

FEATURED ARTICLE

TECHNICAL ADVANCE

Using spontaneous photon emission to image lipid oxidation patterns in plant tissues

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SUMMARY

Plants, like almost all living organisms, spontaneously emit photons of visible light. We used a highly sensitive, low-noise cooled charge coupled device camera to image spontaneous photon emission (autoluminescence) of plants. Oxidative stress and wounding induced a long-lasting enhancement of plant autoluminescence, the origin of which is investigated here. This long-lived phenomenon can be distinguished from the short-lived chlorophyll luminescence resulting from charge recombinations within the photosystems by pre-adapting the plant to darkness for about 2 h. Lipids in solvent were found to emit a persistent luminescence after oxidation *in vitro*, which exhibited the same time and temperature dependence as plant autoluminescence. Other biological molecules, such as DNA or proteins, either did not produce measurable light upon oxidation or they did produce a chemiluminescence that decayed rapidly, which excludes their significant contribution to the *in vivo* light emission signal. Selective manipulation of the lipid oxidation levels in *Arabidopsis* mutants affected in lipid hydroperoxide metabolism revealed a causal link between leaf autoluminescence and lipid oxidation. Addition of chlorophyll to oxidized lipids enhanced light emission. Both oxidized lipids and plants predominantly emit light at wavelengths higher than 600 nm; the emission spectrum of plant autoluminescence was shifted towards even higher wavelengths, a phenomenon ascribable to chlorophyll molecules acting as luminescence enhancers *in vivo*. Taken together, the presented results show that spontaneous photon emission imaged in plants mainly emanates from oxidized lipids. Imaging of this signal thus provides a simple and sensitive non-invasive method to selectively visualize and map patterns of lipid oxidation in plants.

Keywords: lipid peroxidation, imaging, spontaneous photon emission, biophoton, oxidative stress.

INTRODUCTION

Living organisms, including plants, spontaneously emit light. This spontaneous photon emission, also called ultra-weak photon emission, biophoton emission or autoluminescence, occurs at wavelengths from visible to near infrared, without any external excitation or administration of chemiluminescence agents (Abeles, 1986; Devaraj *et al.*, 1997; Havaux, 2003). It has an extremely low intensity, up to

some hundred of photons per second, and is invisible to the naked eye. The mechanism of emission is believed to be the generation of metastable excited states resulting from oxidative metabolic reactions (Duran and Cadenas, 1987), which closely relates spontaneous photon emission to the oxidation status of the organism. Spontaneous chemiluminescence is distinct from the bioluminescence phenomena

observed, for example, in fireflies and luminescent bacteria, jellyfish or fish. Bioluminescent organisms generate luminescence through an enzymatic reaction system (luciferin–luciferase) that has a high quantum yield (Wilson and Hastings, 1998). The intensity of bioluminescence is about three orders of magnitude higher than that of spontaneous photon emission.

Due to its extremely weak intensity, the recording and analysis of autoluminescence requires sensitive instrumentation. In general, photomultiplier tubes and photon counting are employed for this purpose, and spontaneous photon emission has been measured in a variety of plant samples, including roots (Mathew and Roy, 1992; Makino *et al.*, 1996; Hossu *et al.*, 2010), leaves (Havaux, 2003; Kobayashi *et al.*, 2007; Winkler *et al.*, 2009), seeds (Suzuki *et al.*, 1991; Ohya *et al.*, 2002) and isolated cells and organelles (Hideg and Inaba, 1991a; Rastogi and Pospisil, 2010). The signal was found to be strikingly amplified by a large range of conditions that induce reactive oxygen species (ROS) and oxidative stress, such as light stress (Havaux, 2003), hydrogen peroxide treatment (Rastogi and Pospisil, 2007), heavy metal stress (Collin *et al.*, 2007), pathogen infection (Makino *et al.*, 1996; Bennett *et al.*, 2005; Kobayashi *et al.*, 2007), freezing (Mathew and Roy, 1992), drought (Ohya *et al.*, 2002) and herbicide treatment (Hideg and Inaba, 1991b). The responsiveness of spontaneous photon emission to ROS-generating conditions led to the idea that this signal constitutes an internal marker of oxidative stress and has many potential applications.

It is generally assumed that one of the possible sources of spontaneous photon emission from living organisms is lipid peroxidation (Abeles, 1986; Sies, 1987; Halliwell and Gutteridge, 1989; Havaux, 2003). During peroxidation of membrane lipids, different side-reactions can lead to the formation of light-emitting compounds, especially triplet carbonyls and singlet oxygen ($^1\text{O}_2$). The latter molecule is produced through reactions between two lipid peroxy radicals, while the bimolecular reaction of alkoxy radicals and the cleavage of dioxetanes lead to the formation of excited triplet carbonyls. However, the involvement of those reactions in spontaneous photon emission *in vivo* is hypothetical, being mainly based on correlations observed between autoluminescence intensity and various biochemical indices of lipid peroxidation. Direct evidence for the implication of lipid peroxidation in the spontaneous photon emission of plants is still lacking.

Analysis of the spatial distribution of ultraweak spontaneous luminescence was shown to be achievable using highly sensitive imaging systems, such as two-dimensional photon counting tubes (Suzuki *et al.*, 1991; Makino *et al.*, 1996; Kobayashi *et al.*, 1999; Ohya *et al.*, 2002). However, most images obtained using such an approach have a rather low spatial resolution. More recently, highly sensitive charge-coupled-device (CCD) cameras have been used to improve autoluminescence imaging in plants and in other organisms

including humans (e.g. Takeda *et al.*, 2004; Van Wijk *et al.*, 2006; Kobayashi *et al.*, 2009). To achieve a satisfactory signal-to-noise ratio, and hence to enable measurements of faint light by signal integration, cooling of the sensor is required, using thermoelectric modules or liquid N_2 . For instance, using a cooled CCD detector coupled to optical fibers, Flor-Henry *et al.* (2004) imaged wound-induced ultraweak photon emission from detached Arabidopsis leaves and tentatively interpreted this phenomenon in terms of lipid peroxidation products. Using a liquid N_2 -cooled CCD camera, the intensity of photon emission was correlated with the sensitivity of Arabidopsis mutants to photooxidative stress, to the formation of malondialdehyde, a secondary end-product of lipid peroxidation, and to the levels of hydroxy fatty acids (Havaux *et al.*, 2006; Johnson *et al.*, 2007). The hypersensitive reaction of plants to a pathogen was also imaged with CCD image sensors and correlated with the production of ROS and NO (Bennett *et al.*, 2005; Kobayashi *et al.*, 2007). Although those previous studies did not investigate in detail the origin of ultraweak spontaneous photon emission, they demonstrate the great potential of this signal for the non-invasive visualization of oxidative stress patterns in plants.

