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Gibberellic Acid (GA_3) Inhibits ROS Increase in Chloroplasts During Dark-Induced Senescence of *Pelargonium* Cuttings

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Abstract The temporal and spatial changes in reactive oxygen species (ROS) during dark treatment of *Pelargonium* cuttings and the effect of gibberellic acid (GA_3) on ROS levels were studied. ROS-related fluorescence was detected in mitochondria and cytoplasm of epidermal cells and in chloroplasts. By monitoring dichlorofluorescein (DCF) fluorescence, an initial decrease in ROS was observed under darkness in the epidermal cell cytoplasm and the chloroplasts, which was followed by an increase on the third day. Following 3 days under darkness, the size and the structure of the chloroplasts also changed, and they became more sensitive to illumination as judged by a higher accumulation of ROS. Pretreatment of leaves with GA_3 did not prevent the structural changes in the chloroplasts, but it inhibited the increase in ROS levels in all cell compartments, including the chloroplasts. It is suggested that the inhibition of ROS increase by GA_3 prevented complete disintegration of chloroplasts during dark-induced senescence and thereby enabled the maintenance of chlorophyll levels in the tissue.

Keywords Chloroplasts · GA_3 · H_2DCF-DA · Reactive oxygen species · Senescence

Introduction

Dark-induced senescence occurs during storage and shipment of agricultural products, including *Pelargonium* cuttings (Purer and Mayak 1989). This process shares some similarities with natural senescence, which suggests that dark-induced cell death is a consequence of an active program that involves the participation of signaling molecules, transcription factors, and catabolic enzymes (Kleber Janke and Krupinska 1997; He and others 2001; Buchanan-Wollaston and others 2003).

Reactive oxygen species (ROS) can act as toxic molecules, but also as signaling molecules that activate cell death programs (Thompson and others 1987; Breusegem and Dat 2006; Zentgraf and Hemleben 2008). In several cell death programs, including those activated by pathogens, ultraviolet light (UV), and ozone, ROS were hypothesized to initiate the program (Grant and Loake 2000; Langebartels and others 2002; Mittler 2002; Mittler and others 2004; Foyer and Noctor 2005). However, in other abiotic stresses and in senescence processes, the above role of ROS has not been established. Nevertheless, the role of ROS in senescence has been investigated in

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many studies, which reported increased ROS levels during senescence (Thompson and others 1987; Zimmermann and Zentgraf 2005; Zentgraf and Hemleben 2008). Hydrogen peroxide and lipid peroxidation products, associated with elevated ROS levels, increased gradually during natural senescence in tobacco leaves (Dhindsa and others 1981), as well as during dark-induced senescence of watercress and parsley leaves (Philosoph-Hadas and others 1994), wheat and rye leaves (Kar and Feierabend 1984), and *Pelargonium* leaves (Rosenvasser and others 2006). Other studies have identified changes in ROS levels and antioxidative enzymes during senescence, which occurred in specific organelles such as peroxisomes (Pastori and Rio 1994, 1997; del Rio and others 1998) and mitochondria (Jimenez and others 1998; Guo and Crawford 2005; Zentgraf and Hemleben 2008).

The earliest structural changes observed during senescence occur in the chloroplasts, and they were found to be under the control of the nucleus (Orzaez and Granell 2004; Gan and Amasino 1997, and reference therein). The chloroplasts were reported to undergo sequential changes that involve the breakage of thylakoids and increase in the number and size of the plastoglobuli (osmophilic globules) (Simeonova and others 2000). These processes coincided with chlorophyll degradation, which was executed by several chlorophyll-degrading enzymes (Hortensteiner 1999; Tanaka and Tanaka 2006). Pheophorbide a oxygenase (PaO), an enzyme involved in the opening of the porphyrin macrocycle of pheophorbide, has been found to be responsible for chlorophyll degradation in vivo during senescence (Pruzinska and others 2005). Whereas the changes in chloroplast structure have been studied quite extensively, the changes in ROS levels in these organelles during senescence have been rarely examined. An increase in ROS, determined by a direct measurement of electron spin resonance, has been detected in chloroplasts isolated from *Phaseolus* leaves during natural senescence, and incubating isolated chloroplasts under darkness caused a temporary increase in ROS (McRae and Thompson 1983). However, the kinetics of ROS changes in the chloroplasts, particularly during dark-induced senescence, has not been recorded.

Although it has been known for many years that gibberellic acid (GA_3) delays leaf senescence in various plants (Nooden 1988), its mode of action has seldom been studied, despite the fact that application of GA_3 has a major practical importance. For example, GA_3 is the only known material that is effective in delaying the dark-induced senescence of products such as *Pelargonium* cuttings (Purer and Mayak 1989). The GA-delaying mechanisms of senescence have been investigated mainly in *Alsromeria* (Jordi and others 1993, 1994; Kappers and others 1998). GA_3 did not prevent the fast decrease in leaf carbohydrates in darkness (Jordi and others 1993), a phenomenon that has been hypothesized to cause dark-induced senescence (Gan and Amasino 1997). In addition, GA_3 delayed chlorophyll breakdown, but it did not prevent the decline in the photosynthetic activity (Jordi and others 1994).

