

# Membrane lipid peroxidation and its conflict of interest: the two faces of oxidative stress

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**Membranes are the most vital structure for all organisms which not only control molecular trafficking but also perceive environmental cues and transduce it in response. Membrane lipid peroxidation, which is normally associated with natural course of ageing, senescence and environmental stresses, is mechanistically important as it is one of the very few examples of carbon-centered radical production in cell. Chemically, it involves the formation and propagation of lipid radicals, the uptake of molecular oxygen and arrangement of double bonds in the unsaturated lipids and eventually their destruction, with subsequent production of a variety of breakdown products, including alcohol, ketones, alkanes, aldehydes and ethers. The process is considered as the main event involved in oxidative damage to cell, which may eventually cause cell death. A significant proportion of oxidized lipids are electrophilic in nature. Recent studies suggest that reactive lipid species formed through lipid peroxidation can benefit cells in a number of ways. There are strong evidences in support of the view that reactive lipid species-mediated signalling participates in several physiological pathways including apoptosis, induction of antioxidative defence, membrane repair, proteosomal pathway, etc. The activation of cell signalling pathways by reactive lipid species is hierarchical and largely depends on intrinsic chemical reactivity of electrophiles, thiol-containing signalling domains and the subsequent signalling cascades. An effort has been made to provide an update on membrane lipid peroxidation while addressing the conflicting roles of membrane lipid peroxidation in deteriorative oxidative damage and adaptive cell signalling.**

**Keywords:** Adaptive response, cell signalling, membrane lipid peroxidation, oxidative damage, reactive lipid species.

PLASMA membrane senses various environmental stimuli and transduces them to downstream intracellular and intercellular signalling networks. Exposure to both abiotic and biotic stresses causes changes in membrane architecture. In fact, membranes must respond to environmental stresses (extremes of temperatures, drought, salinity,

infection, etc.). It is indeed a matter of great surprise how a fast response to a broad spectrum of different environmental stimuli is being perceived and transduced into response. Probably, the easiest way is to initiate a non-specific response by transformation of cell membrane to signalling compounds with a small time-frame by minute expenditure of energy. Polyunsaturated fatty acids (PUFA), being the most oxygen-sensitive molecules, are ideal compounds to satisfy this condition<sup>1</sup>. All plants contain PUFA in their membranes, which may be stored in the surface of the cell or organelle as free PUFA or remain conjugated as phospholipid, galactolipid or sphingolipid. Peroxidation of membrane lipid, primarily the PUFAs, associated with membrane phospholipid or glycolipids is mechanistically very important from the perspective of production of reactive oxygen species (ROS) and considered as one of the few examples of carbon-centred radical production in cells<sup>2</sup>.

For a long time, peroxidation of membrane lipids, particularly the O<sub>2</sub>-sensitive membrane-associated PUFAs, is considered as the main molecular mechanism involved in oxidative deterioration of the cell architecture which may eventually lead to cell death<sup>3-6</sup>. Redox imbalance under unfavourable environmental conditions, along with high concentrations of PUFAs and the presence of transition metal ions, may be considered as a perfect situation for lipid peroxidation (LPO)<sup>7-9</sup>. In fact, presence of transition metals and high concentration of membrane-associated PUFAs under oxidative stress largely act as trigger to initiate the process of LPO. In plant cells, peroxidation of free fatty acids can occur both in enzymatic and non-enzymatic ways with the generation of a variety of breakdown products, including alcohols, ketones, alkanes, aldehydes and ethers<sup>3</sup>. This broad range of oxidation products is often used as marker of oxidative stress associated with pathogenesis, environmental stress, ageing and senescence<sup>10,11</sup>. LPO products have been detected using an array of techniques, which often shows positive correlation with adverse environmental conditions and pathogenesis<sup>12-14</sup>. Utilization and application of these analytical techniques led to a different concept of reactive lipid species (RLS), which include breakdown products of oxidized lipids that are electrophilic in nature and are capable enough to react with cellular nucleophiles such as amino acids cysteine, lysine, histidine, etc. Several workers

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on this avenue of oxidative stress biology involving RLS strongly suggest that this group of radical species does not simply comprise the byproducts of membrane lipid peroxidation (MLPO) but intermediates in the MLPO pathway under multiple pathophysiological conditions with putative unique attributes. Importantly, several MLPO products are electrophilic; which allows them to form stable covalent adducts with nucleophilic residues on proteins as free amino acids<sup>10,15,16</sup>. This event of RLS addition to nucleophiles is extremely important since the thiol groups associated with proteins or amino acids act as redox-switches controlling cell signalling, metabolism or even gene expression<sup>10,17</sup>. The concept seems to have emerged in recent times where the function of thio-reactive signalling intermediates seems to be modulated with the addition of biologically active electrophile or RLS. Thus, oxidative modification mediated by either H<sub>2</sub>O<sub>2</sub> or lipid peroxidases (breakdown product of MLPO) to sulphinic or sulphenic acids, which were initially being recognized as manifestation of oxidative stress, are not suggesting their role in cell signalling and defence. So, a more refined view of the process of LPO is emerging which suggests that apart from its bonafide role in oxidative deterioration, oxidized lipids can elicit different cellular effects depending on their concentration, reactivity, types and target molecules. In this review an effort has been made to explain the process of LPO in context of its chemistry, mechanisms of formation of RLS through tandem action of enzymatic and nonenzymatic LPO, their involvement in oxidative deterioration of cell and the new emerging role of LPO in cell signalling.

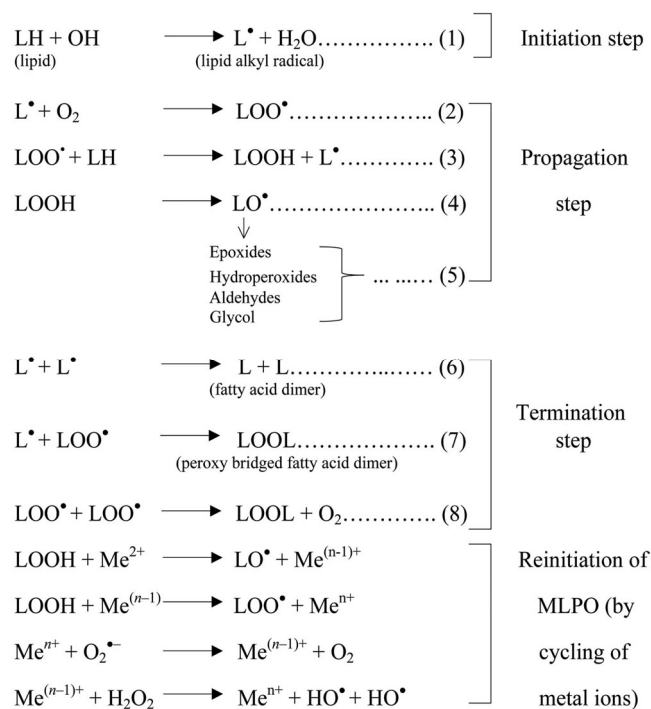
### Chemistry of membrane lipid peroxidation

