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Formation Kinetics and H_2O_2 Distribution in Chloroplasts and Protoplasts of Photosynthetic Leaf Cells of Higher Plants under Illumination

I. A. Naydov, M. M. Mubarakshina, and B. N. Ivanov*

Institute of Basic Biological Problems, Russian Academy of Sciences, 142290 Pushchino, Moscow Region, Russia; E-mail: ivboni@rambler.ru

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Abstract—The dye H_2DCF-DA, which forms the fluorescent molecule DCF in the reaction with hydrogen peroxide, H_2O_2, was used to study light-induced H_2O_2 production in isolated intact chloroplasts and in protoplasts of mesophyll cells of *Arabidopsis*, pea, and maize. A technique to follow the kinetics of light-induced H_2O_2 production in the photosynthesizing cells using this dye has been developed. Distribution of DCF fluorescence in these cells in the light has been investigated. It was found that for the first minutes of illumination the intensity of DCF fluorescence increases linearly after a small lag both in isolated chloroplasts and in chloroplasts inside protoplast. In protoplasts of *Arabidopsis* mutant *vtc2-2* with disturbed biosynthesis of ascorbate, the rate of increase in DCF fluorescence intensity in chloroplasts was considerably higher than in protoplasts of the wild type plant. Illumination of protoplasts also led to an increase in DCF fluorescence intensity in mitochondria. Intensity of DCF fluorescence in chloroplasts increased much more rapidly than in cytoplasm. The cessation of cytoplasmic movement under illumination lowered the rate of DCF fluorescence intensity increase in chloroplasts and sharply accelerated it in the cytoplasm. It was revealed that in response to switching off the light, the intensity of fluorescence of both DCF and fluorescent dye FDA increases in the cytoplasm in the vicinity of chloroplasts, while it decreases in the chloroplasts; the opposite changes occur in response to switching on the light again. It was established that these phenomena are connected with proton transport from chloroplasts in the light. In the presence of nigericin, which prevents the establishment of transmembrane proton gradients, the level of DCF fluorescence in cytoplasm was higher and increased more rapidly than in the chloroplasts from the very beginning of illumination. These results imply the presence of H_2O_2 export from chloroplasts to cytoplasm in photosynthesizing cells in the light; the increase in this export falls in the same time interval as does the cessation of cytoplasmic movement.

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Reactive oxygen species (ROS) in photosynthetic plant cells under light are mainly formed in chloroplasts where in an oxygen-containing atmosphere O_2 molecules are reduced to superoxide anion radicals during the transfer of electrons through the photosynthetic electron transport chain (PETC) [1]. Under physiological conditions this process (the Mehler reaction) can play a significant role and amount up to 30% of the overall electron flow in the PETC [2]. Superoxide is transformed to H_2O_2 as a result of dismutation or reduction, for example, by ferredoxin in chloroplast stroma, and by reduction by plastohydroquinone in thylakoid membrane [3]. Hydrogen peroxide inhibits the Calvin cycle even in low concentrations. As chloroplasts have no catalase, the level of H_2O_2 is supposed to be regulated by membrane-bound and soluble ascorbate peroxidases [4]. ROS can not only inhibit enzymes and destroy biomolecules, but they also may have signaling functions. H_2O_2 is viewed as the most universal signaling molecule [5].

One of the unsolved and crucial questions in analyzing the mechanisms of intracellular signaling involving H_2O_2 remains the ascertainment of transfer of these mol-

Abbreviations: BSA, bovine serum albumin; DCF, dichlorofluorescein; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (diuron); FDA, fluorescein diacetate; H_2DCF-DA, dihydrodichlorofluorescein diacetate; PETC, photosynthetic electron transport chain; PS I(II), photosystem I(II); ROS, reactive oxygen species.

* To whom correspondence should be addressed.

ecules from chloroplasts to cytoplasm [5]. Our previous study [6] indicated the exit of H_2O_2 molecules from intact isolated chloroplasts into the incubation medium as detected using electron paramagnetic resonance (EPR) spectroscopy. We also obtained the first evidence of their exit from chloroplasts inside cells. In the present study the H_2DCF-DA (dihydro-dichlorofluorescein diacetate) dye was used to detect H_2O_2 in cells. In a recent review [7] results obtained with this method were concluded to be adequate, but the authors stressed the need to consider the conditions of measurement. Upon entry into a cell, this dye is de-esterified, turning into H_2DCF (dihydro-dichlorofluorescein), which passes membranes with much more difficulty and thus it remains in cellular compartments where it reacts with H_2O_2, forming the fluorescent product DCF (dichlorofluorescein) [8]. Taking into account the significant level of superoxide dismutase in chloroplasts and its elevated local concentration (1 mM) on the stromal surface of thylakoid membrane [4], we can exclude possible reaction of H_2DCF with superoxide radicals in stroma.

