

Khalid Rehman Hakeem  
Reiaz Ul Rehman  
Inayatullah Tahir  
*Editors*

# Plant signaling: Understanding the molecular crosstalk

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# Plant signaling: Understanding the molecular crosstalk

 Springer

*Editors*

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## Foreword

Through the ages, plants have evolved and used intricate networks of interacting molecules for regulating growth and propagation and also to mount defence mechanisms to overcome and adapt to adverse conditions brought about by abiotic (environmental) and biotic (pathogenic) stresses. In recent times, environmental stresses have intensified due to global warming and climate change-induced adverse conditions such as salinity, submergence, drought and temperature fluctuations. Due to loss of biodiversity and over-reliance on chemicals, there has also been an increase in infection-related stresses. These have had an adverse effect on agricultural production. To ensure food security, it may be necessary to intervene genetically to speed up the defence and adaptation processes. For this, it is very important to understand the molecular mechanisms that underlie defence and adaptation to biotic and abiotic stresses. This new book “Plant Signaling: Understanding the Molecular Crosstalk” will be a very good introduction to the subject.

The environmental and pathogenic stress signals are recognized by stress-specific sensor proteins embedded in the plant cell membrane that help to transmit the exogenous signals from the cell surface to the nucleus where they stimulate different regulatory genes and transcription factors responsible for the expression of specific protective and adaptive proteins. Plant infection triggers production of resistance proteins that bind to pathogen-derived factors. This interaction initiates a signal transduction cascade that ultimately causes localized cell and tissue death at the site of infection and imparts non-specific immunity to the rest of the cell. Understanding the host-pathogen interactions at the molecular level could help with the engineering of disease-resistant transgenic crops.

“Plant Signaling: Understanding the Molecular Crosstalk” is a very timely and relevant anthology of review chapters that outline the current understanding of the myriad ways in which different molecules interact directly and indirectly within various regulatory networks to provide protection and adaptive capability to plants. Different chapters of the book review the current knowledge of different exogenous and intracellular factors involved in stress recognition, signal transduction and expression of genes involved in disease resistance and immunity and the activation of specific transport channels and adaptive molecules. The references will be particularly helpful in guiding researchers and students to original literature that provide information on the various molecular crosstalks that help the plants to adapt to adverse conditions and infections.

Some of the signalling cascades and signalling molecules described in detail include cyclic nucleotide-gated channels involved in multiple pathways related to immunity and stress, plant-specific *Rab* GTPases involved in membrane trafficking and several pathways and reactive oxygen species (ROS) which in optimum amounts help to overcome external stresses but in larger amounts can be harmful to plants. A number of chapters provide details of cell signalling pathways that could help plants to adapt to salinity, drought, heat and cold stress. Plant sugars could also be involved in cell signalling through interaction with plant stress hormones such as ethylene. A number of chapters also demonstrate how plants utilize a large number of signalling molecules and pathways for their own survival, growth and maintenance of biodiversity. These include membrane-active phosphoinositols and “SNARES”, the pathways involved in self-incompatibility (for rejection of self-pollen), senescence, nitrogen regulation and signalling and symbiotic and nonsymbiotic associations between plants, fungi and bacteria.

Unravelling in detail the *N*-signalling pathway could result in the construction and production of high *N*-efficient genotypes of important food crops. Understanding the molecular signalling mechanisms and the functional genomics of important agricultural crops and also of wild-type plants and organisms with important resistance traits could help in the identification of novel pathogenic and stress-resistance genes that could be useful in the production of useful transgenic plants resistant to infections or adapted to climate change-induced stresses. “Plant Signaling: Understanding the Molecular Crosstalk” will be a very valuable addition to academic libraries and a handy source of information to researchers involved in research and innovation in the field of food security and biodiversity.

