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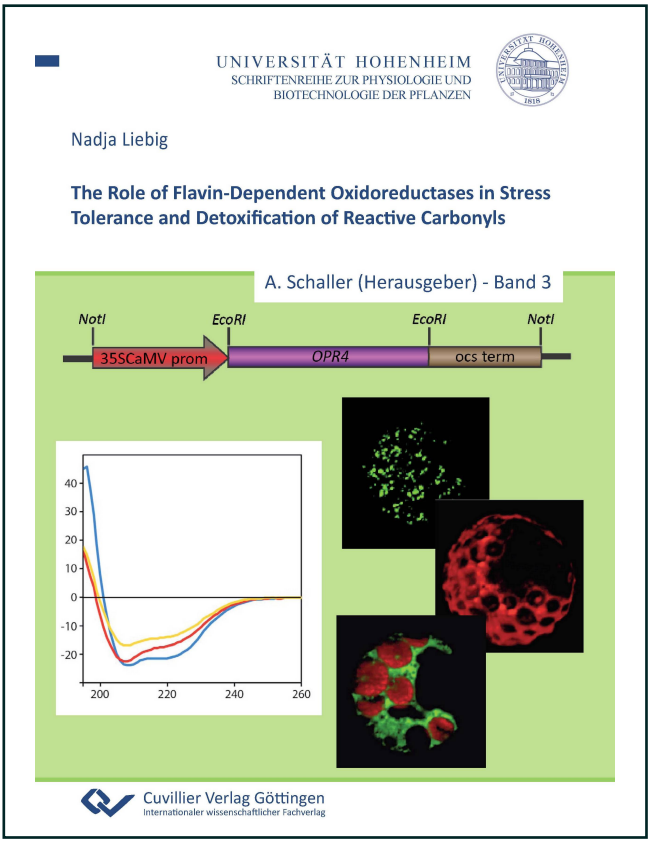
The Role of Flavin-Dependent Oxidoreductases in Stress Tolerance and Detoxication of Reactive Carbonyls



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1 Introduction

1.1 Biotic and abiotic stress in plants

Plants basically need light, water, adequate temperature, carbon and nutrients for growth and reproduction. If these factors deviate from an optimum range, abiotic stress results, whereas damage by other living organisms leads to biotic stress. Both kinds of stress represent the most limiting factors for agricultural productivity (Mahajan and Tuteja, 2005). Research in stress biology is thus of great importance to improve yield and quality in plant production.

1.1.1 Reactive oxygen species (ROS)

One major result of biotic and abiotic stress is an increase in reactive oxygen species (ROS). The four common types are singlet oxygen ($^1O_2$), superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($^\cdot OH$). They are all highly reactive and toxic because of their oxidizing capacity (figures 1.1 and 1.2; Apel and Hirt 2004). Plants produce ROS continuously as a by-product of aerobic metabolism, but stress exposure leads to the so-called "oxidative burst", a strong increase in ROS as the primary response of the cell (Bhattacharjee, 2005). ROS damage membranes, proteins and nucleic acids and are a trigger of cell death (Van Breusegem and Dat, 2006).

In plant cells, chloroplasts are the main production sites of ROS. Chloroplasts generate $^1O_2$ at photosystem II (PS II). Under conditions in which all electrons subtracted from water are used for CO_2 assimilation, the excited P680 reaction center diverts its electrons to its primary electron acceptors pheophytin, Q_A and Q_B and becomes easily reduced by the withdrawal of electrons from H_2O. If light intensity is too high or CO_2 assimilation is decreased because of another stress the electron transport chain is overreduced and the excess energy leads to the formation of $^3P680^*$ which decays to $^1P680$ while $^1O_2$ is generated from triplet ground state molecular oxygen ($^3O_2$) (figure 1.1; Asada 2006; Krieger-Liszkay *et al.* 2008). Singlet oxygen is thus the major ROS formed under photooxidative stress (Gonzalez-Perez *et al.*, 2011)

At photosystem I (PS I) and II superoxide is generated via direct photoreduction of O_2 by reduced components of the electron transport chain associated with the two photosystems.

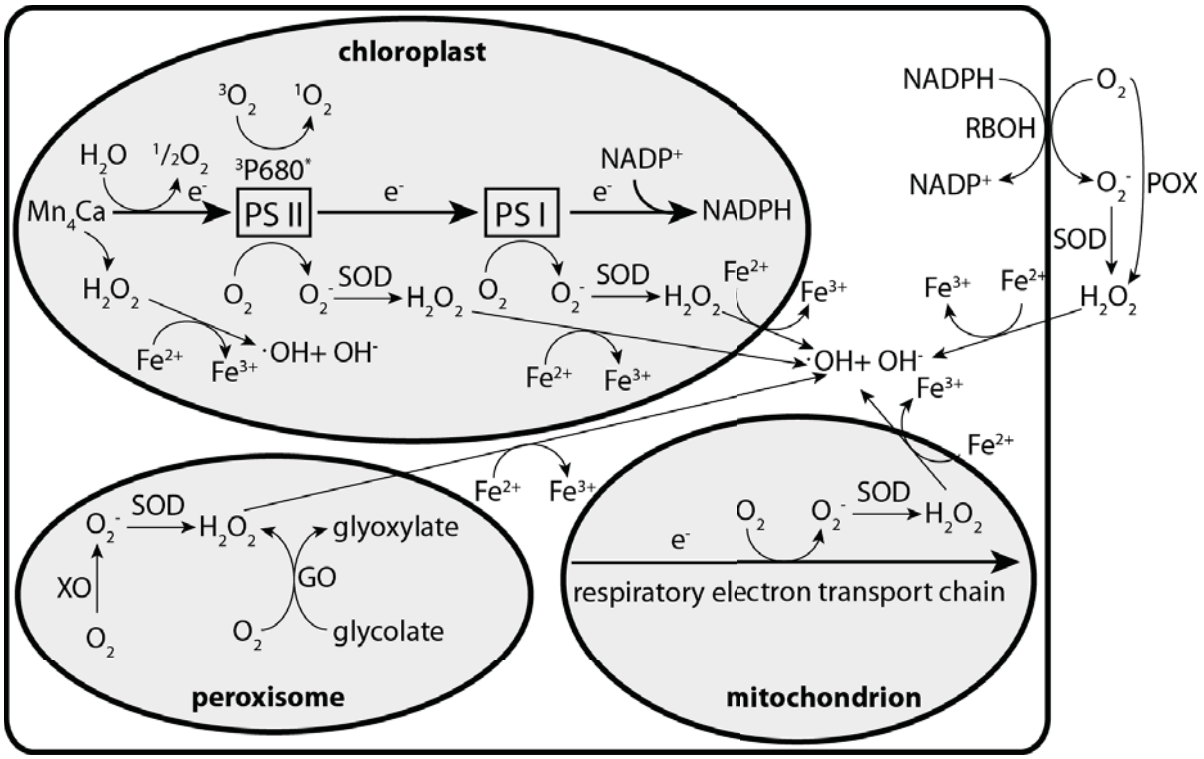

Figure 1.1: ROS production sites in plants. chloroplast: PS I = photosystem I, PS II = photosystem II; peroxisome: GO = glycolate oxidase, XO = xanthine oxidase, apoplast: POX = peroxidase, RBOH = respiratory burst oxidase homolog; SOD = superoxide dismutase.

