1. Introduction

One of the most important challenges higher plants are facing throughout their lives is to cope with a wide range of environmental stresses. Environmental factors can be of abiotic and/or biotic nature. Abiotic factors such as excessive light, drought, salinity and extreme temperatures are the major environmental factors that limit plant productivity, due to the series of negative morphological, physiological and molecular changes they inflict on the development of plants. Biotic factors include the physical damages caused by insects or herbivores, and diseases that develop from pathogenic bacteria and fungi (Lamb and Dixon, 1997; Bolwell and Wojtaszek, 1997).

Combination of several stress factors is the normality for a plant and is referred to as multiple stress (Mittler, 2006; Newton et al., 2011).

Plants are resident in place and have limited ability to escape environmental stresses. Therefore, it is essential for them to generate various types of successful resistance responses, which often partly overlap (Mullineaux et al., 2000; Valcu et al., 2009; Huang et al., 2011). Most plant species, due to continuous evolution and/or targeted selection, have developed to some extent the ability to adapt to those unfavourable stresses.

The response to natural stress involves a complicated signal transduction network that is activated by sensing the stimuli, and is characterized by the synthesis of stress-related proteins and signaling molecules such as hormones and reactive oxygen species (ROS), and finally the transcriptional activation of specific stress-responsive genes to counteract the stress (see for review Xiong et al., 2002; Suzuki et al., 2012). These signals substantially induce the expression of sets of specific defense genes that lead to the organization of the overall defense reaction.

Plants cellular responses, which are always associated with various biotic and abiotic stimuli, may share common mechanisms; for example the alteration of the plant cell redox balances (Jambunathan et al., 2010, Foyer and Noctor, 2009; Mullineaux and Baker, 2010).

1.1 ROS production in different organelles

In plant cells, ROS arise via a number of routes. The major sources of ROS production are those compartments with high rate of electron flow or high oxidizing metabolic activity such as chloroplasts, mitochondria and peroxisomes (Gill and Tuteja, 2010).

In chloroplasts, superoxide anion ($O_2^{\bullet}$) and singlet oxygen ($^1O_2$) are generated at photosystem I (PSI) and photosystem II (PSII) respectively, resulting in the formation of other ROS such as H_2O_2 and hydroxyl radical ($HO^{\bullet}$) by stepwise monovalent reduction of superoxide anion (Figure 1.1) (see for review Mittler et al., 2004; Sharma et al., 2012).

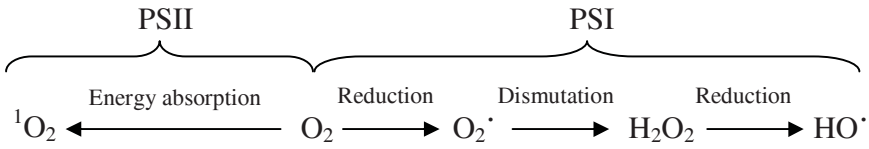


Figure 1.1. Mechanisms of O_2 activation and ROS production. Transfer of excitation energy to oxygen forming $^1O_2$ in the light-harvesting complexes at PSII. Successive reduction of molecular oxygen to $HO^{\bullet}$ via $O_2^{\bullet}$ and H_2O_2 occurs on the acceptor side of PSI.

The ROS formation is favored under conditions of high-light stress coupled with low CO_2 fixation due to limited water availability during different environmental stresses such as salinity, freezing and drought (Nishiyama et al., 2006). The reduction of oxygen can occur on the thylakoid membrane-bound primary electron acceptor of chloroplasts at the level of PSI, as a result of the photosynthetic transport of electrons, triggering the formation of $O_2^{\bullet}$, by one electron diversion from ferredoxin to molecular oxygen via Mehler reaction. $O_2^{\bullet}$ can be further converted to the intermediates, H_2O_2 and $HO^{\bullet}$ in step-wise fashion (for review see Mullineaux et al., 2006; Gill and Tuteja, 2010; Sharma et al., 2012). Moreover, $^1O_2$ can be produced by triplet chlorophyll, which is formed in the antenna system and in the reaction centre of PSII. $^1O_2$ may be responsible for light-induced loss of PSII activity by reacting with the D1 protein of PSII as a target, or inhibiting the PSII protein repair (Krieger-Liszkay, 2005; Krieger-Liszkay et al., 2011).

In mitochondria, significant quantities of ROS can be generated from over-reduced mitochondrial electron transport system. The major site of $O_2^{\bullet}$ generation occurs in two

segments of the respiratory chain: in the flavoprotein region of NADH dehydrogenase segment and the ubiquinone-cytochrome region (Sweetlove et al., 2002).

Direct reduction of molecular oxygen results in $O_2^{\bullet}$ by transfer of a single electron from ubisemiquinone radical. $O_2^{\bullet}$ in turn is reduced to H_2O_2 (Rhoads et al., 2006; Møller et al., 2007). Under water stress conditions, enhanced mitochondrial ATP production could potentially contribute to the generation of ROS by transferring electrons from the cytochrome C_1 electron transport system to O_2 (Turrens, 2003; Norman et al., 2004).

Similar to mitochondria and chloroplasts, peroxisomes also produce ROS as by-products of their normal metabolism such as the glycolate oxidase reaction, fatty acid β-oxidation, and the enzymatic reaction of flavin oxidases (Corpas et al., 2001; Palma et al., 2009). Furthermore, peroxisomes are considered as the major sites of intracellular H_2O_2 production and detoxification. The ROS amount within peroxisomes is regulated by a delicate balance between production and scavenging (reviewed by Maurino and Flügge, 2008; Miller et al., 2010; Sharma et al., 2012).

In addition to those compartments, ROS are also produced in plants in the cytoplasm, the endoplasmic reticulum, and in the apoplast, where they are either generated at the plasma membrane level or extracellularly (Gill and Tuteja, 2010).

Following biotic stress, especially infection with necrotrophic fungi such as *Botrytis cinerea, Sclerotinia sclerotiorum* and *Alternaria brassicicola*, increased level of hydrogen peroxide (H_2O_2) and superoxide ($O_2^{\bullet}$) is an early event in pathogen response (Alvarez et al., 1998; Muckenschnabel et al., 2002; Gechev et al., 2004). For example, six hours after inoculation of *Arabidopsis* leaves with germinated spores of *Botrytis*, the ROS-producing areas not only spread along with the progress of infection, but were several cell layers ahead of the fungal hyphae (Govrin and Levine, 2000), suggesting also signal properties for ROS.