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## Preface

The ever-growing population of the world will have an adverse effect on the agricultural productivity. Besides, many other factors are responsible for a decline in crop productivity including the over-exploitation of bioresources, mal-agricultural practices and deleterious abiotic environmental stresses. For meeting the increasing food demands and overcoming the losses incurred by various factors, researchers are always on the look for the development of novel strategies. The new scientific innovations are reached upon through interdisciplinary approaches and by broadening the research area. Plants have unique abilities to grow in varied locations all over the world, and they show remarkable developmental plasticity for sustaining in these continually changing environments. Unlike other organisms, plants are sessile and they experience a plethora of biotic as well as abiotic stresses such as pathogens (viral, bacterial, insects, etc.), drought, salinity, temperature and metal toxicity. The plants have adapted themselves against these stresses by developing specialized mechanisms. They respond to the external cues by changing their morphologies, and these signals are recognized with the help of some membrane protein sensors that in turn are transduced to the nucleus. Ultimately, the nuclear transcription factors and genes are stimulated to form the product leading to plant adaptations at various levels and thus assisting them to sustain and surpass the adverse conditions.

Over the last 20 years, the concept of signalling is being continually modified, especially the molecular crosstalk associated with it. It has taken a shape of a new field due to the complexities and broadening of the knowledge base in the subject. Furthermore, during the last 10 years, the understanding in the field of plant signalling has been greatly enhanced by the development of various bioinformatic tools. The mapping of various plants especially the *Arabidopsis thaliana* has greatly assisted in identification of hormonal, developmental and environmental signal transduction pathways and the existing crosstalk between them. Signalling pathways utilize a complex network of interactions to orchestrate biochemical and physiological responses such as flowering, fruit ripening, germination and photosynthetic regulation, besides shoot or root development. Signalling agents that appear to be common to many different pathways include  $\text{Ca}^{2+}$ , inositol phospholipids-proteins, cyclic nucleotides, protein kinases and protein phosphatases. Any signal transduction pathway may involve the coordination of multiple signalling agents operating in multiple cellular structures. These agents never work in isolation, but always in networks that intersect multiple signalling pathways. The hormone-mediated



pathways (GA, IAA, ABA, JA, Br and SA) are involved in developmental responses. The cyclic nucleotide-gated channels (CNGCs) in plants are a part of complex signalling network that conduct cations and are responsible for diverse responses from stress tolerance, transpiration and fertilization. Phosphoinositides (PIs) comprise a family of minor membrane lipids which play important roles in many signal transducing pathways in the cell. Signalling through various PIs has been shown to mediate cell growth and proliferation, cytoskeleton organization, vesicle trafficking, regulation of ion channels and nuclear signalling pathways in various eukaryotic models. Membrane trafficking is a highly regulated process in which various molecular machineries are involved. It involves vesicle formation, tethering and finally fusion. SNAREs are involved in these membrane-trafficking events, and these interact with several proteins such as *Rabs* that act as regulators of SNARE-complex formation, and together they gate channels' potential signalling processes. Amongst the environmental factors which are involved in signalling are the reactive oxygen species (ROS) generated during cell metabolism. ROS are spontaneously produced in the cell by auto-oxidation reactions. Some of these ROS are physiologically useful and in fact necessary for life but can also be harmful if present in excess or in inappropriate amounts. The NO-mediated pathways have a role in plant defence and immunity. The cold stress tolerance mechanisms involve the sensing followed by expression of cold-responsive genes and transcription factors leading to formation of compatible solutes or osmoprotectants. The environmental factors are also responsible for the initiation of senescence process that is coordinated through common signalling networks involving various phytohormones acting as signalling molecules (ethylene, ABA, SA and JA). The biotic stresses (pathogens) constantly challenge the plant defence, and the disease development is rare due to resistance mechanisms which are interconnected signalling networks.

The plant signalling is an ocean of information, and it is a challenge to comprehend and cover the entire aspects of a subject at the same place. The present book is providing the detailed coverage of a broad range of aspects of signalling and emphasizing the critical importance of such signalling to plant and associated systems.

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# Plant Signalling: Response to Reactive Oxygen Species

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Bisma Malik, Tanveer Bilal Pirzadah, Inayatullah Tahir,  
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and M.Z. Abdin

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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.