It has previously been found that GA_3 inhibits senescence by two possible pathways: one involving inhibition of ROS accumulation and the other involving an unknown ROS-independent pathway (Rosenvasser and others 2006). The present study aimed to characterize the temporal and spatial changes in ROS levels during dark-induced leaf senescence in *Pelargonium* cuttings and to examine in which of the cell compartments GA_3 inhibits ROS accumulation.

Materials and Methods

Plant Material and Treatments

Pelargonium mother plants were grown in a greenhouse under natural light (300-800 μmol m^{-2} s^{-1}) and at a temperature range of 21–24°C from October to June. All experiments were performed on freshly harvested *Pelargonium* (*Pelargonium* × *hortorum* cv. 'Kira') cuttings that were composed of two internodes with two mature leaves, two young leaves, and an apical meristem. The cuttings were incubated in darkness at 21°C in a moist atmosphere of 99% relative humidity (RH). Mature leaves were used in all experiments during the various days of the dark treatment.

GA_3 treatment was performed by dipping the cuttings in solutions containing 10^{-5} M GA_3 and 0.01% Triton X-100 for 5 min. The time of application and GA_3 concentration were chosen based on previous studies (Rosenvasser and others 2006). The control solution contained Triton X-100 only.

Determination of ROS Levels

To determine ROS levels in the epidermal and mesophyll cells in vivo, the cuttings were incubated under darkness for various lengths of time. Following 3 days under darkness, yellow sections appeared at the rim of the leaves. Leaf discs were excised from the green areas of the leaves. Either the epidermal layer was removed from the abaxial side of the discs to enable viewing of the mesophyll cells, or the discs were kept intact and the abaxial epidermal cells were examined. Leaf discs were placed in Petri dishes containing double-distilled water (DDW) for 30 min under darkness at room temperature, after which they were transferred to MES buffer (50 mM pH 6.2) containing

20 μM H_2DCF-DA for an additional 30 min. Measurement of DCF fluorescence is an assay for quantifying general oxidative stress (Halliwell and Whiteman 2004). The reduced form of the membrane-penetrating dye is hydrolyzed by intracellular esterases and then reacts with oxidants, resulting in the highly fluorescent DCF. H_2DCF detects a broad spectrum of oxidizing molecules rather than a specific ROS form (Hempel and others 1999), and it was used for localization of ROS within plant cells (Joo and others 2005).

Preliminary examinations showed that there was no increase in fluorescence during the first 100 min following excision of the discs. The stained discs were viewed with an inverted laser scanning confocal Olympus IX 81 microscope controlled by FluoView 500 software (Olympus, Tokyo, Japan).

Fluorescence related to H_2DCF-DA was observed in the rim of the leaf discs, and images were acquired by using a BA 515-525 filter following excitation at 488 nm. Images of mesophyll cells are of one optical section, and images of epidermal cells are combined from 20 optical sections. Excitation exposure in most of the experiments, unless otherwise stated, lasted for 1.13 s. The energy of the excitation beam and the sensitivity of the photomultiplier tube detector (PMT) were kept constant during each of the experiments. To examine the effect of light on ROS-related fluorescence of tissues darkened for various lengths of time or treated with GA_3, the leaf discs were subjected to successive illuminations of the same intensity. In these experiments the PMT was adjusted to a lower sensitivity than that used in the experiments in which discs were exposed to one short illumination.

Chlorophyll autofluorescence was detected by using a BA 660 IF emission filter, following excitation at 488 nm. Transmitted light images were obtained with Nomarski differential interference contrast (DIC) equipment. The images were usually color-coded green for H_2DCF-DA and red for chlorophyll autofluorescence.

The H_2DCF-DA fluorescence intensity in chloroplasts and cytoplasm was analyzed with FluoView 500 (Olympus) and SigmaScan Pro5 (Jandel Scientific, USA) software. This analysis was technically impossible for mitochondria. Two leaf discs were analyzed in each time point, and two to four pictures were taken for each leaf disc.

Cell membranes were stained by immersing leaf discs in MES buffer (50 mM pH 6.2) containing 5 μg/ml FM4-64 (Molecular Probes, USA) together with H_2DCF-DA. The FM4-64 probe is specific to membranes (http://probes.invitrogen.com/media/pis/mp34653.pdf). Images of light emission related to FM4-64 were acquired via a BA 610 IF filter following excitation at 515 nm. Mitochondria were stained with 0.5 μM MitoTracker Red (Molecular Probes) and images obtained following excitation at 543 nm (HeNe laser) and the emission light were collected via a BA 610IF filter.