1.2 Oxidative damage

A common denominator of most environmental stresses is the elevated production and accumulation of dangerous ROS inside the cells. These toxic molecules can react with biomolecules and can damage a wide range of macromolecules. Indeed, ROS are causing oxidative damage by being involved in oxidation of proteins and enzyme inhibition, peroxidation of lipids, damage of nucleic acids and activation of the programmed cell death pathway, if they are not effectively and rapidly removed from cells (Shah et al., 2001; Meriga et al., 2004; Chen et al., 2012).

1.2.1 Protein oxidative damage

Oxidative damage to proteins occurs when ROS or by-products of oxidative stress attack them, causing covalent protein modification possibly changing their activity through nitrosylation, carbonylation, glutathionylation, and formation of disulphide bonds (Gill and Tuteja, 2010; Sharma et al., 2012). Thiol groups of the sulphur containing residues Cys and Met are more susceptible to oxidation especially with $^1O_2$ and $HO^{\cdot}$. The oxidation of amino acids side-chains, particularly from Arg, His, Lys, Pro, Thr and Trp causes the formation of free carbonyl groups which may inhibit or change protein activity (Møller et al., 2007). These carbonyls then increase the protein susceptibility towards proteolytic attack.

Enhanced modification of proteins has been demonstrated in plants subjected to various stresses (Romero-Puertas et al., 2002; Tanou et al., 2009). Proteins can be indirectly modified by conjugation with breakdown products of fatty acid peroxidation (Yamauchi and Sugimoto, 2010). These compounds modify proteins and nucleic acids via either Micheal-addition to nucleophiles such as Cys, Lys or His residues or the formation of a Schiff-base between the carbonyl group and the amino group (West and Marnett, 2006).

1.2.2 Lipid peroxidation

Peroxidation of polyunsaturated fatty acids takes place when ROS reach above-threshold levels inside the cells, leading to the production of lipid-derived radicals initiating a chain reaction. Lipid peroxides decompose to small hydrocarbon fragments like ketones,

aldehydes, alkanes, lipid epoxides, and alcohols (Davoine et al., 2006), many of which are characterized by an α,β-unsaturated carbonyl moiety that readily forms Michael-type adducts at the β carbon with cellular nucleophiles, including thiol and amino groups of peptides and proteins (Davoine et al., 2006). Among these reactive electrophile species (RES) are aldehydes, such as 2(E)-alkenals like acrolein and 2(E)-hexenal, or ketones, such as fatty acid ketodienes, ketotrienes and 12-oxophytodienoic acid (OPDA) (Figure 1.2) (Yamauchi et al., 2008).

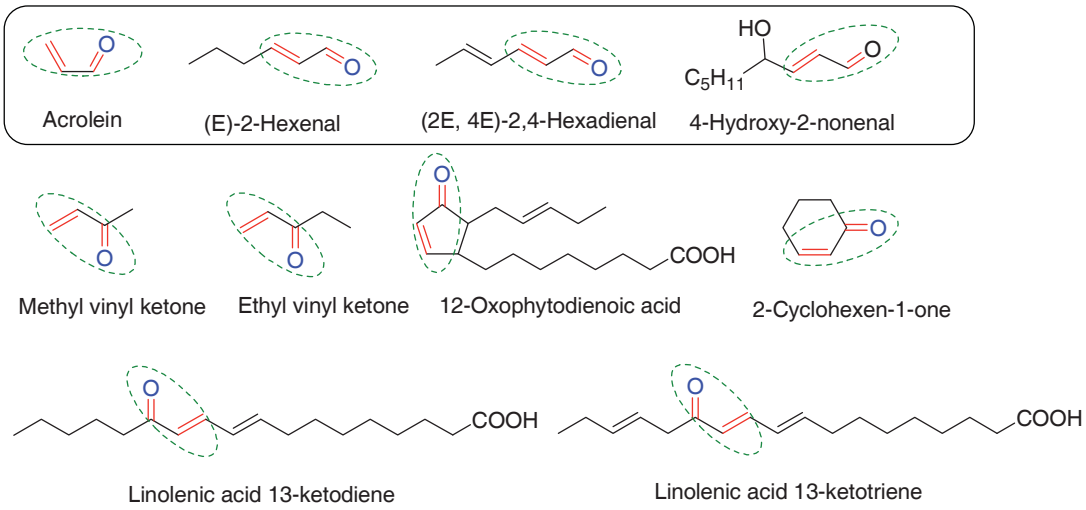


Figure 1.2. Example of α,β-unsaturated aldehydes/ketones. These compounds are commonly observed in plant cells and generated through multistep polyunsaturated fatty acid peroxidation. The α,β-unsaturated carbonyl groups are red in color and encircled with dashed lines

Other sequences of spontaneous lipid peroxidation reactions create cyclopentenone compounds named phytoprostanes, analogous to isoprostanes in animals (Mueller, 2004). These compounds are biologically active and contain a reactive α,β-unsaturated carbonyl structure, classifying them as RES (Mueller et al., 2008).

Lipid peroxidation can also be initiated enzymatically, e.g. by lipoxygenase; the activity of this enzyme generates unstable fatty acid hydroperoxides, which in turn can decompose to a great variety of products. The hydroperoxides serve as substrates for several enzymes including allene oxide synthase (AOS), which is involved in jasmonic

acid biosynthesis (Vick and Zimmermann, 1987; Mosblech et al., 2009; Schaller and Stintzi, 2009).

Because of their reactivity, increased levels of α,β-unsaturated carbonyl compounds are highly cytotoxic. The plant photosynthetic machinery is highly sensitive to elevated levels of these compounds, which strongly inhibit both thylakoid and stromal photosynthetic reactions (Alméras et al., 2003; Mano et al., 2010). High levels of lipids peroxidation have been reported in plants suffering from different forms of biotic and abiotic stresses including bacterial infection (Muckenschnabel et al., 2001), excess light, drought and oxidative stress (Sharma and Dubey, 2005; Kotchoni et al., 2006; Levesque-Tremblay et al., 2009; Mano et al., 2009).

