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# Programmed Cell Death in Plants: Protective Effect of Mitochondrial-Targeted Quinones

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**Abstract**—Ubiquinone or plastoquinone covalently linked to synthetic decyltriphenylphosphonium ($DTPP^+$) or rhodamine cations prevent programmed cell death (PCD) in pea leaf epidermis induced by chitosan or $CN^-$. PCD was monitored by recording the destruction of cell nuclei. $CN^-$ induced the destruction of nuclei in both epidermal cells (EC) and guard cells (GC), whereas chitosan destroyed nuclei in EC not in GC. The half-maximum concentrations for the protective effects of the quinone derivatives were within the pico- and nanomolar range. The protective effect of the quinones was removed by a protonophoric uncoupler and reduced by tetraphenylphosphonium cations. $CN^-$-Induced PCD was accelerated by the tested quinone derivatives at concentrations above $10^{-8}$-$10^{-7}$ M. Unlike plastoquinone linked to the rhodamine cation (SkQR1), $DTPP^+$ derivatives of quinones suppressed menadione-induced $H_2O_2$ generation in the cells. The $CN^-$-induced destruction of GC nuclei was prevented by $DTPP^+$ derivatives in the dark not in the light. SkQR1 inhibited this process both in the dark and in the light, and its effect in the light was similar to that of rhodamine 6G. The data on the protective effect of cationic quinone derivatives indicate that mitochondria are involved in PCD in plants.

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*Key words*: programmed cell death, mitochondrial-targeted quinones, mitochondria, chitosan, reactive oxygen species, pea

Programmed cell death (PCD) is a physiological response aimed to carry out the program of an organism's ontogeny and secure cell differentiation, maintenance of tissue homeostasis, and the elimination of infected, functionally exhausted, aged, and unclaimed cells, as well as cells undergoing malignant degeneration or affected by mutagens and other stressors [1]. Mitochondria produce apoptogenic factors including cytochrome *c*, the flavoprotein AIF, and reactive oxygen species (ROS) [1-5] and, therefore, are essential for animal and human PCD. PCD in plants implicates the release of cytochrome *c* from the mitochondria into the cytoplasm [6-10]. However, mitochondrial cytochrome *c* is not mandatory for controlling PCD in plants [11].

It was revealed in our earlier works that chloroplasts are involved in plant PCD [12-16]. Experiments were conducted with pea leaf epidermis, a monolayer composed of stoma guard cells (GC) containing both mitochondria and chloroplasts and basic epidermal cells (EC) containing only mitochondria. Cyanide induced PCD that was followed by monitoring the disintegration of GC and EC nuclei. Illumination accelerated this process in GC, but not in EC [12-14]. The destruction of GC and EC nuclei was prevented by anaerobiosis or by the addition of antioxidants (α-tocopherol, butylated hydroxytoluene, and mannitol) [13, 14]. $CN^-$ induced PCD with the symptoms of apoptosis such as chromatin condensation and margination, vacuolar volume increase, cytoplasmic volume decrease [16], and internucleosomal fragmentation of DNA [17]. The $CN^-$-induced death of GC proceeds via a combined scenario involving both

*Abbreviations*: BQ, *p*-benzoquinone; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DAPI, 4′,6-diamidino-2-phenylindole dihydrochloride; DCF, 2′,7′-dichlorofluorescein; DCMU, 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; $DTPP^+$, decyltriphenylphosphonium cation; EC, basic epidermal cells; GC, stoma guard cells; MitoQ, 10-(6′-ubiquinonyl)decyltriphenylphosphonium; NBT, nitroblue tetrazolium; PCD, programmed cell death; ROS, reactive oxygen species; SkQ1, 10-(6′-plastoquinonyl)decyltriphenylphosphonium; SkQ3, 10-(6′-methylplastoquinonyl)decyltriphenylphosphonium; SkQR1, 10-(plastoquinonyl)decylrhodamine 19; $TPP^+$, tetraphenylphosphonium cation; Δψ, transmembrane electrical potential difference.

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apoptosis and autophagy and is sensitive to cycloheximide (an inhibitor of protein synthesis by cytoplasmic ribosomes) and to lincomycin (an inhibitor of protein synthesis in chloroplasts and mitochondria) [18]. DCMU (3-(3′,4′-dichlorophenyl)-1,1-dimethylurea), an inhibitor of electron transfer between plastoquinones $Q_A$ and $Q_B$ in Photosystem II, suppressed the $CN^-$-induced destruction of GC nuclei in the light. The same effect was produced by dinitrophenyl ether of 2-iodo-4-nitrothymol (DNP-INT) and stigmatellin that prevented plastoquinol binding at site *o* of the chloroplast cytochrome $b_6f$ complex. PCD in plants seems to be regulated by the redox state of plastoquinone of the chloroplast cytochrome $b_6f$ complex [13, 14].

By inactivating ribulose-1,5-bisphosphate carboxylase and suppressing NADPH utilization in the Calvin cycle, $CN^-$ causes the depletion of the chloroplast $NADP^+$ pool, stimulates the generation of ROS by Photosystem I, and induces PCD in plants [13, 14]. Electron acceptors such as menadione, benzoquinone, diaminodurene, N,N,N′,N′-tetramethyl-*p*-phenylenediamine, 2,6-dichlorophenolindophenol, or methyl viologen suppressed the $CN^-$-induced destruction of nuclei in GC, but not in EC [13, 14]. In the presence of $CN^-$, the reduction of plastoquinone at site *o* of the $b_6f$ cytochrome complex of chloroplasts presumably activates a protein kinase which initiates PCD [13, 14].

$CN^-$ is not a specific PCD inducer. It produces multiple effects and impairs mitochondria and chloroplasts. Other effectors have been revealed. They are signal compounds (elicitors) produced by phytopathogens. Elicitors mimic the interaction of pathogens and plants and initiate defense responses that include ROS generation and the apoptosis of infected plant cells (the hypersensitive response) [19, 20]. An efficient elicitor is chitosan, a poly(β-1,4)-N-acetylglucosamine. It results from incomplete deacetylation of chitin, a component of the fungal cell wall. The plasma membrane of various plants contains a receptor that binds chitin oligosaccharides with high affinity. This receptor is a 75-kDa glycoprotein [21]. Knockdown of the receptor results in the suppression of chitosan-induced $H_2O_2$ generation [21]. In the capacity of an elicitor, chitosan causes the hypersensitive response [22], cell death-mediated antiviral effects in plants [23, 24], and the internucleosomal fragmentation of the nuclear DNA [24-26]. Chitosan causes the destruction of nuclei in EC but not in GC [26]. The chitosan effect as PCD inducer was prevented by anaerobiosis or by antioxidants under aerobic conditions. Exogenous $H_2O_2$ also caused the destruction of the EC nuclei [26].

Compounds selectively taken up by mitochondria hold much promise with respect to research on their role in PCD. These compounds include synthetic penetrating ions, e.g. methyltriphenylphosphonium cations. They are pumped into animal mitochondria by the membrane potential ($\Delta\psi$) gradient with a "minus" sign inside the inner membrane [27, 28] generated by respiration or ATP hydrolysis. The $\Delta\psi$ with a "minus" sign inside is also generated in energized plant mitochondria [9, 11]. The $H^+$-ATPase of the plasma membrane in plant cells generates $\Delta\psi$ (with a "minus" sign inside) of 150-200 mV [29-32]. $H^+$ cations pumped by the ATPase decrease the pH value in the apoplast to 5-6 [31] and below 5 [32]. The combination of $\Delta\psi$ and $\Delta pH$ in the plasma membrane is the driving force for transport processes in plant cells that involve various protein transporters.