Treatment with diphenyleneiodonium (DPI) was performed by submerging leaf discs in 30 μM DPI in 50 mM MES buffer for 30 min prior to staining with H_2DCF-DA.

Experiments were repeated at least three times and a representative experiment is shown for each of the experiments.

Chloroplast Isolation

Crude chloroplast extracts were prepared by homogenizing leaf tissue (5 g) in 20 ml of buffer containing 0.33 M sorbitol, 0.2 mM $MgCl_2$, 20 mM MES pH 6.5, and 0.2% BSA (CB buffer). The homogenate was filtered through four layers of cheesecloth and centrifuged at 1000 *g* for 7 min. The chloroplasts were further cleaned by density-gradient centrifugation on a discontinuous gradient of Percoll (Plama and others 1986), which yields intact chloroplasts (Napier and Barnes 1996). The pellet was gently suspended in 2 ml of CB buffer, and the suspension was layered on the surface of 40-80% Percoll gradient. Intact chloroplasts formed a band at the interface between the 40 and 80% Percoll layers. The chloroplasts were collected, resuspended in three volumes of the extraction buffer without BSA, and centrifuged at 1700×*g* for 1 min. The pellet was resuspended in 50 μl of buffer, and the chloroplasts were used for further analysis.

For size determination, photographs of isolated chloroplasts exhibiting chlorophyll autofluorescence were taken following excitation at 488 nm, and light emission was collected using a filter enabling detection at 660 nm. In each photograph, 20-30 chloroplasts were measured and averaged for five photographs. The chloroplast size was determined with the FluoView 5000 (Olympus) and SigmaScan Pro5 (Jandel Scientific) software.

Electron Microscopy

Tissues were fixed in 3.5% glutaraldehyde in PBS, and then rinsed and postfixed in 1% OsO_4 in PBS. Following several washes in PBS, the tissues were stained with uranyl acetate. The samples were dehydrated by passing them through an ethanol series and acetone and were then embedded in Agar100 epoxy resin (Agar Scientific, Cambridge, UK). Thin sections were cut, treated with uranyl acetate/lead citrate, and examined with a Tecnai G^2 Spirit transmission electron microscope (TEM) (FEI, Phillips, Netherlands). Representative photos are presented.

Results

Localization of DCF Fluorescence in *Pelargonium* Leaves

Staining leaves with H_2DCF-DA resulted in fluorescence emanating from several tissues and from several cell compartments. In the epidermal layer, fluorescence associated with ROS was evident in discrete spots and was colocated with the mitochondria-specific dye (Fig. 1A, B). Another source of fluorescence was localized in the rim of the epidermal cells (Fig. 1C). To determine whether it originated in the apoplast or the cytoplasm, the H_2DCF-DA-treated discs were co-stained with the membrane-specific dye FM4-64 (Fig. 1D). This procedure revealed that the DCF staining was encompassed by the membrane staining, indicating that ROS fluorescence in the rim of the cells emanated from the cytoplasm and not from the cell wall. Fluorescence was also observed in patches within the chloroplasts of the mesophyll cells, which were identified by autofluorescence of the chlorophyll (Fig. 1E).

Following treatment of H_2DCF-DA-stained tissue with H_2O_2, the fluorescence in the cytoplasm and mitochondria of epidermal cells and in chloroplasts increased, but no such increase was observed in the cytoplasm of mesophyll cells. A subsequent dithiothreitol (DTT) treatment reduced the fluorescence of the chloroplasts and the epidermal cells to a level similar to that of untreated tissues (Fig. 2). It is noteworthy that DTT was less efficient in reducing ROS levels in the stomata following H_2O_2 treatment compared with its effect on epidermal cells. The DTT and H_2O_2 results indicate that the dye can detect ROS changes in various subcellular locations *in planta*.

GA_3 Reduced ROS Levels in Epidermal Cytoplasm and Chloroplasts *in planta*

We previously observed ROS reduction followed by an increase in intact leaves during darkness and GA_3 treatment prevented that increase (Rosenvasser and others 2006). In the present study, we monitored the changes in

