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Role of Intracellular Hydrogen Peroxide as Signalling Molecule for Plant Senescence

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1. Introduction

All aerobic organisms use molecular oxygen as terminal oxidant during respiration. Oxygen is neither very reactive nor harmful, but it has the potential to be only partially reduced, leading to the formation of very reactive and therefore toxic intermediates, like singlet oxygen ($^1O_2$), superoxide radical ($O_2^{\cdot-}$), hydroperoxylradical ($HO_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxylradical ($\cdot OH$). These forms are called "reactive oxygen species" (ROS). All ROS are extremely reactive and may oxidize biological molecules, such as DNA, proteins and lipids. However, these reactive molecules are unavoidable by-products of an aerobic metabolism. It is known that reactive oxygen species may have a dual role in plant stress response (Dat et al. 2000). Whereas high concentrations of hydrogen peroxide are toxic for the cell, low concentrations may act as signal which triggers the plant response upon a variety of biotic and abiotic stresses (Dat et al., 2000; Grant & Loake, 2000). It has been known for many years that common signal transduction molecules like MAPKs and calmodulin play an important role in some of these ROS signal transduction pathways.

Mitochondria are an important origin of ROS. During respiration, the ubiquinone pool is the main source for superoxide production. The alternative oxidase (AOX) could be identified in plants and protists, e.g. Trypanosoma, fungi, like *Neurospora crassa* and *Hansenula anomala* and in green algae, e.g. in Chlamydomonas (McIntosh, 1994). It acts as a quinoloxidase by transferring electrons from the reduced ubiquinone directly to molecular oxygen forming water (Siedow & Moore, 1993). AOX mediates an energy-wasteful form of respiration, but its physiological significance is still a matter of intense debate (Rasmusson et al., 2009; Vanlerberghe et al., 2009; Millar et al., 2011). The plant alternative oxidases form homodimers (Moore et al., 2002) and are encoded by a small gene family. In *Arabidopsis thaliana* five genes are known, *AOX1a*, *AOX1b*, *AOX1c, AOX1d* and *AOX2*, each exhibiting organ specific expression (Saisho et al., 1997; https:/www.genevestigator.com). Among these five *AOX* genes in *Arabidopsis thaliana*, *AOX1a* is the major isoform expressed in leaves (Clifton et al., 2006). One important function of the alternative oxidase is to prevent the formation of excess of reactive oxygen molecules (Maxwell et al., 1999). AOX ensures a low reduction status of the ubiquinone pool by oxidizing ubiquinol. Thus, the electron flow is guaranteed (Millenaar & Lambers, 2003). This reaction is necessary, if the cytochrome *c* dependent pathway is restricted by naturally occurring cyanide, NO, sulphide, high concentrations of CO_2, low temperatures or phosphorus deprivation (Millenaar & Lambers, 2003) as well as wounding, drought, osmotic stress, ripening and pathogen infection

(McIntosh, 1994; Moore et al., 2002). Photo-oxidative stress of chloroplasts is also involved in *AOX* up-regulation (Yoshida et al. 2008). Moreover, ascorbate biosynthesis in mitochondria is linked to the electron transport chain between complexes III and IV (Bartoli et al., 2000) and leaves of the *AOX*-overexpressing lines accumulate more ascorbic acid than wild-type leaves (Bartoli et al., 2006). A lack of AOX can lead to an up-regulation of transcripts of the antioxidant defense system at low temperature (Watanabe et al, 2008). Therefore, it is likely that AOX is an important component in antioxidant defense mechanisms.

In addition, it is proposed that AOX also has important functions outside the mitochondria (Arnholdt-Schmitt et al., 2006; Clifton et al., 2006; Van Aken et al., 2009). Furthermore, a beneficial role for AOX in illuminated leaves has been suggested and AOX-deficient *aox1a* mutant showed a lowered operating efficiency of photosystem II and an enhanced activity of cyclic electron transport around photosystem I (CET-PSI) at high irradiance (Yoshida et al., 2011). However, in most cases, transgenic plants with altered levels of AOX exhibited no obvious variation in plant growth phenotype (Vanlerberghe et al., 2009), implying that AOX does not severely affect photosynthetic carbon gain and biomass productivity**.** In addition**,** AOX also has an effect on the control of NO levels in plant cells (Wulff et al., 2009).

There is some evidence that alternative respiration is correlated with senescence and longevity. Aging potato slides showed a decline in the capacity of cytochrome *c* dependent respiration whereas the alternative respiration as well as the protein content of AOX increased (Hiser & McIntosh, 1990). Expression of *AOX1a* of Arabidopsis is highest in rosette leaves at the onset of senescence (https:/www.genevestigator.com). Interestingly, the inactivation of subunit V of the cytochrome *c* oxidase complex in the fungus *Podospora anserina* led to the exclusive use of the alternative respiration pathway and to a decline in ROS formation in these mutants. This inactivation of the cytochrome *c* oxidase resulted in an extraordinary longevity of this fungus (Dufour et al. 2000). There are several lines of evidence that beside mitochondria also chloroplasts and peroxisomes trigger leaf senescence. For peroxisomes a ROS-mediated function in leaf senescence has been described (del Río et al. 1998). Tobacco deficient in the thylacoid Ndh complex showed a delay in leaf senescence. It was discussed that the senescence delay was achieved by lower ROS production (Zapater et al., 2005).

