Imaging the production of singlet oxygen in vivo using a new fluorescent sensor, Singlet Oxygen Sensor Green®

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Abstract
Singlet oxygen is known to be produced by cells in response to photo-oxidative stresses and wounding. Due to singlet oxygen being highly reactive, it is thought to have a very short half-life in biological systems and, consequently, it is difficult to detect. A new commercially available reagent (singlet oxygen sensor green, SOSG), which is highly selective for singlet oxygen, was applied to a range of biological systems that are known to generate singlet oxygen. Induction of singlet oxygen production by the addition of myoglobin to liposome preparations demonstrated that the singlet oxygen-induced increases in SOSG fluorescence closely followed the increase in the concentration of conjugated dienes, which is stoichiometrically related to singlet oxygen production. Applications of photo-oxidative stresses to diatom species and leaves, which are known to result in the production of singlet oxygen, produced large increases in SOSG fluorescence, as did the addition of 3-(3,4-dichlorophenyl)1,1-dimethylurea (DCMU) to these systems, which inhibits electron transport in photosystem II and stimulates singlet oxygen production. The conditional fluorescent (flu) mutant of Arabidopsis produces singlet oxygen when exposed to light after a dark period, and this coincided with a large increase in SOSG fluorescence. Wounding of leaves was followed by an increase in SOSG fluorescence, even in the dark. It is concluded that SOSG is a useful in vivo probe for the detection of singlet oxygen.

Key words: Arabidopsis, diatoms, flu mutant, herbicides, liposomes, photo-oxidative stress, singlet oxygen, wounding.

Introduction
Singlet oxygen (1O₂) is a highly reactive molecule that is potentially damaging to biological systems. It will rapidly oxidize molecules containing carbon-carbon double bonds to form hydroperoxides or endoperoxides; in proteins 1O₂ will specifically oxidize cysteine, histidine, methionine, and tryptophan residues and, on interaction with membrane lipids, it can stimulate rapid lipid peroxidation (Halliwell and Gutteridge, 1999). When ground (triplet) state oxygen (3O₂) interacts with an excited photosensitizer molecule (P*) 1O₂ can be produced (Halliwell and Gutteridge, 1999):

P + light \rightarrow P* 

P* + 3O₂ \rightarrow P + 1O₂ 

In chloroplasts excited chlorophyll can act as a photosensitizer (Owens, 1996). If the chlorophyll triplet states are not quenched by photochemistry or carotenoids, they will interact with 3O₂ to produce 1O₂ (Owens, 1996). Inhibition of photosynthetic electron transport resulting from environmental stresses can result in the production of 1O₂ that has been implicated in damage to the D1 protein of the PSII reaction centre, and if prolonged, in chlorophyll bleaching (Asada, 1996; Krieger-Liskay, 2005).
Production of $^{1}\text{O}_2$ by photosensitizers is a well-established, important component of plant defence mechanisms (Knox and Dodge, 1985). Many secondary metabolites in plants are phototoxic as a result of producing $^{1}\text{O}_2$ (Downum and Wen, 1995), for example, some phenalenone-like phytoalexins and phytoanticipins (Lazzaro et al., 2004; Flors et al., 2005). Phenalenone is a very efficient photosensitizer molecule that can generate $^{1}\text{O}_2$ with a quantum yield of close to 1 (Schmidt et al., 1994). Such light-stimulated production of $^{1}\text{O}_2$ has been implicated in the antifungal properties of phenylphenalenone phytoalexins (Lazzaro et al., 2004).

Recently, $^{1}\text{O}_2$ has been shown to be involved with inducing stress-responsive gene expression in plants (op den Camp et al., 2003; Wagner et al., 2004; Danon et al., 2005). It is widely believed that, in biological systems, $^{1}\text{O}_2$ has a very short half-life of around 200 ns (Gorman and Rodgers, 1992), which would preclude its direct involvement in signalling responses in response to adverse environmental conditions or wounding. In this case, the hydroperoxides and endoperoxides formed when $^{1}\text{O}_2$ oxidizes biological molecules are more likely to be implicated in such signalling responses. Alternatively, chlorophyll degradation products may act as signalling molecules (Krieger-Liszkay, 2005). However, recent observations suggest that the half-life of $^{1}\text{O}_2$ in cells may be much longer than 200 ns, thus allowing diffusion over appreciable distances and across cell membranes (Skovsen et al., 2005), in which case $^{1}\text{O}_2$ may itself act as a signal molecule.