Current methods available for imaging oxidative stress in plants involve the use of fluorescent probes sensitive to $^1\text{O}_2$ and/or reduced forms of oxygen. This fluorescence imaging approach can provide useful information on specific ROS and on their sites of production in plant tissues (Fryer *et al.*, 2002; Hideg, 2008). Another strategy involves pairing of an imaging reporter gene with a complementary imaging agent to visualize the expression of ROS-induced gene expression (Grant *et al.*, 2000; Fryer *et al.*, 2002; Kimura *et al.*, 2003; Baruah *et al.*, 2009). For instance, the fusion of the luciferase open reading frame and the promoter of a gene known to be selectively activated by $^1\text{O}_2$ was used to monitor changes in gene expression by $^1\text{O}_2$ in luciferin-sprayed Arabidopsis plantlets (Baruah *et al.*, 2009). However, luciferase imaging can allow the formation of specific ROS to be detected, but it cannot be used for the purpose of ROS quantification. A new approach based on redox-sensitive green fluorescent proteins (GFPs) has recently emerged for non-invasive monitoring of redox changes in living cells (Jiang *et al.*, 2006; Schwarzlander *et al.*, 2008; Rosenwasser *et al.*, 2010). Oxidation of cysteine residues close to the chromophore causes changes in the GFP fluorescence properties, thus allowing visualization of the oxidation of specific proteins or organelles. All those imaging methods have their own merits and demerits, but none of them is directly related to oxidative damage and a fortiori to lipid peroxidation.

Here, we characterize the ultraweak luminescence signal imaged in plants pre-exposed to oxidative stress or to wounding using a liquid N_2 -cooled CCD camera, and we compare it with chemiluminescence images obtained from biological molecules oxidized *in vitro*. We have also analyzed mutant plants that are affected in lipid hydroperoxide

metabolism. The results show that lipid oxidation is the major mechanism involved in the autoluminescence signal emitted *in vivo* by plant leaves. Consequently, the present study validates and illustrates the use of plant autoluminescence imaging as a sensitive method for visualizing lipid peroxidation patterns.

RESULTS AND DISCUSSION

Imaging plant autoluminescence with a CCD camera

The photons spontaneously emitted by a control Arabidopsis plant placed in complete darkness in a dark cabinet were recorded for 20 min with a liquid N₂-cooled CCD camera. Subsequently, luminescence intensity declined, and no signal could be detected with our imaging system after 2 h in the dark (Figure 1c). Figure 1(e) shows that the decay of leaf autoluminescence was rather fast, with an almost complete extinction of the signal within 20 min (i.e. after

recording the first image). In contrast, a persistent emission of photons, which lasted for at least several hours, was measured in the leaves of Arabidopsis plants pre-exposed to a photooxidative stress treatment (Figure 1c,e). Some of the light-emitting leaves exhibited visual symptoms of oxidative stress, such as chlorophyll bleaching and loss of turgor (Figure 1a), confirming that the long-lasting spontaneous photon emission of plants originates from light-emitting species produced during oxidation reactions.

The luminescence imaged immediately after transfer from light to darkness is attributable to chlorophyll luminescence, as illustrated by the absence of photon emission from non-green sectors of variegated leaves (Figure 1d). The chlorophyll luminescence of photosynthetic systems, also called delayed chlorophyll fluorescence, is light emission by chlorophyll molecules secondarily excited as a result of charge recombinations in photosystem-II reaction centers (Lavorel, 1975), the decay of which comprises several phases with lifetimes ranging from nanoseconds to seconds (Goltsev *et al.*, 2005). Another, relatively slower phenomenon exists, the so-called afterglow chlorophyll luminescence, which lasts several tens of seconds and which is due to back electron transfers from reducing compounds in the stroma of chloroplasts to the plastoquinone pool in thylakoids (Ducruet *et al.*, 2005). Thus, the lifetime of delayed chlorophyll fluorescence and afterglow chlorophyll luminescence is consistent with the fast decay rate of leaf autoluminescence measured in Arabidopsis plants with our imaging system. Moreover, when photosystem II was destroyed by exposing leaves to high temperature (15 min at 65°C), no luminescence could be imaged following transfer from light to darkness (Figure 1d), confirming that functional photosystems are required for the short-lived luminescence signal to occur. Images taken from control leaves after prolonged dark adaptation (2 h) did not reveal any significant light emission, indicating that the sensitivity of our CCD-based imaging system is not sufficient to monitor the extremely weak chemiluminescence associated with redox reactions mediated by the mitochondrial and plastidial respiration pathways (Hideg, 1993). Moreover, we exposed the chloroplastic NAD(P)H dehydrogenase (NDH)-deficient Arabidopsis mutant *ndho*, in which the afterglow chlorophyll luminescence is strongly inhibited (Havaux *et al.*, 2005), to photooxidative stress, and no significant difference was observed in the intensity of the long-lasting luminescence between the mutant and the wild type (WT) (data not shown). This observation allows us to exclude the participation of the afterglow chlorophyll luminescence in the photon emission measured after prolonged dark adaptation of plants pre-exposed to oxidative stress. Hereafter, autoluminescence images of plants were systematically recorded after 2 h of dark adaptation in order to eliminate interference of the short-lived chlorophyll luminescence

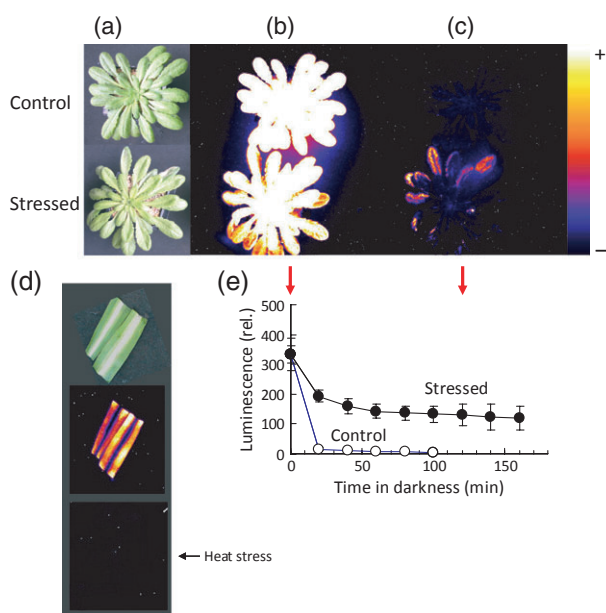


Figure 1. Spontaneous photon emission from control and light-stressed Arabidopsis plants.

(a) Photograph of control and light-stressed Arabidopsis plants. Stressed plants were exposed to high light at low temperature ($1400 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$ at 7°C for 2 days).

(b) Autoluminescence image of the Arabidopsis plants shown in (a) taken immediately after transfer to darkness. Acquisition time 20 min.

(c) Autoluminescence image of plants taken 2 h after transfer to darkness. Acquisition time: 20 min. The color scale indicates signal intensity from 0 (blue) to saturation (white). The same scale was used for (b) and (c) so that the initial luminescence (b) was saturating and thus appeared white.

(d) Luminescence emission from variegated *Tradescantia* leaves taken immediately after transfer to darkness before (middle) and after a heat stress treatment (15 min at 65°C) (bottom). Acquisition time: 20 min. Top: photograph of the leaves.

(e) Plot of the autoluminescence signal intensity emitted by control and stressed Arabidopsis leaves as a function of the time in darkness. Time 0 corresponds to the time at which the plants were placed in the dark cabinet.