Mitochondria selectively accumulate MitoQ (10-(6′-ubiquinonyl)decyltriphenylphosphonium), a ubiquinone residue covalently linked to the decyltriphenylphosphonium ($DTPP^+$) cation [33]. MitoQ interacts with the respiratory chain of mitochondria. It is an effective antioxidant that inhibits membrane lipid peroxidation and displays antiapoptotic properties [33-35].

A number of new antioxidants called SkQs have been synthesized. Their molecules include plastoquinone, a penetrating cation, and a decane or pentane linker [36]. Using planar bilayer phospholipid membranes, SkQ derivatives with the highest penetrating ability were selected, such as plastoquinonyl decyltriphenylphosphonium (SkQ1), plastoquinonyl decylrhodamine 19 (SkQR1), and methylplastoquinonyl decyltriphenylphosphonium (SkQ3). Anti- and prooxidant properties of these compounds and also of ubiquinone and MitoQ were tested on mitochondria. Cationic quinones are prooxidants at high (micromolar) concentrations. They display antioxidant activity at lower (submicromolar) concentrations. The antioxidant activity varies in the sequence SkQ1 = SkQR1 > SkQ3 > MitoQ, so that the concentration difference between the anti- and prooxidant effects is minimal for MitoQ. SkQ1 is rapidly reduced by complexes I and II of the mitochondrial respiratory chain, i.e. it is a rechargeable antioxidant. Extremely low concentrations of SkQ1 and SkQR1 completely arrest $H_2O_2$-induced apoptosis in human fibroblasts and HeLa cells ($C_{1/2} = 1 \cdot 10^{-9}$ M for SkQR1). Higher concentrations of SkQs are required to block ROS-induced necrosis. In mice, SkQ1 decelerates the development of accelerated aging (progeria) as well as normal aging, and this effect is especially demonstrative at early stages of aging. The same effect is produced by SkQ1 in such invertebrates as drosophila and the daphnia. In mammals, SkQ1 slows the development of such age-related diseases as osteoporosis, thymus involution, cataract, and retinopathy [36]. These studies were continued in works [37-41].

This work was aimed at elucidating the effects of $DTPP^+$- or rhodamine cation-bound ubiquinone and plastoquinone on the programmed death of plant cells. We revealed that mitochondrial-targeted quinones protected GC and EC nuclei against the destruction induced by $CN^-$ or chitosan. Data on the intracellular content of $H_2O_2$ suggest that the protective effect of these compounds is due to their antioxidant properties.

## MATERIALS AND METHODS

The structure of the tested compounds is given in Fig. 1. Low viscosity chitosan (Fluka, Germany) used in our experiments was a product of partial alkaline deacetylation of chitin from crab shells. Chitosan is an N-acetylglucosamine–glucosamine heteropolymer that is inhomogeneous in terms of molecular weight.

The experiments were conducted with peels of leaf epidermis of pea (*Pisum sativum* L. cv Alpha) seedlings grown for 7-15 days at 20-24°C with 16 h light/8 h dark photoperiods. The light intensity of the luminescent lamps used in experiments was ~100 $\mu E \cdot m^{-2} \cdot sec^{-1}$. The epidermis was separated with tweezers and placed into distilled water. Vacuum infiltration at a pressure of 1-2 mm Hg for 1-2 min was used for the rapid influx of added reagents into the cells. As experiments with pea seedlings demonstrated, this treatment was not inhibitory to their further growth. Infiltration methods were described in work [42]. The samples were placed into polystyrene plates and incubated in distilled water with additives (the composition is given in the figure legends) at room temperature either in the dark or under illumination.

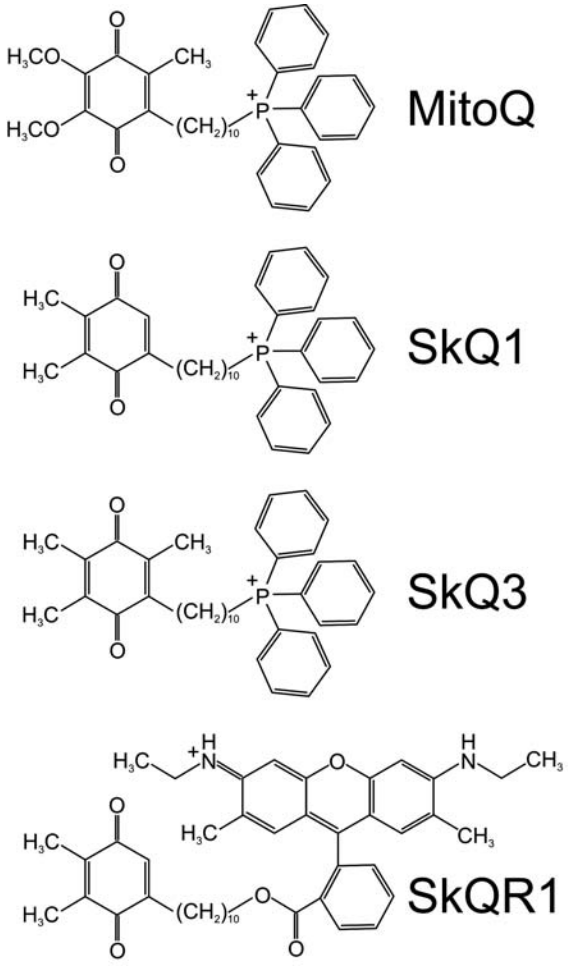


**Fig. 1.** Structures of ubiquinone (MitoQ) and plastoquinone (SkQ1, SkQ3 and SkQR1) derivatives.

Following the incubation, the samples were treated with Battaglia fixative, a chloroform–96% ethanol–glacial acetic acid–40% formaldehyde mixture (5 : 5 : 1 : 1), for 5 min, washed in ethanol for 10 min to remove the fixing mixture, incubated for 5 min in water, and stained with Carazzi's hematoxylin for 20 min. The stained peels were washed with tap water for light microscopy with a Carl Zeiss Laboval 4 microscope (Germany). The number of cells with destroyed or lacking nuclei was determined by examining 300-500 cells (for each epidermal peel) in a light microscope. Fluorescent microscopy was done using a Carl Zeiss Axiovert 200M microscope (Germany).

The intracellular content of $H_2O_2$ was estimated from the fluorescence of 2′,7′-dichlorofluorescein (DCF) [43] measured with a VersaFluor fluorimeter (Bio-Rad, USA). Isolated epidermis was fixed on its intact surface on a polystyrene plate, submerged into the solution of 50 μM 2′,7′-dichlorofluorescin (DCFH) diacetate, incubated for 10 min in the dark, washed with distilled water, and placed into the sample cuvette with 25 mM Hepes-NaOH solution, pH 7.2. DCFH diacetate diffuses across the cell plasma membrane and is hydrolyzed by intracellular esterases to non-fluorescent DCFH that accumulates inside the cells. DCFH is oxidized to DCF by $H_2O_2$ in a peroxidase-involving reaction [43]. DCF fluorescence was excited by light with λ 485-495 nm and recorded at 515-525 nm.