Using H_2DCF-DA for detecting H_2O_2 formation in plants usually provides a static picture of DCF fluorescence in cells, fibrils, and whole leaves [9]. In [10, 11] H_2DCF-DA was used for analyzing processes that initiate and accompany apoptosis in guard cells of stomas in epidermis of pea leaves. In [12] the CM-H_2DCF-DA dye with similar properties was used. H_2O_2 was added to preparations of onion epidermis, and it showed a direct dependence of DCF fluorescence level on H_2O_2 concentration as well as a dependence of rate of DCF fluorescence growth in epidermis cells and whole leaves on temperature and light intensity.

Earlier we found a significant difference in changes in DCF fluorescence under illumination in chloroplasts inside guard cells of stomas and inside mesophyll cells in leaves [13]. The former are known to possess specific carbon metabolism [14], and the latter are where the major part of photosynthesis occurs. Thus, studying the formation and transport of H_2O_2 in mesophyll leaf cells is necessary for analyzing the mechanisms of regulation of this important process.

The main problem in investigations of H_2O_2 formation *in vivo* in experiments with whole leaves or plant tissues with the use of H_2DCF-DA is to guarantee dye entry into cells. Even under vacuum infiltration of leaves the DCF fluorescence is observed only in cells adjacent to fibrous bundles and in epidermal lesions. Protoplasts – the cells without a cellular wall – are a good object for studying processes *in vivo* as long as they remain viable.

This study describes the characteristics of using H_2DCF-DA and confocal microscopy for investigating the dynamics of H_2O_2 production in chloroplasts and mesophyll cell protoplasts in the light and provides recommendations for conducting suitable experiments with these objects. Chloroplasts and cytoplasm of *Arabidopsis* mutants with defective ascorbate biosynthesis were found to accumulate hydrogen peroxide faster than chloroplasts of wild type plants. The investigation of DCF fluorescence distribution in protoplasts of photosynthetic cells in the light showed the presence of flow of H_2O_2 molecules from chloroplasts to cytoplasm under illumination. The increase in the rate of H_2O_2 accumulation near chloroplasts was found to occur simultaneously with the arrest of cytoplasmic movements.

MATERIALS AND METHODS

Protoplast isolation. The middle part of leaves was cut into strips 1 mm wide in extraction medium, incubated for 5 min, and then put in 5 ml of medium with enzymes and incubated for 2 h at a temperature of 28°C under light with intensity of 80 $\mu E \cdot m^{-2} \cdot sec^{-1}$. After transferring to the basal medium, protoplasts were extracted from them, and the suspension was filtered through a nylon cloth with a pore size of 80 μm. The filtrate was centrifuged for 5 min at 30*g*, and the pellet was resuspended in a small amount of basal medium containing 0.5 M sorbitol, 5 mM $CaCl_2$, and 5 mM MES-KOH, pH 5.5. The extraction medium additionally included 5 mg/ml polyvinylpyrrolidone (to prevent the destructive effect of phenolic compounds), 0.2% BSA (to prevent proteases from functioning), and 0.25 mM EDTA (to bind heavy metal ions), and the fermentation medium additionally contained 1% cellulose (Sigma, USA), 0.2% Macerozyme R10 (Serva, Germany), and 10 mM of sodium ascorbate. The process of protoplast extraction is stressful for the cell and can lead to its death. Dead protoplasts preserved an intact outer membrane, but no cytoplasmic movement was observed, and the mitochondria were round and gathered in groups (see "Results and Discussion"). The mentioned additions, especially ascorbate, allowed a significant quantity of viable protoplasts to be obtained. The suspension of protoplasts was kept at room temperature to prevent temperature stress. DCMU (3-(3,4-dichlorophenyl)-1,1-dimethylurea or diuron), nigericin, and valinomycin were added to the protoplast suspension before taking an aliquot of suspension for microscopy.

Isolation of intact chloroplasts. Chloroplasts were isolated from spinach leaves and separated by centrifuging in a step gradient of 40 and 80% Percoll [15, 16] with some modifications. The medium with 40% Percoll contained: 3.33 mM EDTA, 1.66 mM $MgCl_2$, 83.3 mM Hepes (pH 7.6), and 0.55 M sorbitol. The medium with 80% Percoll contained: 10 mM EDTA, 5 mM $MgCl_2$, 250 mM Hepes (pH 7.6), and 1.65 M sorbitol. The chloroplasts concentrated in the lower level were washed free from Percoll in incubation medium, and the percentage of intact organelles was estimated [17]. Suspensions with intact percentage near 100% were used in the experiments. The medium for suspension and incu-

bation under the microscope contained 0.4 M sorbitol, 5 mM $MgCl_2$, 20 mM NaCl, and 25 mM HEPES-KOH (pH 7.6).

Dye administration. Active H_2DCF-DA solution was prepared by dilution of the 20 mM solution in dimethylsulfoxide with incubation medium to the concentration of 100 μM. A 4-μl sample of protoplast or chloroplast suspension was put on a glass slide, an equal volume of H_2DCF-DA was added, and a coverglass was placed on top. The thickness of liquid layer under the coverglass with a side of 18 mm is less than 40 μm, so to prevent compression and crushing of protoplasts they were put on the slide between spacers of appropriate thickness. The protoplasts were viewed in passing green light (light filter ZS 11) to prevent photoinduced formation of H_2O_2 before the beginning of observations. In confocal mode the protoplasts were located by their chlorophyll fluorescence. A minimal number of scan cycles was used so that the first registered frames showed the "dark" state of the protoplasts. MitoTracker Red dye was added to the protoplast incubation medium to final concentration 0.4 μM 20 min before the microscopic observations. Hoechst33258 was added to the protoplast extraction medium at concentration 10 μg/ml 1 h before the end of incubation and was washed off during precipitation of the protoplasts and their resuspension in basic medium.

