicase domain of TMV replicase (p50 protein) [7, 9].

PAMPs, DAMPs, and pathogen effectors are combined by the term “elicitors”. They are the agents inducing immune response in plants. An important element of the immune system is hypersensitive response (HR), when cell death is initiated in the place of pathogen penetration and the resultant barrier of dead cells prevents the spread of infection in plant tissues. Some features of HR-related cell death allow it to be considered as a specific form of programmed cell death (PCD) typical of plants, together with its main forms – vacuolar cell death and necrosis [10-12].

Some researchers believe that the PTI-activating PAMP recognition does not lead to the development of HR accompanied by cell death in an infected plant. They believe that PCD is initiated at the next stage of immune response as a result of interaction between the *R*-gene products and pathogen effectors (ETI), with a stronger immune response of a plant compared to PTI [10, 13, 14].

HR is accompanied by an “oxidative burst”, i.e. intensive reactive oxygen species (ROS) production by plant cells. ROS ( $O_2^-$ ,  $H_2O_2$ ,  $OH^\cdot$ ) are strong oxidants that can react with different cell components (lipids, DNA, proteins), disturbing their functions and causing cell death in both pathogen and plant. ROS production during HR occurs as a result of activation of NADPH oxidase of the plant plasma membrane, as well as with the involvement of the cell wall peroxidase [15-18].

Mitochondria play a key role in apoptosis, one of PCD forms typical of animal cells. On one hand, the following proteins initiating or promoting cell death are released from mitochondria – cytochrome *c*, flavoprotein AIF (apoptosis inducing factor), Smac/DIABLO (second mitochondrial activator of caspases/direct IAP binding protein with low pI), serine protease HtrA2/Omi, and endonuclease G [19]. On the other hand, the mitochondrial respiratory chain generates dangerous for the cell ROS, which cause apoptosis [20, 21]. ROS are also generated by plant mitochondria [22].

Can PCD during HR in plants be regulated by mitochondria and ROS produced in them? Plant cell death, including that during HR, depends on the redox state of ubiquinone in mitochondria and is suppressed by mitochondrial alternative oxidase, which catalyzes electron transfer from ubiquinol to  $O_2$  bypassing respiratory chain complexes III and IV (activation of the alternative oxidase inhibits PCD) [23-26].

Mitochondria-targeted quinones have been synthesized that contain a ubi- or plastoquinone and its derivatives covalently bound to membrane-penetrating cations via a decane or pentane linker. They include 10-(6'-plastoquinonyl)decyl triphenylphosphonium (SkQ1) and 10-(6'-methylplastoquinonyl)decyl triphenylphosphonium (SkQ3). Uptake along the gradient of the membrane potential ( $\Delta\psi$ ) allows the accumulation of these compounds in energized mitochondria [27, 28]. Mitochondria-targeted quinones at low (nanomolar) concentrations demonstrated antioxidant properties and protected animal cells from ROS-induced death [29]. They can be used as a tool for studying the role of mitochondrial ROS in PCD.

The goal of this work was to test the elicitors of various pathogens (fungi, bacteria, and viruses) as PCD inducers in plants. The involvement of mitochondrial ROS in plant cell death was assessed by studying the effects of mitochondria-targeted quinones SkQ1 and SkQ3 on PCD caused by pathogen elicitors.

## MATERIALS AND METHODS

The experiments were performed with the *Nicotiana tabacum* cv. Samsun NN tobacco leaves carrying the *N*-gene and with the epidermal peels isolated from the lower leaf surface of *N. tabacum* and *N. benthamiana* or the pea (*Pisum sativum* L.). The plants were grown in daylight or under periodic illumination with fluorescent lamps or a metal-halide lamp of  $\sim 100 \mu E \cdot m^{-2} \cdot s^{-1}$  (light, 16 h; darkness, 8 h) at 23-28°C. Epidermal peels were separated from the leaves with forceps and put into distilled water; compositions of the reagents added to them are given in figure captions. Chitosan treatment was performed by mixing epidermal peels in chitosan suspension on a magnetic stirrer. Epidermal peels were incubated with the reagents in polystyrene plates at 22-25°C.

After the incubation, the epidermis was treated with Battaglia fixative (chloroform, 96% ethanol, glacial acetic acid, and 40% formalin, 5 : 5 : 1 : 1 v/v) for 5 min, washed with ethanol for 10 min to remove the fixative, incubated in water for 5 min, and stained with Carazzi's hematoxylin for 20 min. The stained epidermal peels were washed with tap water and examined under a light microscope. The portion of the cells with destroyed nuclei and without nuclei was determined per 300-500 of studied cells (in 3-4 epidermal peels). The experiments were

repeated 2-3 times. Typical data obtained in one of the repeated experiments are presented.

Protein kinase activities were determined by the inclusion of [ $^{32}$ P]phosphate from  $\gamma$ [ $^{32}$ P]ATP in myelin basic protein. The epidermal peels in Tris buffer (pH 6.8) with glycerol, SDS, and mercaptoethanol were heated at 95°C for 15 min and electrophoresed in 10% polyacrylamide gel with 0.25 mg/ml of myelin basic protein. Then the gel was washed in 25 mM Tris buffer (pH 7.5) with phosphatase inhibitors (5 mM NaF and 0.1 mM Na<sub>3</sub>VO<sub>4</sub>), 0.5 mM dithiothreitol (DTT), 0.1% Triton X-100, and 0.5 mg/ml of bovine serum albumin three times for 30 min, incubated in renaturing 25 mM Tris buffer (pH 7.5), 5 mM NaF, 0.1 mM Na<sub>3</sub>VO<sub>4</sub>, 1 mM DTT overnight, and preincubated in 20 mM Hepes (pH 7.4), 50 mM KCl, 5 mM MnCl<sub>2</sub> for 30 min; then 50  $\mu$ Ci (~15 pM) of  $\gamma$ [ $^{32}$ P]ATP was added, followed by 1-h incubation. Phosphorylation of myelin basic protein was stopped by a solution containing 0.5-1% sodium pyrophosphate and 5% trichloroacetic acid. The radioactive label of the myelin basic protein in the gel was detected by autoradiography.