Peroxidation of membrane lipids is mechanistically important from a free radical production perspective, as it is one of the few examples of carbon-centred radical production in plant cell<sup>18</sup>. MLPO involves three different stages, which include initiation, progression and termination. It is basically a chain reaction which is initiated by hydrogen abstraction (Figure 1, reaction 1) or addition of an oxygen radical, resulting in oxidative breakdown of membrane-associated PUFA. Since PUFAs are the most oxygen-sensitive molecules as compared to saturated fatty acids, it is obvious that activated methylene (LH) bridge represents a critical target site and the initiation process is usually stimulated by radical species with sufficient reactivity. In the propagation step, molecular oxygen rapidly adds on to carbon-centred radical (L•), formed by initiation process, yielding peroxy radical (LOO•) (Figure 1, reactions 2–5). Formation of LOO• leads to production of organic hydroperoxides, which in turn can abstract H from PUFA (analogous to initiation reaction, Figure 1). This step is termed as propagation, implying that initiation of one might result in the

conversion of numerous PUFAs to lipid hydroperoxides and subsequently can generate alkyl, peroxy and alkoxy radicals.

Finally, the alkyl radical may be stabilized by rearrangement into conjugated diene (fatty acid dimer, Figure 1), which is relatively a stable product of LPO. So, lipid hydroperoxides are the first stable product of LPO. However, the situation where LPO is continuously initiated (under severe oxidative stress), radical termination occurs with the destruction of two radicals (Figure 1, reactions 6–8), forming peroxy-bridged dimers.

Transition elements or metal complexes, especially Fe and Cu, have profound influence on the initiation event of LPO. The role of these metal complexes lies in the fact that either they form an activated oxygen complex that can also abstract allelic hydrogens or as a catalyst in the decomposition of existing lipid hydroperoxides. ROS such as O<sub>2</sub><sup>•-</sup> and H<sub>2</sub>O<sub>2</sub> are capable to initiate LPO, but OH• being extremely reactive, the initiation process is mainly mediated by OH•. Loosely bound Fe is also able to catalyse the decomposition of lipid hydroperoxides resulting in the formation of alkoxy and peroxy radicals, which may further stimulate chain reaction (Figure 1). It is also likely that the physical structure of plant membranes which places the fatty acid side chains in close proximity facilitates autocatalytic propagation of LPO. As a transition metal that can exist in several valences which can be bound up to six ligands, Fe is an important



**Figure 1.** Chemistry of MLPO showing initiation, progression and termination steps and mechanism of re-initiation of LPO by redox cycling of metal ions (details in the text).

component for redox reaction<sup>19</sup>. There are several corroborative facts on the role of transition metal in LPO. Some transition metal ions such as Fe, Pb, Cd and Cr can generate LPO effect in the *in vitro* condition<sup>20</sup>. The Fenton reaction occurs *in vitro* at a very slow pace and hence cannot contribute substantial formation of OH<sup>•</sup> in the cellular system. However, in presence of metal ions, OH<sup>•</sup> can be found through these reactions (Figure 1). Nanomolar concentration of free transition elements in cellular system seems enough for the catalysis of Fenton reaction *in vitro* at the physiological level of H<sub>2</sub>O<sub>2</sub> (ref. 21).

### Enzymatic and nonenzymatic lipid peroxidation and their tandem action

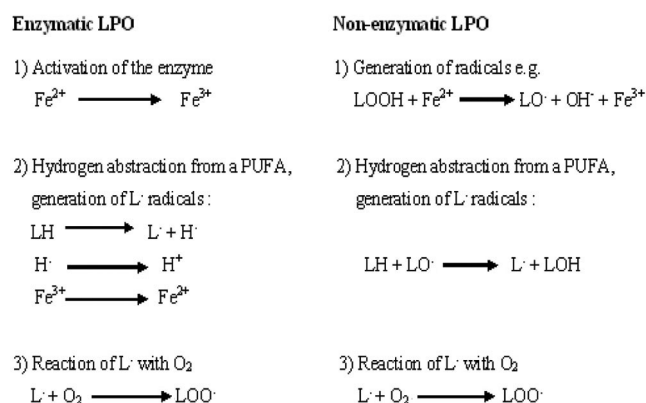
LPO in plant cells can also be mediated by the enzyme lipoyxygenase. This enzyme is able to initiate the formation of fatty acid hydroperoxides, ensuing peroxidation<sup>22</sup>. The enzyme lipoyxygenase (LOX) which is found to be activated particularly under stress and senescence oxidizes specific PUFAs. PUFAs which are characterized by the presence of *cis*, *cis*-1,4-pentadiene moiety (–CH=CH–CH<sub>2</sub>–CH=CH–) become the target of LOX and transformed into lipid hydroperoxides (LOOHs)<sup>22</sup>. The product (LOOH) being unstable is decomposed to a great variety of products. LOX are also found to cleave in a regiospecifically and stereospecifically controlled reaction, an H-atom from a double allylically activated –CH<sub>2</sub> group of PUFA. While still bound to LOX, the H atom reacts with complex-bound Fe<sup>3+</sup> in the active LOX by the formation of H<sup>+</sup> and Fe<sup>2+</sup> ions. Lipid radical, formed (L<sup>•</sup>) subsequently, adds oxygen and generates a peroxy radical (LOO<sup>•</sup>). Ultimately, an electron migrates from Fe<sup>2+</sup> LOO<sup>•</sup>, producing peroxy anion (LOO<sup>–</sup>), which combines with H<sup>+</sup> to produce LOOH<sup>23</sup>. It is important to note that during this process of LOX-mediated LPO, the peroxy radical is not able to escape from the enzyme complex<sup>24</sup>.

In plants, multiple isoforms of LOX have been characterized which can be distinguished by differences in their kinetic properties and other parameters as well as their localization and developmental stages<sup>25</sup>, and which fulfil different functions in plants. The function of LOX-mediated LPO ranges from mobilization of storage lipids during germination, formation of ROS in senescing tissue, stress responses and pathogen defence<sup>26,27</sup>. The connection of senescence with LPO is corroborated by an increase in LPO products and ROS with age<sup>24,28</sup>.