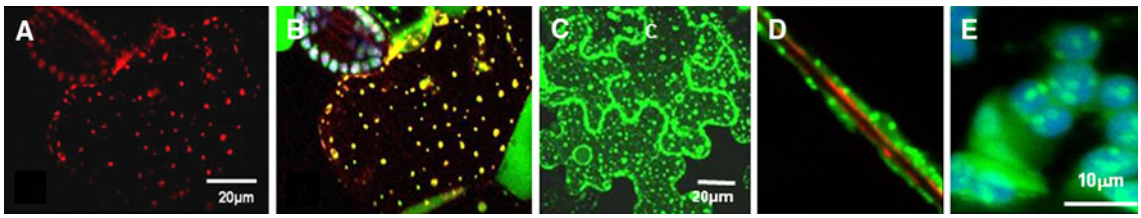

Fig. 1 Localization of ROS in the mitochondria (**A**, **B**), cytoplasm (**C, D**), and chloroplasts (**E**) in *Pelargonium* epidermal cells (**A–D**) and mesophyll cells (**E**). **A** MitoTracker labeling of mitochondria (*red*). **B** Staining with H_2DCF-DA and MitoTracker staining (*yellow*) and autofluorescence (*blue*) of chloroplasts in the stomata. **C** H_2DCF-DA staining of 3-day-darkened tissues exhibiting accumulation of ROS in the rim of the cells. **D** FM4-64 (*red*) and H_2DCF-DA (*green*) staining of epidermal cells. The membrane staining localized out of the ROS staining. **E** Autofluorescence (*blue*) and H_2DCF-DA staining (*green*) of chloroplasts. (Color figure online)

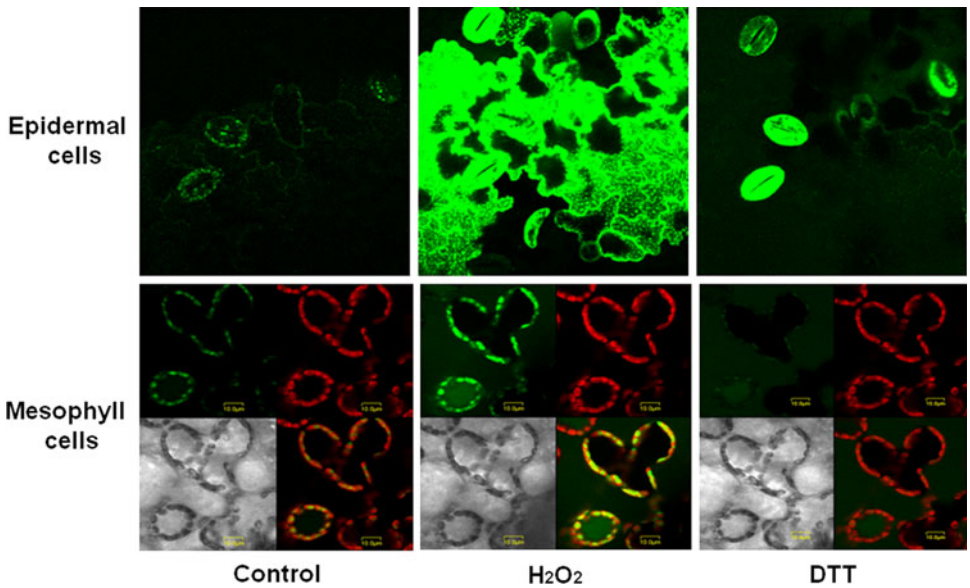

Fig. 2 Effects of DTT and H_2O_2 on DCF fluorescence. Epidermal (*upper panel*) and mesophyll cells (*lower panel*) of *Pelargonium* leaves were stained with H_2DCF-DA and viewed under a confocal microscope before and after addition of 10 mM H_2O_2. Ten mM DTT was added following H_2O_2 application. Photographs of either the top or the bottom panels are of the same cells. On *bottom panel* pictures in a clock-wise direction starting from the top left are: DCF fluorescence, autofluorescence, transmitted light and DCF plus autofluorescence

ROS levels in the chloroplasts and the epidermal cytoplasm during the dark period. A reduction in ROS was observed on the second day of darkness in both the epidermal cytoplasm (Fig. 3A) and the chloroplasts (Fig. 4). On the third day of darkness ROS increased in both the epidermal cytoplasm (Fig. 3) and the chloroplasts (Fig. 4), and remained high during the following days (until day 5) (Table 1).

ROS increase on the third day might have resulted from increased activity of NADPH oxidase associated with tissue injury (Orozco-Cardenas and Ryan 1999). To clarify this issue, leaf discs were treated with DPI, an NADPH oxidase inhibitor. The increase in ROS in the cytoplasm on the third day was apparent also when the tissues were pretreated with DPI, indicating that the increase in ROS did not result from NADPH oxidase activity (Fig. 3B).