With the increasing awareness of the potential importance of $^{1}\text{O}_2$ generation in plants during photo-oxidative stress, pathogen attack and wounding, there is an increasing need for rapid, sensitive, and readily available techniques for detecting $^{1}\text{O}_2$ in intact plant tissues and locating the site of its production. The short lifetime of $^{1}\text{O}_2$ in biological systems makes it difficult to study. Methods such as spin trapping EPR spectroscopy (Hideg et al., 1994), chemical trapping (Telfer et al., 1994), and phosphorescence at 1270 nm (Macpherson et al., 1993) have been used to detect $^{1}\text{O}_2$ in isolated biological preparations. Recently, microscopic detection of $^{1}\text{O}_2$ in single cells by monitoring its 1270 nm phosphorescence has been reported (Snyder et al., 2004). However, such methods are not suitable for routine studies to detect $^{1}\text{O}_2$ in leaves. A dansyl-based fluorescence sensor, dansyl-1,2,2,5,5-tetramethyl-2,5-dihydro-1H-pyrrole (DanePy), has been successfully infiltrated into leaves and quenching of its fluorescence used to detect $^{1}\text{O}_2$ production in situ (Hideg et al., 2002). However, the lack of commercial availability of DanePy, its variable stability in some biological treatments, and the need for a highly sensitive fluorescence detection system to determine the quenching induced by $^{1}\text{O}_2$ limits its usefulness as a routine probe. Recently, a Singlet Oxygen Sensor Green (SOSG) reagent\textsuperscript{8}, which is highly selective for $^{1}\text{O}_2$ and does not show any appreciable response to hydroxyl radicals or superoxide, has become commercially available (Molecular Probes, 2004). SOSG exhibits weak blue fluorescence with excitation peaks at 372 nm and 393 nm and emission peaks at 395 nm and 416 nm (Molecular Probes, 2004). In the presence of $^{1}\text{O}_2$ SOSG emits a green fluorescence with excitation and emission peaks at 504 nm and 525 nm, respectively (Molecular Probes, 2004), which can be readily detected. In this study, the effectiveness of the SOSG reagent for detecting the production of $^{1}\text{O}_2$ in a liposome preparation, algal cells, and the leaves of higher plants are examined.

Materials and methods

Spectral properties of SOSG and its reaction product

Absorption and fluorescence emission spectra between 400 and 650 nm were determined for SOSG before and after reaction with $^{1}\text{O}_2$ (Fig. 1). Phenalenone (1 $\mu$M) was used as a photosensitizer to generate $^{1}\text{O}_2$ in a 1.4 $\mu$M aqueous SOSG solution (containing 2% methanol) using a 5 ns pulse (0.5 mJ per pulse) of 355 nm irradiation produced from a Continuum Surelite I-10 Nd:YAG laser. The absorption spectra were measured using a Varian Cary 4E spectrophotometer. Fluorescence emission spectra were measured with a Jobin-Yvon Spex Fluoromax-2 spectrophotometer using an excitation wavelength of 480 nm. Clearly, from the spectra shown in Fig. 1 excitation of SOSG at 480 nm with monitoring of fluorescence at 530 nm will allow assessment of the reaction of SOSG with $^{1}\text{O}_2$.

