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Chitosan-Induced Programmed Cell Death in Plants

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Abstract—Chitosan, CN^-, or H_2O_2 caused the death of epidermal cells (EC) in the epidermis of pea leaves that was detected by monitoring the destruction of cell nuclei; chitosan induced chromatin condensation and marginalization followed by the destruction of EC nuclei and subsequent internucleosomal DNA fragmentation. Chitosan did not affect stoma guard cells (GC). Anaerobic conditions prevented the chitosan-induced destruction of EC nuclei. The antioxidants nitroblue tetrazolium or mannitol suppressed the effects of chitosan, H_2O_2, or chitosan + H_2O_2 on EC. H_2O_2 formation in EC and GC mitochondria that was determined from 2′,7′-dichlorofluorescein fluorescence was inhibited by CN^- and the protonophoric uncoupler carbonyl cyanide *m*-chlorophenylhydrazone but was stimulated by these agents in GC chloroplasts. The alternative oxidase inhibitors propyl gallate and salicylhydroxamate prevented chitosan- but not CN^--induced destruction of EC nuclei; the plasma membrane NADPH oxidase inhibitors diphenylene iodonium and quinacrine abolished chitosan- but not CN^--induced destruction of EC nuclei. The mitochondrial protein synthesis inhibitor lincomycin removed the destructive effect of chitosan or H_2O_2 on EC nuclei. The effect of cycloheximide, an inhibitor of protein synthesis in the cytoplasm, was insignificant; however, it was enhanced if cycloheximide was added in combination with lincomycin. The autophagy inhibitor 3-methyladenine removed the chitosan effect but exerted no influence on the effect of H_2O_2 as an inducer of EC death. The internucleosome DNA fragmentation in conjunction with the data on the 3-methyladenine effect provides evidence that chitosan induces programmed cell death that follows a combined scenario including apoptosis and autophagy. Based on the results of an inhibitor assay, chitosan-induced EC death involves reactive oxygen species generated by the NADPH oxidase of the plasma membrane.

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Key words: programmed cell death, apoptosis, autophagy, chitosan, plants, epidermal cells

Apoptosis, necrosis, and autophagy are the main forms of cell death. The main distinctive features of apoptosis include the activation of caspases (paracaspases in animals and metacaspases in fungi and plants [1]), internucleosome DNA fragmentation, phosphatidylserine redistribution in the plasma membrane, a decrease in cytoplasm volume, intense cell vacuolization, and the formation of vesicle-like protrusions of the plasma membrane that convert into apoptotic vesicles (such vesicles are not formed in plants) [2]. Necrosis is accompanied by cell swelling resulting in the rupturing of the plasma membrane and the extrusion of the cell content into the intercellular space. Autophagy involves engulfing cytoplasm components by membrane vesicles (autophagosomes) followed by their fusion with lysosomes or vacuoles whose enzymes cause the hydrolysis of the autophagosome content. The forms of cell death often occur in combination. This is exemplified by programmed (controlled) necrosis [3-5] or an autophagy–apoptosis combination [4]. Apoptosis, controlled necrosis, and autophagy (all the three phenomena are forms of programmed cell death) require gene expression, depend on protein synthesis [3-5], and the term "programmed cell death" (PCD) applies to all of them.

Mitochondria play a key role in the PCD of animals as sources of a number of apoptogenic factors including cytochrome *c*, flavoprotein AIF (apoptosis-inducing factor), and reactive oxygen species (ROS) [6, 7]. Mitochondrial cytochrome *c* is released into the cyto-

Abbreviations: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DAPI, 4′,6-diamidino-2-phenylindole; DCF, 2′,7′-dichlorofluorescein; DCF-DA, 2′,7′-dichlorofluorescein diacetate; DPI, diphenylene iodonium; EC, epidermal cells; GC, guard cells; NBT, nitroblue tetrazolium; PCD, programmed cell death; ROS, reactive oxygen species; TMRE, tetramethylrhodamine ethyl ester.

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plasm of plant cells during PCD [8]. However, the release of cytochrome *c* into the cytoplasm is not a mandatory stage of plant PCD [9].

We demonstrated in [10-12] that chloroplasts are involved in plant PCD. The studies were conducted with pea leaf epidermis. The epidermis is a monolayer consisting of stoma guard cells (GC) that contain mitochondria and chloroplasts and bulk epidermal cells (EC) that contain mitochondria only. PCD can be induced by cyanide in plants [13, 14]. CN^- caused the death of GC and EC that was detected by monitoring the destruction of their nuclei. Illumination accelerated this process in GC but not EC [10-12]. Anaerobic conditions prevented ROS generation and suppressed cyanide-induced PCD, in an analogy to the effect of antioxidants. CN^- induced GC death with the symptoms of apoptosis, such as chromatin condensation and marginalization, an increase in vacuole volume [15], and internucleosome DNA fragmentation [16]. Cyanide-induced GC death follows a combined scenario including apoptosis and autophagy and depends on proteins synthesized in the cytoplasm, chloroplasts, and mitochondria [16]. 3-(3′,4′-Dichlorophenyl)-1,1-dimethylurea, an inhibitor of electron transfer between plastoquinones Q_A and Q_B in photosystem II, abolished the light-dependent stimulation of CN^--induced destruction of GC nuclei. The same effect was produced by dinitrophenylether of iodonitrothymol and stigmatellin, competitive inhibitors of plastoquinol oxidation at site *o* of chloroplast b_6f-cytochrome complex. PCD in plants is apparently subject to regulation by the redox state of the plastoquinone of b_6f-cytochrome complex [11, 12].

