Plant Signalling: Response to Reactive Oxygen Species

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Abstract

It is noteworthy to mention how the last 20 years have modified the concept of signalling in plants, especially the molecular crosstalk associated with it. Plants have the ability to show remarkable developmental plasticity to sustain in a continually changing environment. In response to various environmental stresses such as drought, salinity, metal toxicity, temperature and pathogens, plants defend themselves by developing some special defence mechanisms. Plants recognise these environmental signals with the help of some membrane protein sensors and then transduce these signals to the nucleus which ultimately stimulates various transcription factors and genes to form the product that ultimately leads to plant adaptation and assists the plant to sustain and surpass the adverse conditions. Amongst the environmental factors which are involved in signalling is the reactive oxygen species (ROS) generated during cell metabolism. ROS are spontaneously produced in the cell enzymatically through the action of various soluble membrane-bound enzymes and nonenzymatically by autoxidation reactions. Some of these ROS (e.g. superoxide dismutase, hydrogen peroxide and nitric oxide) are physiologically useful and in fact necessary for life but can also be harmful if present in excess or in inappropriate amounts. Current research in this regard focuses more on the

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development of transgenic plants with enhanced tolerance to ROS by using genetic approaches and analytical techniques. In particular nitric oxide (NO), a reactive radical, may be involved in the defence mediated by the ROS such as defence gene activation, hypersensitive response cell death and phytoalexin biosynthesis. By using biotechnological approaches NO together with ROS activates a stronger response and tolerance to various stresses in plants.

Keywords

Signal transduction pathways • Defence mechanism • ROS • Oxidative stress • NO • Transgenic plants

1.1 Introduction

Plants are sessile organisms and are constantly being exposed to a plethora of biotic as well as abiotic stress conditions such as temperature, drought, heavy metal and salinity stresses (Mahajan and Tuteja 2005; Hakeem et al. 2012) (Figs. 1.1 and 1.2). These stress conditions greatly influence the plant growth, development, distribution and productivity. Besides, these stress conditions influence various metabolic reactions that lead to the production of different toxic compounds such as ROS formation which have deleterious effects on the physiology of plants. However during the course of evolution, plants have developed an array of mechanisms manifested through modified physiology to sustain environmental abiotic stress and resist hurdles originating from biotic stress. Plant acclimatisation to abiotic stress conditions usually depends upon the stimulation of cascades of molecular channels involved in stress perception, signal transduction and the expression of particular stress-related genes and metabolites (Guo-Tao et al. 2012). These abiotic as well as biotic signalling agents typically bring about their effect by means of sequences of biochemical reactions, termed as signal transduction pathways, that greatly amplify the original signal and ultimately result in the stimulation or repression of genes. Signal transduction is the term generally used to define the distinct array of biochemical mechanisms that control cellular physiology. The term "signal transduction" became attractive in the early 1980s, and now it is considered to be one of the most intensively studied areas that is spawning increasing interest worldwide. Although much information is known about the animal and fungal signal transduction pathways, analysis of signalling pathways in plants has come from since the pioneering studies which identified phytochrome as the first receptor in plants. These phytochromes were the only known receptors in plants until the early 1990s when a number of putative receptors were identified. Signal fluctuations vary from time to time both qualitatively and quantitatively. However, these signals are carried out by various cellular components such as xylem and phloem, the circulatory system, which can accommodate very large and rapid fluxes. The two fundamental components of signal transduction pathways involve intracellular Ca^{+2} and protein kinases, enzymes that phosphorylate and thereby alter the function of target proteins. Changes in protein phosphorylation patterns were observed after exposure of plant cells to abiotic as well as biotic factors like temperature stress, fungal elicitors and hormones (Felix et al. 1991; Raz and Fluhr 1993) and during establishment of freezing tolerance (Monroy et al. 1993). Recently, progress has been made in identifying primary signal reception mechanisms and early events in signalling cascades in higher plants. Intracellular signalling proteins and second messengers are often used to characterise a readily diffusible molecule involved in transmitting signals from an extracellular source to the

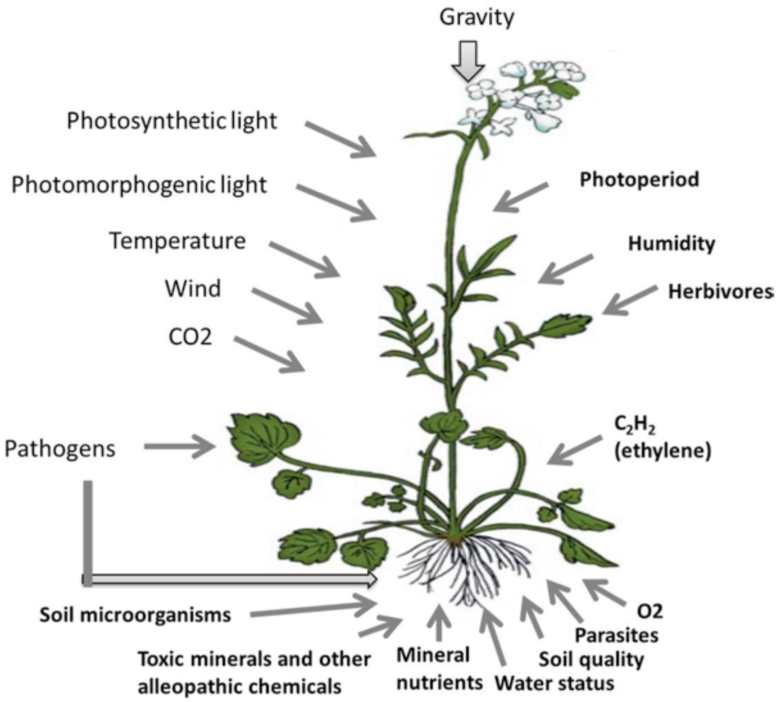

Fig. 1.1 External signals that affect plant growth and development include many aspects of the plant's physical, chemical and biological environments. Some external signals come from other plants. Apart from gravitropic signals, all other signals vary in intensity, often from minute to minute

premier target enzymes within the cell. In plants, cytosolic Ca^{+2} transduces many signals and is an eminent second messenger. Thus, it must be retained in the cytoplasm at concentrations many orders of magnitude lower than the Ca^{+2} in the cell wall. Ion channel-mediated signal transduction in higher plants has notable differences from signalling mechanisms in animal systems. Of the many types of ion channels found in higher plants, recent findings have indicated that an ion channels, along with Ca^{2+} channels, play critical and rate-limiting roles in the mediation of early events of signal transduction. Signals are mostly perceived membrane proteins, and therefore transmembrane events are the likely route for signal generation and transduction. In plants, the well-characterised plasma membrane-based receptors include transmembrane receptor enzymes (usually kinase), ROS sensors and G-protein-coupled receptors (GPCRs). Currently in plants, the G-protein-coupled receptors (GPCRs) are reported to be involved in processes such as ion channel and abscisic acid signalling and modulation of cell proliferation (Wang et al. 2001). Moreover, the signal transduction pathways in plants under abiotic stresses have been categorised into three main categories: (1) osmotic/oxidative stress signalling that involves mitogen-activated protein kinase (MAPK) modules, (2) Ca^{+2}-dependent signalling that leads to activation of LEA-type genes such as dehydration responsive elements (DRE)/cold-responsive sensitive transcription factors (CRT) class of genes and (3) Ca^{+2}-dependent salt overly sensitive (SOS) signalling that results in ion homeostasis (Xiong et al. 2002). In particular, current progress in this area has emphasised the role of Ca^{+2}- and Ca^{+2}/CaM-regulated transcription in plant cell response to stresses (Reddy et al. 2011). The two main objectives of engineering signalling pathways are to understand how natural networks function and to build synthetic

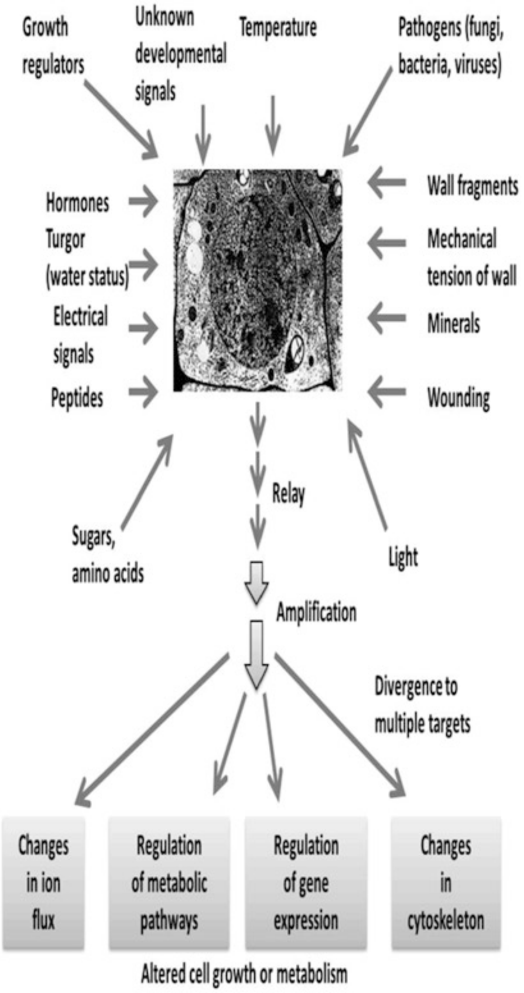

Fig. 1.2 A variety of internal signals modify plant cell metabolism, growth, and development. The ability of cells to respond to these signals is not confined to cells that are still growing and developing. Mature cells, too, can initiate metabolic responses and can even reinitiate growth and division in response to signal information

networks with specific applications or functionalities. Understanding the knowledge about the specificity of the stimulus, the biochemical nature of the receptor and the specificity of the responses is of paramount importance to better understand the diverse signalling pathways in plants. The main aim of this review is to discuss the mechanisms of signalling in plants, the ROS and NO signalling processes and their components, which commence thereafter and the resulting tolerance mechanisms.

1.2 General Features of Signal Transduction

The Signal transduction pathways are remarkably specific and exquisitely sensitive. The interaction between signal and receptor molecules is specific which happens due to complementarity amongst them. Specificity is achieved by precise molecular complementarity between the signal and receptor molecules (Fig. 1.3a). This process is mediated by the same kinds of weak (non-covalent) forces that mediate enzyme-substrate and antigen-antibody interactions. In the multicellular organisms the specificity is provided only by certain types of cells which contain the signalling receptors and intracellular targets of the signalling. Various genes are involved with the signalling process that are necessary for the plant cell to maintain the fate of development in the leaves and roots. A chain of various signalling molecules must be present within the transduction mechanism for the development of the cell because cells undergo changes during growth and they must maintain the internal and external conditions properly even in chaotic environmental conditions. The plant signalling pathway involves the “transducons” that are likely to be Ca^{2+} receptors such as calmodulin, calmodulin-binding proteins (e.g. kinase or microfilament-organising proteins) and calcium-dependent protein kinases. In single cell, the response of the entire plant must not be neglected. The plant cells and tissues individually require the co-ordination between them and complex mechanism of signal communication. Proteins, peptides and RNAs, growth factors, modulation factors, sugar and the mechanical signals are involved in tissues and cell communication (Trewavas 2002). The factors which are responsible for the sensitivity of signal transducers include receptors affinity for signal molecules, cooperativity in the ligand-receptors interaction and finally signal amplification by enzyme cascades.