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**Keywords**

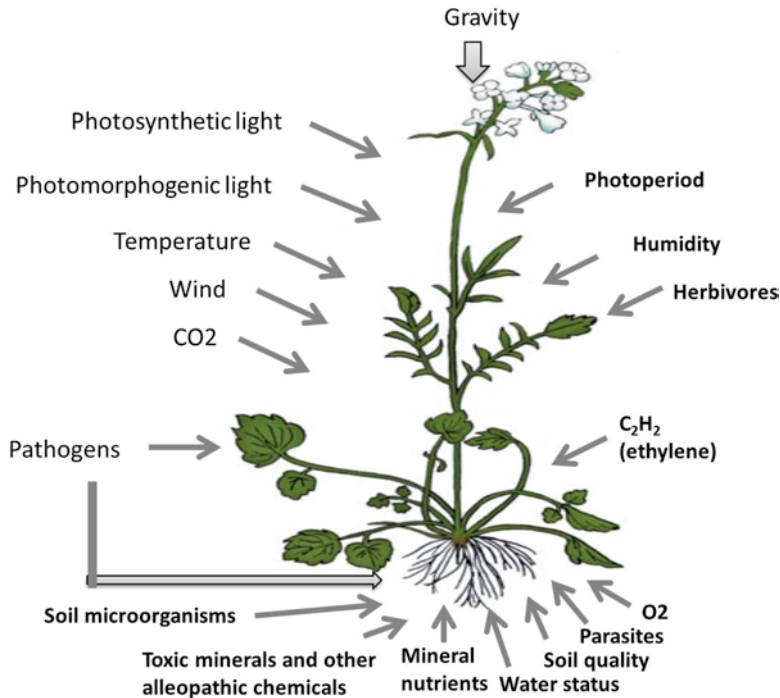
Signal transduction pathways • Defence mechanism • ROS • Oxidative stress • NO • Transgenic plants

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## 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

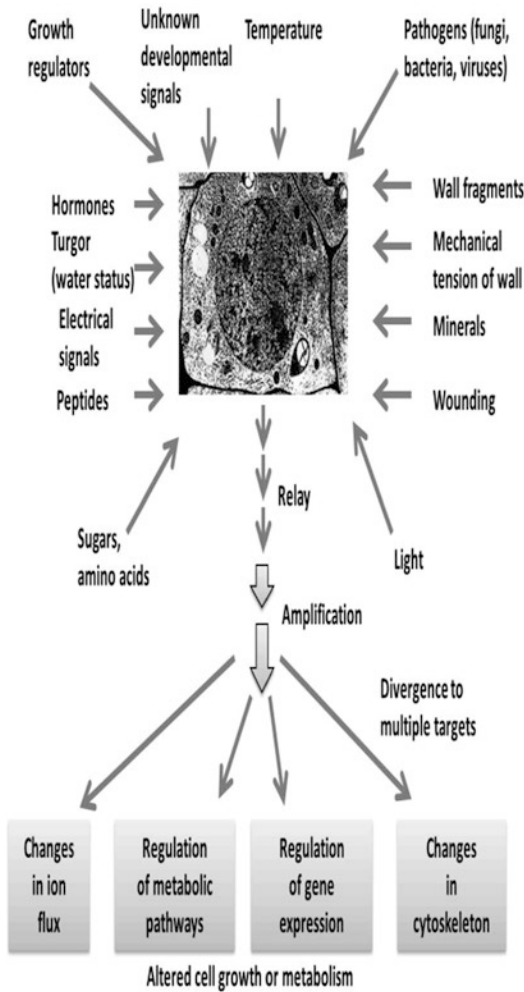


**Fig. 1.1** External signals that affect plant growth and development include many aspects of the plant's physical, chemical and biological environments. Some external sig-

nals 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<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup> 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<sup>2+</sup>-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<sup>2+</sup>-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<sup>2+</sup>- and Ca<sup>2+</sup>/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