CN^- is not a specific PCD inducer. It also inhibits peroxidases, catalase, chloroplast Cu,Zn-superoxide dismutase, and mitochondrial cytochrome *c* oxidase. Moreover, CN^- inactivates chloroplast ribulose-1,5-diphosphate carboxylase [17]. By suppressing NADPH utilization in the Calvin cycle, CN^- causes the depletion of the $NADP^+$ pool and, therefore, stimulates ROS formation by chloroplast photosystem I. Hence CN^- disrupts the operation of mitochondria and chloroplasts.

Other substances are known to induce PCD. These are signal compounds of plant pathogens (elicitors). By imitating the interaction of the pathogen with the plant, elicitors induce protective responses including ROS formation and apoptosis (a hypersensitive response) [18, 19]. An effective elicitor is chitosan, poly(β-1,4)-N-acetylglucosamine, which is a product of incomplete deacetylation of chitin, a component of the cell wall of fungi. The cell plasma membrane of various plants contains a receptor that binds chitin oligosaccharides with high affinity [20-22]. The receptor is a glycoprotein with a molecular weight of 75 kDa [23]. Knocking down the receptor protein suppresses chitin-induced H_2O_2 formation [23]. As an elicitor, chitosan induces a hypersensitive response, produces an antiviral effect on plants [24-27], and causes the internucleosomal fragmentation of the nuclear DNA [27, 28]. However, the DNA fragmentation was not detected by other researchers [26].

The goal of this work was to investigate chitosan-induced cell death in plants and to compare chitosan with H_2O_2 and CN^- in terms of their PCD-inducing activity.

MATERIALS AND METHODS

The studies were conducted with the epidermis isolated from the lower surface of the leaves of 12-17-day-old pea seedlings (*Pisum sativum* L., cv Alpha). The plants were grown under 16 h illumination/8 h darkness at 23-28°C, using luminescent lamps with an intensity of 100 μE/m^2 per sec. Epidermal peels were separated with tweezers, placed in distilled water, and supplemented with reagents whose composition is given in figure captions. The peels were treated with chitosan by stirring them in a chitosan suspension with a multiposition magnetic mixer. The epidermal peels were incubated on polystyrene plates at 22-25°C with reagents that were added as aqueous solutions.

After the incubation, epidermal peels were treated for 5 min with Battaglia fixative (a mixture of chloroform, 96% ethanol, glacial acetic acid, and 40% formalin at a ratio of 5 : 5 : 1 : 1), washed with ethanol for 10 min to remove fixative, incubated for 5 min in water, and stained with Carazzi hematoxylin for 20 min. The stained epidermal peels were washed with tap water and examined using a light microscope. Cell death was detected by monitoring the destruction of cell nuclei. The numbers of cells with damaged nuclei and without nuclei per 300-500 tested cells were determined in 3-4 epidermal peels. Two or three repeats of each experiment were conducted. Typical data obtained in one of the repeats are given below.

Fluorescence studies were conducted with epidermal peels that were fixed, washed to remove the fixative as described above, and stained with 0.2 μM 4′,6-diamidine-2-phenylindole (DAPI) for 20 min. The samples were examined with a Carl Zeiss Axiovert 200M microscope (Germany) in the transmitted light and fluorescent modes. DAPI fluorescence was excited with a mercury lamp with a G365 absorption light filter that transmits light with λ = 300-390 nm and registered with an LP420 absorption light filter that transmits light with λ > 420 nm.

Epidermal peels or leaf parts were prepared for examining using a confocal microscope by treating them with dichlorofluorescein diacetate (DCF-DA) and tetramethylrhodamine ethyl ester (TMRE). The studies were conducted with a Carl Zeiss Axiovert 200M microscope equipped with an LSM 510Meta confocal adapter (Germany). Upon penetrating into a cell, DCF-DA is deacetylated by intracellular diesterases and oxidized by H_2O_2, yielding DCF (2′,7′-dichlorofluorescein), an

intensely fluorescent compound [29, 30]. The fluorescence of chlorophyll (Chl) in chloroplasts, DCF, and TMRE was excited at 633, 488, and 543 nm and detected in the 650-710, 500-530, and 565-615 nm ranges, respectively.

DNA was isolated from pea epidermis by the method described in [31] with minor modifications. From 50 to 100 mg of tissue was ground under liquid nitrogen. The DNA was extracted for 30 min at 65°C in 100 mM Tris-HCl buffer (pH 8.0) supplemented with 20 mM EDTA, 1.4 M NaCl, 2% cetyltrimethylammonium bromide, 1% polyvinylpyrrolidone, and 0.75% (v/v) 2-mercaptoethanol; the mixture was stirred. Cell debris were removed by centrifuging the mixture for 10 min at 15,000*g*; proteins were removed from the supernatant with a mixture of phenol, chloroform, and isoamyl alcohol at the ratio of 25 : 24 : 1. The DNA was precipitated with cold isopropanol, treated with RNase A (100 μg/ml) for 30 min at 37°C, and reprecipitated with ethanol. The resulting DNA was dissolved in 20 μl of 10 mM Tris-HCl buffer (pH 8.0) containing 1 mM EDTA. The DNA content was determined with a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). The DNA (1.2 μg) was subjected to electrophoresis for 40 min at 5 V/cm in 1.5% agarose gel prepared in 40 mM Tris-acetate buffer (pH 7.4) supplemented with 1 mM EDTA. The DNA was stained with ethidium bromide, whose fluorescence was monitored with a GelDoc It Imaging System camera (UVP, USA).