Cooperativity in receptor-ligand interactions results in large changes in receptor activation with small changes in ligand concentration. Amplification by enzyme cascade results when

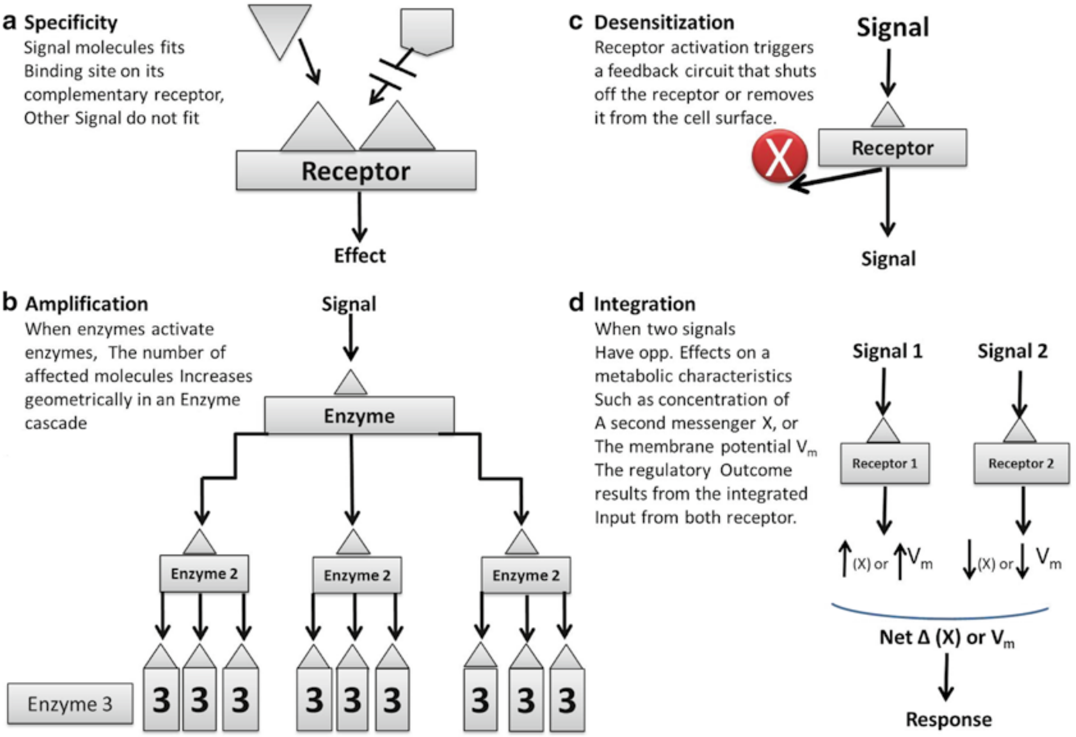

Fig. 1.3 Four features of signal-transducing systems (**a**) *Specificity*: Signal molecules fits binding site on its complementary receptor, Other signal do not fit. (**b**) *Amplification*: When enzymes activate enzymes, The number of affected molecules increases geometrically in an enzyme cascade. (**c**) *Desensitization*: Receptor activation triggers a feedback circuit that shuts off the receptor or removes it from the cell surface. (**d**) *Integration*: When two signals have opp. Effects on a metabolic characteristics such as concentration of a second messenger X, or the membrane potential $V_{m.}$ The regulatory outcome results from the integrated input from both receptor

an enzyme associated with a signal receptor is activated and, in turn, catalyses the activation of many molecules of a second enzyme, each of which activates many molecules of third enzyme and so on (Fig. 1.3b). Such cascade can produce amplifications of several orders of magnitude within milliseconds. The response to a signal must also be terminated such that the downstream effects are in proportion to the strength of the original stimulus. The sensitivity of a receptor system is subject to modification. When a signal is present, continuous desensitisation of the receptor system results (Fig. 1.3c); when the stimulus falls below a certain threshold, the system again becomes sensitive. A final noteworthy feature of signal-transducing systems is integration (Fig. 1.3d), the ability of the system to receive multiple signals, and produces a unified response appropriate to the needs of the cell or organism. Different signalling pathways converse with each other at several levels, generating a wealth of interactions that maintain homeostasis in the cell and the organism.

1.3 Role of Signal Transduction in Plants

Signal transduction in plants plays an essential role to combat multiplex of environmental and hormonal stresses. The abiotic and biotic stresses such as salinity, drought, temperature, pathogens and water stress possess ill effects on plant growth and development. In order to defend themselves, plants have developed some endogenous defence mechanisms to counteract such stresses. Plants have the unique ability to cope up these stresses and survive under various stressful conditions. In this mechanism, the signals are initially perceived by various membrane-bound sensors which in turn get amplified and then transduce these signals to the nucleus in order to stimulate the cascade of reactions that ultimately lead the cell to function in the appropriate manner. Although there are various characteristics that make the plant resistant to environmental changes, some plants show plasticity towards these factors that adds an extraordinary layer of molecular and biochemical complexity that is a unique character of plants. There are many other growth substances like cytokinin, abscisic acid, ethylene and gibberellic acid, brassinosteroids, jasmonates and peptide hormone that act as agents in the signalling pathway and as such help to enhance the plasticity in plants. There are at least 600 receptor kinases present in *Arabidopsis,* and the majority of the receptor kinases are membrane bound. The receptor kinase in the plants plays an important role in incompatibility and disease defence signal transduction. The mechanism of signal transduction in plants involves many kinase cascades and transcription factors that regulate and complete the signalling pathway. A better knowledge of signalling/response coupling mechanisms in plants includes recognition of the stimulus by protein sensors that activates the gated ion channels like G-protein-coupled receptors (Fairchild and Quail 1998; Jones et al. 1998; White 2000), and generation of second messenger by hydrolysis of membrane components, viz. Phosphoinositides (Sanders et al. 1999; Poovaiah et al. 1999). Besides, it also involves enzyme stimulation especially protein kinases and transient increase in calcium influx. Moreover, phosphorylation and dephosphorylation of proteins is a ubiquitous mechanism to maintain the biological function of the cell. Recent progress has been achieved in identifying the signalling mechanism in higher plants. It has been revealed that the gated ion channels along with second messengers play an essential role in mediating intracellular responses in higher plant signal transduction. Through various studies it has been found that anion channels along with Ca^{2+} sensors, MAP kinases and ROS play crucial and rate-limiting roles in mediating plant responses and is now an emerging field of scientific research that finds important application in biotechnology and agriculture (Yinong et al. 2012). Some of the components that play a crucial role in plant signal transduction with recent advancements are as follows:

1.4 Role of ROS in Plant Signal Transduction

Free radicals which are defined as independent chemical species with one or more unpaired electrons can be formed from a diverse group of chemicals and are generally considered to be highly reactive. The term reactive oxygen species (ROS) is now generally used to denote all the oxygen-centred radicals as well as compounds containing chemically reactive oxygen functional groups such as singlet oxygen, hydrogen peroxide, hypochlorous acid and peroxide, hydroperoxide and epoxide metabolites of endogenous lipids and xenobiotics (Giri et al. 1999). ROS are generated spontaneously in a living cell during several metabolic pathways including biological electron transport system (photosynthetic, mitochondrial, microsomal), various enzymes and biomolecules: xanthine oxidase, cyclooxygenase, lipoxygenase, autoxidation of catecholamines etc. (Chandna et al. 2012; Halliwell 1999). Regulation of the multiplex redox and ROS signals in plants requires a high degree of co-ordination and balance between signalling and metabolic pathways in different cellular components (Nobushiro et al. 2012). The process of oxidation occurs in any oxygen-rich environment where substrates are exposed to light and heat. These processes are all mediated by highly reactive oxygen radicals (Harman 2000). During physiological processes, oxygen is involved in oxidation of substrates to generate energy which results in the production of oxygen radical. They also have a beneficial role in phagocytes where they protect the cell against bacteria and parasites. Various ROS such as superoxide radical, hydrogen peroxide and hydroxyl free radical can cause

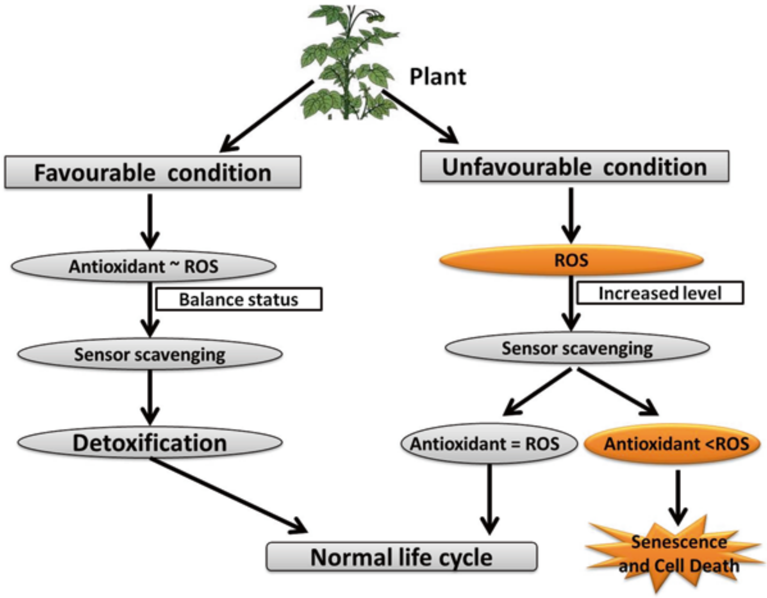

Fig. 1.4 Antioxidants and redox signaling in plants

damage to respiring cells. These ROS are highly toxic, mutagenic and reactive (Halliwell 1997). The production of ROS is a physiological process due to oxidative metabolism of the cell. A serious imbalance between reactive oxygen species and antioxidants causes oxidative stress. Oxidative stress is caused by antioxidant deficiency or by increased production of reactive oxygen species (Fig. 1.4); by environmental stresses such as toxins, light and pathogens; or by inappropriate activation of defence responses (Halliwell 1997). Extreme production of ROS or inappropriate removal leads to oxidative stress which results in the malfunctioning of various physiological processes and damage to biological macromolecules (Chopra and Wallace 1998). In order to combat oxidative stress, plants exhibit an internal defence mechanism such as antioxidants: enzymatic or nonenzymatic or low molecular weight antioxidants. If ROS formation is high and antioxidant level is low, it results in the accumulation of free radical molecules in the cell, causing oxidative stress (O'Brien et al. 2012). The generation of free radicals and oxidative stress has been found to be involved as factors in development of a large number of diseases (Gambhir et al. 1997). Majority of diseases caused by the oxidative stress involves generation of not only ROS but also nitrogen reacting species (NRS) including free radicals (Beckman and Ames 1998; Gutteridge and Halliwell 2000). Besides having an important role in intra- and extracellular signalling, these reactive molecular species may start damaging metabolic reactions (Halliwell 1999). It has been found that oxidative stress may be responsible for biochemical events that results in the cancer formation such as activation of oxidative DNA damage and alternations in intracellular signal transduction (Allen and Tresini 2000; Martin and Barett 2002). Environmental sources such as ultraviolet irradiation, ionising radiations and pollutants also produce ROS (Halliwell 1997), and injured cells and tissues can activate the production of free radicals (Spiteller 2001) in plants. ROS can be formed in foods via lipid oxidation and photosensitisers exposed to light (Boff and Min 2002). In biological systems, ROS can be formed by pro-oxidative enzyme systems, lipid oxidation, irradiation, inflammation, smoking, air pollutants and glycoxidation (Steif 2003). As the plants are exposed to different environmental conditions such as biotic and abiotic stresses including pathogens attack which results in the enhanced production of ROS in plants due to breakdown of cellular homeostasis (Srivastava and Dubey 2011). When the cell is said to be in a state of oxidative stress and the ROS level is enhanced considerably in response to environmental stresses, it results in the lipid peroxidation, oxidation of proteins, DNA damage, enzyme inhibition and activation of programmed cell death (PCD) pathway that ultimately leads to apoptosis (Mishra and Dubey 2011). Irrespective of their destructive activity, ROS are considered to be the second messengers in the cellular process including tolerance to environmental stress (Yan et al. 2007). Depending upon the equilibrium between ROS and antioxidant scavenging activity that whether ROS act as damaging or signalling molecules, the equilibrium between ROS and scavenging activity is very necessary to maintain by the cells in order to combat any oxidative stress. The scavenging activity is achieved by the antioxidant defence system that comprises enzymatic and nonenzymatic antioxidants (Pallavi et al. 2012). The enzymatic antioxidant defence system includes superoxide dismutase (SOD), catalase (CAT), guaiacol peroxidase (GPX), glutathione reductase (GR), whereas ascorbate (ASA), glutathione (GSH), carotenoids, tocopherols and phenolics serve as potential nonenzymatic antioxidants within the cell (Noctor and Foyer 1998). Recent studies have reported that the oxidative stresses in plants system are decreased by increasing the activity of enzyme of the antioxidant defence system which is only possible by maintaining an extreme antioxidant activity to quench the toxic ROS and which in turn increases the plants tolerance to environmental stress (Q. Chen et al. 2010). In order to improve the stress tolerance of crops against environmental stress, considerable efforts have been made by developing transgenic lines with altered levels of antioxidants (Faize et al. 2011). Multiple expression of antioxidant enzyme than single or double expression has been found more effective for developing transgenic plants (Table 1.1) with enhanced tolerance to environmental stresses which in turn results in the increased crop productivity in agriculture (Lee et al. 2007).

1.4.1 Types of ROS, Their Generation and Effects

ROS are generated at various cellular compartments in the cell like chloroplast, mitochondria, plasma membrane, peroxisomes, apoplast, endoplasmic reticulum and cell walls under both stressed and unstressed cells (Fig. 1.5). Reactive oxygen species can be classified into oxygen-centred radicals and oxygen-centred non-radicals. Oxygen-centred radicals are superoxide anion ($O_2^{\bullet -}$), hydroxyl radical ($^{\bullet}$OH), alkoxyl radical (RO$^{\cdot}$) and peroxyl radical (ROO$^{\bullet}$), whereas oxygen-centred non-radicals are hydrogen peroxide (H_2O_2) and singlet oxygen ($^1O_2$). Other ROS are nitrogen-containing species such as NO$^{\bullet}$ and nitric dioxide (NO_2) and peroxynitrite ($OONO^-$) (Simon et al. 2000; Huang et al. 2005).