MitoQ, SkQ1, SkQ3, and SkQR1 were used as $10^{-4}$-$10^{-12}$ M aqueous solutions prepared from starting 2-100 mM solutions in ethanol or dimethyl sulfoxide before experiments. All experiments were repeated 3-5 times. Representative data are shown.

## RESULTS

Figure 2a shows the generation of $H_2O_2$ in the GC and EC of the pea leaf epidermis that was determined from the fluorescence of DCF. DCF fluoresces in spherical structures that represent mitochondria [26]. DCF fluoresces also near the plasma membrane, which is more prominent in EC. The generation of $H_2O_2$ is suppressed by SkQ1. Figure 2b demonstrates (1) a GC image in transmitted light, (2) the fluorescence of chlorophyll in chloroplasts, and (3) the fluorescence of SkQR1. SkQR1 as a cationic derivative accumulates in mitochondria, but not in chloroplasts.

Chitosan caused chromatin condensation and margination and subsequent destruction and disappearance of EC nuclei in epidermis isolated from pea leaves; the cell walls of EC were unaffected by chitosan [26]. The chitosan-induced destruction of EC nuclei was prevented

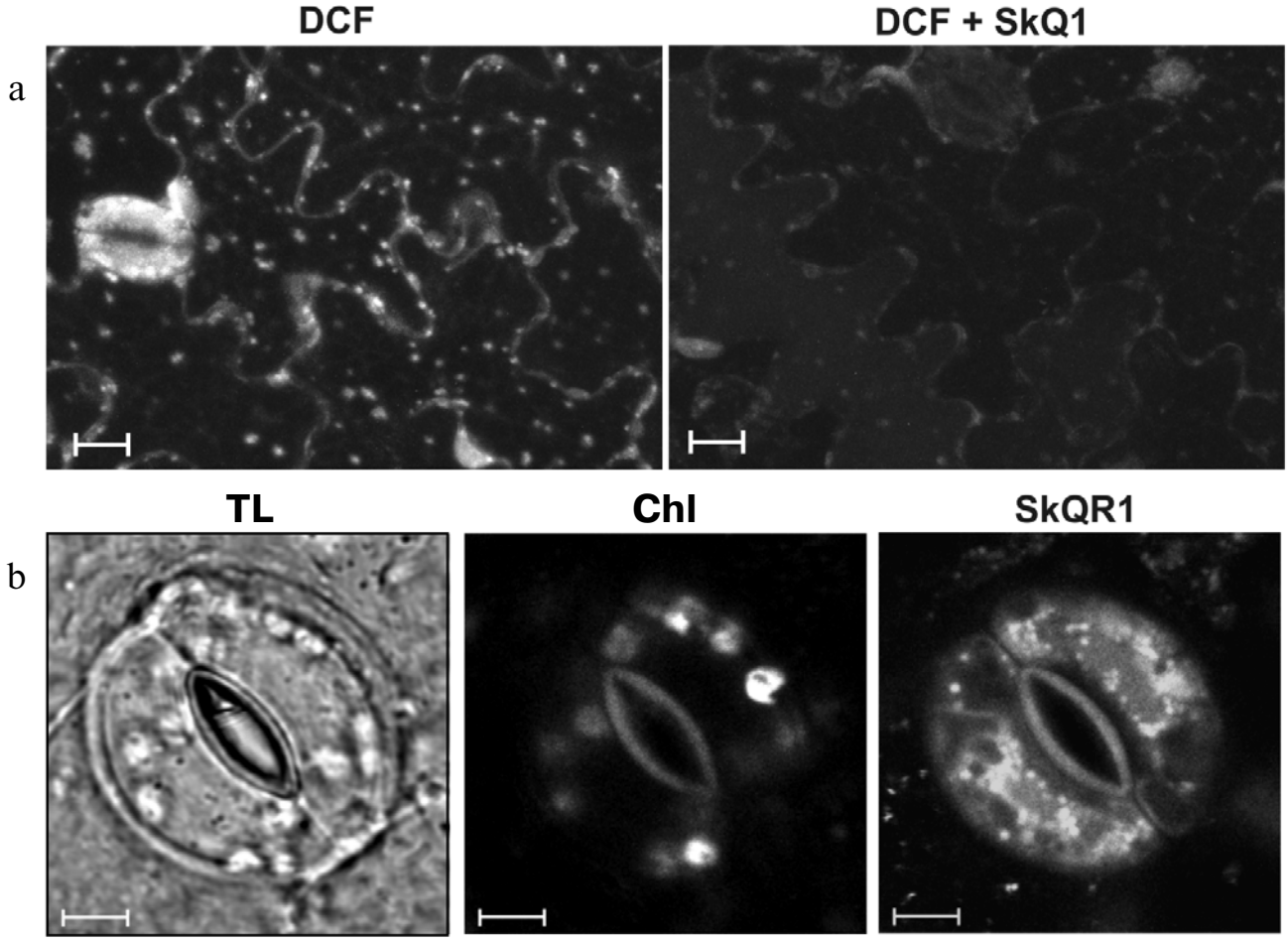


**Fig. 2.** Effect of SkQ1 on DCF fluorescence yield in pea leaf GC and EC (a) and SkQR1 accumulation in GC of pea leaf epidermis (b). a) Leaf fragments were supplemented with 100 nM SkQ1, incubated for 1 h, and stained with 20 μM 2′,7′-dichlorofluorescin diacetate for 20 min. DCF fluorescence was measured near the boundary of the leaf fragments. The images represent the maximum projection of DCF fluorescence that was captured from 20 optical slices. The distance between the optical slices was 1 μm. Scale bars, 10 μm. b) Epidermal peels in 2 ml of distilled water were supplemented with 100 nM SkQR1 and incubated for 1 h. TL, the image in transmitted light; Chl, fluorescence of chlorophyll; SkQR1, fluorescence of SkQR1. Scale bars, 5 μm. DCF, SkQR1, and chlorophyll fluorescence was excited with light with wavelengths of 488, 543, and 633 nm, respectively, and measured within the 500-530, 565-615, and 650-710 nm ranges, respectively.

by mitochondrial-targeted quinones exemplified by SkQ1 (Fig. 3). Chitosan-treated GC nuclei remained intact (Fig. 3b) even after 3 days.

Figure 4a shows that SkQ1 prevents the destructive effect of chitosan on EC nuclei. In the absence of chitosan, quinone derivatives produced no effect on EC nuclei. SkQ1 at concentrations below $10^{-8}$ M exerted a protective influence in this system. In contrast, SkQ1 enhanced the $CN^-$-induced destruction of GC nuclei at higher concentrations (Fig. 4b). The proapoptotic effect of SkQ1 and other tested quinones at concentrations above $10^{-8}$-$10^{-7}$ M manifested itself in the presence of $CN^-$, which is known to inhibit catalase and peroxidases. It also occurred with EC if PCD was induced by $CN^-$ (data are not shown), but was lacking both in EC and in GC in the absence of $CN^-$ (Fig. 4, a and b). The protective effect of $DTPP^+$-linked quinone derivatives in GC manifested itself in the dark (Fig. 4b), not in the light (Fig. 4c). Only rhodamine cation-linked SkQR1 prevented the $CN^-$-induced destruction of GC nuclei both in the dark and in the light (Fig. 4d).