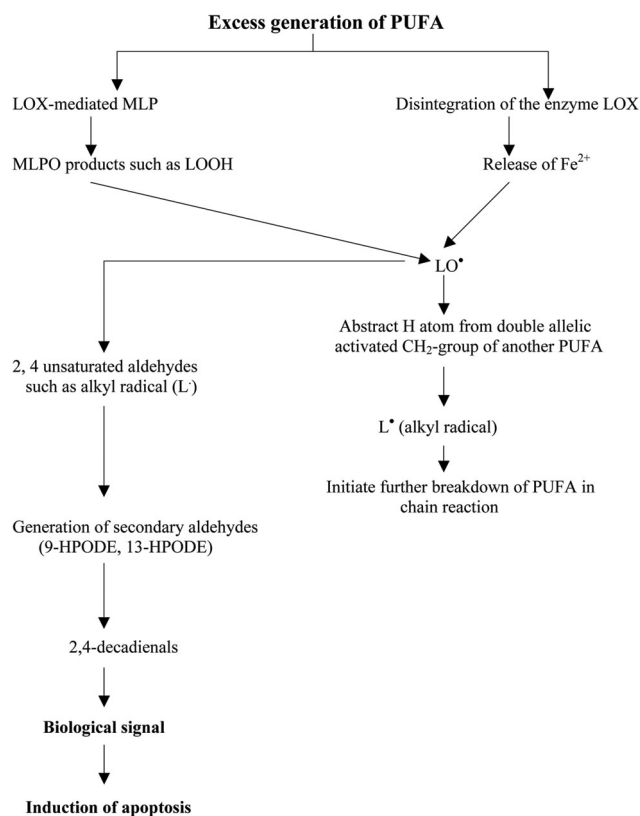
Nonenzymatic LPO which is nonspecific in nature, proceeds through a similar chain reaction composed of three main steps (initiation, propagation and termination) as already discussed. However, in enzymatic LPO, initialization is controlled and stereospecific and propagation does not occur frequently<sup>10</sup>. In fact, the production of specific LPO intermediates is controlled by enzymatic pathway where release of nonenzyme radical intermediate

is maintained. In nonenzymatic LPO, the allelic H atom in PUFA is readily abstracted by radical species such as OH<sup>•</sup>, HO<sub>2</sub><sup>•</sup> and H<sub>2</sub>O<sub>2</sub><sup>•</sup>; and results in the formation of lipid radicals which subsequently react with O<sub>2</sub> when available. Diverse products are generated in this process, depending on the fatty acid oxidized. Once nonenzymatic LPO is initiated, lipid alkoxyl radical (LO<sup>•</sup>) and lipid peroxy radical (LOO<sup>•</sup>) capable of abstracting H from another fatty acid molecule are generated, thus contributing to the propagation of LPO (Figure 2). In biomembranes, the presence of proteins can result in transfer of lipid radicals to protein side chains with subsequent adduct formation, thereby causing the proteins to be active participants in the propagation of LPO. Nonenzymatic LPO generally terminates in radical–radical reactions<sup>29</sup>.

Several experimental evidences strongly favour the fact that LOX-mediated LPO often switches over to a non-enzymatic LPO, when supply of substrate (PUFA) exceeds a certain limit<sup>30,31</sup>. A tandem action of both enzymatic and nonenzymatic LPO was observed in case of programmed cell death. In fact, when supply of PUFA is significantly higher, it is found that LOX not only catalyse MLPO but also commit suicide by catalysing disintegration of its own molecule<sup>32</sup>. This causes the release of enzyme-bound Fe-ion, which subsequently reacts with end product of LOX-mediated LPO (i.e. LOOH) to produce LO<sup>•</sup> in a Fenton type reaction<sup>30</sup>. Lipid radicals formed in this manner then abstract a H atom from double allylic activated CH<sub>2</sub> group of another PUFA, forming a new lipid radical (L<sup>•</sup>), thus inducing once again the chain reaction (Figure 3). The alkoxyl radical is generated primarily by reaction of LOOH with Fe<sup>2+</sup>, although most of the LOOH are reduced by peroxidases to corresponding OH<sup>•</sup>. Alkoxyl radicals generated, are subsequently decomposed to 2,4-unsaturated aldehydes (mainly 2,4-dienols) and alkyl radicals (R<sup>•</sup>). The 2,4-dienal may be further converted to secondary aldehyde compounds (Figure 3).



**Figure 2.** Enzymatic (lipoyxygenase-mediated) and non-enzymatic MLPO and their tandem action in plants.



**Figure 3.** Events showing oxidative degradation of membrane-bound PUFAs (having *cis*, *cis*-1,4-penta diene moiety), producing lipid hydroperoxide and alkoxy and alkyl radicals which initiate further break down of other PUFAs and generate a variety of secondary oxidative products representing non-specific 'biological signals'.

In a study with the simplest PUFA, linoleic acid (generally, a preferred substrate for LOX induced MLPO), transformation during MLPO causes formation of 9-hydroxyperoxy-10,12-octadecadienoic acid (9-HPODE) and 13-hydroxyperoxy-9,11-decadienoic acid (13-HPODE). HPODEs are subsequently reduced to their corresponding alcohols. These classes of compounds represent the major MLPO products that accumulate due to tandem action of both enzymatic and nonenzymatic LPO in senescing and dehydration stressed plant tissues<sup>33</sup>.

### MLPO: adverse consequence and physiological significance

MLPO, which is natural and essential, can occur in enzymatic and/or nonenzymatic fashion in plant cell. Although MLPO, particularly the nonenzymatic process, is viewed as deleterious; extensive study of this process in the last decade has proved other functions of this process as plant effectors. Recent evidences suggest that during stress, both LPO and generated RLS can benefit cells in many ways<sup>5,7,8</sup>.

The classical view of oxidative stress associated membrane LPO that indiscriminately destroys biomolecules

(both plasma membranes and organellar membranes) has undergone a shift, in which several positive biological roles of this process are gradually emerging. When the O<sub>2</sub>-sensitive PUFA associated with biomembranes gets peroxidized under oxidative stress (largely mediated by unfavourable environmental cues), it produces several secondary products with biological roles. The secondary products may induce stress acclimation by stimulating expression of genes encoding detoxification functions and can be associated with the repair of damaged membrane, etc.