O_2^- is rapidly disproportionated to H_2O_2 and O_2 by superoxide dismutase (SOD) (figure 1.1). This mechanism is called Mehler reaction (Asada, 2006; Pospíšil, 2009). Pospíšil (2012) reported that the dismutation might also occur spontaneously by interaction of two O_2^-. Transfer of electrons to oxygen protects from photoinhibition by an overreduced electron transport chain during environmental stresses, because the excess energy can be diverted to an alternative electron acceptor (Ort and Baker, 2002). Nevertheless, damage can occur, mainly by modifications of the acceptor sites in the photosystem like the D1 protein (Krieger-Liszkay *et al.*, 2008). A second site is the Mn_4Ca catalytic site of the water oxidizing complex. Under excess light conditions it is unable to keep up with the rate of withdrawal of electrons by $P680^+$ which results in the accumulation of oxidizing radicals at PSII (figure 1.1; Vass 2012).

Other important production sites of O_2^- and H_2O_2 are peroxisomes. In these organelles ROS are generated e.g. during phosphorespiratory glycolate metabolism or by xanthine oxidase coupled to superoxide dismutase (figure 1.1; Corpas *et al.* 2008; Foyer and Noctor 2009). In mitochondria ROS are generated at the electron transport chain by direct reduction of oxygen (figure 1.1; Moller 2001. While in mammalian cells mitochondria are the main sites of ROS generation they probably contribute little to total ROS production in plants (Apel and Hirt, 2004).

·OH is the chemically most reactive species but not well characterized yet. It is generated by the Fenton type reaction in which iron ions react with hydrogen peroxide (figure 1.1; Chen and Schopfer 1999):

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + {\cdot}OH + OH^-$$

There are also some enzymes described to be involved in ROS production. The best known ones are respiratory burst oxidase homologs (RBOHs, homologs of mammalian NADPH-oxidases) which catalyze the generation of O_2^- by using NADPH as an electron donor (figure 1.1; Sagi and Fluhr 2006. Alternatively, peroxidases produce hydrogen peroxide (figure 1.1; Apel and Hirt 2004).

1.1.2 Protection from ROS

Because of the toxicity of ROS, it is essential for the survival of a plant to keep their levels low. One strategy is to avoid ROS production. This includes trapping of excess excitation energy at the photosystems by non-photosynthetic pigments like anthocyanins (Chalker-Scott, 1999) and increased thickness of the leaf (Frohnmeyer and Staiger, 2003). Another strategy is the induction of conformational changes of the PS II supercomplexes to adapt to changed light intensities (Fristedt and Vener, 2011). In addition, several ROS scavenging mechanisms have evolved and can be classified as non-enzymatic and enzymatic scavenging mechanisms.

Non-enzymatic scavengers

The low molecular weight molecules ascorbate and glutathione (GSH) belong to the non-enzymatic scavengers. ROS can be directly reduced and thereby detoxified by these two compounds which in turn are converted to monodehydroascorbate, dehydroascorbate and oxidized glutathione (GSSG), respectively. Oxidized ascorbate and glutathione can be recycled by the ascorbate-glutathione cycle using NADPH as reducing power (Foyer and Noctor, 2011). Vitamins are other non-enzymatic scavengers. α-Tocopherol (vitamin E) is predominantly localized in membranes of the chloroplast and quenches chemically and physically $^1O_2$ and ·OH (Munné-Bosch, 2005). Pyridoxin and other vitamin B_6 derivatives are quenchers of singlet oxygen as well. Also localized in the chloroplast, they are components of the protecting network under photooxidative stress (Havaux *et al.*, 2009; Raschke *et al.*, 2011). Along with α-tocopherol and pyridoxin, β-carotin is the major scavenger for singlet oxygen in plants (Krieger-Liszkay, 2005). Other carotinoids like neoxanthin and zeaxanthin are also involved in detoxification of ROS (Dall'Osto *et al.*, 2007; Havaux *et al.*, 2007). Flavonoids accumulate during many environmental stresses. However, the relevance of the antioxidative activity of these compounds in plants is still a matter of debate (Hernández *et al.*, 2009).

Enzymatic scavengers

The second group of ROS scavengers are enzymes. The most famous ones are superoxide dismutases (SODs) and catalases (CATs). They catalyze the conversion of O_2^- to H_2O_2 and H_2O_2 to H_2O and O_2, respectively. Based on their metal co-factor, SODs can be classified into three groups: FeSODs are localized in chloroplasts and MnSOD in mitochondria and peroxisomes. Cu/ZnSOD is the most abundant SOD and can be found in the cytosol, in the extracellular space and in the chloroplast (Abreu and Cabelli, 2010). Three catalases have been found in Arabidopsis. They exhibit different tissue specific expression patterns and are predominantly localized in peroxisomes (Mhamdi *et al.*, 2010). Ascorbate peroxidases (APXs) and glutathione peroxidases (GPXs) detoxify H_2O_2 using ascorbate and glutathione as the reducing power (see also the non-enzymatic scavenger section) (Foyer and Noctor, 2011). There is also a report showing that GPXs use thioredoxin as their preferred reductant (Iqbal *et al.*, 2006). Another enzymatic scavenger of hydrogen peroxide are peroxiredoxins whose thiol groups become oxidized during the reduction step (Dietz, 2011).