The table summarizes our data on the protective effects of cationic quinone derivatives and contains their concentrations ($C_{50}$) at which the destruction of nuclei is 50% reduced. The effects of quinones on EC and GC were characterized by nanomolar or still lower $C_{50}$ values. Because the tested quinone derivatives attach to glass and other surfaces, glass vessels with a polycationic covering made of polyvinyl-N-decylpyridinium were also used in these experiments. The polycationic covering that prevented the adsorption of the cationic derivatives lowered the $C_{50}$ values except for SkQ3. The $C_{50}$ values for MitoQ, SkQ1, and SkQR1 were decreased 20-, 30-, and 110-fold if chitosan was used as PCD inducer.

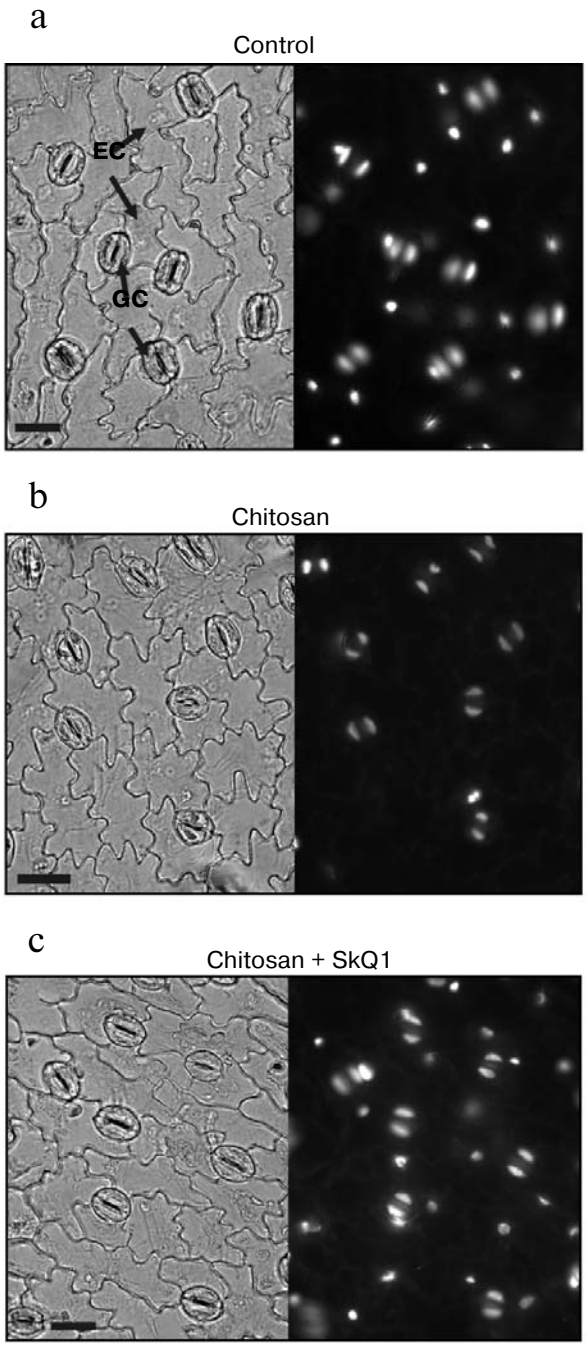


**Fig. 3.** Transmitted light (on the left) and fluorescence (on the right) microscopy of pea leaf epidermis EC and GC. a) Control (without additives); b) 100 μg/ml chitosan; c) 100 nM SkQ1 and 100 μg/ml chitosan. Epidermal peels (~50 μg of protein) were placed in 2 ml of distilled water, supplemented with SkQ1, incubated for 2 h in the dark, treated with 100 μg/ml chitosan on a magnetic stirrer for 0.5 h, and thereupon incubated for 5 h without stirring in the dark. Pea leaf epidermis was treated by the fixative, washed, and stained with 0.2 μM aqueous 4′,6-diamidino-2-phenylindole (DAPI) for 15 min. DAPI fluorescence was excited with light within the 300-390 nm wavelength range and monitored at $\lambda > 420$ nm. Scale bar, 20 μm.

Quinone derivative concentrations that cause half-maximum prevention ($C_{50}$) of chitosan- or KCN-induced destruction of EC and GC nuclei in pea leaf epidermis incubated in the dark and in the light*. The maximum effect was estimated from the difference between the nucleus destruction induced by chitosan or $CN^-$ and the nucleus destruction in the control (without additions). Mean values of five repeats of experiments are shown. The experiments were conducted as described in Fig. 4

| Quinone derivatives | $C_{50}$ for chitosan-induced destruction of EC nuclei, M | $C_{50}$ for KCN-induced destruction of GC nuclei, M |
|---|---|---|
| MitoQ | $(1.1 \pm 0.7) \times 10^{-8}$ | $(4.3 \pm 1.4) \times 10^{-10}$ |
| SkQ1 | $(2.5 \pm 1.9) \times 10^{-9}$ | $(1.7 \pm 1.1) \times 10^{-9}$ |
| SkQ3 | $(9.1 \pm 3.4) \times 10^{-13}$ | $(2.0 \pm 1.4) \times 10^{-10}$ |
| SkQR1 | $(4.4 \pm 1.6) \times 10^{-10}$ | $(1.3 \pm 0.5) \times 10^{-8}$ |
| SkQR1* | | $(1.6 \pm 0.5) \times 10^{-8}$ |

Carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of oxidative and photosynthetic phosphorylation, removed the protective effect of SkQ1 on chitosan-induced destruction of EC nuclei (Fig. 5a). In the absence of chitosan, CCCP caused a negligible destruction of nuclei and did not influence the chitosan effect. The effect of SkQ1 on chitosan-induced destruction of EC nuclei was almost unaffected by $TPP^+$ (tetraphenylphosphonium cation) at a concentration equimolar to that of SkQ1 (0.1 μM). It was abolished upon increasing the $TPP^+$ concentration to 5 μM (Fig. 5b). $TPP^+$ cations (5 μM) per se caused an insignificant destruction of EC nuclei.

CCCP and $TPP^+$ produced a similar effect on the $CN^-$-induced destruction of GC cells (Fig. 5, c and d). CCCP considerably promoted the $CN^-$-induced destruction of GC nuclei, but had no influence in the absence of $CN^-$ and abolished the protective effect of SkQ1. $TPP^+$ (5 μM) lowered the protective effect of SkQ1 (10 nM). The SkQ1 protective effect was eliminated by CCCP, irrespective of whether it was added with SkQ1 or after the incubation of the epidermal peels with SkQ1 for 1-2 h.

Figure 6 (line 1) deals with the $H_2O_2$-dependent generation of dichlorofluorescein (DCF) in epidermal peels. Catalase inhibited and prevented the $H_2O_2$-dependent enhancement of DCF fluorescence. Menadione also caused an increase in fluorescence (line 2) that was inhibited by nitroblue tetrazolium (NBT) (line 3). The quinone derivatives MitoQ, SkQ1, and SkQ3 inhibited the menadione-induced DCF fluorescence increase. However, they produced no effect in the $H_2O_2$-induced fluorescence test (Fig. 6, lines 4-9).