### MLPO: a potential source of ROS in plant cell under stress

Loss of redox homeostasis, largely caused by imposition of unfavourable environmental stress and natural course of senescence in plants triggers both nonenzymatic and enzymatic LOX<sup>5,18</sup>. LOX-induced MLPO plays a significant role in plant senescence by not only causing membrane leakiness but also contributing towards exaggeration of redox imbalance by upregulating the production of radical species<sup>34,35</sup>. A significant role for nonenzymatic LPO in leaf senescence has been proposed<sup>35</sup>. In the non-enzymatic MLPO, lipid peroxides are unstable and readily decompose to form a complex series of breakdown products that display a wide variety of damaging actions. For example, aldehyde molecules generated during MLPO have been proved to be extremely cytotoxic and exhibit direct reactivity with important biomolecules<sup>36</sup>.

Malondialdehyde (MDA) and hydroxyalkenals (HAEs) produced from PUFA by LPO process, form a variety of adducts with lysine residues of proteins or with amines of phospholipid heads of membranes, thereby impairing normal activities or functions of those proteins and functional moieties<sup>36</sup>. Mechanistically, the deleterious effect of MLPO is largely due to its capability to generate a host of toxic radical species<sup>6,8,12</sup>. MLPO, catalysed either by oxidants or enzymatically by LOX, causes the formation of several radical species including alkoxy, peroxy, etc. which may further stimulate chain reactions of MLPO, thus initiating autocatalytic deteriorative cascades, leading to loss of membrane architecture and function<sup>6,18,34,37</sup>. LOX may play a central role in promoting oxidative injury during environmental stress induced oxidative deterioration through initiation of LOX-induced MLPO<sup>18,34</sup>. Under natural conditions, plant cells are used to produce ROS at low rates and MLPO is found to be sluggish. Severe abiotic and biotic stresses induce decontrolled MLPO, which in turn generate several radical species as byproducts that in turn further accelerate MLPO<sup>18</sup>. The role of heavy metals, salinity, extremes of temperature, etc. in the induction of peroxidation of membrane lipids and subsequent loss of membrane integrity has been well-established<sup>1,6</sup>.

## MLPO perturbs membrane assembly causing changes in membrane fluidity and generates toxic messenger OH<sup>-</sup> alkenals

Among the targets of oxidative stress, decontrolled MLPO is considered as the most damaging, because it not only generates a host of ROS, but also perturbs membrane architecture directly. The membrane lipid containing PUFAs are prone to peroxidation reaction due to abstraction of H from methylene group ( $-\text{CH}_2$ ), which holds a solitary electron, leaving behind an unpaired electron on the carbon ( $-\dot{\text{C}}\text{H}_2$ ). Further, the presence of double bond in the fatty acid weakens the C–H bonds on the carbon atom near the double bond and thus facilitates H subtraction. The initial reaction of most potent ROS,  $\cdot\text{OH}$  with PUFA produces a lipid radical ( $\text{L}\cdot$ ) that in turn reacts with  $\text{O}_2$ , producing lipid peroxy radical ( $\text{LOO}\cdot$ ), which can further abstract H from adjacent fatty acid to produce lipid hydroperoxides (LOOH) and a secondary lipid radical<sup>38</sup>. LOOH formed may be cleaved by transition metal ions, producing alkoxy radicals ( $\text{LO}\cdot$ ). Ultimately, generation of both peroxy and alkoxy radicals can stimulate chain reaction of LPO by abstracting other H atom<sup>39</sup>. Therefore, the whole episode of MLPO perturbs the assembly of the membrane, causing changes in fluidity and permeability which may ultimately manifest in decontrolled molecular trafficking across the membrane and inhibition of metabolic processes<sup>40</sup>.

MLPO of PUFAs, one of the most vulnerable events of oxidative stress that contributes towards cascading auto-catalytic chain reaction, causes extensive damage to membrane architecture. As a consequence of MLPO, a great variety of aldehydes is formed when lipid hydroperoxides are broken down in the cellular system. Some of these secondary breakdown products are highly reactive and when accumulated, they can be considered as secondary toxic messengers of the cell. In fact, dissemination of these secondary toxic chemicals can initiate further episodes of ROS-mediated oxidation events<sup>41</sup>. The aldehydes, most extensively studied in this regard, are MDA, 4-hydroxy-2-hexenal (HHE) and 4-hydroxy-2-nonenal (HNE). HNE is recognized as the principal aldehyde generated during MLPO of *n*-6 PUFA (linoleic acid and arachidonic acid). HHE, on the other hand is produced as a result of MLPO of *n*-3 PUFAs (linolenic acid and docosahexanoic acid). Therefore, 4-hydroxyl-2-alkenals represent the most important aldehyde breakdown products generated during MLPO (Figure 3). Assessment of implication of HNE and HHE accumulation revealed their cytotoxic role<sup>42</sup>.

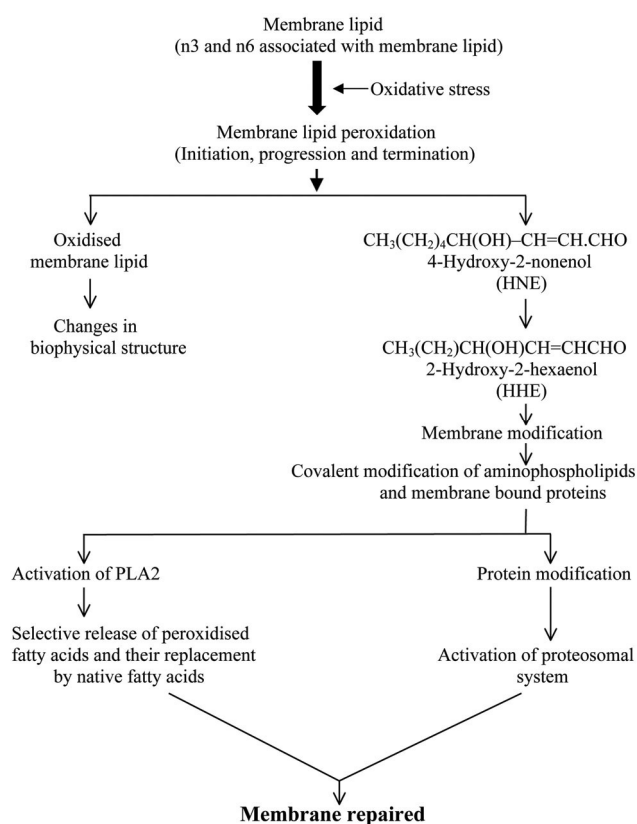
Out of the different products of MLPO, HNE has been demonstrated as the most effective product of the process. HNE apart from its cytotoxic effect, exhibits inter-cellular signalling, modulating the expression of genes, affecting cell proliferation and differentiation<sup>21</sup>. The high reactivity of HNE is related to its molecular architecture;

it possesses hydroxyl groups close to its carbonyl groups that enable it to react with thiol and amine groups of the target molecules during oxidative stress<sup>21,38</sup>. Its ability to diffuse into cellular environment further consolidates its signalling role. Further, high reactivity of these secondary aldehydes with major macromolecules of cell such as DNA, protein and phospholipid generates intra and intermolecular adducts<sup>21</sup>. Protein damage caused by HNE is largely due to the addition of aldehyde compounds with  $-\text{SH}$  groups of cysteine, lysine amino group or histidine imidazole groups<sup>21</sup>.