1.1.3 ROS signaling

Because ROS are highly reactive their lifespan is short. $^1O_2$ and O_2^- have a half life of 1-5 µsec. H_2O_2 is less reactive and thus more stable, with a lifetime of up to 1 msec. It is the only ROS that is able to cross membranes, directly or through aquaporines (Hatz *et al.*, 2007; Bhattacharjee, 2012). ROS reactions are thus restricted to a distinct cellular compartment or single cells. However, their production leads to massive changes in the transcriptome and they can trigger programmed cell death (PCD) (Apel and Hirt, 2004). Therefore, ROS production upon stress exposure does not only cause oxidative damage but also acts as a signal (figure 1.2). By a process called retrograde signaling, the stimulus that is generated by ROS in a certain cellular compartment is transferred to the nucleus in which gene expression is altered (Fernández and Strand, 2008).

Mutants that fail to generate or overproduce a specific ROS revealed ROS-specific patterns of gene induction. The *flu* mutant is an excellent model to examine the signaling effect of singlet oxygen. In the dark, this mutant accumulates protochlorophyllide, a precursor of chlorophyll that can act as a photosensitizer. After a dark-to-light shift energy is transferred to molecular oxygen, generating high amounts of $^1O_2$. This leads to massive changes in gene expression followed by growth arrest and cell death (Meskauskiene *et al.*, 2001; op den Camp *et al.*, 2003). *flu* mutants grown under continuous light do not overproduce $^1O_2$. When these plants are treated with paraquat, a H_2O_2 generating herbicide, a different subset of genes is up- and downregulated (op den Camp *et al.*, 2003), indicating multiple retrograde signaling pathways.

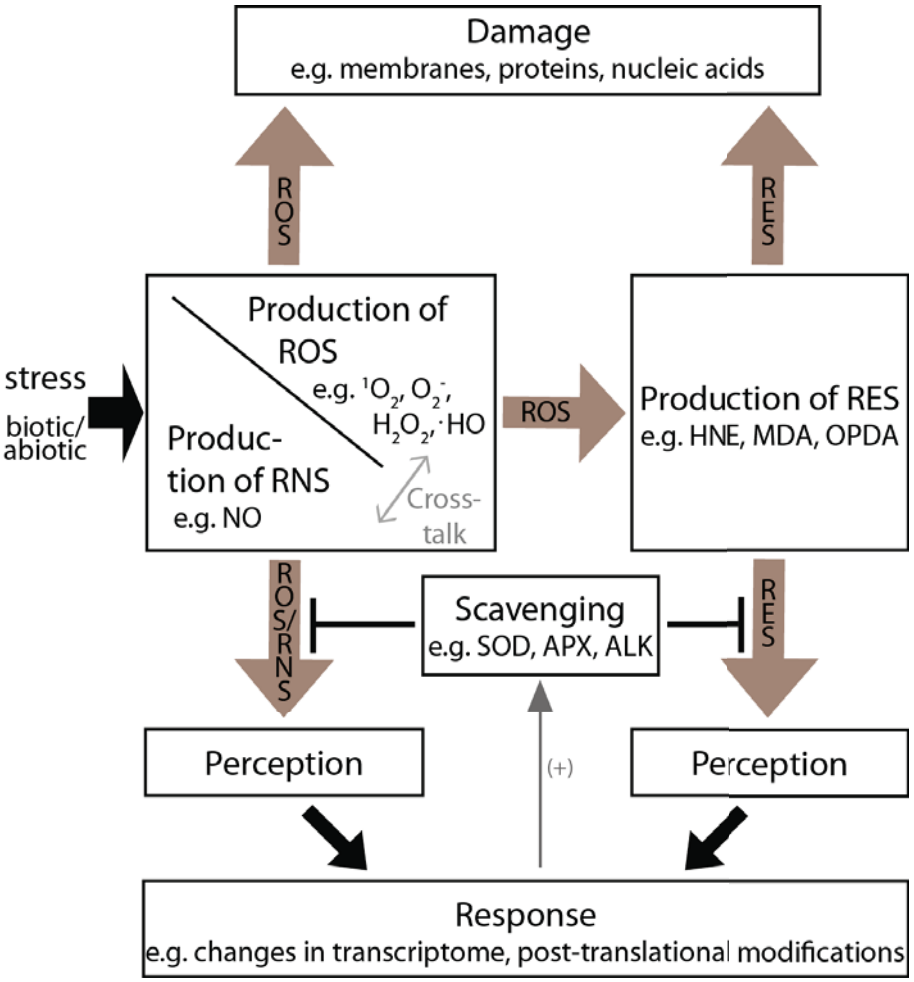

Figure 1.2: Response to biotic and abiotic stress. The first result of environmental stress is an increase in reactive oxygen and nitrogen species. They can damage cells, generate reactive electrophile species and act as signaling molecules. RES are also highly toxic and modify cellular components. The interplay of ROS, RNS and RES signaling results in a stress-specific response.

The phenotype of the dark-to-light shifted *flu* mutant is absent in plants that carry additional mutations in the chloroplastic proteins *EXECUTER (EXE) 1* and *2*, implying that it is not the toxicity of singlet oxygen but its signaling activity that leads to growth arrest and cell death. EXE1 and 2 are part of the $^1O_2$ signaling pathway (Lee *et al.*, 2007). Based on the occurence of cell death, changes in the transcriptome, and the degree of non-enzymatic and enzymatic lipid peroxidation, Kim *et al.* (2008) distinguished three different levels of $^1O_2$ activity. Already low levels of $^1O_2$ result in altered gene expression, without any visible phenotypic changes. Intermediate amounts cause growth arrest and cell death, solely mediated by ROS signaling. Only at high levels, $^1O_2$ has an additional cytotoxic effect.

An ethyl methane sulfonate (EMS) screen for mutants with altered regulation of the *AAA-ATPase* which is exclusively inducible by $^1O_2$, revealed that singlet oxygen signaling does not operate as an isolated linear pathway. Instead, several different cellular components mediate $^1O_2$ derived signals (Baruah *et al.*, 2009). In addition to the EXE1/2-dependent pathway, the blue light photoreceptor CRY1 forms a branch of the $^1O_2$ signaling network. The receptor is necessary for the induction of PCD in the *flu* mutant (Danon *et al.*,

2006). Carotenoids were also shown to be mediators of singlet oxygen signaling. Chemical quenching of $^1O_2$ by β-carotene, lutein, and zeaxanthin generates various aldehydes and endoperoxides that can alter expression of singlet oxygen responsive genes (Ramel *et al.*, 2012a,b). While these $^1O_2$ responsive genes are upregulated in the *soldat10/flu* double mutant, these loss-of-function mutants do not show singlet oxygen mediated cell death, implying that the effects of retrograde signaling are more complex (Meskauskiene *et al.*, 2009).