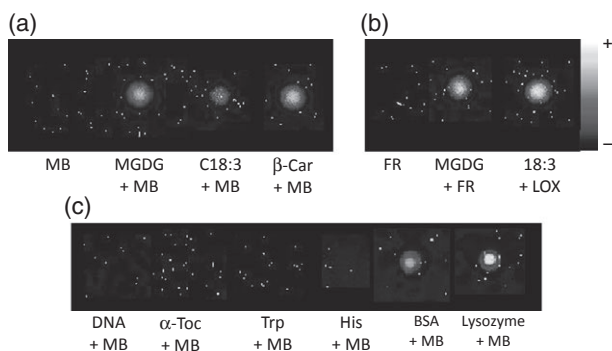


Figure 2. Chemiluminescence of oxidized biological molecules. (a) Luminescence of various lipids oxidized by singlet oxygen ($^1\text{O}_2$): monogalactosyldiacylglycerol (MGDG), linolenic acid (C18:3), β -carotene. Oxidation was induced by illumination for 20 min in the presence of methylene blue (MB), and chemiluminescence was measured immediately after the treatment. Lipid chemiluminescence before oxidation was very low, representing <10% of the luminescence intensity of oxidized lipids. For instance, chemiluminescence before and after oxidation was 7 ± 3 and 101 ± 6 for C18:3, 2 ± 1 and 146 ± 3 (arbitrary units) for β -carotene, respectively. (b) Luminescence of MGDG oxidized by free radicals (FR) induced by the combination of H_2O_2 and iron, and luminescence generated by the enzymatic oxidation of linolenic acid by soybean lipoxygenase (LOX). Linolenic acid was incubated with LOX for 2 h. Luminescence was measured immediately after the LOX and FR treatments. (c) Luminescence of DNA, α -tocopherol (α -Toc), tryptophan (Trp), histidine (His), bovine serum albumin (BSA) and lysozyme oxidized by singlet oxygen produced by MB and light. Luminescence was measured immediately after the treatments. The randomly distributed white spots represent cosmic ray-induced background noise. Acquisition time was 20 min for all experiments. Data are representative images of at least four separate experiments.

with the longer-lived luminescence associated with oxidative stress.

Chemiluminescence of oxidized biological molecules

A major source of light-emitting molecules during oxidative stress is believed to be lipid peroxidation (Abeles, 1986; Sies, 1987; Havaux, 2003), although protein and amino acid oxidation has also been reported to generate chemiluminescence (Barnard *et al.*, 1993). To explore this idea, we screened a range of biological molecules for light emission after oxidation. Light emission from all tested molecules was extremely weak and hardly measurable before oxidation (data not shown). Similarly, methylene blue, a $^1\text{O}_2$ generator that we used to oxidize biomolecules *in vitro*, did not emit luminescence after illumination (Figure 2a). In contrast, lipidic molecules, such as the galactolipid monogalactosyldiacylglycerol (MGDG), the fatty acid linolenic acid and the carotenoid β -carotene, became luminous after oxidation by $^1\text{O}_2$ (Figure 2a). Chemiluminescence was also observed when lipid oxidation was induced by free radicals (generated by the combination of hydrogen peroxide and iron; the Fenton reaction) instead of $^1\text{O}_2$ or when oxidized products were generated enzymatically by the reaction of lipoxygenase (LOX) on linolenic acid (Figure 2b). Luminescence images were also recorded after *in vitro*

oxidation of other types of (macro)molecules, such as tocopherol, DNA, aromatic amino acids and proteins (Figure 2c), which are known to be cellular targets of ROS (Ravanat *et al.*, 2001; Davies, 2003; Kobayashi and DellaPenna, 2008). In particular, tryptophan and histidine have been shown to be highly reactive to $^1\text{O}_2$, bringing about the production of light-emitting intermediates (Barnard *et al.*, 1993; Pollet *et al.*, 1998; Alarcon *et al.*, 2007). However, none of those compounds were found to produce luminescence measurable with our CCD camera. As far as aromatic amino acids are concerned, the absence of measurable luminescence is likely to be due to the short lifetime (<2 min) of their chemiluminescence following reaction with $^1\text{O}_2$ (Alarcon *et al.*, 2007). Oxidized proteins rather than oxidized free amino acids emit chemiluminescence with a more complex and significantly slower decay (Alarcon *et al.*, 2007). Accordingly, oxidation of proteins, such as bovine serum albumin (BSA) and lysozyme, was associated with a marked increase in luminescence (Figure 2c). This finding raises the question that concerns the extent of participation of oxidized proteins in the *in vivo* autoluminescence signal of plants.

Common features of plant autoluminescence and chemiluminescence of oxidized lipids

When oxidized by $^1\text{O}_2$, linolenic acid emitted chemiluminescence that declined by about 50% during the first hour of darkness, followed by a much slower intensity decrease that had a half-time of about 200 min (Figure 3a). A similar kinetic was observed for leaf autoluminescence. In contrast, the decline of chemiluminescence from oxidized lysozyme (or oxidized BSA, not shown) was much faster, with an almost complete loss of photon emission after 100 min. The half time of the protein chemiluminescence decrease was about 20 min. Chemiluminescence associated with reaction between proteins, including BSA and lysozyme, and $^1\text{O}_2$ has been previously reported to decay with a half-time of several minutes (Alarcon *et al.*, 2007), in line with the rapid loss of oxidized protein chemiluminescence recorded with our imaging system.

The persistence of the luminescence signal generated by the oxidation of lipid molecules suggests that the chemical species responsible for light emission are continuously produced by a slow spontaneous decomposition of lipid oxidation products, which appeared to proceed at a much slower rate than for emission by oxidized proteins. Lipid peroxide decomposition is strongly enhanced in alkaline media. Using HPLC, we checked that hydroperoxy fatty acids generated by the enzymatic oxidation of linolenic acid were degraded when incubated for 30 min at high pH (approximately 12.5) (data not shown). Consistently, the chemiluminescence of oxidized linolenic acid was strongly amplified by increasing pH (Figure 3b): increasing pH from 7.5 to 9 or to 12.5 enhanced luminescence by a factor of about 5 or 50, respectively.

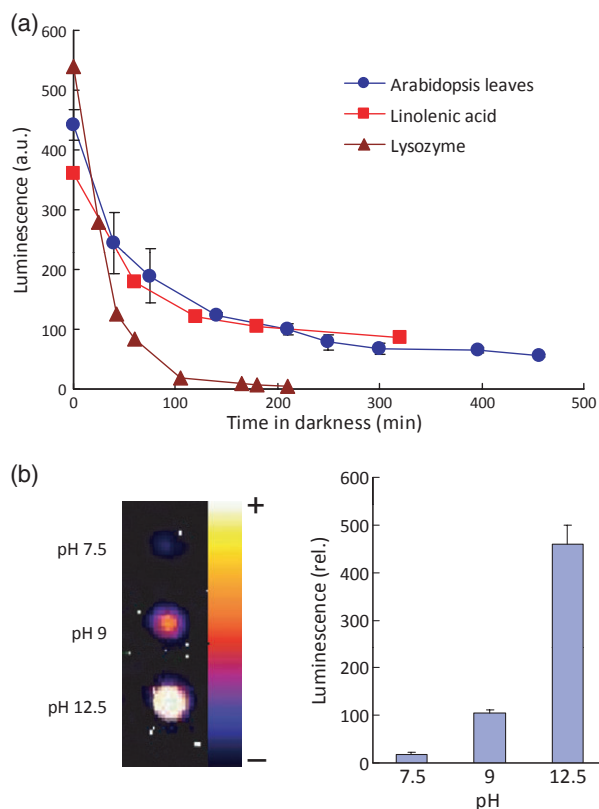


Figure 3. Comparison between chemiluminescence of oxidized macromolecules and plant autoluminescence. (a) Plot of the intensity of the luminescence of oxidized linolenic acid (C18:3) or lysozyme and the autoluminescence of an Arabidopsis plant versus time in darkness. Linolenic acid and lysozyme were oxidized by singlet oxygen ($^1\text{O}_2$) generated by methylene blue and light. The Arabidopsis plant was pre-exposed to photooxidative stress ($1400 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$ at 7°C). (b) Luminescence of oxidized linolenic acid at three different pHs. Different amounts of 5 N NaOH were added to the linolenic acid solution previously oxidized by $^1\text{O}_2$ generated by illumination in the presence of methylene blue. Data are mean values of five separate measurements \pm SD.