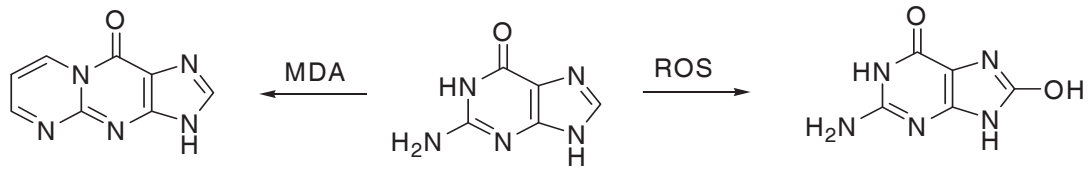
The exogenous application of RES is increasingly used as a tool to simulate oxidative stress and to assess specific and common responses towards different types of α,β-unsaturated aldehydes/ketones. The small volatile electrophiles, acrolein (ACR) and methyl vinyl ketone (MVK) are potent inducers of a pathogenesis-related (*PR4*) gene expression in *Arabidopsis* leaves (Alméras et al., 2003), and leaves of *Cyclamen persicum* infiltrated with these compounds produce necrotic lesions (Kai et al., 2012). These studies provide *in vivo* evidence for the cause-effect relationship between RES and cytotoxic damage.

On the other hand, lipid peroxidation products play an important role as signaling molecules that regulate gene expression (Sattler et al., 2006; Eckardt, 2008). The best characterized example is the OPDA which accumulates in response to various stresses, in particular wounding and pathogen infection (Stintzi et al., 2001; Taki et al., 2005). Additionally, phytoprostanes were also shown to function as signaling molecules. They induce the expression of genes related to detoxification, stress responses, and secondary metabolism (Farmer and Davoine, 2007; Mueller et al., 2008).

1.2.3 DNA oxidative damage

ROS and RES can also react with, and damage DNA mainly by nucleotide base modification, the most commonly observed being the production of 8-hydroxyguanine. They also act indirectly through the conjugation of malondialdehyde (MDA) with

guanine creating an extra ring, pyrimido[1,2-α]-purin-10(3H)-one (Figure 1.3) (Cooke et al., 2003; Jeong et al., 2005).



Pyrimido[1,2-α]-purin-10(3H)-one Guanine 8-Hydroxyguanine

Figure 1.3. Commonly observed DNA modifications in plant cells. The DNA oxidation caused either by reactive oxygen species ($^1O_2$, $HO^{\cdot}$) or modification by malondialdehyde (MDA) (modified after Møller et al., 2007).

The overall effects of plant oxidative damage are the increase in membrane fluidity, permeability and the inactivation of membrane receptors, enzymes and ion channels (Davoine et al., 2006; Triantaphylides et al., 2008; Przybyla et al., 2008).

1.3 ROS scavenging

To protect themselves against ROS, plant cells and their organelles such as chloroplast, mitochondria and peroxisomes evolved efficient antioxidant defense systems composed of non-enzymatic and enzymatic components (Miller et al., 2010; Sharma et al., 2012).

The non-enzymatic components include low-molecular-weight antioxidants such as the major cellular redox buffers ascorbate (AsA) and glutathione (GSH) (both water soluble) as well as tocopherol and carotenoids (lipid soluble). These antioxidants interact with ROS and act as a metabolic interface that modulates the appropriate induction of acclimation responses (Mittler et al., 2004; Sharma et al., 2012). Mutants with decreased non-enzymic antioxidant contents have been demonstrated to be overly sensitive to stress (Semchuk et al., 2009; Page et al., 2012).

1.3.1 The non-enzymatic antioxidants

Ascorbic acid (vitamin C) is an important antioxidant that acts to prevent or reduce the damage caused by ROS in plants. It is considered as the most powerful antioxidant

because of its ability to donate electrons in a number of enzymatic and non-enzymatic reactions (Asada, 1999; Miller et al., 2010). AsA accumulates in the majority of plant tissues, organelles and in the apoplast (Shao et al., 2008a), and is found to be particularly abundant in mature leaves with fully developed chloroplast and highest chlorophyll contents (Smirnoff, 2000). It can also provide protection to membranes by directly scavenging the $O_2^{\cdot}$ and $HO^{\cdot}$, and by regenerating α-tocopherol from tocopheroxyl radical (Noctor and Foyer, 1998).

Glutathione is a tripeptide, low molecular weight, non-protein thiol found abundantly in all cell compartments in its reduced form (Foyer and Noctor, 2005). It is considered as the most important intracellular defense component against ROS-induced oxidative damage. Additionally, the balance between GSH and glutathione disulfide GSSG (oxidized form) is a crucial mechanism in maintaining cellular redox state during H_2O_2 degradation to water and oxygen (Shao et al., 2008b). GSH can react chemically with H_2O_2, $O_2^{\cdot}$ and $HO^{\cdot}$ (Noctor and Foyer, 1998) and, therefore, can function directly as a free radical scavenger. GSH can protect cellular macromolecules either by the formation of adducts directly with reactive electrophiles or by acting as hydrogen donor in the presence of ROS or organic free radicals (Sharma et al., 2012).

Tocopherol (vitamin E) is a lipophilic antioxidant that can scavenge ROS, lipid peroxy radicals, and $^1O_2$ (Li et al., 2008). Tocopherols are present only in green parts of plants, and localized in the thylakoid membrane of chloroplasts. They are known to protect the structure and function of PSII by reacting chemically with $^1O_2$ in chloroplasts (Krieger-Liszkay and Trebst, 2006). Regeneration of oxidized tocopherol back to its reduced form can be achieved by AsA and GSH (Fryer, 1992). Overexpression of *A. thaliana* tocopherol cyclase, an enzyme involved in vitamin E synthesis, in transgenic tobacco plants enhanced tocopherol contents and drought stress tolerance (Liu et al., 2008).

Carotenoids are pigments that belong to the group of lipophilic antioxidants and are able to quench excessive free radicals and ROS generated from photooxidation (Young, 1991). Carotenoids are found in plants and microorganisms and they are localized primarily in the thylakoid membrane of chloroplasts. Their critical functions are to harvest light between 400 and 550 nm of the visible spectrum and pass the captured

energy to chlorophyll (Tracewell et al., 2001). They also protect the photosynthetic apparatus from oxidative damage by quenching the triplet excited state of chlorophyll and reactive $^1O_2$, and by dissipating excess energy (Mittler, 2002).