## MLPO and repair of damaged membrane

Plant cell possesses mechanisms to repair lipoperoxidized membrane by cleavage of peroxidized fatty acid residues and their subsequent replacement by native fatty acids<sup>43,44</sup>. A number of studies reveal that MLPO stimulates phospholipase A2 (PLA2)-mediated release of fatty acids and their subsequent repair<sup>43,44</sup>.

One of the consequences of oxidative stress is the formation of oxidized proteins which in the cellular system is being recognized and hydrolysed by 20S proteasomes<sup>45</sup> (Figure 4). An interesting finding, which strongly corroborates the coordinated function of proteosomal system



**Figure 4.** Scheme of events showing MLPO-associated modification and repair of damaged membrane by secondary LPO products HNE and HHE (details in the text).

and repair function of PLA<sub>2</sub> of peroxidized membrane, is the abundance of oxidized proteins near the cell membrane. Therefore, simultaneous MLPO-mediated stimulation of PLA<sub>2</sub>-mediated liberation of free fatty acids and activation of proteosomal system are conjugately responsible for replacement of peroxidized fatty acids with native fatty acids and proteins, causing membrane repair.

**MLPO produces lipid peroxidized electrophiles that initiate cell signalling and adaptive response**

MLPO, both enzymatic and nonenzymatic, are inherent features of the biological system that aggravate under unfavourable environmental cues and natural course of ageing and senescence. Out of the various breakdown products of LPO, a significant proportion of oxidized lipid products are electrophilic in nature and reactive; and are capable of reacting with cellular nucleophiles such as amino acid histidine, cysteine, lysine, etc.<sup>10,17</sup>. As the field of study related to MLPO evolves, it is gradually evident that the process is also endogenously regulated and has multiple functions, depending on the site, condition and mechanism of oxidation<sup>5,10</sup>. Both enzymatic and nonenzymatic MLPO have many important biological mediators<sup>46</sup>. In fact, peroxidation of membrane-associated PUFAs results in the formation of RLS which are electrophilic in nature (Figure 5). For example, peroxidation of arachidonic acid (a major substrate in MLPO process) results in the formation of several products, including a subset of electrophiles. Examples of nonenzymatic MLPO of PUFA which are electrophilic in nature are MDA, HNE, acrolein, isoprostanes, etc.<sup>47</sup>.

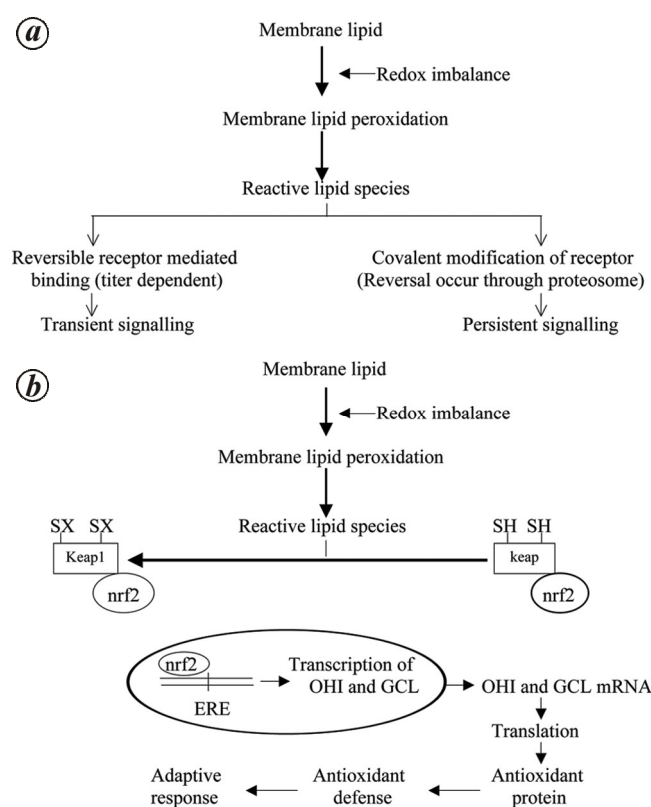
Another source of LPE in plants is LOX-mediated MLPO. In plants, LOX are classified as 9-LOX or 13-LOX, depending on the position of oxygenation of hydrocarbon backbone. Such oxygenation leads to the formation of 9(S) or 13(S) hydroperoxy derivatives of PUFAs which are ultimately metabolized into products that confer plant defence against pathogens<sup>48,49</sup>.

Several studies proposed that LOX-mediated breakdown of octadecadienoic acid (linoleic acid) are basic mediators of defence mechanisms<sup>50,51</sup>. Moreover, LOX-dependent MLPO pathway has been shown to regulate hypersensitive reaction (HR) in plants<sup>51</sup>. While explaining these defence processes in plants through PCD or HR, it is found that a series of complex breakdown products formed during MLPO, some of which are RLS, exhibit a direct reactivity with biomolecules<sup>36</sup> and exert damaging effect. As discovered earlier, oxidation of PUFA generates MDA and hydroxyalkanols among other products which are RLS and form a variety of adducts with lysine residues of proteins or amines containing heads of membrane phospholipids<sup>36,52</sup>.

In recent times, RLS have been found to participate in several physiological pathways including cell death,

induction of antioxidative defence, modification of cell signalling proteins, etc.<sup>10,53</sup>. RLS can mediate biological responses basically either by irreversible covalent modification of receptors or through reversible binding. In fact, in several animal cells, some RLS function as ligands for specific receptors<sup>17,54</sup> and mediate biological effects through reversible receptor-ligated interaction and subsequent signalling event. Irreversible covalent modification of nucleophilic amino acid residues of proteins can also be mediated by RLS<sup>17,55</sup>.

