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# Effect of $Ca^{2+}$ on Programmed Death of Guard and Epidermal Cells of Pea Leaves

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**Abstract**—The effect of $Ca^{2+}$ on programmed death of guard cells (GC) and epidermal cells (EC) determined from destruction of the cell nucleus was investigated in epidermis of pea leaves. $Ca^{2+}$ at concentrations of 1-100 μM increased and at a concentration of 1 mM prevented the $CN^-$-induced destruction of the nucleus in GC, disrupting the permeability barrier of GC plasma membrane for propidium iodide (PI). $Ca^{2+}$ at concentrations of 0.1-1 mM enhanced drastically the number of EC nuclei stained by PI in epidermis treated with chitosan, an inducer of programmed cell death. The internucleosomal DNA fragmentation caused by $CN^-$ was suppressed by 2 mM $Ca^{2+}$ on 6 h incubation, but fragmentation was stimulated on more prolonged treatment (16 h). Presumably, the disruption of the permeability barrier of plasma membrane for PI is not a sign of necrosis in plant cells. Quinacrine and diphenylene iodonium at 50 μM concentration prevented GC death induced by $CN^-$ or $CN^-$ + 0.1 mM $Ca^{2+}$ but had no influence on respiration and photosynthetic $O_2$ evolution in pea leaf slices. The generation of reactive oxygen species determined from 2′,7′-dichlorofluorescein fluorescence was promoted by $Ca^{2+}$ in epidermal peels from pea leaves.

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*Key words*: reactive oxygen species, calcium, programmed cell death, necrosis, guard cells, epidermal cells, leaves, pea

Programmed cell death (PCD) is a physiological response that serves for the execution of the ontogeny program and cell differentiation, maintains tissue homeostasis, eliminates infected, functionally exhausted, aged, and unclaimed cells, premalignant cells, or cells exposed to mutagenic agents and other stress influences [1]. Formation of reactive oxygen species (ROS), chromatin condensation and margination, protease activation, DNA fragmentation, and accumulation of phosphatidylserine in the external monolayer of the plasma membrane accompany PCD.

ROS are products of one-, two-, and three-electron $O_2$ reduction: $O_2^{\cdot-}$, $H_2O_2$, and $OH^{\cdot}$. Their sources in plant cells are the electron transfer chains of mitochondria [2] and chloroplasts [3, 4], NADPH oxidases of plasma membrane [5] and of the cell nucleus [6-8], and apoplast peroxidase [8-11]. Singlet oxygen ($^1O_2$) is also related to ROS that is formed *in vivo* mainly in two ways [12]: photochemically, through the light excitation of endogenous photosensitizer followed by intermolecular transfer of electron excitation energy from triplet donor to triplet oxygen molecule ($^3O_2$), or in dark reactions of inflammatory response in animals with the participation of myeloperoxidase:

$$H_2O_2 + Cl^- \rightarrow OCl^- + H_2O,$$

$$H_2O_2 + OCl^- \rightarrow Cl^- + H_2O + {}^1O_2.$$

There are different PCD inducers of biological and non-biological nature. Mass PCD is caused by $CN^-$ [13, 14]. In isolated epidermis of pea leaves, $CN^-$ induced nucleus destruction in guard cells (GC) and in epidermal

*Abbreviations*: DCF, 2′,7′-dichlorofluorescein; DCFH-DA, 2′,7′-dichlorofluorescin diacetate; DPI, diphenylene iodonium; EC, epidermal cells; GC, guard cells; PCD, programmed cell death; PI, propidium iodide; ROS, reactive oxygen species.

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cells (EC) [15]. Elicitors, signal compounds of plant pathogens [16, 17], are PCD inducers. Chitosan is an effective elicitor; it is a product of incomplete chitin deacetylation, which is a compound of fungal cell wall [18-21]. Chitosan, an inducer of programmed death of EC, caused destruction of nuclei in EC followed by internucleosomal DNA fragmentation, but it did not influence the nuclei of GC [22].

Elicitors are inducers of ROS formation in plant cells that is preceded by fast increase in cytoplasmic $Ca^{2+}$ concentration. $Ca^{2+}$ is a universal agent transferring signals in cells, regulating their vital activity, and taking part in cell death [23]. $Ca^{2+}$ content in cytoplasm that was originally 40-110 nM increased to ~1 μM after 2 min of fungal oligopeptide elicitor exposure on parsley cells, and it decreased to ~0.3 μM in the subsequent 10-40 min [24]. A similar response of $Ca^{2+}$ was induced by acetylchitoheptaose [24] or chitosan [25]. Chitosan-induced PCD was suppressed by verapamil, which blocks $Ca^{2+}$-channels in membranes [26]. EDTA or EGTA chelating $Ca^{2+}$, ruthenium red inhibiting intracellular $Ca^{2+}$ transfer, as well as $Ca^{2+}$-channel blocker nifedipine and $Zn^{2+}$ ($Ca^{2+}$ antagonist) prevented internucleosomal DNA fragmentation in oat leaves treated by the fungal toxin victorin [27]. In contrast, 10 mM $Ca^{2+}$ stimulated DNA fragmentation in tomato protoplasts that was induced by the fungal toxin fumonisin $FB_1$ [13].

In plant cells $Ca^{2+}$ can be bound by pectins, pectates, oxalic acid, some cytoplasmic proteins (calnexin, calsequestrin, calreticulin, calmodulin, $Ca^{2+}$-dependent protein kinases, protein phosphatases, and phospholipases), and $Ca^{2+}$ is found in cell wall (apoplast), plasma membrane, endoplasmic reticulum, and chloroplasts. Most of the water-soluble $Ca^{2+}$ is situated in vacuoles [28]. $Ca^{2+}$ homeostasis is influenced by $Ca^{2+}$ accumulation in mitochondria that is dependent on their energized state [29]. The concentration ratio of $Ca^{2+}$ at the intra- and extracellular surfaces is $\sim 10^{-4}$. Removal of $Ca^{2+}$ from the cell is ATP-dependent process. $Ca^{2+}$ is taken up by the cell through $Ca^{2+}$-channels in the plasma membrane [28].