RESULTS

Chitosan caused the destruction of EC nuclei in pea leaf epidermis under aerobic conditions (Fig. 1). In analogy to the PCD inducer CN^- [11], anaerobiosis prevented the chitosan-induced destruction of cell nuclei. The number of EC with damaged nuclei increased with an increase in chitosan treatment time and reached 80% after 5 h of incubation with chitosan. The effect of chitosan under aerobic conditions was prevented by nitroblue tetrazolium (NBT), an $O_2^{\cdot-}$ quencher [32, 33] and, to a lesser extent, by pyruvate, which was converted to acetate via non-enzymatic oxidative decarboxylation [34]. The chitosan effect was abolished with mannitol, a trap for hydroxyl radical [35, 36]. H_2O_2 (100 μM) per se caused the destruction of EC nuclei to a somewhat larger extent than chitosan. Mannitol and NBT suppressed the effects of H_2O_2 and chitosan + H_2O_2 (Fig. 1). Chitosan caused chlorophyll condensation and marginalization (Fig. 2, a-d) followed by the decay and disappearance of EC nuclei, while the cell walls were not affected (Fig. 2, e

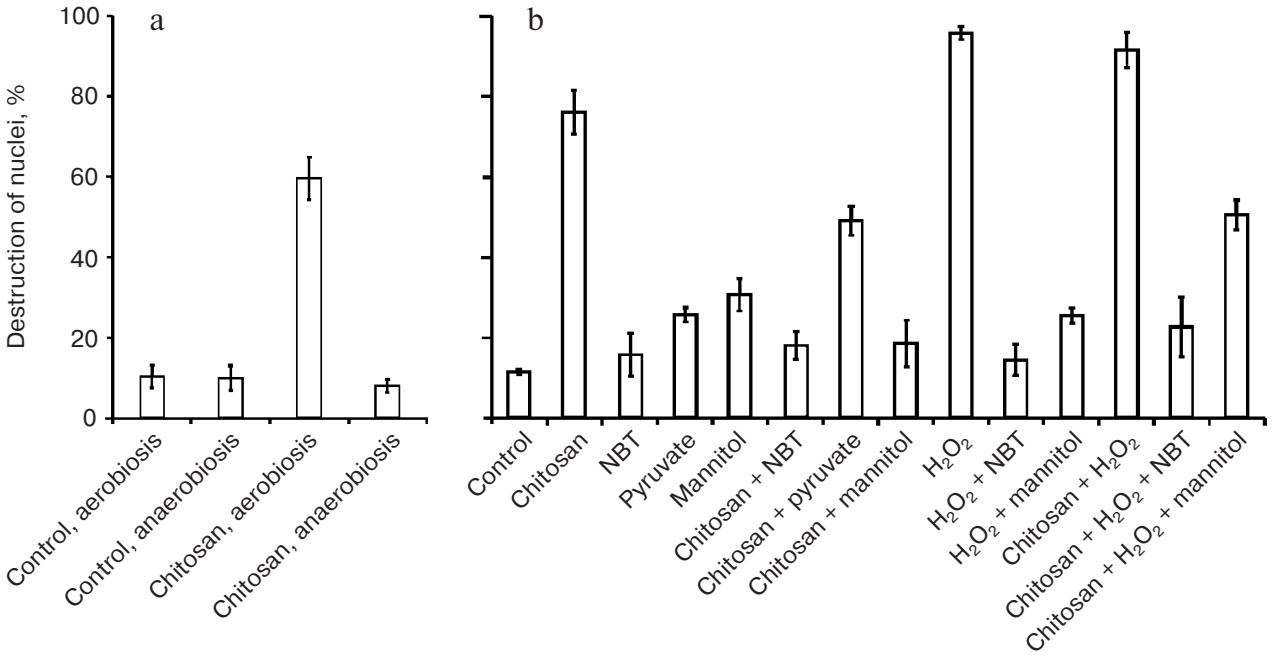

Fig. 1. Effects of anaerobiosis (a) and antioxidants and H_2O_2 (b) on the chitosan-induced destruction of EC nuclei in pea leaf epidermis. a) To create anaerobic conditions, a 3-5 mm thick layer of sunflower oil was applied on distilled water (2 ml) containing epidermal peels, 50 mM glucose, 0.1 μg/ml glucose oxidase, and catalase (2 activity units per ml). The system was incubated for 30 min, supplemented with a chitosan suspension (100 μg/ml), and re-incubated for 30 min with a magnetic stirrer and then for 3 h in the light without stirring. Thereupon, the epidermal peels were fixed and cells with damaged nuclei were counted; b) epidermal peels were supplemented with 0.2 mM NBT, 5 mM potassium pyruvate, or 125 mM mannitol, or 100 μM H_2O_2 and incubated for 20 min. Chitosan (100 μg/ml) was added to the system that was then re-incubated for 30 min with a magnetic stirrer and then for 3 h in the dark without stirring.