Production of $^{1}\text{O}_2$ and conjugated dienes by liposomes reacting with myoglobin

Liposomes were prepared from soybean lecithin (50 mg; type II-S, 19% phosphatidycholine; Sigma-Aldrich, Poole, UK) as previously described (Reeder and Wilson, 2005). Liposomes (200 $\mu$g ml\textsuperscript{-1}) were reacted with horse myoglobin (1 $\mu$M; Sigma-Aldrich, Poole, UK) in 50 mM sodium phosphate buffer pH 7.4. Oxidation of liposomes was measured by the appearance of lipid-based conjugated dienes measured optically at 234 nm using an Agilent HP8453 diode array spectrophotometer. To correct for changes in light scattering, a

![Fig. 1. Spectral properties of SOSG before and after reaction with $^{1}\text{O}_2$. Absorption (a, c) and fluorescence emission (b, d) spectra of SOSG before (a, b) and after (c, d) reaction with $^{1}\text{O}_2$. SOSG was dissolved in water containing 2% methanol. SOSG fluorescence emission was produced using an excitation wavelength of 480 nm.](https://academic.oup.com/jxb/article-abstract/57/8/1725/523548)
three-point baseline drop was calculated from optical measurements taken at 220, 234, and 255 nm. The lipid hydroperoxide 13(S)-hydroperoxy-9,11(cis,trans)-octadecadienoic acid (HPODE) was used as a standard for conjugated diene concentration (\(E_{234\text{nm}}\) 2.5x10^{-4} M^{-1} cm^{-1}; Egmond et al., 1976). Using the three-point baseline drop method the extinction coefficient for conjugated diene concentration was calculated to be 1.45x10^{4} M^{-1} cm^{-1}. HPODE was prepared from linoleic acid as previously described (Reeder and Wilson, 1998).

Singlet oxygen sensor green (SOSG) reagent was dissolved in methanol to make a stock solution of 500 mM. SOSG (final concentration 50 \(\mu\)M) was added to liposomes (2 mg ml^{-1}) in 0.5 M sodium phosphate buffer pH 7.4 in a Petri dish (2 cm diameter). Lipid oxidation was started by the addition of ferric horse myoglobin (10 \(\mu\)M). Following excitation of SOSG at 480 nm using a 75 W xenon lamp (Leica UK Ltd, Milton Keynes, UK) with a 480 nm interference filter and heat filter, images of the fluorescence from SOSG were produced as described for the liposomes. Detection of hydrogen peroxide and the leaves were then allowed to transpire for 2.5 h in the dark. For this dark period leaves were detached and infiltrated with 260 \(\mu\)M SOSG in the dark as described above, and then kept in the dark or transferred to a PPFD of 150 \(\mu\)mol m^{-2} s^{-1}. Some plants were kept in continuous light then leaves were detached and infiltrated in the light with SOSG.

**1O2 production in leaves of the Arabidopsis flu mutant**

Seeds of the Arabidopsis flu-1 mutant were kindly provided by Dr K Apel, (Institute of Plant Sciences, Plant Genetics, Swiss Federal Institute of Technology, CH 8092 Zürich, Switzerland). Plants were grown from seed under continuous light (PPFD 150 \(\mu\)mol m^{-2} s^{-1}) for 4 weeks before being transferred into a 16 h dark period. After this dark period leaves were detached and infiltrated with 260 \(\mu\)M SOSG in the dark as described above, and then kept in the dark or transferred to a PPFD of 150 \(\mu\)mol m^{-2} s^{-1}. Some plants were kept in continuous light then leaves were detached and infiltrated in the light with SOSG.

**1O2 production in leaves in the dark following wounding**

Plants of Arabidopsis were grown from seed as described above for 5 weeks. Fully expanded leaves were detached and infiltrated with 260 \(\mu\)M SOSG as described above. The leaves were wounded by crimping in two places with blunt-end forceps. Leaves were then either kept in the dark or in the growth PPFD of 150 \(\mu\)mol m^{-2} s^{-1}, and, after 30 min, the SOSG fluorescence imaged as described above. Celery (Apium graveolens var. Giant Pascal) plants were grown from seed under controlled environmental conditions (PPFD 200 \(\mu\)mol m^{-2} s^{-1} during a 16 h photoperiod at day/night temperatures of 26/18°C in a relative humidity of c. 70%). Leaves were used for experiments when plants were c. 1 month old and were wounded by cutting with a razor blade at the edge. After wounding, the cut areas of the leaves were immersed in a solution of 50 \(\mu\)M SOSG for 20 min. Leaves were then dried in the dark before imaging the SOSG fluorescence as described above.

