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Review Article

The Language of Reactive Oxygen Species Signaling in Plants

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Reactive oxygen species (ROS) are astonishingly versatile molecular species and radicals that are poised at the core of a sophisticated network of signaling pathways of plants and act as core regulator of cell physiology and cellular responses to environment. ROS are continuously generated in plants as an inevitable consequence of redox cascades of aerobic metabolism. In one hand, plants are surfeited with the mechanism to combat reactive oxygen species, in other circumstances, plants appear to purposefully generate (oxidative burst) and exploit ROS or ROS-induced secondary breakdown products for the regulation of almost every aspect of plant biology, from perception of environmental cues to gene expression. The molecular language associated with ROS-mediated signal transduction, leading to modulation in gene expression to be one of the specific early stress response in the acclamatory performance of the plant. They may even act as "second messenger" modulating the activities of specific proteins or expression of genes by changing redox balance of the cell. The network of redox signals orchestrates metabolism for regulating energy production to utilization, interfering with primary signaling agents (hormones) to respond to changing environmental cues at every stage of plant development. The oxidative lipid peroxidation products and the resulting generated products thereof (associated with stress and senescence) also represent "biological signals," which do not require preceding activation of genes. Unlike ROS-induced expression of genes, these lipid peroxidation products produce nonspecific response to a large variety of environmental stresses. The present review explores the specific and nonspecific signaling language of reactive oxygen species in plant acclamatory defense processes, controlled cell death, and development. Special emphasis is given to ROS and redox-regulated gene expression and the role of redox-sensitive proteins in signal transduction event. It also describes the emerging complexity of apparently contradictory roles that ROS play in cellular physiology to ascertain their position in the life of the plant.

1. Introduction

Environmental stresses such as extremes of temperature, salinity, drought, heavy metals, herbicides and pathogens greatly affect plant metabolism and productivity [1]. Because of environmental stress, the yield potential of the crops is hardly realized. It has been estimated that a country like USA is being able to harvest approximately one fourth of the genetic potential of the crop [1, 2]. To survive plant have developed a complex signaling network involving different endogenous growth regulators that sense and protect them from environmental stresses. One of the common responses to different environmental stresses, both abiotic and biotic, is the accelerated generation of reactive oxygen species (ROS), including superoxide ($O_2^{\bullet -}$), perhydroxy radical ($HO_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), alkoxy radical ($RO^{\bullet}$), peroxy radical ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), organic hydroperoxide (ROOH), and so forth [2–5]. Accumulation of ROS imposes ultimately oxidative stress, exacerbating cellular damages [4, 6]. ROS are generally produced as a byproduct of normal aerobic metabolism, involving largely the membrane-linked electron transport processes, redox-cascades, and metabolisms, whose production are aggravated under the influence of unfavorable environmental cues. In all aerobic organisms, the concentration of ROS is tightly controlled by ROS-scavenging pathways that metabolize ROS. However, an imbalance in generation and metabolism of ROS leads to a variety of physiological challenges by

Table 1: The important ROS in plant tissues and their basic properties (half life-in biological system, migration capacity-Distance traveled in one half-life time, if the diffusion coefficient is assumed to be $10^{-9}\,m^{-2}s^{-1}$).

ROS	Half life	Migration capacity	Source	Reacts with			References
				DNA	Protein	Lipid, carbohydrate	
Superoxide ($O_2^{\bullet -}$)	1–4 μs	30 nm	Membranes, chloroplast (Mehler reaction), mitochondria	No	Yes (Fe-centre)	Hardly	[2, 5, 9]
Hydrogen peroxide (H_2O_2)	1 ms	1 μm	Membranes, chloroplast, mitochondria, peroxysome	No	Yes (Cysteine)	Hardly	[2, 8]
Hydroxyl radical ($OH^{\bullet}$)	1 μs	1 nm	Chloroplast, membranes, mitochondria	Rapidly	Rapidly	Rapidly	[2, 8]
Singlet oxygen ($^1O_2$)	1–4 μs	30 nm	Chloroplast, membranes, mitochondria	Yes (Guanine)	Trp, His, Tyr, Met, Cys.	PUFA	[8, 10]
Alkoxy radicals ($RO^{\bullet}$)	?	1 nm	Membrane lipid peroxydation	No	Yes	PUFA	[2, 8]
Peroxy radicals ($ROO^{\bullet}$)	?	1 nm	Membrane lipid peroxydation	No	Yes	PUFA	[8, 10]

disrupting redox homeostasis of cell, which is collectively known as "oxidative stress." Plant possesses an efficient antioxidative defense that protects the cell from oxidative damage caused by oxidative stress. This is executed by redox-sensing and signaling pathways which largely regulate and control spatiotemporal titer of ROS by modifying the production and scavenging mechanisms of ROS. In fact, it is correct to assume that plant might possess the mechanisms that regulate the concentration of ROS according to cell's need. In this aspect, comprehensive overviews of extensive plant oxidative stress-response literature are available [2–6]. However although the concept of redox-regulation is an old one in enzymology, there is ample of work in recent times that suggest the significance of ROS in cell signaling and redox sensing mechanisms, particularly for the survival of the plant under environmental stress [3–5]. Retrograde signaling mediated by autopropagating waves of ROS that travels at a rate of more or less $8.4\,cm\,min^{-1}$ from the organelles-like chloroplast or mitochondria to nucleus has been proposed for abiotic stress perception and systemic responses [5]. The present review explores the different signaling languages to understand emerging complexity of apparently contradictory roles that ROS play in cellular physiology to ascertain their position in the life of the plant.

2. Chemistry and Sites of Generation Reactive Oxygen Species in Plants

All the present form of life lives in oxidizing environment where oxygen supports aerobic life with great energy output. The very molecule which sustains aerobic life can act as lethal contaminant in mildly reduced cellular environment through endless formation of ROS. The term "ROS" comprises of ions or small molecules consisting of oxygen ions or free radicals of inorganic or organic forms. Oxygen itself is a strong oxidant since it possesses two unpaired electrons in its outermost π orbital. The reduction of oxygen by nonradical species needs transfer of two electrons having parallel spins to oxygen in order to fit with parallel spins of two unpaired electrons. Oxygen, therefore, got converted to ROS by univalent reduction (transfer of electron) or by energy transfer. The common ROS produced in plant include superoxide ($O_2^{\bullet -}$), perhydroxy radical ($HO_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), alkoxy radical ($RO^{\bullet}$), peroxy radical ($ROO^{\bullet}$), singlet oxygen ($^1O_2$), organic hydroperoxide (ROOH), and so forth [2–5].

Superoxide radical is generated in plant cell at the onset of oxidative burst of cell. Protonated form of $O_2^{\bullet -}$, $HO_2^{\bullet}$ is more reactive than superoxide itself, but in plant cells at physiological pH, a very small proportion of $O_2^{\bullet -}$ would be in this form [2, 7]. However, superoxide can dismutate to form H_2O_2. Much more reactive $OH^{\bullet}$ can be formed from $O_2^{\bullet -}$ and H_2O_2 through Fe catalyzed Haber-Weiss reaction [2, 7]. Singlet oxygen, an electronically excited species of O_2, is also very toxic and its significance has been realized due to the development of methods for its generation, free from other contaminants as well as its detection [8]. In addition, peroxy and alkoxy radicals formed as intermediates in membrane lipid peroxidation are also very toxic at high concentration and poses threat to several biomolecules. $O_2^{\bullet -}$ is a moderately reactive, short-lived ROS (Table 1) with a half-life of approximately 1–4 μs [9]. $O_2^{\bullet -}$ cannot pass through biological membranes as it is readily dismutated to H_2O_2. $^1O_2$ can either transfer its excitation energy to other biological molecules or continue with them, thus forming endoperoxides or hydroperoxides [8]. $^1O_2$ can last for nearly 4 μs in water and 100 μs in polar solvent [10].

H_2O_2, on the contrary, is moderately reactive (Table 1) and has relatively long half-life (1 ms) and can diffuse some distances from its site of production [11]. Hydrogen peroxide can react with other molecules in sites different from those where it has been produced for its capability to cross-biomembranes, probably through aquaporins of cellular membranes [11]. H_2O_2 may inactivate enzymes by oxidizing their thiol groups [12]. Hydroxy radiocals with half life of 1 μs is the most harmful ROS, because its strong instability

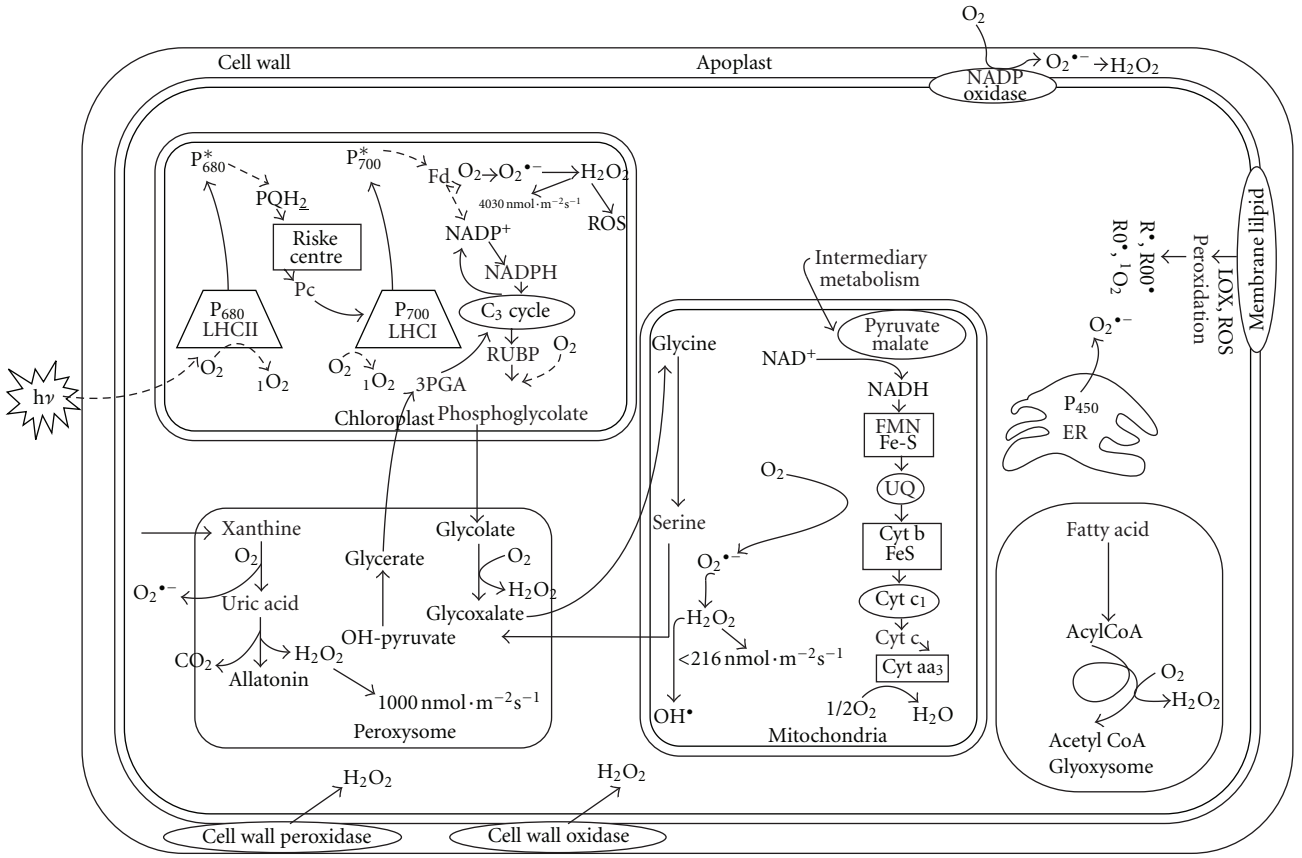

Figure 1: Sites and typical rates of generation of reactive oxygen species (ROS) in plant cell.

leads to combine rapidly with whatsoever cellular component present in vicinity.