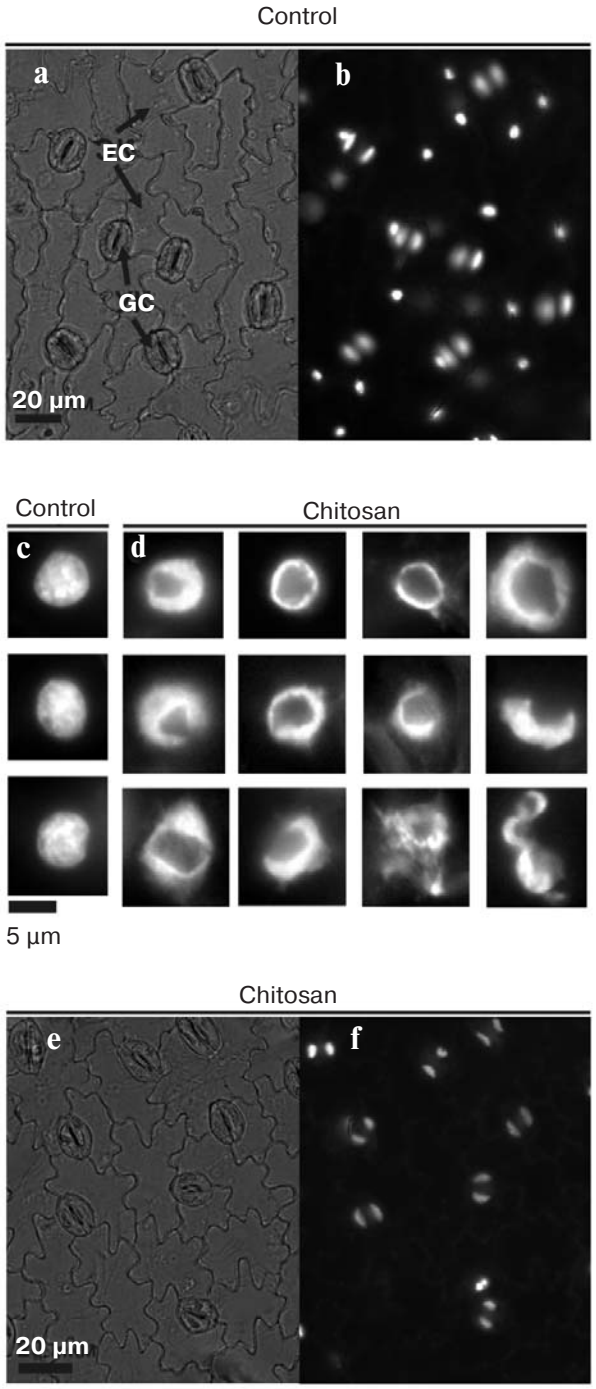

Fig. 2. Light (transmitted light images (a, e)) and fluorescence (b-d, f) microscopy of EC and GC (a, b, e, f) and EC nuclei (c, d) in the epidermis of pea leaves. a, b) Control (no additions), 5 h of incubation; c) control (no additions), 2 h of incubation; d) various forms of chitosan-induced destruction of EC nuclei, 2 h of incubation; e, f) chitosan added, 5 h of incubation with chitosan. Epidermal peels (~50 μg of protein) in 2 ml of distilled water were supplemented with chitosan (100 μg/ml), incubated for 30 min with a magnetic stirrer, and then for 2 or 5 h without stirring in the dark. After the incubation, fixed epidermal peels were stained with DAPI and examined in a microscope (see "Materials and Methods").

and f). The GC remained intact (Fig. 2, a, b, e, and f) even 3 days after the chitosan treatment. Chitosan induced the destruction of cell nuclei in various plants. In our studies, it caused the destruction of (i) EC nuclei in the leaf epidermis of maize and tobacco and (ii) cell nuclei in the leaves of the aquatic plants Canadian pondweed and eelgrass (data not shown).

Since mitochondria are ROS generators, we tested the effects of respiratory chain inhibitors. Cyanide, which inhibits mitochondrial cytochrome oxidase, caused a rapid destruction of EC nuclei: the number of nucleus-lacking EC after 30 min of incubation with KCN exceeded that after 3 h of incubation with chitosan (Fig. 3). The destruction of EC nuclei in the presence of chitosan was insignificantly stimulated with CN^- and suppressed by propyl gallate or salicylhydroxamate, which inhibit the CN^--resistant alternative oxidase of mitochondria [37]. Propyl gallate and salicylhydroxamate slightly inhibited the cyanide-induced destruction of EC nuclei.

Diphenylene iodonium (DPI) and quinacrine are employed as inhibitors of the NADPH oxidase of the plasma membrane of plant cells [38-41]. These compounds can affect other flavin enzymes. However, DPI and quinacrine at concentrations up to 100 μM did not influence respiration and photosynthetic O_2 evolution in pea leaf fragments and inhibited CN^--induced destruction of GC nuclei [42]. Chitosan-induced destruction of EC nuclei was prevented by DPI and quinacrine (Fig. 4a). Quinacrine (20-100 μM) did not influence cyanide-induced destruction of EC nuclei [42]. DPI had no effect on this process either (Fig. 4b).

As shown earlier [43], CN^--induced destruction of GC nuclei was enhanced by treating the epidermis with cycloheximide, an inhibitor of protein synthesis in the cytoplasm of eukaryotes, and suppressed by lincomycin, an inhibitor of protein synthesis in mitochondria and chloroplasts. Chitosan- and H_2O_2-induced destruction of EC nuclei was reduced by cycloheximide and suppressed by lincomycin and the lincomycin–cycloheximide combination (Fig. 5). 3-Methyladenine, an inhibitor of autophagy [44], caused a considerable destruction of EC nuclei by itself. It did not influence H_2O_2-induced but prevented chitosan-induced destruction of EC nuclei (Fig. 5).

Figure 6a contains the data on GC obtained by means of fluorescence microscopy including (i) chlorophyll fluorescence and (ii) DCF fluorescence. This enables locating chloroplasts and H_2O_2 formation sites in the cell, respectively. Figure 6a also includes the superposition of images (i) and (ii) and a transmitted light GC image. It is evident that H_2O_2 is generated in spherical structures and cell nuclei, but not in chloroplasts. KCN prevented H_2O_2 formation in spherical structures and reduced it in nuclei. It induced H_2O_2 formation in chloroplasts (Fig. 6b). The protonophore CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) abolished