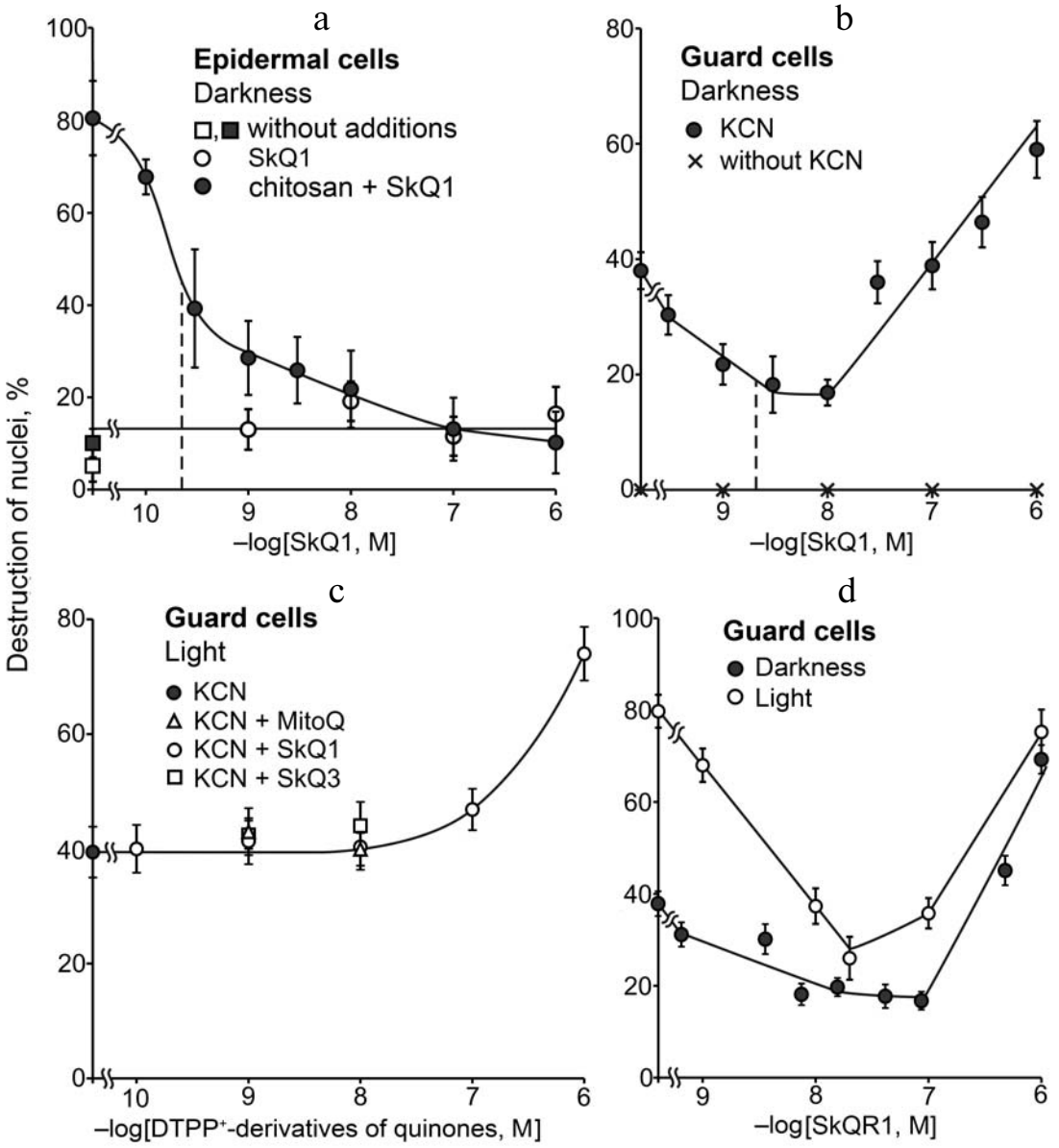


**Fig. 4.** Effect of quinone cationic derivatives on the chitosan-induced destruction of EC nuclei (a) and on the $CN^-$-induced destruction of GC nuclei (b-d) in pea leaf epidermis in the dark and in the light. a) Epidermal peels in 2 ml of distilled water were supplemented with SkQ1, incubated for 2 h in the dark, and treated by 100 μg/ml chitosan on a magnetic stirrer for 0.5 h and then for 5 h without stirring in the dark. b-d) Epidermal peels in 2 ml of distilled water were supplemented with SkQ1, exposed to vacuum infiltration for 1 min, incubated for 2 h in the dark, supplemented with 2.5 mM KCN, once more exposed to vacuum infiltration for 1 min, and incubated for 22 h in the dark. No destruction of GC nuclei occurred in the control systems (without additions) of experiments (b-d). Dotted lines, SkQ1 concentrations eliciting the half-maximum protective response.

In accordance with the data presented in [44, 45], $H_2O_2$ stimulated the $CN^-$-induced destruction of GC nuclei (Fig. 7a). $H_2O_2$ failed to induce the destruction of GC nuclei without $CN^-$ at concentrations up to 10-50 mM [44], apparently due to the high catalase and peroxidase activity of GC. The destructive effect of $CN^-$ and $CN^- + H_2O_2$ was lacking in the presence of compounds that oxidize the photosynthetic and respiratory electron transfer chains [13, 14, 44]. We used *p*-benzoquinone (BQ; 100 μM) as an oxidant that removed the destructive effect of $CN^-$ and $CN^- + H_2O_2$ (Fig. 7a). SkQ1 (10 nM) diminished the destructive effect of $CN^-$ on GC nuclei, but was inefficient in the $CN^- + H_2O_2$ system. The combination of BQ (100 μM) and SkQ1 (10 nM) removed the effect of $CN^- + H_2O_2$. An increase in SkQ1 concentration up to 10 μM (approximating the BQ concentration) abolished the protective effect of SkQ1 and enhanced the destructive influence of $CN^- + H_2O_2$ (Fig. 7a). Quinone derivatives prevented the chitosan or $H_2O_2$ influence on EC, but were inefficient in the chitosan + $H_2O_2$ system (Fig. 7b).