ROS production in the cells was assessed using 2',7'-dichlorofluorescein diacetate (DCFH-DA). Epidermal peels were stained with 20  $\mu$ M DCFH-DA for 30 min. While penetrating the cells, DCFH-DA is deacetylated by intracellular esterases and oxidized by ROS, mainly by H<sub>2</sub>O<sub>2</sub> with the involvement of peroxidases, transforming into intensively fluorescing 2',7'-dichlorofluorescein (DCF). DCF formation may involve transition metal ions, e.g. Fe<sup>2+</sup>, and cytochrome *c* possessing an activity similar to that of peroxidase [30, 31]. DCF fluorescence was excited with a laser beam of 488 nm and recorded using a Carl Zeiss Axiovert 200M fluorescence microscope with an LSM 510 Meta confocal module (Carl Zeiss, Germany) at 500-530 nm.

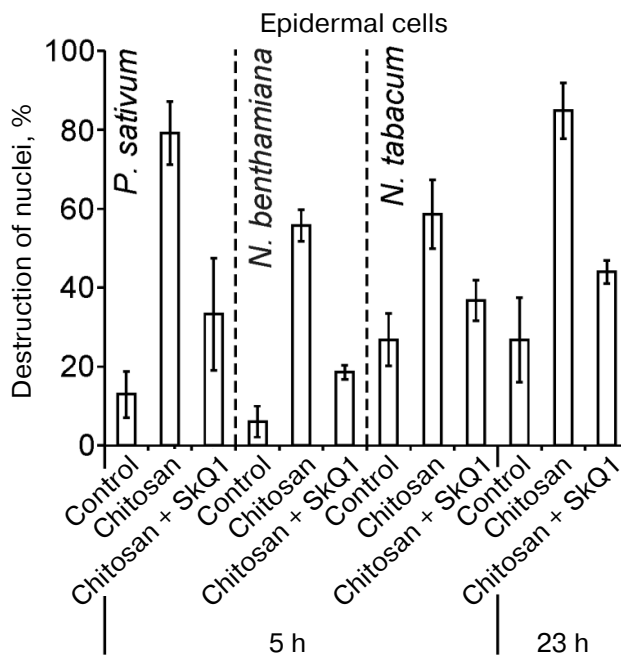
The state of the plasma membrane of the cells was assessed using propidium iodide (PI), a fluorescent dye binding to DNA and not penetrating through undamaged cell membranes. Epidermis was stained with 2  $\mu$ M PI for 20 min and examined under the Carl Zeiss Axiovert 200M fluorescence microscope with LSM 510 Meta confocal module. PI fluorescence was excited with a mercury lamp at 525-565 nm or with a laser at 543 nm and recorded at 575-640 or 565-615 nm in the fluorescent or confocal mode, respectively.

The following agents were tested as elicitors: low-viscosity chitosan from crab shells (Fluka, Switzerland), LPS from *Escherichia coli* 0127:B8 (Sigma, USA), PG from *Bacillus subtilis* (Sigma), and viral protein p50 (the helicase domain of TMV replicase) synthesized in tobacco cells as a result of their transformation by *Agrobacterium tumefaciens* carrying genetic constructions with the *p50* gene [32]. The suspension of *A. tumefaciens* cells with plasmid pLH with or without (control) *p50* gene was grown for 16 h on a shaker at 28°C in LB medi-