The decay of protein and lipid peroxides is influenced by temperature, with low temperature decreasing the rate of decay (Gebicki and Gebicki, 1993). Similarly, luminescence emission imaged from oxidized fatty acid showed a marked dependence on temperature: heat ($>40^\circ\text{C}$) strongly stimulated chemiluminescence while photon emission was almost suppressed at low temperature (4°C) (Figure 4a). The same phenomena were observed in leaves (Figure 4b). Luminescence from oxidized proteins was also dependent on temperature. However, although low temperature reduced light emission by oxidized proteins, a significant luminescence was still measured at 4°C (Figure 4c), in contrast with oxidized linolenic acid.

From our results, we can conclude that lipid oxidation can lead to intermediates whose slow decomposition can produce long-lasting luminescence. Thus, similarities between features of luminescence emitted by oxidized lipids and

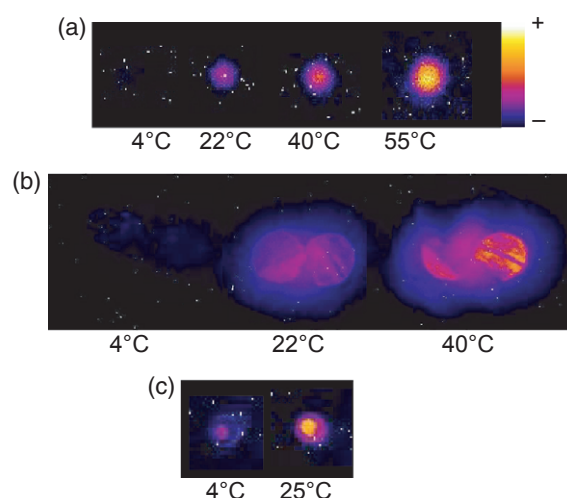


Figure 4. Temperature dependence of spontaneous photon emission *in vitro* and *in vivo*.

Effect of temperature on (a) the luminescence of oxidized linolenic acid (18:3), (b) the autoluminescence of leaf disks taken from Arabidopsis plants pre-stressed for 2 days in high light at low temperature ($1700 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$ at 7°C) and (c) lysozyme. Linolenic acid and lysozyme were oxidized by singlet oxygen produced by methylene blue in the light (20-min exposure), and chemiluminescence was measured immediately after the treatment. Data are representative images of three separate experiments.

by plants point to oxidized lipids as contributors to plant autoluminescence. Obviously, amino acid and protein oxidation products are less stable than oxidized lipids, leading to a rapid decomposition and loss of photon emission. Therefore, when the luminescence images are recorded after a 2-h delay in darkness (as done in Figure 1c), it is unlikely that protein oxidation contributes significantly to the leaf autoluminescence signal. Other cellular targets of ROS, such as DNA for example, either did not produce light upon oxidation or did generate luminous intermediates but with a very short lifetime, so that their radiative decomposition could not be monitored by our luminescence imaging method. Accordingly, Watts *et al.* (1995) were unable to detect light emission from isolated DNA or nucleotides subjected to oxidation. Tocopherols are major $^1\text{O}_2$ quenchers *in vivo*, but the only oxidation product of tocopherol found in light-stressed leaves was α -tocopherol-quinone (Kobayashi and DellaPenna, 2008), which is not expected to be a light-emitting species.

Autoluminescence of mutants affected in lipid hydroperoxide metabolism

Oxidation of lipids into lipid hydroperoxides can occur in plants either via non-enzymatic (ROS-induced) or enzymatic (LOX-mediated) pathways (Feussner and Wasternack, 2002).

Wounding treatment. When wounded, plant leaves spontaneously emit photons (Suzuki *et al.*, 1991; Flor-Henry

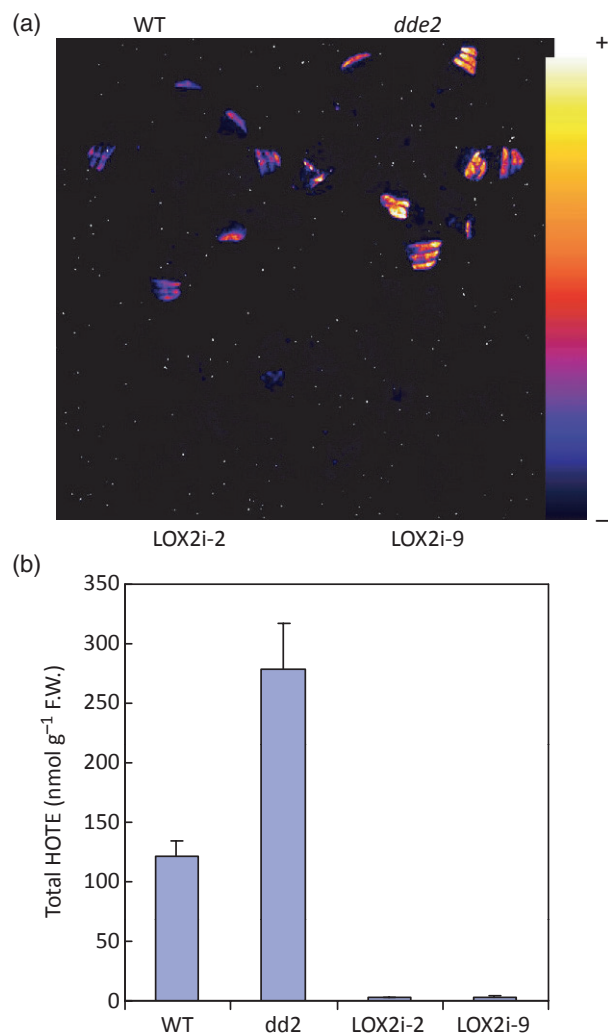


Figure 5. Arabidopsis leaf autoluminescence induced by wounding. (a) Autoluminescence imaging of Arabidopsis plants induced by leaf wounding (three wounds per leaf). The image was taken 2 h after wounding (acquisition time, 20 min). Wild-type (WT) Arabidopsis plants are compared with *dde2* mutant plants and LOX2-RNA interference (RNAi) plants (LOX2i-2 and LOX2i-9). (b) Accumulation of total hydroxy octadecatrienoic acid (HOTE) in WT, *dde2* and LOX2-RNAi Arabidopsis leaves induced by wounding. Leaves were harvested 2 h after wounding. Data are mean values of three separate experiments + SD.