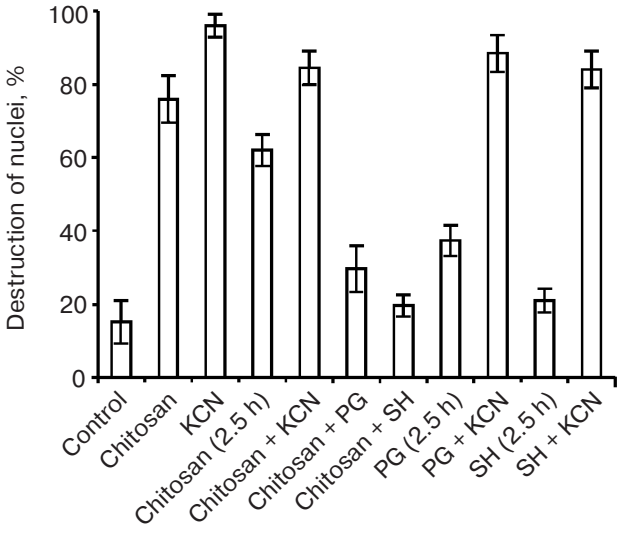

Fig. 3. Effects of propyl gallate (PG) and salicylhydroxamate (SH) on chitosan- and CN^--induced destruction of EC nuclei in pea leaf epidermis. Epidermal peels were treated with chitosan (100 μg/ml) for 30 min with a magnetic stirrer. Then 50 μM PG or 1 mM SH was added, and the system was incubated for 3 h without stirring in the dark. KCN (2.5 mM) was added 30 min before the end of the incubation.

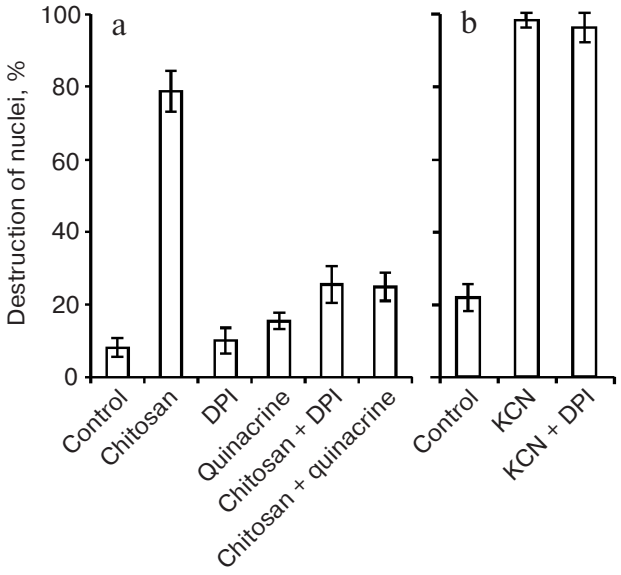

Fig. 4. Effects of DPI and quinacrine on chitosan- and CN^--induced destruction of EC nuclei in pea leaf epidermis. a) After treating epidermal peels with 100 μg/ml chitosan for 30 min with a magnetic stirrer, 25 μM DPI or 50 μM quinacrine was added; the system was incubated for 4 h without stirring in the dark. b) Epidermal peels preincubated with DPI for 5 min were supplemented with 2.5 mM KCN and incubated for 30 min in the dark.

DCF fluorescence in spherical structures, had no effect on it in nuclei, and induced DCF fluorescence in chloroplasts (Fig. 6c). TMRE cations accumulated in spherical structures and caused their fluorescence. No TMRE accumulation occurred in nuclei and chloroplasts (Fig. 6d). The superposition of the TMRE and DCF images reveals that the fluorescence of both dyes occurs in the same spherical structures. Based on this criterion in con-

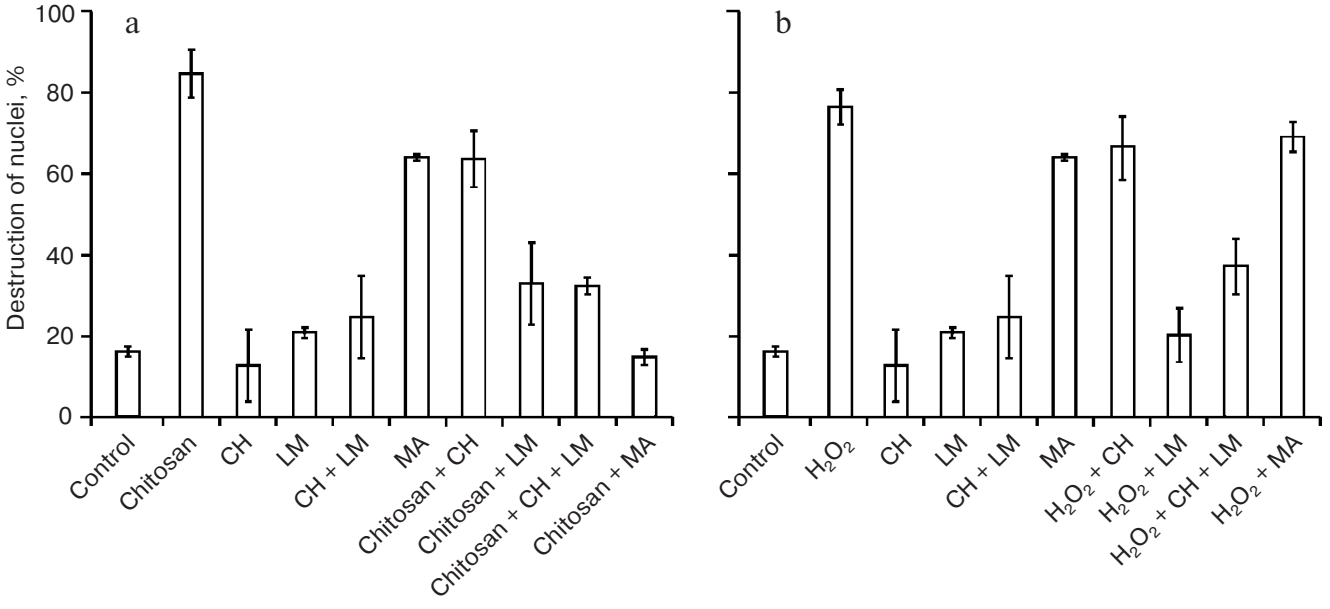

Fig. 5. Effects of cycloheximide (CH), lincomycin (LM), and 3-methyladenine (MA) on chitosan- and H_2O_2-induced destruction of EC nuclei in pea leaf epidermis. a) After treating epidermal peels with 100 μg/ml chitosan for 30 min with a magnetic stirrer, CH, LM, and MA were added; the system was incubated for 3 h without stirring in the dark. b) Epidermal peels were supplemented with H_2O_2, CH, LM, and MA and incubated for 3 h in the dark. The H_2O_2, CH, LM, and MA concentrations were 100 μM, 200 μM, 2 mM, and 5 mM, respectively.