Observation setup. For this study, we used a Leica TCS SPE confocal microscope (Germany). To prevent destructive processes, less than 10% of laser capacity was used. Both DCF and chlorophyll fluorescence are quite visible at this level, but the latter does not bleach and the light does not cause cell death. Further decrease in laser intensity requires further amplifying of the signal, which reduces the image quality. Fluorescence was registered by successively scanning the field of vision by appropriate lasers. Chlorophyll fluorescence was observed with the following parameters: excitation at 635 nm, registration in the 640-750-nm range; for DCF fluorescence: excitation at 488 nm, registration in the 504-543-nm range; for Hoechst33258 fluorescence: excitation at 405 nm, registration in the 410-480-nm range; for MitoTracker Red fluorescence: excitation at 488 nm, registration in the 550-605-nm range. The object was scanned with a 3-sec interval. Observation time in experiments with protoplasts without compression reached 10 min. Lasers of the microscope were the source of photosynthesis-initiating light.

Oil immersion lenses were used in this study. They provided a well-defined picture of fluorescence distribution in areas close to the lens. During observation of deeper planes the shape of object becomes distorted along the vertical axis and fluorescence intensity is decreased. A water immersion lens does not distort the picture, but significantly decreases resolution of the microscope.

Characteristics of water medium observation and corrections for movements of the objects. During the observation water evaporates from under the cover glass, leading to a decrease in thickness of the liquid layer between the glasses. As a result, the chloroplast-containing layer of protoplast adjacent to the cover glass moves 5-8 μm down in 5 min, thus going out of the focal plate. To measure dynamics of DCF fluorescence in a selected chloroplast plane – so-called XYT series, where X and Y are coordinates on the plane, and T is time – this movement should be compensated by continuous focus correction either manually, guided by the picture of chlorophyll fluorescence and adjusting the focus with the fine-tuning screw, or automatically, if the microscope allows it. With manual correction of the focus there is a danger to select different focal planes at different stages of observation. We used the automatic correction of focus, in which the microscope performs several scans of the object before the registration of each new frame along the vertical axis within a predetermined range relative to the focal position in the previous frame, and finds the best focal position for the next frame or a series of frames in accordance with the chosen algorithm. With scan interval of 3 sec, 5-10 scans in the range of 1 μm (0.5 μm above and below the previous position of the focal plane) are enough to find the best focus. It should be taken into account that searching for the best focus by laser scanning means additional illumination, which leads to H_2O_2 formation.

Movements of chloroplasts inside protoplasts, as well as movements of protoplasts in the space between glasses, can influence the detected values when using XYT series. Dynamics of changes in fluorescence can be registered in the chloroplast volume – in XYZT series where Z is the vertical axis. In XYZT experiment series fluorescence is recorded in the whole layer of chloroplasts, and their movements do not affect the dynamics of fluorescence registered. However, in this case the interval between frames is several times longer and the object moves a greater distance, which requires increases in the range and number of scans in search for the best focus, therefore decreasing the time resolution of fluorescence dynamics recorded.

Estimation of mean DCF fluorescence intensity inside and around chloroplasts. We used a macro for the ImageJ program to reveal specific dynamics of DCF fluorescence in chloroplasts. Based on chlorophyll fluorescence pattern for each frame, we created a mask for the area of the image where the chlorophyll fluorescence is at least 25% of the maximum value (chloroplasts region). The fluorescence values only from pixels present in the mask were used to calculate the average DCF fluorescence in chloroplasts in each frame. Values were averaged over the area of the mask of a single optical slice in the case of XYT series, or over all Z layers in the case of XYZT series. This approach eliminates the influence of chloroplast movement and changes in their relative area in the frame during the monitoring on the measured fluorescence values. An additional mask, based on the chloroplasts mask, was built to estimate the fluorescence around the chloro-

plasts; it included the pixels within a short distance from the border of the chloroplasts mask, and not overlapping with it.

RESULTS AND DISCUSSION

Objects observed in protoplasts of mesophyll leaf cells. Areas corresponding to chloroplasts were identified in the field of view based on chlorophyll fluorescence (Fig. 1; see color insert). It is seen that the DCF fluorescence after 5-min illumination of protoplasts of mesophyll cells of *Arabidopsis* (Fig. 1a′) and maize (Fig. 1b′) leaves is concentrated in the chloroplasts. A dark field in the protoplast – area "V" in Fig. 1b′ – is the vacuole, where living protoplasts never demonstrated DCF fluorescence increase under illumination.