In contrast to the highly specific activation of genes by distinct ROS, some genes, mainly transcription factors (TF), are induced by several stresses (Vanderauwera *et al.*, 2005). Additionally, there is evidence for a cross-talk between different ROS. *Flu* mutants that overexpress the thylakoid-bound ascorbate peroxidase (tAPX), a H_2O_2 specific scavenger, exhibit reduced levels of H_2O_2 in addition to the accumulation of $^1O_2$ after a dark-to-light shift. The singlet oxygen mediated growth arrest and cell death were more severe in these double mutants, and genes specifically induced in the *flu* parental line were markedly more upregulated in the *flu/tapx* mutant, indicating that H_2O_2 antagonizes $^1O_2$-mediated signaling (Laloi *et al.*, 2007).

Genes upregulated by ROS are mostly related to antioxidative defense and inducible stress proteins. Examples are heat shock proteins, enzymes involved in anthocyanin production and ROS scavengers (Rizhsky *et al.*, 2002; Vanderauwera *et al.*, 2005; Alboresi *et al.*, 2011, etc.). In contrast, genes involved in growth are often downregulated (Alboresi *et al.*, 2011). This is consistent with the observation of an attenuation of plant growth and development during stress. In addition to the multitude of ROS-responsive genes identified by microarray experiments, tiling arrays revealed many pseudogenes and transposons to be regulated by ROS (Zeller *et al.*, 2009).

1.1.4 Lipid peroxidation products

ROS that are not scavenged by the protecting network oxidize many different cellular components. The polyunsaturated fatty acids (PUFAs) of membrane lipids are one major target (figure 1.2; Blokhina 2003). While lipid oxidation may serve as a ROS scavenging mechanism (Mène-Saffrané *et al.*, 2009), it results in the formation of highly reactive lipid peroxidation products, which in turn can damage the cell (Esterbauer *et al.*, 1991). Oxidation of PUFAs can occur by different ROS: (1) singlet oxygen attacks the double bonds of PUFAs to form an endoperoxide which is converted to a lipid hydroperoxide (LOOH). This is the major way of lipid peroxidation during high-light stress (Triantaphylidès *et al.*, 2008). (2) Hydroxyl or superoxide radicals can abstract a hydrogen from the double bond. The resulting radical forms a peroxyl radical with O_2, which is then converted to

LOOHs. LOOHs are further processed to highly reactive carbonyl species, which are often referred to as reactive electrophile species (RES), due to their high electrophilicity. Even though many different products are formed, most of them share one common structure: an α,β-unsaturated carbonyl group (reviewed in Mano 2012). Beside this non-enzymatic pathway, lipid oxidation can also be catalyzed by enzymes. Lipoxygenases form LOOHs which are further processed to various products by a large number of different enzymes (Feussner and Wasternack, 2002)

Common plant RES are 4-hydroxy-2-nonenal (HNE), methyl vinyl ketone (MVK), acrolein and malondialdehyde (MDA) , but also larger compounds like OPDA and phytoprostanes (Alméras *et al.*, 2003; Uchida, 2003; Eckardt, 2008). Toxicity increases with decreasing number of C atoms and increasing number of double bonds (Alméras *et al.*, 2003; Mueller, 2004). Different stresses lead to different structural patterns of RES. For instance, levels of pentenal are especially increased under photooxidative stress (Mano, 2012) while MDA is a predominant carbonyl formed during heat stress (Yamauchi *et al.*, 2008). Stress-specific formation of RES may be induced by stress-specific ROS composition. 10-hydroxy octadecadienoic acid, 12-hydroxy octadecadienoic acid, 10-hydroxy octadecatrienoic acid and 15-hydroxy octadecatrienoic acid have been identified as primary products of singlet oxygen mediated non-enzymatic peroxidation, a ROS predominantly produced during high-light stress (Przybyla *et al.*, 2008). Vu *et al.* (2012) measured lipid peroxidation products in response to different biotic and abiotic stresses like wounding, pathogen infection and low temperature simultaneously and could show that the composition and amount of oxidized lipids vary depending on the type and duration of stress application. From these results it can be concluded that a stress-specific signature of RES exists.

α,β-unsaturated carbonyls contribute to cellular toxicity by their electrophilicity (Esterbauer *et al.*, 1991). Targets of RES are other fatty acids. This results in a chain reaction that produces large amounts of oxidized lipid peroxidation products within a short period of time (Farmer and Davoine, 2007). Other targets are nucleic acids and proteins in which especially the thiol and amino groups of amino acids like cysteine, lysine and histidine form Michael adducts or Schiff bases, respectively, with the α,β-unsaturated carbonyls. The affected proteins are modulated in their activity or degraded (Farmer and Davoine, 2007; Yamauchi *et al.*, 2008; Mueller and Berger, 2009; Mano, 2012). RES interact directly with several proteins, particularly in mitochondria and chloroplasts (Millar and Leaver, 2000; Winger *et al.*, 2007; Yamauchi *et al.*, 2008; Mano *et al.*, 2009, etc.).

The photosynthetic apparatus is one major target of RES. Small reactive carbonyls such as acrolein and MVK strongly damage photosystem II, indicating that RES mediate photooxidative injury in leaf cells. Alternatively, RES can damage the cell indirectly by

lowering the cellular glutathione pool because GSH is used to detoxify these reactive compounds (Mano *et al.*, 2009).

1.1.5 RES scavengers

Like ROS, RES can be scavenged in different ways (figure 1.2). They can be inactivated upon conjugation with GSH (Marrs, 1996; Davoine *et al.*, 2006). Glutathione-S-transferases and peroxiredoxins can catalyze the reduction of RES to their corresponding monohydroxyalcohols (Dixon *et al.*, 2009; Dietz, 2011). α-tocopherol detoxifies RES by reducing the lipid peroxyl radicals to the corresponding hydroperoxides (Munné-Bosch, 2005).

In addition to these detoxification steps which are common for many different cellular compartments, α,β-unsaturated carbonyls can be scavenged by reduction/oxidation of the carbonyl group or saturation of the double bond (figure 1.3). Aldehyde dehydrogenases (ALDHs) catalyze the oxidation of aldehyde groups resulting in the formation of the corresponding carboxylates (Mitchell and Petersen, 1987). There are several reports about ALDHs that enhance stress tolerance probably by scavenging RES. Kirch *et al.* (2001), Sunkar *et al.* (2003), Kotchoni *et al.* (2006) and Stiti *et al.* (2011) reported different ALDHs from *A. thaliana* and *Craterostigma plantagineum* that detoxify reactive aldehydes in different cellular compartments.