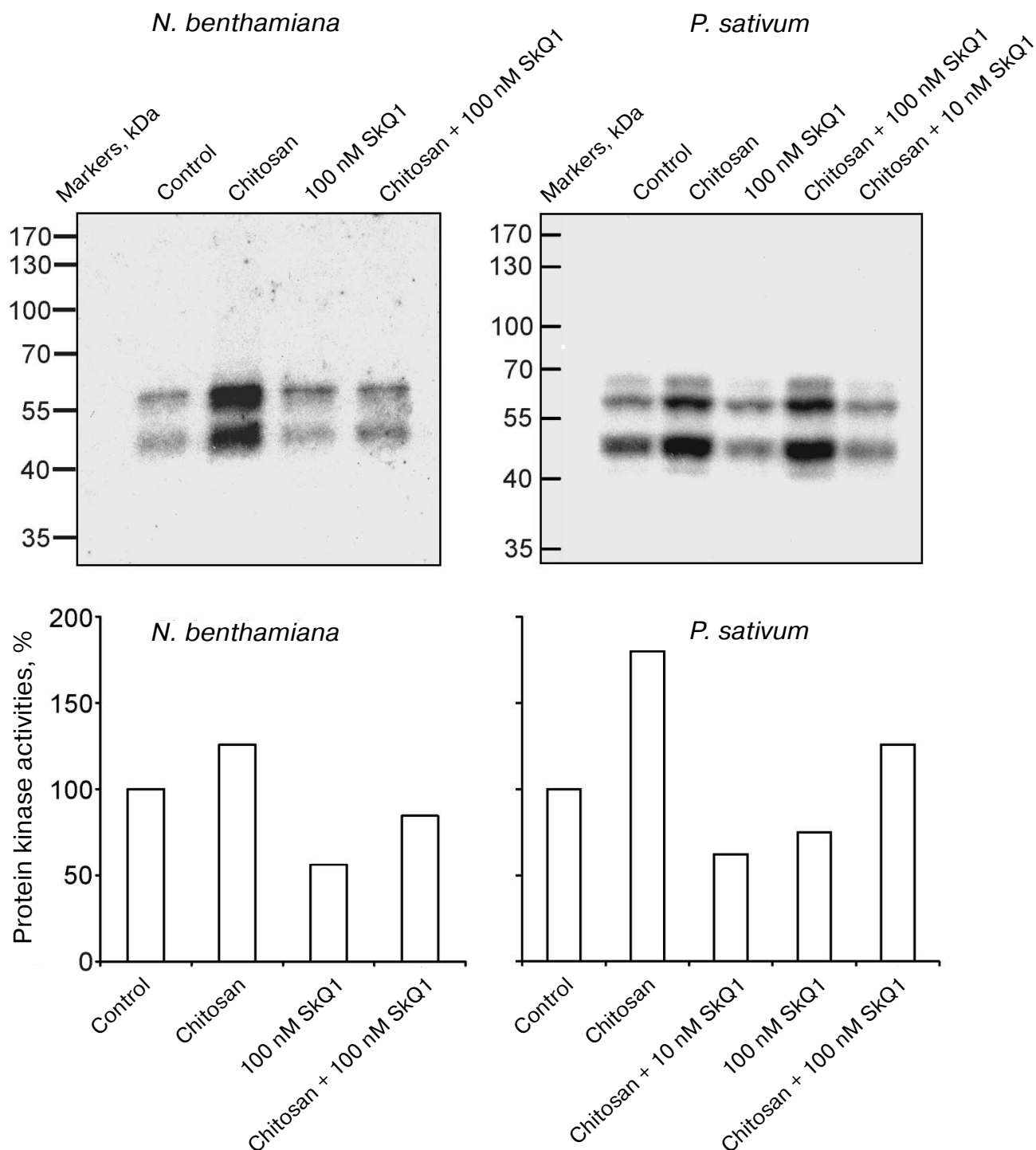
um (10 g/liter tryptone, 5 g/liter NaCl, 5 g/liter yeast extract), with sterile antibiotics (rifampicin, streptomycin, and spectinomycin, 100  $\mu$ g/ml of each; the plasmid pLH carries the genes of resistance to these antibiotics), 10 mM MES buffer, pH 5.5, and 20  $\mu$ M acetosyringone (4'-hydroxy-3',5'-dimethoxyacetophenone). Acetosyringone is a phenol compound released from damaged plant cells and recognized by the VirA protein. As a result the virulence genes (*vir*-genes) are activated in the Ti-plasmid of *A. tumefaciens* to ensure the process of agrobacterial transformation – the transfer of the region of the Ti-plasmid (T-DNA) into the cells and its integration into the plant genome [33, 34]. *Agrobacterium tumefaciens* cells were precipitated by centrifugation for 15 min at 3000 rpm and incubated for 3 h for transformation in 10 mM MES buffer (pH 5.5) with 10 mM MgCl<sub>2</sub> and 150  $\mu$ M acetosyringone.

## RESULTS

Chitosan (poly( $\beta$ -1,4)-*N*-acetylglucosamine) formed as a result of incomplete deacetylation of chitin, a component of fungal cell walls, induced cell death detected by the destruction of cell nuclei. Chitosan caused destruction of EC (epidermal cell) nuclei in the epidermal peels from pea leaves, without affecting GC (guard cells) [35]. Anaero-



**Fig. 1.** Effect of SkQ1 on chitosan-induced destruction of EC nuclei in epidermis from pea (*P. sativum*) and tobacco (*N. benthamiana* and *N. tabacum*) leaves. 100 nM SkQ1 was added to epidermal peels, incubated for 1 h, treated with 0.1 mg/ml of chitosan for 30 min on a magnetic stirrer, and incubated for 5 or 23 h in the dark.