There is no indication in the literature on the nature of the bright green DCF fluorescence areas within chloroplasts that are clearly visible in Fig. 1a′. Comparison of DCF fluorescence intensity in chloroplasts of *Arabidopsis*, kept one or two days under continuous illumination, which promotes the formation of starch (Fig. 2a; see color insert), or the same time in the dark, which encourages the use of starch in plant metabolism (Fig. 2b; see color insert), as well as the absence of such zones in the chloroplasts of mesophyll cells of maize, which are not able to form starch (Fig. 1b′), identified these areas as starch grains. Higher level of DCF fluorescence was detected in starch grain-containing chloroplasts due to nonspecific adsorption and less shielding of DCF from the excitation light by chlorophyll. This should be considered when comparing DCF fluorescence in cells with different number and size of starch grains in chloroplasts, as different levels of fluorescence may not reflect actual differences in H_2O_2 formation. The concentration of DCF in starch grains can distort significantly the results of static experiments, when DCF fluorescence is measured only once after an experimental influence.

Previously, using MitoTracker Red dye, we identified small organelles that are visible in Fig. 2b between the chloroplasts as mitochondria [18]. The fact that mitochondria can be observed when recording DCF fluorescence is because DCF fluorescence is growing in these organelles when the protoplasts are illuminated, albeit more weakly than in the chloroplasts. The most likely explanation for this growth is increased H_2O_2 formation in the mitochondria as a result of photorespiration [18]. In protoplasts, which were chosen for observations, the mitochondria were elongated and moved quickly with cytoplasmic flow. The movement of the cytoplasm, cyclosis, is an important characteristic of intact plant cells. In protoplasts that were isolated from leaves with the precautions described in Methods we observed, using dye Hoechst33258 that binds to DNA, a compact nucleus with clear boundaries characteristic of living cells (not shown).

Dynamic of DCF fluorescence in chloroplasts under illumination. Isolated intact chloroplasts as well as chloroplasts inside protoplasts exhibit nearly linear increase in DCF fluorescence after illumination, following a small lag period (Fig. 3). Due to fast destruction of chloroplasts in the microscopic field of view, only a few chloroplasts exhibited DCF fluorescence for 2 min, as shown in Fig.

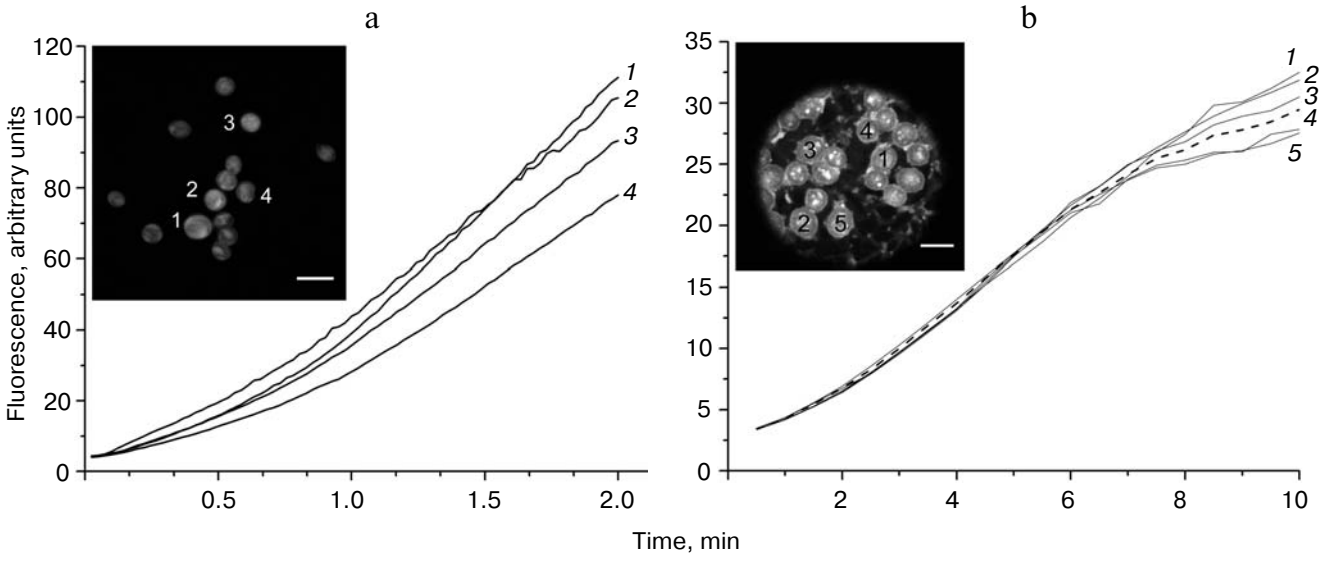

Fig. 3. a) Change in DCF fluorescence under light in isolated intact spinach chloroplasts (curves *1-4* correspond to chloroplasts that are marked in the inset). b) Change in DCF fluorescence under light in chloroplasts inside the protoplast (curves *1-5* correspond to the chloroplasts that are marked in the inset) and DCF fluorescence changes, averaged over all chloroplasts of the protoplast (dashed curve). Protoplasts were in a medium with pH 5.5. Insets show images after 2 min (a) and 10 min (b) of illumination, the brighter regions corresponding to higher DCF fluorescence intensity; the scale is 10 μm. The moment of switching on the illumination corresponds to the beginning of the horizontal axis.