So, any condition in which cellular redox homeostasis of the cell is disrupted that is manifested in the form of an imbalance in which the redox steady state of the cell is altered in the direction of prooxidants can be defined as oxidative stress. It has been estimated that 1% of O_2 consumed by plants is diverted to produce ROS in various subcellular loci [7, 13]. In fact, the most cellular compartments have the potential to become the source of ROS (Figure 1). The peroxysomal and chloroplastic H_2O_2 may be 30 to 100 times faster than the formation of H_2O_2 in mitochondria as evident from whole leaf point of view (Figure 1) [2, 13].

The reactive oxygen species arises in plant cells via a number of routes. Most ROS in plant cells are formed via dismutation of superoxide, which arises as a result of single electron transfer to molecular oxygen in electron transfer chains principally during the Mehler reactions in chloroplast [14]. The dearth of $NADP^+$ in PSI due to redox imbalance causes spilling of electron on to molecular oxygen triggering the generation of $O_2^{\bullet -}$. The majority of $O_2^{\bullet -}$ *in vivo* is thought to be produced via electron spilling from reduced ferridoxin to oxygen. Superoxide formed then undergoes dismutation either spontaneously or facilitated by SOD. Superoxide radicals generated by one electron reduction of molecular oxygen by Mehler reaction in PSI are rapidly converted into hydrogen peroxide by chloroplastic Cu-Zn-superoxide dismutase. It has been suggested that photoreduction of O_2 to water by ascorbate-peroxidase pathway (Halliwell Asada pathway) in intense light may involve about 30% of total electron transport [15]. This also suggests that O_2 plays an important role as an alternative electron acceptor in photoprotection and photooxidative acclimation. Therefore, production of large amount of ROS is an inevitable consequence under excess photochemical energy, and plants evolved efficient strategies by devising and integrating antioxidative defense mechanism with normal photosynthetic pathway to adjust to the imposed oxidative stress.

Singlet oxygen is continuously produced during photosynthesis involving mainly PS II. The reaction centre complex of PS II consists of heterodimer of D1 and D2 proteins apart from cytochrome b_{559} enabling the binding of functional prosthetic groups (chlorophyll P_{680}, pheophytin, Q_A, Q_B, etc.). Under excess photochemical stress or light energy, the redox state of plastoquinone pool and Q_A and Q_B are overreduced oxidized P_{680} recombined with reduced pheophytin. This condition favors the formation of triplet state of P_{680}, leading to the generation of singlet oxygen by energy transfer. It is found that excess photochemical energy that leads to photoinhibition of PS II causes significant enhancement in the generation of singlet oxygen [16].

In most of the C_3 plants, ROS (H_2O_2) may be generated during the oxidation of glycolate through PCOC (photosynthetic carbon oxidation cycle) in peroxysome (Figure 1).

In case of PCOC exhibited by C_3 plants, oxygenation of RuBP by Rubisco constitutes a major alternative sink of electrons, thereby sustaining partial oxidation of PSII acceptors and preventing photoinactivation of PSII when CO_2 concentration is reduced. Rubisco favors oxygenation compared to carboxylation as temperature increases. The oxygenation reaction leads to generation of glycolate which is translocated from chloroplast to peroxysomes. The subsequent metabolic fate of glycolate causes its oxidation, producing the major portion of H_2O_2 produced in photosynthesizing cells [17]. Another potential source of generation of ROS in plant is chlororespiration. It describes the reduction of molecular oxygen resulting from the presence of respiratory chain consisting of an NADPH dehydrogenase and a terminal oxidase in chloroplast that competes with electron transport chain for reducing equivalents. Although this process is more prevalent in algae but more recently the evidence of chlororespiration in the form of presence of respiratory chain in chloroplast is also noticed in higher plants [18].

Mitochondrial electron transport system is also a potential source of ROS (Figure 1) including superoxide, hydrogen peroxide, hydroxyl radicals [19]. Direct reduction of O_2 to $O_2^{\bullet -}$ anions takes place in flavoprotein region of NADH dehydrogenase segment of respiratory chain. Oxygen radical during mitochondrial electron transport is markedly enhanced in presence of Antimycin A, which blocks electron flow after ubiquinone. This results in the accumulation of reduced ubiquinone which may undergo autooxidation, resulting in the production of $O_2^{\bullet -}$. Several observations reveal ubiquinone as a major H_2O_2 generating locations of mitochondrial electron transport chain *in vitro* and it would appear that $O_2^{\bullet -}$ is a major precursor of H_2O_2 [20].

Superoxides are known to be produced during NADPH-dependant microsomal electron transport [21]. Two possible loci of $O_2^{\bullet -}$ production in microsomes are auto-oxidation of oxycytochrome-P-450 complex that forms during microsomal-mixed function oxidase (MFO) reactions and/or auto-oxidation of cytochrome P-450 reductace [22], a flavoprotein that contains both FAD and FMN.

Cell wall peroxidase is able to oxidize NADH and in the process catalyzes the formation of $O_2^{\bullet -}$. This enzyme utilize H_2O_2 to catalyze the oxidation of NADH to NAD^+, which in turn reduces O_2 to $O_2^{\bullet -}$ [23]. Superoxide consequently dismutates to produce H_2O_2 and O_2.

Other important sources of ROS in plants that have received little attention are detoxification reactions catalyzed by cytochrome P_{450} in cytoplasm and ER. ROS are also generated in plants at plasma membrane level or extracellularly in apoplast. Plasma membrane NADPH-dependent oxidase (NADPH oxidase) has recently received a lot of attention as a source of ROS for oxidative burst, which is typical of incompatible plant-pathogen interaction. In phagocytes, plasma membrane-localized-NADPH oxidase was identified as a major contributor to their bactericidal capacity [22]. In addition to NADPH oxidase, pH-dependent cell wall peroxidases, germin-like oxalate oxidases, and amine oxidases have been proposed as a source of H_2O_2 in apoplast of plant cell. pH-dependent cell wall peroxidases are activated by alkaline pH and in presence of a reductant produce H_2O_2. Alkalization of apoplast upon elicitor recognition precedes the oxidative burst and the production of H_2O_2 by pH-dependent cell wall peroxidases has been proposed as an alternative way of ROS production during biotic stress [23].

3. ROS in Acclamatory Stress Tolerance and Signaling in Plants

Accumulating evidences suggest that ROS, especially H_2O_2, is an active signaling molecule and its accumulation (oxidative stress) through redox sensing leads to variety of cellular responses [6, 24–26]. In fact, plant responses to ROS are dose dependent. High concentration of ROS results in cellular damage or even hypersensitive cell death [24, 27], whereas low concentration of ROS functions as developmental signal, controlling various aspect of plant biology [4, 6, 24]. Additionally, preexposure to abiotic and biotic stresses that induce an "oxidative burst" can trigger a protective function or immunize plants against environmental stresses, thus playing a role in acclamatory stress tolerance [6, 9, 24, 28].

One of the mechanisms contributing to oxidative stress induced signaling is the activation of defense genes, making specific acclamatory responses [29, 30]. For example, *Arabidopsis* plants respond to ROS signaling by boosting the antioxidative defense through upregulating the expression of antioxidative genes as well as activating the genes of inducible stress proteins [31, 32]. To corroborate this specific effect of ROS-mediated signaling involving expression of specific genes, several workers identified various oxidative stress responsive elements including specific promoters and transcription factors [33, 34]. However, the redox-sensing mechanisms and the associated signaling pathways induced by ROS are still obscure.

Another important avenue of ROS-associated signaling, as evident from the effort of various workers include production of various lipid peroxidation product as "biological signals" which do not require preceding activation of genes [35, 36]. Various radical species generated as a result of oxidative membrane damage (alkoxy radicals, peroxy radicals) and their subsequent resulting generated products (2,4-dienals, 2,4-decadienals, etc.) may initiate signaling event ultimately inducing programmed cell death, hypersensitive reaction, and so forth [37]. However, the redox-sensing mechanisms and signaling pathways induced directly by ROS as "signaling molecule" or indirectly by "secondary products" of oxidative lipid peroxidation that largely regulate the plant responses of environmental stresses requires further refining.

Plant adapt to environmental stresses through specific genetic responses. Molecular mechanisms associated with acclamatory stress tolerance, leading to the expression of genes as an early stress response, are largely unknown. However, it became gradually evident that the gene expression associated with acclamatory responses is highly sensitive to the redox state of the cell. In fact, plant cells have embraced the potential interactions with oxygen for metabolic regulations [38]. Surprisingly, ROS are important metabolites which participate in metabolism, growth, and morphogenesis of plant cells. The imposition of abiotic and biotic stresses can further increase the level of ROS [24, 39, 40].