Aldo-keto reductases (AKRs) and aldehyde reductases (ADRs) convert carbonyls to alcohols (Petrash, 2004). Oberschall *et al.* (2000) was the first who reported a plant ADR that is induced by several stresses and, upon overexpression, provides enhanced tolerance to paraquat, heavy metals and drought by reducing the concentrations of lipid peroxidation products. In Arabidopsis, at least two ADRs and two AKRs exist which are involved in RES scavenging. They exhibit broad substrate specificity to many typical lipid peroxidation products. The two ADRs are localized in the chloroplast and accept saturated as well as α,β- unsaturated long chain aldehydes. Substrates of AKR4C8 and AKR4C9/*At*ChlAKR include also some ketones and smaller α,β-unsaturated aldehydes. However, all enzymes differ in their affinity to various RES substrates (Simpson *et al.*, 2009; Yamauchi *et al.*, 2011).

As saturated aldehydes are still reactive because of their carbonyl group, it might be questionable as to whether the reduction of the double bond of α,β-unsaturated carbonyls constitutes a detoxification step. However, Haynes *et al.* (2000) reported that carbonyls are markedly less toxic without the double bond. The enzymes that catalyse this reducing step are called alkenal/one oxidoreductases (AORs) or 2-alkenal reductases (AERs). In contrast to ALDHs, AKRs and ADRs most AORs accept preferentially ketones as substrates. For instance, two AORs from cucumber and their homolog in Arabidop-

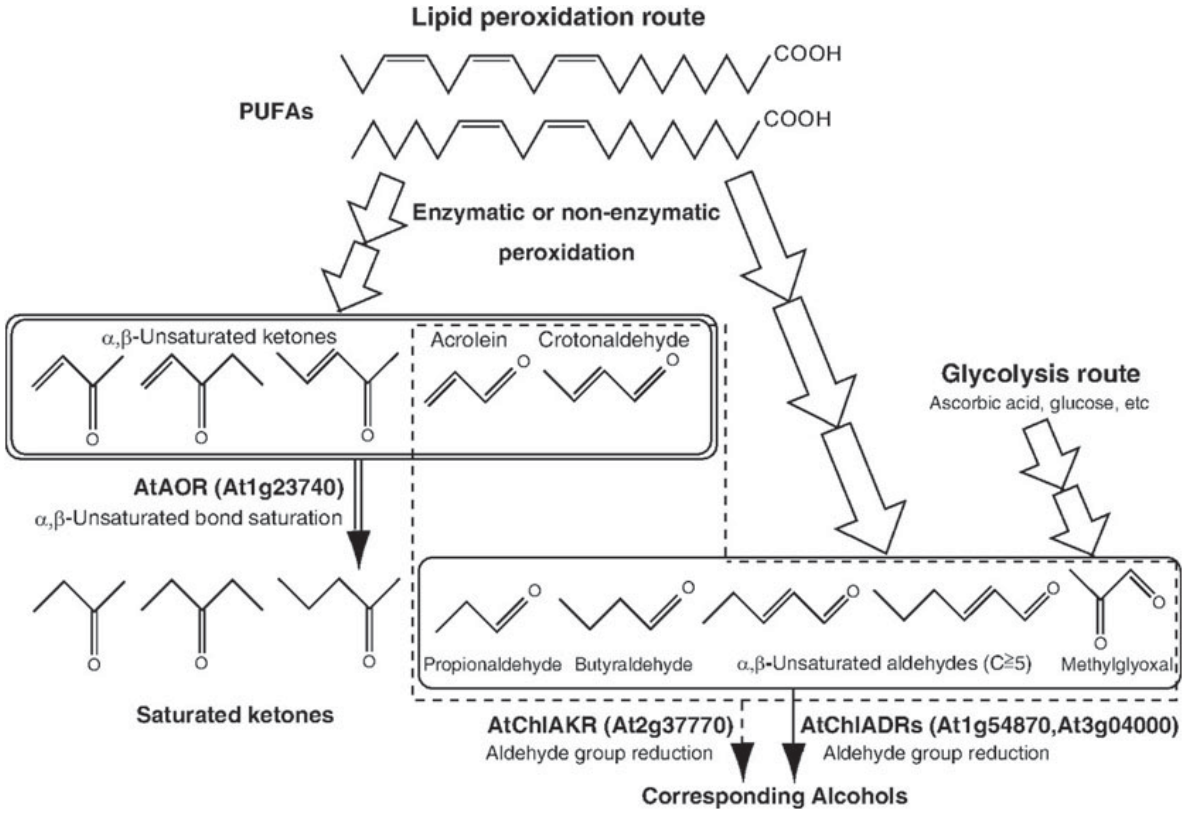

Figure 1.3: Detoxification pathways of RES in Arabidopsis. Polyunsaturated fatty acids (PUFAs) can undergo enzymatic or non-enzymatic peroxidation. The resulting products belong to the group of RES and do often contain an α, β-unsaturated carbonyl group. For detoxification alkenal/one reductases (AORs) reduce the double bond that result in the formation of the corresponding saturated aldehydes and ketones. Alternatively, aldehyde reductases and aldo-keto reductases reduce the carbonyl group to the corresponding alcohols. Substrates for *At*AOR-, *At*ChlADR- and *At*ChlAKR) are circumscribed by double, solid, and dashed lines, respectively. Adapted from Yamauchi *et al.* (2011), with kind permission of the American Society for Biochemistry and Molecular Biology.

sis catalyse the saturation step of small α, β-unsaturated carbonyls, especially ketones (Yamauchi *et al.*, 2011). Another AOR from Arabidopsis also accepts several long chain α, β-unsaturated aldehydes, and its overexpression improves tolerance to various stresses (Mano *et al.*, 2005; Yin *et al.*, 2010).

Most of the carbonyl reducing enzymes use NADPH as a reductant. In crude extracts from cucumber and Arabidopsis, carbonyl-reducing activity using NADPH is 10 times greater than using NADH (Yamauchi *et al.*, 2011). Altogether, it can be proposed that ALDHs, AKRs, ADRs and AORs form a NADPH-dependent network throughout the cell in which they cooperatively scavenge RES.