1.3.2 The enzymatic antioxidants

Enzymatic antioxidants include an array of ROS-scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), as well as associated antioxidant enzymes such as glutathione reductase (GR). These enzymes function in different subcellular compartments and play important roles in the scavenging of stress-induced ROS.

Superoxide dismutases are metalloenzymes that constitute the first line of defense in the detoxification of active oxygen in all aerobic organisms. The SOD enzyme catalyzes the dismutation of $O_2^{\cdot}$, one $O_2^{\cdot}$ being reduced to H_2O_2 and another oxidized to O_2 (Gill and Tuteja, 2010). SOD is present in most of the subcellular compartments prone to ROS-mediated oxidative stress, but there are no reports of extracellular SOD enzymes in plants. Three isozymes of SOD are known in plants: the copper/zinc (Cu/Zn-SOD), the manganese (Mn-SOD) and the iron (Fe-SOD) (Mittler, 2002). Increased SOD activity has been detected in plants subjected to abiotic stresses, including salinity and metal toxicity (Harinasut et al., 2003; Skorzynska-Polit et al., 2003). Overexpression of SOD in transgenic plants has been reported to result in enhanced abiotic stress tolerance (Lee et al., 2007).

Catalase was the first antioxidant enzyme to be discovered and characterized. It has the potential to directly catalyze the dismutation of two molecules of H_2O_2 into water and oxygen. H_2O_2 is mainly generated in peroxisomes by oxidases involved in β-oxidation of fatty acids, photorespiration and purine catabolism (Sharma et al., 2012). CATs are unique as they do not require cellular reducing equivalents. CAT isozymes have one of the highest reported turnover rates (one molecule can convert about 6 million molecules of H_2O_2 to water and oxygen per minute). In addition to peroxisomes, they are localized in the cytosol, chloroplasts, and mitochondria (Gill and Tuteja, 2010). Significant increase in CAT activity has been reported under salt treatment (Eyidogan and Oz, 2005). Additionally, CAT-deficient plants show high susceptibility to paraquat, salt and ozone

(Willekens et al., 1997), these plants allowed to assess H_2O_2-dependent and -independent high light-triggered transcriptional responses during a sustained H_2O_2-stress over time (Vanderauwera et al., 2005). On the other hand, the ectopic expression of a CAT gene in tobacco enhanced its tolerance to cadmium induced oxidative stress (Guan et al., 2009).

Ascorbate peroxidase is thought to play an essential role in the control of intracellular ROS levels and protecting cells in higher plants. APX is a central component of ascorbate-glutathione cycle, and is involved in the scavenging of H_2O_2. There are at least five chemically and enzymatically distinct isoenzymes of APX with different subcellular localizations including the thylakoid (tAPX), glyoxisome membrane (gmAPX), chloroplast stroma (sAPX), and the cytosol (cAPX) (Davletova et al., 2005; Narendra et al., 2006). It has been demonstrated that expression of APX was enhanced in plants growing under unfavorable environmental conditions. Moreover, isoforms of APX have much higher affinity for H_2O_2 (μM range) than CAT (mM range) (Sharma and Dubey, 2005; Miller et al., 2007). Overexpression of APX in *Nicotiana tabacum* chloroplasts resulted in high tolerance toward salt and water deficit (Badawi et al., 2004).

Glutathione reductase is a NAD(P)H-dependent flavoenzyme, which plays an essential role in defense against ROS by catalyzing the reduction of glutathione disulfide to GSH, therefore, maintaining high cellular reduced/oxidized GSH ratio. GR as well as GSH are involved in detoxification of H_2O_2 generated by the Mehler-peroxidase reaction in chloroplast PSI. GR is localized in chloroplasts, mitochondria and cytosol but the main GR activity in photosynthetic tissues is reported for the chloroplastic isoforms (Edwards et al., 1990). Several studies have reported increased activity of GR under different environmental stresses (Skorzynska-Polit et al., 2003; Eyidogan and Oz, 2005; Sharma and Dubey, 2005).

Depending on the ROS levels inside of the cells (or the balance between their production and scavenging), ROS could serve as versatile signaling molecules to sense stress (Wagner et al., 2004; Suzuki et al., 2012) or cause oxidative damage by direct peroxidation of unsaturated fatty acids and oxidization of proteins and DNA.

1.4 RES scavenging

Both, the cytotoxicity as well as the gene-regulatory activities of RES have been attributed to their particular properties as strong electrophiles, which require a tight control to balance these activities and maintain coordination (Wagner et al., 2004; Weber et al., 2004; Sattler et al., 2006). GSH conjugation with RES plays an essential role in the non-enzymatical scavenging of a variety of RES in plants (Esterbauer et al., 1991).

Moreover, plant cells contain multiple enzymes or enzyme systems capable of detoxifying RES or metabolizing them into less reactive compounds. The targets in scavenging are saturation of the double bond and reduction/oxidation of the carbonyl group of the α,β-unsaturated aldehydes and ketones (Alméras et al., 2003; Mano, 2012).

1.4.1 Flavin-dependent oxidoreductases

The α,β-unsaturated carbonyl compounds and cyclic enones are substrates for the Old Yellow Enzyme family of flavin-dependent oxidoreductases (OYE) (Raine et al., 1994; Williams and Bruce, 2002). OYE was the first flavin-dependent enzyme identified and isolated from brewer's yeast (Warburg and Christian, 1933). It catalyzes the reduction of the double bond at the expense of NADPH. OYE was found to be composed of two essential parts for enzyme activity; a colorless apoprotein and a yellow pigment. The pigment was found to be similar in nature to vitamin B_2 (riboflavin), thus OYE provided the first biochemical role for a vitamin (Theorell, 1935). The yellow cofactor was identified as riboflavin 5'-phospate (flavin mononucleotide; FMN) (Williams and Bruce, 2002).

New members of the OYE family were discovered and an increasing number of homologous proteins have been identified of both prokaryotic and eukaryotic origin; in bacteria (*Bacillus subtilis*) (Fitzpatrick et al., 2003), in yeasts (*Saccharomyces cerevisiae*) (Niino et al., 1995), and in plants (*Arabidopsis thaliana*) (Schaller and Weiler, 1997), but not in animals.

