Lipoxygenase in singlet oxygen generation as a response to wounding: *in vivo* imaging in *Arabidopsis thaliana*

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Wounding, one of the most intensive stresses influencing plants ontogeny and lifespan, can be induced by herbivory as well as by physical factors. Reactive oxygen species play indispensable role both in the local and systemic defense reactions which enable “reprogramming” of metabolic pathways to set new boundaries and physiological equilibrium suitable for survival. In our current study, we provide experimental evidence on the formation of singlet oxygen (1\textsuperscript{O}_2) after wounding of Arabidopsis leaves. It is shown that 1\textsuperscript{O}_2 is formed by triplet-triplet energy transfer from triplet carbonyls to molecular oxygen. Using lipoxygenase inhibitor catechol, it is demonstrated that lipid peroxidation is initiated by lipoxygenase. Suppression of 1\textsuperscript{O}_2 formation in lox2 mutant which lacks chloroplast lipoxygenase indicates that lipoxygenase localized in chloroplast is predominantly responsible for 1\textsuperscript{O}_2 formation. Interestingly, 1\textsuperscript{O}_2 formation is solely restricted to chloroplasts localized at the wounding site. Data presented in this study might provide novel insight into wound-induced signaling in the local defense reaction.

Various factors are known to affect deleteriously the growth and development in plants1–3. Wounding can be related to both biotic and abiotic stresses, as it is caused either by herbivory or plants exposure to environmental mechanical injury4–6. Physiological responses of plants to wounding have been categorized based on their timing or spatial distribution. Local responses includes oxidative burst linked with cell wall reorganization or cell death7, 8 while systemic response, imply activation of defense related genes9, deposition of callose, accumulation of defensive proteins (mostly with enzymatic activity) and lectins10, 11. These damage-induced changes are mediated by complex signaling networks, which include receptors, calcium (Ca\textsuperscript{2+}) influx, ATP release, kinase cascades, reactive oxygen species (ROS), reactive nitrogen species12 and oxylipin signaling pathways8, 13, 14. Detailed studies revealed that mechanical injury is tightly linked with variation in electric potential15, 16 and chemical signals such as terpenes, methyl salicylate, methyl benzoate, ethylene, and especially jasmonic acid16.

Evidence has been provided that wounding leads to the release of polyunsaturated fatty acids from the cell membranes and the accumulation of unsaturated fatty acid at the site of wounding17. The activation of phospholipases/other lipases is associated with the release of fatty acids from cell membrane18. The released polyunsaturated fatty acid has been known to act as a substrate for lipoxygenase leading to the production of hydroperoxy polyunsaturated fatty acids (lipid hydroperoxide, LOOH)19. This enzyme produces precursors for several compounds important for defense reactions, including the plant hormone jasmonic acid20, 21. Plant lipoxygenase or linoleate:oxygen 13-oxidoreductase (EC 1.13.11.12) present in different isoforms22 is a non-heme iron containing dioxygenase which catalyzes the addition of molecular oxygen to polyunsaturated fatty acid to produce a polyunsaturated fatty acid hydroperoxide. The oxygenation reaction comprises of hydrogen abstraction, radical rearrangement, oxygen insertion and proton addition to polyunsaturated fatty acid23. The phospholipase and lipoxygenase activity is categorized as the local response (also referred to as immediate or fast response) which occurs within minutes of wounding in plants which is then followed by the systemic response (also referred to as delayed response) including activation of genes related to phospholipase and lipoxygenase24.

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Under reducing conditions, LOOH is reduced by transition metals to lipid alkoxyl radical (LO•) which might further abstract hydrogen from another polyunsaturated fatty acid forming another lipid radical (L•) and hydroxy polyunsaturated fatty acids (lipid hydroxide, LOH)\(^2\). Under oxidizing condition, LOOH is oxidized to lipid peroxyl radical (LOO•) by oxidized transition metals, ferric heme iron of cytochrome c, peroxyxinitrite, chloroperoxide, and hypochlorous acid. The cyclization of LOO• is known to form a cyclic endoperoxide (dioxetane), whereas recombination of the two LOO• forms a linear tetroxide \(^2\)\(^6\), \(^2\)\(^7\). These high energy intermediates decompose to triplet carbonyls (3L=O\(^*\)) which might transfer triplet energy either to pigments forming excited pigments or molecular oxygen forming singlet oxygen (1O\(_2\)). In addition, tetroxide might decompose directly to 1O\(_2\) by Russell mechanism\(^2\)\(^8\)–\(^3\)\(^0\) (Fig. 1).