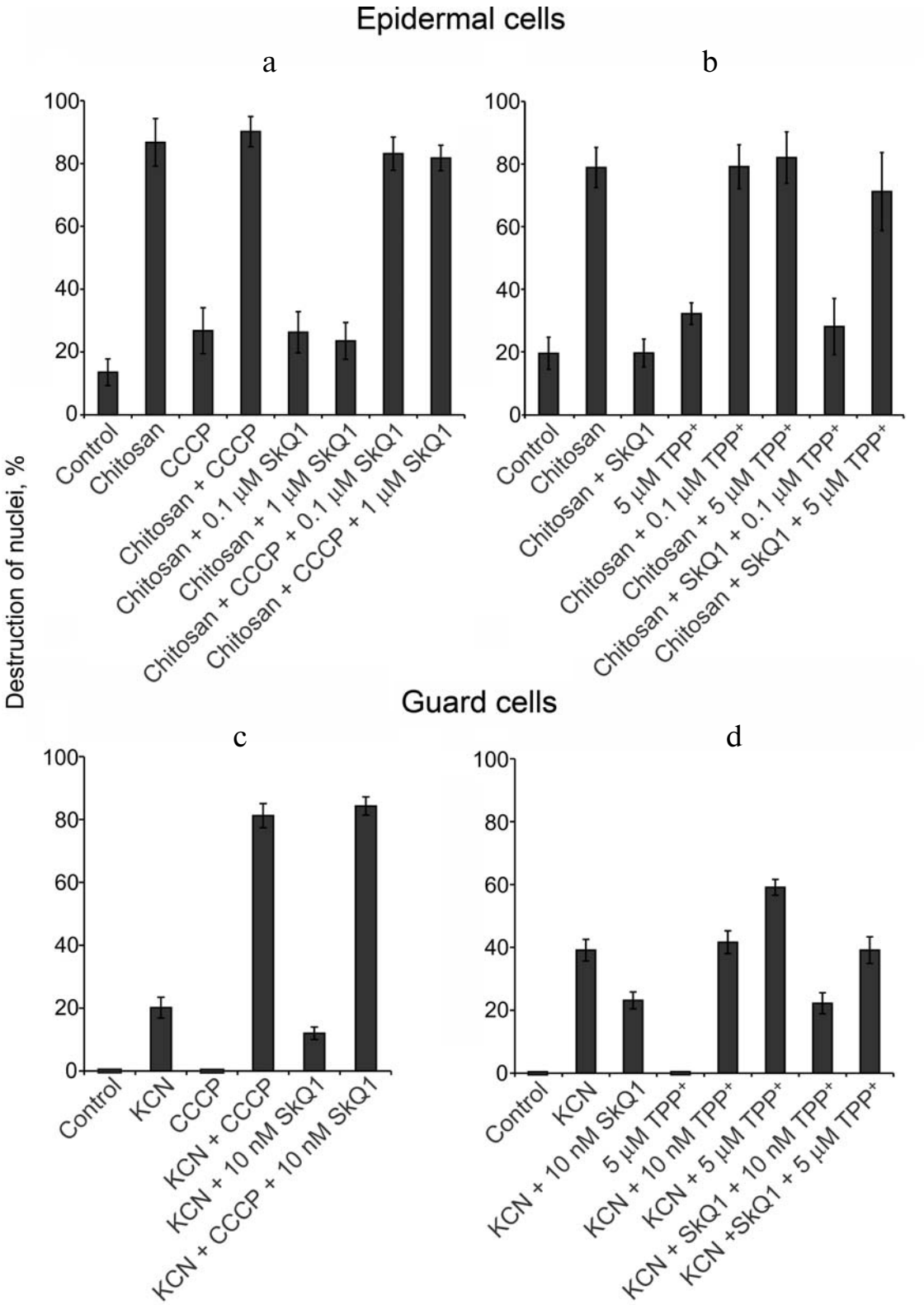


**Fig. 5.** Effect of SkQ1, the uncoupler CCCP, and penetrating $TPP^+$ cations on the chitosan-induced destruction of EC nuclei (a, b) and on the $CN^-$-induced destruction of GC nuclei (c, d) of pea leaf epidermis in the dark. Epidermal peels were placed in 2 ml of distilled water, supplemented with 10 μM CCCP and SkQ1 (a) or $TPP^+$ and 0.1 μM SkQ1 (b), incubated for 1 h, treated by 100 μg/ml chitosan on a magnetic stirrer for 0.5 h, and then incubated for 5 h without stirring in the dark. The epidermal peels in 2 ml of distilled water were supplemented with 10 μM CCCP and 10 nM SkQ1 (c) or $TPP^+$ and 10 nM SkQ1 (d), exposed to vacuum infiltration for 1 min, incubated for 2 h in the dark, supplemented with 2.5 mM KCN, once more exposed to vacuum infiltration for 1 min, and incubated for 15 h (c) or 24 h (d) in the dark.

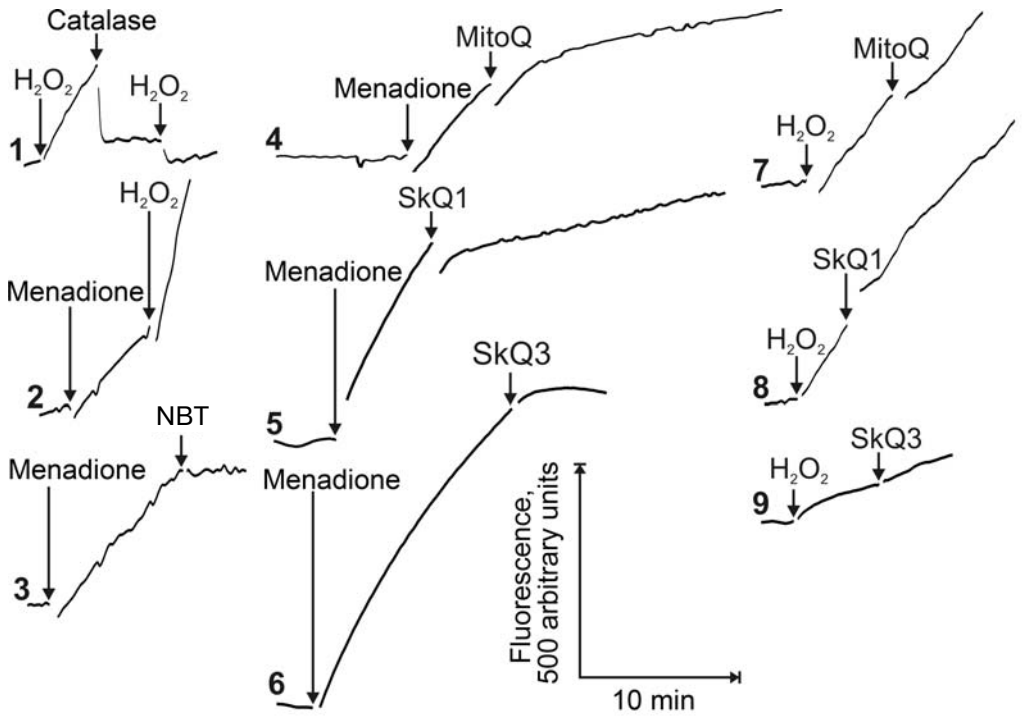


**Fig. 6.** Dichlorofluorescein (DCF) fluorescence in epidermal peels from pea leaves. Additions: 30 μM (lines 1, 2) or 100 μM $H_2O_2$ (lines 7, 8, 9), 2 units/ml catalase, 100 μM menadione, 200 μM NBT, 0.55 nM (line 4) and 5.5 nM MitoQ (line 7), 1 nM (line 5) and 10 nM SkQ1 (line 8), 10 nM SkQ3 (lines 6, 9).

SkOR1 reduced the $CN^-$-induced destruction of GC nuclei both in the dark and in the light (Figs. 4d and 8a). Since light-excited SkQR1 can produce singlet oxygen, the effect of rhodamine 6G was tested. Rhodamine 6G decreased the $CN^-$-induced destruction of GC nuclei in the light and was without effect in the dark (Fig. 8a).