In a recent study (Macheroux et al., 2011), 374 flavin-dependent enzymes were analyzed with regard to their function, structure and distribution among 22 prokaryotic and eukaryotic genomes, which reveled that almost all the enzymes are oxidoreductases. 75%

of the analyzed flavin-dependent enzymes bind FAD, whereas only 25% utilize FMN as cofactor (for structure see Figure 1.4). The majority of enzymes bind the cofactor noncovantly. The flavoprotein structural topologies are quite diverse compared with other cofactor-dependent enzymes. The FAD-binding enzymes are dominated by proteins of the Rossmann fold, whereas the FMN-binding enzymes exhibit the classic eight-stranded ß/α -(TIM) barrel structure, comprised of alternating parallel β-sheets and α-helices, and the flavodoxin-like fold (Macheroux et al., 2011).

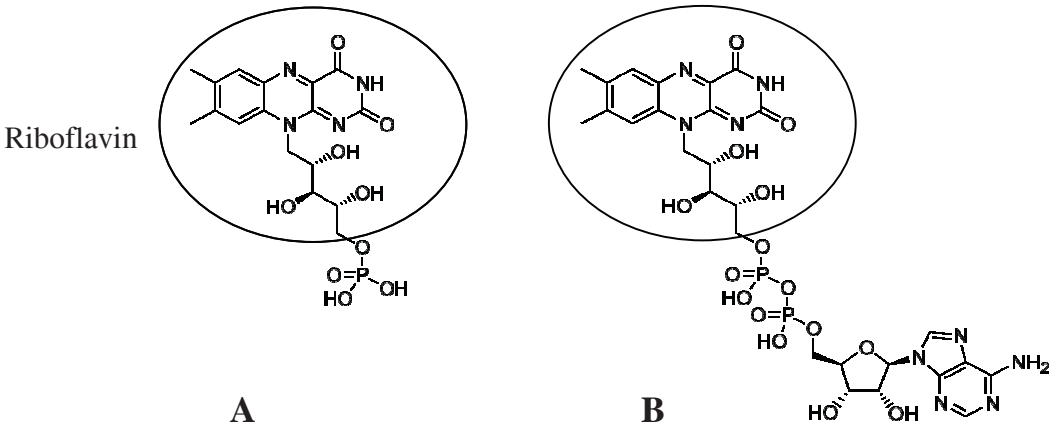


Figure 1.4. The chemical structure of flavin mononucleotide (A) and flavin adenin dinucleotide (B) cofactors. The yellow vitamin B_2, or riboflavin structure is circled.

1.4.2 Yeast Old Yellow Enzymes (OYEs)

The first characterization of OYE was performed for the enzymes from *S. carlsbergensis* (OYE1) and *S. cerevisiae* (OYE2 and OYE3). Proteins from these natural sources have been shown to be homo-dimers. Each monomer of around 45 kDa binds FMN non-covalently, which is rapidly reduced by NADPH. OYE has served as a model for enzyme-catalyzed redox reactions, and led to a clear understanding of how OYEs accept electrons from NADPH onto their flavin mononucleotide cofactor and transfer these electrons to diverse ligands (Niino et al., 1995; Karplus et al., 1995).

The OYEs are characterized by binding tightly with aromatic and heteroaromatic compounds that have an ionizable hydroxyl group, such as phenol, and forming charge

transfer complexes. The formed complexes show dramatic changes in the flavin absorption spectra, including the development of strong absorbance maxima in the 500-800 nm range which arise from the charge transfer interaction between the phenolate anion and neutral flavin (Abramovitz and Massey, 1976; Chateauneuf et al., 2001).

Physiological roles of OYEs *in vivo* have remained uncertain, despite the fact that several members of the OYE family have been extensively studied (Kohli and Massey, 1998; Trotter et al., 2006; Odat et al., 2007; van Dillewijn et al., 2008). However, OYE2 and 3 have been reported to mediate resistance in *S. cerevisiae* to small α,β-unsaturated aldehydes such as acrolein, a product of lipid peroxidation in biological systems (Trotter et al., 2006). Moreover, the overexpression of OYE2 in yeast significantly lowers ROS levels generated by organic prooxidants (Odat et al., 2007).

Analysis of OYE2 overexpression and knock-out indicated its antioxidant and detoxification activities. On the other hand, OYE2 activities are opposed by its homolog OYE3, which functions as a prooxidant promoting H_2O_2-induced programmed cell death. Therefore, Old Yellow FMN oxidoreductases are firmly placed in the signaling network connecting ROS generation and PCD modulation (Odat et al., 2007). In addition, recent interest in OYEs as biocatalysts has illustrated that the enantioselectivity of OYE enzymes was very useful in organic synthesis for the asymmetric reduction of C=C double bonds with chemo- and stereoselectivity of up to 99% ee (Swiderska and Stewart, 2006; Hall et al., 2008; Oberdorfer et al., 2011).

These unique characters and the fact that no endogenous physiological substrates of OYE have been identified so far increased the attention on the physiological functions of OYEs and their homologs in other organisms. They were postulated to be good candidates participating in the detoxification of RES and therefore relief from the oxidative stresses.

1.4.3 Old Yellow Enzyme homologues from bacteria

OYE homologues have been characterized in Gram-positive and Gram-negative bacteria including the xenobiotic reductases from *Pseudomonas putida* and *P. fluorescens*, pentaerythritol tetranitrate reductase (PETN) from *Enterobacter cloacae*, *N*-ethylmaleimide reductase from *Escherichia coli* and *Shewanella* Yellow Enzyme (SYE)

from *Shewanella oneidensis* (French et al., 1996; Miura et al., 1997; Blehert et al., 1999; Brigé et al., 2006). The first OYE homologue from Gram-positive bacteria was isolated from *Bacillus subtilis* designated as YqjM (Fitzpatrick et al., 2003).

Recently, a novel thermostable OYE homologue named chromate reductase (CrS) from *Thermus scotoductus SA-01* was described (Opperman et al., 2008). This enzyme is able to reduce the carcinogen hexavalent chromium to the innocuous trivalent oxidation state using NADPH as a reductant (Opperman et al., 2008).