1.1.6 RES as signaling molecules

In addition to their toxicity, low levels of RES are biologically active by acting as signaling molecules (figure 1.2). It was shown that gene expression is strongly affected by RES (Bate and Rothstein, 1998; Vollenweider *et al.*, 2000; Alméras *et al.*, 2003; Weber *et al.*,

2004; Sattler *et al.*, 2006; Mueller and Berger, 2009). Mueller *et al.* (2008) reported 157 upregulated and 211 downregulated genes in Arabidopsis after treatment with the reactive phytoprostane PPA_1. Many of the upregulated transcripts are known to be involved in defense mechanisms, like GST1 (Vollenweider *et al.*, 2000), HEL (Alméras *et al.*, 2003) and HSC70-1 (Weber *et al.*, 2004). RES and ROS induce substantially overlapping but also clearly distinct genes (Mueller and Berger, 2009). Likewise, overlapping but not identical sets of genes are activated by different RES. MDA preferentially activates abiotic stress-related genes, while MVK enhances transcripts related to biotic stress (Alméras *et al.*, 2003; Weber *et al.*, 2004). In addition, it was shown that only α,β-unsaturated carbonyls activate defense gene expression, their saturated forms not (Vollenweider *et al.*, 2000; Alméras *et al.*, 2003).

Abundant and especially thiol-rich proteins are a major target of RES signaling. These proteins are often redox-regulated and the thiol modifications alter the activity of these proteins (Mueller and Berger, 2009). These modified proteins may in turn be able to induce transcription of stress related-genes, as it is commonly known in animals (reviewed in Schopfer *et al.* (2011)). In plants, this direct interaction has not been proven yet, but the existence of a TGA transcription factor binding site in about 50 % of genes upregulated upon phytoprostane treatment strongly suggests that TGA is either a direct or indirect target of RES (Mueller *et al.*, 2008).

1.1.7 Reactive nitrogen species

Not only ROS and RES play an important role during biotic and abiotic stress, but also reactive nitrogen species (RNS), which cause nitrosative stress (figure 1.2; Delledonne *et al.* 1998; Corpas *et al.* 2009; Airaki *et al.* 2012). The most important RNS is nitric oxide (NO), a gaseous, lipophilic radical that is able to diffuse through membranes (Crawford, 2006). NO can be generated enzymatically and non-enzymatically as reviewed in Corpas *et al.* (2011). Nitric oxide synthases (NOS) catalyse the formation of NO from L-arginine and are the most prominent NO producers in animals (Nathan and Xie, 1994). Recently, NOS-like proteins were identified in plants and shown to be involved in enzymatic NO production as well as nitrate reductases (NRs) (Corpas *et al.*, 2011). Results from tobacco suspension cultures also suggest NO formation from nitrosylamines by SOD (Ruemer *et al.*, 2009).

NO modulates the activity of many proteins by s-nitrosylation of thiol groups, by nitration of tyrosines, and by interacting with iron-containing proteins. This altered protein activity causes nitrosative stress and can induce cell death (Neill *et al.*, 2003; Radi, 2004; Lindermayr *et al.*, 2005). Besides cGMP-dependent signaling, NO-dependent sig-

naling cascades also contain nitrosylated proteins (Arasimowicz and Floryszak-Wieczorek, 2007). NO treatment leads to large changes in the transcriptome. Mainly stress responsive genes are induced, like PR proteins, alternative oxidase AOX1, redox-regulated genes like GSTs, and genes involved in phytohormone production and regulation (Palmieri *et al.*, 2008; Ahlfors *et al.*, 2009; Besson-Bard *et al.*, 2009).
Comparison of transcript patterns revealed an RNS-ROS crosstalk (figure 1.2). Zago *et al.* (2006) demonstrated that NO and H_2O_2 activate a similar subset of genes. This might be linked to the fact that H_2O_2 enhances NO levels by activating NRs (Lin *et al.*, 2012). Tanou *et al.* (2009) could show that NO and H_2O_2 activate overlapping signaling pathways during salt stress. Nitric oxide reacts with O_2^- forming $ONOO^-$ (peroxynitrate) (Delledonne *et al.*, 2001). Peroxynitrate is highly reactive and treatment of soybean with this RNS leads to enhanced levels of lipid peroxidation products and oxidized proteins (Jasid *et al.*, 2006). Together with the ability of H_2O_2 and NO to modify thiol groups of proteins, these examples emerge as the most important cross-talking sites (Molassiotis and Fotopoulos, 2011).
NO can provoke both, harmful and beneficial effects in plant cells. Locally restricted concentrations of NO might be responsible for this duality (Arasimowicz and Floryszak-Wieczorek, 2007). RNS play an antioxidant role during plant stress. Indeed, mutants deficient in the nitric oxide producing enzyme NOS1 exhibit enhanced levels of hydrogen peroxide, superoxide, oxidized lipids and oxidized proteins, an effect that could be reversed by the treatment with a NO donor (Guo and Crawford, 2005; Zhao *et al.*, 2007). In turn, application of NO donors to wild-type plants decreases ROS production, lipid peroxidation and delays PCD (Beligni *et al.*, 2002; Jasid *et al.*, 2006; Velikova *et al.*, 2008).

1.1.8 Cytoplasmic foci which develop under stress

Biotic and abiotic stress can inhibit translation and alter the composition of protein-mRNA complexes (messenger ribonucleoproteins (mRNPs)) (Balagopal and Parker, 2009) . mRNPs in eukaryotic cells exist in three different states: translated mRNPs (figure 1.4 A), stalled mRNPs (figure 1.4 B) and mRNPs under degradation (figure 1.4 C; Eulalio *et al.* 2007; Garneau *et al.* 2007; Parker and Sheth 2007). Whenever the exchange of components between these states is interrupted, stalled mRNPs accumulate in distinct cytoplasmic foci called processing bodies (PBs) (figure 1.4 D) and stress granules (SGs) (figure 1.4 E; Kedersha *et al.* 2005). PBs are membraneless protein aggregates that contain decapping enzymes like DCP1 and 2, activators of decapping and the exonuclease XRN1 (XRN4 in Arabidopsis) as well as the RISC (RNA-induced silencing) complex. mRNAs in PBs can be sequestered for translational arrest and become degraded (figure 1.4 F).

Alternatively, these mRNAs reenter the translation machinery (figure 1.4 G) suggesting that PBs are also important for storage of translationally inhibited mRNAs (Brengues *et al.*, 2005; Anderson and Kedersha, 2006; Eulalio *et al.*, 2007; Parker and Sheth, 2007; Balagopal and Parker, 2009).

