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Role of Chloroplast Photosystems II and I in Apoptosis of Pea Guard Cells

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Abstract—We investigated the CN^--induced apoptosis of guard cells in epidermal peels isolated from pea (*Pisum sativum* L.) leaves. This process was considerably stimulated by illumination and suppressed by the herbicides DCMU (an inhibitor of the electron transfer between quinones Q_A and Q_B in PS II) and methyl viologen (an electron acceptor from PS I). These data favor the conclusion drawn by us earlier that chloroplasts are involved in the apoptosis of guard cells. Pea mutants with impaired PS I (Chl-5), PS II (Chl-I), and PS II + PS I (Xa-17) were tested. Their lesions were confirmed by the ESR spectra of Signal I (oxidized PS I reaction centers) and Signal II (oxidized tyrosine residue Y_D in PS II). Destruction of nuclei (a symptom of apoptosis) and their consecutive disappearance in guard cells were brought about by CN^- in all the three mutants and in the normal pea plants. These results indicate that the light-induced enhancement of apoptosis of guard cells and its removal by DCMU are associated with PS II function. The effect of methyl viologen preventing CN^--induced apoptosis in wild-type plants was removed or considerably decreased upon the impairment of the PS II and/or PS I activity.

Key words: apoptosis, chloroplasts, Photosystem II, Photosystem I, DCMU, methyl viologen, *Pisum sativum* L., mutants

Programmed cell death (PCD) is a process of cellular autodestruction that, in contrast to unprogrammed cell death, necrosis (e.g., under the influence of deleterious physical and chemical environmental factors), is subject to regulation at the genetic level. PCD is involved in the implementation of individual developmental programs of multicellular organisms, immune responses to pathogen invasions, the maintenance of tissue homeostasis, and responses of organisms to stress factors.

There are two forms of PCD: apoptosis and paraptosis [1]. The result of apoptosis in animals is cell destruction with the formation of apoptotic vesicles that are engulfed by macrophages and neighboring cells. Apoptosis in animals results in cell elimination. This is not the case with plants. The rigid plant cell wall prevents phagocytosis, and plants lack specialized phagocyte cells. The outcome of apoptosis in plants varies depending on the kind of tissue involved. Instead of autodestruction, apoptosis in many systems results in the formation of essential plant structures, including the pollen tube, the root cap, the xylem, and the phloem. If a plant is submerged into water, apoptosis enables it to form a special air-containing tissue referred to as the aerenchyma. The hypersensitive response of infected cells, the senescence and the fall of leaves, are regarded as PCD manifestations in plants (reviewed in [2, 3]). The morphological changes in plant cells during apoptosis are similar to those in animal cells. Phosphatidylserine appears in the outer monolayer of the cytoplasmic membrane, the oligonucleosomal fragmentation of nuclear DNA occurs, the chromatin condenses, the nucleus disintegrates and disappears, the protoplast shrinks, and vesicular protrusions form on the cytoplasmic membrane. The plasmodesms are ruptured to prevent the spreading of pathogens from the infected cell to adjacent intact cells.

Cysteine proteases termed caspases are the main tools used during apoptosis by animal cells (reviewed in [4]). There also is an alternative pathway not involving caspases. This kind of apoptosis depends on a mitochondrial protein, the apoptosis-inducing factor (AIF) that directly activates the nuclease cleaving the nuclear DNA [5]. As for plants, no direct evidence for the involvement of caspases in PCD has yet been presented, but some circumstantial data have been obtained using specific

Abbreviations: DCMU) 3-(3′,4′-dichlorophenyl)-1,1-dimethylurea; DNP-INT) dinitrophenyl ester of iodonitrothymol; MV) methyl viologen; PCD) programmed cell death; PS I, PS II) photosystems I and II.

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tetrapeptide caspase inhibitors (reviewed in [6]). The addition of such inhibitors results in the suppression of PCD in plants. The expression of cystatin, an endogenous inhibitor of cysteine proteases, suppresses the apoptosis of soybean cells.

Caspases exist in an inactive form (as procaspases) in normal cells. There are various strategies for activating procaspases in animals and humans [3, 4, 7], such as the cytoplasmic membrane receptor Fas-triggered pathway (involving caspase-8), the mitochondrial cytochrome *c*-dependent pathway (involving caspase-9), etc. Another PCD form, paraptosis, is characterized by cytoplasm vacuolization and the involvement of a special form of caspase-9 [1]. Paraptosis and also necrosis, in contrast to apoptosis, is not accompanied by the following phenomena: nuclear DNA fragmentation, nucleus disintegration, and the formation of subcellular apoptotic vesicles [1].