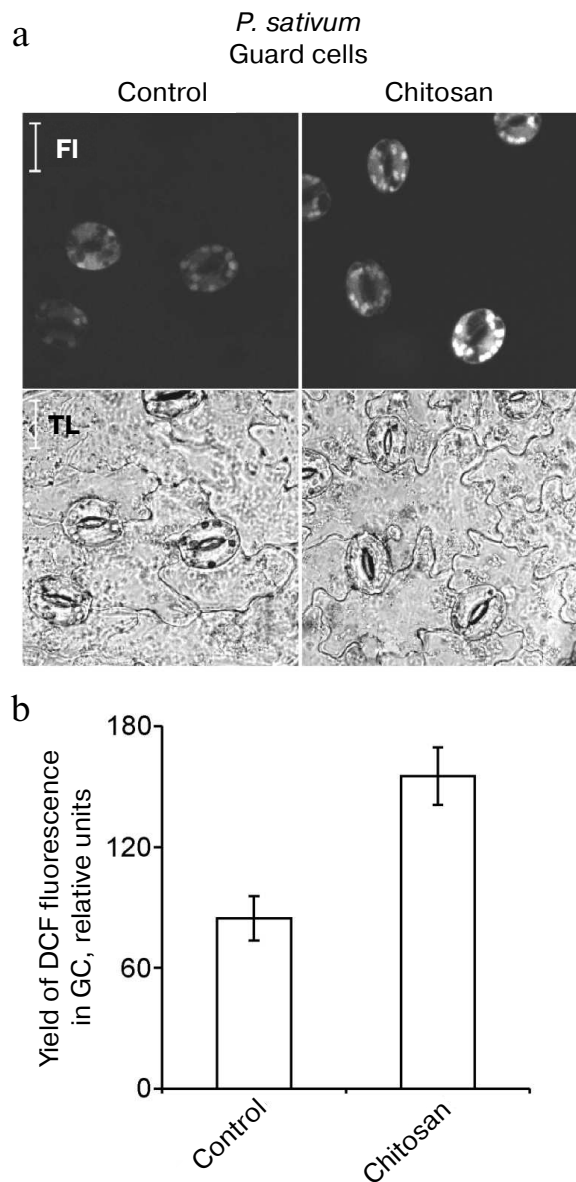


**Fig. 2.** Effects of chitosan and SkQ1 on protein kinase activities in epidermal peels from *N. benthamiana* and *P. sativum* leaves. SkQ1 was added to epidermal peels, incubated for 1 h, treated with 0.1 mg/ml of chitosan for 30 min on a magnetic stirrer, and incubated for 1 h in the dark. The figure shows the results of autoradiography of the gel with myelin basic protein after electrophoresis of the proteins from epidermal peels and its treatment with  $\gamma$ [ $^{32}$ P]ATP (top) and the specific protein kinase activity (bottom) calculated with TotalLab by the ratio of the total optical densities of bands on the upper figures to the total optical densities of Coomassie-stained protein bands in the same region of the gel. The activity of protein kinases in the control (without the additives) was taken as 100%.

biosis, antioxidants, and mitochondria-targeted quinones (SkQ) prevented chitosan-induced destruction of pea EC nuclei [35, 36]. Chitosan and mitochondria-targeted quinones had similar effects on the cells from leaves of *N.*

*benthamiana* and *N. tabacum*: chitosan induced destruction of nuclei in ECs that was prevented by SkQ1 (Fig. 1).

In *N. benthamiana* and *P. sativum* plants, it was demonstrated that chitosan stimulated and SkQ1 sup-



**Fig. 3.** Effect of chitosan on the yield of DCF fluorescence in pea leaf epidermal cells. Epidermal peels were incubated for 3 h in distilled water in the light, stained with DCFH-DA, washed from excessive dye in distilled water, and treated with 1 mg/ml of chitosan for 20 min on a magnetic stirrer. FI, DCF fluorescence; TL, transmitted light image; scale bar, 20  $\mu$ m (a). b) Yield of DCF fluorescence in GC as the mean of measurements in 40-63 cells.

pressed the activation of protein kinases detected by the incorporation of radioactive isotope  $^{32}$ P from  $\gamma$ [ $^{32}$ P]ATP into myelin basic protein as a result of its phosphorylation in gel by the protein kinases from plant cells after electrophoresis. SkQ1 at concentrations of 10 and 100 nM inhibited chitosan-dependent activation of protein kinases (Fig. 2).

Chitosan did not induce destruction of GC nuclei [35], but it stimulated ROS generation in GC from pea leaves, which was detected by DCF fluorescence using

confocal microscopy (Fig. 3). The treatment of epidermal peels from pea leaves with chitosan at a concentration of 0.1-1 mg/ml resulted in disruption of the permeability barrier of the GC plasma membrane for PI staining the DNA (Fig. 4, a and b). At the same time, GC nuclei remained intact. SkQ1 had no effect on chitosan-induced disruption of the permeability barrier of the GC plasma membrane for PI (Fig. 4c). The effect of H<sub>2</sub>O<sub>2</sub> at a concentration of 0.3-10 mM on the pea GC was similar to that of chitosan (Fig. 4d).

The following components of the outer membrane of Gram-negative bacteria and bacterial cell wall were tested as cell death inducers: LPS from *E. coli* and PG from *B. subtilis*. LPS, similar to chitosan, at a concentration of 20-200  $\mu$ g/ml induced the destruction of EC nuclei in pea leaf epidermis (Fig. 5a). At higher concentrations (1-2 mg/ml), the effect of LPS vanished. GC with nuclei not destroyed by chitosan [35] were also resistant to LPS. PG did not induce the destruction of EC and GC nuclei in pea leaf epidermis in a broad range of its concentrations and under different conditions of treatment (Fig. 5b). SkQ1 prevented the LPS-induced destruction of EC nuclei (Fig. 5c).