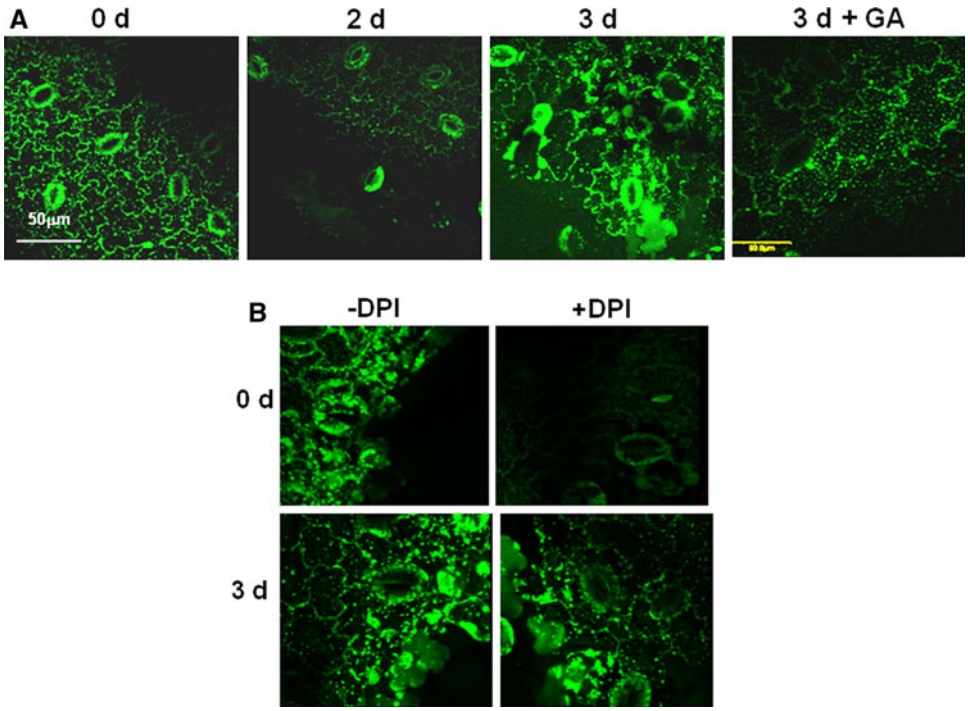

Fig. 3 Effect of dark treatment, GA_3, and DPI on ROS levels in the epidermal cells. Epidermal cells of *Pelargonium* leaves were stained with H_2DCF-DA and viewed under constant excitation light intensity. GA_3-treated or nontreated cuttings were subjected to dark treatment before staining. Leaf discs were excised from the rim of the leaves. The numbers above the photographs indicate the number of days under darkness (**A**). DPI treatment was applied to leaf discs excised from nondarkened (0 d) or 3-day-darkened (3 d) tissues before staining with H_2DCF-DA (**B**)

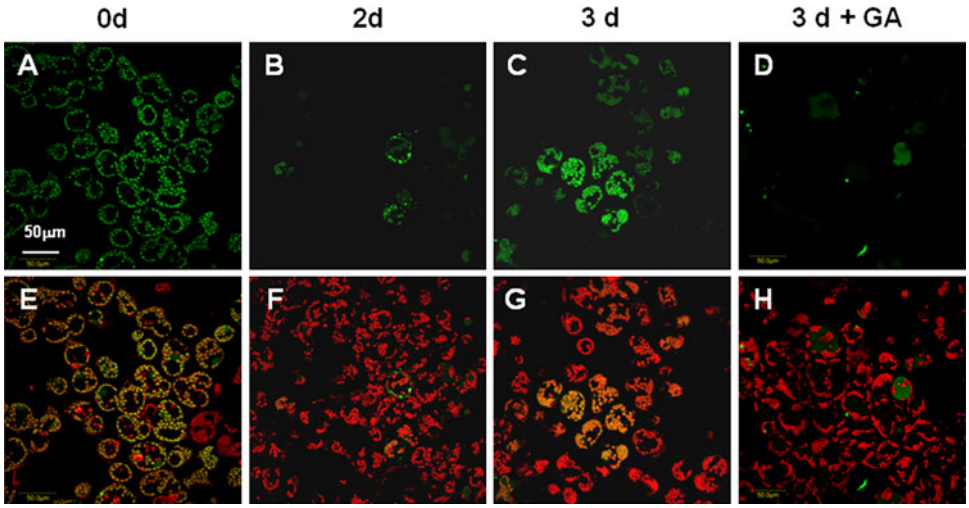

Fig. 4 Changes in ROS levels in the chloroplasts during dark treatment and the effect of GA_3 on ROS levels. Mesophyll cells of *Pelargonium* leaves were stained with H_2DCF-DA and viewed under constant excitation light intensity. Leaf discs were excised from the rim of the leaves. GA_3-treated or nontreated cuttings were subjected to dark treatment before staining. The numbers above the photographs indicate the number of days under darkness. *Upper-panel* photographs present the fluorescence of H_2DCF-DA staining, and the lower ones present autofluorescence together with DCF fluorescence

Table 1 Increase in ROS during dark treatment and the effect of GA_3 on ROS Levels

Cellular site	Treatment	Days 3-5/Day 2
Chloroplasts	$-GA_3$ (4)	2.39 ± 0.84
	$+GA_3$ (3)	0.46 ± 0.09
Epidermal cell cytoplasm	$-GA_3$ (6)	2.06 ± 0.63
	+ GA_3 (4)	0.66 ± 0.06