Considerable progress has been made in elucidation of mechanisms of cell signalling mediated by RLS in animal systems as compared to the plant system. As revealed by various studies, it is pertinent that RLS generally functions through reversible binding to cellular receptors and subsequently initiates cell signalling (Figure 5). RLS-receptor binding, which depends on the concentration of RLS, results in the formation of a transient signal<sup>56</sup>. Electrophilic RLS also participates in cell signalling through covalent modification. Many RLS also function through G-protein-coupled receptors<sup>57</sup>. Apart from this, some RLS can also act through peroxisome-proliferator-activated receptor  $\gamma$  (PPAR  $\gamma$ ).



**Figure 5.** Basic receptor-mediated reversible and nonreversible signalling events and subsequent modification of protein targets by oxidized lipids of MLPO. *a*, Receptor-mediated reversible signalling (transient) as compared with signalling mediated by covalent modification (persistent) by oxidized lipids. *b*, Events showing modifications of protein target (Keap1) that lead to release of transcription factor Nrf2 and its subsequent transferred to nucleus, upregulating the expression of associated with antioxidative defence.

Recently, some electrophilic-responsive proteomes have been identified which are modified upon exposure to electrophilic RLS, subsequently orchestrating a signalling event. The mechanism of reactivity between RLS and nucleophiles (particularly amino acids) depends on the hard/soft acid–base principle<sup>58</sup>. The ‘hard electrophiles’ which include RLS generally react with ‘soft nucleophiles’ such as protein thiol or GSH<sup>59</sup>. In contrast, a few hard electrophiles such as isoketals adduct to harder nucleophiles such as DNA and lysine-NH<sub>2</sub> group. Since thiol residues are redox-sensors, any modification of thiol residues by RLS is biologically significant to cell signalling<sup>59</sup>.

Apart from modification of ‘thiol redox-sensors’, RLS-mediated signalling can also operate through thiol-dependent post-translational modification (S-glutathionylation, S-nitrosylation, disulphide formation, etc.)<sup>60,61</sup>. Different target proteins of RLS, which subsequently operate the signalling pathway, include HSP70, HSP90, ATP synthase, thioredoxin, thioredoxin reductase, k-Ras, cytochrome oxidase, 26S proteasome, etc.<sup>59,60</sup>. The amino acid residue of target protein confers specificity in reaction with lipid electrophiles, which in turn become the determinant factor whether RLS elicits an adaptive signalling response or contributes to cell death<sup>10,62</sup>. Oxidative stress induced formation of RLS often contributes towards adaptive response of organisms. One of the important ‘protein target/sensor’ is keap1, which is an adaptor protein normally attached to transcription factor Nrf2. Electrophilic attack of RLS (HNE) to keap 1 causes release of Nrf2 and subsequent translocation of transcription factor to the nucleus (Figure 5). In the nucleus, Nrf2 binds to electrophile response element (ERE) or antioxidative response element (ARE); and genes responsible for antioxidative protein glutamate–cysteine ligase and AO-1 are transcribed, up-regulating ultimately the antioxidative defense and acclamatory response.

### Assessment of MLPO: sensitive biomarker of oxidative stress

MLPO, an event associated with oxidative stress, can be determined by measuring either the primary peroxidation products or accumulation of secondary products<sup>63,64</sup>. Estimation of loss of unsaturated fatty acids can also be a parameter for assessing MLPO<sup>65</sup>. Out of these processes, measuring the end products of MLPO is the most widely accepted factor for the estimation of oxidative stress and associated oxidative damage of the plant tissue<sup>66</sup>. Loss of redox homeostasis due to overaccumulation of ROS causes peroxidation of PUFAs, producing  $\alpha,\beta$ -unsaturated aldehydes such as 4-hydroxyenal, MDA, etc.<sup>67</sup>. These products of MLPO are the accepted markers of oxidative stress in plants<sup>63</sup>. Several analytical techniques can be

used to assay MLPO and understand the extent of oxidative stress suffered by the plant tissues under environmental odds (Table 1)<sup>68–78</sup>.

A well-accepted thiobarbituric acid (TBA) assay for MDA, is based on its reaction with TBA followed by measuring absorbance at 532 nm (refs 68–70). TBA assay and its modification are used as a sensitive test for MLPO in temperature stress, heavy metal stress, salinity stress, UV irradiation stress of plants<sup>79–82</sup>. Although used widely for assessing MLPO and oxidative stress of plants, there are a few drawbacks in this process; there may be formation of thiobarbituric acid reactive substances (TBARS) which are not related to MLPO<sup>64</sup>. In fact, the TBA test works well with microsomal and liposomal membrane systems, but its application to other membrane preparations poses a problem<sup>83</sup>. Aldehydes other than MDA can also form chromogens with the same absorbance at 532 nm as TBARS test<sup>84</sup>. Another weakness of the process is that TBA test rarely measures free MDA content of the system; instead most if not all MDA measured is generated by decomposition of lipid peroxides during acid heating of the test<sup>85</sup>. In addition, the peroxide decomposition produces radicals that can start peroxidation of other fatty acids during the assay, thereby causing amplified response<sup>86</sup>. Endogenous H<sub>2</sub>O<sub>2</sub> is also found to exert false positive results when TBA test is applied to microsomal membrane<sup>87</sup>.

Recent progress in MS techniques caused the development of more accurate GC–MS based process for the estimation of 4-HNE and MDA<sup>74,88</sup>. A highly sensitive LC–MS-based detection of 2,4-dinitrophenyl hydrazine derivative of 4HNE and MDA has been practised since the late nineties<sup>73,89</sup>. One significant advantage of LC–MS and GC–MS bound test is the ability to identify individual lipid species targeted by ROS<sup>90</sup>.

The conjugated diene structures formed during the peroxidation of unsaturated fatty acids, absorbs UV in the range of 230–235 nm. Therefore, by measuring UV absorbance at 230–235 nm, one can indirectly measure the LPO of pure lipids<sup>91</sup>. Several researchers used HPLC to separate the UV-absorbing diene-conjugates for further analysis<sup>92</sup>.