Like animals, plants use the PCD pathway involving mitochondrial cytochrome *c* and implicating the activation of caspase-like proteases (reviewed in [8]). Apoptosis in plants can be also performed using vacuolar hydrolytic enzymes [9]. Cyanide is an apoptosis inducer in plants [10, 11]. CN^- produces multiple effects on cell metabolism. It inhibits heme catalase and peroxidases, mitochondrial cytochrome *c* oxidase, and chloroplast ribulose-1,5-bisphosphate carboxylase. The impact of CN^- as a PCD inducer was investigated in experiments with epidermal peels from pea leaves [12]. The epidermis is a monolayer consisting of guard and epidermal cells that differ in structural and functional terms. The essential difference between them is that guard cells contain both chloroplasts and mitochondria, whereas epidermal cells contain mitochondria only. Illumination enhanced the CN^--induced destruction of nuclei in guard cells, not in epidermal cells. This process was prevented by antioxidants [12]. Compounds oxidizing the quinone components of the photosynthetic and the respiratory chain prevented the CN^- effect on guard cells [13]. DCMU, an inhibitor of the electron transfer between plastoquinones Q_A and Q_B in Photosystem II, abolished the light-dependent stimulation of CN^--induced destruction of nuclei in guard cells. A similar effect was produced by the quinone analog DNP-INT, a competitive inhibitor of plastoquinone oxidation at the Q_o site of chloroplast cytochrome b_6f complex [13]. These data attest to the involvement of chloroplasts in the CN^--induced destruction of nuclei in guard cells. The process depends on reactive oxygen species and seems to be governed by the redox state of plastoquinone Q_o of the cytochrome b_6f complex of the photosynthetic electron transfer chain [13].

The goal of this work was to elucidate the role of Photosystems II and I in CN^--induced destruction of nuclei in guard cells. We used pea mutants with impaired Photosystem II, Photosystem I, or both photosystems.

MATERIALS AND METHODS

Pea mutants. In this work, we used pea plants (*Pisum sativum* L., cultivar Capital) and lethal pea mutants of this species from the collection of the Department of Genetics, School of Biology, Lomonosov Moscow State University. The mutants were obtained by ethylmethanesulfonate treatment of pea seeds with subsequent screening for plants with disruptions at various stages of photosynthesis. Lethal mutants were reproduced using heterozygous plants. The Chl-1 mutant of the pea cultivar Capital was characterized by a high chlorophyll content (70% of that of the original cultivar) and an impaired PS II [14, 15]. The mutant phenotype was due to a mutation in one nuclear gene [16]. The Chl-5 mutant also obtained from the pea cultivar Capital had a high chlorophyll content (80% of that of the original cultivar) and an impaired PS I [15, 16]. The phenotype of mutant Chl-5 also resulted from a mutation in one nuclear gene [16]. The Xa-17 mutant obtained from the pea cultivar Rannyi Zelenyi (Early Maturating, Green) was characterized by a low chlorophyll content (1% of that of the original variety) and disrupted early stages of formation of the chloroplast structure [15, 17]. The mutant phenotype of Xa-17 was due to a mutation in one nuclear gene [16].

ESR spectroscopy. ESR spectra of pea leaves were recorded with a RE-1307 radiometer at 20°C. The leaves were placed in a flat cuvette into the resonator of the radiometer. The power of the UHF radiation was 20 mW, the modulation amplitude was 0.2 mT. We measured the ESR signal of the photooxidized P700 reaction centers of Photosystem I and Signal II of the oxidized tyrosine Y_D (tyrosine-161) residue of subunit D2 of Photosystem II. The pea leaves were illuminated directly in the resonator of the ESR spectrometer using an incandescent lamp with a power of 300 W.

Epidermal peels and the experimental protocol. The studies were conducted with peels obtained from the lower leaf epidermis of 7- to 15-day-old pea seedlings. The seedlings were grown hydroponically under continuous illumination (~1000 lx) at 24-25°C. Epidermal peels were separated with tweezers and placed into distilled water. To secure quick entry of reagents into the cells of the detached epidermis, we employed the infiltration method: the samples were incubated *in vacuo* for 1-2 min. The samples were thereupon placed on polystyrene plates with six wells and incubated in distilled water supplemented with the substances given in figure captions at room temperature in the dark or under a luminescent lamp at a light intensity of ~1000 lx.