In different Arabidopsis mutants a tight correlation between extended longevity and tolerance against oxidative stress has been observed (Kurepa et al., 1998). The most extended longevity mutant of this collection which also showed the highest tolerance against paraquat treatment was *gigantea3*. GIGANTEA acts in blue light signaling and has biochemically separable roles in circadian clock and flowering time regulation (Martin-Tryon et al., 2007). However, the link between this nuclear localized protein and resistance to oxidative stress is still unclear. CATALASE2 (CAT2) and CATALASE3 (CAT3) enzymes, which are expressed under the control of the circadian clock, might be good candidates. They exhibit a higher activity in the *gigantea3* mutant which might be responsible for the elevated oxidative stress tolerance (Zentgraf & Hemleben, 2007). In contrast, the delayed leaf senescence mutants of Arabidopsis *ore1, ore3,* and *ore9* also exhibit increased tolerance to various types of oxidative stress but the activities of antioxidant enzymes were similar or lower in the mutants, as compared to wild type providing evidence that oxidative stress tolerance is also genetically linked to control of leaf longevity in plants (Woo et al., 2004).

In addition, the expression of many SAGs is enhanced by increased levels of reactive oxygen species (Miller et al., 1999; Navabpour et al., 2003) indicating that elevated levels of ROS

might be used as a signal to promote senescence. In Arabidopsis the coordinate regulation of the hydrogen peroxide scavenging enzymes catalase (CAT) and ascorbate peroxidase (APX) leads to a defined increase of hydrogen peroxide content during bolting time (Ye et al., 2000; Zimmermann et al., 2006). Removing the bolt and thereby delaying the decrease in APX activity led to a delay in chlorophyll degradation and senescence (Ye et al., 2000). Since APX enzyme activity appears to be regulated on the posttranscriptional level (Panchuk et al., 2005; Zimmermann et al., 2006) and appears to be inhibited by hydrogen peroxide itself in this developmental stage, the initial event to create the hydrogen peroxide peak during bolting time at the onset of senescence is the transcriptional down-regulation of *CAT2*. The transcription factor responsible for this down-regulation was isolated by a yeast-one hybrid screen and turned out to be a member of the bZIP transcription factor family, namely GBF1. If *GBF1* is knocked out by a T-DNA insertion, the down-regulation of *CAT2* during bolting time is abolished, the hydrogen peroxide peak during bolting time disappears and senescence is delayed (Smykowski et al., 2010). This hydrogen peroxide peak is discussed to trigger senescence induction by activating the systemic expression of the senescence-related transcription factors e.g. *WRKY53* (Miao et al., 2004).

In order to understand the correlation between mitochondrial ROS production and senescence in *Arabidopsis thaliana*, we treated cell cultures and whole Arabidopsis plants with antimycin A, an inhibitor of cytochrom *c* oxidase, and measured hydrogen peroxide production and senescence parameters. In addition, two different genes encoding the peroxisomal enzyme catalase have been knocked-out and the single knock-out plants *cat2* and *cat3* have been crossed to produce double knock-out plants *cat2/3*. In these plants also the consequences on hydrogen peroxide levels and leaf senescence were analysed.

2. Results and discussion

2.1 Changes in mitochondrial hydrogen peroxide production

Dufour and others (2000) characterized an almost immortal mutant of the fungus *Podospora anserina* carrying a mutation in the gene encoding subunit V of the cytochrom *c* oxidase complex. These mutants exclusively used the alternative respiration pathway thus clearly leading to a lower content of reactive oxygen species than in normal growing fungi. In Arabidopsis resistance to oxidative stress and longevity are also tightly correlated (Kurepa et al., 1998; Woo et al., 2004). Therefore, we wanted to analyse Arabidopsis plants and cells with increased alternative respiration for mitochondrial ROS production and a delay in senescence.

2.1.1 Antimycin A treatment of cell cultures and whole plants

The alternative respiration in plants can be induced by application of antimycin A (Vanlerberghe & McIntosh, 1992), which was isolated from *Streptomyces* sp. and inhibits specifically the electron transport between cytochrome *b* and c_1. To investigate the influence of antimycin A on the production of ROS, we analysed antimycin A treated *Arabidopsis thaliana* cell cultures for their hydrogen peroxide contents. Two hours after treatment with 5 µM antimycin A or 0.02 % ethanol as control the H_2O_2 concentration slightly increased, whereas further incubation clearly lowered H_2O_2 content in antimycin A treated cells in comparison to control cells (Fig. 1 A). The transient increase in H_2O_2 levels might be a result of the inhibition of cytochrome *c* oxidase as it was already shown by Maxwell and

coworkers (1999) for tobacco cells. Here an initial hydrogen peroxide production after antimycin A treatment could be localized almost exclusively to the mitochondria using laser scanning microscopy of H_2DCF-DA and mitotracker double-labelled cells. Dot blot analyses of 10 µg total RNA isolated from Arabidopsis culture cells and subsequent hybridization revealed that alternative oxidase 1a (AOX 1a) was induced by antimycin A as well as by hydrogen peroxide treatment (Fig. 1 B). This was also observed in tobacco cells, where antimycin A led to a more efficient alternative respiration capacity (Maxwell et al., 2002) and subsequently to a reduced mitochondrial ROS formation (Maxwell et al., 1999).