### Results and discussion

In the presence of molecular oxygen, carbon-centred lipid radicals (\(L^*\)) can form lipid peroxide radicals (LOO\(^*\)) and induce a cascade reaction in which an autocatalytic cycle results in further lipid oxidation (Halliwell and Gutteridge, 1999):

\[
L^* + O_2 \rightarrow LOO^* \tag{3}
\]

\[
\text{LH} + \text{LOO}^* \rightarrow L^* + \text{LOOH} \tag{4}
\]

Ferryl myoglobin, formed from the oxidation of ferric haem protein by trace lipid peroxides, can initiate lipid oxidation by virtue of its pseudoperoxidase activity. This results in a radical cascade reaction in which lipid conjugated dienes are formed and in the presence of molecular oxygen produce LOO\(^*\) (Reeder et al., 2004). Self termination of a fraction of LOO\(^*\) results in the formation of lipid alcohol (LOH) and lipid ketone (LO) and \(1O_2\) is produced (Halliwell and Gutteridge, 1999):

\[
\text{LOO}^* + \text{LOO}^* \rightarrow \text{LOO} \rightarrow \text{OOL} \rightarrow \text{LOH} + \text{LO} + 1O_2 \tag{5}
\]

Consequently, there is a stoichiometric relationship between the rate of lipid oxidation, lipid conjugated diene formation, and the generation of \(1O_2\). Using such a lipid oxidation system the development of SOSG fluorescence was evaluated as an indicator of \(1O_2\) formation. A liposome preparation was made from soybean lecithin and myoglobin added to initiate oxidation of the lecithin in the presence of SOSG. Increases in SOSG fluorescence...
were observed 60 s after the addition of the myoglobin and increased progressively over 120 min (Fig. 2). The kinetics of appearance of lipid conjugated dienes was monitored by absorbance spectroscopy in parallel with the increase in SOSG fluorescence (Fig. 3). The increase in SOSG fluorescence closely followed the increase in the concentration of conjugated dienes, demonstrating that increases in SOSG fluorescence were a good indicator of increases in \( ^1\text{O}_2 \) formation.

It is well established that when photosynthetic organisms are exposed to light levels in excess of those that can be used for photosynthesis, photo-oxidative stress occurs which can result in the production of \( ^1\text{O}_2 \) (Krieger-Liskay, 2005). Photo-oxidative stress was imposed on cultures of three diatom species (Navicula perminuta, Nitzschia epithemoides, and Thalassiosira pseudonana) by exposing cells that had been grown at a \( \text{PPFD} \) of 50 \( \mu\text{mol} \text{m}^{-2} \text{s}^{-1} \) to an increased \( \text{PPFD} \) of 1000 \( \mu\text{mol} \text{m}^{-2} \text{s}^{-1} \). Dark-adapted cell cultures were placed in Eppendorf tubes, SOSG was added, and then the cells were either kept in the dark or exposed to 1000 \( \mu\text{mol} \text{m}^{-2} \text{s}^{-1} \) for 20 min. SOSG fluorescence was considerably greater in the high-light-treated samples than the dark controls (Fig. 4A), demonstrating the production of \( ^1\text{O}_2 \) during the photoinhibitory treatment. The kinetics of the development of SOSG fluorescence during exposure of the cells to high light were examined by placing aliquots of the cultures in Petri dishes. The cultures were kept in the dark prior to exposure to the high light. SOSG was added and then half of the dish was exposed to a \( \text{PPFD} \) of 1000 \( \mu\text{mol} \text{m}^{-2} \text{s}^{-1} \) while the other half of the dish was covered to protect the cells from direct light. For all three species exposure to high light progressively induced increases in SOSG fluorescence over 1 h (Fig. 4B). The halves of the cultures that were kept covered exhibited considerably less fluorescence, although it was noticeable that there were some increases in fluorescence detected from the covered regions of the Petri dishes, most noticeably in the N. perminuta and N. epithemoides cultures. There is likely to be some light scatter within the Petri dishes from the lighted side to the covered regions, which could account for the small increases in SOSG fluorescence in the covered halves of the dishes. However, both N. perminuta and N. epithemoides are diatom species that are highly motile (Round et al., 1990) and it is known that motile diatoms can migrate away from high \( \text{PPFDs} \) (Perkins et al., 2002; Cohn et al., 2004). Consequently, it is likely that cells of N. perminuta and N. epithemoides will migrate from the illuminated halves of the Petri dishes to the covered half with time, and this will result in an increase in SOSG fluorescence in the covered areas. Cells of T. pseudonana are not motile and considerably less SOSG fluorescence was observed in the covered region of dishes of this species.