Singlet oxygen imaging with SOSG showed that 1O\(_2\) is formed in the wounded Arabidopsis leaves\(^3\)\(^1\). The authors proposed that 1O\(_2\) formation is accompanied by recombination of two LOO\(^*\) via Russell mechanisms formed during enzymatic lipid peroxidation. Nevertheless, no experimental data supporting this proposal have been published yet. In this study, we provide experimental evidence on the role of lipoxygenase in 1O\(_2\) formation after wounding of Arabidopsis plants. Formation of 1L=O monitored by ultra-weak photon emission and 1O\(_2\) detected by the Singlet Oxygen Sensor Green (SOSG) fluorescence assessed by a laser confocal scanning microscopy was studied in lox2 mutant, which lacks chloroplast lipoygenase. Our data revealed that in wounded Arabidopsis plants, the lipoxygenase plays a key role in the formation of 1L=O and 1O\(_2\).

Material and Methods

Chemical Reagents. All chemicals were purchased of analytical grade from Sigma Aldrich GmbH (Germany) and Molecular Probes Inc. (Eugene, OR, USA).

Arabidopsis plants. *Arabidopsis thaliana* WT (Columbia-0) and lox2 mutant lacking chloroplast LOX2 were obtained from Nottingham Arabidopsis Stock Centre\(^2\)\(^2\), University of Nottingham (Loughborough, United Kingdom). Plants were potted in growing pots with a peat substrate (Klasmann, Potground H) after 4 days of...
soaking in distilled water. The plants were grown at a photoperiod of 16 h light/8 h dark (photon flux density 100 µmol photons m⁻² s⁻¹) for 6 weeks at a temperature of 25 °C and at 60% relative humidity. The wounding of plant leaves was performed under diffused green light using sharp blade while any external mechanical pressure was avoided at other parts on the surface of plant leaves. Measurement was performed 20 min after wounding.

**Charge coupled device imaging.**  Highly sensitive CCD camera VersArray 1300B (Princeton instruments, Trenton, NJ, USA) was used for two-dimensional photon imaging. Dark current of the CCD camera was achieved by cooling it down to −110 °C using a liquid-nitrogen cooling system. Spectral sensitivity of the CCD camera was within the range of 350–1000 nm. The quantum efficiency was almost 90% in the visible range of the spectrum. The measurement was done in the image format of 1340 × 1300 pixels and the data correction was done by subtracting the background noise from every measurement. The following CCD camera parameters were used: scan rate, 100 kHz; gain, 2 and accumulation time of 20 min, based on the parameters described in Prasad et al. The CCD camera was situated in a black box located in an experimental dark room with an approximate dimension of 3 m × 1.5 m × 5 m. To avoid any possible interference by external light, the data recording computer was installed in the outer dark room. Prior to the two-dimensional ultra-weak photon emission measurements, the Arabidopsis plant was dark-incubated for approximately 2 h duration to prevent any intervention of delayed luminescence. The data accumulation from Arabidopsis plants and leaves were started 20 min after wounding.

**Confocal laser scanning microscopy.**  Imaging of ¹O₂ was achieved using Singlet oxygen sensor green (SOSG). Wounding of Arabidopsis leaves was exerted by a sharp razor blade used to cut ca 5 × 5 mm pieces from marginal leaf lamina while avoiding other mechanical injury. These cuts were incubated in the presence of 50 µM SOSG for 30 min, washed in 40 mM HEPES buffer (pH 7.5) and forthwith visualized by Fluorview 1000 confocal laser scanning microscope (Olympus Czech Group, Prague, Czech Republic). The excitation was achieved by a 488 nm line of an argon laser and the emission recorded using a 505–525 nm band-pass filter. Negative controls were treated with 5 mM catechol during the staining procedure. Integral distribution of fluorescence signal intensity corresponding to singlet oxygen localization within figures was visualized in FV10-ASW 4.0 Viewer software (Olympus).