Figure 8b demonstrates the menadione- and $H_2O_2$-dependent DCF fluorescence increase. SkQR1 did not lower the menadione- or $H_2O_2$-induced DCF fluorescence yield. Moreover, SkQR1 slightly increased (Fig. 8b, line 1) or induced (lines 3, 4, 6) DCF fluorescence in the presence of menadione. The DCF fluorescence increase was also initiated by tetramethylrhodamine ethyl ether (TMRE) and rhodamine 6G (Fig. 8b, lines 7 and 8). NBT inhibited the DCF fluorescence increase.

## DISCUSSION

Cyanide induces apoptosis in leaf epidermal cells [12-17] that may be accompanied by autophagy [18]. Both GC and EC perished in the presence of $CN^-$. The elicitor chitosan that induces apoptosis in plants [24-26] causes the death of EC and the destruction of their nuclei (Fig. 3), whereas GC remain intact.

Plant stomata, microscopic surface openings in the epidermis of leaves, regulate gas exchange (photosynthesis and respiration) and water transpiration (heat regulation). An additional important function concerns their involvement in the plant innate immune response: stomata close upon detecting microbial pathogens to prevent their penetration into the intercellular spaces in the leaf interior [46]. Infectious agents evolve specific virulence factors that cause stomatal reopening [46].

The $CN^-$-induced death of GC and EC was prevented by anaerobiosis and increased by the addition of $H_2O_2$ [13, 14, 44]. The chitosan-induced destruction of EC nuclei was also prevented by anaerobiosis and antioxidants [26]. Both types of cell death involve ROS. The efficient concentrations of the antioxidants α-tocopherol and butylated hydroxytoluene were 100 μM. They exceeded 100 mM with mannitol [12-14].

The cationic derivatives of the quinones tested in this study are far more effective than all other known compounds possessing antioxidant (Figs. 2a and 6) and antiapoptotic properties (table). Their concentrations required for a half-maximum prevention of PCD were within the pico- and nanomolar range (table). SkQ3 (a plastoquinone with a supplementary methyl group) was the most efficient agent in terms of preventing chitosan-induced PCD, and the effects of the tested substances decreased in the SkQ3 > SkQR1 (plastoquinone attached to the rhodamine cation) > SkQ1 (the plastoquinone derivative) > MitoQ (the ubiquinone derivative) sequence. The strong influence exerted by these com-

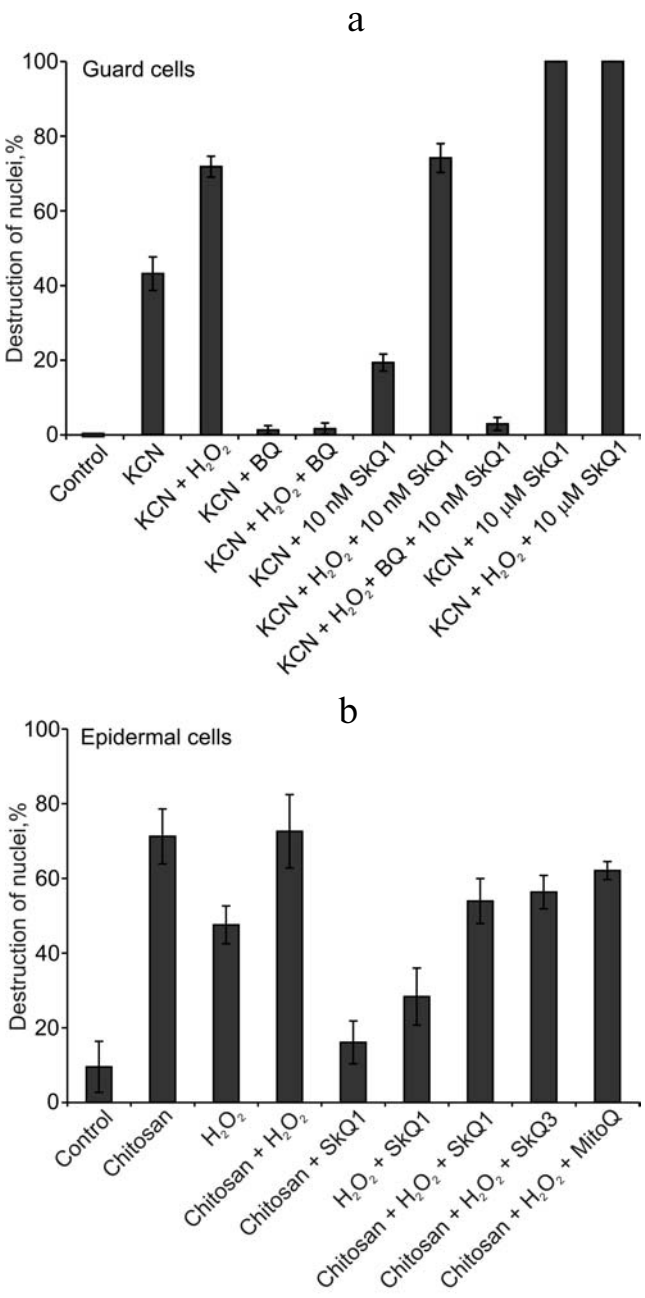


**Fig. 7.** Effects of quinone derivatives on the $CN^-$-induced destruction of GC nuclei (a) and the chitosan-induced destruction of EC nuclei (b) in pea leaf epidermis in the dark. a) Epidermal peels were placed in 2 ml of distilled water, supplemented with SkQ1, exposed to vacuum infiltration for 1 min, incubated for 1.5 h in the dark, supplemented with 100 μM *p*-benzoquinone (BQ), incubated for another 0.5 h in the dark, supplemented with 2.5 mM KCN, once more exposed to vacuum infiltration for 1 min, supplemented with 100 μM $H_2O_2$, and incubated for 24 h in the dark. b) Epidermal peels in 2 ml of distilled water were supplemented with 100 nM SkQ1, 10 nM SkQ3 or 55 nM MitoQ, incubated for 2 h, treated with 100 μg/ml chitosan on a magnetic stirrer for 0.5 h, supplemented with 100 μM $H_2O_2$ and incubated for 5 h without stirring in the dark.

pounds is due to their accumulation inside cells and, specifically, in energized mitochondria (Fig. 2).

In the presence of $CN^-$, the protective effect of quinones with respect to GC (table) decreased in the SkQ3 > MitoQ > SkQ1 > SkQR1 sequence, i.e. the arrangement of MitoQ and SkQR1 is reversed compared to the chitosan-containing system. The reason for this discrepancy is unclear. Of relevance might be the fact that $CN^-$, an apoptosis inducer in GC, inhibits mitochondrial cytochrome oxidase.

The antiapoptotic effect of quinone derivatives is removed by a protonophoric uncoupler (Fig. 5). This is to be expected because the effect of $DTPP^+$-derivatives involves the mitochondrial matrix or the coupling membrane *per se*. In addition to the inner mitochondrial membrane, the plasma membrane of a plant cell generates an ion potential difference that is a prerequisite for carrying out the osmotic work necessary for the transport of metabolic substrates and products [29-32]. The primary uptake of positively charged compounds is due to the $\Delta\psi$ generated across the plasma membrane (with a "minus" sign inside); the bulk of these compounds therefore accumulate in the mitochondria where higher $\Delta\psi$ values are attained. Furthermore, the solubility of the quinone derivatives in the lipid phase of membranes considerably exceeds that in water. The distribution coefficient of SkQ1 between lipid and water is about 13,000 : 1 [36, 37]. Overall, the concentration gradient between the extracellular medium and the inner monolayer of the inner mitochondrial membrane reaches $1.3 \cdot 10^8$ [36, 37]. This implies that the SkQ1 concentration in the inner monolayer exceeds $1 \cdot 10^{-2}$ M at its concentration of $1 \cdot 10^{-10}$ M in the medium (table). Cationic quinones are reduced by the mitochondrial respiratory chain, and the rate of their reduction is higher than the rate of their oxidation [36, 37]. Thus, various forms of SkQ and MitoQ are mitochondrial-targeted rechargeable antioxidants.