Crystal structures of YqjM and CrS were resolved in the enzyme holoform and in complex with the ligand *p*-hydroxybenzaldehyde, showing overall structural relation to the OYE family of enzymes, with a classical TIM barrel fold and each enzyme having one molecule of flavin mononucleotide bound non-covalently (Kitzing et al., 2005; Opperman et al., 2010)

The general substrates for the characterized bacterial OYE homologues are quinones and α,β-unsaturated aldehydes and ketones, in which the olefinic bond is reduced through the oxidation of NAD(P)H. In addition, they can also catalyse the slow aromatization of cyclic enones, such as cyclohexenone (Fitzpatrick et al., 2003).

Moreover, bacterial OYEs were found to be reactive against explosive chemicals such as trinitrotoluene (TNT) (Khan et al., 2004). PETN reductase from *Enterobacter cloacae PB2* and xenobiotic reductase B from *Pseudomonas fluorescens* and *P.putida* catalyze the reduction of the TNT aromatic ring by hydride addition, yielding hydride- and dihydride-Meisenheimer complexes (French et al., 1996; French et al., 1998; Williams et al., 2004; Khan et al., 2004; van Dillewijn et al., 2008).

Studies on bacterial OYE expression under conditions of oxidative stress revealed that *Bacillus subtilis* YqjM and *Shewanella oneidensis* SYE4 proteins, were rapidly induced 2–4 fold after stress application (e.g. paraquat, acrolein, NEM and 2-cyclohexen-1-one) (Fitzpatrick et al., 2003; Brigé et al., 2006). Taken together, the rapid induction and the substrate specificty of bacterial OYEs provide strong evidence for the direct involvement of these proteins in the oxidative stress response as a cellular defense mechanism against oxidative injury (Williams and Bruce, 2002).

1.4.4 12-Oxophytodienoic acid reductases (OPRs)

In higher plants, several OYE homologs have been identified so far, and they are usually encoded by a multigene family. The first plant member of the OYE family was identified in *Arabidopsis thaliana* and designated as 12-Oxophytodienoate reductase (OPR) (Schaller and Weiler, 1997).

The OPR name is derived from the isozyme OPR3 identified from *A. thaliana* (Schaller et al., 2000; Stintzi and Browse, 2000) and *Solanum lycopersicum* (Strassner et al., 2002). OPR3 catalyzes the reduction of the cyclopentenone (9S,13S)-12-oxophytodienoate [(9S,13S)-OPDA] to the corresponding cyclopentanone (OPC-8:0) in the biosynthesis of the plant hormone jasmonic acid (JA) (Vick and Zimmerman, 1987; Schaller et al., 2000; Stintzi and Browse, 2000).

In parallel, other OPR members were identified including three isoforms in *Solanum lycopersicon* (Straßner et al., 1999; Strassner et al., 2002), and six OPR genes in *A. thaliana*, three of which have been characterized, namely OPR1, OPR2 and OPR3 (Figure 1.6) (Schaller et al., 1998; Abu-Romman, 2008; Modjesch, 2008). *At*OPR1, 2, 4 and 5/6 genes are located on *Arabidopsis* chromosome I and do not possess any of the known organelle-target sequences. OPR3, on the other hand, is located on chromosome II and contains a C-terminal Ser-Arg-Leu type 1 peroxisome targeting sequence (Stintzi and Browse, 2000; Strassner et al., 2002, for review see Schaller and Stintzi, 2009).

Thirteen OPRs are encoded in the *Oryza sativa* genome, and fall into five subfamilies (subs. I-V) according to a phylogenetic relationship (Agrawal et al., 2003; Sobajima et al., 2003; Li et al., 2011). Eight OPR genes were identified in *Zea mays* by genomic sequences analysis (Zhang et al., 2005). Recently, OPRs were also identified in *Solanum tuberosum* and *Hordeum vulgare* and named *StOPR3* and *HvOPR1*, respectively (Díaz et al., 2012; Abu-Romman, 2012a).

There is an essential sequence conservation between the OPRs and the yeast enzymes, including two of the three amino acids involved in substrate binding, namely Tyr376 and His192, whereas Asp195 is substituted by His in most of the OPR plant sequences (for review see Schaller et al., 2005). In addition, like in the OYE family, the OPRs have been

shown to form charge transfer complexes with a series of phenolic ligands (Straßner et al., 1999). Furthermore, OPR family members from different plants were shown to reduce a variety of α,β-unsaturated carbonyl compounds *in vitro* (Schaller and Weiler, 1997; Straßner et al., 1999; Sobajima et al., 2003; Li et al., 2011)

The OPRs within the plant are differentially regulated in response to stress hormones, such as jasmonic acid (JA), wounding or pathogen infection (Abu-Romman, 2008) and distinct OPR transcripts accumulate differentially in diverse maize organs (Zhang et al., 2005).

1.4.4.1 OPR substrate specificity

Studies on *Le*OPRs and *At*OPRs enzymatic activity revealed distinct substrate preferences. *In vitro,* OPR3 is able to reduce not only the (9S, 13S)-OPDA but also its enantiomer (9R, 13R)-OPDA (Schaller et al., 1998; Schaller et al., 2000). However, the other family members such as OPR1/2 are highly selective; able to only reduce the enantiomeric (9R, 13R)-OPDA but not (9S, 13S)-OPDA (Schaller et al., 1998; Straßner et al., 1999; Breithaupt et al., 2009) (Figure 1.5). Therefore, OPR3 is unique in its ability to reduce the C=C double bond of the conjugated enone moiety in (9S, 13S)-OPDA, which is the only among the four possible OPDA stereoisomers that is a precursor of biologically active JA. Therefore, *Le*OPR3 and *At*OPR3 rather than OPR1/2 are the enzymes involved in JA biosynthesis (Schaller et al., 2000; Stintzi and Browse, 2000; Strassner et al., 2002).

Depending on their substrate specificity (i.e. enantioselectivity); OPRs have been classified into two groups: subgroup I OPRs (Figure 1.5) are selective, preferentially catalyzing the reduction of the (9R,13R)-OPDA enantiomer (Schaller et al., 1998). Subgroup II, on the other hand, exhibit a rather broad substrate specificity and reduces both the (9S,13S)- and the (9R,13R)-enantiomers of OPDA such as *Le*OPR3, *At*OPR3 and OPR3 orthologs in other species (Figure 1.6) (Strassner et al., 2002; Zhang et al., 2005). The remarkable difference in substrate stereoselectivity (i.e. difference in their ability to reduce the four stereoisomers of OPDA) has been explained by the comparative analysis of OPR1 (subgroup I) and OPR3 (subgroup II) crystal structures (Breithaupt et al., 2001; 2006).