et al., 2004; Winkler *et al.*, 2009). This was confirmed in this study using Arabidopsis plants (Figure 5a). The wounds were luminous, and the luminescence emission appeared to be restricted to the injured tissues and did not propagate to the intact parts of the leaves. A number of key genes are induced upon wounding treatment, including *AtLOX2*, the gene encoding the chloroplastic lipoxygenase 2 (Park *et al.*, 2002; Glauser *et al.*, 2009). Indeed, leaf wounding initiates the LOX pathway and enhances lipid metabolism leading to the production of hydroxy-fatty acids and resulting in jasmonate (Gfeller *et al.*, 2010). Accordingly, wounded Arabidopsis plants accumulated six times more hydroxy fatty

acids [hydroxy octadecatrienoic acid (HOTE), approximately 120 nmol g⁻¹; Figure 5b] compared with control leaves (approximately 20 nmol g⁻¹, data not shown; see also Levesque-Tremblay *et al.*, 2009). The accumulation of HOTES is directly and exclusively attributable to the enzymatic activity of LOX since this phenomenon was cancelled in RNA-interference mutant plants (LOX2i) deficient in the LOX2 lipoxygenase (Seltmann *et al.*, 2010) (Figure 5b). The latter enzyme catalyzes the oxygenation of linolenic acid to 13-hydroperoxylinolenic acid. As shown here, this enzyme is entirely responsible for the accumulation of 13-LOX products in Arabidopsis leaves after wounding. Concomitantly, no luminescence could be measured in the LOX2i wounded mutant leaves, indicating the involvement of enzymatic rather than ROS-induced oxidation of lipids in the autoluminescence of wounded leaves. A previous study (Aoshima *et al.*, 2003) corroborated our data as the addition of LOX on sweet potato slices reinstated photon emission.

We then studied a mutant (*dde2*) incapable of hydroxy-fatty acid metabolism. This mutant is deficient in the *ALLENE OXIDE SYNTHASE* gene encoding a key enzyme of jasmonic acid biosynthesis (von Malek *et al.*, 2002). Allene oxide synthase converts 13-hydroperoxylinolenic acid to an unstable epoxide which is further catabolized to 12-oxo-phytodienoic acid (OPDA), the first biologically active compound of the jasmonate pathway. Because the *dde2* mutant is affected in the metabolism of oxidized lipids, when wounded it accumulated much higher amounts of HOTES than the WT (Figure 5b). Increased levels of oxidized lipids were associated with enhanced autoluminescence intensity (Figure 5a). Thus, selective manipulation of oxidized lipid levels by the *dde2* mutation and the LOX2 RNA interference (RNAi) demonstrates the link between leaf autoluminescence intensity and lipid oxidation status.

Photooxidative stress. Another condition that can cause the accumulation of metabolites generated by the LOX pathway is high light (Montillet *et al.*, 2004). We exposed Arabidopsis plants to a photooxidative stress treatment using a combination of high light and low temperature. As previously shown in Figure 1(c), this treatment led to an increased autoluminescence of plants (Figure 6a) which was accompanied by an accumulation of the total HOTES in leaves (Figure 6b). Light-induced oxidative stress was heterogeneous, affecting mainly the tip and the margins of the leaves. Again, the LOX2i mutants responded very differently from WT plants: the hydroxy fatty acid levels in whole plants after high light stress, exclusively due to ROS-induced lipid peroxidation, remained low compared with WT levels resulting from both ROS-induced lipid peroxidation and LOX-mediated lipid peroxidation. As shown in Figure 6(a), the reduced accumulation of lipid peroxidation products in the LOX2i mutant was accompanied by a decrease in plant autoluminescence. Compared with the WT, the number of

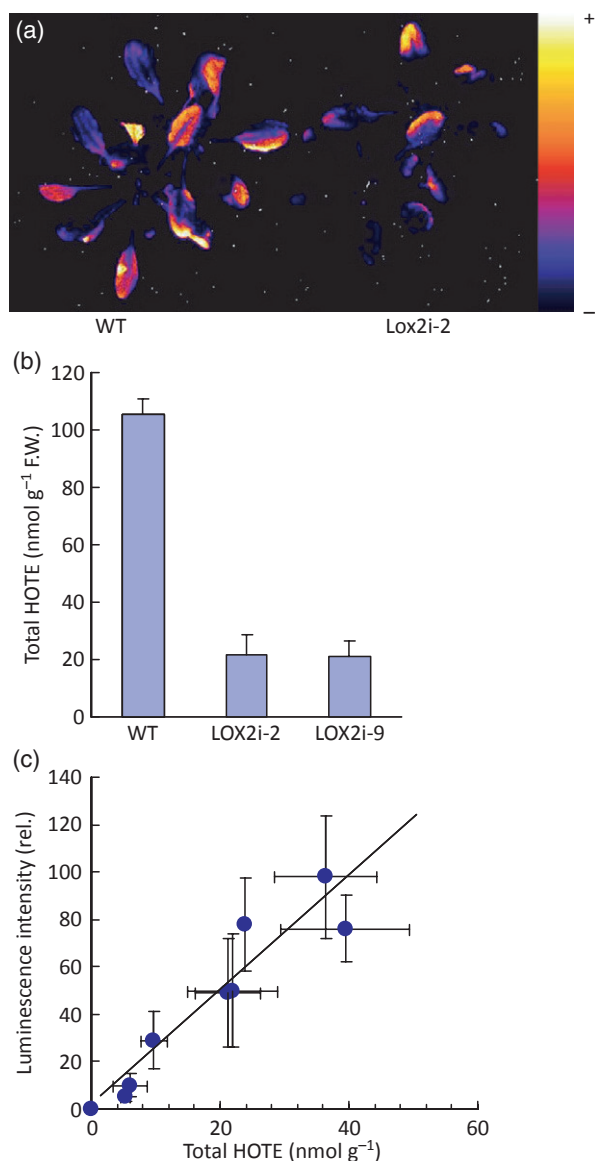


Figure 6. Arabidopsis leaf autoluminescence induced by photooxidative stress. (a) Autoluminescence imaging of Arabidopsis plants after high light stress at low temperature ($1400 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$ at 7°C). Two wild-type (WT) Arabidopsis plants are compared with two LOX2-RNA interference (RNAi) plants (LOX2i-2). (b) Accumulation of total hydroxy octadecatrienoic acid (HOTE) in leaves of high-light stressed WT and LOX2-RNAi Arabidopsis plants. Data are mean values of three separate experiments \pm SD. (c) Correlation between autoluminescence intensity and hydroxy fatty acid (HOTE) content of leaves of Arabidopsis plants (WT and LOX2i mutant) exposed to photooxidative stress ($1400 \mu\text{mol photons m}^{-2} \text{sec}^{-1}$ at 7°C) for different times. Data are mean values of three and six separate measurements (for HOTE and luminescence, respectively) \pm SD.

LOX2-deficient leaves exhibiting an increase in autoluminescence intensity was much lower. Under photooxidative stress, it is clear that various biomolecules besides lipids can be oxidized, including proteins (Davies, 2003). However, even in this case, a correlation was obtained between lipid

peroxidation levels and leaf autoluminescence levels, as also illustrated in Figure 6(c) where we compiled the data from WT and mutant leaves exposed to different photooxidative stress treatments. The results obtained with the LOX2-deficient mutants indicate that *in planta* lipid oxidation does generate photons, and, in the case of wounding, the selective oxidation of lipids by LOX activity is the exclusive source of photon emission. Taken together, the *in vitro* and *in vivo* experiments identify lipids as a major source of persistent photon emission by plants after oxidative stress. Consequently, we can consider that CCD imaging of spontaneous photon emission, as performed in this study, provides a useful monitoring method for lipid peroxidation that accompanied the generation of ROS in living tissues or cells.