Based on current assay techniques for the estimation of MLPO, it can be said that in order to determine the real occurrence of MLPO in plant samples, it is important to use techniques that extend specific chemical information on the product of the process. Thus, more emphasis has to be given to separation of MLPO products. This is often achieved by processes based on HPLC (assaying peroxides, aldehydes), GC (assaying hexanals) and LC (assaying dienes)<sup>74,75,93</sup>. However, sample preparation is of utmost importance and extreme care has to be undertaken to ensure that loss of oxidized material and artificial peroxidation can be avoided during sample preparation.

**Table 1.** Common methods of detection and estimation of MLPO

Procedure	Test and sensitivity of the process	References
TBA test	Thiobarbituric acid reactive substances (TBARS) such as different aldehydes, malondialdehyde react with TBA at low pH and form [TBA]-MDA adduct (pink chromogen with absorbance at 532 nm). Standardized test, sensitive for microsomal and liposomal MLPO test. Rarely measures the free MDA content of lipid system. TBA reactivity depends on the lipid content of the sample	64, 68–70
Fluorescence test for aldehyde and end product of MLPO	MDA and other aldehydes, the end products of MLPO are measured. Aldehydes react with $-NH_2$ group forming Schiff's bases in acidic pH. At neutral pH fluorescent dihydropyridines are formed. Aldehydes are polymerized to form fluorescence products and fluorescence is measured. Highly sensitive method, although formation of fluorescence products takes place in a minor complex reaction.	65, 71
Diene conjugation test	Diene conjugated structures, formed by oxidation of PUFAs as the intermediates of MLPO are assessed. Absorbance in the UV range (230–235 nm) useful for the measurement of MLPO of pure lipids. The process requires specific extraction techniques.	72, 65
LC–MS/GC–MS-based techniques	Aldehydes and lipid peroxides produced by MLPO are identified and estimated. DNPH-derivatives of MDA/4HNE have been utilized in these processes. Peroxidation products are extracted and reduced to alcohols, which are subsequently separated and identified by MS techniques.	73–75
HPLC-based process	Estimation of loss of fatty acids by MLPO. Extremely useful for the estimation of LPO stimulated by metal ions.	64, 76
Spin trapping method	Intermediate radical species formed by MLPO are identified.	77, 78
Glutathione peroxidase (GPX) method	GPX made to react with hydroperoxides and $H_2O_2$ forming oxidized glutathione (GSSG), which subsequently react with NADPH to regenerate reduced glutathione (GSH). Consumption of NADPH in the reaction is estimated as the rate of LPO. However, membrane-associated peroxide needs to be separated first for the estimation of MLPO.	65

### Omic approach for elucidation of RLS-associated signalling mechanism in plants

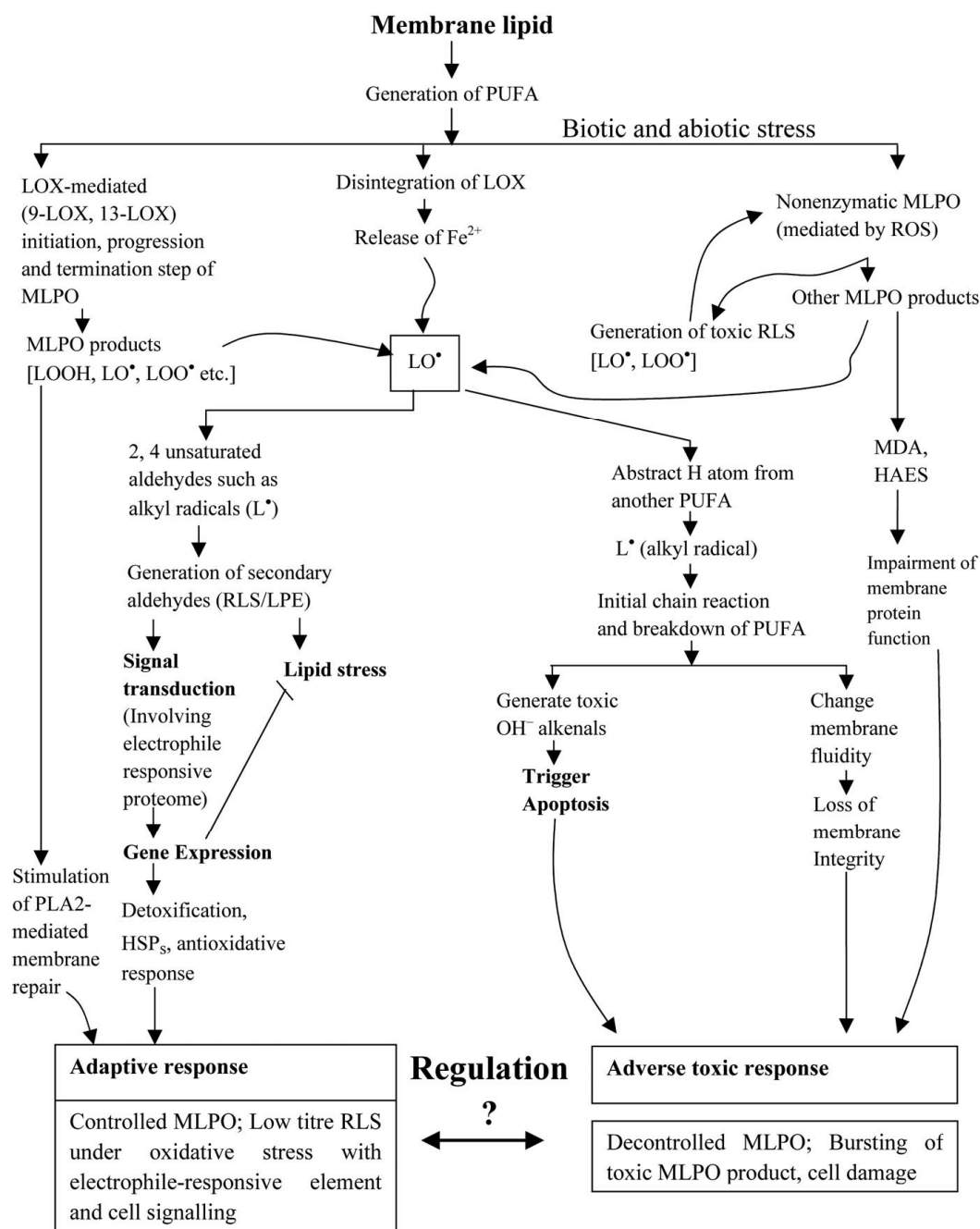
Recently, combined transcriptomic and proteomic approaches suggest that electrophilic compounds generated from MLPO could induce gene expression<sup>94,95</sup>. In *Arabidopsis*, biotic stress, caused by *Pseudomonas syringae* results in the accumulation of linoleic acid 9- and 13-ketodienes, which in turn stimulates the expression of glutathione-S-transferase 1 gene (*GST1*). In fact, the  $\alpha,\beta$ -carbonyl motifs of ketodienes were found to be responsible for the stimulation of the expression of the *GST1* gene. MDA, the inevitable product of MLPO, also stimulates the expression of *GST1*<sup>96</sup>. Several transcriptome studies have been undertaken in recent times with MLPO products and oxylipins such as MDA, *cis*-jasmonate, phyto-prostane A<sub>1</sub> (a product of nonenzymatic fatty acid oxidation), etc. to elucidate their effect on gene expression<sup>97–99</sup>. These studies revealed that two of the dominant categories of genes are upregulated by RLS and are involved in stress amelioration. Regulation of stress amelioration genes involves either signalling through TGA transcription factors or direct activation of heat shock factors<sup>100</sup>.