These ROS are generated due to the leakage of electrons onto O_2 from the electron transport chain or as an output of several biochemical pathways localised in various cellular compartments. These are five possible species: superoxide anion radical ($O_2^{\bullet -}$), hydroperoxyl radical $\left(HO_2^{\bullet}\right)$, peroxide ion $\left(HO_2^{-}\right)$, hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}$OH).

$$O_2 \longrightarrow O_2^{\bullet -}$$
$$O_2^{\bullet -} \longrightarrow H_2O_2$$
$$H_2O_2 \xrightarrow{Fe^{2+}/Cu} {}^{\bullet}OH + OH^-$$

The $O_2^{\bullet -}$ and H_2O_2 so formed in presence of metal catalyst such as Cu^+/Fe^{2+} may lead to formation of most reactive $^{\bullet}$OH. Synthesis of $O_2^{\bullet -}$ and H_2O_2 leads to the generation of $^{\bullet}$OH which oxidises lipids and results in lipid peroxidation (Chessman and Slater 1993). A study described the role of H_2O_2, $^1O_2$, $O_2^{\bullet -}$ and the products of lipid peroxidation signalling molecules in the processes of stress signalling transduction in plants and also suggests that the redox regulators such as protein kinases/protein phosphatases and transcription factors play a critical role in the functioning of ROS-dependent signalling system in the plants (Kreslavski et al. 2012). Some of the reactive oxygen species are briefly discussed below:

Table 1.1 Genetic engineering approaches to achieve cold tolerance by over expression of antioxidant enzymes

Enzyme	Reaction catalysed	Transgenic plant against cold stress	References
Super oxide dismutase (SOD)	$O_2^- + O_2^- + 2H^+ \leftrightarrow 2H_2O + O_2$	Cu/Zn SOD from *Pisum sativum* to *Nicotiana*	Gupta et al. (1993)
		Fe-SOD from *A. thaliana* to *Medicago sativa*	McKersie et al. (2000)
		Mn-SOD in *Gossypium hirsutum*	Payton et al. (2001)
Catalase (CAT)	$2H_2\,O_2 \leftrightarrow 2H_2\,O + O_2$	Rice (*Oryza sativa*)	Matsumura et al. (2002)
Glutathione reductase (GR)	NADPH + GSSG ↔ NADP +2GSH	Tobacco (*N. tabacum*)	Le Martret et al. (2011)
		From *A. thaliana* to *Gossypium hirsutum*	Payton et al. (2001) and Kornyeyev et al. (2003b)
Dehydro-ascorbate reductase (DHAE)	$AA + H_2\,O_2 \leftrightarrow DHA + 2H_2O$	From human to Tobacco (*N. tabacum*)	Kwon et al. (2003)
		Tobacco (*N. tabacum*)	Le Martret et al. (2011)
Ascorbate peroxidase (APX)		From *Pisum sativum* to *Gossypium hirsutum*	Kornyeyev et al. (2001, 2003a, b)
		From *Spinacia oleracea* to *N. tobaccum*	Yabuta et al. (2002)
		From *Pisum sativum* to *Lycopersicum esculentum*	Wang et al. (2005)
		Tomato (*Lycopersicum esculentum*) *StAPX* gene in Tobacco (*N. tabacum*)	Sun et al. (2010)

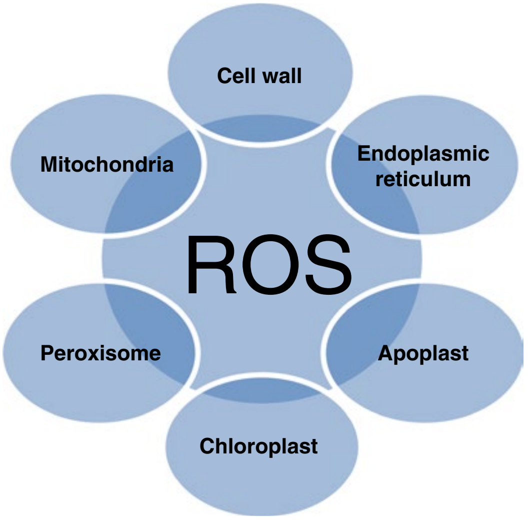

Fig. 1.5 Sites of production of reactive oxygen species (*ROS*) in plants

1.4.1.1 $^1O_2$

In the reaction centre of photosystem II, the highly reactive $^1O_2$ can be generated via triplet chlorophyll (Chl) production in the antenna system in the presence of light (Krieger-Liszkay 2005). The Chl triplet state can react with $^3O_2$ to give up very highly reactive ROS$^1O_2$

$$\mathrm{Chl} \xrightarrow{\text{light}} {}^3\mathrm{chl}$$
$$\mathrm{Chl} + {}^3\,O_2 \longrightarrow \mathrm{Chl} + {}^1\,O_2$$

$^1O_2$ formed thus reacts with biomolecules and directly leads to the oxidation of proteins, unsaturated fatty acid and DNA (Wagner et al. 2004). It causes nucleic acid modification by reacting with deoxyguanosine (Kasai 1997). It is also found to be highly responsible ROS which may trigger cell death (Krieger-Liszkay et al. 2008). $^1O_2$ can be quenched by nonenzymatic antioxidant or with D1 protein of photosystem II (Krieger-Liszkay 2005).

1.4.1.2 $O_2^{\bullet-}$

The oxygen radicals are highly reactive, producing hydrogen peroxides with enes and dienes

(Salim 1987). Moreover few particular amino acids such as histidine, methionine and tryptophan can be oxidised to $O_2^{\bullet-}$ (Knox and Dodge 1985), will cause lipid peroxidation in a cellular environment and lead to the disruption of cell membrane (Halliwell and Gutteridge 1989). By the process of enzyme or metal catalysed reactions a primary ROS $O_2^{\bullet-}$ is formed in the cell which in turn starts a chain of reactions to generate "secondary" ROS (Valko et al. 2005) depending upon the type of cell or cellular components. $O_2^{\bullet-}$ is a moderate reactive nucleophilic reactant species which is short-lived ROS having both oxidising and reducing properties (Halliwell 1997). It causes the oxidation of enzymes containing 4fe-4s clusters and reduces cytochrome *C* (McCord et al. 1977).

1.4.1.3 H_2O_2

It is formed when $O_2^{\bullet-}$ accepts one electron and two protons. Nonenzymatic or SOD-catalysed reaction can easily dismute to H_2O_2 (hydrogen peroxide)

$$2O_2^{\bullet-} + 2H^+ \longrightarrow H_2O_2 + O_2$$
$$2O + 2H^+ \longrightarrow H_2O_2 + O_2$$

H_2O_2 is produced easily in the cells under normal or stressful condition such as drought, chilling, UV irradiation, exposure to light, wounding and pathogen attacks. H_2O_2 is generally produced during electron transport chain of chloroplast, mitochondria, ER and plasma membrane or by β-oxidation of fatty acid and photorespiration. H_2O_2 is moderately reactive and long-lived molecule (Mittler and Zilinskas 1991). Besides other types of free radicals, H_2O_2 can easily cross the membrane and lead to the oxidative damage in the cell. It is beneficial as well as relatively stable than the molecules which are associated with H_2O_2 in the regulation of particular metabolic processes and activate the defence system in plants (Yan et al. 2007). High concentration of H_2O_2 can inactivate the enzymes involved at a specific level, and the enzymes lose 50 % of their activity (Dat et al. 2000). It also causes oxidation of protein enzymes and transcription factor and also triggers programmed cell death. It has been found that transgenic plants with increased levels of H_2O_2 due to the constituent overproduction of glucose oxidase or suppression of peroxisomal catalase were more resistant to pathogen accumulated SA and expressed PR genes and protein (Chammongpol et al. 1998). Pretreatment of maize seedling with H_2O_2, a SOD-generating compound that activates tolerance to chilling (Prasad et al. 1994). Plants regenerated from potato nodal explant treatment with H_2O_2 are found to be more resistant to temperature than the controlled plants (Lopez- Delgado et al. 1998). A recent study indicates that H_2O_2 promotes seed germination of various plants such as *Arabidopsis thaliana, Hordeum vulgare, Oryza sativa, Triticum aestivum* and *Helianthus annuus* (Yushi et al. 2013).

1.4.1.4 OH

OH is the most highly reactive molecule amongst all ROS. It has a single unpaired electron; thus, it can react with oxygen in triplet ground state. $^{\bullet}OH$ interacts with all biochemical molecules and causes extensive cellular damages such as lipid peroxidation, DNA and protein damage and membrane disruption (Foyer et al. 1997). As cells do not exhibit any defence system to abolish $^{\bullet}OH$ and thus its excess generation subsequently leads to apoptosis (Pinto et al. 2003). $^{\bullet}OH$ is produced under illumination, via Fenton reaction at the active site of the enzyme (RbcL) which results in its breakdown in chloroplast lysates (Luo et al. 2002). $^{\bullet}OH$ involves two essential reactions, viz. addition of $^{\bullet}OH$ to organic molecules or elimination of hydrogen atom from it. As $^{\bullet}OH$ are short-life period molecules having an extensive positive redox potential (close to +2V) of "free" $^{\bullet}OH$, the sites at which they react are nearer to the point at which they are formed (Elstner 1982). In this context, organic oxygen radicals such as alkoxy, peroxy, semiquinones, reduced hydrogen peroxide and hydrogen peroxide-electron donor complexes (crypto-OH), as well as metallo-oxygen complexes, have been described as the ultimately active species despite destructive-free $^{\bullet}OH$ (EF Elstner 1987).

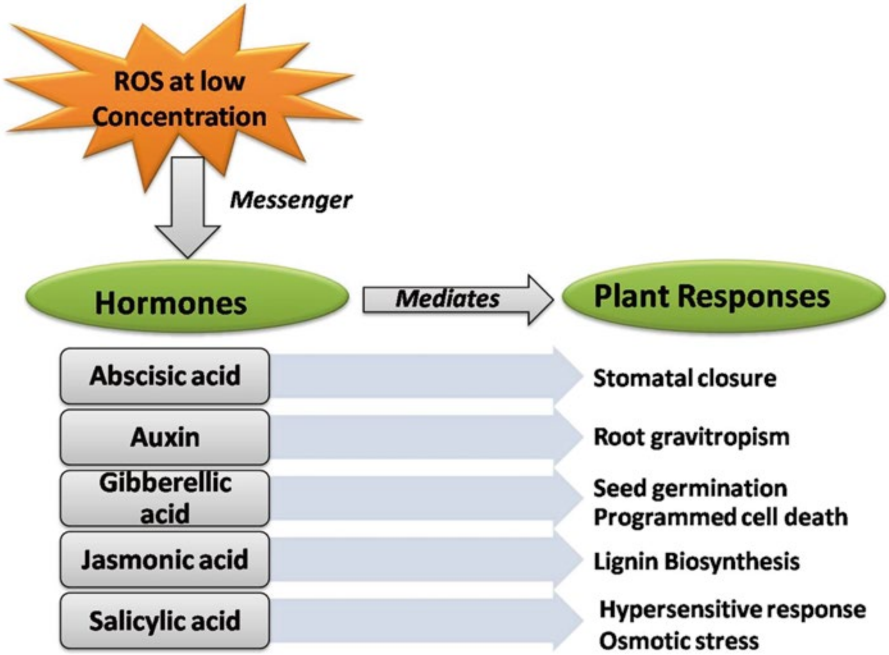

Fig. 1.6 Reactive oxygen species (*ROS*) as second messengers in several plant hormone responses

1.4.2 ROS as Second Messenger in Plants

ROS has been involved as a second messenger in intracellular signalling cascade as they activate various response in plants cells which includes tolerance to biotic and abiotic stresses (Miller et al. 2008), gravitropism (Jung et al. 2001), stomatal closure (Yan et al. 2007) and programmed cell death (PCD) (Mittler 2002). It has also been found that ROS mediates the hormonal response in plant as second messenger (Fig. 1.6). With the help of some redox-sensitive proteins, calcium mobilisation, protein phosphorylation and gene expression, the plant analyses transduce and allocate ROS signals into appropriate responses. ROS can be directly sensed by tyrosine phosphate signalling proteins (Xiong et al. 2002). Many component activities can be modulated by ROS and also interacts with other signalling molecules that regulate the response of downstream ROS by the pathway that forms a part of the signalling network (Neill et al. 2002). Miller and co-workers, using a mutant deficient in key ROS-scavenging enzymes, isolate a signalling pathway that is stimulated by the synthesis of ROS in the cells (Miller et al. 2008), and the important molecules implicated in this pathway are zinc finger proteins, WRKY transcription factors, that mediate the abiotic stresses which include temperature, salinity and osmotic stresses. ROS are referred to as the second messenger in the abscisic acid (ABA) transduction mechanism in guard cells (Yan et al. 2007). The plasma permeable channel reduces, which ultimately loss to mediate stomatal closure signal which is induced by ABA (Pel et al. 2000). It has been found that the constitutive increase of H_2O_2 is induced by ABA functions in stomatal closure (Jannat et al. 2011). The biosynthesis of drought-induced abscisic acid in plants by ROS plays an important role and suggests that they may be the signals through which the plant can “sense” drought conditions. The metals Cd^{2+} and Cu^{2+} induce activation of MAPK through ROS-generating system by using pharmacological inhibitors (Yeh et al. 2007). ROS can also function as second messenger in root gravitropism.