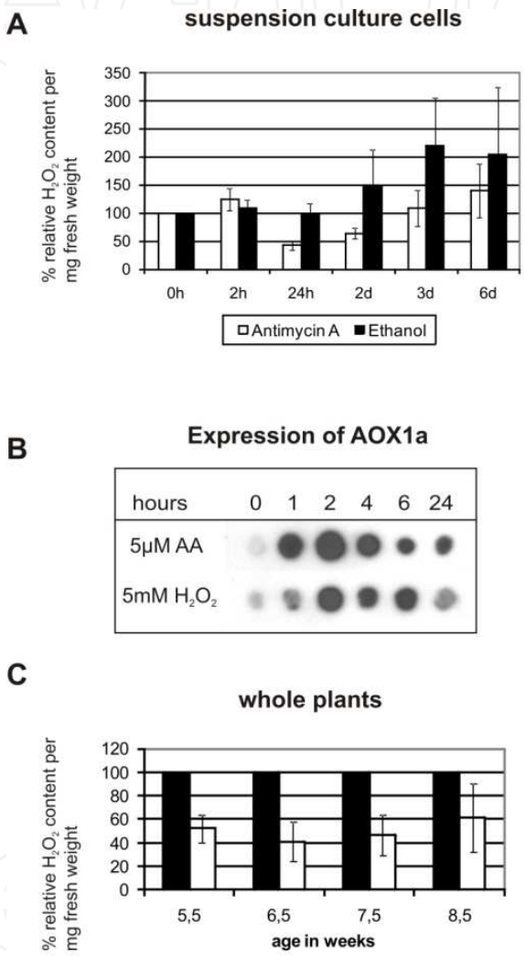

Fig. 1. *Short term treatment of cell cultures and whole plants with antimycin A*.
A) *Arabidopsis thaliana* cell cultures were treated with 5 µM antimycin A or 0.02 % ethanol as control and were analysed for their hydrogen peroxide content. The 0 h value was referred to as 100%. The error bars indicate the standard deviation of 4 independent experiments.
B) Hybridization of 10µg of total RNA isolated from antimycin A or H_2O_2 treated culture cells dotted on a nylon filter with an *AOX1a* specific probe. **C)** *Arabidopsis thaliana* plants of different developmental stages were watered with 10 ml of a 20 µmol antimycin A solution whereas control plants were treated with 0.8 % ethanol and were analysed for their hydrogen peroxide content after 24 hours. The values of ethanol treated plants were referred to as 100%. The error bars indicate the standard deviation of 4 independent experiments.

Since we were interested in analyzing the induction of senescence in whole Arabidopsis plants, we watered plants of different developmental stages with antimycin A and measured the hydrogen peroxide content 24 h after the treatment. In all developmental stages the hydrogen peroxide content was significantly lower in leaves of antimycin A treated plants (Fig. 1 C) indicating that in all developmental stages AOX and alternative respiration was induced to reduce mitochondrial ROS production.

2.1.2 Long term treatment of plants with antimycin A

In order to elucidate the long term effects of alternative respiration on plant development and senescence, soil grown Arabidopsis plants were watered over a time period of five weeks with 10 ml 20 µmol antimycin A solution every second day beginning with 5-week-old plants. Control plants were treated with 0.8 % ethanol in which antimycin A was dissolved. Since it was possible to reduce the hydrogen peroxide levels by the induction of the alternative pathways in all developmental stages, we assume that these plants grew under conditions favouring the alternative respiration from week 5 on. We have chosen this experimental design in order to guarantee that plant growth and development is not impaired in early stages by the lack of a functional cytochrome *c* pathway and the ATP it generates. Therefore, we did not use cytochrome *c* oxidase knock-out mutants, which appear to be impaired in growth and development from early on (data not shown).

The hydrogen peroxide content of antimycin A watered and control plants was measured weekly and the H_2O_2 level at the beginning of the experiment was set as 100 % (Fig. 2A). H_2O_2 concentrations of the control plants exhibit a peak in 7-week-old plants during the time of bolting and an increase in late stages of development as it was already shown before (Miao et al. 2004; Zimmermann et al. 2006).