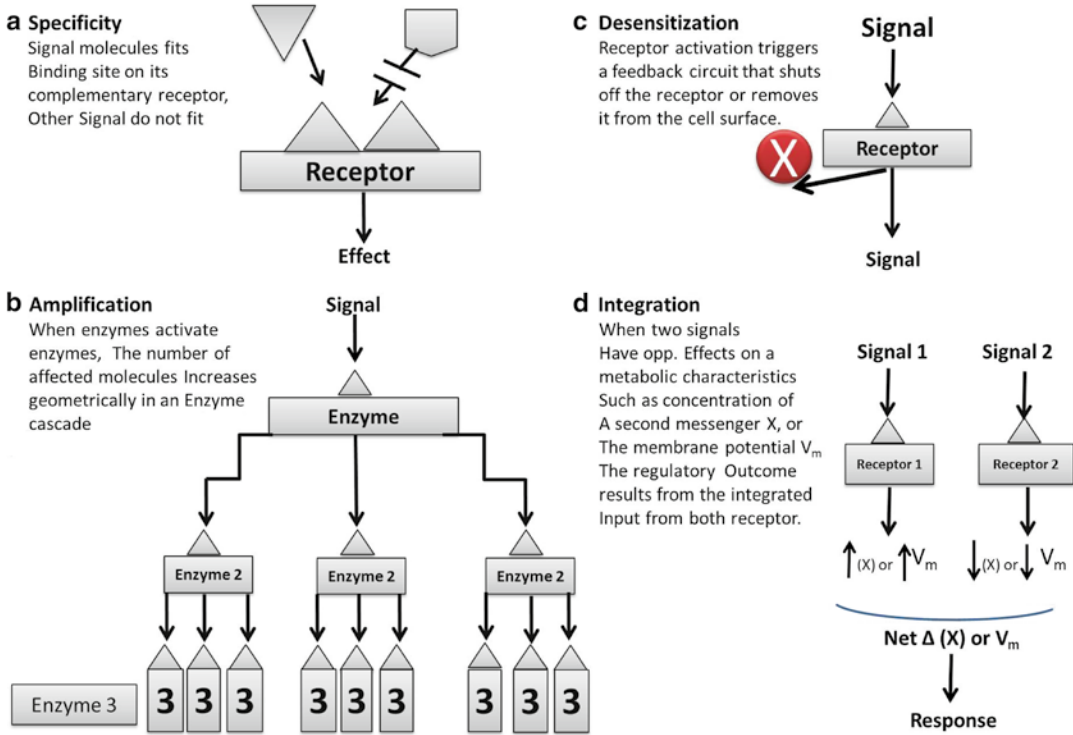
**Fig. 1.2** A variety of internal signals modify plant cell metabolism, growth, and development. The ability of cells to respond to these signals is not confined to cells that are still growing and developing. Mature cells, too, can initiate metabolic responses and can even reinstate growth and division 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 “transducers” that are likely to be  $\text{Ca}^{2+}$  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



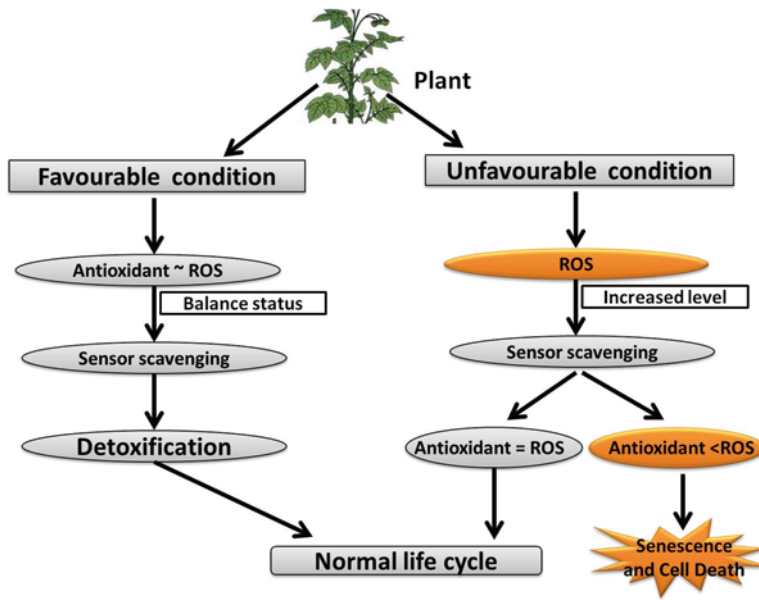
stresses. Plants have the unique ability to cope up these stresses and survive under various stressful conditions. In this mechanism, the signals are initially perceived by various membrane-bound sensors which in turn get amplified and then transduce these signals to the nucleus in order to stimulate 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  $\text{Ca}^{2+}$  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:

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#### 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).