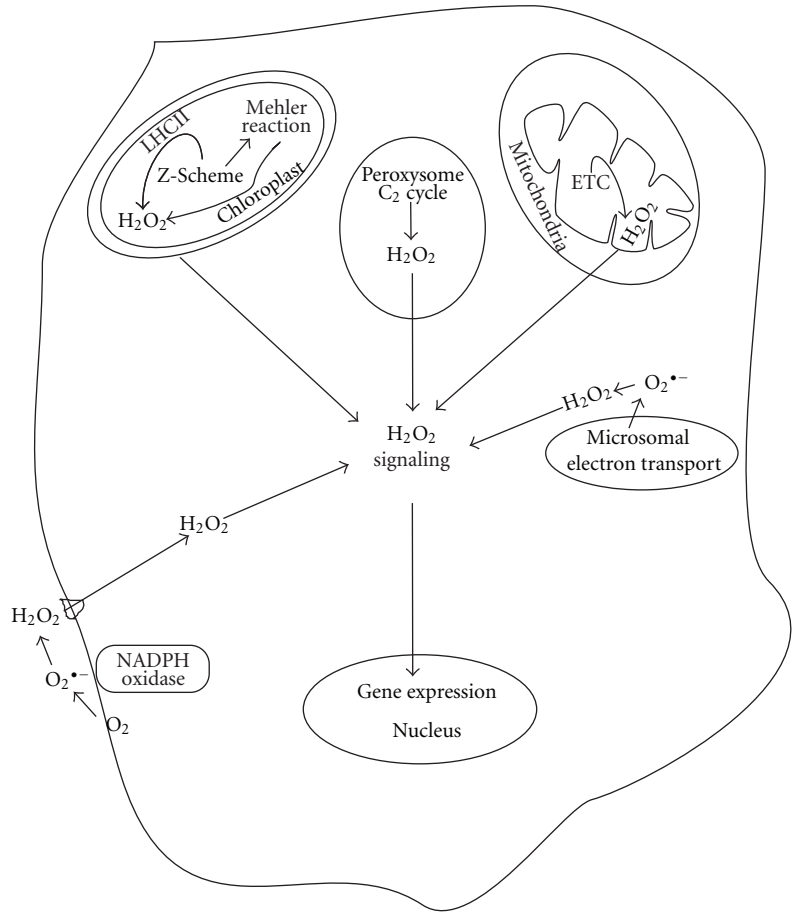

FIGURE 2: H_2O_2 generation in photosynthetic green cell. H_2O_2 produced by Mehler Reaction in chloroplast, ETC in mitochondria, C_2 cycle in Peroxisome, microsomal electron flow and specific enzyme-mediated reactions (NADPH oxidase) acts as a signaling molecule. The oxidant signal is transduced in the nucleus for triggering gene expression.

ROS are, therefore, implicated in most, if not all stress responses. Being highly reactive, most of the ROS can cause membrane damage, inhibit enzyme activities, and therefore when accumulated they are not compatible with cell function and considered to be deleterious and harmful. While $O_2^{\bullet-}$, $OH^{\bullet}$, and $^1O_2$ have very few well-characterized role in plant cells, except perhaps in senescence, H_2O_2 may have important metabolic roles [41–43].

The steady-state level of ROS in a cell is largely determined by the efficiency of the antioxidative systems [38, 44]. When the production of ROS exceeds significantly the capacity of the tissue to scavenge them, oxidative stress is favored. Much of the injury caused by the exposure of the abiotic and biotic stresses is associated with oxidative damage at cellular level. Augmentation of antioxidative defenses, therefore, plays a pivotal role in preventing stress-induced injuries and toxicities. Various efficient low-molecular weight antioxidants and quenchers like glutathione, ascorbate together with the activities of the antioxidant enzymes are generally increased in plants under stressful conditions and correlate significantly with enhanced tolerance [40, 45]. However, little evidences are available on the molecular mechanisms underlying the induction of defense genes.

There happen to be many putative bonafide signal transducing molecules under stress, for example, ethylene, ABA, salicylic acid [36–38]. Surprisingly, ROS like H_2O_2, $^1O_2$, and antioxidant molecule glutathione make important contributors to the redox state of the plant cell and are implicated in the activation of the genes that lead to the acclimation, stress tolerance, and other defense responses [38, 46]. It becomes gradually clear that gene expression associated with acclamatory stress responses is largely sensitive to the redox state of the cell. Of many components that contribute to the redox balance of the cell thiol/disulphide exchange reactions, particularly involving glutathione pool and the generation of ROS like H_2O_2 are the central components of the signal transduction in both environmental and biotic stresses.

Since H_2O_2 is an endogenous oxidant with moderately higher half-life and diffusible, that accumulates in many stress situations [38, 40, 47], a central role for this metabolite as a diffusible signal for selective induction of defense genes has been envisaged (Figure 2) [40, 48, 49].

4. Signaling Role of ROS in Systemic Acclimation to Photo-Oxidative Stress

In higher plants, dissipation of excess photochemical energy (EPE) is an immediate and finely tuned response which

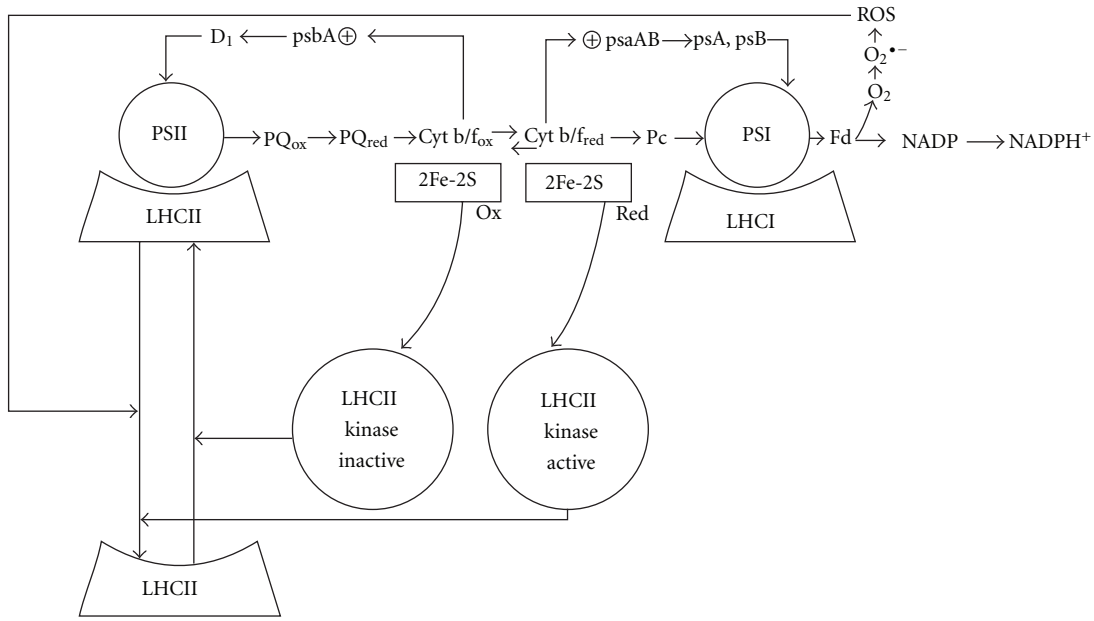

Figure 3: Mechanism of redox sensing in chloroplast. In presence of excess photochemical energy (EPE), plastoquinone became predominantly reduced (PQ_{red}), resulting in the activation of LHCII kinase via structural changes around Rieske centre (2Fe–2S) protein of cytochrome bf complex. The kinase in turn phosphorylates LHCII and PSII, resulting in migration of LHCII away from PSII and subsequently reducing light absorption by PSII. Oxidation of PQ_{red} reverses structural changes of cyt bf and leads to kinase inactivation. Phosphatase mediated dephosphorylation of mobile LHCII leads to reassociation of PSII and further increase in light energy by PSII. Alternatively, redox state of PQ controls adjustment of stoichiometry of PSI and PSII by transcriptional regulation of chloroplastic genes that encode apoprotein PSI (psA and psB proteins) and PSII (D_1 proteins) reaction centers. ROS produced under EPE utilization may cause physical separation of PSII from LHCII by degrading D_1 proteins, thus reducing light-energy absorption.

occurs through heat irradiation, alternative sinks for photosynthetic electrons, and ultimately downregulation of Photosystem II [50, 51]. The photoreduction of molecular O_2 is an alternative sink, especially when there is an acute dearth of $NADP^+$. But the spilling of electron to molecular O_2 is always associated with the formation of ROS, such as $O_2^{\bullet -}$, H_2O_2, $OH^{\bullet}$, and O_2 [40, 50]. If accumulation of ROS under conditions of EPE exceeds the capacity of enzymatic and nonenzymatic antioxidant systems to scavenge them, then photooxidative damage to photosynthetic apparatus ensues (Mehler reaction), which leads to cell death. This is manifested at whole plant level by the appearance of chlorotic lesions on damaged leaves. However, ROS may also play a positive role in response to EPE by initiating an increase in rate of degradation of DI protein, a key component of LHCII (Figure 3). This causes photoinhibition of photosynthetic electron flow, which may be a protective mechanism in such conditions. The possibility of both positive and negative roles for ROS under EPE suggests that it is more appropriate to view equilibrium between the processes that produce ROS and antioxidative defense which destroy them rather than the levels of these antagonists perse [50]. However, the immediate responses to EPE may lead to a whole plant acclimation which could include an alteration to the photosynthetic capacity of new leaves in which ROS also could play a role [38, 51]. Therefore, in natural environment, how well plants tolerate different abiotic stresses may be determined by their ability to deal with EPE before ROS seriously poses problems to cellular structures especially chloroplasts [52].

In recent years, several works highlight the impact of oxidative stress in response to fluctuating environmental conditions which elicit EPE on acclamatory responses of plants [53–55]. When a leaf experiences a set of conditions that promote EPE, such as EL (Excess Light) conditions, the immediate response is an intracellular signaling of antioxidant defense genes elicited by redox changes in the proximity of PS II, which still followed by a subsequent increase in H_2O_2 level. Prolonged exposure under such condition leads to the death of such leaves. However, leaves suffering EPE also produce a systemic signal, a component of which is H_2O_2, which set up an acclamatory response in unstressed regions of the plant. The signaling, mediated by H_2O_2, leads to an increased capacity to tolerate further episodes of EPE-induced photooxidative stress by remote activation of antioxidant defenses, that is, systemic acquired acclimation.

Experimentally, excess light (EL) applied to low-light (LL) adopted *Arabidopsis* for up to one hour causes rapid EPE and subsequently a burst in the titer of ROS, leading to the reversible photoinhibition [56]. Surprisingly, it was found that such a chloroplast-localized oxidative stress only induced genes encoding key components of cytosolic ROS scavenging systems. One of these genes APX 2, an ascorbate peroxidase isoform is induced only under EL. The induction of this nuclear gene is also found to be regulated by changes

in the activity of PS II, by rapid changes in the redox status of plastoquinone pool (PQ). Moreover, the major cellular antioxidant glutathione [57] blocked the induction of this gene by EL, implying that redox change in the cellular redox pool may play a role in chloroplast to nuclear communication.

Treatment of leaves with H_2O_2 and then exposure to EL caused significantly greater induction of APX2 than control EL alone. This surprising observation was investigated in more detail in a series of time course experiments which revealed that detached leaves pretreated with H_2O_2 showed a slower decline in maximal PS II efficiency under EL conditions than control leaves, indicating that prooxidant status of leaf may be a crucial factor in adapting EPE. Protective effects of H_2O_2 have been described for maize seedlings, chilled in dark [54], and have been explained by triggering various stress defense mechanisms [50].

Though all those data do not indicate whether H_2O_2 has a direct or indirect effect but strongly emphasize a role of this ROS in the acclimation to conditions which invoke EPE. The opposite effects of exacerbated PS II inhibition upon treatment with antioxidant GSH [41], which reduces H_2O_2 by enzymatic and nonenzymatic reactions, are consistent with such a role of H_2O_2.