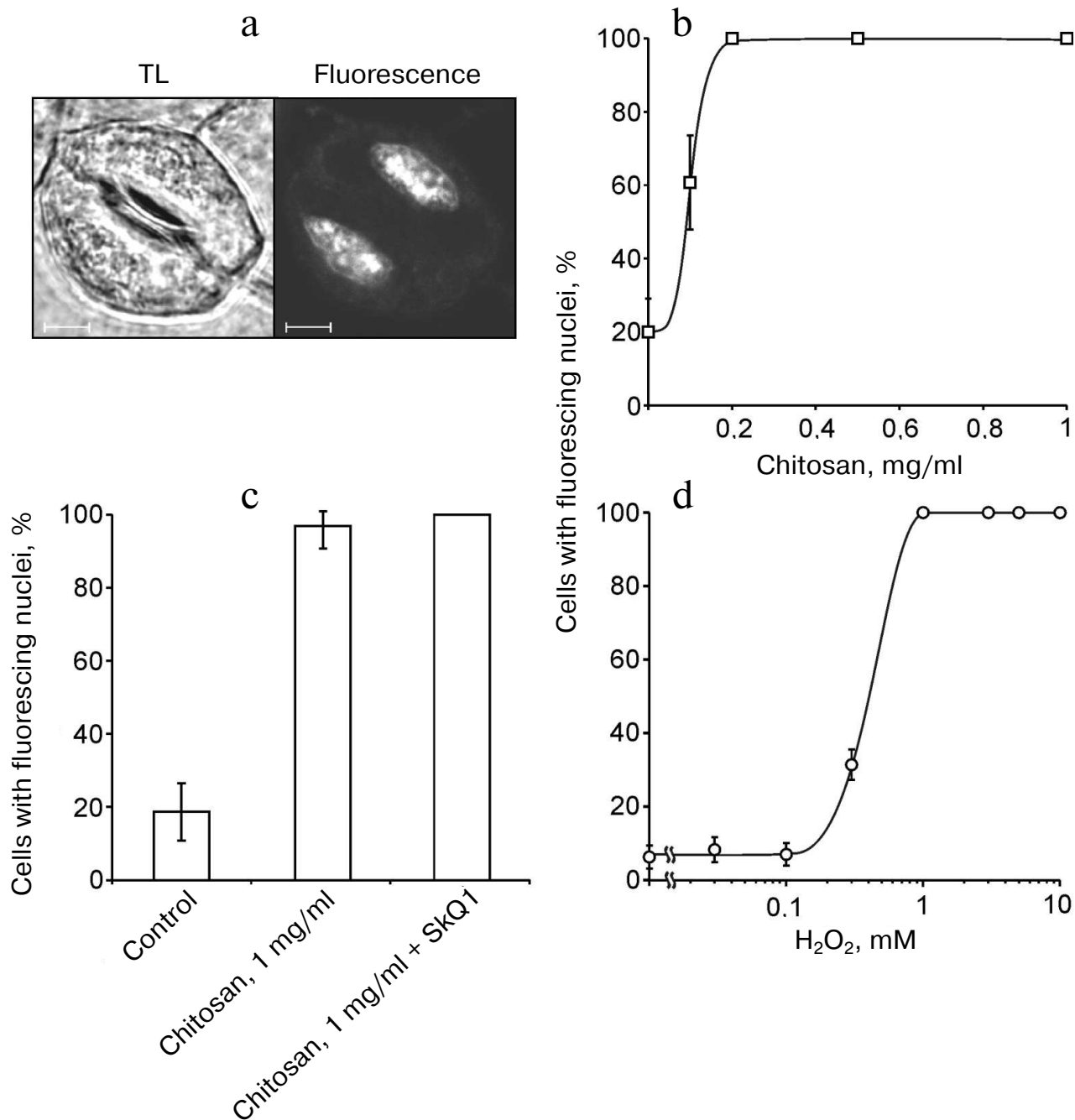
The interactions between the plants and the viral elicitor were simulated as follows: the tobacco (*N. tabacum*) leaves carrying the *N*-gene of TMV resistance were transformed by agrobacteria. *Agrobacterium tumefaciens* contained the pLH plasmid with the *p50* gene encoding the helicase domain of TMV replicase. The transformation of tobacco cells by *A. tumefaciens* with the *p50* gene resulted in the conditions for recognition of the viral protein p50 by the *N*-gene product of the plant and for HR development. *Agrobacterium tumefaciens* with the pLH plasmid but without the *p50* gene was used as a control.

The cell suspension of *A. tumefaciens* with the pLH plasmid carrying the *p50* gene injected into a tobacco leaf caused, after 5 days, the yellowing of the treated section of a leaf due to chlorophyll degradation – the common symptom of leaf aging that precedes its death [37, 38]. After 8 days, the section of the leaf treated with *A. tumefaciens* cells died off – it thinned and was dehydrated, turning into a fragile light-brown film (Fig. 6).

In this experiment, the mitochondria-targeted quinone was SkQ3 containing methylated plastoquinone, in contrast to the ordinary plastoquinone in SkQ1. The concentrations of SkQ3, when it protects plant cells from cyanide- or chitosan-induced death, are lower than the concentrations of other tested mitochondria-targeted quinones [36]. Pretreatment of a section of a tobacco leaf with SkQ3 at a concentration of 1 nM for 2 h before injection of the suspension of *A. tumefaciens* cells with the pLH plasmid carrying the *p50* gene inhibited its yellowing and prevented its die-off (Fig. 6).

The p50 protein induced the destruction of EC nuclei in epidermal peels from *N. tabacum* leaves with the