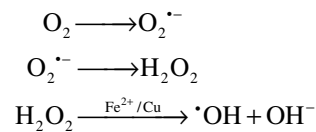
Majority of diseases caused by the oxidative stress involves generation of not only ROS but also nitrogen reacting species (NRS) including free radicals (Beckman and Ames 1998; Gutteridge and Halliwell 2000). Besides having an important role in intra- and extracellular signalling, these reactive molecular 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 Barrett 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 glycooxidation (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 ( $O_2^{\cdot-}$ ), hydroxyl radical ( $\cdot OH$ ), alkoxy radical ( $RO\cdot$ ) and peroxy radical ( $ROO\cdot$ ), whereas oxygen-centred non-radicals are hydrogen peroxide ( $H_2O_2$ ) and singlet oxygen ( $^1O_2$ ). Other ROS are nitrogen-containing species such as  $NO\cdot$  and nitric dioxide ( $NO_2$ ) and peroxynitrite ( $OONO^-$ ) (Simon et al. 2000; Huang et al. 2005).

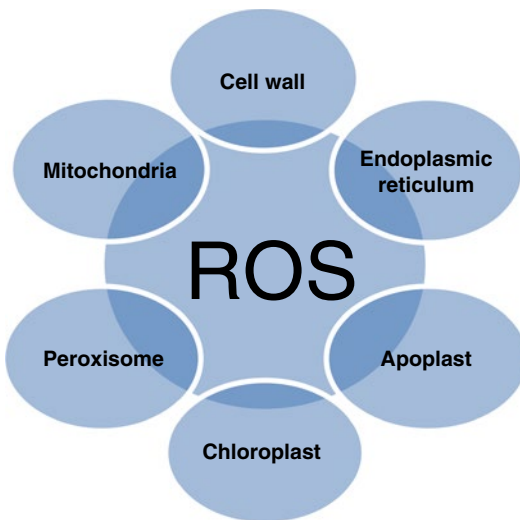
These ROS are generated due to the leakage of electrons onto  $O_2$  from the electron transport chain or as an output of several biochemical pathways localised in various cellular compartments. These are five possible species: superoxide anion radical ( $O_2^{\cdot-}$ ), hydroperoxyl radical ( $HO_2^{\cdot}$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



The  $O_2^{\cdot-}$  and  $H_2O_2$  so formed in presence of metal catalyst such as  $Cu^+/Fe^{2+}$  may lead to formation of most reactive  $\cdot OH$ . Synthesis of  $O_2^{\cdot-}$  and  $H_2O_2$  leads to the generation of  $\cdot OH$  which oxidises lipids and results in lipid peroxidation (Chessman and Slater 1993). A study described the role of  $H_2O_2$ ,  $^1O_2$ ,  $O_2^{\cdot-}$  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