Joo et al. proposed that the mobilisation of auxin in plants by gravity stimulates generation of ROS to mediate gravitropism (Jung et al. 2001). Root gravitropism is inhibited by the scavenging activity of ROS by antioxidants (Jung et al. 2001). ROS is also involved in dormancy alleviation, and under normal conditions, gibberellic acid (GA) signalling and ROS content are lower in dormant barley grains under controlled condition. A pronounced effect has been found on GA signalling by exogenous H_2O_2 but does not have any effect on ABA biosynthesis and signalling and results in germination by activating a change in hormonal balance (Bahin et al. 2011). ROS have been found to play a critical role in programmed cell death in barley aleurone cells that are activated by GA. One of the studies has revealed that ROS are constituents of the hormonally mediated cell death mechanism in barley aleurone cells by observing that GA-treated aleurone protoplasts are less resistant to internally generated or externally applied H_2O than ABA-treated protoplasts (Bethke and Jones 2001). Increased generation of ROS is observed in the early onset of plant-pathogen communication and plays an essential signalling role in the pathogenesis of signal transduction regulators (Nanda et al. 2010). In HR, SA is thought to be potential ROS signalling molecules (Klessig et al. 2000). Expression of a salicylate hydroxylase (NahG) gene of transgenic *Arabidopsis* that is resistant to the increased osmotic stress may result from the reduced SA-stimulated production of ROS (Borsani et al. 2001). In response to wounding the ROS are found to act as second messenger for the activation of defence genes in tomato plants (Orozco-Cárdenas et al. 2001). In the leaves of tomato, ROS were produced close to the cell wall of vascular bundle cells in response to wounding and lead in the generation of H_2O_2 from wounding inducible polygalacturonase that acts as a second messenger for the activation of defence genes in mesophyll cells but not for signalling pathway genes in vascular bundle cells (Orozco-Cárdenas et al. 2001). Lignin plays a key role in mediating defence responses of plants to environmental changes. Through the interaction between jasmonic acid and ROS, a metabolic network that facilitates the plants to stimulate the accumulation of lignin in response to damage of cell wall has been recently characterised (Denness et al. 2011). ROS also plays an important role in heavy metal signal transduction pathway (Yan et al. 2007). ROS upregulates the genes implicated in osmotic stress signalling that include transcription of DREB2A and a histidine kinase (Desikan et al. 2001). It has been revealed that MAPK AtMPK6 can be stimulated by low temperature in *Arabidopsis* cell cultures and osmotic stress could also be activated by oxidative stress (Yuasa et al. 2001).

1.4.3 Adverse Effects of ROS

The production of ROS is a biochemical process due to oxidative metabolism of the cell. A serious imbalance between ROS formation and antioxidants levels causes oxidative stress. Oxidative stress is caused by the presence of low levels of antioxidant or by the increased production of ROS by environmental stresses such as salinity, drought, pathogen attacks and toxicity due to heavy metal (Halliwell 1997). Extensive formation or inadequate removal of ROS results in oxidative stress which may cause various metabolic malfunctions and extreme damage to biological macromolecules (Chopra and Wallace 1998). Oxidative stress leads to the production of extremely reactive oxygen species that are lethal to the cell, specifically the cell membrane in which these reactive molecules intercommunicate with lipid bilayer and generate lipid peroxides. The oxidative stress causes enzyme inhibition, damage to protein synthesis, DNA-strand breakage and lipid peroxidation (Fig. 1.7) that finally leads to apoptosis (Devasagayam et al. 1999).

1.4.3.1 Lipids

The oxidative deterioration of membrane lipid is called lipid peroxidation LPO (Horton and Fairhurst 1987). Lipid in biological systems undergoes autoxidation through a cascade of reactions involving three steps: initiation, propagation and termination. The first step involves

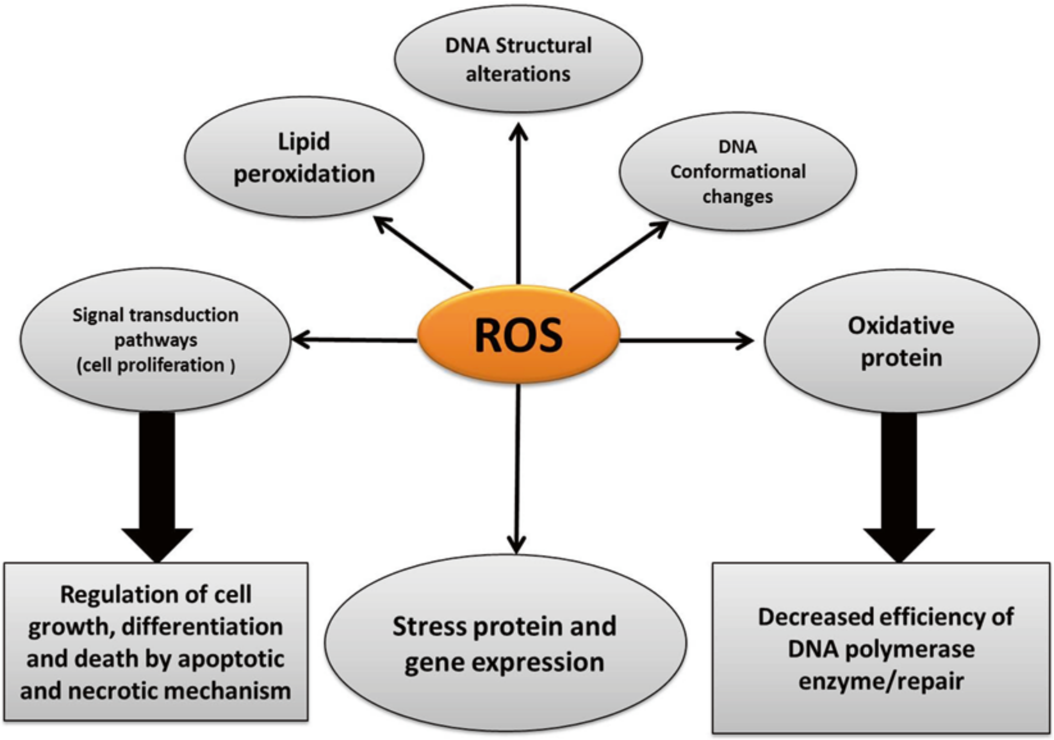

Fig. 1.7 Adverse effects of ROS

lipid peroxidation which is initiated by the reaction of an activated free radical such as singlet oxygen ($^1O_2$, $O_2^{\bullet-}$, or $^{\bullet}OH$) with a lipid substrate (LH) to produce extremely reactive carbon-centred lipid radical ($^{\bullet}L$). In the second step of LPO, molecular oxygen adds quickly to generate lipid peroxyl radical ($LOO^{\bullet}$). The $LOO^{\bullet}$ eliminates a hydrogen atom from another lipid molecule (LH), generating lipid hydroperoxide (LOOH) and another extremely reactive carbon-centred radical ($L^{\bullet}$) which then elongates the chain reaction, and the third step involves termination of lipid peroxidation that occurs through coupling of any two radicals to form non-radical products (NRP). NRP are stable but not able to propagate lipid peroxidation reactions (Porter et al. 1995). Transition metal ions such as copper and iron are essential in LPO (Fig. 1.8). Besides increasing the generation of initiating hydroxyl radicals, ferrous (Fe^{2+}) and ferric (Fe^{3+}) can catalyse the elongation of LPO chain by degrading LOOH. The resulting alkoxyl ($LO^{\bullet}$) and peroxyl ($LOO^{\bullet}$) radicals are able to induce new radical chains by interacting with additional lipid molecules.

Lipid peroxidation (LPO) of the cell membrane has been implicated in a number of physiological processes such as increased membrane rigidity, reduced cellular deformity and lipid fluidity in erythrocytes (Matkovics et al. 1998). When ROS formation crosses the equilibrium level in both cellular and organellar membranes, lipid peroxidation takes place spontaneously which results in affecting normal cellular functioning. Lipid peroxidation results in the generation of lipid free radicals that interacts with biomolecules and leads to the damage of proteins and DNA. It has been found that in plants under stressed condition, increased degradation of lipids takes place along with enhanced generation of ROS (Mishra and Dubey 2011). Malondialdehyde (MDA) one of the lipid oxidation by-products is responsible for the damage of cell membrane (Halliwell 1989). The polyunsaturated fatty acid present in phospholipids is very sensitive to ROS attack.

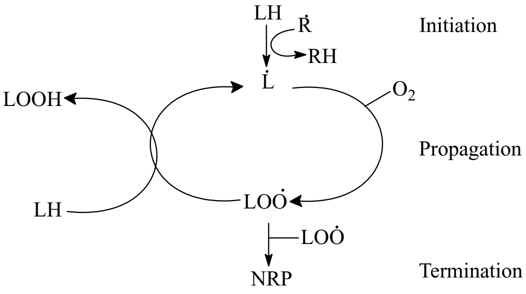

Fig. 1.8 Overview of lipid per-oxidation (Waldeck and Stocker 1996)

1.4.3.2 Proteins

There are direct and indirect effects of ROS on proteins which results in the alteration in various ways. Direct modification of protein activity takes place through nitrosylation, carboxylation, disulphide bond formation and glutathionylation, and the indirect modification takes place through conjugation with breakdown products of fatty acid peroxidation (Yamauchi et al. 2008). Extreme generation of ROS and modified electric charge elevates the sensitivity of proteins to proteolysis. Oxidative stress causes injury to tissues which generally contain high concentration of carbonylated proteins, commonly used markers of protein oxidation (Moller and Kristensen 2004). The plants under stress result in enhanced modification of proteins (Tanou et al. 2009). Oxidation of iron-sulphur centres by $O_2^{\bullet -}$ is invariable and results in the suppression of enzyme (Gardner and Fridovich 1991). Due to metal treatment, oxidised proteins were more effectively decomposed and the proteolytic activity increased by 20 % (Romero-Puertas et al. 2002). Several studies have described that more increase in damage leads to the inactivation of protein enzymes (Grune et al. 1997).

1.4.3.3 DNA

ROS contributes as a major source of DNA damage (Imlay and Linn 1988). DNA is the genetic material of the cell, and damage to the DNA can lead to the malfunctioning of many important proteins. The oxidative damage caused by ROS particularly hits nuclear, mitochondrial and chloroplastic DNA. The attack of ROS on DNA results in the strand lesions, sugar oxidation, elimination of nucleotides and DNA protein interlinks. Moreover alteration in the nucleotides on both the strands results in subsequent mutations. Environmental stresses such as salinity (Liu et al. 2000) and metal toxicity (Meriga et al. 2004) lead to the enhanced degradation of DNA in plants. Both the sugar and base components of DNA are more sensitive to oxidation by ROS. Mutagenic alteration can be caused by the attack of ROS on DNA (Fink et al. 1997). ROS attack to DNA sugar leads to strand breakage (Evans et al. 2004). Because of the absence of shielding proteins, histones and near location of ROS, nuclear DNA was less sensitive to oxidative stress than mitochondrial and chloroplastic DNA (Richter 1992). Extensive alterations induced by ROS result in the unaltered changes of DNA with extreme effects for the cell, despite the repairing system is already present for the destructed DNA.

1.4.4 Defence Mechanism in Plants

Oxidative stress generated during pathophysiological conditions may be effectively neutralised by enhancing the cellular defences in the form of antioxidants (Devasagayam et al. 1999). Antioxidants are a group of substances, when present at low levels, in relation to oxidisable substrates, considerably suppress or delay oxidative processes, while often being oxidised themselves. The content of protective antioxidants present under normal metabolic conditions is sufficient only to combat with physiological rate of free radical production. It is obvious, therefore, that any additional burden of free radicals from environment or generated within the body can disrupt the free radical (pro-oxidant) and anti-free radical (antioxidant) balance (Davies 1995). Depending upon their mechanisms of action, antioxidants act in two different ways as radical chain breaking antioxidants and preventive antioxidants. Several compartments of antioxidative defence mechanism that have been implicated in ROS quenching activity have been altered, overexpressed or downregulated that adds to the current information and understanding the role of the antioxidant defence mechanisms. The defence mechanism of plant system comprises of enzymatic and nonenzymatic defence systems shown as follows:

1.4.4.1 Nonenzymatic Defence System

The nonenzymatic defence system includes the major cellular redox buffer ascorbate (ASA) and glutathione (GSH) as well as tocopherols, carotenoids and phenolic compounds. They have a crucial role in defence and as enzyme co-factor, as they interact with many cellular components; these antioxidants influence the plant growth and development by regulating process from mitosis and cell elongation to senescence and apoptosis (De Pinto and De Gara 2004). It has been found that the mutants with decreased nonenzymatic antioxidant content are hypersensitive to stress (Semchuk et al. 2009).

Ascorbate (ASA)

ASA is the most commonly found, low molecular weight antioxidant that plays an essential role against oxidative damage caused by the increased production of ROS. It has the capability to donate electrons in a wide variety of enzymatic and nonenzymatic reactions. It has also played a key role in plant growth, differentiation and metabolism. Ascorbate exists in diverse plant cell types, organelle and apoplast (Smirnoff et al. 2004). Ninety percent of ASA is localised in cytoplasm but majority is present in apoplast. It has been found that apoplastic ASA shows basal defence against external oxidants (Barnes et al. 2002). ASA also functions as a co-factor of violaxanthin de-epoxidase when present in reduced state in chloroplast thus sustaining the dispersion of extensive excitation energy and also protects macromolecules from oxidative damage (Smirnoff 2000). It directly reacts with $O_2^{\bullet -}$, H_2O_2 and generating α-tocopherols and preserves the activity of enzymes (Noctor and Foyer 1998) and also provides protection to membranes. ASA plays a crucial function by eliminating H_2O_2 through AsA-GSH cycle (Pinto et al. 2003). It has been observed that the content of ASA depends on the balance between the rate and capacity of ASA accumulation and output of antioxidant demand under stress conditions (Chaves et al. 2002). Overexpressing of enzymes that have been implicated in ASA accumulation results in abiotic stress resistance in plants. In tomato plants, overexpression of two members of the GME gene family leads to the elevated biosynthesis of ascorbate and resistance to abiotic stress (Zhang et al. 2011). A recent study has observed that the overexpression of strawberry D-galacturonic acid reductase in potato plants results in the biosynthesis of ASA and increase abiotic stress resistance (Hemavathi et al. 2009). Similarly stress tolerance in Arabidopsis has been shown by increased content of ASA (Wang et al. 2010). The Vtc-1 mutant has shown high susceptibility to supplementary UV-B treatment than wild type (Gao and Zhang 2008) as it lacks the function of GDP-mannose pyrophosphorylase (Wheeler et al. 1998).