$Ca^{2+}$ activates NADPH oxidase of plant plasma membrane [5, 30]. Diphenylene iodonium (DPI), an inhibitor of NADPH oxidase, did not affect changes of $Ca^{2+}$ concentration in cytoplasm of *Arabidopsis thaliana* leaf cells in response to treatment with phytopathogenic *Pseudomonas syringae*, but DPI suppressed the formation of $H_2O_2$ and PCD [31]. On the contrary, $La^{3+}$ (a $Ca^{2+}$-channel blocker) suppressed increase in $Ca^{2+}$ in cytoplasm, formation of $H_2O_2$, and PCD. Binding of $Ca^{2+}$ with NADPH oxidase of *A. thaliana* and its phosphorylation with the participation of protein kinase synergistically activates ROS formation [32].

The goal of this study was to investigate the effect of $Ca^{2+}$ on ROS formation and programmed death of GC and EC.

## MATERIALS AND METHODS

The studies were performed with epidermis isolated from the lower surface of leaves of 7-15-day-old seedlings of the pea (*Pisum sativum* L., cv. Alpha). Plants were grown under conditions of periodic illumination with intensity ~100 μE/($m^2$·sec) (light 18 h, darkness 6 h) at 23-28°C. Epidermis was separated with forceps and put in distilled water, and then reagents (the composition is presented in figure legends) were added. To incorporate the reagents, the epidermal peels were subjected to vacuum infiltration for 1 min. The epidermal peels then were incubated in polystyrene plates at room temperature.

Upon completion of the incubation, the epidermal peels were treated for 5 min with Battaglia fixer (mixture of chloroform, 96% ethanol, glacial acetic acid, and 40% formalin in the ratio 5 : 5 : 1 : 1), washed with ethanol for 10 min to remove the fixer, incubated in water for 5 min, and stained with Carazzi's hematoxylin for 20 min. The stained peels were washed with tap water and analyzed with a light microscopy. Cell death was determined as destruction of the cell nucleus. The share of cells with destroyed nuclei and cells without nuclei were determined on the basis of 300-500 studied cells (in 3-4 epidermal peels). The experiments were replicated 3-5 times. Typical data obtained from one of the replications are given.

Epidermis was stained with 2 μM PI for 20 min for fluorescence microscopy. The cells were observed using a Zeiss LSM 510 Meta laser scanning confocal microscope

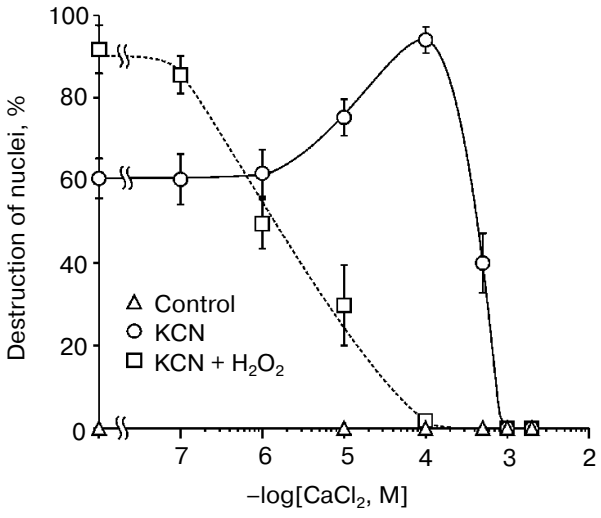


**Fig. 1.** Effect of $Ca^{2+}$ on $CN^-$- and ($CN^-$ + $H_2O_2$)-induced destruction of nuclei in GC in epidermis of pea leaves. Epidermis with added $CaCl_2$ was exposed to vacuum infiltration for 1 min and then incubated for 20 min, then supplemented with 2.5 mM KCN and again exposed to vacuum infiltration for 1 min, then supplemented with 0.1 mM $H_2O_2$ and incubated for 23 h with $CN^-$ or 16 h with $CN^-$ + $H_2O_2$ in the light.