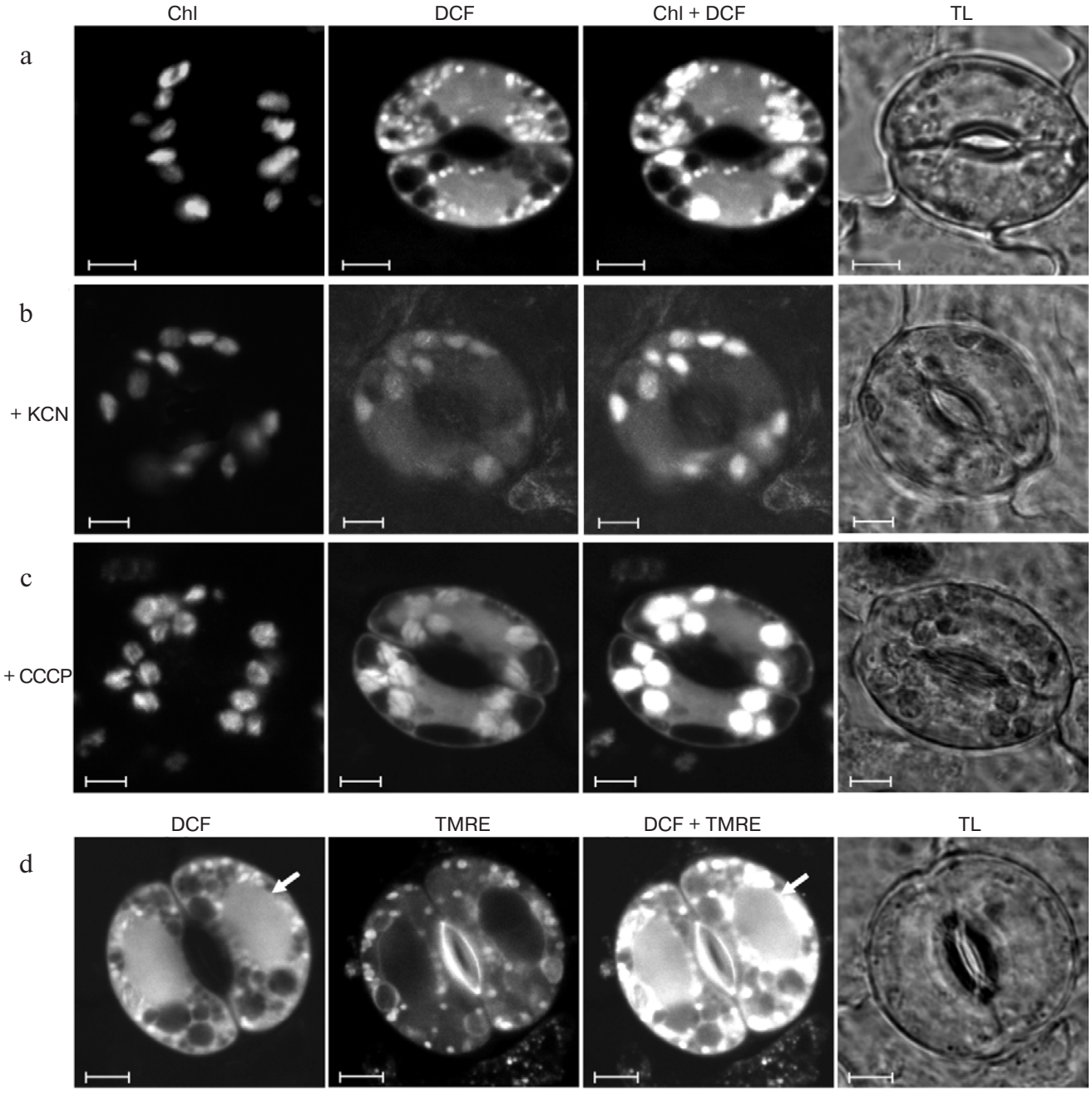

Fig. 6. Effects of KCN and CCCP on DCF and TMRE fluorescence in GC of pea leaf epidermis. The designations chlorophyll (Chl), DCF, and TMRE refer to the fluorescence of these compounds. Chl + DCF and DCF + TMRE, the superposition of the images; TL, a transmitted light image. Epidermal peels were incubated with 20 μM DCF-DA and 2.5 mM KCN for 15-20 min, with 10 nM CCCP for 30 min, and with 1 μM TMRE for 1 h. Arrow, cell nucleus. Bar, 5 μ.

junction with the fact that CN^- and CCCP prevent the DCF fluorescence, we conclude that these structures are mitochondria.

After increasing the sensitivity of the fluorescence detector, DCF and TMRE fluorescence was revealed in the mitochondria of the EC of isolated epidermis (Fig. 7a). H_2O_2 formation can be directly determined from DCF fluorescence in EC mitochondria in pea leaf fragments (Fig. 7b). CCCP quenched DCF fluorescence (Fig. 7, b and c). The fluorescence of cell walls was also decreased.