Spectral characteristics of *in vitro* and *in vivo* spontaneous photon emission

Chemiluminescence images of oxidized lipids were recorded in different spectral ranges using colored glass filters. Oxidation of linolenic acid produced mainly red light ($>600 \text{ nm}$), with a major contribution in the wavelength range $640\text{--}695 \text{ nm}$ (Figure 7a). Nevertheless, some light emission was found below 600 nm , representing $<20\%$ of the total emission. Light emission by oxidized proteins also occurred in the red and blue/green wavelength domains. However, compared to lipids, emission in the blue/green spectral region appeared to be enhanced relative to the emission at wavelengths $>640 \text{ nm}$, reaching approximately 50% of total emission (Figure S1 in Supporting Information), in agreement with previous data showing that protein chemiluminescence occurs predominantly between 490 and 580 nm (Pollet *et al.*, 1998; Alarcon *et al.*, 2007). The wavelength dependence shown in Figure 7(a) is consistent with the emission spectrum of autooxidized oil (Miyazawa *et al.*, 1982) or the enzymatic oxidation of linoleic acid by LOX (Hideg, 1993). Chemiluminescence associated with lipid peroxidation is usually attributed to excited carbonyls and $^1\text{O}_2$, which are both by-products of the decomposition of lipid peroxides and endoperoxides (Sies, 1987; Havaux, 2003). Reactions leading to those light-emitting compounds are dioxetane breakdown and self-reactions between lipid peroxy and alkoxy radicals. Upon relaxation, excited carbonyls emit light in the blue wavelength region ($350\text{--}480 \text{ nm}$) while bimolecular emission of $^1\text{O}_2$ occurs in the red region ($703\text{--}706$ and 640 nm) (Duran and Cadenas, 1987). The spectral characteristics of the imaged chemiluminescence of oxidized lipids are compatible with the supposed contribution of excited carbonyls and $^1\text{O}_2$ (Figure 7a). The involvement of the dimol luminescence of $^1\text{O}_2$ is further supported by the inhibiting effect of the $^1\text{O}_2$ quencher, NaN_3 , on the luminescence intensity of oxidized linolenic acid solutions (Figure S2). The contribution of excited carbonyls to the chemiluminescence of oxidized lipids seems to be rather

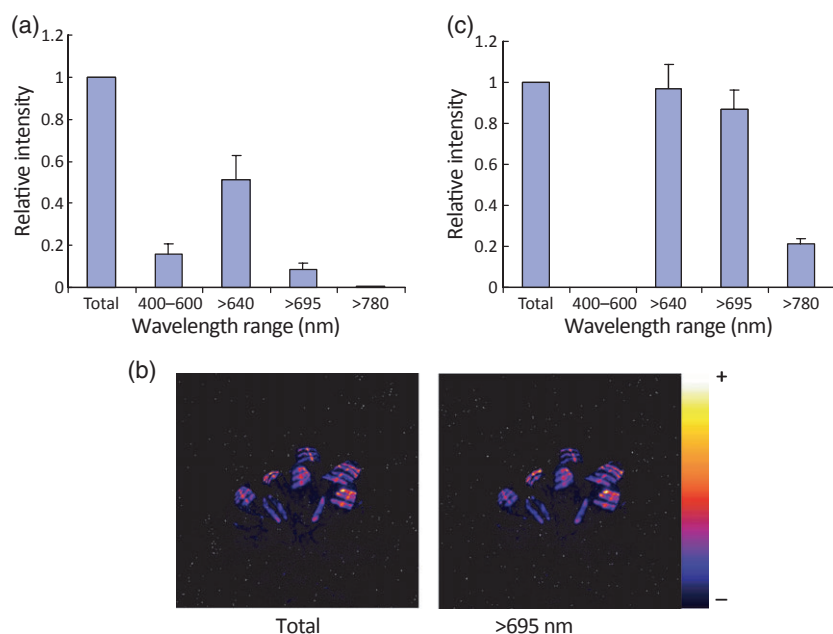


Figure 7. Spectral dependence of chemiluminescence emission from oxidized lipids and of leaf autoluminescence.

(a) Relative luminescence intensity of oxidized linolenic acid in different wavelength regions: 400–600 nm, >640 nm, >695 nm, >780 nm.

(b) Autoluminescence images of wounded plants measured without an optical filter or measured in the red spectral region (>695 nm).

(c) Relative autoluminescence intensity of wounded leaves in different wavelength ranges: 400–600 nm, >640 nm, >695 nm, >780 nm. 'Total' means that no filter was placed in front of the camera. Data are mean values of four separate experiments +SD.

low (Figure 7a), but we cannot exclude a more significant contribution to leaf autoluminescence through efficient transfer of excitation energy to chlorophylls (see below).

Leaf autoluminescence induced by wounding was also found to occur in the red spectral domain (>640 nm) (Figure 7b,c). However, the emission appeared to be shifted towards higher wavelengths compared with oxidized lipid chemiluminescence, since almost 100% of the signal was emitted above 695 nm and about 20% above 780 nm. In contrast to oxidized fatty acid solutions, no leaf autoluminescence could be measured at wavelengths <600 nm. Thus, although photon emission induced upon wounding resulted from enzymatic oxidation of lipids (Figure 5), the luminescence recorded *in fine* had a spectrum that was distorted compared with the luminescence spectrum of oxidized lipids. Similar emission spectra, centered on the far-red wavelength region of the visible spectrum, were previously measured in leaves during pathogen infection (Kobayashi *et al.*, 2007) or wounding (Flor-Henry *et al.*, 2004).

The range of wavelengths (640–780 nm) at which leaf autoluminescence was observed to occur resembled the spectrum of *in vivo* chlorophyll fluorescence (see, e.g., Ilik *et al.*, 2000). Transfer of excitation from triplet carbonyl species to chlorophyll has been reported *in vitro* (Brunetti *et al.*, 1983; Bohne *et al.*, 1986), and it has been suggested in the literature that chlorophyll could be the final emitter of the autoluminescence signal of chlorophyll-containing materials (Hideg, 1993). Moreover, the absorption spectrum of chlorophyll coincides with the emission spectrum of oxidized lipids, particularly in the red domain, so that chlorophyll molecules can act as an absorbing screen. As shown in Figure 8(a), the addition of chlorophyll *a* to linolenic acid

after *in vitro* oxidation by $^1\text{O}_2$ was found to strongly enhance the luminescence emission. In Figure 8(b), wounding-induced luminescence was analyzed in green and non-green leaf sectors of the *Arabidopsis thf1* mutant. The latter mutant is affected in chloroplast biogenesis, leading to variegated leaves containing white sectors deficient in chlorophyll (Wang *et al.*, 2004). Autoluminescence was observed in both white and green sectors, indicating that chlorophyll is not indispensable for *in vivo* spontaneous photon emission from leaves. Moreover, this is also illustrated by the fact that oxidative stress-related luminescence was imaged in various organisms or organs which do not contain chlorophyll, such as non-photosynthetic bacteria and plant seeds (see below). Autoluminescence intensity was higher in green sectors relative to non-green sectors, consistent with an amplifying effect of chlorophyll. However, we cannot exclude a differential LOX activity or fatty acid composition between green and non-green leaf sectors which could generate a differential accumulation of lipid oxidation products. Even in the non-green leaf sectors, no blue–green light emission (400–600 nm) could be measured (Figure 8c). Non-photosynthetic materials, such as plant roots treated with hydrogen peroxide, emit light in the same wavelength range with no contribution of wavelengths <500 nm (Rastogi and Pospisil, 2010). This suggests that chlorophyll is probably not the exclusive factor responsible for the absence of leaf autoluminescence in this wavelength range. For instance, leaf epidermis is known to contain various UV- and blue light-absorbing compounds (Hutzler *et al.*, 1998) which can absorb photons at wavelengths below 500 nm (Merzlyak *et al.*, 2008). Nevertheless, upon wounding, the luminescence spectrum from non-green leaf sectors is distinguished from green sectors by the fact that its