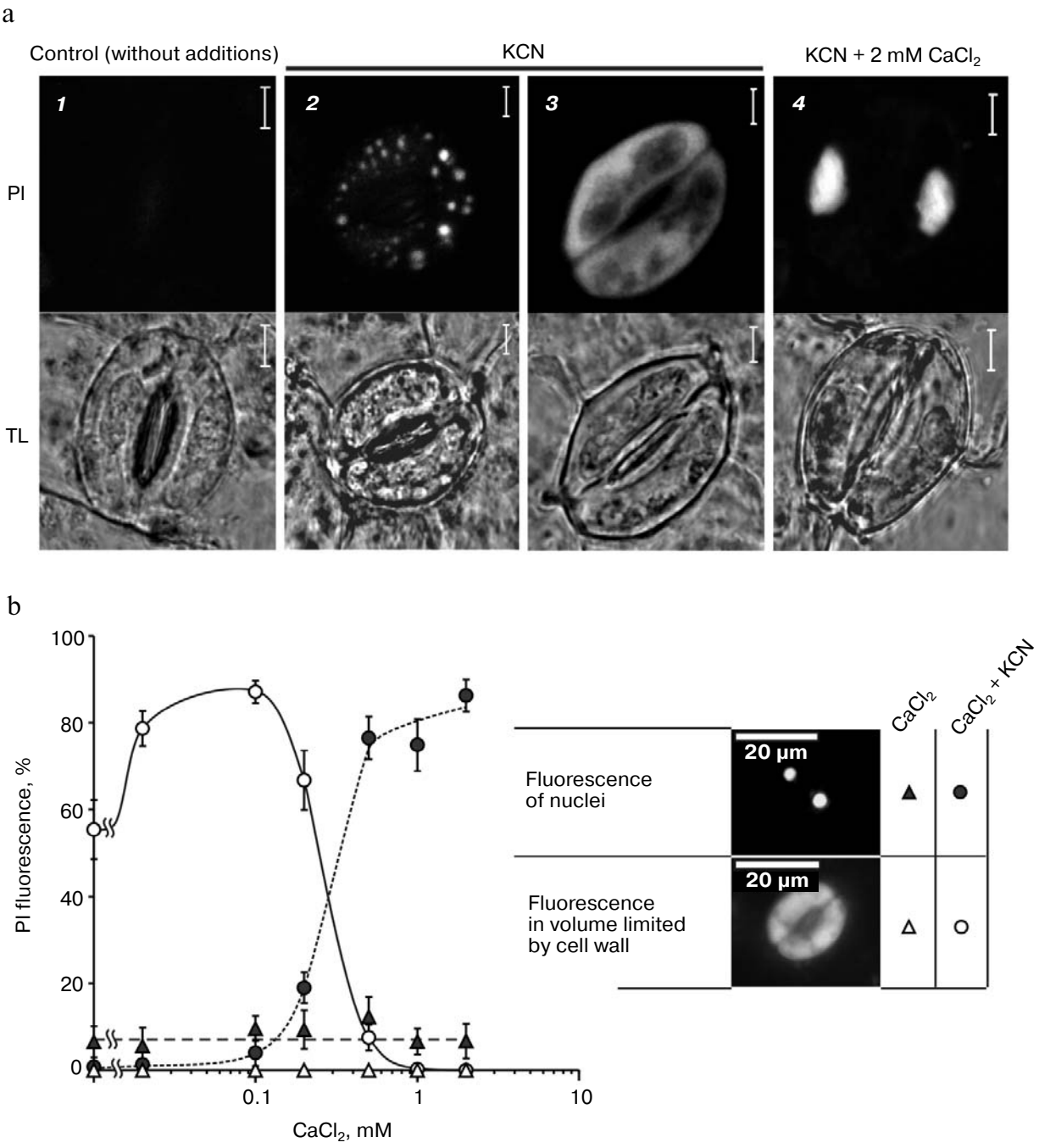


**Fig. 2.** Effect of $CN^-$ and $Ca^{2+}$ on GC nuclei and on the permeability of GC plasma membrane for PI in epidermis of pea leaves. Epidermis with added $CaCl_2$ was exposed to vacuum infiltration for 1 min with following incubation for 20 min, then supplemented with 2.5 mM KCN and again exposed to vacuum infiltration for 1 min with following incubation for 19 h in the light, and finally stained with PI. a) Confocal microscopy of GC. PI, fluorescence of PI; TL, image of GC in transmitted light; scale 5 μm. b) PI fluorescence in GC. The fraction of cells with fluorescing nuclei or their fragments in the volume limited by cell wall is noted.

equipped with inverted Zeiss Axiovert 200M (Carl Zeiss, Germany) in fluorescence or confocal mode. In fluorescence mode, fluorescence of PI was excited at 525-565 nm and registered at 575-640 nm, and in confocal mode fluorescence of PI was excited at 543 nm and registered at 565-615 nm.

DNA for electrophoretic separation was isolated from pea epidermis using the method described in study [33] with small modifications. Tissue (50-100 mg) was ground in liquid nitrogen. DNA was extracted for 30 min at 65°C in 100 mM Tris-HCl buffer, pH 8.0, containing 20 mM EDTA, 1.4 M NaCl, 2% cetyltrimethylammoni-

um bromide, 1% polyvinyl pyrrolidone, and 0.75% (*v*/*v*) 2-mercaptoethanol with constant stirring. Cell debris was removed by centrifugation for 10 min at 15,000*g*, and the supernatant was deproteinized with a mixture of phenol, chloroform, and isoamyl alcohol in the ratio 25 : 24 : 1. DNA precipitated with cold isopropanol was treated with RNase A (100 μg/ml) for 30 min at 37°C, and then it was again precipitated with ethanol. Extracted DNA was dissolved in 20 μl 10 mM Tris-HCl buffer, pH 8.0, containing 1 mM EDTA. DNA concentration was determined using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, USA). DNA (1.2 μg) was subject to electrophoresis (40 min, 5 V/cm) in 1.5% agarose gel prepared in 40 mM Tris-acetate buffer, pH 7.4, containing 1 mM EDTA. DNA was stained with ethidium bromide; its fluorescence was detected using a GelDoc It Imaging System camera (UVP, USA).

Evolution and consumption of $O_2$ by pea leaf slices was measured with a closed Clark-type Pt-electrode. Incubation medium for leaf slices (10 mg/ml with chlorophyll concentration 45 μg/ml) contained 10 mM Hepes-KOH, pH 7.0, 25 mM KCl, and 5 mM $NaHCO_3$. White light with saturating intensity (~600 μE/($m^2$·sec)) was used. Chlorophylls *a* and *b* were extracted from leaves using 80% aqueous acetone solution [34].

Fluorescence of 2′,7′-dichlorofluorescein (DCF) was measured with a VersaFluor fluorometer (BioRad, USA). Intact surface of epidermis was fixed with silica gel on a polystyrene plate, and then it was dipped into 50 μM 2′,7′-dichlorofluorescin diacetate (DCFH-DA) solution and incubated for 10 min in darkness. Then it was washed with distilled water and put in a cuvette with solution of 25 mM Hepes-NaOH, pH 7.2, at an angle of 45° to illuminator and fluorescence radiation detector. Fluorescence of DCF was excited at 485-495 nm and registered at 515-525 nm. Fluorescent microscopy of pea leaf epidermis treated with 10 μM DCFH-DA for 10 min was performed on a Zeiss LSM510 META laser scanning confocal microscope equipped with inverted Zeiss Axiovert 200M (Carl Zeiss) with fluorescence excitation and emission wavelengths 488 and 500-530 nm, respectively.

## RESULTS

Cyanide caused nuclear fragmentation and subsequent disappearance of nuclear fragments of GC and EC without affecting on their cell walls [15]. According to the data of bright-field microscopy of pea leaf epidermis stained with nuclear dye hematoxylin, $CaCl_2$ at concentrations less than 0.1 mM stimulated the $CN^-$-induced destruction of nuclei in GC, and further increase in $CaCl_2$ concentration suppressed this process and prevent-