3a. Chloroplast destruction is visible as complete vanishing of DCF fluorescence in separate chloroplasts between two scans (3 sec). In spots where DCF fluorescence disappeared, a compact zone of chlorophyll fluorescence remained for a long time (not shown). Discrete disappearance of DCF fluorescence indicates breaching of the chloroplast membrane and dye exit into the medium where in becomes diluted. When glutathione cycle components get washed away, the amount of accumulated hydrogen peroxide should increase, but it cannot be observed because of dilution. Fast destruction of isolated intact chloroplasts limits their use for analyzing H_2O_2 production in these organelles.

Kinetics of DCF fluorescence increase in chloroplasts inside protoplasts (Fig. 3b) in the beginning of illumination is similar to that in isolated chloroplasts. It was practically the same when protoplasts were put into medium with pH 5.5 (Fig. 3b), which imitates the conditions of apoplast that surrounds cells in natural conditions, as well as in medium with pH 7.6, which was used in experiments with intact chloroplasts (not shown). When chloroplasts were exposed to medium with pH 7.6 due to breaching of protoplasts, DCF fluorescence continued in chloroplasts for almost 5 min. Breaching was discovered only when distinct borders of protoplasts disappeared. In the medium with pH 5.5, complete disappearance of DCF fluorescence inside chloroplasts after breaching of the plasma membrane occurred in less than 30 sec. This allowed keeping track of protoplast intactness during observations.

We showed earlier that under illumination in the presence of DCMU, an inhibitor of photosynthetic electron transport, no DCF fluorescence increase occurred under the conditions used, neither in isolated chloroplasts, nor in chloroplasts and mitochondria inside protoplasts [13]. This indicated not only a dependence of observed H_2O_2 formation on photosynthetic electron transport, but also an absence of ROS generation as a result of photodynamic processes, which occurs in the light in the presence of dyes. Deceleration of increase in DCF fluorescence in chloroplasts after 5-6 min of illumination (Fig. 3b) was not a result of dye depletion, as indicated by a linear increase in fluorescence after a two-fold increase in light intensity (not shown). This decrease could be due to acceleration of exit of H_2O_2 molecules from chloroplasts as they accumulate in the chloroplast stroma.

Effect of ascorbate content in plant on DCF fluorescence under illumination of photosynthetic cell protoplasts. The kinetics of DCF fluorescence increase in chloroplasts under illumination reflects the rate of accumulation of H_2O_2 molecules, reacting with H_2DCF, which competes with other reactions of these molecules. The H_2O_2 content in chloroplasts *in vivo*, according to established conceptions, is kept at a very low level by the functioning of soluble and membrane forms of ascorbate-peroxidase [4]. The activity of these peroxidases is high, and the efficiency of H_2O_2 removal, if current theories are correct, should depend primarily on the amount of available ascorbate [19]. Therefore, the growth rate of DCF fluorescence should depend not only on the rate of H_2O_2 production, but also on the degree of competition of ascorbate and H_2DCF for H_2O_2 molecules. Indeed, the accumulation of DCF was much higher in the chloroplasts of protoplasts extracted from *Arabidopsis* mutant *vtc2-2* leaves with a low content of ascorbic acid in the cells (10% of its content in wild-type cells) [20] than in wild type after the same period of illumination (Fig. 4; see color insert). In previous work it was found that the addition of ascorbate to protoplasts suspension of this mutant decreases the rate of DCF fluorescence in their chloroplasts, becoming the same as that of wild-type [18]. These results demonstrate for the first time by *in vivo* observations the role of ascorbate as primary antioxidant in chloroplasts and suggest that rate and level of H_2O_2 accumulation in these organelles in its absence may increase significantly. In protoplasts from *vtc2-3* mutant leaves, which contain up to 30% of the amount of ascorbate in wild type, the growth rate of DCF fluorescence in chloroplasts was not significantly different from that observed in wild type for 5 min (data not shown). This fact shows that even this content of ascorbic acid is sufficient during the observation period to maintain steady-state concentration of H_2O_2 at low levels, and supports the view that chloroplasts contain an "excess" amount of ascorbate, judging from the values of K_m reactions in which it participates [21]. It is obvious that an excess of ascorbate is a necessary safety margin in case of malfunction of its regeneration system, taking into account the fact that it can be used in chloroplasts for detoxification of not only H_2O_2, but also other ROS forms.

Changes in DCF fluorescence intensity in chloroplasts and cytoplasm during light–dark and dark–light transitions. Outflow of H_2O_2 from intact spinach chloroplasts (capable of CO_2 fixation) into the incubation medium in the light was shown in [6]. Increase in DCF fluorescence intensity, which should reflect the accumulation of H_2O_2, in the cytoplasm around the chloroplasts was low in illuminated *Arabidopsis* protoplasts (Figs. 1a and 2). However, DCF fluorescence increased near the chloroplasts after extinguishing the light, but decreased in response to the reintroduction of light, while increasing in the chloroplasts simultaneously (Fig. 5; see color insert).