5. Signaling Role of ROS in Acclimation to Other Environmental Stresses

Application of reactive oxygen species (ROS), specifically H_2O_2, can induce stress tolerance in plants. Treatment of winter wheat with low concentrations of H_2O_2 and inhibitor of catalase induced the synthesis of polypeptides similar to those synthesized under chilling stress [58]. Prasad et al. (1995) [59] also reported that maize seedlings became more chilling tolerant following pretreatment with H_2O_2. A transient increase in H_2O_2 was suggested to activate signal activation of protective mechanisms for acclimation to chilling [49, 59, 60]. Doke et al. (1994) [61] proposed that H_2O_2 generation should be considered as a central trigger for defense metabolism following exposure to abiotic and biotic stresses. H_2O_2 also found to be responsible for the expression for chilling responsive genes [60]. In *Arabidopsis*, treatment with H_2O_2 altered cytosolic calcium concentrations similar to those observed during cold acclimation [62].

In numerous studies, suspension cultures of *Arabidopsis thaliana* is used as model system to explain the signaling processes required for both the generation of H_2O_2 and subsequent cellular responses that it induces. Treatment of such cultures with harpin, a proteinaeous bacterial elicitor, induces rapid oxidative burst that require both protein phosphorylation and calcium influx [63]. In this case, as suggested by Doke et al. [61], H_2O_2 arises primarily from dismutation of $O_2^{\bullet -}$, which is formed via single electron reduction of molecular O_2 catalyzed by a plasma membrane-located enzyme similar to NADPH oxidase. In *Arabidopsis* suspension cultures both biochemical and pharmacological evidences are consistent with the activity of NADPH oxidase [64] and homologues of gp91, the key redox component of the enzyme complex [65].

Various lines of evidence support the existence of cross-tolerance, that is, induction of tolerance to a particular kind of environmental stress involving oxidative stress that also increases the tolerance to one or more other kind of stresses including biotic stresses. For example, O_3 exposure to *Arabidopsis thaliana* induces resistance to virulent *Pseudomonas* strains [66]. In this case, O_3 exposure resulted in the expression of a number of pathogenesis-related (PR) proteins and genes. Similarly, cotton plants exposed to water deficit were found to be more resistant to paraquat than water-depleted plants [67]. All these results provide evidence supporting the hypothesis that common redox signals are involved in the induction of acclamatory responses to both abiotic and biotic stresses. Evidences for the involvement of H_2O_2 and GSH in the signal transduction and regulation of gene expression is provided by reports of acquisition of stress tolerance by exposure to abiotic stresses with accompanying changes in gene expression [38, 68].

6. Signaling Role of ROS under Biotic Stress

In incompatible plant-pathogen interactions, H_2O_2 has been implicated in the elicitation of variety of defense responses [69]. Among these, the most significant is the induction of GSTs and GP_X [70]. ROS, particularly H_2O_2, can influence the recycling of GSSG and GSH, thereby have an impact on redox status of the cell (by changing thiol/disulphide ratio) which ultimately can instigate redox signaling of cell (Figure 4) [71].

In addition to oxidative burst, expression of defense-related genes is also induced by harpin in *Arabidopsis* suspension cultures. Such genes include PAL, encoding phenyl alanine ammonia lyase, a key enzyme of phenyl propanoid metabolism and GST, encoding glutathione-s-transferase, required for detoxification of lipid hydroperoxides generated during oxidative stress. The expression of these defense genes can be induced by H_2O_2 in a time and dose-dependent manner [72]. Experiments related to the identification of catalase as a salicylic-acid- (SA-) binding protein along with other relevant experiments suggest that H_2O_2 is downstream from SA in the pathogenesis-related (PR-1) gene induction [73].

Through a series of experiments, it was demonstrated that H_2O_2 is a diffusible signal in the induction of plant defense genes, such as glutathione-s-transferase and glutathione peroxidase (GP_x) [69]. A catalase trap, placed between soybean cells inoculated with an avirulent pathogen and uninfected cells, blocked the diffusible signal that originated from infected cells and was necessary for defense-gene induction [69]. Transgenic plants with elevated levels of H_2O_2 due to constitutive overproduction of glucose oxidase or repression of peroxisomal catalase were more resistant to pathogens, exhibited accumulated salicylic acid, and expressed pathogenesis-related genes [74–76]. Accumulation of H_2O_2 in terms of catalase-deficient tobacco plants was sufficient to induce the production of defense proteins (GP_x, PR-1), not only locally, but also systematically [76].

A general notion is that H_2O_2 is a diffusible molecule with half-life of only 1 ms, which essentially excludes of it being mobile signal for the induction of defensive responses

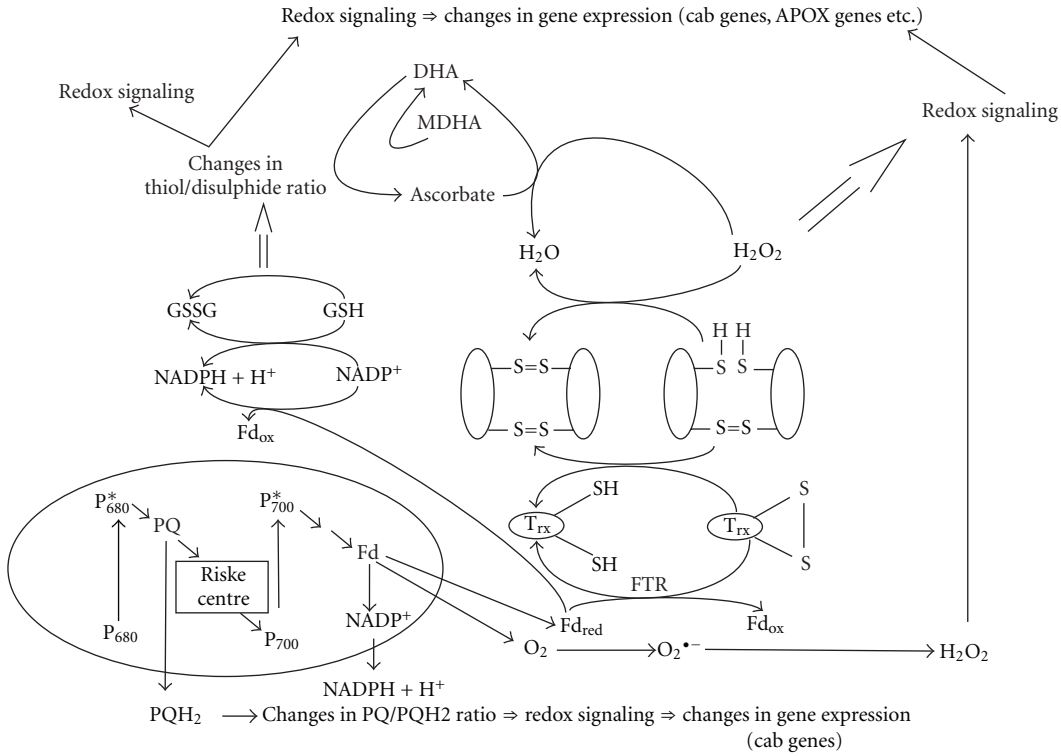

Figure 4: Thiol-disulphide, plastoquinone-plastoquinol interactions in chloroplast leading to activation of enzymes and redox signaling. In chloroplasts, reduced ferridoxin (Fd_{red}) reduces the regulatory protein thioredoxin (T_{rx}) via enzyme ferridoxin-thioredoxin reductase (FTR). This, in turn, activates chloroplastic enzymes. Molecular oxygen is a natural oxidant that reverses this activation via formation of superoxide ($O_2^{\bullet -}$) and H_2O_2·H_2O_2 is diffusive and moderately stable oxidant and inactivates these enzymes. H_2O_2 production particularly from the electron flow of Z-scheme of photosynthesis is exacerbated during stress. Ascorbate-glutathione cycle, on the other hand, destroys H_2O_2. Turning over of GSH pool is central to the processes. The accumulation of H_2O_2 together with changes in thiodisulfide, plastoquinone-plastoquinol ratio of cell, provides the redox signals leading to changes in gene expression.

in systemic tissues. But the work of Alvarez et al. (1998) [77] and Park et al. (1998) [78] proposed that this problem of short half-life of H_2O_2 may be overcome by a relay of H_2O_2-generating microbursts, including NADPH oxidase, as a mechanism for the reiteration of these microbursts. Such a model was proposed based on the observation of microscopic HR lesions that appear throughout the *Arabidopsis* plants upon infection with avirulent bacterial pathogens. This micro-HRs is correlated with acquirement of resistance and expression of defense genes (GST, PR-2) and at the same time could be blocked by the application of DPI, an inhibitor of NADPH oxidase. Moreover, application of H_2O_2 generating system, such as glucose oxidase to plants was sufficient to induce these responses [77].

Superoxide ($O_2^{\bullet -}$) or its derived products is also capable of inducing a signaling event, triggering defense responses. Phytoalexin synthesis in soybean cells in response to pathogens or elicitors is blocked by DPI and SOD but not by catalase [78–80]. Similarly, $O_2^{\bullet -}$, but not H_2O_2, is necessary and sufficient to induce lesion formation and PR-1 mRNA accumulation in "lesion-stimulating disease resistance response" mutant (lsd1) of *Arabidopsis* [82-83]. Furthermore, one of the members of tomato multigene family that encodes extensin is transcriptionally induced upon treatment with the $O_2^{\bullet -}$-generating compounds digitonin or xanthine oxidase, but not with H_2O_2 [81]. Bacteria and yeast induce distinct defense proteins in response to either $O_2^{\bullet -}$ or H_2O_2, although a considerable overlap exists between two responses. So, it is clear that $O_2^{\bullet -}$ can also act as signaling molecule in defense responses to execute its acclamatory function independently of H_2O_2. In spite of all these developments, there is emerging complexities in ROS production and its implication in cell signaling during plant-pathogen interaction [82].

7. Signaling Role of ROS in Programmed Cell Death and Senescence

H_2O_2 has been implicated as a key factor mediating programmed cell death (PCD) that occurs during HR in plants and also suspension cultures [83]. PCD is induced in *Arabidopsis* suspension cultures by harpin and this effect is mediated at least partially by H_2O_2 [64, 84]. Exogenous H_2O_2 induces cell death in a dose- and time-dependent manner. That such cell death is programmed is indicated by the requirement of transcription and translation and for a "presentation time" for exposure to H_2O_2 [65].

Data regarding intracellular signaling processes mediating H_2O_2 responses leading to cell death are scanty. Oxidative

stress results in increased cytosolic calcium [85] and H_2O_2-induced PCD in soybean cultures was dependent on Ca^{2+} influx and protein phosphorylation [86]. Mitogen-activated protein kinase (MAP) is activated in response to a number of abiotic and biotic stresses [87]. Neill et al. (1999) [88] reported that harpin induced the rapid activation of a 44 KDa protein kinase with the characteristics of an MAP kinase and that the same or similar kinase is also activated by H_2O_2. However, it remains yet to be established whether or not activation of protein kinase is absolutely essential for H_2O_2-induced PCD and gene expression.