Pelargonium cuttings were treated as described in Figs. 3 and 4. Leaf discs were excised from the rim of leaves darkened for 2 days and longer. ROS-related fluorescence analyses of the photographs were performed as described in Materials and Methods. The data are expressed as the ratio between fluorescence intensity on days 3-5 and that of nontreated tissue on day 2 ± SE. Numbers in parentheses indicate the number of independent experiments used for data calculation

Pretreatment of cuttings with GA_3 inhibited ROS increase in the epidermal cell cytoplasm and the chloroplasts, which occurred following 3 days of darkness (Figs. 3, 4; Table 1). Table 1 summarizes several experiments that examined the increase in ROS in the cytoplasm and the chloroplasts from the third to the fifth days under darkness compared with the ROS levels in untreated cuttings on the second day, and the effect of GA_3 on this increase. The data show that GA_3 treatment prevented ROS increase in darkened tissues. It even decreased the ROS levels below those of untreated tissues on the second day of darkness. GA_3 treatment had no effect on ROS levels on earlier days under darkness (data not shown).

To examine how chloroplasts of GA_3-treated or nontreated tissues respond to illumination following darkness, tissues darkened for 2 and 4 days and stained with H_2DCF-DA were exposed to a sequence of short illuminations under the microscope (Fig. 5). This experiment simulates the transfer of cuttings to light following an extended dark period to which they are subjected during shipment. Successive illuminations revealed that in 2-day-darkened tissues ROS-related fluorescence was released to the cytoplasm potentially from chloroplasts. However, in 4-day-darkened tissues ROS-related fluorescence accumulated in the chloroplasts (Fig. 5, Supplementary Data). Figure 5 represents a combined photograph of the first 20 pictures of this illuminations sequence. It is clear that in tissues darkened for 2 days only, the ROS-related fluorescence accumulated in the cytoplasm, whereas in 4-day-darkened tissues it accumulated in the chloroplasts. GA_3 pretreatment of the cuttings reduced the levels of ROS accumulated due to extended illumination in the chloroplasts of 4-day-darkened tissues (Fig. 5). This suggests that GA_3 inhibited ROS increase related to darkness as well as ROS increase related to illumination of darkened tissues.

Changes in the Structure and Size of Chloroplasts During Darkness and the Effect of GA_3

The structural changes have been examined in chloroplasts from either GA_3-pretreated or untreated tissues that had been subjected to extended dark periods. The chlorophyll content decreased in *Pelargonium* leaf tissues darkened for more than 3 days (Rosenvasser and others 2006), and these leaves contained both yellow and green areas. Samples taken from yellow areas revealed complete destruction of the chloroplasts, as shown in a representative photograph (Fig. 6, yellow). However, chloroplasts in the green areas still appeared to be intact, but their shape changed from elongated to round, and they contained fewer grana than those of nondarkened tissues. Chloroplasts in GA_3-pretreated tissues appeared similar to those observed in the green areas of 3-day-darkened tissues. Hence, GA_3 did not prevent the modification of shape that occurred due to dark treatment.

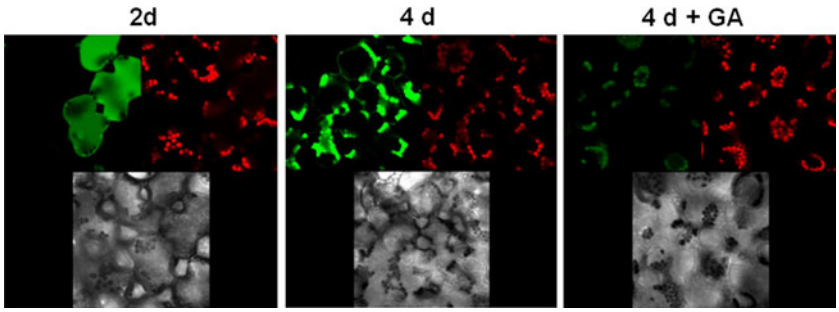

Fig. 5 Effect of successive illuminations on ROS levels in the chloroplasts of GA_3-pretreated and nontreated mesophyll cells. GA_3-treated and nontreated *Pelargonium* leaves darkened for various lengths of time were viewed following staining with H_2DCF-DA. Cells were pulsed successively 40 times at 488 nm as detailed in the text. The pictures present the 20th image for each treatment. (The movies showing changes in ROS during extended illumination are presented in supplementary data.) The numbers above the photographs indicate the number of days under darkness. H_2DCF-DA staining (*green—left panel*), autofluorescence (*red—right panel*), and transmitted light (*gray—bottom panel*). (Color figure online)