The analysis revealed that the OPR3 crystal structure is relatively similar to that of OPR1 (Figure 1.7), exhibiting a classical TIM barrel fold but the active site is more open, allowing formation of a wider substrate binding pocket that can accept both the (9S,13S)- and (9R,13R)-enantiomers (Breithaupt et al., 2001; Fox et al., 2005; Breithaupt et al., 2006).

Furthermore, the crystal structure of the OPR1 enzyme/substrate complex confirmed the mode of substrate binding proposed by OYE, which suggests a hydrogen bonding of the substrate carbonyl group to His187 and 197, which leads to polarization of the olefinic bond, therefore facilitating the hydride transfer from the reduced flavin to the substrate ß carbon. The substrate α carbon is then protonated from the opposite side by Tyr192 (Breithaupt et al., 2001; Breithaupt et al., 2009).

Recently, a structure/function analysis of *Le*OPR1/*Le*OPR3 was conducted to unravel the molecular basis of the difference in enantioselectivity. Based on structural comparison, site-directed mutagenesis was performed followed by biochemical characterization of *Le*OPR1 and 3 with ρ-hydroxybenzaldehyde as a substrate analogue revealing two critical active-site residues, Tyr78 and Tyr246 in *Le*OPR1, and Phe74 and His244 in *Le*OPR3, for substrate filtering and selectivity (Breithaupt et al., 2009).

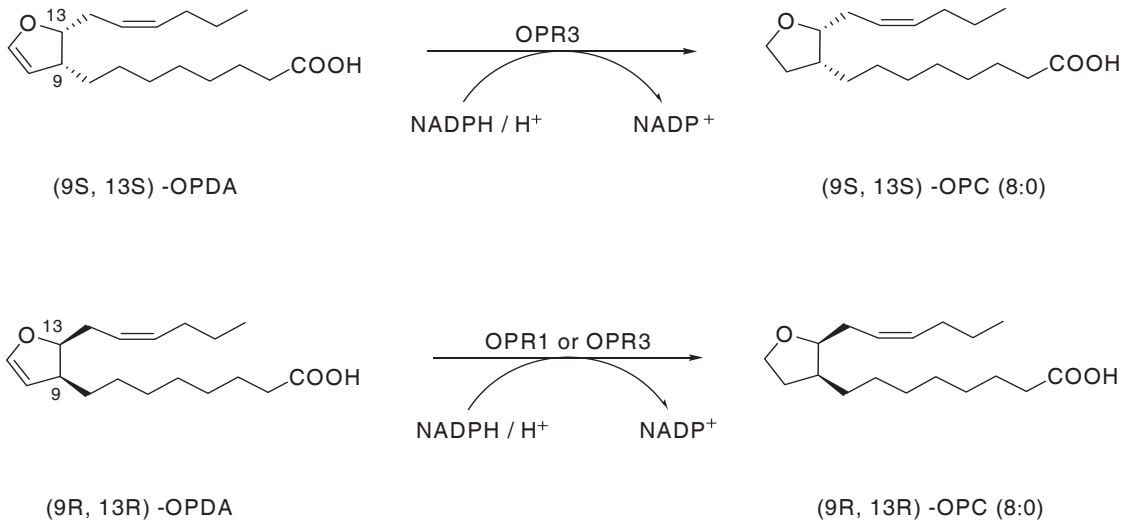


Figure 1.5. The OPRs isoform substrate specificity. OPR1 can reduce only (9R,13R)-OPDA but OPR3 reduces both OPDA enantiomers (modified after Schaller et al., 1998).

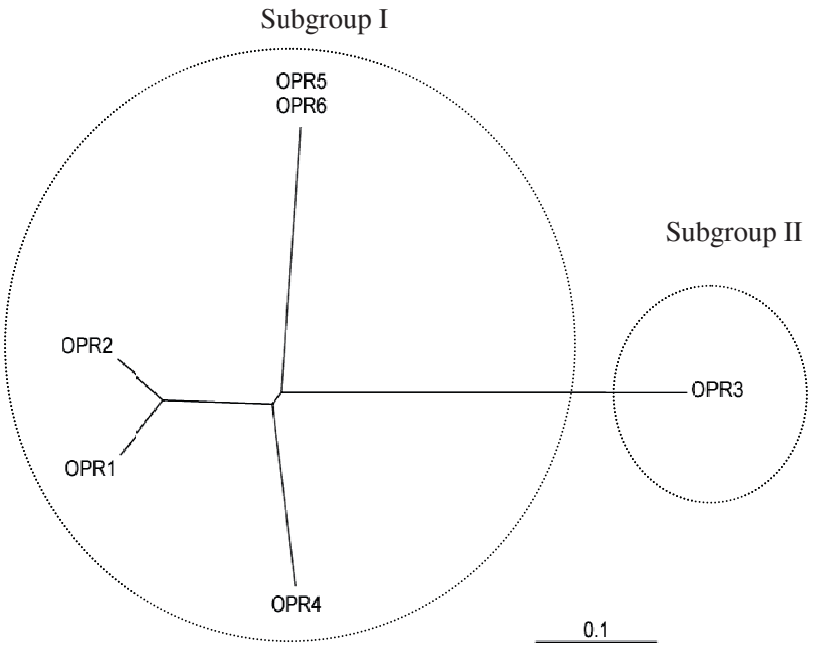


Figure 1.6. Phylogenetic analysis of *Arabidopsis* 12-oxophytodienoate reductases deduced amino acid sequences. The family is divided into two subgroups according to amino acid sequences. Subgroup I and II type *At*OPRs are indicated by dashed circles.