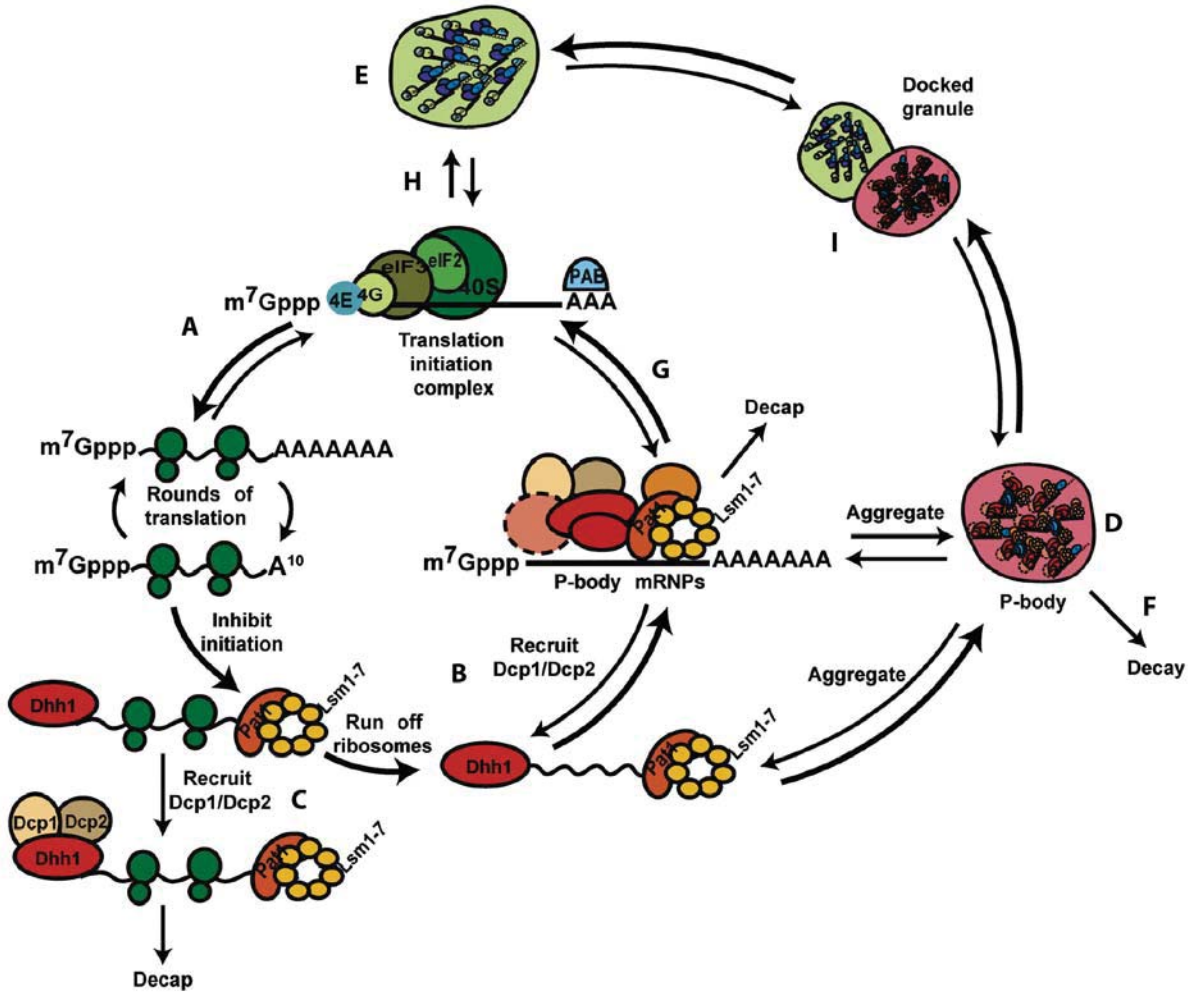

Figure 1.4: Model integrating processing bodies (PB) and stress granules (SG) into an messenger ribonucleoprotein (mRNP) cycle. mRNPs can be translated (A), stalled (B) or degraded (C). Stalled mRNPs can aggregate in PBs (D) and SGs (E). The sequestered mRNAs in PBs can be degraded (F) or they can reenter the translational machinery (G). SGs act as a sorting site, where mRNAs are either degraded, or recycled in a translation complex (H). PBs and SGs can dock to each other and exchange their components (I). Modified from Balagopal and Parker (2009), with kind permission of Elsevier.

In contrast, SGs are composed of mRNAs that are stalled in the process of translation initiation (figure 1.4 E). They contain translation initiation factors, 40S ribosomal subunit and poly(A) binding proteins and act as a sorting site. In SGs it is decided whether the mRNA becomes degraded or recycles in a translation complex (figure 1.4 H; Anderson and Kedersha 2008; Balagopal and Parker 2009; Buchan and Parker 2009; Buchan *et al.* 2011). The functional consequence of the assembly of mRNPs into PBs and SGs remains unclear, because manipulations that lead to the loss of visible PBs do not result in im-

paired mRNA decay. And despite the fact that several repressors of translation initiation are part of SGs, there is no clear evidence that the assembly of mRNPs into SGs is important for translational repression (Erickson and Lykke-Andersen, 2011). One reason for the assembly of PBs and SGs might be that the formation of the granules increases a local concentration of factors which may be especially important under stress conditions when certain translational resources are limited. Additionally, aggregation of mRNAs or mRNPs in PBs and SGs may limit the interaction of some mRNAs with degradation enzymes or polysomes (Anderson and Kedersha, 2002; Buchan and Parker, 2009).

PBs and SGs are distinct and independent cytoplasmic foci. There are many proteins which could be exclusively observed in PBs and others only in SGs. However there are also several components which are present in both types of granules (Kedersha *et al.*, 2005; Hoyle *et al.*, 2007; Buchan and Parker, 2009). This suggests that PBs and SGs might exchange these proteins and their incorporated mRNAs when they are docked (figure 1.4 I; Kedersha *et al.* 2005). Other hints that PBs and SGs are highly related come from the fact that many (but not all) stimuli induce assembly and disassembly of both granules. For example, arsenite and sodium azide treatment results PB and SG formation while cells treated with clotrimazol form only SGs. Disassembly of both, PBs and SGs, is enforced by chemicals which inhibit translational elongation and block the disassembly of polysomes, thereby preventing the translocation of mRNAs into PBs or SGs. Assembly, disassembly, kinetics and composition of PBs and SGs can vary in a stress-specific manner, implying that also these types of granules contribute to the specific response upon a distinct stress treatment (Kedersha *et al.*, 2005; Buchan *et al.*, 2011).