Glutathione

γ-Glutamyl-cysteinyl-glycine (GSH) is a low molecular weight nonprotein thiol playing an essential role in combating oxidative stress mediated by ROS. It is virtually present in cytosol, chloroplast of ER, vacuoles and mitochondria (Foyer and Noctor 2003). Cellular redox state was maintained by balancing the GSH and glutathione disulphide (GSSH). GSH plays an important role due to its reducing power in many biological processes, including signalling, conjugation of metabolites, enzymatic regulation, accumulation of proteins and nucleic acid and the expression of stress responsive genes (Foyer et al. 1997). It acts as a scavenger because it chemically reacts with $O_2^{\bullet -}$, $^{\bullet}OH$ and H_2O_2. GSH can protect proteins, lipids and DNA either by the generation of adducts or by functioning as proton donor in the presence of ROS yielding GSSH (Asada 1994). Plants under various stresses like salinity (Hefny and Abdel-Kader 2009), chilling (Radyuk et al. 2009) and metal toxicity (Mishra and Dubey 2011) have been found that altered ratios of GSH/GSSH are present. In the GSH biosynthesis, the overexpression of enzyme glutathione synthetase is unable to show an impact on GSH formation and observed that it was incapable to induce ozone resistance (Strohm et al. 1999) and resistance to photo inhibition (Foyer et al. 1995) in hybrid proteins. Overexpression of γ-ECS results in less susceptibility towards cadmium stress in Indian mustard (Zhu et al. 1999) and increased resistance towards chloroacetanilide herbicide in polar plants (Gullner et al. 2001). With the high level of reduced glutathione in transgenic potato, it results in higher protection against oxidative damage stimulated by several abiotic stresses (Eltayeb et al. 2010).

Tocopherols

Different types of α-, β-, γ- and δ-tocopherols belong to a group of lipophilic antioxidants implicated in oxygen free radical, lipid peroxyl radicals and $^1O_2$-scavenging activity (Diplock et al. 1989). Amongst tocopherols α-tocopherols contain the highest antioxidant activity (Kamal-Eldin and Appelqvist 1996). The main function of tocopherols is to protect lipids and other membrane compartments and hence defend the structure and function of PSII by metabolically scavenging and biochemically reacting with chloroplast O_2 (Ivanov and Khorobrykh 2003). Accumulation of α-tocopherols in different plant species found to activate resistance to chilling, water deficit and salinity (Bafeel and Ibrahim 2008). One of the studies has described that expression level of genes encoding enzyme of AsA-GSH cycle were stimulated, viz. APX, DHAR and MDHAR (Li et al. 2010a). Transgenic rice plant with Os-VTE1 RNA interference show high susceptibility to salt stresses, while as in transgenic plants overexpressing Os-VTE1 results in extreme resistance to salt stress (Ouyang et al. 2011).

Carotenoids

Carotenoids also represent a group of lipophilic antioxidant that is able to remove several types of ROS (Young 1991). Carotenoids as an antioxidant quench $^1O_2$ to suppress oxidative stress. Carotenoids also act as precursors to signalling molecules that controls plant development and abiotic/biotic stress response (Li et al. 2008). It has been found that extreme carotenoids amount shows good adjustment of sugar cane plants under salinity stress (Gomathi and Rakkiyapan 2011).

Phenolic Compounds

Phenolic compounds are varied secondary metabolites (flavonoids, tannis, hydroxylcinnamate esters and lignin) which exhibit antioxidant activity and found in plant tissues (Grace and logan 2000). Polyphenols contain an aromatic ring with $^-$OH or OCH_3 substituent that acts synergistically which contributes to their metabolic functions including antioxidant activity. Polyphenols also change lipid packing order and reduce membrane permeability (Arora et al. 2000). In response to different stresses, there are evidences for the activation of phenolic metabolism in plants (Michalak 2006). It has been observed that ROS might involve in the biosynthesis of total phenolic compound in dark-grown lentil roots (Janas et al. 2009); with increased amount of flavonoid concentration, the transgenic potato plant showed higher antioxidant activity (Lukaszewicz et al. 2004).

Proline

α-Amino acid is an antioxidant and potential inhibitor of programmed cell death. It has been suggested that free proline acts as osmoprotectant, a protein stabiliser, a metal chelator, an inhibitor of lipid peroxidation and $OH^{\bullet}$ and $^1O_2$ scavenger. Increased proline accumulation appears especially during salt, drought and metal stresses (Trovato et al. 2008). Thus, proline is not only an essential signalling molecule but also an efficient ROS scavenger. It has been found that the important role of proline is in potentiating pentose-phosphatase pathway activity as essential compartment of antioxidative defence system (Hare and Cress 1997).

1.4.4.2 Enzymatic Defence System

The enzymatic components of the antioxidant mechanism that includes various antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR) (Noctor and Foyer 1998) (Fig. 1.9).

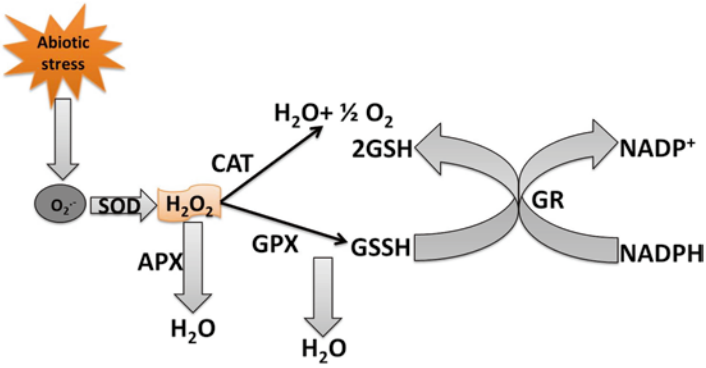

Fig. 1.9 ROS and enzymatic antioxidant defense mechanisms

SOD

Superoxide anion is a reduced form of molecular oxygen evolving by receiving an electron. Superoxide anion an initial free radical plays an essential role in the generation of reactive oxygen species in biological systems (Steif 2003). SOD converts superoxide anion into hydrogen peroxide and oxygen. There are three types of SOD: a Mn-containing SOD, a Cu/Zn-dependent SOD and extracellular SOD (Racchi et al. 2001). SOD plays a critical role in defence mechanism against oxidative stress in all aerobic organisms (Scandalios 1993). Mn-SOD is localised in chloroplast (Jackson et al. 1978), and Cu/Zn SOD is found in cytosol, chloroplast, peroxisomes and mitochondria (Del Rio et al. 1998). Due to drought and metal toxicity, the activity of SOD has been found to enhance in the plants (Mishra et al. 2011) and has been found often mutually related with elevated resistance of plants to environmental stresses. SOD can be used as an indirect selection criterion for screening drought-resistant plant material (Zaefyzadeh et al. 2009). Increased oxidative stress resistance in plants has been reported by the overproduction of SOD (Gupta et al. 1993).

Catalase

Catalase, a tetrameric enzyme, is localised mainly in peroxisomes, while reduced levels also exist in mitochondria and cytosol. The human erythrocytes are rich in catalase (Aebi 1983). Catalase is involved in cellular detoxification and converts H_2O_2 to water and oxygen. Catalase protects cells from H_2O_2 production within them. Although catalase is not crucial for some cell types under normal conditions, it plays a critical role in developing resistance to oxidative stress in adaptive response of cells (Grazioli et al. 1998). Amongst antioxidant enzymes, catalase (CAT) was the first enzyme to be characterised. CAT has extreme specificity for H_2O_2 but less activity against organic peroxides. CATs are rare enzymes as they do not need cellular reducing equivalent, and plants exhibit various types of H_2O_2 decomposing enzymes. CAT exhibits less affinity for H_2O_2 than APX but higher turnover rate. During photorespiratory oxidation, CAT scavenges H_2O_2 further β-oxidation of fatty acid and other enzymes such as XOD coupled SOD (Corpas et al. 2008). One report proposed grouping of CATs based on the expression profile of tobacco genes. Class I CATs are mediated by light and expressed in photosynthetic tissues, Class II CATs are expressed in vascular tissues, whereas Class III CATs are expressed in seeds (Willekens et al. 1995). H_2O_2 is degraded by CAT in an energy efficient manner (Mallick and Mohn 2000). It has been found that environmental stress either enhanced or reduced activity of CAT depending on the magnitude of strength and type of stress (Moussa and Abdel-Aziz 2008). Stress analysis has described that enhanced sensitivity of CAT-deficient plants to paraquat salt and ozone but not chilling (Willekens et al. 1997). CAT is crucial for regulating the redox balance during oxidative stress that is indicated by CAT activity which shows biosynthesis of GSSH and a 4-fold reduction in ASA in transgenic tobacco plant having 10 % wild type (Willekens et al.1997). A CAT gene isolated from *Brassica juncea* introduced and overexpressed in tobacco plants increased its resistance to cd-induced oxidative stress (Guan et al. 2009). Catalase was found to be receptor and when SA binds it inactivates CAT. Catalase inactivation results in H_2O_2 synthesis, which was shown to act as a secondary messenger to stimulate pathogen-related (PR) gene (Chen et al. 1993). By using the sense and antisense technology in transgenic Nicotiana lines (CATIAS), only 10 % of the residual catalase activity was retained (Chamnongpol et al. 1996). In *Arabidopsis thaliana*, *Nicotiana plumbaginifolia*, *Oryza sativa* and *Zea mays,* cDNAs that code for three different classes have been isolated (Frugoli et al. 1998). *N. plumbaginifolia* contains three active catalase-encoding genes (cat1, cat2, cat3); two of which are expressed in mature leaves (Willekens et al. 1994). Increased susceptibility against ozone and salt stress of the CATIAS plants indicates that catalase activity is crucial for cellular defence against the environmental stress that cause H_2O_2 generation from photorespiration (Willekens et al. 1997). It has been found that in *Arabidopsis* mutants, phytoalexin biosynthesis in parsley cell suspension and lesion formation is particularly activated by SOD not by H_2O_2 (Jabs et al. 1996). There is spontaneous reduction in CAT1, CAT2 transcript and protein levels and decrease in total catalase action which is correlated with the accumulation of H_2O_2 in the tobacco cells that sustains HR upon infiltration with fungal elicitors (Dorey et al. 1998).

Glutathione Reductase

Glutathione peroxidase is an essential hydrogen peroxide-removing enzyme present in the membranes. Glutathione disulfide reductase, a flavoprotein, allows the transformation of oxidised glutathione (GSSG) to reduced glutathione (GSH) by oxidation of NADPH to $NADP^+$ (Papas 1999).

$$2O_2^{\bullet -} + 2H^+ \xrightarrow{SOD} H_2O_2 + O_2$$

$$2H_2O_2 \xrightarrow{CAT} 2H_2O_2 + O_2$$

$$2GSH + H_2O_2GP_x \longrightarrow GSSG + 2H_2O$$

$$GSSG + NADPH + H^+ \xrightarrow{GR} 2GSH + NADP^+$$

Superoxide anion itself is not a strong oxidant, but it reacts with protons in water solution to form hydrogen peroxide, which then serves as substrate for the production of highly reactive species such as hydroxyl radicals and singlet oxygen (Steif 2003). As an antioxidant GR plays an essential role in enzymatic as well as nonenzymatic redox cycle in which oxidation of GSH to GSSH takes place. GR represents a group of flavoenzymes and exhibits an important disulphide group (Ghisla and Massey 1989). Two steps involved in catalytic mechanism of GR, viz. (1) the moiety is reduced by NADPH and (2) reduction of GSSH via thiol disulphide interchange reaction (Ghisla and Massey 1989). It is present in chloroplast, cytosol, mitochondria and peroxisomes, and 80 % of GR activity is found to be present in photosynthetic tissues and chloroplast isoforms (Edwards et al. 1990). Environmental stresses increase the activity of GR (Maheshwari and Dubey 2009), and it has been found that antisense-mediated depletion of tomato chloroplast GR has been shown to enhance susceptibility to chilling (Shu et al. 2011). Extreme amount of foliar ASA and increased resistance to stress have been shown by overexpression of the GR in *N. tobacco* and *populous* plant (Foyer et al. 1995). As the ROS detoxification system is very complex, it has been observed that overexpressing of antioxidant defence system may not change the capability of the entire pathway (Lee et al. 2009). Several studies have shown that in transgenic plants the overexpression of combination of antioxidant enzymes has synergistic effect on stress resistance (Kwon et al. 2002). Overexpression of genes of SOD and APX in chloroplast, (Kwak et al. 2009) SOD and CAT in cytosol (Tseng et al. 2008) and SOD and GR in cytosol (Aono et al. 1995) has been shown to result in increased resistance to different environmental stresses. It is important to note that much importance is now given to generate transgenic plants with overexpression of different types of antioxidants for achieving resistance in order to combat environmental stresses.