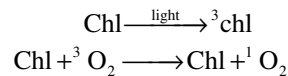
Enzyme	Reaction catalysed	Transgenic plant against cold stress	References
<i>Super oxide dismutase (SOD)</i>	$O_2^- + O_2^- + 2H^+ \leftrightarrow 2H_2O + O_2$	Cu/Zn SOD from <i>Pisum sativum</i> to <i>Nicotiana</i>	Gupta et al. (1993)
		Fe-SOD from <i>A. thaliana</i> to <i>Medicago sativa</i>	McKersie et al. (2000)
		Mn-SOD in <i>Gossypium hirsutum</i>	Payton et al. (2001)
<i>Catalase (CAT)</i>	$2H_2 O_2 \leftrightarrow 2H_2 O + O_2$	Rice ( <i>Oryza sativa</i> )	Matsumura et al. (2002)
<i>Glutathione reductase (GR)</i>	$NADPH + GSSG \leftrightarrow NADP + 2GSH$	Tobacco ( <i>N. tabacum</i> )	Le Martret et al. (2011)
		From <i>A. thaliana</i> to <i>Gossypium hirsutum</i>	Payton et al. (2001) and Kornyejev et al. (2003b)
<i>Dehydro-ascorbate reductase (DHAE)</i>	$AA + H_2 O_2 \leftrightarrow DHA + 2H_2O$	From human to Tobacco ( <i>N. tabacum</i> )	Kwon et al. (2003)
		Tobacco ( <i>N. tabacum</i> )	Le Martret et al. (2011)
<i>Ascorbate peroxidase (APX)</i>		From <i>Pisum sativum</i> to <i>Gossypium hirsutum</i>	Kornyejev et al. (2001, 2003a, b)
		From <i>Spinacia oleracea</i> to <i>N. tabacum</i>	Yabuta et al. (2002)
		From <i>Pisum sativum</i> to <i>Lycopersicum esculentum</i>	Wang et al. (2005)
		Tomato ( <i>Lycopersicum esculentum</i> ) <i>StAPX</i> gene in Tobacco ( <i>N. tabacum</i> )	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  $^1O_2$



$^1O_2$  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).

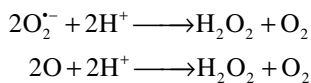
#### 1.4.1.2 $O_2^-$

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  $O_2^{\cdot-}$  (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  $O_2^{\cdot-}$  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.  $O_2^{\cdot-}$  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  $O_2^{\cdot-}$  accepts one electron and two protons. Nonenzymatic or SOD-catalysed reaction can easily dismutate to  $H_2O_2$  (hydrogen peroxide)

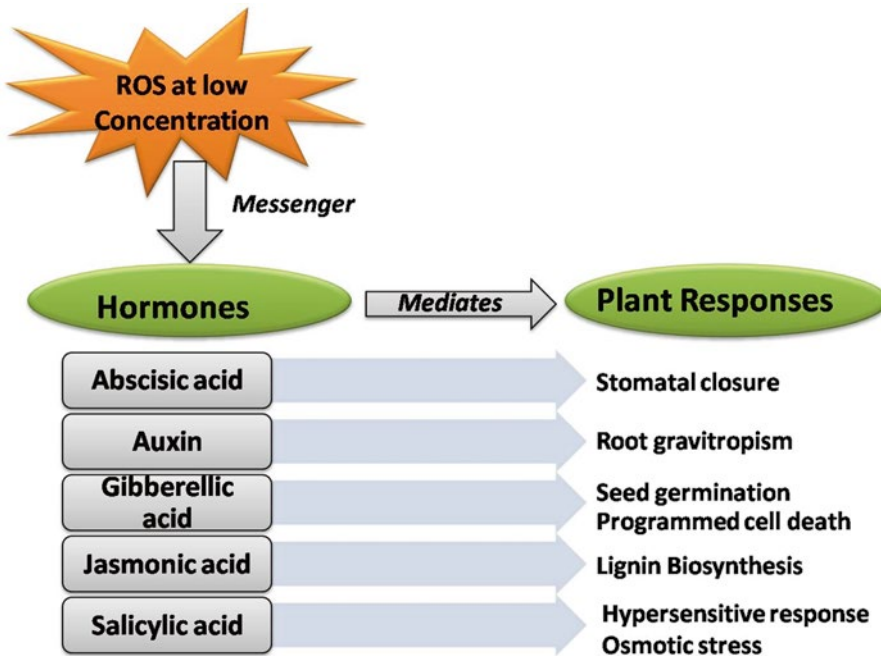


$H_2O_2$  is produced easily in the cells under normal or stressful condition such as drought, chilling, UV irradiation, exposure to light, wounding and pathogen attacks.  $H_2O_2$  is generally produced during electron transport chain of chloroplast, mitochondria, ER and plasma membrane or by  $\beta$ -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.  $\cdot OH$  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  $\cdot OH$  and thus its excess generation subsequently leads to apoptosis (Pinto et al. 2003).  $\cdot OH$  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).  $\cdot OH$  involves two essential reactions, viz. addition of  $\cdot OH$  to organic molecules or elimination of hydrogen atom from it. As  $\cdot OH$  are short-life period molecules having an extensive positive redox potential (close to +2V) of “free”  $\cdot OH$ , 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  $\cdot OH$  (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