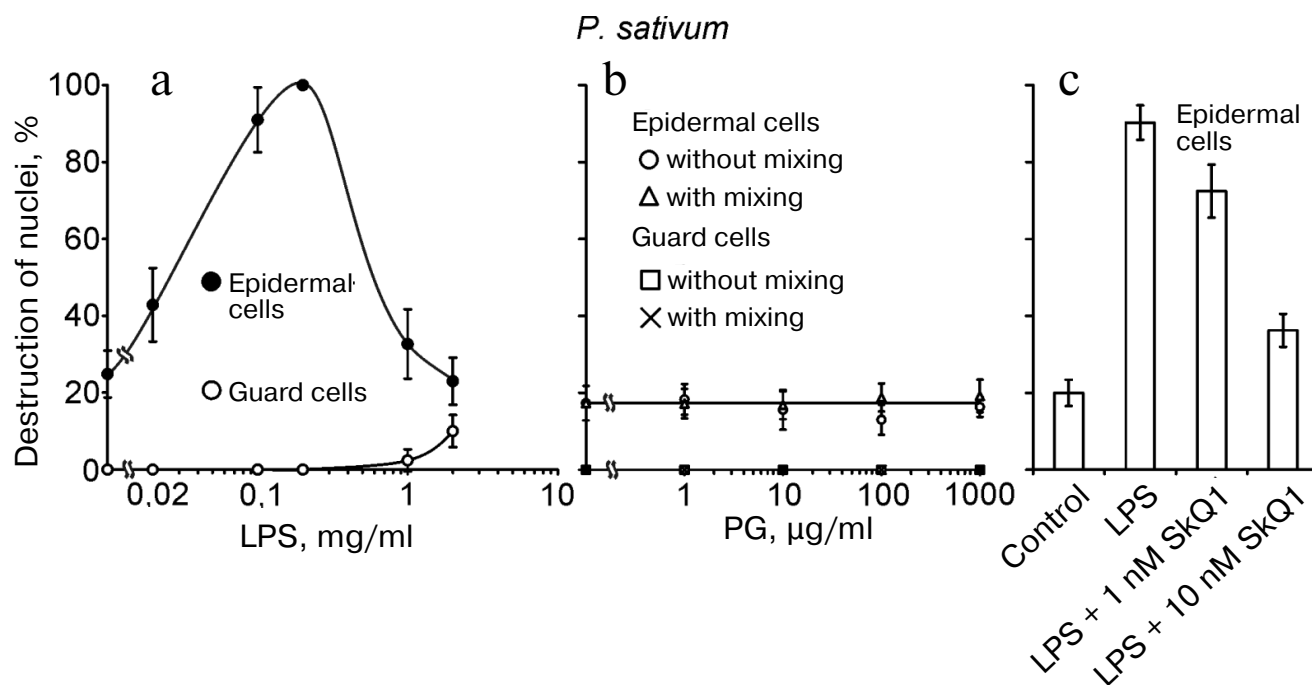
*P. sativum*  
Guard cells



**Fig. 4.** Effects of chitosan (a-c) and H<sub>2</sub>O<sub>2</sub> (d) on the GC plasma membrane permeability in pea leaf epidermis detected by the PI fluorescence in cell nuclei. a-c) Epidermal peels were put into chitosan solutions and, after the addition of 10 nM SkQ1, incubated for 22 h with mixing on a magnetic stirrer. TL, transmitted light image; scale bar, 5  $\mu$ m (a). d) Epidermal peels were put into H<sub>2</sub>O<sub>2</sub> solutions and incubated for 23-24 h. After the incubation, the peels were stained with PI. The portion of the cells (b-d) with PI-stained nuclei was determined as in (a).

*N*-gene on application of the suspension of *A. tumefaciens* cells carrying the *p50* gene (Fig. 7). In the control experiments, epidermal peels were treated with *A. tumefaciens* cells containing the pLH plasmid without the *p50* gene. The difference between the control and the variant with

*p50* is statistically significant. It is greater when the incubation time is longer. GC nuclei were not destroyed in epidermis isolated from the leaves after the treatment with *A. tumefaciens* cells. SkQ1 at a concentration of 100 nM per se had no effect on cell nuclei but prevented



**Fig. 5.** Effects of LPS from *E. coli* (a) and PG from *B. subtilis* (b) on GC and EC nuclei; effect of SkQ1 on LPS-induced destruction of EC nuclei (c) in pea leaf epidermis. a, b) Epidermal peels with the added LPS or PG were incubated for 22 h in the dark without mixing (a, b) or with mixing (b) on a magnetic stirrer 10 times for 2 min during 1 h for each experimental variant. c) Epidermal peels with the added SkQ1 were incubated for 2 h and then, after the addition of 0.2 mg/ml LPS, incubated for 22 h in the dark without mixing.

the destruction of tobacco EC nuclei induced by *A. tumefaciens* with the *p50* gene (Fig. 7). SkQ3 prevented the destruction of EC nuclei at a concentration of 10 nM.

## DISCUSSION

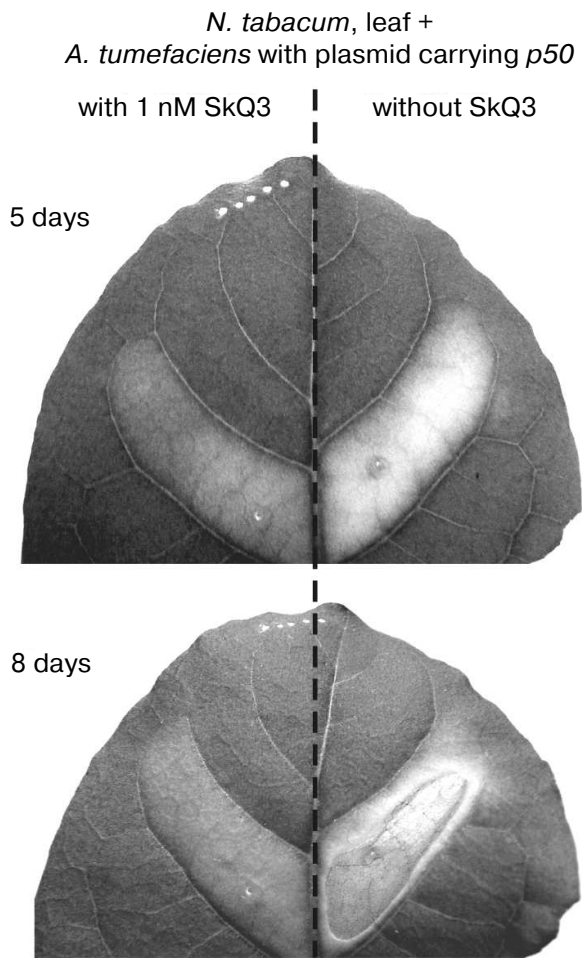
The effects of mitochondria-targeted quinones on cyanide- or chitosan-induced destruction of pea GC and EC nuclei have been studied previously [36]. These quinones at nanomolar concentrations prevented the destruction of cell nuclei in the dark but not in the light. Their effects were eliminated by carbonyl cyanide *m*-chlorophenylhydrazone, an uncoupler of oxidative phosphorylation. Mitochondria-targeted quinones accumulated in the mitochondria of pea cells and prevented ROS production. The quinones reduced menadione-induced ROS generation but had no effect on H<sub>2</sub>O<sub>2</sub>-dependent ROS generation. Mitochondria-targeted quinones are antioxidants that selectively accumulate in mitochondria [36]. Predictably, SkQ1 prevented chitosan-induced destruction of nuclei in tobacco EC (Fig. 1).