Transcriptomic data from *Arabidopsis* revealed that the dominant gene families encoding glutathione-S-transferase,

cytochrome P450s and UDP-glucosyl transferases<sup>101</sup> are upregulated by RLS. MLPO-derived RLS induce significantly the expression of heat shock genes. Under hyperthermia, wild type plants accumulate RLS significantly. RLS formation is strongly enhanced in mutants defective in heat shock protein (HSP) synthesis and antioxidative metabolism<sup>102</sup>. Recently, it is suggested that induction of heat shock genes by RLS is not necessarily related to hyperthermia. Instead, the inducible HSPs, which act as chaperones, might insulate damaged proteins to prevent illegitimate hydrophobic binding by oxidized lipids, thereby changing a protein function or even tagging it for proteolysis. So, the heat shock and lipid stress genes in many cases overlap and are co-inducible in nature.

Transcriptome analysis also revealed genes that are downregulated by RLS. In general, genes associated with auxin signalling, cell division and cell wall formation are downregulated<sup>99</sup>. Genes responsible for coding enzymes necessary for the pectin, cellulose and expansions are found to be repressed under the accumulation of RLS<sup>97,99</sup>. RLS are also found to have a significant negative effect on the expression of genes that encode cyclins such as *CYCD3*, affecting cell division<sup>103</sup>. Several genes associated with auxin signalling such as *PINI*, *AUX/IAA 3,4,5,13* are downregulated in *Arabidopsis* under the influence of





**Figure 6.** Pictorial image summarizing the events associated with MLPO (enzymatic and non-enzymatic), its adverse consequence, role in cell signalling and biological implication (details in the text).

RLS<sup>101</sup>. A recent genomic and proteomic study also revealed that most of the RLS-inducible genes contain a TGA motif (TGACG) in their promoter elements which acts as putative binding site for basic leucine zipper transcription factors of TGA family<sup>104</sup>.

### The paradox

The most noteworthy experimental finding on MLPO till date is their contradictory roles under oxidative stress and

the emerging regulatory mechanisms to finetune the process for a definite purpose. The MLPO paradox is indeed the paradox of evolution itself. In fact, evolutionary pressure has made the best out of a bad situation by generating a mechanism to curtail the unwanted toxic effect of the process, which is an unavoidable consequence of aerobic life. Indeed, evolution has co-opted membrane-bound PUFAs to serve necessary and useful purposes in the cell under oxidative stress. Focus must now be placed on a more thorough understanding of redox-regulation of

MLPO. How RLS-mediated signals are perceived and transduced into adaptive response under stress is still not clear and deserves special attention.

The current paradigm is that controlled and low level of MLPO which generates low levels of RLS over time can specifically modify cysteinyl thiols to modulate protective cell signalling pathways, exhibiting adaptive response. MLPO also stimulates phospholipase A2 (PLA2)-mediated release of fatty acids and subsequent repair of the damaged membrane, thereby demonstrating a defensive strategy. In contrast, high level of RLS can modify other nucleophilic residues in a less specific manner, resulting in non-target damage of proteins. Decontrolled MLPO, which is also the characteristic feature of stress-induced plant tissue and natural course of ageing, is also the vital source of ROS, which in turn further instigates the same process, thereby forcing the cell to enter a vitiating cycle of autocatalytic oxidative cascades. Failure of the cell to contend the rate of MLPO or decrease the level of RLS and subsequent repair or removal of damaged membrane is likely to lead to deleterious consequences for the cell and the development of serious symptoms of oxidative stress. It will be interesting to see how this concept develops to address the conflict of interest associated with MLPO as sophisticated analytical techniques allow the identification of specific cellular targets for RLS in a time and dose-dependent manner.

### Conclusion and perspective

MLPO is thus a natural and essential process and mechanistically important as it is one of the few examples of carbon-centered radical production in cell. Decontrolled MLPO, which is associated with senescence and environmental stress, exerts cytotoxic effect on cells, whereas regulated MLPO functions as effector (Figure 6). Indeed, evolution has co-opted MLPO to serve necessary significant purposes such as communication with external environment, modification of biophysical structure of membrane, programmed cell death, etc.

Although the process is apparently deleterious and associated with the generation of ROS, a close look reveals its physiological significance. It is associated with repair of damaged membrane, can generate toxic messengers required for initiating HR, modify lipid–lipid interaction and change fluidity of membranes, etc. However, the most significant role of the process is the contribution of RLS to the modulation of cell function and signalling. Development of a sensitive mass spectrometric technique is required for the identification and characterization of oxylipidomes, an important subset of lipidomes formed through MLPO under oxidative stress. Development of techniques to monitor specific RLS-protein (sensor) adducts to define electrophile-responding proteome for specific RLS will help us understand the paradigm of cell

signalling mechanisms. It is now clear that RLS reacts with discreet electrophile-responsive proteome and subsequently sponsors signalling events which evoke adaptive response.

In summary, the current paradigm is about how the cells exploit MLPO and their products particularly under different physiological conditions. Accumulation of different levels of RLS plays a crucial role as low titre of RLS can modify specific target protein (sensor) in contrast to high titre where RLS can modify other cellular macromolecules in a less specific manner. Thus, failure to control or finetune the process always leads to deteriorative events and ultimately the lethal consequence. So, it is extremely important to critically unfold the process of regulation of MLPO and identification of specific cellular targets of RLS in the physiology of organisms. It would be extremely interesting and challenging to identify changes in gene expression initiated by MLPO through RLS. Such a global analysis of effect of RLS on the transcriptome of plants has not yet been attained, but with emergence of post-genomic technologies, it is not far off.

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