Guaiacol Peroxidase

Guaiacol peroxidase, a haeme-containing protein, is commonly found in microbes, plants and animals and is mostly oxidised by guaiacol and pyrogallol electron donors at the cost of hydrogen peroxide. It has been found that guaiacol (anionic) peroxidase exhibits about 90 % of peroxidase activity in plants (Foyer et al. 1994). This haeme-containing protein is found to be involved in the accumulation of lignin and suppression in the formation of indole-3-acetic acid (IAA) and provides defence system against pathogen that inhales H_2O_2 (Kobayashi et al. 1996). It has been revealed that several isoenzymes of GPX are found to be present in tissues that are mobilised in vacuoles, the cell wall and the cytosol of the plant (Asada 1992). It has been found that GPX plays an efficient role by scavenging of O_2 and peroxyl radical under stressful conditions and commonly termed as "stress" enzyme (Vangronsveld and Clijsters 1994). In response to biotic and abiotic stresses, there is a rapid stimulation of GPX activity in plants (Moussa and Abdel-Aziz 2008). One of the studies carried out by Radotic et al. (2000) suggested that during oxidative stresses in response to metal toxicity, GPX can be used as biomarker for not so lethal toxicity of metals in plants by correlating increased activity of GPX. Current studies have described that GPX exhibits an essential role against salt resistance of safflower plants which is increased by increasing the GPX activity in the plants (Tayefi-Nasrabadi et al. 2011).

1.4.4.3 Enzymes of Ascorbate-Glutathione Cycle

In the cell, there is change in the ratio of AsA to DHA and GSH to GSSG which is critical state for the cell to identify oxidative stress and functions in an appropriate manner. The AsA-GSH cycle also termed as Halliwell-Asada pathway is the recycling pathway of AsA and GSH production which also eliminates H_2O_2 and is present in four different subcellular locations, including the cytosol, chloroplast, mitochondria and peroxisomes (Jiménez et al. 1997). The AsA-GSH pathway involves extensive redox reactions of AsA, GSH and NADPH catalysed by the enzymes APX, MDHAR, DHAR and GR. During environmental stresses AsA-GSH pathway plays an essential role in overcoming oxidative stress (Sharma and Dubey 2005).

Ascorbate Peroxidase

Ascorbate peroxidase (APX) is a main component of AsA-GSH cycle and plays an important role in mediating internal responses of ROS levels. It belongs to a class-I superfamily of haeme peroxidase (Welinder 1992) and is mediated by redox signals and H_2O_2 (Patterson and Poulos 1995). The reaction involves the use of two molecules of ASA to reduce H_2O_2 to water with a simultaneous generation of two molecules of MDHA. APX exists in cytosol, apoplast and other organelles playing an important role in scavenging H_2O_2 within the organelle, whereas cytosolic APX detoxifies H_2O_2 generated in the cytosol, apoplast or that diffused from organelles (Mittler and Zilinskas 1992). During stressful conditions CAT makes APX less effective in mediating scavenging activity to eliminate H_2O_2, whereas isoforms of APX have much higher affinity for H_2O_2 and are known as one of the most commonly found antioxidant enzyme in the plant cell (Wang et al. 1999). Several studies have observed that in response to abiotic stresses such as drought, salinity, chilling, metal toxicity and UV irradiations, the APX activity is accelerated considerably (Hefny and Abdel-Kader 2009). It has been found that in transgenic tomato plants, overexpression of a cytosolic APX gene isolated from pea decreases the oxidative injury induced by chilling and salt stress (Wang et al. 2005). Similarly in *Nicotiana tobaccum* or *Arabidopsis thaliana,* overexpression of tAPX gene increased resistance to oxidative stress.

Monodehydroascorbate Reductase

In plants, the monodehydroascorbate reductase (MDHAR) is an enzymatic component of the glutathione-ascorbate cycle that is one of the major antioxidant systems of plant cells for the protection against the damages by reactive oxygen species (ROS). The MDHAR activity has been observed in several cell compartments, such as chloroplasts, cytosol, mitochondria, glyoxysomes and leaf peroxisomes, and various isoenzymes of MDHAR have been found to exit in various cellular compartments like chloroplasts (Hossain et al. 1984). Various studies have described that during environmental stresses the activity of MDHAR is enhanced in plants (Maheshwari and Dubey 2009). It has been found that in tobacco, overexpression of *Arabidopsis* MDHAR gene results in the increased resistance to salt and polyethylene glycol stresses (Eltayeb et al. 2007) and similarly in transgenic *Arabidopsis* overexpression of tomato chloroplastic MDHAR increased its resistance to temperature and methyl viologen-mediated oxidative stresses (Li et al. 2010a, b).

Dehydroascorbate Reductase

Dehydroascorbate reductase involves in the stimulation of the symplastic and apoplastic ascorbate pool size and redox state of the cell. The reduction of dehydroascorbate to ascorbate is catalysed by DHAR at the expense of glutathione in the reaction. Thus, dehydroascorbate reductase catalyses the regeneration of ascorbate from its oxidised state and serves as an important regulator of ascorbate recycling. In plants overexpression of DHAR has been found that guard cells contain lower levels of H_2O_2 and decreased responsiveness to H_2O_2 or abscisic acid signalling, which results in higher stomatal opening (Chen and Gallie 2005). On the contrary, suppression of DHAR expression in guard cells results in higher levels of H_2O_2 and increased stomatal closure under normal growth conditions or following water deficit. It has been found that enhanced expression of DHAR results in the increased resistance to ozone in plants, while decreased expression of DHAR in plants results in the reduced rate of CO_2 assimilation with less growth and reduced biomass accumulation (Chen and Gallie 2005). One of the recent studies shows that overexpression of cytosolic Arabidopsis AtDHAR1 results in the enhanced resistance to herbicide, drought and salt stresses in transgenic potato plants (Eltayeb et al. 2011).

In addition to the above discussion, NO is one of the reactive oxygen species that have diverse functions in plant physiology. NO may be implicated in some of the defence responses mediated by the reactive oxygen species (ROS) such as defence gene activation, the hypersensitive cell death and phytoalexin biosynthesis (Durner et al. 1998). Nitric oxide (NO) is a ubiquitous signal molecule that has been implicated in a multiplex of plant responses to environmental stress. In the current years, the regulating role of NO on heavy metal toxicity in plants is realised increasingly, but knowledge of NO in alleviating aluminium (Al) toxicity is quite limited. There are many evidences that describe the role of NO in increasing Al toxicity in plants via activating defence mechanism to eliminate reactive oxygen species (Huyi et al. 2012). It has been suggested that ROS is not always sufficient to mediate a strong disease resistance response in plants, and their combination with NO can act synergistically to activate a stronger response. Many studies have reported the isolation of the enzymes that catalase NO synthesis and its involvement in many process includes its signalling functions at the molecular level (Lamotte et al. 2004). However, much information has been gaining in understanding the role of NO in plants over the past decade. NO functions in many metabolic processes that include generation, root growth, stomatal closing and adaptive responses to various environmental stresses (Delledonne 2005) in plants. Let us briefly discuss the role of NO in plant signal transduction and its function together with ROS in plant immunity.

1.5 Role of Nitric Oxide (NO) in Plant Signal Transduction

Nitric oxide (NO) is an inducible molecule or free radical reactive gas which has been found to be present in animals and other biological systems that play an essential role in various metabolic processes (Schmidt and Walter 1994). NO is a small molecule, having short half-life period, absence of charge and high diffusivity that would serve as an ideal inter- and intracellular signalling molecule in plant defence mechanisms. Many experiments suggest that NO is not generated only from nitrite but can be also produced from L-arginine (Corpas et al. 2006). Understanding the pathway, mechanism underlying NO synthesis and signalling activities in plants is still rudimentary. However, many studies have shown the essential function of NO as a signalling molecule in plant systems. Nitric oxide (NO) is a reactive radical molecule and during plant signalling it has been synthesised in response to biotic and abiotic stresses. During life cycle of plants, it has been described that many essential developmental processes that can be mediated by this inducible molecule which includes plant growth and development, de-etiolation and gravitropism response are well defined. Besides the existing information about NO, the pathway of NO signalling is not yet fully elucidated, but there are evidences that explained the importance of NO as a stimulator of plant growth and stress is rising significantly. It has also been found that NO plays a crucial role in upregulating antioxidant defence system that contributes to increased resistance against high-temperature-induced oxidative damage in wheat (Bavita et al. 2012). In tomato plants it has been found that the primary root (PR) growth is lowered by NO, while lateral root (LR) development is accelerated (Correa-Aragunde et al. 2004). A study showed that NO inhibits the growth of roots and has been utilised as a morphological tool for screening of hypersensitive mutants of NO (He et al. 2004). By using this property of NO, it became possible to isolate NO-overproducer mutant nox1 and also found that CUE1 underexpressed as the mutant gene. It has been found that differentiation of NO takes place in a dose-dependent manner and also suggested the growth is inhibited by endogenous application of the extreme levels of NO, while the low levels promote it (He et al. 2004). Similar results have been found in plant gravitropic response in which addition of high level of NO on the upper side promoted elongation, while low levels of NO on the lower sides suppress it, thus effecting gravitropic bending (Hu et al. 2005). In *Arabidopsis* by using different analysis approach, it has been found that NO operates during onset of plant growth, but it also has been reported that PR growth of wild-type *Arabidopsis* seedlings is suppressed by NO (Fernandez-Marcos et al. 2011). One of the recent studies has observed that in *Euphorbia pekinensis* suspension cells, the endophytic fungal elicitor stimulates elevation in the amount of NO and generation of SA which in turn increases the biosynthesis of isoeuphpekinensin and also describes that ROS are not involved in the endophytic fungus-host interaction signalling pathway (Fu-Kang et al. 2012). One of the studies has also revealed that in *Catharanthus roseus* cells, the extracellular application of NO through its donor sodium nitroprusside (SNP) results in the generation of catharanthine (Xu et al. 2005). Mao-Jun et al. (2005) studied the impact of NO scavenger 2- to 4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPITO), nitric oxide synthase inhibitor S, S′-1, 3-phenylene-bis (1,2-ethanediyl)-bis-isothiourea and inhibitors of the octadecanoid pathway on elicitor-induced NO generation, JA biosynthesis and hypericin production. The results revealed a causal relationship exists between elicitor-induced NO production, JA biosynthesis and hypericin generation in *H. perforatum* cells and indicates a sequence of signalling events from NO to hypericin production within which NO mediates the elicitor-induced hypericin biosynthesis at least partially via a JA-dependent signalling pathway. The constitutive part of common signal transduction in plant defence mechanism contains JA and its methyl ester methyl jasmonate (MeJA) that are well-defined signalling molecules (Mueller et al. 1993). Many studies have been carried out and demonstrate that when the plant tissues and cells are subjected to external treatment of JA or MeJA, they have been found to imitate the impact of wounding and elicitor to stimulate the corresponding responses like accumulation of secondary metabolites and proteinase inhibitor enzymes (Gundlach et al. 1992) and rapid production of H_2O_2. One of the recent studies suggested that NO plays a crucial role in few JA-stimulated or JA-mediated defence mechanisms, viz. the inhibition of wounding-induced H_2O_2 production and proteinase inhibitor synthesis in tomato leaves (Orozco-Cárdenas and Ryan 2002) and MeJA-induced accumulation of H_2O_2 and malondialdehyde (MDA) in rice leaves (Hung and Kao 2004). Jian and Jian (2005) found a similar result in *Taxus chinensis* cell cultures in which nitric oxide (NO) plays an active role in MeJA-mediated plant defence responses and secondary metabolism. Similarly, it has been found

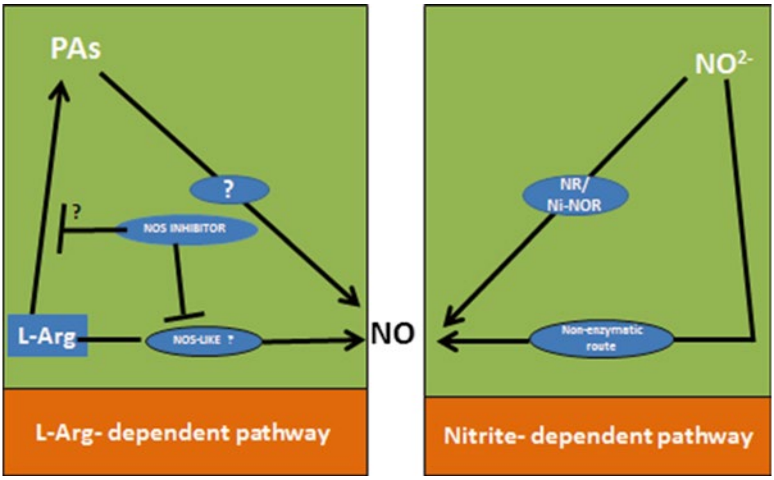

Fig. 1.10 Nitric oxide (*NO*) synthesis in plants: It comprises of two pathways L-Arg dependent pathway that involved NOS like enzyme and Nitrite-dependent pathway that involves NR, Ni- NOR and non-enzymatic pathway for the production of NO (Angelique Besson-Bard et al. 2008)

that in *Arabidopsis thaliana* JA and wounding activates a strong NO burst (Huang et al. 2004).