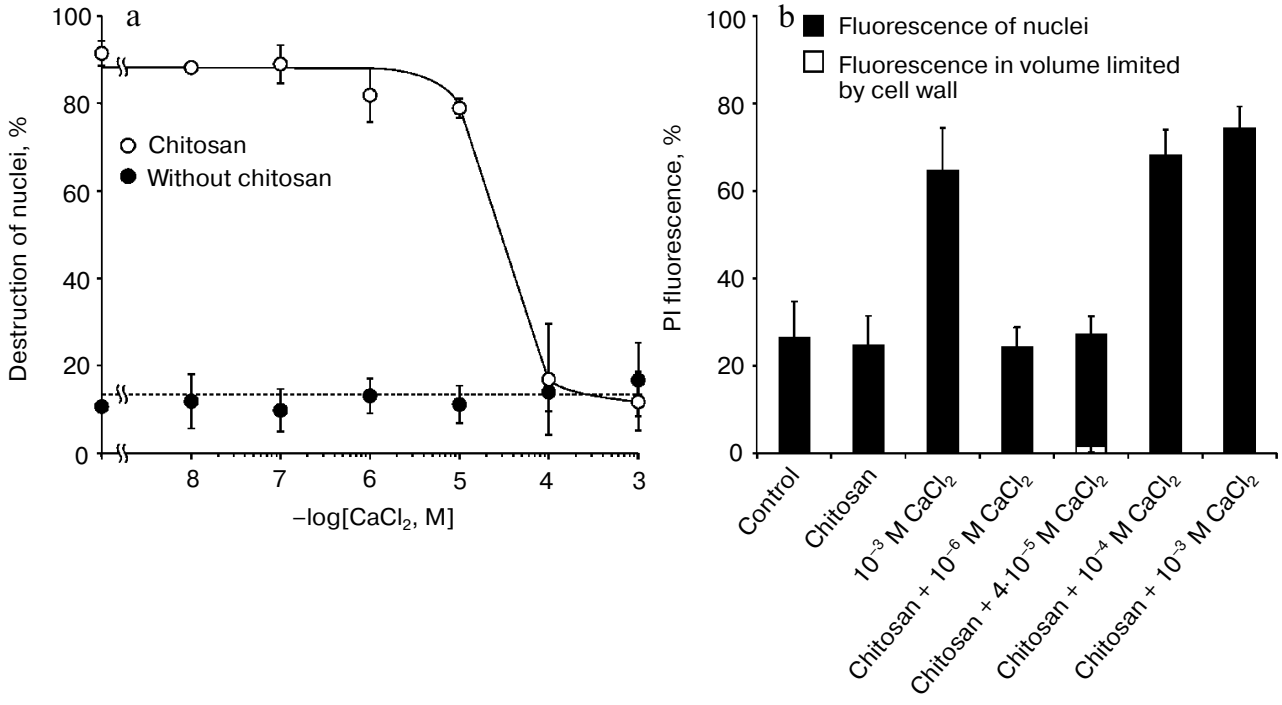


**Fig. 3.** Effect of $Ca^{2+}$ on chitosan-induced destruction of nuclei in EC of pea leaf epidermis (determined with hematoxylin staining (a)) and on PI fluorescence in EC nuclei (b). Epidermal peels, where indicated, were treated with a suspension of chitosan (100 μg/ml) in distilled water for 30 min with magnetic stirring, then supplemented with $CaCl_2$ and incubated for 3 h in darkness without stirring. b) EC were stained with 2 μM PI for 20 min. Fragments of EC nuclei had IP fluorescence in the volume limited by the cell wall (light column).

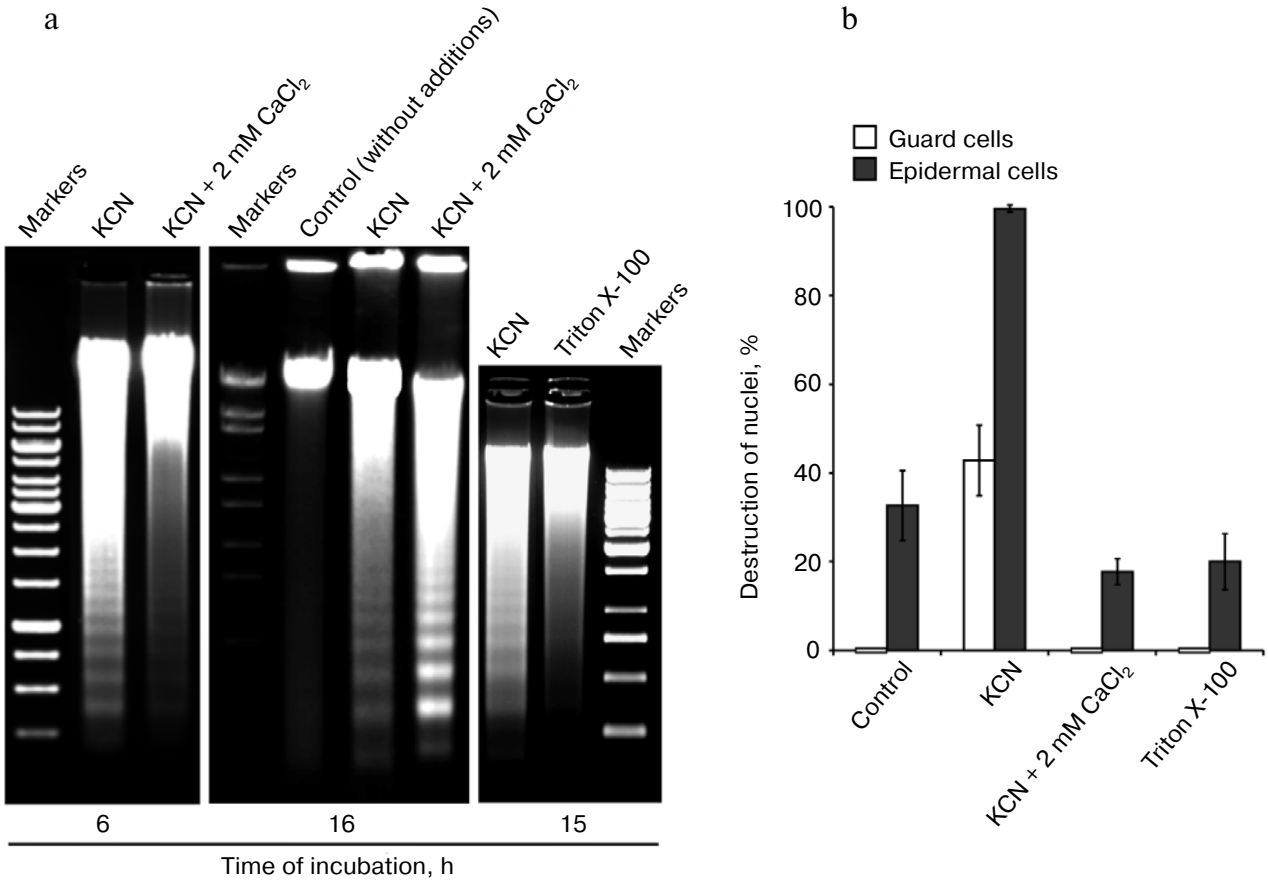


**Fig. 4.** Electrophoretic separation of DNA fragments (a) and state of nuclei in GC (light columns) and in EC (dark columns) (b) in epidermis of pea leaves. Epidermal peels were supplemented with 2.5 mM KCN, 2 mM $CaCl_2$, or 5 mM Triton X-100 and then exposed to vacuum infiltration for 1 min with following incubation for 6 (a), 15 (a, b), or 16 h (a) in the light.

ed it at a concentration of 1 mM (Fig. 1). Inhibiting action of $CaCl_2$ was drastically enhanced on treatment of epidermis with $CN^-$ and 0.1 mM $H_2O_2$: destruction of nuclei in GC was arrested when the concentration of $CaCl_2$ was 0.1 mM. In the absence of $CN^-$ or $CN^-$ + $H_2O_2$, $CaCl_2$ had no influence on GC nuclei.