The protein kinase cascade in plant cells is activated under the influence of factors of different nature, including abiotic factors such as high salinity, drought, cold, mechanic damage of plant tissues, or biotic factors (phytohormones, pathogen elicitors) [39, 40]. The best-studied of pathogen elicitors that can induce the activation of

MAP kinase cascade are the protein of the bacteria flagellum (flagellin), bacterial elongation factor EF-Tu, chitin oligosaccharides, and the helicase domain of TMV replicase [41, 42].

The relationship between the MAP kinase cascade and ROS generation in HR is still unclear. ROS (H<sub>2</sub>O<sub>2</sub>, ozone) activated the MAP kinase cascade in plant cells. Diphenyleiodonium, the inhibitor of plasma membrane NADPH oxidase, suppressed ROS production induced by the fungal elicitor but did not prevent the activation of MAP kinases. The activation and inactivation of MAP kinases stimulated and suppressed ROS generation, respectively. Thus, HR with the involvement of plasma membrane NADPH oxidase and the MAP kinase cascade in plants after the reception of pathogen elicitor proceed independently of each other but may be cross-regulated [39, 40, 42]. SkQ1-dependent suppression of ROS production in plant mitochondria apparently results in the inactivation of chitosan-induced protein kinase cascade (Fig. 2). The activation of protein kinases is probably initiated by the ROS formed as a result of the action of chitosan on plant cells (Fig. 3). Chitosan-mediated stimulation of ROS production is in agreement with the data that chitosan initiated H<sub>2</sub>O<sub>2</sub> production in rice cell suspension culture [43].

The results demonstrate the similar effects of chitosan and LPS on plant cells as PCD inducers. The identical effects of LPS and chitin oligosaccharides on plant



**Fig. 6.** Effect of SkQ3 on *N. tabacum* leaf tissue die-off caused by the treatment with the suspension of *A. tumefaciens* cells with the pLH plasmid carrying the gene of the helicase domain of TMV replicase (*p50*). Time of incubation with *A. tumefaciens* is indicated in the figure. Preincubation with 1 nM SkQ3 (before introduction of *A. tumefaciens*) lasted 2 h. The suspension of *A. tumefaciens* cells (optical density 0.5 at 600 nm) was injected into leaf through the lower epidermis with a syringe.

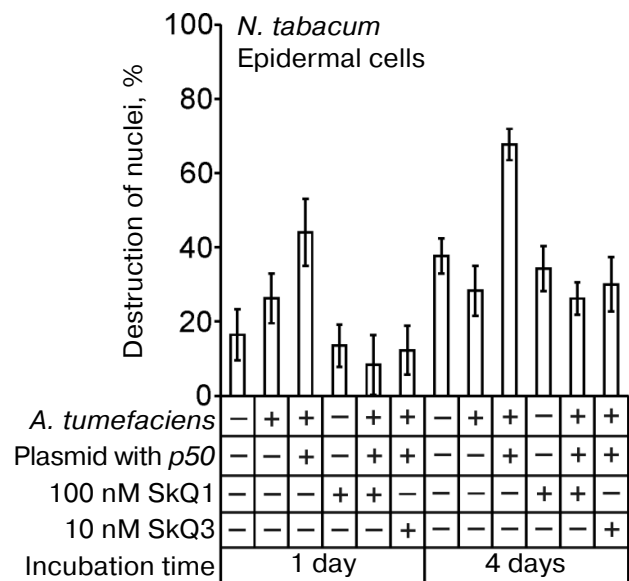
cells were shown previously [44]. Both chitosan- and LPS-dependent cell death was suppressed by SkQ1 ([36], Figs. 1 and 5c).

PG did not demonstrate the properties of a cell death inducer in our experiments (Fig. 5b), probably due to the lesser availability of PG for recognition by cell receptors because of its insolubility in water. PG can show anti-apoptotic properties with animal cells: PG stimulated the production of the inhibitor of apoptosis cIAP-2 in keratinocytes and inhibited their death induced by the tumor necrosis factor TNF $\alpha$  [45]. PG recognition seems to involve the same system (with the receptor-like kinase CERK1), which recognizes also chitin oligosaccharides [46].