Electrophoresis of the DNA isolated from epidermal peels after 48 h of incubation with chitosan revealed internucleosomal DNA fragmentation that is peculiar to apoptosis (Fig. 8). DNA fragmentation was not detectable if the time of incubation with chitosan was

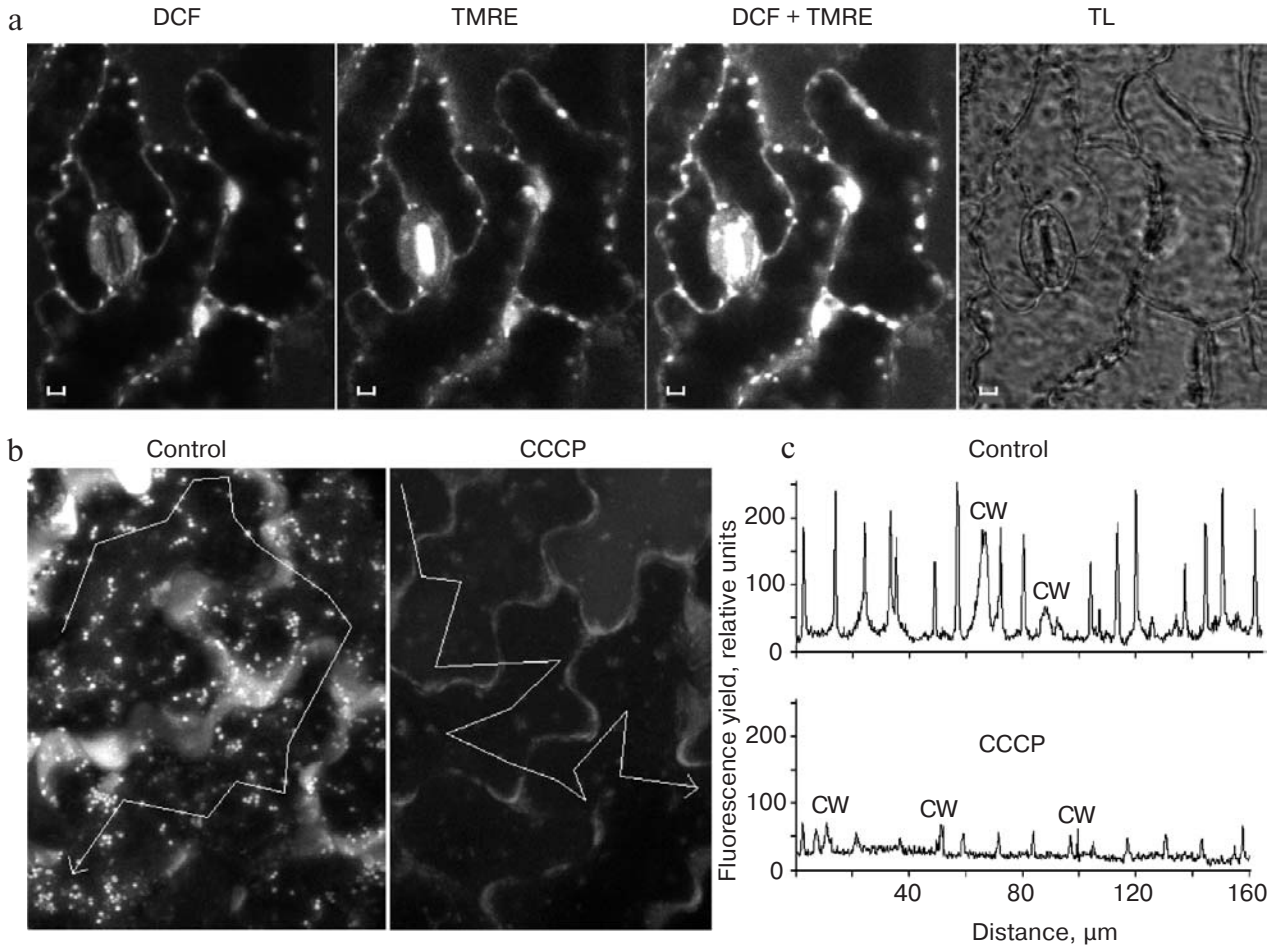

Fig. 7. DCF and TMRE fluorescence in EC and GC of epidermal peels from pea leaves (a) and the effect of CCCP (1 μM) on DCF fluorescence in EC (b, c) of a pea lamina. a) The epidermis was treated with 20 μM DCF-DA for 15-20 min and with 1 μM TMRE for 1 h; DCF + TMRE, superposition of the images; TL, a transmitted light image; bar, 5 μ. b) Total image (maximum projection) of DCF fluorescence based on 20-30 optical slices. c) DCF fluorescence yield along the broken lines in Fig. 7b that were drawn across the fluorescence images of intracellular mitochondria. CW, cell wall. The bar in Fig. 7b corresponds to the horizontal axis of Fig. 7c. Leaf fragments were incubated with 20 μM DCF-DA and with CCCP for 10 and 40 min, respectively.

decreased. However, nuclear DNA fragmentation manifested itself as early as after 6 h of incubation with KCN as an apoptosis inducer according to [16].

DISCUSSION

The suppression of chitosan-induced destruction of EC nuclei by anaerobiosis and the antioxidants NBT, pyruvate, or mannitol (Fig. 1) indicates that this process is ROS-dependent. The destruction of EC nuclei was caused by H_2O_2 added at a concentration of 100 μM. This effect was not produced on GC even at H_2O_2 concentrations of 10-50 mM [42]. Importantly, the effect of H_2O_2, similar to chitosan or chitosan + H_2O_2, was removed by NBT [32, 33] or mannitol [35, 36]. The H_2O_2 effect apparently involves an additional mechanism of ROS formation.