It has previously been demonstrated, by monitoring the decrease in DanePy fluorescence, resulting from the reaction of DanePy with \(^1\text{O}_2\), to form the non-fluorescent DanePyO, that \( ^1\text{O}_2 \) is produced in Arabidopsis leaves which were grown at a \( \text{PPFD} \) of 200 \( \mu\text{mol} \text{m}^{-2} \text{s}^{-1} \) and exposed to a \( \text{PPFD} \) of 650 \( \mu\text{mol} \text{m}^{-2} \text{s}^{-1} \) for 1 h (Fryer et al., 2003). SOSG was applied to similarly grown Arabidopsis leaves to examine the kinetics of \( ^1\text{O}_2 \) production. Leaves were detached from the plants, infiltrated with SOSG in the dark and then the top half of the leaves were exposed to a \( \text{PPFD} \) of 650 \( \mu\text{mol} \text{m}^{-2} \text{s}^{-1} \); the lower half of the leaves were kept in the dark. Within 5 min of exposure to the elevated \( \text{PPFD} \) an increase in SOSG fluorescence was detected in the upper half of the leaves (Fig. 5). In all leaves a large non-light-dependent SOSG fluorescence was observed at the cut end of the petiole, presumably in response to the wounding (see below). The SOSG fluorescence increased progressively over 50 min in the upper regions of the leaves exposed to the elevated \( \text{PPFD} \) (Fig. 5). In a similar experiment where leaves were infiltrated with DanePy to detect \(^1\text{O}_2\), quenching of DanePy fluorescence was not observed until after 60 min exposure to the high light (Fryer et al., 2003; also other data not shown) suggesting that SOSG is a much more sensitive detector of \( ^1\text{O}_2 \) in leaves than DanePy.

The herbicide 3-(3',4'-dichlorophenyl)1,1-dimethylurea, commonly referred to as DCMU or diuron, binds specifically to the \( Q_b \) site on the D1 protein of the PSII reaction centre, thereby preventing electron transfer from \( Q_A \) to \( Q_b \) (Bowyer et al., 1991). In the light, binding of DCMU to the PSII reaction centre results in the formation of the

![Fig. 2. Changes in SOSG fluorescence with time after the addition of myoglobin to liposomes.](https://academic.oup.com/jxb/article-abstract/57/8/1725/523548)
radical pair \( \text{P}^+_{680} \text{Pheophytin}^- \), whose recombination produces the triplet state of \( \text{P}^*_{680} \), which reacts with \( \text{O}_2 \) to form \( \text{^1O}_2 \) (Asada, 1996). Under such conditions the probability for the formation of the triplet states of PSII antennae chlorophylls also increases, and these react with oxygen resulting in more \( \text{^1O}_2 \) production. Consequently, infiltrating a leaf with DCMU in the light will result in a large increase in \( \text{^1O}_2 \) production. The increase in SOSG fluorescence on infiltrating an \( \text{Arabidopsis} \) leaf at the growth \text{PPFD} of 150 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) is shown in Fig. 6. A low level of SOSG fluorescence was detected in control leaves exposed to this growth \text{PPFD}, indicating that some \( \text{^1O}_2 \) is being produced at this low growth \text{PPFD} in control leaves. In DCMU-treated leaves exposed to a \text{PPFD} of 350 \( \mu \text{mol m}^{-2} \text{s}^{-1} \), SOSG fluorescence was detected 30 min after exposure to illumination and increased with time of exposure (Fig. 7). On transfer of a DCMU-treated leaf into the dark after exposure to the \text{PPFD} of 350 \( \mu \text{mol m}^{-2} \text{s}^{-1} \)

Fig. 3. Changes in the concentration of conjugated dienes (open circles) and SOSG fluorescence (filled circles) with time after the addition of myoglobin to liposomes.