**Results**

**Triplet carbonyl formation in WT Arabidopsis monitored by ultra-weak photon emission.**  Formation of ³L→O⁻ in WT Arabidopsis plants subjected to wounding was monitored by two-dimensional imaging of ultra-weak photon emission. It is well established that singlet chlorophylls (‘Chl’) contribute predominately to photon emission in plant tissue. As ‘Chl’ is formed solely by excitation energy transfer from ³L→O⁻ to chlorophylls, ultra-weak photon emission might serve as an indirect indicator of ³L→O⁻ formation. Figure 2 shows a photograph (A) and two-dimensional image of ultra-weak photon emission (B) measured in non-wounded and wounded leaves. Spontaneous ultra-weak photon emission observed from the non-wounded area of Arabidopsis plants is caused by oxidative metabolic processes. The wounding of Arabidopsis plant (Fig. 2A, red circles; Supplementary data 1) resulted in the enhancement of ultra-weak photon emission from the cut edges of the leaves which are caused by wound-induced oxidative processes (Fig. 2B, red circles). The spatial profile of photon emission shows a higher photon emission at the injured part (Fig. 2C). This observation indicates that wounding of Arabidopsis plants results in ³L→O⁻ formation restricted solely to the wounded areas of plant. To verify the contribution of the propagative reaction and associated ultra-weak photon emission, we have measured photon emission up to 2 h after wounding (Supplementary data 2). It can be observed that the ultra-weak photon emission as a response to wounding last in the scale of hours. Thus, it can be hypothesized that the ultra-weak photon emission originates at the initial stage due to burst of oxidative reactions after injury and propagative reaction continuing up to few hours.

**Effect of catechol on triplet carbonyl formation in WT Arabidopsis.**  To study the involvement of lipoxigenase in ³L→O⁻ formation, the effect of lipoxigenase inhibitor catechol on ultra-weak photon emission was studied in WT Arabidopsis leaves. It is well established that binding of catechol to the ferric non-heme iron of lipoxigenase leads to inactivation of the enzyme active site. Figure 2 shows a photograph (D) and two-dimensional imaging of ultra-weak photon emission (E) measured in wounded Arabidopsis leaves in the absence (left) and presence (right) of catechol. The topical application of catechol on the wounded areas of the Arabidopsis leaves suppressed significantly ultra-weak photon emission (Fig. 2E and F). The spatial profile of photon emission measured in the presence of catechol (red dotted rectangle) shows that photon emission was decreased to the level of photon emission observed in the non-wounded site (Fig. 2F). As a control, the exogenous application of catechol (n = 3) was also tested in the non-wounded leaves which showed no changes in ultra-weak photon emission (Supplementary data 3). These results reveal that lipoxigenase is involved in ³L→O⁻ formation in Arabidopsis leaves exposed to wounding.