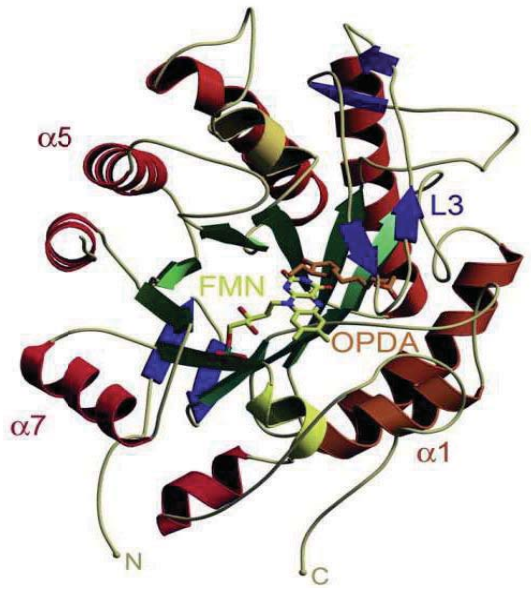


Figure 1.7. Graphical representation of *Sl*OPR1 structure. The *Sl*OPR1 structure in complex with (9R,13R)-OPDA. FMN and (9R,13R)-OPDA are shown as stick models. Secondary structures complementing the $\alpha_8\beta_8$ barrel are shown in blue. (Figure adapted from Breithaupt et al., 2009).

1.4.4.2 OPR subgroup II: *A. thaliana* OPR3

JA biosynthesis is initiated in the chloroplast and is catalyzed by several enzymes, namely lipoxygenase (LOX) (Bell et al., 1995), allene oxide synthase (AOS) (Froehlich et al., 2001) and allene oxide cyclase (AOC) (Ziegler et al., 2000), and terminates in the peroxisome, where OPR3 is located (Schaller et al., 2000; Stintzi and Browse, 2000; Strassner et al., 2002). OPR3 action is followed by three cycles of β-oxidation to synthesize the active form of JA (Delker et al., 2007; Schaller and Stintzi, 2009)

The final proof of OPR3 being the only OPR member involved in JA biosynthesis in *A. thaliana* came from a genetic approach (Sanders et al., 2000; Stintzi and Browse, 2000). The *opr3* loss-of-function mutant is characterized by jasmonate deficiency and male sterility. It shows a characteristic set of developmental defects, including shorter anther filaments that are unable at anthesis to place the anther locules above the stigma, delayed dehiscence of the anther locules, and reduced viability of the pollen, with ≈ 4% of mutant pollen grains germinating compared to 97% for wild type (McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001). However, chemical complementation of *opr3* mutants by the application of exogenous JA but not OPDA restore fertility (Sanders et al., 2000; Stintzi and Browse, 2000), indicating that OPR3 is the only isoform of OPRs able to reduce the correct stereoisomer of OPDA to produce JA required for male gametophyte development (Stintzi and Browse, 2000).

On the other hand, the jasmonate-inducible defense response is not impaired in *opr3,* and OPDA which accumulates in the *opr3* mutant was shown to induce many jasmonate-dependent genes, allowing the activation of defense responses against pathogenic fungi and insect pests. Moreover, a subset of defense genes specifically responded to OPDA but not to JA (Stintzi et al., 2001; Taki et al., 2005). Since the phenotype of the *opr3* mutant indicates that OPR3 can not be substituted by any one of the other five OPRs isoforms in JA biosynthesis (Stintzi and Browse, 2000), the question arises as to what the physiological function is of the remaining OPRs.

1.4.4.3 *A. thaliana* OPR subgroup I

In the *A. thaliana* OPR subgroup I, OPR1 and OPR2 have been partially characterized (Abu-Romman, 2008; Modjesch, 2008; Beynon et al., 2009).

*At*OPR1 and *AtO*PR2 were demonstrated to catalyze the reduction of a wide range of α,β-unsaturated carbonyls (Abu-Romman, 2008; Modjesch, 2008), as well as unsaturated nitro compounds and nitrate esters (Beynon et al., 2009).

Gene expression studies have found that members of the *A. thaliana* OPR subgroup I are upregulated in response to TNT exposure (Ekman et al., 2003; Gandia-Herrero et al., 2008) and high-light stress (Abu-Romman, 2008), suggesting that OPR subgroup I could be involved in plant responses to oxidative stress.

Interestingly, upon exposure of *opr1* and *2* mutants to high-light stress at reduced temperature, less protective anthocyanin pigments accumulated but higher levels of malondialdehyde were recorded than in wild-type plants (Abu-Romman, 2008), indicating that OPR1 and 2 may have an important role in RES detoxification *in vivo*.

The expression profiles of the individual genes in the *At*OPRs family exhibit tissue specific differences (Abu-Romman, 2008), which may account for non-redundant function of the OPRs enzymes.

However, there are three putative OPRs as yet uncharacterized, namely OPR4, OPR5, and OPR6, with OPR5 and OPR6 being identical in the promoter and coding regions of their genes and thus yield identical transcripts.

1.5 Aim of the present study

The aim of this study was to contribute to an improved understanding and gain knowledge in the area of plant oxidative stress and the possible scavenging role of *A. thaliana* OPR subgroup I (Figure 1.6), with special focus on *At*OPR5/6, since no hints about their physiological function is available.

Therefore, it was the objective of the study to investigate the impact of loss-of-function mutants for OPRs subgroup I on oxidative stress resistance in *A. thaliana*. To reach these goals, the following approaches were taken:

1. ***Generation of AtOPR5/6 loss-of-function mutant plants.***
   In order to explore the biological function of the *AtOPR5*/6 and to overcome the presence of identical transcripts for *OPR5* and *OPR6*, silencing the expression of both *AtOPR5* and *6* by generating *A. thaliana* OPR5/6 RNAi plants was employed.
2. ***Phenotypic characterization of the generated AtOPR5/6 loss-of-function mutants plants.***
   Characterization of the *At*OPR5/6 RNAi plants under abiotic stress compared to wild-type plants was done. Special focus was given to (photo)oxidative stress generated by high-light stress.
3. ***Subcellular localization of AtOPR5/6 protein and activity of its gene promoter.***
   The subcellular localization of *A. thaliana* OPR5/6 was investigated by the transient expression of GFP-fusion protein in onion epidermal cells. In addition, the promoter activity of *AtOPR5/6* gene was characterized in transgenic *A. thaliana* plants expressing GUS.
4. ***Characterization of recombinant AtOPR5/6.***
   In order to assess the biochemical activity of *At*OPR5/6, the protein was expressed either in *E. coli* or in yeast and purified, using specific affinity chromatography purification methods.