In contrast, the antimycin A treated plants showed a slight decrease up to 8 weeks and recovered in older stages to the starting level (Fig. 2A). This coincides with the results of Dufour and coworkers (2000) for the fungus *Podospora anserine*, where long term activated alternative respiration led to lower hydrogen peroxide contents and strongly increased longevity. However, in Arabidopsis no obvious differences in the development and the progression of senescence could be detected phenotypically in antimycin A treated plants (Fig. 2B). In contrast, a transgenic Arabidopsis line overexpressing the senescence-associated transcription factor *WRKY53* exhibited an accelerated senescence phenotype (Fig. 2B; Miao et al., 2004). In accordance with the phenotype, chlorophyll and total protein content differed only slightly between antimycin A and ethanol treated plants, but were reduced earlier in 35S:*WRKY53* plants (Fig. 2C). Northern blot analyses revealed that the senescence-specific cystein protease gene *SAG12* was induced earlier and stronger in the antimycin A treated plants (Fig. 2D). This implies that even though less reactive oxygen species are produced in plants with favoured alternative respiration, development and senescence are not impaired or even slightly accelerated. Maxwell et al. (2002) presented evidence that, besides AOX, different senescence associated genes of tobacco (e.g. ACC, GST and Cystein protease precursor) can rapidly be induced by antimycin A treatment and this rapid induction can be prevented by ROS scavengers (Maxwell et al., 2002). In addition, overexpression of AOX in tobacco culture cells led to a decline in ROS concentration and a reduced expression of antioxidative enzymes, like superoxide dismutase or glutathione peroxidase (Maxwell et al., 1999).

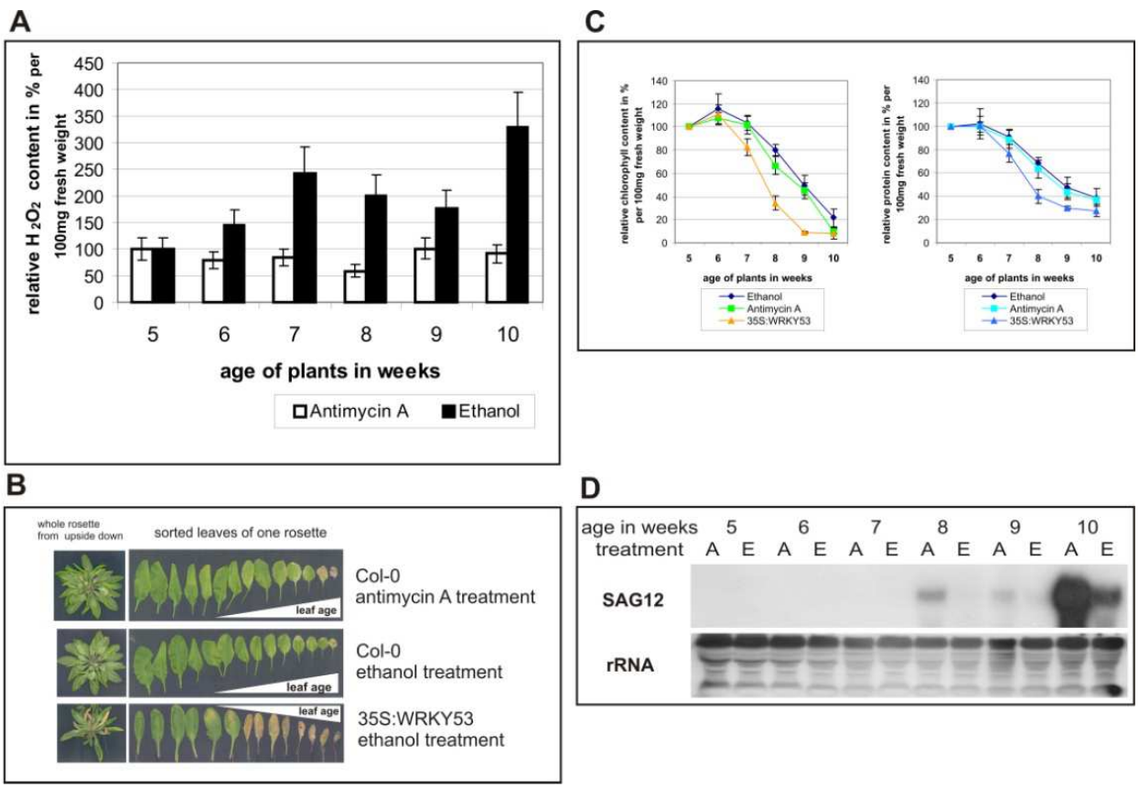

Fig. 2. *Long term treatment of whole plants with antimycin A*.
A) *Arabidopsis thaliana* plants were watered with 10 ml of a 20 µmol antimycin A solution every second day over a time period of five weeks beginning with 5-week-old plants. Control plants were treated with 0.8 % of ethanol. These plants were analysed for their hydrogen peroxide content every week. The values of the 5-week-old plants were referred to as 100%. The error bars indicate the standard deviation of 3 independent experiments. **B)** Phenotypic analyses of antimycin A or ethanol treated 8-week-old wildtype plants and ethanol treated transgenic *WRKY53* overexpressing line (35S:*WRKY53*). Whole plants are shown upside down to visualize older leaves of the rosette. In addition, the leaves were sorted according to their age using a specific colour code. **C)** Chlorophyll (left) and total protein (right) were measured in ethanol treated wildtype plants (Col-0), antimycin A treated wildtype plants and ethanol treated *WRKY53* overexpessing plants (35S:*WRKY53*). The values of 5-week-old plants were referred to as 100%. The error bars indicate the standard deviation of 3 independent experiments. **D)** Northern blot analyses of 15 µg of total RNA isolated from antimycin A (A) or ethanol treated (E) plants. The nylon filters were hybridized with a *SAG12* specific probe. Rehybridization with a 25S rRNA probe was used as loading control.