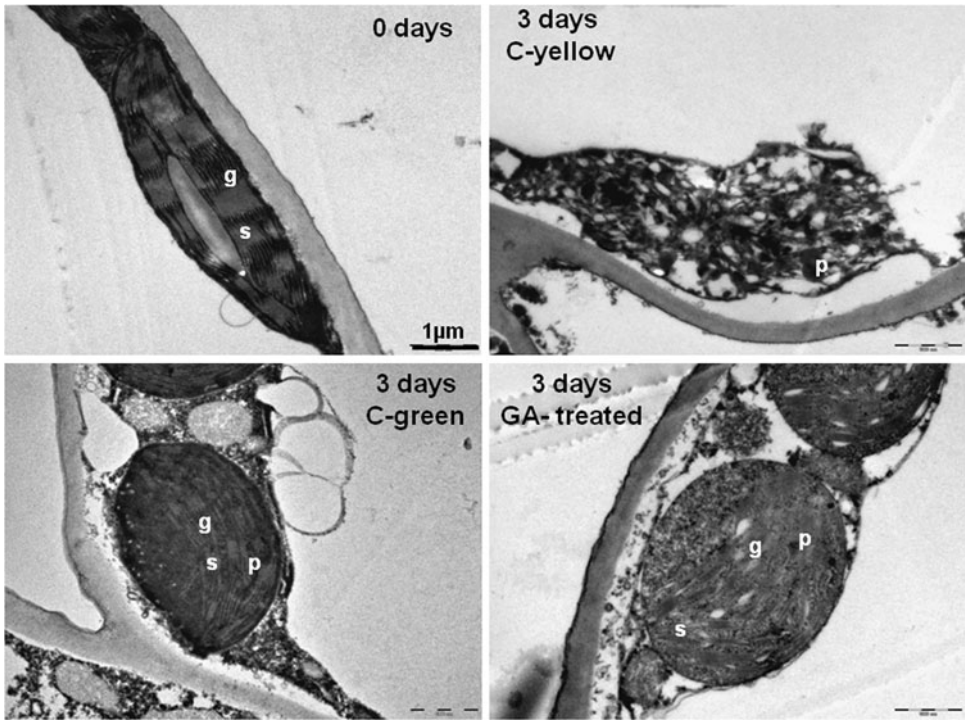

Fig. 6 Structure of chloroplasts of GA_3-treated and nontreated leaves following 3 days of dark treatment. *Pelargonium* leaves were viewed with a transmission electron microscope and a representative picture is presented for nondarkened, green and yellow areas of 3-day-darkened tissue, and GA_3-pretreated tissues darkened for 3 days. g, grana; p, plastoglobuli; s, stroma; C, nontreated control. The numbers indicate the duration of the dark treatment

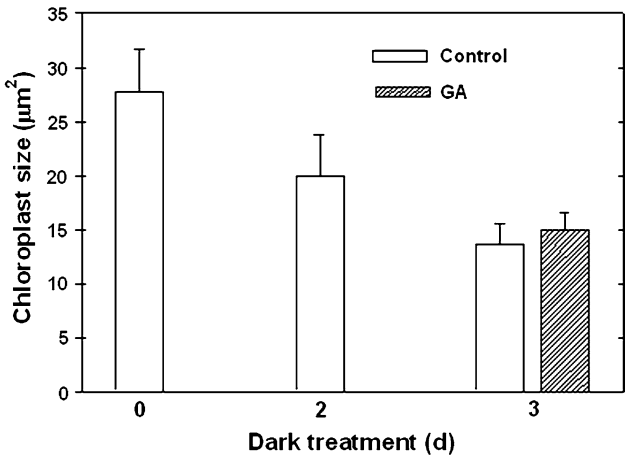

Fig. 7 Effect of dark treatment on chloroplast size in *Pelargonium* leaves. Cuttings were dark-treated for 3 days, and chloroplasts were isolated as described in Materials and Methods at the indicated times. The area of chloroplasts in the pictures was determined with image analysis software. Results are averages for at least five pictures per treatment ± SE

The method used for chloroplast isolation yielded intact chloroplasts (Napier and Barnes 1996). Therefore, the isolated chloroplasts from 3-day-darkened leaves, which had both yellow and green tissues, represented only a fraction of the chloroplast population, and probably not those present in the yellow areas (Fig. 6). Measurement of the dimensions of the isolated chloroplasts showed that their size decreased as the dark period increased, and GA_3 did not prevent this decrease (Fig. 7). These results are consistent with the chloroplast modifications observed by electron microscopy (Fig. 6).

Discussion

Previous studies showed that ROS-related fluorescence in intact tissues increased during dark-induced senescence, and that GA_3 inhibited this increase (Rosenvasser and others 2006). In addition, the increase in ROS paralleled the decrease in chlorophyll content. Increases in ROS in dark-treated tissues have been observed also in other studies (Kar and Feierabend 1984; Philosoph-Hadas and others 1994). An increase in ROS during dark treatment of pea leaves was also observed in isolated peroxisomes (Pastori and Rio 1994; Jimenez and others 1998) and mitochondria (Jimenez and others 1998). However, no temporal or spatial changes in ROS levels in various cell compartments have been recorded in situ. In the present study, the changes in ROS in different subcellular compartments during dark-induced senescence were monitored, and the question of whether GA_3 reduces ROS in all cell compartments during dark-induced senescence was studied.