1.5.1 Pathways

Recent information known about NO signalling in plants revealed that there exists two different enzymatic pathways for the NO production in plants, viz. a nitrate-/nitrite-dependent pathway and an L-Arg-dependent pathway (Fig. 1.10). The enzyme involved in the first pathway is a cytosolic nitrate reductase NR (Yamasaki and Sakihama 2000) and a root-specific plasma membrane nitrite-NO reductase (Ni-NOR) (Stohr and stremlau 2006). The in vitro production of NO was catalysed by NR and uses NAD(P)H as an electron donor through one electron-nitrite reduction (Stohr et al. 2001). In vivo, the basic generation of NO levels in the leaves and roots of various plant species is induced by NR (Vanin et al. 2004), and the process is regulated by the phosphorylation state of enzyme (Lea et al. 2004). It has been found that in ABA-signalling of guard cells NR- induces NO synthesis is the main step; however by observing the transduction mechanism in *Arabidopsis thaliana* nia1, nia2 NR-deficient mutants ABA do not lead to the generation of NO during stomatal closure (Bright et al. 2006). NO formation from nitrite, Ni-NOR, is involved and formation of NO takes place extensively in roots. There is co-ordination between the Ni-NOR and the plasma membrane-bound NR that reduces nitrate to nitrite (Stohr et al. 2001). It has been observed that Ni-NOR is implicated in many physiological processes particularly related to root such as development, response to anoxia and root symbiosis (Stohr and Stremlau 2006), but the actual identity of Ni-NOR is not clear. Although many experiments have been carried out to elucidate the actual process involved and finally scientists have developed a nitrite-dependent mechanism for NO synthesis. These include the following:

(a) At the acidic pH in the apoplasm, there is reduction of nitrite to NO (Bethke et al. 2004).
(b) And production of NO in mitochondria due to reduction of nitrite to NO (Planchet et al. 2005).

However there exists no homology of animal NOS in the Arabidopsis genome (Arabidopsis genome initiative 2000). It has been suggested that NOS-like enzyme may be present in plant tissues and purified organelles (Tian et al. 2007). NO synthesis in plants can be suppressed by the use of mammalian NOS inhibitor and also cell suspension exposed to hormones (Zottini et al. 2007), derived elicitors or pathogen (Vandelle et al. 2006) and salt stress (Zhao et al. 2007). Recent studies have described that a protein which is involved in the accumulation of NO in snail Helix pomatia shows sequence resemblance to the enzyme involves in the reactions of *Arabidopsis* results in the cloning of AtNOS1 gene which encodes mitochondrial enzyme in *Arabidopsis thaliana* (Guo and Crawford 2005). A study revealed that the AtNOS1 enzyme plays a critical role in floral transition (He et al. 2004) and in the signalling pathway of ABA that is the main source of NO (Guo et al. 2003) and lipopolysaccharide (LPS) (Zeidler et al. 2004). However it has been found that in specific environmental conditions such as in response to ABA or LPS, the mutant AtNOS1 shows the presence of reduced levels of NO, and it was recently questioned that how the mutant AtNOS1 exhibited the NOS activity (Zemojtel et al. 2006). It has been currently described that in the synthesis pathway of NO, there is an active involvement of an enzyme that is not known but has been found to play a role in the uninterrupted transformation of polyamines to NO. Recently a specific analysis has been carried out on the amount of amino acid in the leaves of *Arabidopsis* nia1, nia2 NR-deficient mutant grown, and it has been observed that a decrease of tenfold level of L-Arg is observed in presence of ammonia. The above observation suggests that there is an insufficient amount of endogenous substrate present in plants lacking nitrate or NR which is not able to generate NO either from the nitrate/nitrite-dependent pathway or from the L-Arg-dependent pathway.

1.5.2 NO Signalling in Plants

The metal nitrosylation, S-nitrosylation and tyrosine nitration play an important role in NO signalling in plants (Fig. 1.11). Recently it has been described that NO forms complex with metal-containing proteins in plants. In the recent years more focus has been given to clearly elucidate the reaction between NO and haemoglobin. Plants contain three main types of haemoglobin (Hb), viz. symbiotic Hb found in nitrogen-fixing root nodules of leguminous plants (Mathieu et al. 1998), non-symbiotic consists of two classes of Hb both containing extreme and reduced affinity for oxygen and truncated Hb (Dordas et al. 2004). It has been recently found that the root nodules of soya bean, cowpea and alfalfa contain Lbfe^{II} NO and also described the presence of different forms of LB generated in vivo such as oxy Lb and ferryl Lb which are well defined for their involvement in the quenching activity of NO and peroxynitrite ($ONOO^-$) (Herold and Puppo 2005) in vitro. Similarly studies have shown that the different plant species containing class-I Hb catalyse the reaction involving NAD(P)H-dependent transformation of NO to nitrate in vitro (Seregelyes et al. 2004). Perazzolli et al. (2004) have described that *Arabidopsis* class-I Hb is also mediated via S-nitrosylation. Mutagenesis experiment recently has shown that this regulation might not be crucial for quenching of NO (Igamberdiev et al. 2006). One of the recent hypothesis supports that functional interaction between NO and class-I Hb by the use of both defective and overexpressing class-I Hb mutants lowers the intracellular level of NO under hypoxia (Perazzolli et al. 2004) and pathogen attack (Seregelyes et al. 2004). Similarly, transgenic plants overexpressing class-1 Hb lead to the increased resistance to hypoxic stress and lowered necrotic symptoms in response to avirulent pathogens (e.g. tobacco necrosis virus and *Pseudomonas syringae* pv. *phaseolicola*). Therefore, the major activity of class-1 Hb is to provide defence system against lethal effects of nitrosative stress in order to assist and surpass the adverse conditions in plants. In addition to Hb and lipoxygenase, cytochrome oxidases (COX) are presumed targets of NO which is also mediated by metal nitrosylation and results in the loss of activity in biological system (Clark et al. 2000). Moreover, hundreds of studies have described the metabolic role of NO in plants, but no appreciation has been given earlier for the involvement of S-nitrosylation as a presumed post-translational protein modification. Currently advancement in methodologies has been successfully applied in mammalian cells (Greco et al. 2006), and this provides a better technique for isolating plant protein that are S-nitrosylated in vivo (Jaffrey et al. 2001). One

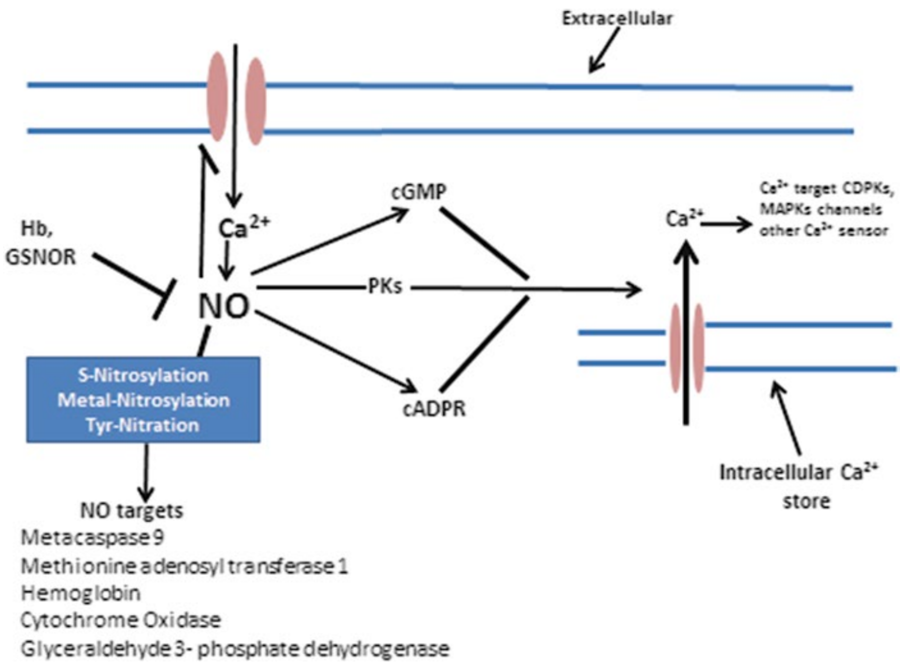

Fig. 1.11 Nitric oxide signaling in plant cells: NO convey its effects through S- Nitrosylation, metal- Nitrosylation and Tyrosine nitration. In these processes there is the involvement of Ca^{2+} channels. Protein kinases, cyclic ADP ribose and GMP mechanisms for NO signaling and switch off mechanisms include NO scavenging by Hb, GSNO (Nitrosoglutathione), reductase (GSNOR) and attenuation of Ca^{2+} influx that stimulates NO synthesis (Angelique Besson-Bard et al. 2008)

of the studies suggested that by the use of biotin switch method, the first S-nitrosylated proteins are isolated from the leaves of *Arabidopsis* and cell suspension extracts in which artificially released NO is applied (Lindermayr et al. 2005) and are found to be involved in metabolism, photosynthesis, redox control and stress response and various amongst these are known to be S-nitrosylated in vitro/in vivo in mammals (Stamler et al. 2001). By using biotin switch approach, the identification of methionine adenosyl transferase (MAT) is possible (Lindermayr et al. 2005). Many studies have revealed that the metabolic description for the NO to downregulate ethylene accumulation and some effects such as senescence forms the basal line of crosstalk between NO and ethylene signalling (Leshem et al. 2000). The interaction between NO and the *Arabidopsis* metacaspase 9 (ATMC9) provides the new insight for the effect of S-nitrosylation on plant protein activity (Belenghi et al. 2007). There are also evidences that show the presence of nitrosoglutathione reductase (GSNOR), conserved between bacteria, animals and plants (Sakamoto et al. 2002), and GSNOR catalyses the oxidation of GSNO to glutathione disulphide (GSSG) and ammonia (Liu et al. 2001) and hence plays an essential role by acting as checkpoints to regulate S-nitrosothiol-mediated effects in plants that are confronted by the pathogen (Rusterucci et al. 2007). The above finding reveals that the S-nitrosylation is an important post-translational reversible mechanism in plants.

Besides the above-defined signalling mechanisms, there are evidences that in animals Tyr nitration is basically involved with the knock-down of protein functions and is a relevant biomarker of NO-dependent oxidative stress (Hanafy et al. 2001), but recent studies have revealed that this plays a key role in post-translation modification in signalling (Schopfer et al. 2003). It has been observed that the protein kinase leads to the inhibition of Tyr phosphorylation, as Tyr nitration acts as antagonist to Tyr phosphorylation during protein kinase-mediated cell signalling (Schopfer et al. 2003). Although in plants, association of ONOO– with Tyr residues in target proteinases has drawn the less information to date as mechanism of NO signalling. However the identification and isolation of highly reactive $ONOO^-$ in biological system have also been described, and various studies have revealed the active involvement of protein in Tyr nitration in plants. One of the recent studies has observed that by using the antibodies that are raised against 3-NO_2-Tyr residues demonstrates that there is 100-fold higher NR-mediated NO emission rate compared with the wild type that results due to the increased protein Tyr nitration in an antisense nitrite reductase tobacco lines. Similar results that have been observed from immunological-based strategy detected Tyr nitration in olive leaves exposed to salt stress (Valderrama et al. 2007) and also in tobacco cells treated with INFA, an elicitor secreted by Phytophthora infestans that promotes defence responses (Saito et al. 2006). All these proteins remain unidentified so far.

1.5.3 Role of NO and ROS in Plant Immunity

There are several evidences that described the role of NO and ROS in plant signal transduction; they act either individually or synergistically and stimulate a stronger defence response against various environmental stresses, and similarly the extensive generation of NO and ROS called as NO burst and ROS burst, respectively, has been identified to be involved in many morphological and metabolic processes such as tolerance against biotic and abiotic stresses and hormonal signalling and also plays an essential role in the plant growth and development (Hong et al. 2008). NO as a reactive radical is found to be involved in various essential processes in both plants and animals, and notably NO in animals is generated by NO synthase (NOS). Currently, NO generation as a radical and its diverse function in plant innate immunity has excited the plant biologist, as NO stimulates mitogen-activated protein kinase cascade (Kumar and Klessig 2000) and results in the increase of defence gene expression and other proteins markedly the enzymes coding for phenylalanine ammonia-lyase and pathogenesis-related proteins (Durner et al. 1998). Similarly the generation of NO in plants involves the reduction of nitrite by nitrate reductase, while oxidation mechanism by NOS of arginine to citrulline is not well defined. However, there are evidences that reveal that plants involve arginine-dependent pathway of NO synthesis, but there is no gene or protein in plants that resembles the mammalian-like NOS (Butt et al. 2003). From *Arabidopsis* a study revealed that the isolation of NOS-like enzyme (AtNOS1) has a sequence resembling a protein which is involved in the accumulation of NO in the snail Helix pomatia (Guo et al. 2003). But after some time it has been revealed that AtNOS1 protein does not exhibit NOS-like activity (Zemojtel et al. 2006), and therefore AtNOS1 was termed as AtNOA1 for NO Associated1 (Crawford et al. 2006), and a recent study reveals that AtNOA1 has circularly transposed GTPase activity in plastides (Moreau et al. 2008). Although *Arabidopsis thaliana* mutant noa1 is found to be useful for various physiological processes, it shows that only low levels of NO mediate plant growth, fertility, hormonal signalling, salt resistance and defence mechanisms (Kato et al. 2008). However, enormous generation of ROS results in the association between AtNOA and NO that is actually the outcome of the multiple effects and malfunctioning carried out by plastids (Gas et al. 2009). Decreasing or loss of function of NOA1 provides one of the simplest techniques for the analysis of NO function. The respiratory burst oxidase homology gene (RBOH) is a plant homology of NOX5 and has been found that in mammalian, NADPH oxidase is found in many plant genomes such as *Arabidopsis thaliana,* rice (*Oryza sativa*), tomato (*Solanum lycopersicum*), potato (*Solanum tuberosum*), tobacco (*Nicotiana tabacum*) and *Nicotiana benthamiana* (Yoshie et al. 2005). As it has been found that RBOH is present in several plants, at the same time it has also been observed that RBOH plays a crucial role in mediating ROS signal transduction that includes development, elongation and defence mechanism of the cell.