Data of fluorescence microscopy of epidermis treated with PI are shown in Fig. 2. PI is a stain that intercalates in DNA helix; it does not enter cells with intact membranes. It is used as indicator of necrotic cell death accompanied with disruption of the plasma membrane barrier properties. PI stains DNA in necrotic cells, but not in intact or apoptotic cells [35]. $CN^-$ disrupted the permeability barrier of GC plasma membrane for PI (Fig. 2a). The cell nuclei were desintegrated into fragments that were stained with PI (*1*, *2*). In most GC in the presence of $CN^-$, PI uniformly stained nuclear fragments in the volume limited by the cell wall (*3*). $CaCl_2$ at concentrations of 1-2 mM prevented the $CN^-$-induced destruction of nuclei in GC, and PI caused fluorescence of undamaged nuclei (*4*). With increasing $Ca^{2+}$ concentration, we observed the following (Figs. 1 and 2b): $CN^-$-induced destruction of nuclei in GC detected with hematoxylin staining, increase in number of GC with PI stained nuclei, and decrease in number of GC with PI stained nuclear fragments in the volume limited by the cell wall. $CaCl_2$ per se had no influence on PI fluorescence in GC (Fig. 2b).

Chitosan-induced destruction of nuclei in EC was reduced and arrested by $CaCl_2$ at concentrations >10 μM and 0.1-1 mM, respectively (Fig. 3a). In the absence of chitosan $Ca^{2+}$ had no effect on GC nuclei. Up to 40 μM $CaCl_2$ did not affect the number of EC nuclei stained by PI in epidermis treated with chitosan, but at concentrations of 0.1-1 mM $CaCl_2$ drastically enhanced it (Fig. 3b). $CaCl_2$ at a concentration of 1 mM (without chitosan) disrupted the permeability barrier of EC plasma membrane for PI causing the fluorescence of nuclei in EC (Fig. 3b).

According to electrophoresis data (Fig. 4), $CN^-$ caused internucleosomal DNA fragmentation. $CaCl_2$ suppressed $CN^-$-induced DNA fragmentation determined after 6 h of incubation, but enhanced it after 16 h of incubation. In control (without additives), internucleosomal DNA fragmentation was not observed. Treatment of epidermis with detergent Triton X-100 (5 mM) solubi-