Sample staining and microscopy. Upon incubation, the samples were kept in distilled water for 5 min and thereupon treated with Battaglio fixative (a mixture of chloroform, 96% ethanol, glacial acetic acid, and 40% formalin at ratio 5 : 5 : 1 : 1). The samples were subsequently washed with ethanol and incubated in water for 10 min.

The samples were stained with the nuclear stain Carazzi hematoxylin for 20 min, washed with tap water, and examined in a light microscope. Upon scanning 500-700 cells, we calculated the percentage of cells with disrupted nuclei and nucleus-lacking cells. We repeated each experiment two or three times.

RESULTS AND DISCUSSION

ESR spectroscopy of pea leaves. Figure 1a shows the ESR spectra of photooxidized P700 reaction centers (spectrum 1) and of the radicals of tyrosine residues Y_D in subunit D2 of Photosystem II of illuminated pea leaves (cultivar Capital). The ESR signal of the oxidized form of Y_D ($Y_D^{\bullet}$) denoted as Signal II is stable in the dark. By oxidizing Mn^{2+} of the oxygen-evolving complex (OEC) of PS II to Mn^{3+}, $Y_D^{\bullet}$ brings about the transition of the OEC from state S_0 to state S_1, stabilizing thereby the Mn cluster of the OEC (reviewed in [18]).

The ESR signal of $P700^+$ is lacking in the leaves of the Chl-5 mutant with impaired PS I (Fig. 1b). ESR Signal II in the Chl-5 mutant is characterized by a low amplitude, and this is consistent with the data that $Y_D^{\bullet}$ can be reduced by the primary plastoquinone Q_A^- of the electron-acceptor branch of PS II and by cytochrome b_{559} [18]. These processes should be promoted by blocking the electron flow from the quinone electron acceptors of PS II to PS I. This is the case with the Chl-5 mutant because the activity of PS I is disrupted.

The leaves of the Chl-1 mutant (Fig. 1c) generate signals of both types although their amplitude is below that in the leaves of the normal pea plants. This indicates that the reaction centers of PS I are functionally active and component Y_D of PS II is capable of oxidation. The ESR signals of $P700^+$ and $Y_D^{\bullet}$ are lacking in the Xa-17 mutant (Fig. 1d) with impaired PS I and PS II.

Light microscopy of guard cells. Figure 2 presents light microscopy data concerning epidermal cells of pea leaves that were preincubated for 24 h in the dark. Guard and epidermal cells with sharp images of nuclei are clearly visible in Fig. 2 (left column). Cyanide, an apoptosis inducer [10, 11], causes the destruction and disappearance of cell nuclei (Fig. 2, middle and right column). Destruction of nuclei in epidermal cells is a more rapid process than in guard cells [12]. Fragmentation of nuclei, a symptom of apoptosis in contrast to necrosis and paraptosis [1], was revealed in the guard cells of all tested pea cultivars. This indicates that the CN^--induced destruction of guard cell nuclei involves the apoptosis mechanism both in the normal pea plants and in the pea mutants.

Effects of DCMU and methyl viologen on the CN^--induced apoptosis of guard cells. Taking into account the data on the involvement of chloroplasts in the apoptosis of guard cells [12, 13], we investigated the effects on this process of agents that interact with PS II or PS I. We employed the herbicides DCMU and methyl viologen that interact with PS II and PS I, respectively.

Light enhanced the CN^--induced destruction of nuclei in the cells of the normal pea plants (Fig. 3a) and the Chl-5 mutant with impaired PS I (Fig. 3b), but it exerted no influence on the cells of the Chl-1 mutant with impaired PS II (Fig. 3c) and of the Xa-17 mutant with impaired PS I + PS II (Fig. 3d). The level of the CN^--induced destruction of nuclei in the dark in the Chl-5