The observed phenomenon can influence the interpretation of observed changes in DCF fluorescence in photosynthetic cells under illumination. Increase in DCF fluorescence in chloroplasts after re-introduction of light might be connected with the beginning of PETC functioning and additional production of H_2O_2, but the decrease of DCF fluorescence near the chloroplasts allows several explanations. To elucidate the mechanism of this phenomenon, we used FDA (fluorescein diacetate)

dye, whose properties are close to those of H_2DCF-DA, but which is fluorescent in its native form, i.e. without reaction with H_2O_2. Distribution of its fluorescence in the cytoplasm without illumination (Fig. 6a; see color insert) was similar to the distribution of DCF fluorescence in Fig. 5a. Intensity of FDA fluorescence under illumination decreased outside chloroplasts and increased in chloroplasts (Fig. 6a′; see color insert), as occurred after the reintroduction of light in the experiments with DCF. DCMU completely prevented the changes in fluorescence of both FDA and DCF in the chloroplasts and around them, indicating the dependence of this phenomenon on electron transport and associated processes. Valinomycin, discharging the transmembrane electrical potential difference, did not affect the changes in FDA fluorescence in the chloroplasts and around them under illumination (data not shown), but these changes were not observed in the presence of nigericin, which prevents the development of transmembrane proton gradient (Fig. 6, b and b′; see color insert).

The intensity of FDA fluorescence depends on pH, and FDA has been used, for example, to estimate pH changes in roots and near-root environment [22]. Fluorescence emerged from monoanion (pK 5.0) and dianion (pK 6.4) forms of this dye (the dianion form has a higher quantum yield), but not from the fully protonated form. Consequently, a significant change in the fluorescence level due to changes in its quantum yield may occur at pH below 7.7, where the fraction of dianion form (95% at this pH) decreases rapidly. The pK value of DCF is lower than that for FDA, and its fluorescence depends strongly on pH at values below 7.0. The effect of nigericin on changes in FDA fluorescence inside and around chloroplasts under illumination suggests that these changes depend on photoinduced pH fluctuations inside and outside chloroplasts. It is known that the pH in stroma of chloroplast located in cytoplasm shifted to the alkaline side under illumination: from 7.3-7.4 to 7.7-7.8 at normal concentration of CO_2 in the air ([23, 24], Heber, personal communication). Growth of DCF and FDA fluorescence in chloroplast stroma under illumination can be partly explained by pH increase there. This applies mostly to FDA, because the quantum yield of DCF fluorescence in this pH range increases very slightly, and DCF fluorescence increase in chloroplasts is apparently caused by H_2O_2 production in the light. It is unlikely that this increase is due to return of some DCF molecules from the cytoplasm (which has pH 7.2-7.4) to chloroplast stroma. Theoretically, anions of weak acids accumulate in the compartment with higher pH, but in this case DCF molecules have to move through the chloroplast membrane.

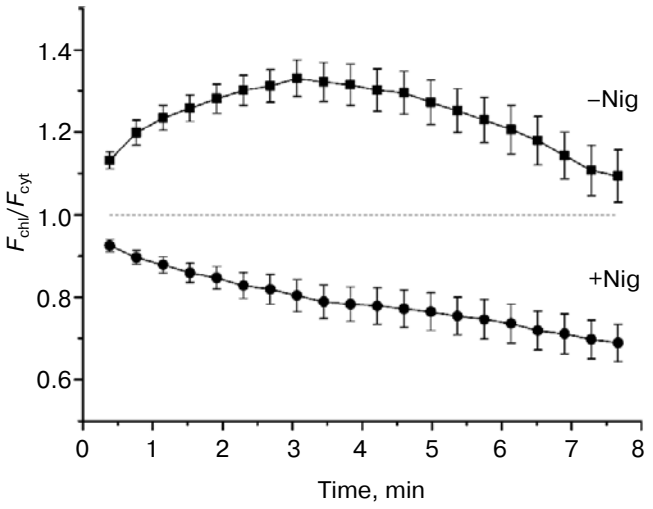

Fig. 7. Effect of 1 μM nigericin on the ratio of DCF fluorescence intensity in chloroplasts (F_{chl}) to DCF fluorescence intensity in the adjacent layer of cytoplasm (F_{cyt}) during illumination of *Arabidopsis* protoplasts. For determination of mean fluorescence intensity of the dye inside and around the chloroplasts, see "Materials and Methods". The moment of switching on the light corresponds to the beginning of the horizontal axis.

A significant fluorescence decrease in the layer adjacent to chloroplasts could not be the result of dye molecule movement into the chloroplast since, as calculations show, their concentration in the cytoplasm is almost without change in response to a slight pH change in the chloroplast stroma, the volume of which is much less than the volume of cytoplasm. It is more likely that the decrease in DCF and FDA fluorescence in the vicinity of the chloroplast under illumination is the result of a significantly lower pH in this zone. The phenomenon of proton outflow from intact chloroplasts to the outside medium has long been known (see review in [25]), although there is no conventional view on its mechanism yet. The proton outflow from the chloroplast may be either the result of functioning of ATPase, located in the membrane of chloroplast and using thylakoid-produced ATP to pump protons from the chloroplast to the cytoplasm [26], or – as suggested in [25] and clearly demonstrated for chloroplasts in cytoplasm [27] – caused by proton diffusion directly from the lumen, where they come from stroma by electron transfer through the PETC in the light. Thus, pH lowering under light near the chloroplast masks proton accumulation in this area, reducing the DCF fluorescence quantum yield. When the light is turned off, the pH increases and DCF fluorescence increases.