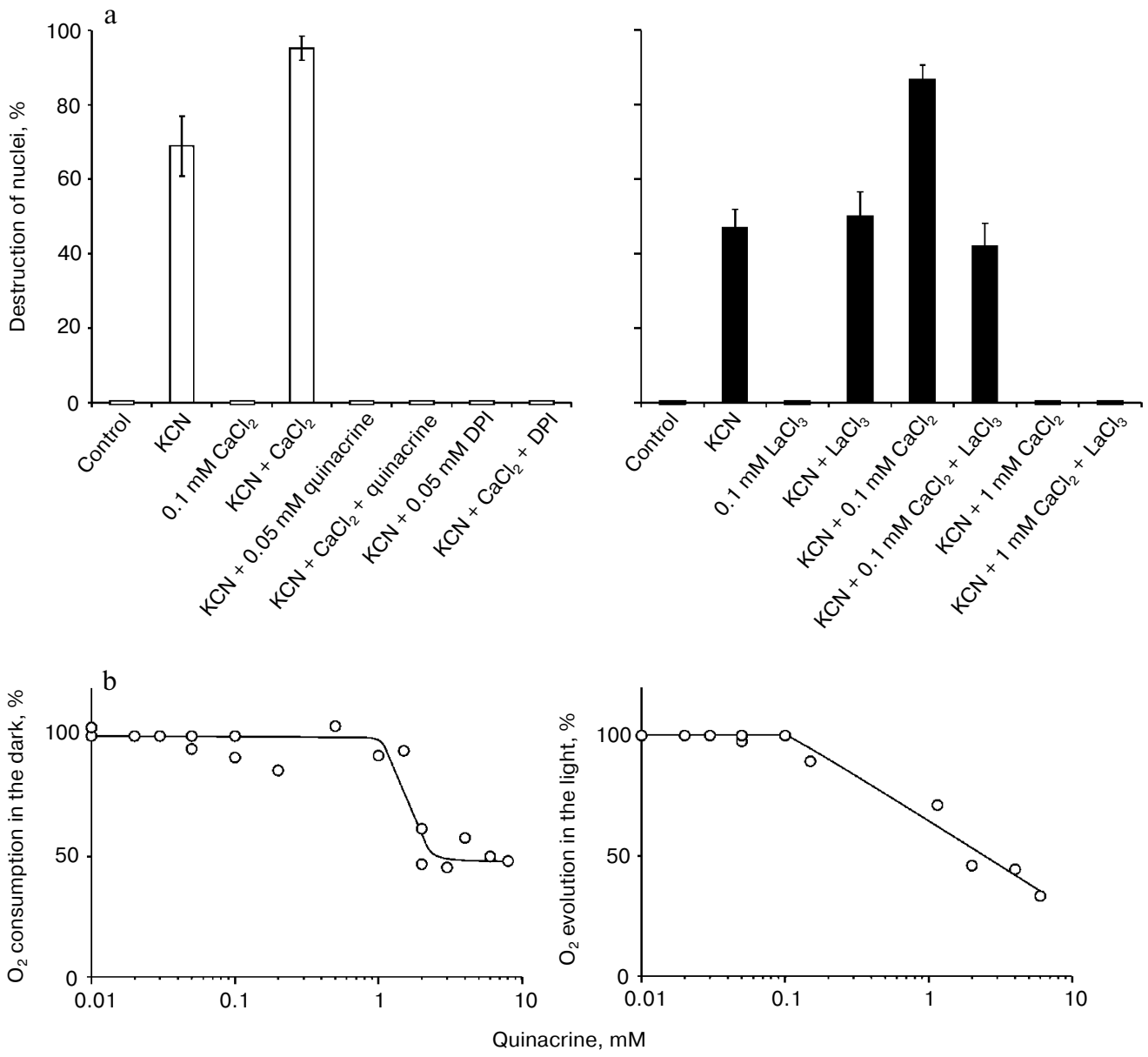


**Fig. 5.** Effect of quinacrine, DPI, and $La^{3+}$ on $CN^-$- and ($CN^-$ + $Ca^{2+}$)-induced destruction of nuclei in GC in epidermis (a) and effect of quinacrine on mitochondrial respiration and photosynthetic $O_2$ evolution in chloroplasts in pea leaf slices (b). a) Epidermal peels with 0.1 mM $CaCl_2$, 50 μM quinacrine, 50 μM DPI, or 0.1 mM $LaCl_3$ were exposed to vacuum infiltration for 1 min with following incubation for 20 min, then supplemented with 2.5 mM KCN, and again exposed to vacuum infiltration for 1 min with following incubation for 24 h in the light. b) 100% $O_2$ consumption and evolution rates were 10.7-11.2 and 26.7-27.3 μmol $O_2$/mg chlorophyll per hour, respectively.

lizing cell membranes did not induce destruction of nuclei in GC and EC and internucleosomal DNA fragmentation (Fig. 4). DNA fragmentation was absent when time of incubation of epidermis with 5 mM Triton X-100 was increased to 42 h and when the concentration of detergent was decreased to 0.25 mM (data not shown).

Quinacrine, an inhibitor of flavin enzymes including NADPH oxidase of plasma membrane, at a concentration of 50 μM suppressed destruction of nuclei caused by $CN^-$ or by $CN^-$ + 0.1 mM $CaCl_2$ (Fig. 5a). It had no influence on the dark respiration of mitochondria in pea leaf slices at concentrations up to 1 mM and on photosynthetic $O_2$ evolution at concentrations up to 0.1 mM (Fig. 5b). Quinacrine at higher concentrations inhibited respiration in the dark and $O_2$ evolution in the light. Another inhibitor of flavin enzymes, DPI, also suppressed destruction of nuclei in GC caused by $CN^-$ or by $CN^-$ + 0.1 mM $CaCl_2$ (Fig. 5a). Previously it was shown [36] that 50 μM DPI had no influence on respiration and photosynthetic $O_2$ evolution in pea leaf slices. $La^{3+}$ as a

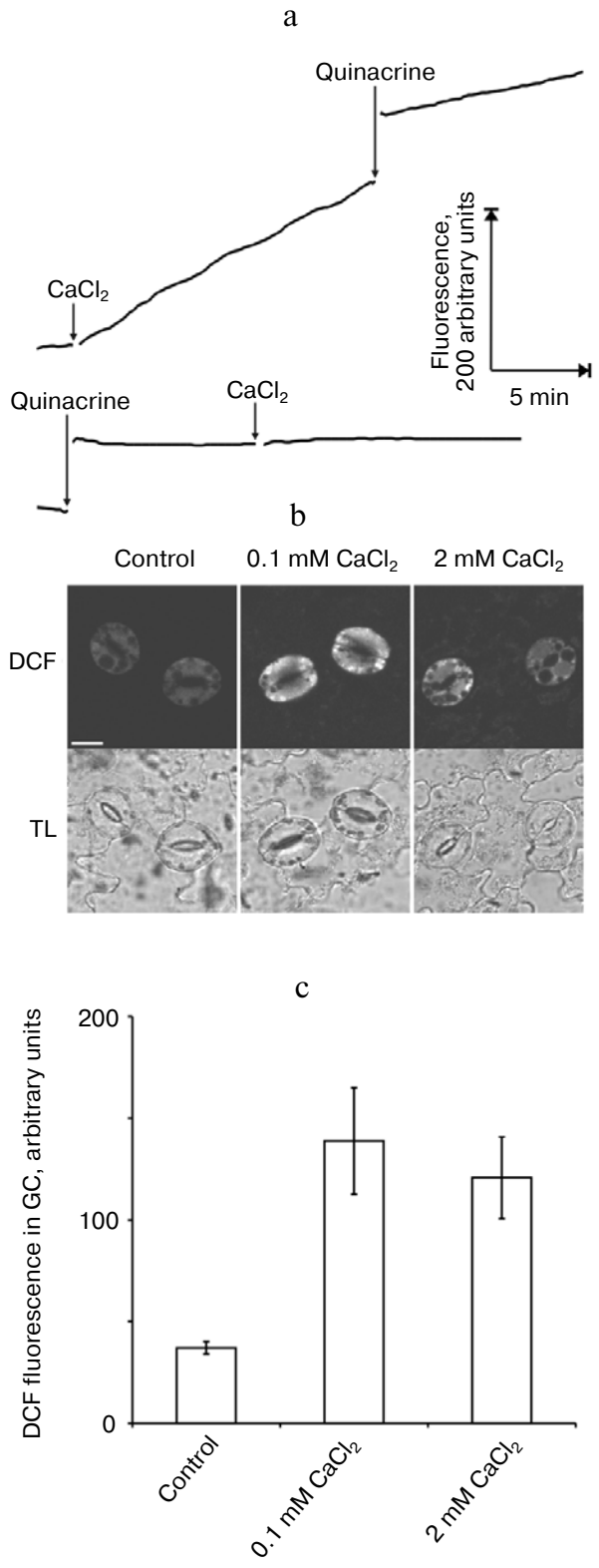


**Fig. 6.** DCF fluorescence in epidermal peels from pea leaves. a) Fluorimetry (additives: 0.2 mM $CaCl_2$, 50 μM quinacrine). b) Fluorescence microscopy of GC in epidermis treated with DCFH-DA with added $CaCl_2$. TL, image in transmitted light. c) Highest values of DCF fluorescence in GC as average of measurements on 30-40 cells.

membrane $Ca^{2+}$-channel blocker removed the stimulatory effect of 0.1 mM $Ca^{2+}$ on $CN^-$-induced destruction of nuclei in GC but had no influence on the effect of $CN^-$ or $CN^-$ + 1 mM $Ca^{2+}$ (Fig. 5a).