Fig. 4. Changes in SOSG fluorescence emission in cultures of \( \text{Navicula perminuta}, \text{Nitzschia epithemioides}, \) and \( \text{Thalassiosira pseudonana} \) on exposure to high light. (A) Cell cultures grown at a \text{PPFD} of 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) were placed in Eppendorf tubes and exposed to a \text{PPFD} of 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (light) or kept in the dark (dark) for 20 min. The effect of adding DCMU to the cultures prior to exposure to the high light is shown (light+DCMU). (B) The development of SOSG fluorescence on exposure of cells to high light. Cell cultures grown at a \text{PPFD} of 50 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) were placed in Petri dishes. The top half of the dishes was covered (dark) and the bottom half exposed to a \text{PPFD} of 1000 \( \mu \text{mol m}^{-2} \text{s}^{-1} \) (light); images were taken after 0, 10, 30, and 60 min.
for 90 min, a strong SOSG fluorescence was maintained over 12 h (Fig. 7), indicating a good stability of the SOSG fluorescence when the light-dependent production of $^1\text{O}_2$ is removed. It would appear that there is an increase in SOSG fluorescence in the dark in the DCMU- and light-treated leaves, which may be accounted for by light-generated $^1\text{O}_2$ resulting in cell damage and associated $^3\text{O}_2$ production in the dark.

Although it is claimed by the manufacturer that SOSG is specific for $^1\text{O}_2$ and does not react with hydroxyl radicals or superoxide anions (Molecular Probes, 2004), the possibility of SOSG in vivo responding to hydrogen peroxide and superoxide, which are often produced in very large amounts in stressed leaves, was examined by determining SOSG fluorescence in leaves treated with paraquat. Paraquat, which is methyl viologen, can be readily reduced by electron transfer from PSI in leaves which results in the production of superoxide anions and hydrogen peroxide (Halliwell, 1991). Exposure of the top half of an Arabidopsis leaf infiltrated with paraquat to a PPFD of 350 $\mu$mol m$^{-2}$ s$^{-1}$ for 120 min resulted in the production of large amounts of hydrogen peroxide and superoxide anions, which were identified by staining with DAB and NBT, respectively (Fig. 8). The DAB and NBT staining in the
illuminated top half of the paraquat-treated leaf was not associated with large increases in SOSG fluorescence (Fig. 8). Some SOSG fluorescence was detected that was slightly greater than observed in the non-paraquat control, and this may be attributable to $^1$O$_2$ production resulting from cell damage induced by the high levels of hydrogen peroxide and superoxide anions.

Fig. 7. The effect of DCMU with time on SOSG fluorescence in Arabidopsis leaves. Leaves were grown and treated as stated in the legend of Fig. 6, except that they were exposed to a PPFD of 350 μmol m$^{-2}$ s$^{-1}$. Images of SOSG fluorescence were taken after 30, 60, and 90 min exposure to the light. After 90 min the leaves were placed in the dark and then imaged again 12 h later (12 h in dark +DCMU). An image of a control leaf that had been kept in the dark for 12 h is also shown.

Fig. 8. SOSG fluorescence in Arabidopsis leaves treated with paraquat. Leaves were grown at a PPFD of 150 μmol m$^{-2}$ s$^{-1}$, detached and infiltrated with SOSG. Leaves were painted with 100 μM paraquat (+paraquat) or water (control) and then the top half of the leaves were exposed to a PPFD of 350 μmol m$^{-2}$ s$^{-1}$ for 120 min. The bottom halves were kept in the dark. Prior to treatment with paraquat and exposure of the top halves of the leaves to a PPFD of 350 μmol m$^{-2}$ s$^{-1}$, some leaves were infiltrated with 5 mM 3,3'-diaminobenzidine (DAB) or 6 mM nitroblue tetrazolium (NBT) in order to detect hydrogen peroxide or superoxide anions, respectively.