When nigericin was introduced in a protoplast suspension under illumination, DCF fluorescence increase around chloroplasts in response to switching on the light was faster than inside the chloroplasts, which is evident from the comparison of the values of fluorescence intensity ratio inside the chloroplasts and in the surrounding layer of cytoplasm in the absence and in the presence of nigericin (Fig. 7). In the absence of nigericin the rate of fluorescence increase in the cytoplasm becomes faster than inside the chloroplasts after 3-4 min of illumination,

as indicated by the beginning of decrease in this ratio, which, however, is still greater than 1. In the presence of nigericin, this trend can be observed from the very first minutes of illumination. Experiments in the presence of nigericin showed that increase in DCF fluorescence in the cytoplasm cannot be the result of the outflow of these molecules to cytoplasm, but reflects their appearance in the reaction of H_2DCF with H_2O_2, which leaves the chloroplasts. In the case when DCF could freely diffuse through the membrane, the inhibition of pH changes near the chloroplast in the presence of nigericin (see above) would have lead to synchronous increase in the rates of increase in DCF fluorescence in the cytoplasm and chloroplasts.

The fact that the ratio of fluorescence intensities in the chloroplast and cytoplasm is less than 1 in the presence of nigericin and that it decreases during illumination shows that H_2O_2 molecules come into the cytoplasm around the chloroplast faster than in the chloroplast stroma. We emphasize that the increase in DCF fluorescence does reflect not the accumulation of H_2O_2, but the accumulation of DCF molecules, emerging in reaction of H_2DCF with the appearing molecules of H_2O_2. It has also been shown in experiments with intact isolated chloroplasts [19] that H_2O_2 exit from the chloroplast to the incubation medium is accelerated in the presence of nigericin. The reason for this is unclear. Perhaps this is caused by functioning of two ways of hydrogen peroxide exit from the chloroplast: 1) diffusion of H_2O_2 molecules from stroma, and 2) the diffusion of H_2O_2 molecules formed in the thylakoid membrane [28] through chloroplast lumen, as was discussed above for protons. Stimulation of the second path in the presence of nigericin would at the same time lead to a decrease in intake of membrane-formed H_2O_2 molecules into the stroma, whose detoxification system operates to further reduce the possibility of their reaction with H_2DCF. Transition from increase to decrease in the ratios of fluorescence intensities in the chloroplast and the cytoplasm observed in the absence of nigericin may also be caused by a gradual increase in outflow to the cytoplasm of membrane H_2O_2 molecules specifically, while the speed of their intake to stroma becomes saturated, which eventually manifests itself in slower growth of DCF fluorescence in chloroplasts (Fig. 3b).

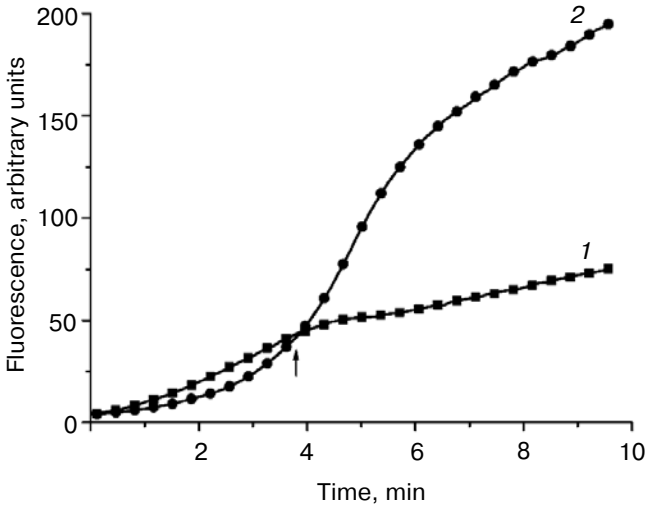

Fig. 8. Effect of cytoplasm flow arrest (arrow) on the kinetics of DCF fluorescence in chloroplasts (*1*) and in the surrounding layer of cytoplasm (*2*). The moment of switching on the light corresponds to the beginning of the horizontal axis.

Thus, a low DCF fluorescence in cytoplasm of protoplasts in the absence of nigericin is related, first of all, to effective scavenging of H_2O_2 by chloroplast ascorbate peroxidase (especially in the first minutes of illumination), which reduces the total outflow of H_2O_2 from chloroplasts. Second, lowering pH around chloroplasts decreases (due to decrease in quantum yield) the fluorescence intensity of DCF molecules emerging in this area in the reaction of H_2DCF with H_2O_2 molecules emerging from the chloroplast. In addition, we cannot exclude degradation of H_2O_2 molecules in chloroplasts by cytoplasmic peroxidases.