Addition of $CaCl_2$ caused ROS formation in epidermal peels from pea leaves detected by the increase in DCF fluorescence. Quinacrine suppressed and prevented $Ca^{2+}$-induced ROS formation (Fig. 6a). $CaCl_2$ at 0.1 and 2 mM concentrations significantly enhanced DCF fluorescence in GC (Fig. 6, b and c).

## DISCUSSION

Enhancement of $CN^-$-induced destruction of nuclei in GC after exposure to $Ca^{2+}$ at low concentrations (Figs. 1 and 2) can be caused by increase in ROS formation because of activation of plasma membrane NADPH oxidase [5, 30]; adding $CaCl_2$ induced increase in DCF fluorescence (Fig. 6), an indicator of ROS, mainly $H_2O_2$ [37, 38]. In the presence of $H_2O_2$ (0.1 mM), $CaCl_2$ already at 0.1 mM concentration prevented $CN^-$-induced destruction of nuclei in GC (Fig. 1). Chitosan-induced destruction of nuclei in EC was also suppressed by 0.1 mM $CaCl_2$ (Fig. 3).

Suppression of $CN^-$- and chitosan-induced cell nucleus destruction with increasing $Ca^{2+}$ concentration (Figs. 1-3) is presumably connected with disruption of barrier characteristics of the plasma membrane. This is confirmed by data obtained using PI. KCN caused fragmentation of nuclei in GC (Fig. 2a) and internucleosomal DNA fragmentation (Fig. 4), which are markers of apoptosis. At the same time, the permeability barrier of the GC plasma membrane for PI was disrupted: nuclei of most GC in control (without additives) were not stained by PI. $CaCl_2$ (0.5-2 mM) suppressed $CN^-$-induced destruction of nuclei and, in parallel, triggered the GC plasma membrane PI permeability (Fig. 2). $CaCl_2$ at concentrations of 0.1 mM and higher had the same effect on EC and chitosan-induced destruction of nuclei in EC (Fig. 3). $Ca^{2+}$ at a concentration of 2 mM prevented $CN^-$-induced DNA fragmentation in epidermis after 6 h of incubation but increased the fragmentation after 16 h of incubation (Fig. 4). $CaCl_2$ (10 mM) enhanced DNA fragmentation in protoplasts of tomato plants treated with the fungal phytotoxin fumonisin $FB_1$ [13].

So the effect of $Ca^{2+}$ at elevated concentrations (>0.1 mM) is not caused by switching from PCD to non-programmed cell death, necrosis, when the plasma membrane is disrupted, intracellular content flows out, and cell nuclei are not destroyed. Internucleosomal DNA fragmentation is the most typical marker of PCD [39-43]. Therefore, the disruption of the permeability barrier of plasma membrane for PI is presumably not a sign of necrosis in plant cells. $CN^-$ also disrupted this barrier (Fig. 2), but in its presence internucleosomal DNA frag-

mentation was observed (Fig. 4). Detergent Triton X-100 solubilizing cell membranes did not cause cell nucleus destruction and internucleosomal DNA fragmentation (Fig. 4). Fragmentation of DNA with cell nuclei remaining morphologically intact was described for different treatments of plant cells [44, 45].

Lack of DNA staining after PI treatment is considered as a sign of plasma membrane integrity [35]. However, plant cells significantly differ from animal cells: plant cells have a rigid cell wall that prevents unlimited dilution and mixing of intracellular proteins and other macromolecular compounds in extracellular aqueous volume. Even PI fluorescing DNA fragments remain in the volume limited by the cell wall (Figs. 2 and 3).

There are some data about internucleosomal DNA fragmentation in *Nicotiana tabacum* cells after exposures certainly causing necrosis, namely, after freezing in liquid nitrogen, mechanical destruction (homogenization), and after treatment with 5% Triton X-100 [46]. However, DNA fragmentation was observed after a long period (1-7 days) of treatment, and it could be the result of action of hydrolytic enzymes that are released from destroyed cells, and also secreted by living cells after their further cultivation.

Quinacrine and DPI suppressing activity of flavin enzymes are used as inhibitors of plant plasma membrane NADPH oxidase [47-50]. Both compounds suppressed $CN^-$- [36] or ($CN^-$ + 0.1 mM $CaCl_2$)-induced destruction of nuclei in GC (Fig. 5) and chitosan-induced apoptosis in EC [22]. $La^{3+}$, a membrane $Ca^{2+}$-channel blocker [31], cancelled the stimulatory effect of $Ca^{2+}$ (0.1 mM) on $CN^-$-induced destruction of nuclei in GC (Fig. 5). Quinacrine suppressed and prevented ROS formation induced by $Ca^{2+}$ (Fig. 6). The data show that NADPH oxidase of plasma membrane, which generates ROS, takes part in $CN^-$- and chitosan-induced plant cell death. $Ca^{2+}$ as a NADPH oxidase activator enhances ROS formation, thereby stimulating PCD.