**Formation of triplet carbonyls in lox2 mutant.**  To further clarify the involvement of lipoxigenase in ³L→O⁻ formation during wounding of Arabidopsis leaves, two-dimensional imaging of ultra-weak photon emission was measured in WT and lox2 mutant lacking chloroplast lipoxigenase LOX2 (Fig. 2G–H; Supplementary data 4). Spontaneous ultra-weak photon emission from non-wounded site of lox2 mutant showed no major difference as compared to WT (Fig. 2H). On the contrary, the ultra-weak photon emission at the site of wounding in lox2 mutant (Fig. 2H, right leaf) was suppressed compared to WT (Fig. 2H, left leaf). The spatial profile of photon emission in lox2 mutant (red dotted rectangle) shows the intensity comparable with non-wounded site while the wounded site in WT shows comparatively higher photon emission (black dotted rectangle) (Fig. 2I). These observations indicate that chloroplast lipoxigenase LOX2 plays a key role in ³L→O⁻ formation in wounded Arabidopsis leaves.
Singlet oxygen formation in WT Arabidopsis detected by confocal laser scanning microscopy. To visualize the formation of $^1\text{O}_2$ in wounded Arabidopsis leaves, we used SOSG which is highly sensitive and specific fluorescent probe for $^1\text{O}_2$. SOSG fluorescence was detected by confocal laser scanning microscopy. In this method, the formation of SOSG endoperoxide by cycloaddition of $^1\text{O}_2$ to SOSG results in the enhancement of SOSG fluorescence. Figure 3 represents the Differential interference contrast; SOSG fluorescence and DIC + fluorescence channel for comparison of tissue/cell details measured at different magnifications (for objectives 10x, 20x and 40x) where the margins indicate the wounding site which reflects higher SOSG fluorescence signal. SOSG fluorescence was pronouncedly higher in the wounded area compared to none or very low SOSG fluorescence in the intact area of Arabidopsis leaf lamina. Highest SOSG fluorescence signal originated mainly from the first layer of cells on the cutting edge (Fig. 3 and Fig. 4I,A–C). However, the number of cell
layers with signal can be influenced by the sharpness of razor blade, i.e. mechanical injury intensity within tissues (Fig. 3, 10X). These observations revealed that wounding in leaves results in \( 1O_2 \) formation at the site of injury restricted predominantly to the first, i.e. most impacted, layer of cells and a limited signal from adjoining cells.

Effect of catechol on singlet oxygen formation in WT Arabidopsis. To confirm the involvement of lipoxygenase in \( 1O_2 \) formation caused by wounding, the effect of catechol on SOSG fluorescence was measured in WT Arabidopsis leaves. Figure 4I shows the Nomarski DIC (D), SOSG fluorescence (E) and SOSG intensity (F) measured in wounded Arabidopsis leaves in the presence of catechol. Topical application of catechol on the wounded area of Arabidopsis leaves caused significant suppression of SOSG fluorescence (Fig. 4I,E) as compared to non-catechol treated Arabidopsis leaves (Fig. 4I,B). The distribution of SOSG fluorescence intensity reveals that SOSG fluorescence in wounded Arabidopsis leaf (Fig. 4I,C) was lower as compared to wounded WT Arabidopsis leaf (Fig. 4I–C). Based on these results, it is concluded that lipoxygenase is involved in the formation of \( 1O_2 \) in Arabidopsis leaves under wounding.

Singlet oxygen formation in lox2 mutant. To identify the involvement of lipoxygenase in the formation of \( 1O_2 \) during wounding in Arabidopsis leaves, SOSG fluorescence was measured in lox2 mutant. Negligible SOSG fluorescence was observed in lox2 mutant at the cut edges of the wounded Arabidopsis leaves (Fig. 4I–H). The distribution of SOSG fluorescence intensity shows that SOSG fluorescence in wounded leaf of lox2 mutant (Fig. 4I–I) is pronouncedly lower as compared to wounded WT Arabidopsis leaf (Fig. 4I–C). These observations reveal that chloroplast lipoxygenase LOX2 play a key role in \( 1O_2 \) formation during wounding in plants. The negligible SOSG fluorescence in few numbers of cells on the cut edge is believed to be contributed by the lipoxygenase located within the cell other than the chloroplasts. Supplementary data 5 and Fig. 4II shows the intensity of SOSG