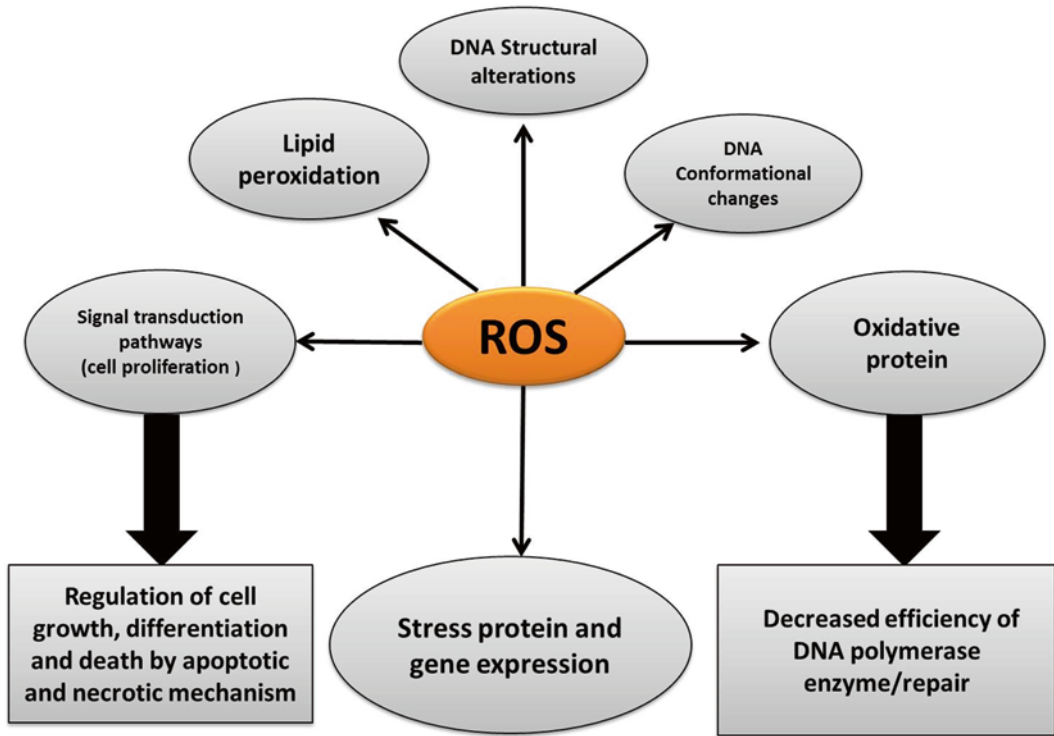
network that facilitates the plants to stimulate the accumulation of lignin in response to damage of cell wall has been recently characterised (Denness et al. 2011). ROS also plays an important role in heavy metal signal transduction pathway (Yan et al. 2007). ROS upregulates the genes implicated in 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