Apart from PCD, another form of cell death that exists in nature is necrosis. Unlike PCD, which is genetically regulated, necrosis results from severe and persistent trauma and considered not to be genetically orchestrated [89]. However, both PCD and necrosis have been found to be just two distinct ends of the same process that can be initiated by the same signal, ROS [79]. The first evidence that ROS act as signaling molecule to initiate a signal transduction pathway towards plant cell death rather than kill cells by reaching toxic levels came from experiments in soybean cell cultures, in which short pulse of H_2O_2 is sufficient enough to activate a cell death mechanism [89]. Accordingly, exogenous H_2O_2 (>5 mM) initiate an active cell death pathway, requiring both DNA and protein synthesis in *Arabidopsis* suspension cultures [65]. Concentrations of H_2O_2 that activated cell death pathway were in both cases higher than the one inducing expression of defense genes [65, 69]. It is believed that H_2O_2 initiates a cell death programme via interplay with other signaling molecules, such as ethylene and salicylic acid. Jabs et al. (1996) [80] showed that $O_2^{\bullet -}$, but not H_2O_2 initiates a cell death phenotype in *Arabidopsis* lsd1 mutant, providing genetic evidence for the role of $O_2^{\bullet -}$ in plant cell death.

Arabidopsis mutant lsd1 grown under long days spontaneously forms necrotic lesion on leaves and is unable to stop spreading of cell death [90]. In front of the spreading zone of cell death, $O_2^{\bullet -}$ is drastically accumulating. Hence, $O_2^{\bullet -}$ seems to be critical signaling agent in cell death processes that is monitored via a "rheostat" LCD1. However, despite contradictory and controversial data $O_2^{\bullet -}$ versus H_2O_2 in cell death activation, ROS has been found to be indisputably the signaling agent who activates genetically regulated cell death programme(s) in plant.

8. Signaling Role of ROS in Growth and Morphogenesis

One of the most important adaptive strategies that plants used to adopt under stress is to slow down growth rate. The ability to reduce cell division under unfavorable environmental conditions may not only allow conservation of metabolic energy for defense purposes but may also reduce the risk of heritable damage. ROS being the ubiquitous messengers of stress responses likely play a signaling role in those adaptive processes. Application of menadione impairs G_1-to-S transition of cell cycle, thereby retarding DNA replication and hence the rate of mitotic cell division [91]. Another important evidence, where application of reduced glutathione (GSH), raises the number of meristematic cells undergoing mitosis, whereas the depletion of GSH has the opposite effect [92]. Although cell cycle progression is under negative control of ROS, H_2O_2 is formed to stimulate somatic embryogenesis [93] and is essential for root gravitropism [94]. In fact, the role ROS in plant growth and development, both under stress and normal environmental condition, is still poorly understood and requires further investigation.

9. Redox Sensing and ROS-Mediated Signal Transduction and Their Targets

Redox signals are the most fundamental forms of information monitored by the plants [95, 96]. More complex aspects of redox control of physiology of plants ultimately through the regulation of gene impression developed with the evolution of higher plants. It is now widely accepted that redox signals are the key regulators of plant metabolism, growth, and development and may even have plenty of cross-talking with other system of signal transduction of parts. In fact, controlled generation of ROS acts as "second messenger" along with other mediators like Ca^{2+}, not only in plant responses to environmental stress but also in hormone signaling [30, 97].

To have a comprehensive idea regarding the ROS mediated signal transduction, one has to develop the primary understanding of the fact that how the increased titer of ROS is sensed. One simple possibility is the direct modification of transcription factor with redox-sensitive groups [98]. Chemistry of ROS sensing dictates that the redox-sensing proteins have a commonality, with active thiol groups as potential ROS target [99]. But, in reality there seems to be much more complex signal transduction routes. In fact, the ROS-associated redox changes in the different organelles (mainly chloroplast and mitochondria) are signaled to the nucleus. Surprisingly the "sensing" function is generally performed by the antioxidative system itself. This system acts as a strong buffer against ROS, maintaining relatively low concentrations of oxidants under normal optimal conditions. The redox balances of the cell in generally perturbed without large changes in the concentration of ROS. This is feasible because of the presence of plethora of components capable of scavenging ROS. In case of catalase mutants placed under photo-oxidative stress or excess photochemical energy, H_2O_2 concentration is not greatly increased to the relative to the wild type but the glutathione pool is found to be massively perturbed [88, 98]. The enhanced availability of ROS may therefore be sensed by the cell as increased oxidative flux through key components, rather than marked increase ROS titer. This view strongly supports the existence of a dynamic system for allowing acclamatory changes through the components of antioxidative system that are integrated into the signal transduction network. It would allow appropriate response to occur as a result of increased flux of ROS, even in absence of mark changes in ROS titer. Apart from that, changes in ROS trigger marked modulation in the expression of gene far beyond their perception by antioxidative systems [95, 96]. This ultimately

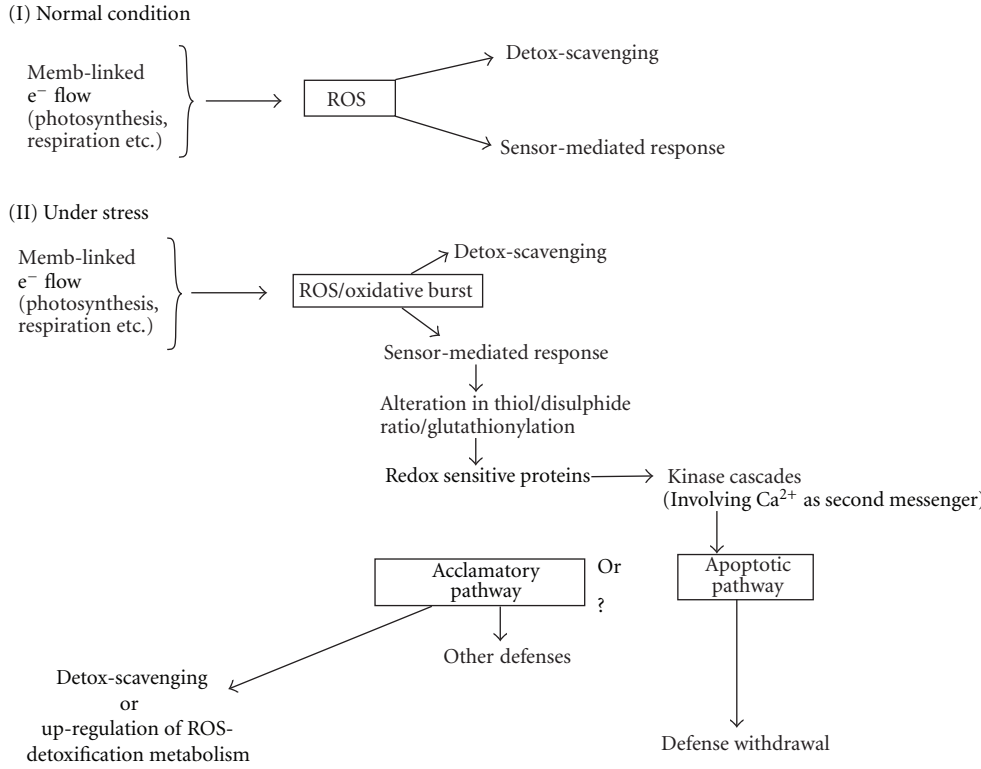

Figure 5: Model explaining the perception of enhanced ROS involving redox components or antioxidant system. Under normal growing condition (I), ROS are produced as a consequence of many metabolic events and are efficiently removed by detox-scavenging of antioxidants. However, under stress (II), increased production of ROS or oxidative burst causes increased oxidation of "sensor-scavenging" redox components locked into signal transduction pathways. Signaling pathway operates via kinase cascades and other secondary messengers that ultimately lead to upregulation of ROS detoxification capacity (acclamatory pathway) or cell death pathway involving defense withdrawal. The decisive factors that determine the feasibility of acclamatory pathway or cell death pathway are yet to found out but could involve the intensity of oxidative stress or ROS signal and their location.

confers upregulation of defense system against environmental stresses. Many studies strongly support the view that some antioxidative compounds have dual role of scavenging and signaling (through sensor scavenging). In these cases, the antioxidants probably exhibit low capacity of antioxidative role compared to classical detoxification scavenging (Figure 5).

Studies related to the nature of "sensor-scavenging component" revealed that haem-based enzymatic antioxidants such as catalase and ascorbate peroxidases and thiol containing antioxidant molecules such as GSH are basically the candidates for such responses. Plants also contain numerous proteins with redox-active thiol groups, some of which have been shown to have activity against peroxides. These include chloroplastic and cytosolic glutathione-peroxidases, chloroplastic and cytosolic peroxiredoxins, glutaredoxins and chloroplastic thioredoxins [100–102]. Although the exact roles of these thiol containing sensor scavengers remain to be elucidated, it is clear that there may be considerable divergence of function within each class of these molecules.

YAP-1 is a basic zipper type transcription factor which induces several genes in response to H_2O_2. Peroxides enhance the accumulation of nuclear YAP-1 by oxidizing cystein residue from intramolecular disulfide bond that appears to trap YAP-1 in the nucleus, thereby enhancing their reactivity [103]. *Arabidopsis* genome does not appear to contain sequence similar to YAP-1, but there may well be functionally similar elements in the initial perception of changes in radox state of plant.

Peroxiredoxins (PRX), otherwise known as thiroredoxin (THX) peroxides, has been shown to reduce peroxides using reductant from thioredoxins (Trx). The enzyme system is closely linked to Z-scheme of photosynthesis via a specific chloroplastic Trx isoform [104]. In fact, the primary role of these thiol containing redox-sensitive proteins is not to detoxify peroxides but to sense enhanced production or in other words to initiate a signaling process by acting as a "signal-scavenging" component (Figure 5).

Another mechanism of redox-signal perception involves oxidation of glutathione pool accompanied by an increase in total glutathione under environmental stresses [101]. The method of sensing this redox perturbation involves protein glutathionylation, in which GSH forms a mixed disulfide with target protein, thereby modifying the activities of enzymes and transcription factors. This process is considered to play an important role in redox signaling and protection of protein structure and function.

Another significant class of thiol-containing protein is certain subclasses of GST super family which is active in reducing peroxides or dehydroascorbates (DHA) or in

catalyzing thiol-transferase reaction [105]. Some of these GSTs are strongly induced by oxidative stress especially under biotic stresses. So, it seems that the power of ROS can be effectively harnessed (as most of these are short lived) to convey redox information. These signals in most of the cases are incorporated into complex redox network that involves electron carriers (plastoquinone, ubiquinone) and electron accepters like (TRX, Fd).