Transgenic tobacco culture cells carrying an antisense construct for *AOX* show an increased ROS formation and an elevated transcript abundance of catalase (Maxwell et al., 1999). In contrast, Umbach and coworkers (2005) observed that in *AOX* overexpressing or *AOX* antisense transgenic Arabidopsis lines transcript levels of the antioxidative enzymes MnSOD, organellar APX, cytosolic and organellar glutathione reductase and peroxiredoxins

were not altered. This indicates that in Arabidopsis the lower production of ROS does not lead to compensatory reduction of oxidative stress enzymes. A senescence phenotype was also not observed in these lines.

2.1.3 Transgenic plants overexpressing *AOX1a*

The analysis of transgenic plants is helpful to gain more information about the function of a gene. For this reason, plants expressing the genes *AOX1a* under the constitutive 35S promoter were generated. This isoform was selected since it is strongly expressed in leaves. Plants of the T2 generation of these overexpressing lines were tested for *AOX1a* expression and three lines were obtained which overexpressed the transgene about 20-fold. The H_2O_2 content of these lines was analysed and a clear reduction in the hydrogen peroxide content in the transgenic lines could be measured (Fig.3B). Again, no obvious senescence phenotype could be detected (Fig. 3A). If at all, a slight acceleration of leaf senescence can be observed in the 35S:*AOX1a* lines. Fiorani et al. (2005) could observe a phenotype in 35S:*AOX1a* lines under low temperature conditions (12°C) with increased leaf area and larger rosettes. This could not be observed in our 35S:*AOX1a* lines under normal growth conditions. However, the cytochrom *c* dependent respiration is still functional in these plants probably masking the effect of increased levels of AOX.

Millenaar and Lambers (2003) describe that there is no clear positive correlation between the concentration of AOX protein and its activity *in vivo*, since an increase in protein formation does not change pyruvate concentration and the reduction state of ubiquinone, which are necessary for the activation of the AOX protein. For example, tobacco leaves infected with tobacco mosaic virus showed an increased AOX protein level but no change in activity of the alternative respiration (Lennon et al., 1997). In the transgenic plants overexpressing AOX, the capacity of the alternative respiration pathway appears to be elevated, but this does not necessarily reflect its activation. In the same line of evidence neither overexpression nor inactivation of AOX caused a change in ROS formation in the fungus *Podospora anserina* (Lorin et al., 2001). There was no effect on lifespan or senescence in the transgenic fungi either. However, in our transgenic lines the ROS production is clearly reduced indicating an activation of the alternative respiration pathway but nevertheless no effect on senescence could be observed.

Overexpression of *AOX* in tobacco culture cells leads to a decline in ROS concentration and a reduced expression of other antioxidative enzymes, like superoxide dismutase or glutathione peroxidase (Maxwell et al., 1999) whereas transgenic tobacco culture cells carrying an antisense construct for *AOX* show an increased ROS formation and an elevated transcript abundance of catalase (Maxwell et al., 1999). This would suggest that the plants would be either more sensitive or more resistant to oxidative stress. In contrast, in transgenic Arabidopsis lines either overexpressing *AOX* or an *AOX* antisense construct transcript levels of the antioxidative enzymes MnSOD, organellar APX, cytosolic and organellar glutathione reductase and peroxiredoxins were not altered (Umbach et al., 2005). In consistence with these findings, no altered resistance against oxidative stress could be observed in the transgenic 35S:*AOX1a* transgenic plants, which we germinated on MS plates and applied oxidative stress by spraying the seedlings with hydrogen peroxide (Fig. 3C).