It has also been revealed that the genes AtRBOHD and AtRBOHF exhibit several properties that function in the generation of ROS during pathogen stresses (Torres et al. 2002) and ABA stimulating stomatal closing in guard cells (Kwak et al. 2003). It has been found that in *N. benthamiana*, the induction of NbRBOH A and NbRBOH B by virus-induced gene silencing (VIGS) results in the reduction of ROS generation that ultimately leads to the tolerance against *Phytophthora infestans* (Asai et al. 2008). Recent study showed that the use of antisense technology in the tomato plants results in the loss of RBOH function that reduces generation of ROS in leaves and leads to the phenotypic abnormalities (Sagi et al. 2004). It has been found that in root hair development AtRBOHC/RHD2 produced ROS which results in the stimulation of Ca^{2+} channels which in turn regulates cell elongation (Foreman et al. 2003). When act synergistically NO and ROS results in the cell apoptosis (Delledonne et al. 1998) while as normal generation of NO and H_2O_2 plays an essential role in hypersensitive response (HR) cell death (Delledonne et al. 2001).

1.5.4 Role of CDPK and MAPK as a Crosstalk in ROS

During pathogen signalling, the first step that arises in the cascade of signalling is the sharp increase of inward current of Ca^{2+} into the cytoplasm that results in the activation of ROS burst and HR cell death (Lecourieux et al. 2006). Recent studies have described the role of CDPKs in ROS and NO signalling mediated by various biotic and abiotic stresses and found that some CDPK genes are activated by certain transcription factors during pathogen signalling (Chico et al. 2002). It has been suggested that CDPK functions upstream in ROS generation (Kobayashi et al. 2007). It has been found that in tomato protoplast, the ectopic expression of AK1 (AtCPK1) in *Arabidopsis thaliana* accelerates NADPH oxidase activity and generation of ROS (Xing et al. 2001). During low abiotic-osmotic and wound signalling, there is an increased expression of NtCDPK2VK that activates generation of ROS and HR-like cell death and also found that under these stresses NtCDPK2VK accelerates the jasmonic acid, 12-oxo-phytodienoic acid and ethylene which has a crosstalk in ethylene signalling with MAPK (Ludwig et al. 2005). During the events like pathogen infection or elicitor treatment, there is quick activation of ROS phase I burst and then extensive ROS phase II burst and also described that a protein synthesis inhibitor is present in potato tubers and leaves that ends in ROS phase II burst (Yoshioka et al. 2001). In earlier studies a gene StRBOHA-D is isolated from potato plants (Yamamizo et al. 2006), and with the help of genetic engineering approaches in potato tubers, one gene StRBOHA is integrally expressed at low levels, and the other gene StRBOHB is activated from P. infestans by treatment with cell wall elicitor (Yoshioka et al. 2001). And also in leaves, the three genes StRBOHA, StRBOHB and StRBOHD are expressed at low levels, while StRBOHC is especially mediated in response to *P. infestans* (Yamamizo et al. 2006). Recent study of promoter analysis of StRBOHC reported that at the transcriptional level MEK2 plays an important role in the stimulation of gene expression and both I and II burst is abolished by NADPH oxidase inhibitor diphenylene iodonium, but another protein inhibitor cycloheximide ends only with phase II burst during the primary treatment of potato tubers (Kobayashi et al. 2007). These evidences suggest that StRBOHA and StRBOHB and StRBOHC contribute to phase I and phase II burst, respectively, and also inhibited by a protein kinase or a calcium inhibitor (Kobayashi et al. 2007). By in-gel kinase assays, it has been observed that StRBOHB acts as an important phosphorylation site and isolated Ser 82 and Ser 97 in the N-terminus of potato by using mutated *N*-terminal proteins of StRBOHB (Kobayashi et al. 2007). Also it has been observed that Ser 82 is phosphorylated in response to pathogen signalling in plants that is shown by using an anti-phosphopeptide (pSer 82) antibody. By cDNA expression screening, StCDPK5 is cloned by using the anti-pSer 82 antibody and found that the cells expressed an N-terminus of StRBOHB, and only Ser 82 and Ser 97 are phosphorylated by

CDPK in the *N*-terminus of calcium dependent which is analysed by mass spectrometry. ROS generation in leaves of *N. benthamiana* is stimulated by ectopic expression of StCDPK5VK, the integrally active mutant of StCDPK5. The ROS generation is stimulated by CDPK and is hindered by the loss of function of NbRBOHB in tobacco. The knockdown is complemented not by the mutant S82A/S97A but by the heterologous expression of wild-type potato StRBOHB. Phosphorylation of Ser 82 in tobacco is activated by the heterologous expression of StCDPK5VK. One of the studies suggests that the phosphorylation of RBOHs is mediated by StCDPK5 which in turn stimulates the ROS burst. These evidences have been analysed by bimolecular fluorescence complementation method which shows that intercommunication of StRBOHB and StCDPK5 has been carried out on the plasma membrane and revealed that the mutation of *N*-myristoylation and palmitoylation sites of StCDPK5 plays a crucial role in the membrane mobilisation, eludes these intercommunication.

Several studies have described that during plant-pathogen intercommunication, the generation of NO and ROS has several impacts on defence mechanism in plants and also exhibits various functions in defence systems that are mediated by these stresses (Fig. 1.12). It has been reported that *Arabidopsis thaliana* lacks the mutant AtNOA1 that results in the reduction of NO generation and, thus, increases the sensitivity of noxious Pseudomonas syringae (Zeidler et al. 2004) indicates that NO is participating in basic defence system during compatible hemibiotrophic bacterial pathogen and Arabidopsis interaction. A study showed that silencing of NbRBOHB in *N. benthamiana* results in the adverse impact on tolerant property against potentiate pathogen *P. infestans* (Asai et al. 2008) but not to *B. cinerea*, and by silencing NbNOA1 it leads in the stimulation of extreme high sensitivity to *B. cinerea* but not to *P. infestans* (Asai and yoshioka 2009). It has been observed that expressing StCDPK5 fused to pathogen-inducible promoter in potato plants shows high tolerance to *P. infestans* but shows high sensitivity to necrotrophic pathogen *Alternaria solani* (Yoshioka et al. 2009). With the help of genetic engineering approaches, it has been found that StCDPK5 is able to generate ROS but incapable of producing NO which implies that ROS may have a negative role in disease resistance to necrotrophic pathogen or a positive role in expansion of disease lesions. It has been observed that expressing StMEK2 (StMEK2^{DD}) and a pathogen-inducible promoter gene StCDPK5VK in the transgenic potato plants show resistance to both *P. infestans* and *A. solani* (Yamamizo et al. 2006). One of the recent studies showed that SIPK is activated by MEK2^{DD} which results in the generation of RBOH-dependent ROS and NO production which is stimulated by NOA1 in *N. benthamiana* (Asai and Yoshioka 2008). As it was found that MAP kinase plays an important role in NO and ROS signalling, they also play a crucial role in the activation and phosphorylation of nitrate reductase (Wang et al. 2010). The above findings revealed that contribution of NO in plants makes them resistant against necrotrophic pathogens *A. solani* and *B. cinerea* besides other diseases, but simultaneously NO together with ROS induces apoptosis which is an advantage over these necrotrophic pathogens. This implies that NO and ROS synergistically mediates a stronger response against these environmental stresses. It has been observed from the earlier studies that NO has several functions along with ROS that contributes to either cell apoptosis or resistance to a number of pathogens depending upon the type of stress that leads to the open question of how NO and ROS changes the physical and cellular metabolism. It is not always possible that generation of ROS leads to increased sensitivity of the cell which depends on the exposure and intensity of ROS formation, but it can also result in the activation of defence responses during various abiotic and biotic stresses.

1.6 Perspectives and Conclusion

Plants have developed various stress-tolerant mechanism during their evolutionary period of development. In recent times plant signal transduction has been in focus and has emerged as an

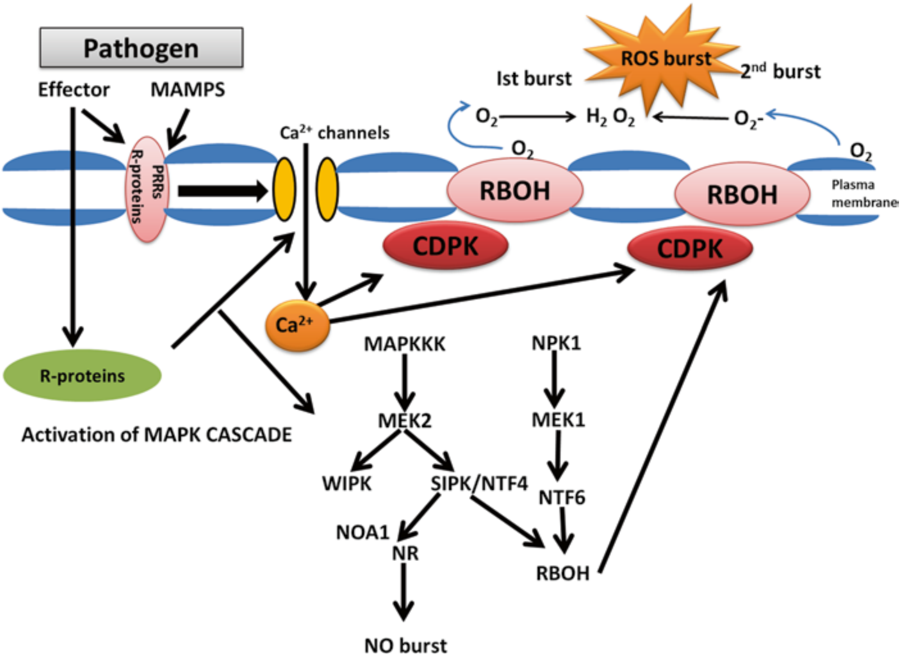

Fig. 1.12 A model showing NO burst and ROS burst that induces Ca^{2+} channels, CDPKs and MAPK cascade that upregulates inducible form of RBOH gene (Hirofumi Yoshioka et al. 2011)

important area of research in biology. The plants mediate responses to these environmental changes through diverse mechanisms that help them to withstand these changes. The role of calcium-mediated signalling pathways in plants has been investigated and shows gross resemblance to that of animal system. As CDPKs play an essential role in signalling pathways, therefore, there is a need for broad and comprehensive knowledge regarding their structural and functional relationships. Through various studies it has been revealed that there is an active involvement of MAP kinase in plant signal transduction. In majority of cases, only indirect proof regarding the involvement of MAP kinase in signalling is known but the responsible genes/ protein kinase that regulates these signalling pathways is still unknown. In addition to this, other types of protein kinases also share some properties of MAP kinases such as substrate, specificity and size. Therefore, utilising the proficient technologies to identify the particular MAP kinases and their respective genes encoding enzymes is of fundamental importance to biology. Therefore, multidisciplinary approaches are required to develop novel methods of analysis just to acquire better understanding about the function of MAP kinase in different processes. Further, identification and characterisation of plant protein kinases and their intercommunication will lead to insights into the mechanism regulating plant growth and development. ROS formation during various metabolic reactions poses a deleterious effect on different cellular components by causing disruption of the electron transport chain. ROS play two different roles in plants, viz. at low concentration they behave like signalling molecules mediating several plant processes, while at high concentration they cause oxidative stress leading to apoptosis. In response to oxidative stress, plants have developed endogenous defence mechanisms comprising of enzymatic and nonenzymatic antioxidants. Despite that much progress has been made in current years, there are still some lacuna in ROS formation and its ill effects on plant growth and development because of their short half-life and high reactivity. With the help of advanced analytical approaches, the fate of ROS formation will assist in developing a broad knowledge about their function in cellular signalling. Last but not least, future research should be focused more on biochemical, molecular and genomic technologies to better understand the signalling pathways in plants that will ultimately lead to the significant advancement in the fields of agricultural, biotechnology and forestry.

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