10. Redox-Sensitive Proteins and Redox Signaling

Plant cell can sense, transducer, and translate the ROS signals into appropriate cellular responses through the involvement of redox-sensitive proteins. Redox-sensitive proteins mainly operate through reversible oxidation/reduction thereby switching "on" and "off" depending on the cellular redox state. ROS can oxidize the redox-sensitive proteins directly [96] or indirectly via some other ubiquitous redox-sensitive molecules like glutathione or thioredoxins which control the cellular redox state [96, 101]. Therefore, redox-sensitive proteins are susceptible to oxidation and reduction that depends on the titer of ROS and, or redox state of the cell. Redox-sensitive proteins further execute their function via downstream signaling components like kinases, phosphatases and transcription factors. In some cases, ROS directly oxidize the target proteins, particularly peroxyredoxins and thioredoxins and subsequently the transcription factors [96, 102]. In fact, most of the redox regulation of gene expression is mediated by a family of protein disulphide oxidoreductases, namely thioredoxins, peroxyredoxins, glutaredoxins, and protein disulphide isomerases [101]. Thioredoxins are small (approximately 12 kDa) portein with S=S reducing activity. They oxidized directly by ROS or indirectly by peroxyredoxins (thioredoxin peroxidase). Thioredoxins may be reduced by thioredoxin reductase, by NADPH dependent enzymes. There are ample of evidences to suggest that thioredoxins and other similar proteins are enzymatic mediators of the regulatory effects of ROS at transcriptional levels [95]. It is found that UV irradiation promote translocation of thioredoxin to the mammalian nucleus where it activates stress-related transcription factors like NF-κB and AP-1 by enhancing DNA binding [95]. Thioredoxin can bind directly with NF-κB p^{50} and interacts indirectly with AP-1 through redox factor 1 [96]. On the other hand, plants possess chloroplastic, mitochondria, and cytosol redox-regulating system for controlling expression of genes [95, 101, 102]. Ferredoxin, being the component of PS I of Z-scheme of electron flow gets photoreduced during photosynthesis. The reducing power of Fd is then subsequently transformed to thioredoxin by Fd-Trx reductase, which then interacts with target enzymes. There are distinct classes of "thioredoxin target" including the "transcription factors." One of the best studied redox-regulatory plant proteins is a class of RNA-binding proteins that control translation or stability of chloroplastic mRNA under the exposure of photochemical energy by the way of ferredoxin-thioredoxin system [95].

11. ROS, Redox Signaling, and Gene Expression

The molecular mechanisms associated with signal transduction, leading to changes in gene expression are early stress response and largely unknown. It is clear, however, that gene expression associated with acclamatory responses is sensitive to redox state of the cell. Of many components which contribute to the redox balance of the cell, two factors have been shown to be crucial in mediating stress responses. Thiol/disulphide exchange reactions, involving glutathione pool and the generation of ROS, particularly H_2O_2, are the central components of signal transduction in both abiotic and biotic stresses.

Plant cells contain considerable amount of nonprotein thiols which are involved in many molecular, metabolic, and physiological functions via thiol/disulphide exchange reactions [13]. Thiols are important antioxidants and are also involved in DNA and protein synthesis as well as activation and inactivation of enzymes. Glutathione is a major low-molecular weight thiol compound in plants [106]. There are ample of evidences that glutathione plays a pivotal role in the defense systems in plants as an antioxidant, in the detoxification of xenobiotics by glutathione-s-transferase [106], as a precursor of phytochelatines and as an inducer of genes [107]. In spite of having compelling lines of evidence, considerably less is known about the role of GSH and its oxidized form, glutathione disulphide (GSSG), in the signal transduction system of plants. Disruption of the gene for γ-glutamylcystine synthase in the glutathione-deficient mutant of yeast is lethal. Similarly, depletion of GSH in *Arabidopsis thaliana* cell suspensions renders them susceptible to oxidative damage [108]. Taken together, these observations suggest that glutathione is an essential component of plant cells. The glutathione pool may regulate gene transcription in response to pathogen attack, an effect exacerbated by H_2O_2.

Thiol modulations of enzymes is a wide-spread mechanism of flux control in plant metabolism. In particular, the pathway of C_3 carbon assimilation is essentially activated and inactivated by –SH group modifications [109]. The activities of key enzymes of C_3 pathway such as fructose-1,6-bisphosphatase, sedoheptulose-1,7-bisphosphatase, are modulated by changes in redox state of the cell. The redox state of cytosol is coordinated with that of chloroplast by metabolite transport across the chloroplast envelope. In addition to control of metabolism, redox state of the cell affects the expression of genes for chlorophyll a/b binding proteins (cab genes, Figure 4) [38]. The redox state of the cell not only regulates gene transcription in responses to abiotic and biotic stresses [45] but has also been implicated in the activation of plant transcription factors [45].

Identification of all changes in gene expression regulated by oxidative stress is of considerable importance for developing stress tolerant plants. However, till date a global analysis of the effect of ROS on the transcriptome of any one plant species has not yet been completely described. In bacteria ROS induces expression of at least 80 genes [82]. In yeast, approximately 300 genes and in plants more than 100 genes are found to be induced by ROS [110] and the numbers are

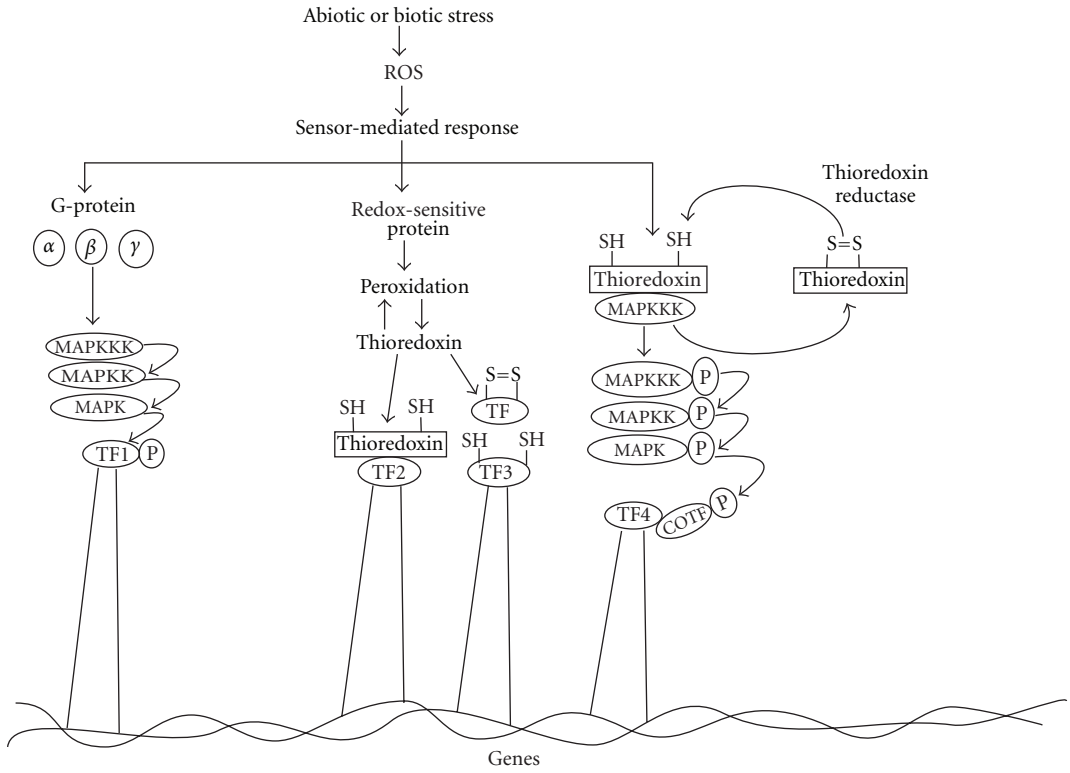

Figure 6: Pathways through which Reactive oxygen species (ROS) can influence gene expression (ROS—reactive oxygen species, MAPK—mitogen activated protein kinase, MAPKK—MAPK kinase, MAPKKK—MAPKK kinase, TF—transcription factor, COTF—transcription cofactor).

growing with the application of cDNA microarray technique to carry out a transcriptomic analysis of oxidative stress-regulated gene expression [111].

Bacterial genes are organized in regulons that are controlled by specific transcription factors. $O_2^{\bullet -}$-induced genes are controlled by the Sox R protein that have Fe–S cluster and upon oxidation induces the expression of a downstream transcription factor called Sox S. H_2O_2-induced genes are controlled by the oxidation of thiol groups present in the transcription factor Oxy R [112]. Alternatively, two-component systems may activate the expression of bacterial genes upon changes in the redox status of the cell. A redox sensor, a membrane-associated phosphoprotein, becomes phosphorylated on histidine when it is either oxidized or reduced by components of electron transport chain. Its substrate, the redox response regulator, is a sequence-specific DNA binding protein that is phosphorylated at an aspartate residue that regulates transcription [113]. In yeast, genes induced by redox signals consist of a complex network of different regulons, so-called stimulons [114]. Activities of one of the best studied redox-sensitive transcription factors, yAPI, are found to be controlled by redox signals at the level of nuclear localization and DNA binding [115]. Induction of oxidative stress causes relocalization of yAPI from cytoplasm to nucleus and its DNA binding capacity increases many fold. In mammalian systems, many studies point to the significance of two classes of transcription factors that are sensitive to redox signals: the nuclear factor κB (NF-κB) and the activator protein-1 (AP-1). The pro-oxidant state in cytoplasm (largely determined by the ratio of GSSG and GSH) or ROS activates these transcription factors and induces their mobilization to nucleus, where a reducing environment is required for proper DNA binding. Thioredoxins and redox factor Ref-1 provide the reducing power for DNA binding [116]. Therefore, two major steps in transcriptional activation of eukaryotic transcription factors seem to be influenced by redox balances: nuclear relocalization and DNA binding.

In plants, generation of ROS occurs under diverse range of conditions, and it appears that ROS accumulation in specific tissues and appropriate quantities is of benefit to plants and can mediate cross-tolerance towards other stresses. ROS, specifically H_2O_2 is found to be involved in plant defense response, affecting both gene expression and activities of proteins such as MAP kinase, which in turn functions as regulators of transcription [117, 118]. In spite of the fact that ROS and cellular redox state are known to control expression of plant genes, the signaling pathway(s) involving transcription factors or promoter elements specific for the redox regulation are still to be identified. There are, however, several candidates for promoter elements as well as DNA-binding factors that act as redox response elements (Figure 6).

One of the examples of induction of defense genes, controlled by redox balance of the cell, is Glutathione-S-transferase (GSTs), which catalyses the conjugation of GSH

to a variety of hydrophobic electrophilic compounds that otherwise attack important cellular macromolecules. Compounds with bound GSH undergo cellular detoxification pathway [119]. The signal by which the expression of GST gene is regulated is believed to be a prooxidant state of the cells, probably resulting from a reduced GSH content [119]. The promoter element responsible for the induction of the Ya subunit in mouse GST by electrophilic compounds consists of two adjacent AP1-like sites [120]. The consensus sequence of this site is AGACA (A/T) (A/T) GC and is called an antioxidant-responsive element or electrophile-responsive element. Two adjacent AP1-like sites are also present in the *Arabidopsis* GST 6 gene and constitute promoter element [121]. This promoter element is at least in part, required for GST 6 inductions by H_2O_2, SA, and Auxins [122]. A single antioxidant-responsive element has recently been identified in the promoter of a maize catalase gene (Cat 1) and was found to bind molecular factors from senescing scutella that accumulate Cat 1 transcripts, probably as a result of oxidative stress [123]. Very recently, production of ROS under the influence of organic volatile compounds has been held responsible to induce a signaling pathway for ACO gene expression in tomato plants [124].