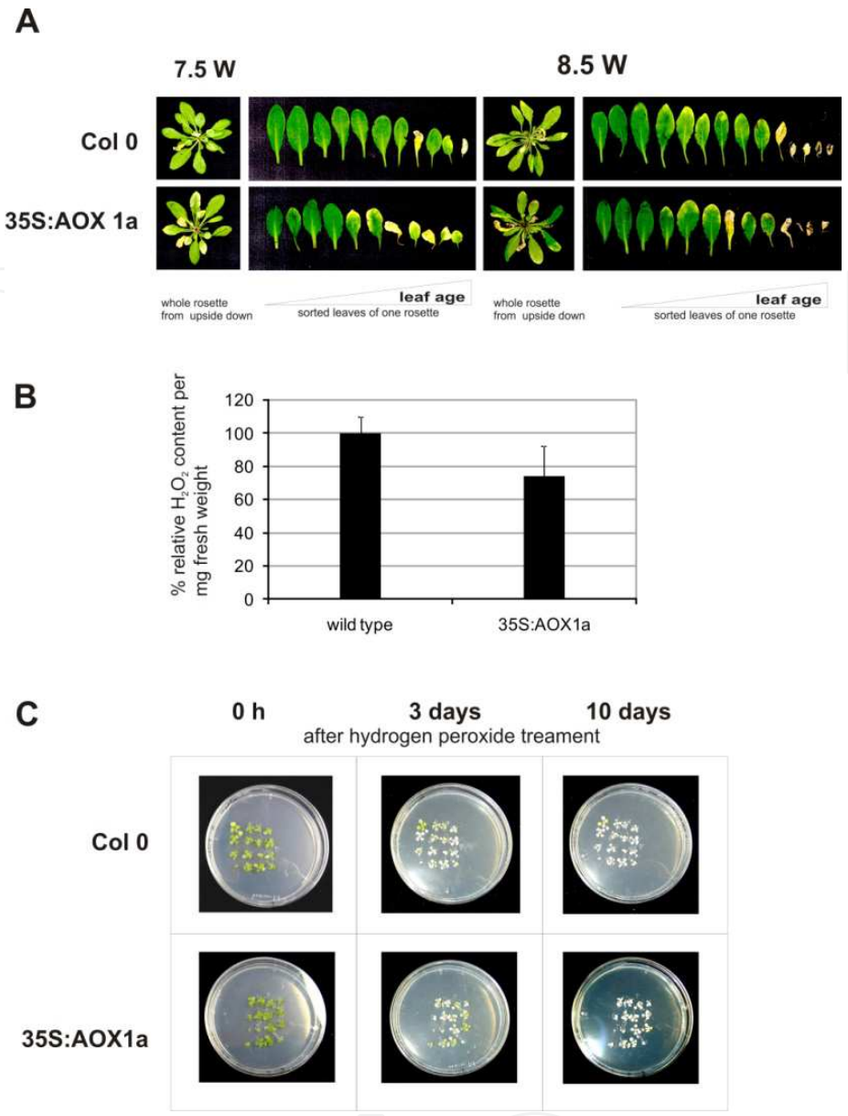

Fig. 3. *Transgenic plants overexperessing AOX1a*
A) Phenotypic analyses of wildtype (Col-0) and 35S:*AOX1a* transgenic plants. Whole plants are shown upside down to visualize older leaves of the rosette. In addition, the leaves were sorted according to their age using a specific colour code. **B)** 4-6 wildtype (Col-0) and 35S:*AOX1a* transgenic plants were pooled and analysed for their hydrogen peroxide content. The values of the wild type plants were referred to as 100%. The error bars indicate the standard deviation of 2 independently collected plant pools. **C)** Phenotypic analyses of hydrogen peroxide treated seedlings of wild type (Col-0) and 35S:*AOX1a* transgenic plants.

2.1.4 Senescence-associated and circadian expression of *AOX1a*

The family of alternative oxidases comprises five genes with an organ specific expression (Saisho et al., 1997; https://www.genevestigator.com). In general, *AOX* is expressed only at a very low level under normal conditions. By using leaf material of plants of different age harvested in the morning hours, a senescence dependent expression of *AOX1a* could be

observed with the highest transcript abundance in old plants. In young, up to 7-week-old plants, no expression could be detected by Northern blot analyses (Fig. 4A). This coincides with genevestigator data and with the *AOX* expression in different stages of the leaf development in potatoes, where an increase in AOX protein from young to mature leaves could be observed (Svensson & Rasmusson, 2001; https:/www.genevestigator.com). Furthermore, there is a *de novo* synthesis of alternative oxidase in aging potato slides (Hiser & McIntosh, 1990). Our *in silico* analysis of about 1500 bp upstream the coding region of the *AOX1a* gene revealed, amongst others, several W-box core elements and one sequence for a circadian element. The W-boxes indicate a regulation by WRKY transcription factors which are involved in senescence or pathogen dependent regulation (Eulgem et al., 2000; Miao et al., 2004) whereas the circadian element points out a clock dependent regulation. Based on these results, we used 8.5-week-old plants to harvest leaf material every three hours over 27 h. A circadian regulation of *AOX1a* could be detected with the maximum of expression in the early morning hours with the beginning of illumination (Fig. 4B). This corresponds to the expression of *AOX* in tobacco (Dutilleul et al., 2003).

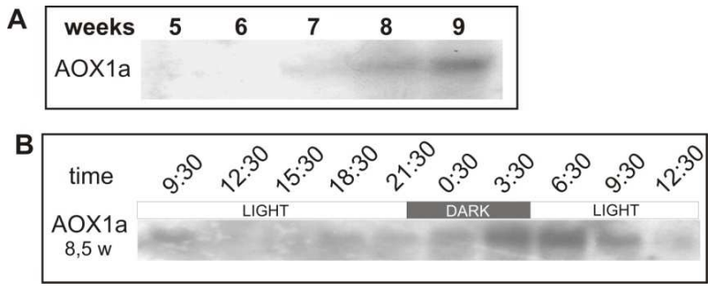

Fig. 4. *Senescence-associated expression of AOX1a*
Northern blot analyses of 15 µg of total RNA isolated from plants of **A)** 5-week-old to 9-week-old plants and **B)** 8-week-old plants at different day times. The nylon filters were hybridized with an *AOX1a* specific probe. Equal loading was controlled by Toluidin blue staining of the membranes.