DCF fluorescence was detected in the mitochondria, cytoplasm, and chloroplasts (Fig. 1), and it increased in these compartments following H_2O_2 application (Fig. 2). Thus, any increase in DCF fluorescence in these compartments during dark-induced senescence can be attributed to the oxidation of the dye by ROS. During dark treatment parallel dynamic changes in ROS were observed in the

epidermal cell cytoplasm and the chloroplasts (Figs. 3, 4). A decrease in ROS was observed on the second day under darkness in both compartments, followed by an increase in ROS on the third day under darkness thereafter (Figs. 3, 4; Table 1).

Chloroplasts are the major source of ROS when tissues are exposed to light. However, darkness, which leads to cessation of electron transfer, causes on one hand, a diminution in the reducing power (Buchanan 1991) and on the other hand, a reduction in ROS levels. Thus, it is possible to assume that the reduction in DCF fluorescence in the chloroplasts on the second day was a result of cessation of ROS production during photosynthesis.

The ROS increase observed on the third day does not seem to be the result of cell injury resulting from disc excision, because DPI, which inhibits NADPH oxidase activity, did not prevent the increase in ROS. ROS increase paralleled structural changes in the chloroplasts, which were manifested as a decrease in size, a change in shape from elongated to round, and a decrease in the number of grana (Figs. 6, 7). Similar changes in the chloroplast structure were also observed in wheat during dark-induced senescence (Zavaleta-Mancera and others 2007). Chloroplasts of tissue darkened for 3 days and longer were also more sensitive to successive illuminations, and they accumulated more ROS than those of 2-day-darkened tissue (Fig. 5). An increase in ROS of 3-day-darkened tissues due to illumination probably occurred because of the excitation of chlorophyll or possibly of its intermediates (Pruzinska and others 2005).

The mechanisms involved in the induction of ROS increase in the chloroplasts under darkness are still not clear. This increase could have resulted from enhanced degradation of membrane lipids and peroxidation of free fatty acids, which have been suggested to occur during natural senescence (McRae and Thompson 1983). The oxidized products could lead to a further increase in the production of ROS, especially alkoxy and peroxy radicals and singlet oxygen (Bhattacharjee 2005).

In *Arabidopsis*, the transcript expression levels of several antioxidative enzymes localized to the chloroplasts were reduced during darkness, including *superoxide dismutase* (*SOD*), *ascorbate peroxidase4* (*APX4*), and *glutathione peroxidase* (*GPX*) (Rosenwasser and Friedman, unpublished). It is still not clear whether the reduction in the expression of these enzymes in *Pelargonium* facilitates the ROS increase in the chloroplasts under darkness.

The observations obtained in the present study did not show an early increase in ROS in a specific compartment, indicating that either the same signal affected all compartments or that the present technique was not sensitive enough to detect small changes occurring in a specific compartment. Hence, our data do not support a signaling role for the increase in ROS in the chloroplasts. Nevertheless, it is possible that the increase in ROS in the chloroplasts during darkness activates a whole cascade of deterioration processes, as suggested for ROS increase in chloroplasts during various biotic and abiotic stresses, especially under light (Foyer and Noctor 2005). However, it is also possible that the death signal is not related to ROS in the chloroplasts, as has been suggested for cell death induced by the bacterial elicitor harpin (Garmier and others 2007).

Application of GA_3 inhibited ROS increase in the epidermal cell cytoplasm and in the chloroplasts that occurred following 3 days of darkness (Figs. 3, 4). It even decreased the ROS level to below that detected on the second day of darkness (Table 1). GA_3 also prevented ROS increase when 3-day-darkened tissues were subjected to successive illuminations (Fig. 5). Our data contrast with those of previous studies that demonstrated that GA_3 increased ROS levels in aleurone cells (Fath and others 2001). In addition, it was suggested that DELLA, the inhibitor of gibberellin action, is responsible for reduction of ROS (Achard and others 2008), and, hence, induction of DELLA degradation, which is induced by GA_3, might lead to enhanced ROS levels.

It is clear that GA_3 did not inhibit the morphological changes observed in the chloroplasts (Figs. 6, 7). However, the mode of action of GA_3 in reducing ROS is still not clear. It is possible that GA_3 either enhances the levels of antioxidants or inhibits processes involved in membrane deterioration to reduce ROS production, both of which lead to reduced ROS levels.

The present findings clearly show that ROS increased concomitantly in several subcellular compartments, including chloroplasts, under prolonged darkness and that the increase in the chloroplasts was not caused by chlorophyll breakdown (data not shown). GA_3 application did not prevent the structural changes that occurred in the chloroplasts during darkness, but it did decrease ROS accumulation. It is possible that this decrease in ROS levels prevented a complete disintegration of the chloroplasts and maintained the chlorophyll levels in GA_3-treated *Pelargonium* leaves.

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