The G box (CACGTG) is a ubiquitous *cis*-element present in many plant genes and is thought to mediate response to diverse environmental stimuli, including primarily redox changes [125]. Together with the H box (CCTACC), the G box functions in the activation of phenyl propanoid biosynthetic genes. Transcription of at least two of these genes that encode phenylalanine ammonia-lyase (PAL) and chalone synthase (CHS) is under redox control (mainly controlled by GSH) [126].

Heat shock elements and heat shock factors also participates in redox-regulated gene expression. Activation of heat shock factor is characterized by the conversion of this factor from monomer to trimeric state, a process induced by heat shock and large variety of conditions that generate abnormally folded proteins. Disulfide-linked aggregates of cellular proteins are formed as a consequence of disturbed intracellular redox homeostasis and are one of the signals required for HSF trimerization [116].

A novel homeodomain protein of HD-zip class isolated from tomato and zinc-finger protein LSD1 from *Arabidopsis* are negative regulators of oxidative cell death and have been proposed to act as transcriptional regulators down-stream of ROS signal [127].

The molecular mechanism underlying the regulation of gene expression through redox-sensitive proteins involves either the oxidation of thiol group of proteins or through the oxidation of Fe–S clusters, both of which may be integral part of target redox sensitive proteins [128, 129]. The former one involves the oxidation of –SH group of redox-sensitive groups by ROS to yield sulphenic (–SOH), sulphinic ($–SO_2H$), or sulphonic ($–SO_3H$) moieties or may form intramolecular or intermolecular disulphide bonds. The changes in the chemistry of –SH groups modify the electronic and steric conformations of cysteine residues, thereby causing conformational changes or protein-protein interactions [130]. All these changes alter the functionability of proteins that have cysteine residues at strategic positions [131]. Another mechanism where an Fe–S cluster of redox-sensitive proteins suffers oxidation by ROS, especially $O_2^{\bullet -}$, inactivates the cluster and affects enzyme activity [131]. Additionally, Fe^{2+} released from Fe–S clusters of redox-sensitive proteins stimulates the formation of $OH^{\bullet}$ by Haber-Weiss reaction. The activity of many PCRC enzymes, glycolytic enzymes and the coupling factors that provide ATP for biosynthetic reactions is under redox control [38, 111].

The classical example of redox-sensitive protein or receptor in plant is the cytochrome bf complex of Rieske centre of PS II of photosynthesis. During the light-harvesting half of photosynthesis, both PS II and PS I work in tandem and the light energy funneled to the two reaction centers must be controlled. When the light harvesting is not balanced by light energy utilization and dissipation, toxic radicals formed, leading to the photooxidative damage. One of the control mechanisms regulate dissociation of LHCPII from PSII, a process controlled by phosphorylation. The kinase responsible for the phosphorylation of LHCPII is activated by reduction of plastoquinone pool, a signal that is transduced to the kinase activations via structural changes in Fe–S protein associated with the cytochrome bf complex [132]. Additionally, the redox status of plastoquinone controls the rate of transcription of chloroplast genes encoding PS II and PS I reaction centre apoproteins [133]. The redox state of plastoquinones and thiol/disulphide ratio also control expression of nuclear genes associated with the synthesis of antioxidative enzyme ascorbate peroxidase (APx 1 and 2, Figure 4) [134].

12. Enzymatic and Nonenzymatic Oxidative Membrane Lipid Peroxidation and Initiation of Nonspecific Signaling Events in Plant

Membranes are the vital structure for all organisms which not only controls molecular trafficking but also perceives of environmental cues. Exposure of abiotic and biotic stresses instantaneously causes change in membrane architecture. In fact, membranes must respond to environmental stresses (extremes of temperature, drought, salinity, infections, etc.). It is a matter of great surprise that how is a fast response to a broad spectrum of different stimuli is being perceived and transduced to a response. The easiest way to address this problem is to initiate a non-specific response by transformation of cell membrane to signaling compounds within a small time frame by minute expenditure of energy. PUFA, being a most O_2-sensitive molecule, is the ideal compound to satisfy these conditions [135]. All plants contain PUFA in their membrane which may be stored in the surface of the cell or organelle as free PUFA or may remain conjugated as phospholipids or galactolipid.

Peroxidation of membrane lipids (primarily the phospho-lipids and galactolipids of plant cell and thylakoid membranes) are mechanistically important from free radical production perspective, as it is one of the few examples of

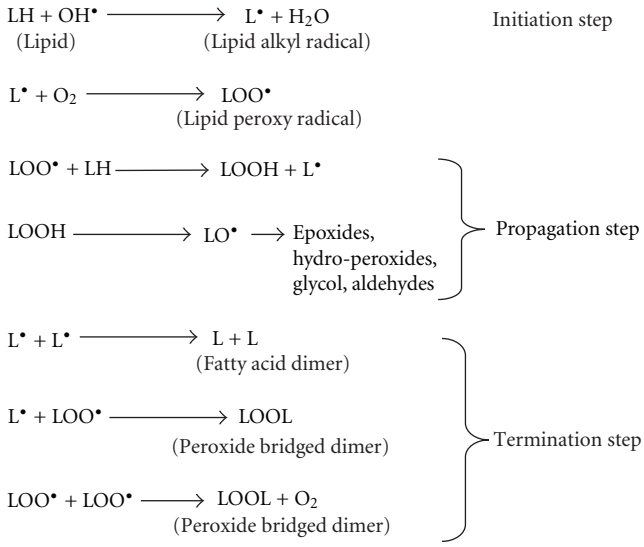

Figure 7: Membrane lipid peroxidation: A potential source of reactive oxygen species in plant cell.

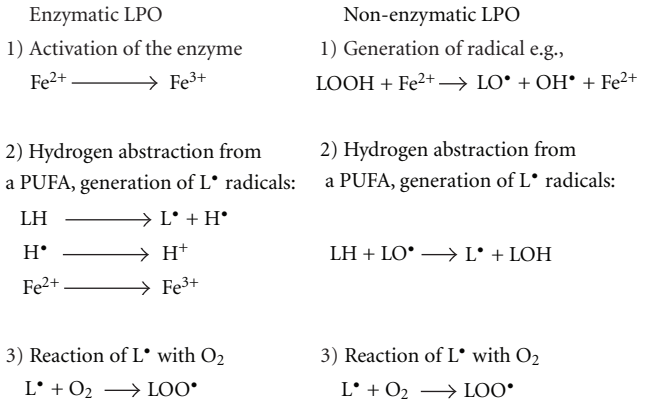

Figure 8: Tandem action of enzymatic (Lipoxygenase mediated) and non-enzymatic membrane lipid peroxidation (MLP) in plants.

carbon-centered radical production in plant cells [136]. Peroxidation of lipids in plant cells appears to be initiated by a number of ROS itself. Essentially, membrane lipid peroxidation involved three distinct stages (Figure 7), which include initiation, progression, and termination. Initiation event involves transition metal complexes, especially those of Fe and Cu. The role of these metal complexes lies in the fact that either they form an activated oxygen complex that can abstract allylic hydrogens or as a catalyst in the decomposition of existing lipid hydroperoxides. Although $O_2^{\bullet -}$ and H_2O_2 are capable to initiate the reactions but as $OH^\bullet$ is sufficiently reactive, the initiation of lipid peroxidation is mainly mediated by $OH^\bullet$. Loosely bound Fe is also able to catalyze the decomposition of lipid peroxides resulting in the formation of alkoxy and peroxy radicals, which further stimulates the chain reactions of lipid peroxidations (Figure 5) [136, 137]. It is likely that physical structures of plant membranes which places the fatty acid side chains in close proximity facilitates autocatalytic propagation of lipid peroxidation.

Lipid peroxidation in plant cells can also be initiated by the enzyme lipoxygenase (Figure 8). The enzyme is able to initiate the formation of fatty acid hydroperoxides and ensuing peroxidation [128]. During senescence, lipoxygenases (LOX) are activated [138, 139]. These are enzymes which oxidize polyunsaturated fatty acids (PUFAs). PUFAs which are characterized by the presence of one or more structural elements –CH=CH–CH_2–CH=CH– became the target of LOX. Lipoxygenase transform PUFAs in a reaction called lipid peroxidation (LPO) to lipidhydroperoxides (LOOHs). The latter are unstable and are decomposed to a great variety of products. LOX removes in a regio- and stereospecifically controlled reaction a hydrogen atom from a double allylically activated CH_2-group of PUFA. While still bound to the enzyme, the hydrogen atom reacts with the complex-bound Fe^{3+} in the active center of LOX by the formation of a proton and a Fe^{2+} ion. The lipid radical $L^\bullet$ adds oxygen and generates a peroxy radical ($LOO^\bullet$). Subsequently, an electron migrates from the Fe^{2+} to the peroxy radical producing a peroxy anion (LOO^-). The latter combines with the proton to LOOH (Figure 8) [140]. It is important to note that during this process of enzyme-catalysed LPO the peroxyl radical is not able to escape from the enzyme complex.

The connection of senescence with LPO is corroborated by an increase in LPO products and reactive oxygen species with age [141]. Identical oxidation products are detectable after pathogen attack and in highly enhanced amounts after mechanical crushing (homogenation) of plant tissue [142]. This is a severe type of wounding and therefore multiplies the responses observed by pathogen attack.

Any kind of stresses cause change in membrane structure which apparently activates membrane bound phospholipases. Phospholipases include a large family of that can be distinguished according to their ability to attack different ester bonds at glycerol backbone [143]. PUFAs are mostly located in position 2 of glycerol backbone. Phospholipids are cleaved exactly at this site by the action of phospholipase A_2 [144]. Phospholipase A_2 has been found to be rapidly activated under stress, wounding, and exposure of elicitors [143], causing hydrolysis of phospholipids and oxidized phospholipids [145].