A conditional fluorescent (flu) mutant of Arabidopsis accumulates protoclorophyllide, a photosensitizer molecule, in the dark (Op den Camp et al., 2003). When the flu mutant is kept in the dark and then exposed to light, photoactivation of protoclorophyllide results in the production of $^1$O$_2$ (Op den Camp et al., 2003) and this results in rapid bleaching and death (Meskauskiene et al., 2001).
Consequently, in order to produce mature plants, this mutant has to be grown under continuous light, otherwise the production of $^1\text{O}_2$ in the light after a dark period is lethal (Op den Camp et al., 2003). When flu mutant plants were grown in continuous light, leaves were detached and placed in the dark for 16 h and infiltrated with SOSG, no SOSG fluorescence was detected (Fig. 9). However, when the detached mutant leaves after the 16 h dark period were transferred into light (PPFD of 150 $\mu$mol m$^{-2}$ s$^{-1}$) for 2 h, a large SOSG fluorescence was observed (Fig. 9). Leaves that had not been kept in the dark and had only received continuous light, which would not be expected to be producing large amounts of $^1\text{O}_2$ in the lamina, exhibited only very low levels of SOSG fluorescence (Fig. 9). However, these leaves did exhibit a very high SOSG fluorescence in the wounded petiole region.

Generation of $^1\text{O}_2$ in germinating soybeans in response to wounding has been reported recently (Chen et al., 2003). When an Arabidopsis leaf that had been infiltrated with SOSG was wounded by crimping and then kept in the dark for 30 min, considerable SOSG fluorescence was observed, which was absent from the dark control (Fig. 10A). If the leaf was kept in growth light after wounding an increase in the extent of $^1\text{O}_2$ was generally observed (Fig. 10A). Wounding of a celery leaf in the dark also resulted in the production of $^1\text{O}_2$ around the area of damage (Fig. 10B). It has been speculated that $^1\text{O}_2$ produced by plants in response to pathogen attack may result from the activation of a plasma membrane NADPH oxidase (Apel and Hirt, 2004), since NADPH-oxidase in mammalian cells can produce $^1\text{O}_2$ in the dark (Steinbeck et al., 1992; Klotz et al., 2003). This suggestion is supported by the observation that Cle, an elicitor found in fungal cell walls triggers the activation of a plasma membrane NADPH oxidase and production of $^1\text{O}_2$ in cultured cells of ginseng (Xu et al., 2005). However, oxidation of linoleic acid by soybean lipoxygenases has been shown to be accompanied by $^1\text{O}_2$ production (Kanofsky and Axelrod, 1986). Consequently, $^1\text{O}_2$ production that accompanies wounding could also

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**Fig. 9.** Light-dependent production of SOSG fluorescence in the Arabidopsis flu mutant. Leaves of the flu mutant, which had been grown in continuous light, were placed in the dark for 16 h and then infiltrated with SOSG (dark). These leaves were then transferred from the dark into a PPFD of 150 $\mu$mol m$^{-2}$ s$^{-1}$ for 2 h (dark/light). Leaves that had been kept in continuous light and infiltrated with SOSG in the light are also shown (continuous light).

**Fig. 10.** Production of SOSG fluorescence in leaves in the dark in response to wounding. (A) Arabidopsis leaves after infiltration with SOSG were wounded and either kept in the dark (dark) or at a PPFD of 150 $\mu$mol m$^{-2}$ s$^{-1}$ (low light) for 30 min. White arrows indicate the points at which leaves were wounded. A control leaf that had not been wounded and kept in the dark is also shown (dark control). (B) A celery leaf after infiltration with SOSG was wounded and kept in the dark for 20 min (SOSG). A similar leaf that had not been infiltrated with SOSG, was similarly wounded and kept in the dark for 20 min is also shown (no SOSG control). White arrows indicate the points at which leaves were wounded.
result from the self-reaction of peroxy radicals which are produced by lipoxygenase activity (Halliwell, 1991).

Using a range of conditions known to result in the production of $^1\text{O}_2$, these studies have demonstrated that SOSC can be used effectively to detect the production of $^1\text{O}_2$ in diatoms and leaves.

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