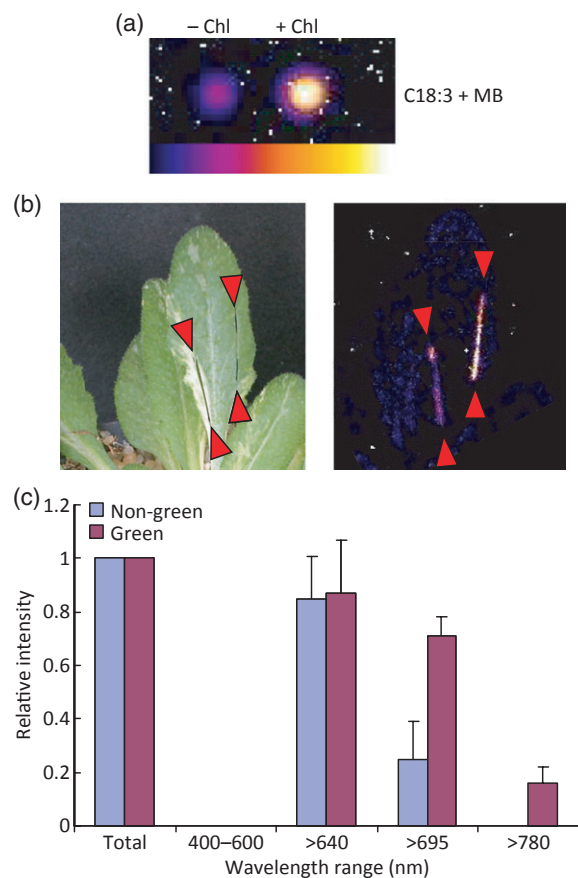


Figure 8. Effects of chlorophyll (Chl) on spontaneous photon emission. (a) Enhancement of the luminescence of oxidized linolenic acid by the addition of chlorophyll *a*. (b) Wounding-induced luminescence in variegated leaves of the *thf1* Arabidopsis mutant. Green and non-green sectors of a leaf were injured with a scalpel. The red triangles indicate the position of the wounds. (c) Spectral dependence of the wound-induced luminescence of green and non-green leaf sectors of *thf1* mutant leaves. 'Total' means that no filter was placed in front of the camera. Data are mean values of four separate measurements \pm SD.

emission occurred mainly in the 640–695 nm wavelength domain (Figure 8c), as was the case for lipids (Figure 7a). As compared with green leaf sectors, autoluminescence of non-green sectors was low at wavelengths above 695 nm. This supports the idea that the upward shift of the spontaneous photon emission spectrum of leaves is related to the presence of chlorophyll molecules in the tissues. The extent of this shift is likely to be dependent on the environment of the oxidized lipids, especially the chlorophyll molecules that can come into close proximity to allow excitation transfer.

Spontaneous light emission from various types of organisms or organs

As shown in a number of previous studies, spontaneous photon emission is not restricted to chlorophyll-containing tissues. In Figure 9, we measured spontaneous light emis-

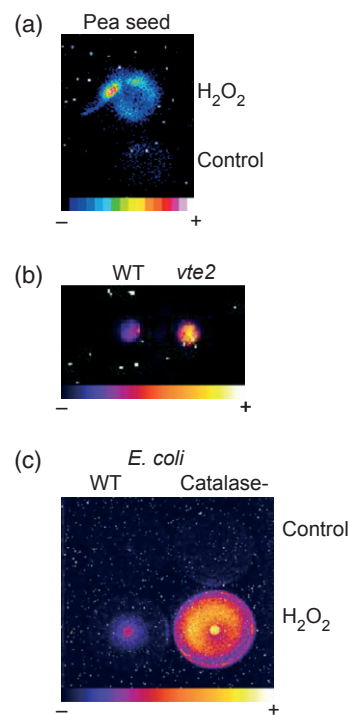


Figure 9. Autoluminescence of different organisms/organs exposed to oxidative stress.

(a) Germinating pea seed treated with H_2O_2 and iron. The radicle of the germinating seed was plunged in 30% H_2O_2 + iron for 1 h before imaging. (b) Arabidopsis seeds upon imbibition. No luminescence was measured before imbibition (not shown). (c) *Escherichia coli* cells [wild type (WT) and catalase mutant] exposed to H_2O_2 . H_2O_2 (30%) was added to a pastille placed at the center of the Petri dish.

sion in various biological samples different from plant leaves. For instance, autoluminescence was measured in germinating pea seeds exposed to hydrogen peroxide: although the whole seed became luminescent, maximal emission was visible at the basis of the root tip (Figure 9a). Similarly, luminescence was induced in Arabidopsis seeds upon imbibition. Interestingly, this light emission was significantly enhanced in seeds of the vitamin E-deficient mutant *vte2* as compared with WT seeds (Figure 9b). The *vte2* mutant accumulated high levels of lipid hydroperoxides relative to WT during germination and early seedling growth (Sattler *et al.*, 2004).

No luminescence could be measured from the bacterium *Escherichia coli* grown on gelose on Petri dishes (Figure 9c). However, when challenged with hydrogen peroxide (Figure 9c), *E. coli* became luminous, and this phenomenon was strongly enhanced in a catalase mutant. This confirms, in bacteria, the link between spontaneous photon emission and oxidative stress.

CONCLUSIONS

Lipid oxidation brings about the formation of peroxides that generate light-emitting species upon decomposition, and

the present study shows that this phenomenon can be imaged *in vitro* and *in vivo* with a high-sensitivity CCD camera. Moreover, our study demonstrates that lipid peroxidation constitutes the main source of spontaneous light emission by plants under oxidative stress. Lipid peroxide decomposition is probably very slow *in planta* since the spontaneous photoemission of plants persists for relatively long periods of time, exhibiting limited decrease on the hour time scale. Oxidation of other major molecular targets of ROS, such as proteins or DNA, either did not produce measurable light emission or it led to a transient emission of photons but with a considerably shorter lifetime compared with ultraweak plant photon emission. Therefore, we can exclude the contribution of these molecules to plant light emission and consider that autoluminescence imaging as performed here provides a new and useful technique to specifically monitor and visualize *in vivo* oxidation patterns of lipids rather than other chemical compounds. As shown in the present study using luminescence imaging, and also in previous studies that used other experimental detection systems (Singh *et al.*, 1996; Albertini and Abuja, 1998; Vavilin and Ducruet, 1998), ultraweak photon emission by plants and other biological samples is well correlated with the extent of lipid peroxidation measured by biochemical analyses. Moreover, luminescence imaging with a cooled CCD camera provides an excellent sensitivity, inherent in light detection techniques, and appears to be an appropriate technique for analyzing the spatiotemporal dynamics of lipid peroxidation and real-time monitoring of oxidative stress.