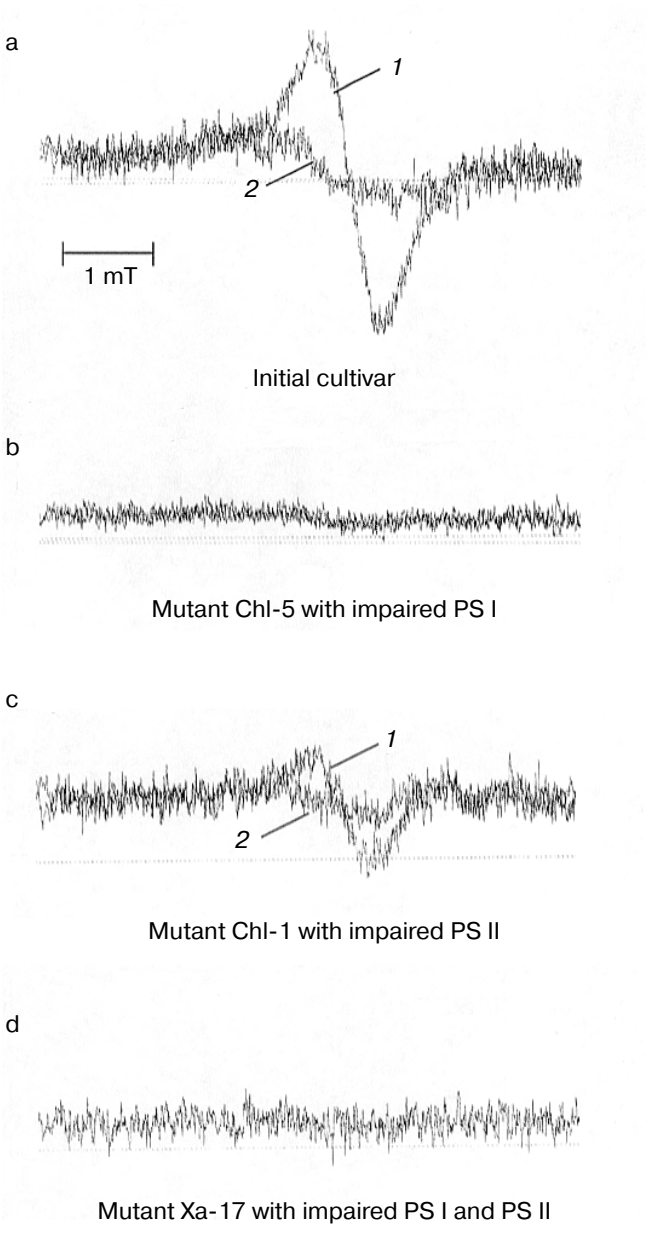

Fig. 1. ESR spectra of photooxidized reaction centers of PS I (*1*) and Signal II of PS II (*2*) in the leaves of the pea cultivar Capital (a) and the Chl-5 (b), Chl-1 (c), and Xa-17 (d) mutants.