2.2 Changes in peroxisomal hydrogen peroxide production

Peroxisomes are organelles encircled by only a single membrane layer embedding an extensive oxidative metabolism. These organelles are found in all eukaryotic organisms. In plants, peroxisomes participate in many physiological processes like seed germination, leaf senescence, fruit maturation, response to abiotic and biotic stress, photomorphogenesis, biosynthesis of the plant hormones jasmonic acid and auxin, and in cell signaling by reactive oxygen and nitrogen species. A specific feature of peroxisomes is their dynamic metabolism meaning that the enzymatic constitution of peroxisomes is adjusted to the organism, cell or tissue-type, and also to a variety of environmental conditions (Palma et al., 2009). One important source for ROS formation, especially for H_2O_2, is photorespiration. During CO_2 fixation, ribulose-1,5-bisphosphate-carboxylase (RubisCO) can use CO_2 to carboxylate ribulose-1,5-bisphosphate but also molecular oxygen to oxygenate ribulose-1,5-bisphosphate forming glycolate. The glycolate is then transported from the chloroplasts into the peroxisomes where it is oxidized generating H_2O_2 as a by-product. Peroxisomes and ROS generated in these organelles were shown to play a central role in natural and dark induced senescence in pea (del Rio et al., 1998) and appear to play an important role as a supplier of

signal molecules like $NO^{\cdot}$ (nitric oxide), $O_2^{\cdot -}$, H_2O_2 and possibly S-nitrosoglutathione (del Rio et al., 1998; 2002; 2003). These signaling molecules can trigger specific gene expression by so far largely unknown signal transduction pathways (Corpas et al., 2001; 2004; del Rio et al., 2002). However, the concentration of these molecules is tightly regulated by a sensitive balance between production and decomposition by different specific scavenging systems. Catalases are the most abundant enzymes in peroxisomes and convert hydrogen peroxide into water and oxygen without the consumption of reducing equivalents. Besides catalases all enzymes of the antioxidant ascorbate-glutathione cycle, also called Foyer-Halliwell-Asada cycle, are present in peroxisomes to detoxify H_2O_2 through the oxidation of ascorbate and glutathione in an NADPH-dependent manner, thus complementing the action of catalase in peroxisomes. If the mitochondrial and the peroxisomal ascorbate-glutathione cycles are compared during progression of senescence, it can be speculated that peroxisomes may participate longer in the cellular oxidative mechanism of leaf senescence than mitochondria, since mitochondria appear to be affected by oxidative damage earlier than peroxisomes (Jiménez et al. , 1998; del Rio et al., 2003).

Catalases are tetrameric heme containing enzymes and are present in all aerobic organisms. Due to a very high apparent Michaelis constant catalases are not easily saturated with substrate and can act over a wide range of H_2O_2 concentrations maintaining a controlled intracellular H_2O_2 concentration. Whereas animals have only one form of catalase, plants have evolved small gene families encoding catalases. The plant catalases can be grouped into three classes depending on their expression and physiological parameters. In *Arabidopsis*, the small catalase gene family has been characterized to consist of three members, the class III catalase *CAT1*, class I catalase *CAT2* and class II catalase *CAT3.* All three Arabidopsis catalases show a senescence-specific alteration in expression and activity (Zimmermann et al., 2006*). CAT2* expression and activity is down-regulated at an early time point when plants are bolting. Subsequently, expression and activity of *CAT3* is up-regulated during progression of senescence. In contrast to *CAT2* expression, which is predominantly located in mesophyll cells, *CAT3* expression is mainly expressed in vascular tissue indicating that the vascular system appears to be protected against oxidative stress during senescence to guarantee the transport of nutrients and minerals out of the senescing tissue into developing parts of the plant like e.g. the seeds (Zimmermann et al., 2006). CAT1 expression and activity is very low during plant development and only increases significantly during germination and in very late stages of senescence. Due to this expression pattern, its activity is discussed to be related to fatty acid degradation which takes place when peroxisomes are converted into glyoxisomes.

Especially the transcriptional down-regulation of *CAT2* appears to be involved in the regulation of the onset of senescence. This down-regulation is executed by the bZIP transcription factor GBF1. Insertion of a T-DNA into the *GBF1* gene revealed a loss of *CAT2* down-regulation and resulted in the loss of a hydrogen peroxide increase during bolting time. These *gbf1* mutant plants exhibit a delayed onset of senescence (Smykowski et al., 2010). Consequently, the idea suggests itself that *CAT2* knock-out plants also have a senescence phenotype. Taking into consideration that *CAT2* is expressed not only in leaves but also in roots, stems and flowers contributing substantially to the regulation of intracellular hydrogen peroxide contents and the protection of the cells against ROS in stress situations, the knock-out of this gene would have severe effects on the plants. The loss of such an important enzyme has to be compensated somehow during development but it

would be expected that these knock-out plants are more sensitive against all stresses implying an increased ROS production and that they most likely show a senescence phenotype. The lack of peroxisomal catalase CTL-2 in *Caenorhabditis elegans* causes a progeric phenotype whereas the lack of the cytosolic catalase CTL-1 has no effect on nematode aging (Petriv & Rachubinski, 2004). In yeast, catalase T activity but not catalase A activity was necessary to assure longevity under repressing conditions on glucose media. However, under derepressing conditions, on ethanol media, both catalases were required for longevity assurance (Van Zandycke et al., 2002) indicating a correlation between CAT activity and longevity in animal systems.