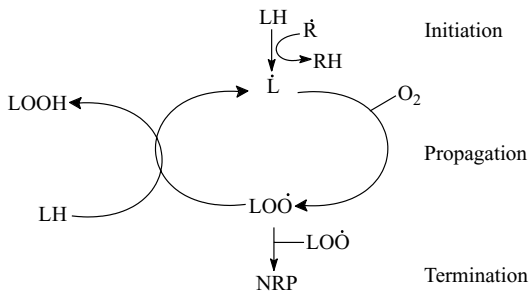
**Fig. 1.7** Adverse effects of ROS

lipid peroxidation which is initiated by the reaction of an activated free radical such as singlet oxygen ( $^1\text{O}_2$ ,  $\text{O}_2^{\cdot-}$ , or  $\cdot\text{OH}$ ) with a lipid substrate (LH) to produce extremely reactive carbon-centred lipid radical ( $\cdot\text{L}$ ). In the second step of LPO, molecular oxygen adds quickly to generate lipid peroxy radical ( $\text{LOO}\cdot$ ). The  $\text{LOO}\cdot$  eliminates a hydrogen atom from another lipid molecule (LH), generating lipid hydroperoxide (LOOH) and another extremely reactive carbon-centred radical ( $\text{L}\cdot$ ) 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 ( $\text{Fe}^{2+}$ ) and ferric ( $\text{Fe}^{3+}$ ) can catalyse the elongation of LPO chain by degrading LOOH. The resulting alkoxy ( $\text{LO}\cdot$ ) and peroxy ( $\text{LOO}\cdot$ ) 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 (Matkovic 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  $O_2^{\cdot -}$  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, over-expressed 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 non-enzymatic 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  $O_2^-$ ,  $H_2O_2$  and generating  $\alpha$ -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

$\gamma$ -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  $O_2^-$ ,  $\cdot OH$  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  $\gamma$ -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  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherols belong to a group of lipophilic antioxidants implicated in oxygen free radical, lipid peroxyl radicals and  $^1\text{O}_2$ -scavenging activity (Diplock et al. 1989). Amongst tocopherols  $\alpha$ -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  $\text{O}_2$  (Ivanov and Khorobrykh 2003). Accumulation of  $\alpha$ -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  $^1\text{O}_2$  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, hydroxycinnamate esters and lignin) which exhibit antioxidant activity and found in plant tissues (Grace and Logan 2000). Polyphenols contain an aromatic ring with  $^-\text{OH}$  or  $\text{OCH}_3$  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).

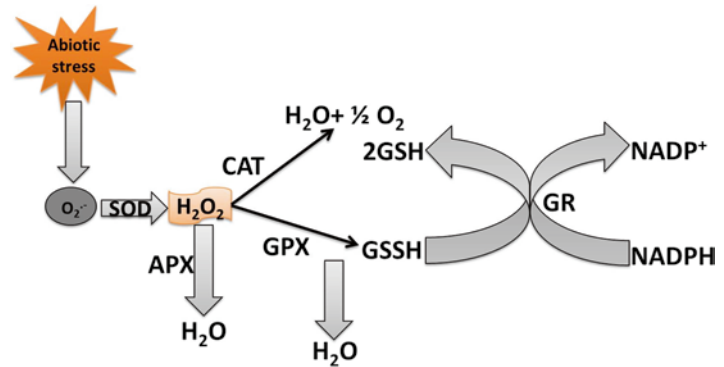
### Proline

$\alpha$ -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  $\text{OH}^*$  and  $^1\text{O}_2$  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

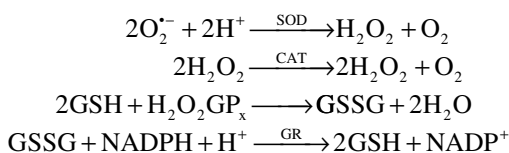
Catalase, a tetrameric enzyme, is localised mainly in peroxisomes, while reduced levels also exist in mitochondria and cytosol. The human erythrocytes are rich in catalase (Aebi 1983). Catalase is involved in cellular detoxification and converts  $H_2O_2$  to water and oxygen. Catalase protects cells 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  $\beta$ -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<sub>2</sub>O<sub>2</sub> 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 (Chamnonpol 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<sub>2</sub>O<sub>2</sub> 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<sub>2</sub>O<sub>2</sub> (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<sub>2</sub>O<sub>2</sub> 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 flavo-protein, allows the transformation of oxidised glutathione (GSSG) to reduced glutathione (GSH) by oxidation of NADPH to NADP<sup>+</sup> (Papas 1999).



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 *populus* 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 peroxy 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 tabacum* 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 exist in various cellular compartments like chloroplasts (Hossain et al. 1984). Various studies have described that during environmental stresses the