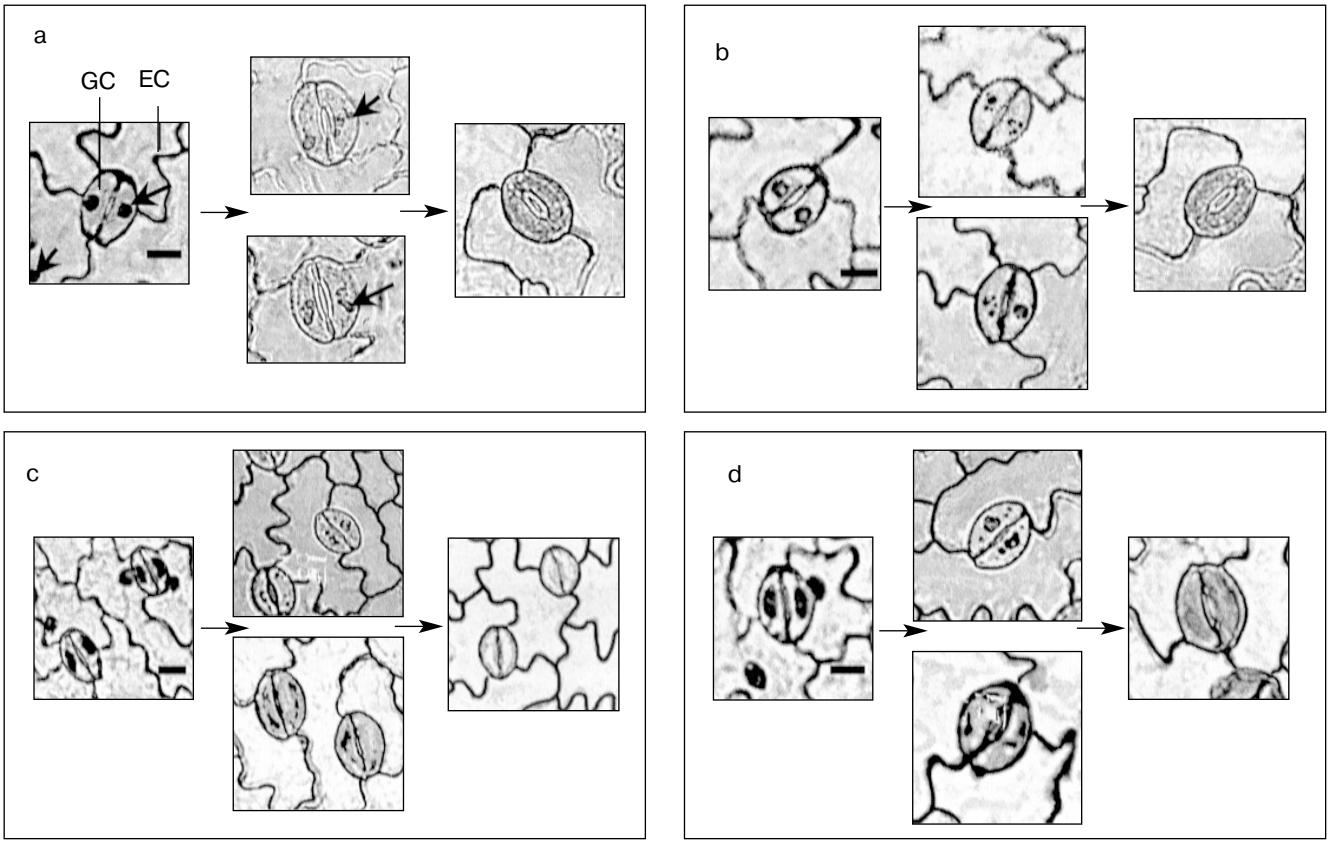

Fig. 2. Light microscopy of guard cells (GC) and epidermal cells (EC) in samples of the epidermis of the leaves of the pea cultivar Capital (a) and of the Chl-5 (b), Chl-1 (b), and Xa-17 (d) mutants. Left column, control samples; middle and right column, samples incubated with 2.5 mM NaCN for 24 h in the dark. Arrows, cell nuclei (left column) and their fragments (middle column). Bar, 10 μ.

mutant and the Chl-1 and Xa-17 mutants was increased twofold and threefold, respectively, in comparison to that of the normal pea plants (cf. Figs. 3a and 3b). Therefore, prerequisite for the light-induced stimulation of CN^--induced destruction of nuclei in guard cells is a functionally active PS II; light fails to stimulate this process upon disruption of PS II.

DCMU, an inhibitor of the electron transfer between plastoquinones Q_A and Q_B in PS II of chloroplasts, exerted no influence *per se* on the nuclei of guard cells. However, it efficiently prevented the light-induced stimulation of the destructive action of CN^- in samples with active PS II, without influencing the CN^--induced destruction of nuclei in the same samples in the dark (Figs. 3a and 3b). In PS II- or PS II + PS I-lacking samples (see Figs. 3c and 3d, respectively) DCMU produced no effect on the destructive action of CN^- in the light or in the dark.

MV, an electron acceptor at the level of FeS center F_b in PS I [19], inactivates stromal and thylakoid-bound ascorbate peroxidase and superoxide dismutase and $NADP^+$-glyceraldehyde-3-phosphate dehydrogenase, but not catalase in chloroplasts [20]. By inactivating ribulose-1,5-bisphosphate carboxylase [21] and depleting the $NADP^+$ pool (with concomitant NADPH accumulation), CN^- converts the components of the electron transfer chain to the reduced state. The components of the mitochondrial respiratory chain are also reduced, because CN^- inhibits cytochrome *c* oxidase. It was shown earlier [13] that, in the presence of CN^-, MV and other electron acceptors elicit the oxidation of the components of the chloroplast photosynthetic chain and thereby prevent the CN^--induced destruction of the nuclei of guard cells in the light. Moreover, the removal of the CN^- effect by MV also occurred in the dark.