Figure 3. SOSG fluorescence imaging in cells of WT Arabidopsis leaves detected by confocal laser scanning microscope. The left panel represents the DIC, the middle panel represents the SOSG fluorescence and the right panel represents DIC + fluorescence channel following 30 min of incubation in SOSG measured at different magnifications (40x, 20x, 10x) where the margins indicate the wounding site. The fluorescence signal was measured with an excitation (\( \lambda_{exc} \)) and emission (\( \lambda_{em} \)) wavelengths of 488 nm and 505–525 nm, respectively.
Figure 4. I. SOSG fluorescence imaging in cells of Arabidopsis leaves detected by confocal laser scanning microscope in WT, WT (with catechol) and lox2 mutant. The left panel represents the Nomarski interference contrast; the middle panel represents the SOSG fluorescence and the right panel represents integral distribution SOSG fluorescence intensity following 30 min of incubation in SOSG. The fluorescence signal was measured with an excitation ($\lambda_{ex}$) and emission ($\lambda_{em}$) wavelengths of 488 nm and 505–525 nm, respectively. II. The intensity of the fluorescence signal in SOSG channel of confocal images (800 × 800 pixels, taken under objective magnification 40x) was exported using FV10-ASW 4.0 Viewer software (Olympus). ¼ of the image area was chosen from the cut edge of the leaves (n = 3–5 per each variant) and brightness levels, i.e. values from 0 to 4095, obtained for each of 160 000 px. Following conversion for Microsoft Excel 2010, the data were processed and presented as mean ± standard deviation, completed by maximal signal intensity value.
fluorescence channel of confocal images from non-injured edge and the injured edge of WT Arabidopsis leaves, respectively. The results indicate an enhancement in intensity of SOSG fluorescence by 3 times in wounded edge of Arabidopsis leaf as compared to non-wounded areas of the Arabidopsis leaves. The effect to catechol was observed to suppress the SOSG intensity close to the value comparable to non-injured area of the Arabidopsis leaf.

Effect of desferal and trolox on singlet oxygen formation in WT and lox2 mutant. The effect of Desferal (deferoxamine mesylate), which is an iron chelator that forms nontoxic ferrioxamine; can attenuate iron-induced oxidative stress and also known to interact with LO• was measured in WT and lox2 mutant of Arabidopsis leaves. Figure 5 shows the Nomarski DIC [left panel (A, E); right panel (C, G)] and SOSG fluorescence [left panel (B, F); right panel (D, H)] measured in wounded Arabidopsis leaves in WT and lox2 mutant, respectively. Topical application of desferal on the wounded area of Arabidopsis leaves caused pronounced suppression of SOSG fluorescence in both WT (Fig. 5F) and lox2 mutant (Fig. 5H) as compared to non-desferal treated Arabidopsis leaves (Fig. 5B and D). As a termination agent for lipid peroxidation, effect of trolox (2-carboxy-2,5,7,8-tetramethyl-6-chromanol) which is a water soluble analogue of vitamin E was measured in WT and lox2 mutant of Arabidopsis leaves. Topical application of trolox on the wounded area of Arabidopsis leaves caused complete suppression of SOSG fluorescence in both WT (Fig. 5J) and lox2 mutant (Fig. 5L) as compared to non-trolox treated Arabidopsis leaves (Fig. 5B and D).

In addition, ultra-weak photon emission imaging was measured in non-wounded (right leaves of the panel) and wounded (left leaves of the panel) of WT and lox2 Arabidopsis leaves, respectively in the presence of desferal and trolox showing pronounced suppression in ultra-weak photon emission (Supplementary data 6). Based on these results, it is concluded that inhibition of the propagation and termination step of lipid peroxidation can lead finally to negligible 1O2 generation.
Localization of singlet oxygen formation in WT Arabidopsis. Figure 6 shows the distribution of SOSG fluorescence within the cells at the edges and provides clear evidence indicating the generation of \( ^1O_2 \) localized predominantly in the chloroplasts. A short videosequence representing Z-stack (2x focusing up and down through the sample) has been presented (Supplementary data 7).

Discussion

In plants, local response to wounding comprises of oxidative damage of lipids and proteins at the wounding site, whereas systemic response mediated by hormones such as jasmonic acid, ethylene, salicylic acid, and abscisic acid is widespread over the plant tissue and organs\(^{35} \). In this study, we provided evidence that \( ^3L = O^* \) formed during lipid peroxidation results in \( ^1O_2 \) formation in the local response to wounding.