The obtained free PUFA having Cis, Cis,-1,4-pentadiene moiety (–CH=CH–CH_2–CH=CH–) became the substrate for the enzyme lipoxygenase (LOX). There are ample of evidences that during senescence and under stress LOX are activated [135]. LOX transforms PUFAs in a chain reaction cascades (Figure 8), called membrane lipid peroxidation (MLP) to lipid hydroperoxides (LOOHs). LOOHs are subsequently converted to a number of great varieties of secondary products. These MLP products and their subsequent resulting generated products thereof represent "nonspecific biological signals" which do not require preceding activation of genes. They are produced as a nonspecific response to a variety of environmental stresses or environmental stimuli which neither need any specific gene expression nor

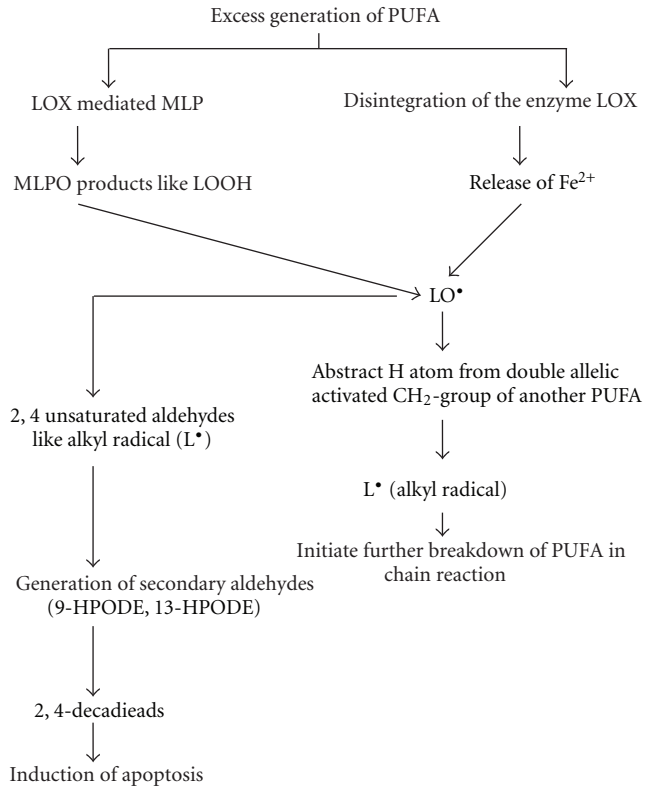

Figure 9: Model of events followed by oxidative degradation of membrane associated PUFA, producing lipid hydroperoxides (LOOH), and subsequently a great variety of secondary products representing "biological signals."

a long downstream signaling cascades for evoking their signals [38, 139].

The product of LOX mediated MLP process alone hardly have any role to play in signaling event and mostly implicated in oxidative degradation of membrane lipid associated with the symptoms of stress associated aging and senescence [139, 142]. Therefore, the event of molecular changes of membrane lipid, starting with the activation of phospholipases followed by LOX and subsequent generation of LOOH has long been advocated as possible mechanism of membrane damage associated with unfavourable environmental cues [146, 147].

However, recent experimental evidence strongly favors the fact that LOX mediated MLP processes often switch to a nonenzymatic MLP [139, 148], when supply of substrates (PUFA) exceeds a certain limit, as evident in case of programmed cell death [149, 150]. In fact, when supply of PUFA is significantly higher, it is found that LOX not only catalyzes MLP but also commits suicide by catalyzing the disintegration of its own molecule. This causes the release of enzyme bound Fe-ion [149, 150], which subsequently reacts with the end product of LOX mediating MLP, that is, LOOH to produce $LO^{\bullet}$ in a Fenton-type reaction [8]. $LO^{\bullet}$ radicals then abstract an H atom from the double allylic activated CH_2-group of another PUFA, forming a new $L^{\bullet}$ radical, thus inducing once again the chain reaction (Figure 9).

Essentially the enzymatic MLP differs from nonenzymatic MLP by the fact that enzymatic MLPO is regio- and stereospecifically controlled. Whereas nonenzymatic MLPO causes formation of all possible stereo isomers by causing ROS attack on PUFA at all double allylic activated CH_2-group. Another significant point of differences based on the consequences of both enzymatic MLPO and nonenzymatic MLPO resides in the fact that in enzymatic MLP process peroxiradicals formed still converted to anion inside the enzyme complex of LOX, while nonenzymatic MPLO produced peroxyradicals due to their hyper chemical reactivity randomly attack other molecules with activated X-H bonds in vicinity (preferentially double allylic activated CH_2-group of free PUFA) [151].

Alkoxy radicals are generated primarily by the reaction of LOOH with Fe^{2+}, although most of the LOOH are reduced by peroxidases to corresponding hydroxyl radicals [152]. Alkoxy radicals generated, subsequently decomposed to 2,4-unsaturated aldehydes (mainly 2,4-dienal) and an alkyl radical ($R^{\bullet}$). The generated 2,4-dienal may be further elevated to a secondary aldehyde molecule [139, 148].

In a study with linoleic acid (the simplest PUFA preferentially acting as substrates for LOX) transformation during MLP, it is found that oxidative degradation causes formation of 9-hydroxyperoxy-10,12-octadecadienoic acid (9-HPODE) and 13-hydroxyperoxy-9, 11-decadinoic acid (13-HPODE). HPODEs are subsequently reduced to their corresponding alcohols. These represent the major MLPO product that accumulates in senescent and dehydrated leaves [153].

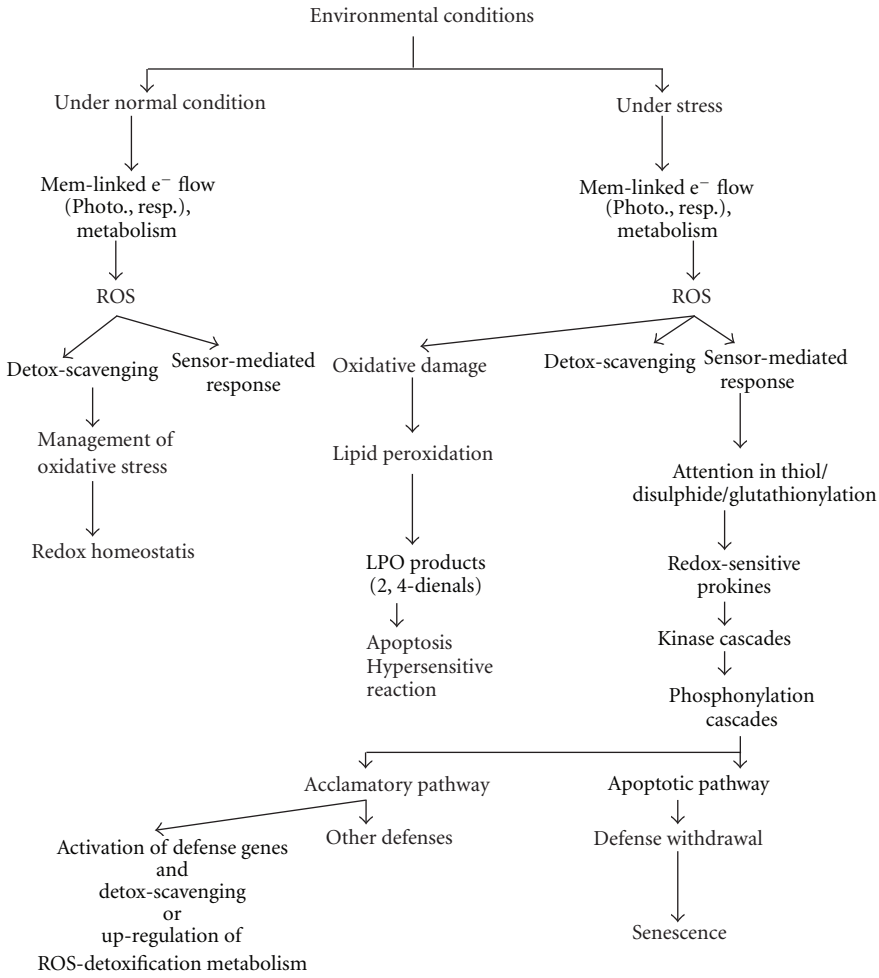

Figure 10: Integrated model of events under different environmental conditions, showing implications of ROS associated with different signaling events.

Alternatively the alkoxy radicals generated by decomposition of 9-HPODE is cleaved to 2, 4-decadenienal which induces apoptosis [154]. For a long time, 2, 4-decadenienal has been known as product of deteriorated fat compounds [155], in spite of their known reactivity. The observation that 2, 4-decadenienal can induce apoptosis is of great importance. These compounds represent the first response of cells to change in membrane architecture under stress. Its generation unlike other signaling agents like C_2H_4, ABA, JA, SA does not require a preceding gene expression. Therefore, as a precursor of death initiating signal, both linoleic acid and 2, 4-decadenienal (the decomposition product of MLP) is physiologically extremely significant. However, the generation of compounds active in signaling is not restricted to MLP products of free PUFAs but also concerns phospholipids in which original PUFA got preferentially oxidized, like degradation products of hydroperoxides of phospholipids [156] of muscle cells. Similar products are also assumed to be produced under oxidative stress triggering downstream signaling event without gene expression ultimately exhibiting a response (Figure 9) [129, 156].

13. Conclusion and Perspective

The rationale of the present chapter describing physiochemical significance of reactive oxygen species formation in plant has its root in myriad of metabolic aberrations in which ROS has been implicated for sensing environmental cues. In fact, ROS and their language of signaling are core regulators of plant cell physiology and cellular responses to environment (Figure 10). Besides exacerbating cellular and macromolecular damage, ROS can act as ubiquitous "signaling molecule" or "second messenger" in normal plant cell function. In fact, ROS are a central component in stress responses and the level of ROS or redox status of the cell determines the type of responses, whereas at high concentrations cell death is initiated, at low concentrations, ROS initiate defense genes and adaptive responses. Numerous sources of ROS and complex regulatory system of antioxidant functions provide the flexibility necessary to allow the dual role of ROS. However, how these regulatory system functions at cellular level to achieve the spatiotemporal level of ROS is still poorly understood.

Plant adapt to environmental stresses through specific genetic responses. The molecular mechanisms associated with signal transduction, leading to changes in gene expression early in the stress response, are largely unknown. It is clear, however, from the present discussion, that gene expression associated with acclamatory responses is sensitive to the redox-state of the cell.

In fact, sublethal amounts ROS acclimate plants to both abiotic and biotic stresses. Of the many components which contribute to the redox-state of the cell, generation of oxidant H_2O_2 is the central component in the signal transduction in both environmental and biotic stresses. Oxidants, especially H_2O_2, act as multifunctional triggers, modulating metabolism and gene expression. H_2O_2 alone or in association with other oxidants act in intracellular and systemic signaling systems to achieve acclimation and tolerance to both abiotic and biotic stresses. Although substantial genome response and activity of many enzymes are known to be affected by ROS, molecular mechanisms underlying the adaptive responses and the signaling network that controls the responses are still elusive. The oxidative membrane lipid peroxidation products and the resulting generated products thereof also represent "biological signals," which do not require preceding activation of genes and produce nonspecific response to a large variety of environmental stresses.

ROS communicate with other signaling molecules and being part of the signaling network may control responses both downstream and upstream of ROS. The facts discussed in the review indicate that we are now going through the initial steps in understanding how oxidants/ROS modulate signal transduction pathway with or without activating genes. So, in spite of tremendous development in our understanding of ROS biology, the exact nature of ROS-signaling network largely remain obscured. Molecular Genetic studies in addition to unique physiological approaches will be required to ascertain the position of ROS in the signal transduction pathways and also to understand how these short-lived endogenous signaling compounds are perceived and transduced to specific and nonspecific responses necessary for survival of plants. This will ultimately help us to screen better performing plants under environmental stress for breeding programme.

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