Quinone derivatives inhibit the generation of $H_2O_2$ induced by menadione via the electron transfer systems (Fig. 6). Menadione is reduced by Photosystem II, the cytochrome $b_6f$ complex, and Photosystem I of chloroplasts [47, 48] and by the mitochondrial NADH:ubiquinone oxidoreductase [49], and cytochrome $bc_1$ complex [50]. Menadione semiquinone is spontaneously oxidized by $O_2$, resulting in $O_2^{\cdot-}$ formation. It thereupon produces $H_2O_2$ by the superoxide dismutase reaction. Menadiol is also oxidized by $O_2$, and $H_2O_2$ is formed [49, 51]. However, the quinone derivatives do not cope with the added $H_2O_2$ (Fig. 6). The $CN^-$- and chitosan-induced destruction of GC and EC nuclei is not inhibited by the quinone derivatives in the presence of $H_2O_2$ (Fig. 7). This fact may be due to the $H_2O_2$-dependent activation of the ROS generation. ROS-induced ROS release by the mitochondrial respiratory chain of heart cells [52], by the NAD(P)H oxidase of the plasma membrane in nonphagocytic cell types of vascular origin [53], and presum-

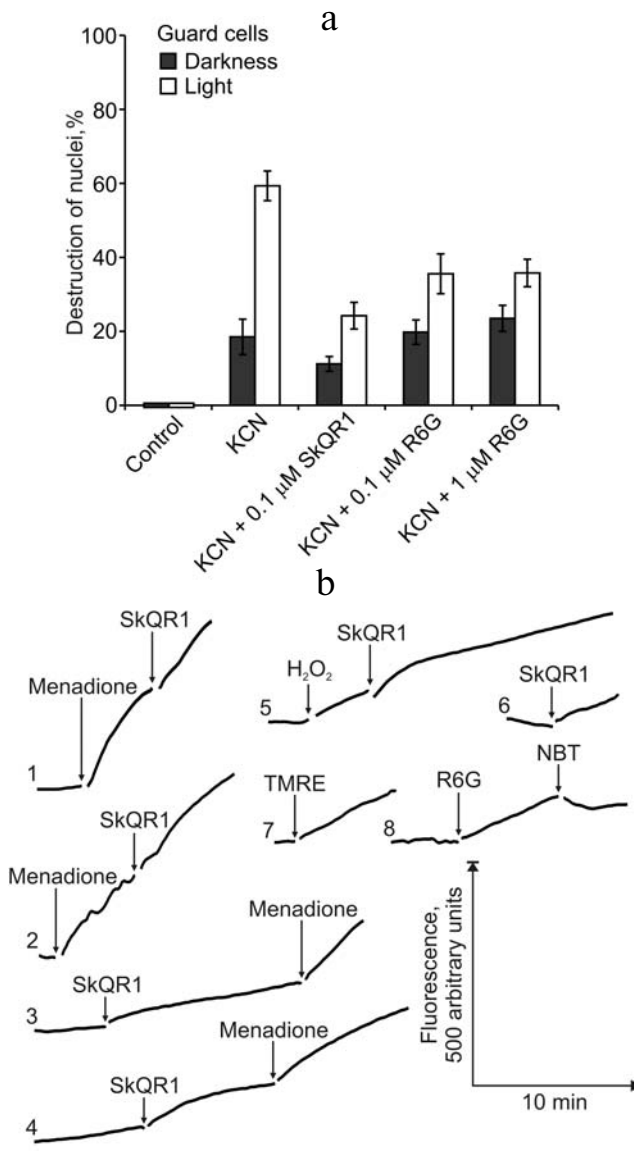


**Fig. 8.** Effect of SkQR1 and rhodamine derivatives on the $CN^-$-induced destruction of GC nuclei (a) and DCF fluorescence (b) in pea leaf epidermis. a) Epidermal peels were placed in 2 ml of distilled water, supplemented with SkQR1 or rhodamine 6G (R6G), exposed to vacuum infiltration for 1 min, incubated for 2 h in the dark, supplemented with 2.5 mM KCN, once more exposed to vacuum infiltration for 1 min, and incubated for 18 h in the dark or in the light. b) Additions: 100 μM menadione, 10 nM (lines 1-5) and 100 nM (line 6) SkQR1, 100 μM $H_2O_2$, 100 nM R6G and ethyl ester of tetramethylrhodamine (TMRE), 200 μM NBT.

ably by the NADPH oxidase of the plasma membranes in epidermis cells of pea leaves [44] have been documented in the literature. Therefore, exogenous $H_2O_2$ is likely to increase the intracellular content of ROS. The antiapoptotic effect of SkQ1 on animal cells did not manifest itself after increasing the $H_2O_2$ concentration to 500 μM [37].

BQ (100 μM) or its combination with SkQ1 (10 nM) removed the effect of $CN^- + H_2O_2$ on GC (Fig. 7). An increase in SkQ1 concentration up to 10 μM enhanced $CN^-$- and ($CN^- + H_2O_2$)-induced apoptosis of GC. The inefficiency of SkQ1 in protecting GC nuclei from $CN^- + H_2O_2$ is probably due to the inability of SkQ1 to operate as an efficient electron acceptor in the chloroplast photosynthetic chain, in contrast to ferricyanide, methyl viologen, menadione, and other compounds. The chloroplast electron transfer chain generates $\Delta\psi$ with a "plus" sign on the inner side of chloroplast thylakoids. Therefore, quinones linked to penetrating cations, while moving down their concentration gradients, do not accumulated inside energized chloroplasts. Conversely, they are expelled from them. Indeed, the protective effect of quinone $DTPP^+$ derivatives on the $CN^-$-induced destruction of the GC nuclei does not manifest itself upon illumination (Fig. 4c).

Unlike other quinone derivatives, SkQR1 produced a protective effect on the $CN^-$-induced destruction of GC nuclei not only in the dark, but also in the light (Figs. 4d and 8, table). Light-excited rhodamine dyes interact with triplet oxygen ($^3O_2$), converting it into the singlet excited state, $^1O_2$ [52, 54-56]. Thereupon, $^1O_2$ induces NAD(P)H-dependent $O_2^{\cdot-}$ generation [57]. By destroying protein D1 of chloroplast Photosystem II [58], $^1O_2$ inhibits the reduction of plastoquinone. The $CN^-$-induced destruction of the GC nuclei in the light was also prevented upon inhibition of Photosystem II by DCMU [13, 14]. The disruption of the operation of chloroplast Photosystem II by rhodamine and its derivatives seems to suppress the $CN^-$-induced destruction of GC nuclei in the light.

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