Analogous data were obtained in this work with the normal pea plants (Fig. 3a). However, MV produced little or no effect on the level of the light-induced destruction of nuclei in the guard cells of mutants with impaired PS I (Figs. 3b and 3d). It slightly mitigated the destructive action of CN^- in mutants with active PS I in the light (Fig. 2c) and had no effect on the CN^--induced destruction of nuclei in all three mutants in the dark. The results indicate that MV prevents the CN^--induced destruction

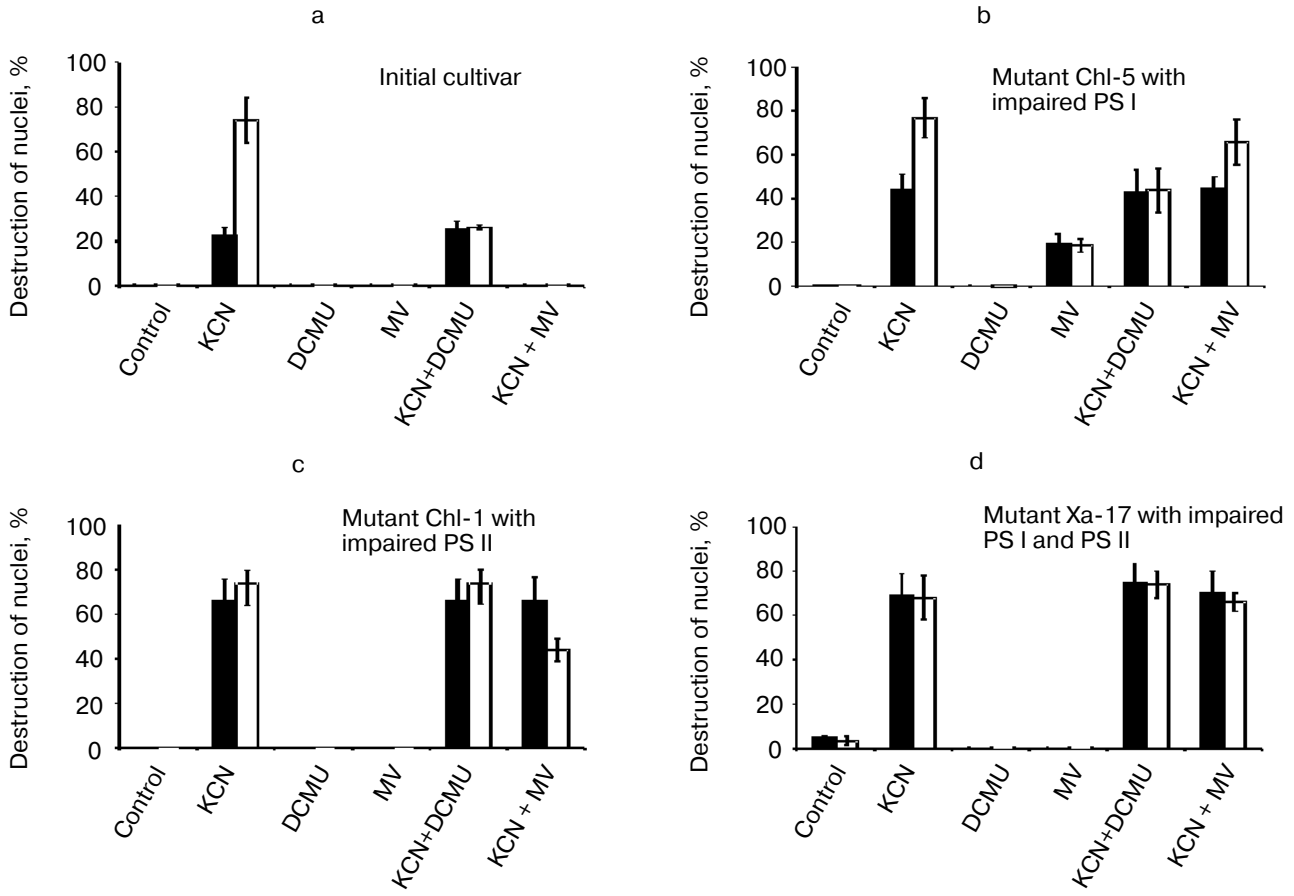

Fig. 3. Effects of DCMU and methyl viologen (MV) on the CN^--induced destruction of nuclei in guard cells from the leaves of the pea cultivar Capital (a) and the Chl-5 (b), Chl-1 (c), and Xa-17 (d) mutants in the dark (black bars) and in the light (white bars). Additions: 2.5 mM KCN, 10 μM DCMU, and 5 mM MV. The incubation time was 20 h.

of the nuclei of illuminated guard cells if PS II and PS I function normally and produces no effect if either PS II or PS I is impaired.

Hence, the light stimulation of CN^--induced apoptosis of guard cells and its removal by DCMU involve the operation of PS II. The effect of MV that prevents the CN^--induced apoptosis of guard cells in normal pea plants is reversed or significantly decreased upon disrupting the activities of PS II and/or PS I.

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