## REFERENCES


1. Samuilov, V. D., Oleskin, A. V., and Lagunova, E. M. (2000) *Biochemistry (Moscow)*, **65**, 873-887.
2. Moller, I. M. (2001) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **52**, 561-591.
3. Foyer, C. H., Lelandais, M., and Kunert, K. J. (1994) *Physiol. Plant.*, **92**, 696-717.
4. Asada, K. (2006) *Plant Physiol.*, **141**, 391-396.
5. Sagi, M., and Fluhr, R. (2006) *Plant Physiol.*, **141**, 336-340.
6. Allan, A. C., and Fluhr, R. (1997) *Plant Cell*, **9**, 1559-1572.
7. Ashtamker, C., Kiss, V., Sagi, M., Davydov, O., and Fluhr, R. (2007) *Plant Physiol.*, **143**, 1817-1826.
8. Samuilov, V. D., Kiselevsky, D. B., Shestak, A. A., Nesov, A. V., and Vasil'ev, L. A. (2008) *Biochemistry (Moscow)*, **73**, 1076-1084.
9. Yokota, K., and Yamazaki, I. (1977) *Biochemistry*, **16**, 1913-1920.
10. Halliwell, B. (1978) *Planta*, **140**, 81-88.
11. Liszkay, A., Kenk, B., and Schopfer, P. (2003) *Planta*, **217**, 658-667.
12. Klotz, L.-O., Kroncke, K.-D., and Sies, H. (2003) *Photochem. Photobiol. Sci.*, **2**, 88-94.
13. Wang, H., Li, J., Bostock, R. M., and Gilchrist, D. G. (1996) *Plant Cell*, **8**, 375-391.
14. Ryerson, D. E., and Heath, M. C. (1996) *Plant Cell*, **8**, 393-402.
15. Samuilov, V. D., Lagunova, E. M., Beshta, O. E., and Kitashov, A. V. (2000) *Biochemistry (Moscow)*, **65**, 696-702.
16. Boller, T. (1995) *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **46**, 189-214.
17. Dangl, J. L., and Jones, J. D. G. (2001) *Nature*, **441**, 826-833.
18. Ito, Y., Kaku, H., and Shibuya, N. (1997) *Plant J.*, **12**, 347-356.
19. Day, R. B., Okada, M., Ito, Y., Tsukada, K., Zaghouani, H., Shibuya, N., and Stacey, G. (2001) *Plant Physiol.*, **126**, 1162-1173.
20. Okada, M., Matsumura, M., Ito, Y., and Shibuya, N. (2002) *Plant Cell Physiol.*, **43**, 505-512.
21. Kaku, H., Nishizawa, Y., Ishii-Minami, N., Akimoto-Tomiyama, C., Dohmae, N., Takio, K., Minami, E., and Shibuya, N. (2006) *Proc. Natl. Acad. Sci. USA*, **103**, 11086-11091.
22. Vasil'ev, L. A., Dzyubinskaja, E. V., Zinovkin, R. A., Kiselevsky, D. B., Lobysheva, N. V., and Samuilov, V. D. (2009) *Biochemistry (Moscow)*, **74**, 1035-1043.
23. Berridge, M. J., Bootman, M. D., and Lipp, P. (1998) *Nature*, **395**, 645-648.
24. Blume, B., Nurnberger, T., Nass, N., and Scheel, D. (2000) *Plant Cell*, **12**, 1425-1440.
25. Zuppini, A., Baldan, B., Millioni, R., Favaron, F., Navazio, L., and Mariani, P. (2003) *New Phytologist*, **161**, 557-568.
26. Iriti, M., Sironi, M., Gomarasca, S., Casazza, A. P., Soave, C., and Faoro, F. (2006) *Plant Physiol. Biochem.*, **44**, 893-900.
27. Tada, Y., Hata, S., Takata, Y., Nakayashiki, H., Tosa, Y., and Mayama, S. (2001) *Mol. Plant-Microbe Interact.*, **14**, 477-486.
28. Hirschi, K. D. (2004) *Plant Physiol.*, **136**, 2438-2442.
29. Duszynski, J., Koziel, R., Brutkowski, W., Szezepanowska, J., and Zablocki, K. (2006) *Biochim. Biophys. Acta*, **1757**, 380-387.
30. Sagi, M., and Fluhr, R. (2001) *Plant Physiol.*, **126**, 1281-1290.
31. Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A., and Mansfield, J. (2000) *Plant J.*, **23**, 441-450.
32. Ogasawara, Y., Kaya, H., Hiraoka, G., Yumoto, F., Kimura, S., Kadota, Y., Nishinuma, H., Senzaki, E., Yamagoe, S., Nagata, K., Nara, M., Suzuki, K., Tanokura, M., and Kuchitsu, K. (2008) *J. Biol. Chem.*, **283**, 8885-8892.
33. Allen, G. C., Flores-Vergara, M. A., Krasynanski, S., Kumar, S., and Thompson, W. F. (2006) *Nature Protocols*, **1**, 2320-2325.
34. Arnon, D. I. (1949) *Plant Physiol.*, **24**, 1-15.

35. Darzynkiewicz, Z., Bruno, S., Del Bino, G., Gorczyca, W., Hotz, M. A., Lassota, P., and Traganos, F. (1992) *Cytometry*, **13**, 795-808.
36. Samuilov, V. D., Kiselevsky, D. B., Sinitsyn, S. V., Shestak, A. A., Lagunova, E. M., and Nesov, A. V. (2006) *Biochemistry (Moscow)*, **71**, 384-394.
37. LeBel, C. P., Ischiropoulos, H., and Bondy, S. C. (1992) *Chem. Res. Toxicol.*, **5**, 227-231.
38. Wrona, M., Patel, K., and Wardman, P. (2005) *Free Radic. Biol. Med.*, **38**, 262-270.
39. Gavrieli, Y., Sherman, Y., and Ben-Sasson, S. A. (1992) *J. Cell Biol.*, **119**, 493-501.
40. Cohen, J. J. (1993) *Immunol. Today*, **14**, 126-130.
41. Proskuryakov, S. Ya., Gabai, V. L., and Konoplyannikov, A. G. (2002) *Biochemistry (Moscow)*, **67**, 387-408.
42. Festjens, N., Vanden Berghe, T., and Vandenabeele, P. (2006) *Biochim. Biophys. Acta*, **1757**, 1371-1387.
43. Krysko, D. V., D'Herde, K., and Vandenabeele, P. (2006) *Apoptosis*, **11**, 1709-1726.
44. Danon, A., Delorme, V., Mailhac, N., and Gallois, P. (2000) *Plant Physiol. Biochem.*, **38**, 647-655.
45. Ning, S.-B., Wang, L., and Song, Y.-C. (2002) *J. Exp. Bot.*, **53**, 651-658.
46. Kuthanova, A., Opartny, Z., and Fischer, L. (2008) *J. Exp. Bot.*, **59**, 2233-2240.
47. Van Gestelen, P., Asard, H., and Caubergs, R. J. (1997) *Plant Physiol.*, **115**, 543-550.
48. Papadakis, A. K., and Roubelakis-Angelakis, K. A. (1999) *Plant Physiol.*, **121**, 197-205.
49. Frahri, G., and Schopfer, P. (2001) *Planta*, **212**, 175-183.
50. Dat, J. F., Pellinen, R., Beeckman, T., van de Cotte, B., Langebartels, C., Kangasjarvi, J., Inze, D., and van Breusegem, F. (2003) *Plant J.*, **33**, 621-631.