The absence of destruction of pea EC nuclei after the treatment with LPS at a high concentration (1-2 mg/ml)

may be due to switching the mode of cell death from programmed to non-programmed, i.e. necrosis, when the permeability barrier of the plasma membrane is disrupted and cell nuclei remain intact. Such disruption of the plasma membrane permeability barrier in pea GC was induced by chitosan at a concentration above 0.1 mg/ml or H<sub>2</sub>O<sub>2</sub> at a concentration above 0.3 mM (Fig. 4). Ca<sup>2+</sup> (1-2 mM) and Triton X-100 had similar effects on plant cells [47].

In cultured cells of soybean (*Glycine max*) and haricot (*Phaseolus vulgaris*), chitosan at a concentration of 0.1 mg/ml and more induced disruption of the plasma membrane permeability barrier detected by the release of electrolytes (conductometrically) and proteins from the cells. Polycations (polylysine, diethylaminoethyl dextran) had the same effect on plant cells. Divalent cations, including Ca<sup>2+</sup>, suppressed polycation-induced disruption of the permeability barrier of the plasma membrane. This property of chitosan located on the outer surface of cell walls of some phytopathogenic fungi, e.g. *Fusarium solani* (the pathogen of pea fusarial wilt) or *Puccinia striiformis* (the pathogen of wheat rust), is supposed to contribute to the outflow of nutrients from plant cells into fungal cells [48]. Thus, the effect of chitosan at concentrations above 0.1 mg/ml on the state of GC plasma



**Fig. 7.** Effect of SkQ1 on destruction of EC nuclei in the epidermis of *N. tabacum* leaves caused by treatment of epidermis with the suspension of *A. tumefaciens* cells with the pLH plasmid carrying the gene of the helicase domain of TMV replicase (*p50*). Epidermal peels were put into the transformation buffer, 100 nM SkQ1 or 10 nM SkQ3 was added, and the suspension of *A. tumefaciens* cells in the same buffer was introduced (optical density 2 at 600 nm), followed by incubation in the dark. Incubation time is indicated on the diagram. Cells of *A. tumefaciens* contained the pLH plasmid with or without the *p50* gene. In the control, epidermal peels were put into the buffer without *A. tumefaciens*.



membrane seems to be determined not by the specific reaction of its recognition by a plant cell receptor but by its properties as a polycation. That is why SkQ1 will certainly have no effect on chitosan-induced disruption of the permeability barrier of the plasma membrane in GC, which is in agreement with our observations (Fig. 4c).

H<sub>2</sub>O<sub>2</sub> can induce both apoptosis and necrosis of cells depending on its concentration. Human oocytes were treated for 1 h with H<sub>2</sub>O<sub>2</sub> at different concentrations: at 50 μM, the cells died mainly through apoptosis; at 100 μM, both apoptosis and necrosis were observed; at 1 mM, mainly necrosis was observed [49]. Similar to chitosan, H<sub>2</sub>O<sub>2</sub> per se did not stimulate the destruction of nuclei (PCD) in pea GC, but already at a concentration of 10 μM it significantly stimulated the destruction of GC nuclei induced by cyanide, the inhibitor of catalase and peroxidases [50]. It can be supposed that the photosynthesizing GC have an antioxidant protection (including catalase and peroxidases), which copes with the low concentrations of H<sub>2</sub>O<sub>2</sub>. When H<sub>2</sub>O<sub>2</sub> concentrations exceeded the capabilities of antioxidant protection of the cells, it induced disruption of the permeability barrier of the plasma membrane, i.e. necrosis (Fig. 4d).

The *N*-gene imparts TMV resistance to tobacco plants. In tobacco cultivars without the *N*-gene, the replicating TMV induces plant diseases (a mosaic of light and dark green spots on leaves) and suppresses plant growth. In cultivars with the *N*-gene, TMV induces HR, which limits further spread of the virus. The helicase domain of the TMV replicase (protein p50) is sufficient to induce the *N*-gene-dependent HR in tobacco. The synthesis of p50 protein was achieved by transgene expression through the introduction of *A. tumefaciens* cells carrying the plasmid with the *p50* gene into the mesophyll of *N. tabacum* leaves [9].

The introduction of *A. tumefaciens* cells with the *p50* gene into a tobacco leaf initiated the development of HR manifesting itself in the wilting and dying-off section of the leaf where the *A. tumefaciens* cells with the *p50* gene were introduced. The treatment of a section of the leaf with *A. tumefaciens* cells containing the plasmid pLH without the *p50* gene did not result in its wilting and dying-off. SkQ1 at the concentrations of 0.1 and 1 nM prevented HR development [32]. In agreement with these data, the treatment of tobacco with the suspension of *A. tumefaciens* cells carrying the *p50* gene induced HR, while SkQ3 at a concentration of 1 nM suppressed HR (Fig. 6). The treatment of tobacco epidermal peels with *A. tumefaciens* cells carrying the *p50* gene induced destruction of EC nuclei, which was prevented by SkQ1 or SkQ3 (Fig. 7). In this respect, the effect of the viral protein p50 is similar to the effects of chitosan and LPS (Figs. 1 and 5c).

The data obtained show that PCD in plant EC is initiated by fungal, bacterial, and viral elicitors – chitosan, LPS, and the p50 protein of TMV. PCD occurs not only during ETI induced by the viral effector p50 but also dur-

ing PTI (the resistance ensured by the recognition of PAMPs, including chitosan and LPS). In both variants, the mitochondria-targeted quinone SkQ1 had a protective effect, which is evidence of the involvement of mitochondrial ROS in PCD induced by pathogen elicitors.

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