The metabolism of ROS in plant mitochondria is discussed in detail in review [45]. ROS formation in the respiratory chain of plants is suppressed by the cytochrome oxidase inhibitor CN^- [46, 47] or by uncouplers of oxidative phosphorylation [48-50]. In fact, H_2O_2 formation in GC and EC mitochondria that was estimated from DCF fluorescence was suppressed by CN^- and the uncoupler CCCP (Figs. 6 and 7) but stimulated by these agents in GC chloroplasts (Fig. 6). However, chitosan-induced destruction of EC nuclei was not inhibited by CN^- (Fig. 3). According to [10-12], CN^- caused the destruction of EC nuclei (Fig. 3), which was prevented by anaerobiosis and antioxidants [10-12].

The mitochondrial alternative oxidase inhibitors propyl gallate and salicylhydroxamate removed the destructive effect of chitosan (Fig. 3). The alternative oxidase intercepts electrons from the main mitochondrial

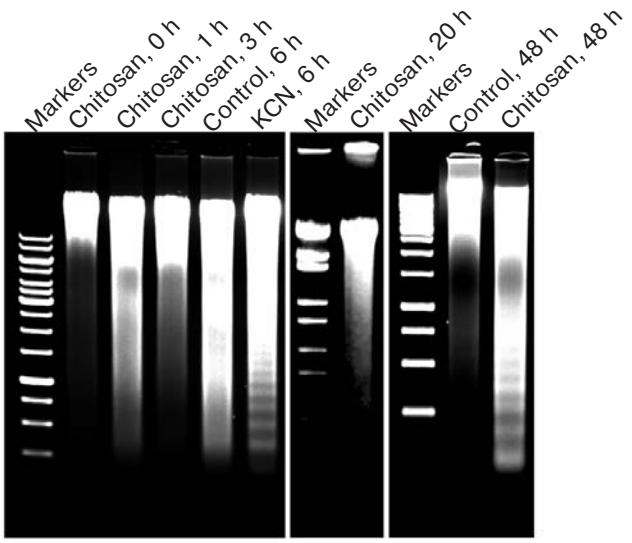

Fig. 8. Electrophoretic separation of DNA fragments from chitosan- or KCN-preincubated epidermal peels of pea leaves. The epidermal peels were treated with 2.5 mM KCN for 6 h or with 100 μg/ml chitosan in distilled water with a magnetic stirrer for 30 min and incubated thereupon for 1, 3, 6, 20, and 48 h without stirring in the dark.

respiratory chain at the ubiquinone level and reduces O_2 to H_2O without translocating H^+ across the inner membrane of mitochondria [37]. The CN^--resistant alternative oxidase received special attention because of its involvement in decreasing ROS formation in the respiratory chain. The alternative oxidase-involving electron transport that is not subject to control by the adenyl system maintains the oxidized state of respiratory chain components, preventing one-electron reduction of O_2 resulting in O_2^- formation [51-53]. The protection from chitosan by alternative oxidase inhibitors (Fig. 3) might be due to the conversion of mitochondrial ubiquinone into the ubiquinol form. Ubiquinol is a natural antioxidant that blocks chain reactions of mitochondrial lipid peroxidation [54, 55]. Inhibiting both terminal oxidases (cytochrome oxidase and alternative oxidase) of the respiratory chain results in high ROS generation rates [48, 49]. Nonetheless, inhibition of respiration by a combination of CN^- and propyl gallate (or salicylhydroxamate) failed to increase CN^--induced destruction of EC nuclei (Fig. 3).

Of special interest is the effect of DPI and quinacrine (Fig. 4), which are used as inhibitors of the NADPH oxidase of the plasma membrane of plant [38-41] and animal [56] cells. They prevented chitosan- but not CN^--induced destruction of EC nuclei.

If the effect of DPI and quinacrine really involves the NADPH oxidase of the plasma membrane, this suggests that it is the enzyme that supplies the ROS required for chitosan-induced EC death. Apparently, DPI (Fig. 4b) and quinacrine [42] do not influence the CN^- effect because the NADPH oxidases of the plasma membrane of plants function in combination with the peroxidase of the cell wall [39], i.e. they are double oxidases [57]. Therefore, CN^- decreases the generation of O_2^- and completely suppresses H_2O_2 generation with their involvement [39]. This might also account for the NBT- or mannitol-preventable H_2O_2 effect (Fig. 1), which possibly implicates the activation of NADPH oxidase by exogenous H_2O_2. In fact, the addition of 200 μM H_2O_2 activated ROS generation by the NADPH oxidase of the plasma membrane in non-phagocytic cells of vascular origin (smooth muscle cells and aorta fibroblasts) and coronary arteries of humans [58].

Lincomycin prevented chitosan- or H_2O_2-induced destruction of EC nuclei (Fig. 5). The effect of cycloheximide was less significant, but it was enhanced by adding cycloheximide in combination with lincomycin. These data indicate that proteins synthesized in mitochondria promote chitosan-induced destruction of EC nuclei to a greater extent than those synthesized in the cytoplasm. The nature of these proteins is still controversial. Lincomycin and cycloheximide produced a different effect on CN^--induced destruction of GC nuclei: lincomycin suppressed, and cycloheximide stimulated this process [43].

Inhibiting autophagy with 3-methyladenine and suppressing protein synthesis in mitochondria (Fig. 5) yield the same result: they prevent chitosan-induced EC death. The elicitor chitosan imitates a pathogen, and the cell attempts to protect itself from it by destroying inadequate cytoplasm parts via autophagy.

Like CN^-, chitosan caused internucleosomal DNA fragmentation in epidermal cells that manifested itself after incubating the epidermis with chitosan for 48 h, but not for 20 h or a still shorter time (Fig. 8). DNA fragmentation occurred in tobacco cells after 48 h of incubation with chitosan [27] and was lacking in soybean cells after 24 h of incubation [26]. In conjunction with the data on the effect of 3-methyladenine, the apoptosis-specific internucleosome DNA fragmentation does not rule out the option that chitosan-induced EC death follows a combined scenario including apoptosis and autophagy.

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