The imaging method presented in this study is applicable to a variety of samples, including bacteria. One of the main advantages of this method is its simplicity of use as it does not require any sample preparation. As far as chlorophyll-containing materials are concerned, the unique requirement is a pre-adaptation to darkness before recording an image in order to eliminate chlorophyll luminescence. Dark adaptation is unnecessary for non-photosynthetic organisms or organs. Because autoluminescence imaging provides biological information without external perturbations or exogenously applied probes, it is expected to produce realistic mapping of lipid peroxidative damage and oxidative stress in plants. To the best of our knowledge, the only existing alternative to directly image lipid peroxidation is based on the probe diphenyl-1-pyrenylphosphine (DPPP) that exhibits increased fluorescence emission when oxidized by lipid hydroperoxides (Okimoto *et al.*, 2000; Matot *et al.*, 2003). Alternatively, luminescent probes which emit photons when exposed to oxidizing agents (e.g. luminol) or by accepting an excitation energy from an excited product (e.g. coumarin) can also be used to image lipid peroxidation (Vladimirov and Proskurnina, 2009), but unfortunately they are not specific to lipid oxidation products. Moreover, these approaches require pre-infiltration of biological tissues with an exogenous probe and therefore they have been applied so far

to cells or organs only (Takahashi *et al.*, 2001; Matot *et al.*, 2003). In addition, the infiltration of living tissues with a chemical probe can have perturbing effects and the distribution of the fluorescent or luminescent probe within the sample can be heterogeneous, which complicates the interpretation of data.

We believe that CCD imaging of ultraweak spontaneous photon emission has the potential to become an important tool in stress physiology for the non-perturbing measurement of the lipid oxidation status of plants and other living organisms. This method is useful not only for quantification purposes but also for an early detection of oxidative stress before damage becomes visible. Clearly, a field that has much to gain from this technique is phytopathology. Induction of LOX activity constitutes one of the primary events in the defense response of plants against pathogen infection, leading to the accumulation of hydroxy fatty acids and derived oxylipins (Feussner and Wasternack, 2002), and this phenomenon could be monitored by the associated luminescence emission. Because of its high sensitivity and ease of use, spontaneous photon emission imaging is also applicable to automated screening tests for tolerance to oxidative stress and for lipid peroxidative destruction. For screening purposes, spatial resolution can be reduced (by pixel binning) in favor of the rapidity of the measurements. The sensitive equipment required for acquiring images of ultraweak light is relatively expensive, but the technique could become more affordable by using a less sensitive, hence cheaper, camera and by stimulating autoluminescence emission by warming, as exemplified in Figure 4. A previous study has shown that ultraweak spontaneous photon emission and high temperature-induced luminescence are basically the same phenomenon (Havaux *et al.*, 2006). We predict that this new and rather simple imaging technique that specifically detects lipid peroxides will complement in the future the panoply of functional imaging tools that are currently available to plant biologists.

EXPERIMENTAL PROCEDURES

Plant material

Arabidopsis plants (*Arabidopsis thaliana*, ecotype Col-0) were grown in a phytotron under controlled environmental conditions: photon flux density (PFD) 200–250 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$; photoperiod 8 h; air temperature 23°C/18°C (day/night). The following mutants were analyzed: the variegated *thf1* mutant affected in chloroplast biogenesis (Wang *et al.*, 2004), the allene oxide synthase-deficient mutant *dde2* (von Malek *et al.*, 2002) and the lipoxigenase 2 RNAi lines LOX2i-2 and LOX2i-9 (Seltmann *et al.*, 2010). Photooxidative stress was induced by transferring plants to high PFD (1500 $\mu\text{mol photons m}^{-2} \text{sec}^{-1}$; photoperiod 8 h) at low temperature (7°C/12°C, day/night). Leaves were mechanically wounded using tweezers; three parallel wounds were made on attached leaves. After wounding, plants were transferred to the dark for 2 h. Then, luminescence was imaged and wounded leaves were harvested, frozen in liquid N₂ and stored at –80°C before analyses.

For comparison purposes, we imaged the luminescence emission from germinating pea seeds (*Pisum sativum*), imbibed Arabidopsis seeds, variegated leaves (*Tradescantia fluminensis*) and cultures of the bacterium *E. coli* on Petri dishes.

In vitro oxidation of biological molecules

Monogalactosyldiacylglycerol (in chloroform), linolenic acid (in ethanol/water), β -carotene (in chloroform/acetone), DNA from salmon testes (in water), tryptophan and histidine (in water), BSA (in water), lysozyme (in water) and tocopherol (in methanol) were supplemented with methylene blue (at a final concentration of 0.1 mM). Oxidation of those compounds by singlet oxygen was induced by exposing the mixtures to white light (PFD 400 $\mu\text{mol photons m}^{-2} \text{ sec}^{-1}$) at 7°C for 20 min. Oxidation of MGDG by free radicals was done by incubating the lipid with H_2O_2 and iron (FeCl_2) (Fenton reaction) for 10 min. Luminescence was measured immediately after the oxidation treatments (acquisition time, 20 min). Five hundred microliters of soybean LOX at a concentration of 3 mg ml^{-1} in 100 mM borate buffer at pH 9 was added to 500 μl linolenic acid (5 mg ml^{-1} in 100 mM borate buffer at pH 9), and luminescence was measured after 2-h incubation of the mixture at room temperature ($\sim 23^\circ\text{C}$).

Luminescence imaging

Luminescence emission from plants, leaf disks, liquid solutions or bacterial cell cultures on Petri dishes was imaged using a highly sensitive, liquid N_2 -cooled CCD camera (VersArray 1300B; Roper Scientific, <http://www.roperscientific.com/>). The camera is mounted with a back-illuminated CCD (CCD36-40; e2v Technologies, <http://www.e2v.com/>) with a 1340×1300 imaging array showing a quantum efficiency (QE) peaking at 560 nm (QE = 0.93) and higher than 0.75 between 425 and 770 nm. Above 770 nm, the sensitivity of the sensor monotonously declines reaching a QE of 0.1 at approximately 1000 nm. The sensor operates at a temperature of -110°C . The sample was imaged on the sensor by a 50-mm focal distance lens with an f -number of 1.2 (F mount Nikkor 50-mm, $f:1.2$; Nikon, <http://www.nikon.com/>) for maximizing light collection. The camera was mounted on a laboratory-built black box and was placed in a dark-room to avoid contamination by external photons. In general, acquisition time was 20 min, and on-CCD binning of 2×2 was used to increase detection sensitivity, so that the resulting resolution was 650×670 pixels. For the imaging of oxidized molecules in solution and bacteria, binning was 4×4 and 5×5 , and acquisition time was 20 and 40 min, respectively. A filter holder was attached to the camera lens, and colored glass filters were used to analyze the spectral characteristics of the measured signal: red and far-red long-pass filters, Corion LG640 (3 mm thick), Schott RG695 (6 mm thick) and Schott RG780 (3 mm thick) showing 50% transmittance at around 640, 695 and 780 nm, respectively; a blue-green bandpass Schott BG 18 filter (thickness, 3 mm) with cut-on and cut-off wavelengths at around 400 and 600 nm, respectively. We checked that those filters were not luminescent.

Hydroxy fatty acid determination

Hydroxy fatty acid (HOTE) analyses were performed using the HPLC method developed by Montillet *et al.* (2004), as illustrated in previous papers (e.g. Levesque-Tremblay *et al.*, 2009).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Spectral dependence of the chemiluminescence emission of lysozyme oxidized by singlet oxygen.

Figure S2. Effect of sodium azide (NaN_3) on the luminescence of linolenic acid oxidized by lipoxygenase (LOX).

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