The composition of mRNAs incorporated in PBs and SGs is largely unknown. In principle, each mRNA that is part of an arrested translation complex may be translocated to these granules. Anderson and Kedersha (2002) speculated that especially mRNAs of housekeeping genes whose expression is downregulated under stress conditions are redirected to PBs and SGs.

While PBs and SGs in yeasts and mammals are well studied, there are few reports about these granules in plants. The first evidence that PBs and SGs also assemble in plants came from Weber *et al.* (2008). They could show that the Arabidopsis homologs of yeast and mammalian PB components DCP1, DCP2 and XRN1 relocate into cytoplasmic foci within seconds after the application of anoxia. On the other hand, elF4E as well as the RNA binding proteins RBP47 and UBP1 which are related to the mammalian SG component TIA-1, accumulate in different granules with altered kinetics. Both, PBs and SGs also form under heat stress and disassemble after cycloheximide treatment. Like in mammals and yeasts, they are often in close proximity, suggesting that plant PBs and SGs exchange their proteins and mRNAs as well. Besides the proteins mentioned above,

there are some additional proteins known to accumulate in plant PBs and SGs. Varicose and DCP5, components of the decapping complex (Xu *et al.*, 2006; Xu and Chua, 2009), the polypyrimidine tract-binding proteins *At*PTB1, 2 and 3 (Stauffer *et al.*, 2010), the tandem zinc finger protein *At*TZF1 (Pomeranz *et al.*, 2010) and SUO, a protein likely required for miRNA-mediated translational repression (Yang *et al.*, 2012), localize in PBs. Inreased Size Exclusion limit 2 helicase (ISE2) is probably localized in SGs (Kobayashi *et al.*, 2007)
T-DNA insertion lines of PB components have severe developmental defects. *Dcp1* and *dcp2* exhibit postembryonic lethality with disorganized veins, swollen root hairs, and altered epidermal cell morphology (Xu *et al.*, 2006). Knock-down mutants of *dcp5* are viable but show visible morphological changes including serration, pointed rosette and cauline leaves and disorganized cotyledonary veins (Xu and Chua, 2009). These severe defects may result from the fact that mRNAs encoding seed storage proteins are not decapped. Therefore, they reenter the translation machinery where they compete with mRNAs necessary for seedling development. In fact, mRNAs of seed storage proteins accumulate in PBs and in the *dcp5* mutants capped mRNAs accumulate (Goeres *et al.*, 2007; Xu and Chua, 2009). These findings suggest that in contrast to yeasts and mammals, plant PBs merely contain development-related mRNAs rather than those of housekeeping genes (Xu and Chua, 2011).

1.1.9 Stress memory

The development of a memory of previous stresses and subsequent enhanced tolerance to following stress treatments is well known in plants (Amzallag *et al.*, 1990; Karpinski, 1999; de Azevedo Neto *et al.*, 2005; Pandolfi *et al.*, 2012). Reprogramming of gene expression plays a key role in this acclimation process (Chinnusamy and Zhu, 2009). Acclimation to biotic and abiotic stress stimuli often involves epigenetic modifications. DNA methylation of cytosines results in repression of promoter activity and downregulation of associated transcripts. Other modifications of the genome without changes in the nucleotide sequence include post-translational modifications of histones, like acetylation or dimethylation of histone H3, which activate or inactivate chromosome sections by reorganization of the chromatin (Chinnusamy and Zhu, 2009).
Stress memory not only lasts for a prolonged period of time in an individual plant, but these epigenetic modifications are also inheritable. Several groups reported about transgenerational tolerance to biotic and abiotic stress treatments in the untreated offsprings. For example, the progenies of Arabidopsis and tomato subjected to herbivory or jasmonic acid (JA) are more resistant to herbivory in the two following generations (Rasmann *et*

al., 2012). Exposure of Arabidopsis to abiotic stresses, including salt, UV-C, cold, heat and flood, also resulted in higher tolerance to stress in the untreated progeny (Boyko and Kovalchuk, 2010). Transgenerational inheritance of increased frequency of homologous recombination is a repair mechanism for damaged DNA during stress. As one of the first groups, Molinier *et al.* (2006) reported that the increase in frequency of homologous recombination after treatment with UV-C or flagellin persists for at least four generations. In contrast, Boyko and Kovalchuk (2010) stated that the memory of hyper-recombination lasts only into the next generation.

Like short-term acclimation processes, stress-induced transgenerational responses depend on altered DNA methylation and post-translational modifications of histones, as shown mainly after infection with pathogens (Akimoto *et al.*, 2007; Luna *et al.*, 2012; Rasmann *et al.*, 2012). The signal depends on small RNAs, as mutations in the dicers DCL 2, 3 and 4 abolish heritable transgenerational effects (Boyko and Kovalchuk, 2010; Molnar *et al.*, 2010; Rasmann *et al.*, 2012). Methylation is sequence-independent and autonomous in the first progeny with a decrease of variability in the second progeny (Verhoeven *et al.*, 2010). However, transgenerational inheritance of stress responses is no general effect. Exposure of Arabidopsis plants to 10 different chemical and physical stress treatments of different strengths resulted in only low and stochastic increases in homologous recombination in the offsprings (Pecinka *et al.*, 2009). Whittle *et al.* (2009) indicated that heat but not cold pretreatment results in enhanced tolerance to theses temperatures in the next two unstressed generations. The control of transgene silencing is heritable but only at small loci and does often not exceed two generations (Lang-Mladek *et al.*, 2010). In addition, transgenerational effects are reset with prolonged seed storage (Lang-Mladek *et al.*, 2010). At the moment it is unclear how it is determined and whether or how long an epigenetic modification is transmitted to next generations.

1.2 Senescence

A developmental process that is highly associated with stress and the generation of stress-related molecules is senescence. Initiation of senescence is age-dependent. Both, the age of the individual leaf as well as the age and developmental stage of the entire plant account for the beginning of this process (Zentgraf *et al.*, 2004). In addition, various unfavorable environmental factors like high salinity and cold can trigger the onset of senescence (Munns, 2005; Masclaux-Daubresse *et al.*, 2007; Balazadeh *et al.*, 2011). The intricate connection of stress and senescence is also apparent from the fact that some genes, like the aminopeptidase *LAP2*, regulate both, senescence and stress responses as the knock-out plants exhibit an early senescence phenotype, as well as an increased sensitivity to various stresses (Waditee-Sirisattha *et al.*, 2011).