Change in DCF fluorescence distribution in chloroplasts and cytoplasm after the arrest of cytoplasmic movement under illumination. It was found in the study of DCF fluorescence distribution in protoplasts from wild-type plants that arrest of the movement of cytoplasm coincides with a sharp acceleration of DCF fluorescence increase in the cytoplasm near the chloroplasts, and a slowdown in DCF fluorescence increase in chloroplasts (Fig. 8). Additionally, besides the fact that mitochondria stop moving with the flow of cytoplasm, they undergo rounding and adhesion, which are the signs of apoptosis [29]. Then the protoplasts, which showed arrest of cyclosis and mitochondrial shape changed, can be regarded as cells with a developing apoptosis process. At the same time, electron transfer reactions in the chloroplasts of these protoplasts continue normally.

Considering the ratio of pH values in the cytoplasm and in chloroplasts in the light (see above), DCF fluorescence increase in the cytoplasm, observed after cyclosis arrest, with simultaneous reduction of its rate of increase in the chloroplast, is most likely a result of increased outflow of H_2O_2 molecules from the chloroplast. This would also explain the decrease in the rate of DCF fluorescence increase in the chloroplast. DCF fluorescence increase near chloroplasts may also be partly caused by increased production of H_2O_2 in mitochondria, which are located in the vicinity of chloroplasts in living cells [30]. Previously, we found that the destruction of the protoplast leads to rapid growth of DCF fluorescence in mitochondria coupled with a decrease in DCF fluorescence in the chloroplasts [31]. Studies of the connection between arrest of cytoplasmic movements and increased H_2O_2

content in cytoplasm may be important for understanding mechanisms of signal transmission from chloroplasts to other systems of cells and to other cells. It is possible that outflow of H_2O_2 molecules from chloroplasts enhances a cell death signal. This assumption corresponds to data obtained in [11] showing that not only mitochondria, but also chloroplasts are involved in the development of apoptosis.

Cytoplasmic flow is related to a phenomenon important for understanding of intracellular regulation described in [32, 33], namely, the appearance of a high-conductivity plasmalemma zone near the illuminated area of a *Chara* algal cell, and on the same side where cytoplasm moves but not on the opposite side [34]. Zones with altered plasmalemma conductance disappear when cytoplasmic flow is stopped [35]. It is possible that molecules of H_2O_2, emerging from the illuminated chloroplasts, are a signal to processes that regulate the opening of membrane channels to plasmalemma. In this case, stopping the cytoplasm reduces the flow of these molecules, which affects the properties of plasmalemma.

The results obtained in this study show the possibility of using H_2DCF-DA to investigate the kinetics of H_2O_2 formation in protoplasts of photosynthetic leaf cells exposed to light. Light is the main source of energy for plants, and photosynthetic cells adjust their metabolism to use this energy optimally. The primary step in such adjustment is H_2O_2 formation in the PETC, which functions not only as a converter of electromagnetic light energy into chemical energy, but as a sensor of both the behavior pattern and the very possibility of this transformation. "Dumping" of electrons to oxygen and H_2O_2 production may reflect a balance between the arrival of the light energy and capacities of the PETC, Calvin cycle, and assimilate transport system to use this energy for CO_2 fixation.

The quantitative measurement of H_2O_2 content in cells *in vivo* is an almost impossible task [36], so the study on the kinetics of its formation can give valuable information about the implementation of the signaling function of these molecules. The use of colored and fluorescent indicators for various ROS is a technique that seemed to hold great promise for study of the signaling role of ROS. However, not only are new opportunities for such studies found, but also their limitations. For example, nitrotetrazolium blue, which could have been applied – and this was suggested in some papers – as a detector of superoxide anion radicals in living cells, is a good indicator of the ascorbate content [20]; we have indeed found that a change in nitrotetrazolium blue color reflects changes in ascorbate content in cell compartments (unpublished). Use of H_2DCF-DA for registration of H_2O_2 formation turned out to be more promising. However, even in this case it was found that there are processes that should be taken into consideration for correct interpretation of the observed H_2O_2 formation kinetics. Some of these processes, which can be referred to as instrumental, are described in the "Materials and Methods" section. The other, biological processes, which obviously not all have been identified, include, for example, the concentration of DCF in starch grains, which is characteristic for plant cells, as well as changes in the fluorescence distribution of dye in response to pH shift in cell compartments.

With the use of methodological improvements developed in the course of this study, as well as computer data processing, we were able to record the phenomenon of H_2O_2 content increase in the cytoplasm after arrest of its movements in the light. There is evidence in the literature that ROS, and in particular H_2O_2 can affect, either directly or through redox-sensitive enzymes, actin cytoskeleton system [37], and that depolymerization of cytoskeleton microtubules under the effect of H_2O_2 initiates the expression of some genes of protection against pathogens [38]. We found a phenomenon consistent with the idea that H_2O_2 specifically is the chemical basis of the primary signal – which is able to transform into cell signals of other nature – that is sent by photosynthetic cells to other cells using the PETC, their most powerful energy converter.

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