Triplet carbonyl formation. It is known that wounding of plant tissue is accompanied by oxidative damage of lipids and proteins\(^{11} \). Two-dimensional imaging of ultra-weak photon emission which is known to be a non-invasive indicator of oxidative stress\(^{36} \) was found to be pronouncedly enhanced at the site of wounded plant tissue (Fig. 2; Supplementary data 1). In agreement with our data, Flor-Henry et al.\(^{37} \) proposed that lipid peroxidation occurs under wounding in detached Arabidopsis leaves using ultra-weak photon emission. More recently, suppression of LOH formation in \( \text{lox2} \) mutant revealed that enzymatic lipid peroxidation is initiated by lipoxygenase\(^{38} \). Oxidative burst characterized by ROS production is known to be generated in plant tissues in response to wounding in plants\(^{17, 39} \). Recent reports on studies involving \( P. sativum \) and other plant models have claimed activation of NADPH oxidase in response to wounding\(^{17, 39–41} \). Evidences have been provided on direct detection of superoxide anion radical (\( O_2^\bullet^- \) ) and hydrogen peroxide (\( H_2O_2 \)) measured during wounding in Arabidopsis leaves as monitored by NBT and DAB staining\(^{42} \). Due to the fact that light enhanced NBT and DAB signals, the authors proposed that \( O_2^\bullet^- \) and \( H_2O_2 \) formation is related to electron transport. In addition, the treatment of Arabidopsis leaves with calcium blockers and calcium chelators after wounding of leaves abolished ROS signal indicating the involvement of calcium in the pathway that couples perception of wounding with the generation of ROS\(^{43} \). It has also been reported that LOX2 can be activated by calcium ion; however, its direct interaction is not sufficiently understood\(^{44–47} \).

Singlet oxygen formation. In vivo imaging of \( ^1O_2 \) using SOSG fluorescence measured by confocal laser scanning microscopy revealed that wounding of Arabidopsis leaves caused \( ^1O_2 \) formation. The observation that lipoxygenase inhibitor catechol completely suppressed \( ^1O_2 \) formation indicates that lipid peroxidation is initiated by lipoxygenase. Suppression of \( ^1O_2 \) formation in \( \text{lox2} \) mutant reveals that lipoxygenase localized in chloroplast is predominantly responsible for \( ^1O_2 \) formation. The observation that \( ^1O_2 \) formation is localized solely at the site of the wounded plant tissue indicates that \( ^1O_2 \) unlikely diffuse to surrounding plant tissue. Under dark conditions, the chloroplasts are known to be situated near the periphery attached to the cell membrane of the cells. In the mechanically injured Arabidopsis leaves, the SOSG fluorescence was observed in the periphery close to the cell membrane indicating the generation of \( ^1O_2 \) localized predominantly in the chloroplasts (Fig. 6). The SOSG fluorescence was observed in layers adjoining the site of mechanical injury indicating that the oxidative radical reaction occurs predominantly close to the site of mechanical injury and that the chain reaction is limited to a close proximity. The termination of chain reaction is likely to occur due to limitation of presence of initiators of the oxidative radical reaction which cannot diffuse to longer distance due to its shorter half-life period. The less probable reason which cannot be neglected completely can be the limited diffusion of the SOSG probe.

Physiological relevance. Based on the results obtained and understanding from our current study, the response of wounding and generation of \( ^1O_2 \) can lead to hypothesis on existence of wound induced signaling pathway mediated by \( ^1O_2 \). The signal observed is predominantly contributed by the chloroplasts which were
found suppressed almost entirely in the lox2 mutant leads to the conclusion that lipoygenase plays a major role in wound-induced $\mathrm{O}_2^-$ production which is in agreement with experiments performed in model system $^{32}$. However, a lower signal as observed using confocal microscopy may indicate the diffusion of $\mathrm{O}_2^-$ to the neighboring cells. The direct contribution of $\mathrm{O}_2^-$ or oxidized biomolecules can thus be hypothesized to play a role in cellular signaling and opens a new perspective in the signaling pathway $^{43}$. It is proposed here that $\mathrm{O}_2^-$ formed during wounding in plants can be involved in oxidation of either lipids or proteins which can act as signaling molecule.

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**Author Contributions**
A.P., P.P. contributed to the conception and design of the work. A.P., P.P. analyzed and interpreted the data. M.S. and R.S.K. participated in experiments. A.P., P.P. drafted the manuscript and all authors approved the final version of the manuscript.

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