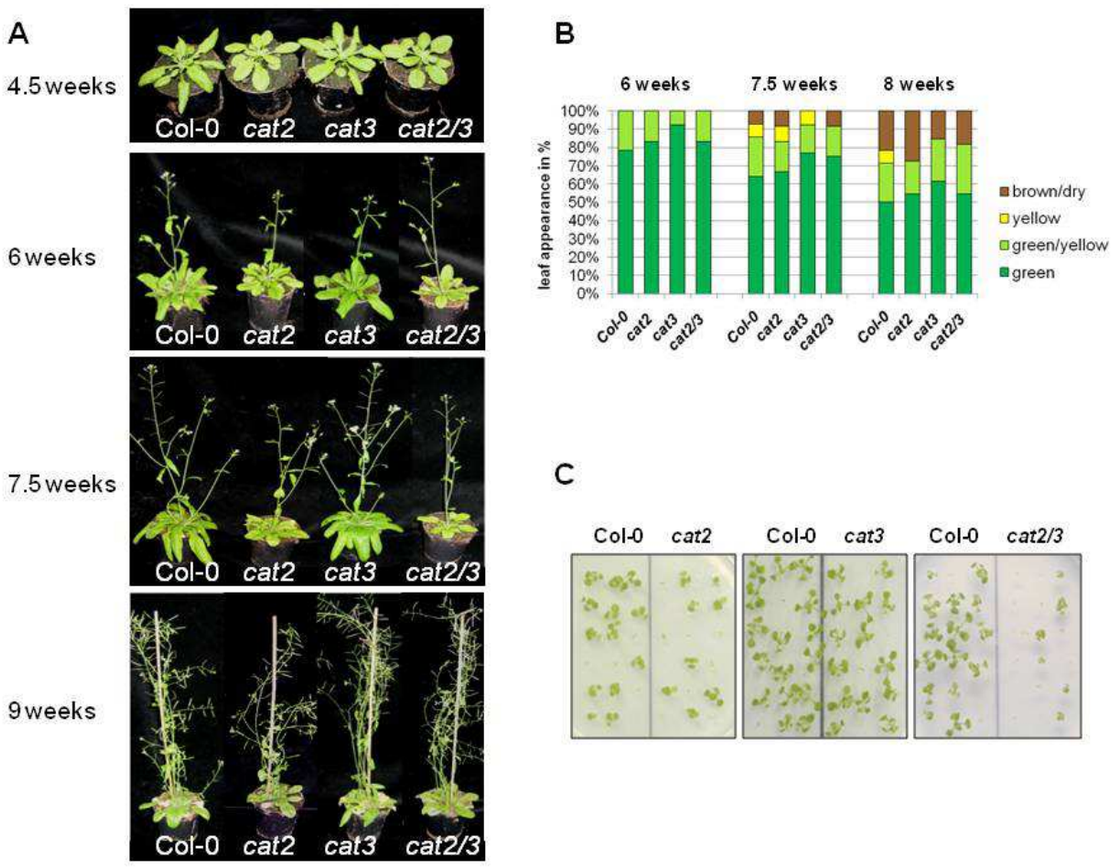

Fig. 5. *Phenotypic analyses of catalase mutants*
A) Plant development, **B)** Percantage of phenotypical appearance of the leaves of ten rosettes, **C)** Germination rate of wild type (Col-0), *cat2*, *cat3*, and *cat2/3* mutant plants.

Surprisingly, *cat2* knock-out plants appear to be more or less inconspicuous. They are slightly impaired in germination (Fig. 5C) but once germinated the plants developed relatively normaly (Queval et al., 2007; Fig. 5A). Photoperiod and CO_2 levels have a high impact on the phenotypic appearance of the plants and on the ascorbate and glutathione contents and their balances of the oxidized and reduced form, respectively. Under high CO_2

conditions no obvious phenotype could be observed whereas growth under ambient air, which favours photorespiration, led to a lower biomass production of the rosette and an altered leaf shape (Queval et al., 2007; Fig 5). We characterized SALK T-DNA insertion lines for *CAT2* and *CAT3* for homozygous insertion of the T-DNA and crossed the homozygous *cat2* and *cat3* mutants and selected the offsprings for a homozygous double knock-out line *cat2/3*. After separation of leaf protein extracts of these lines on native PAGEs, we could confirm that according to the gene knock-out the activity of the respective isoform disappeared (Fig. 6 A). When we analyzed plant development under long day conditions, leaf or plant senescence does not seem to be impaired (Fig. 5A, B); only leaf shape and biomass production were slightly altered in *cat2 and cat2/3* plants. However, the mutant plant populations did not senesce as homogenously as the wildtype populations. If the hydrogen peroxide content was measured, the profiles appeared to be not much different indicating that a very efficient compensation of the loss of CAT activity has been activated (Fig 6A).

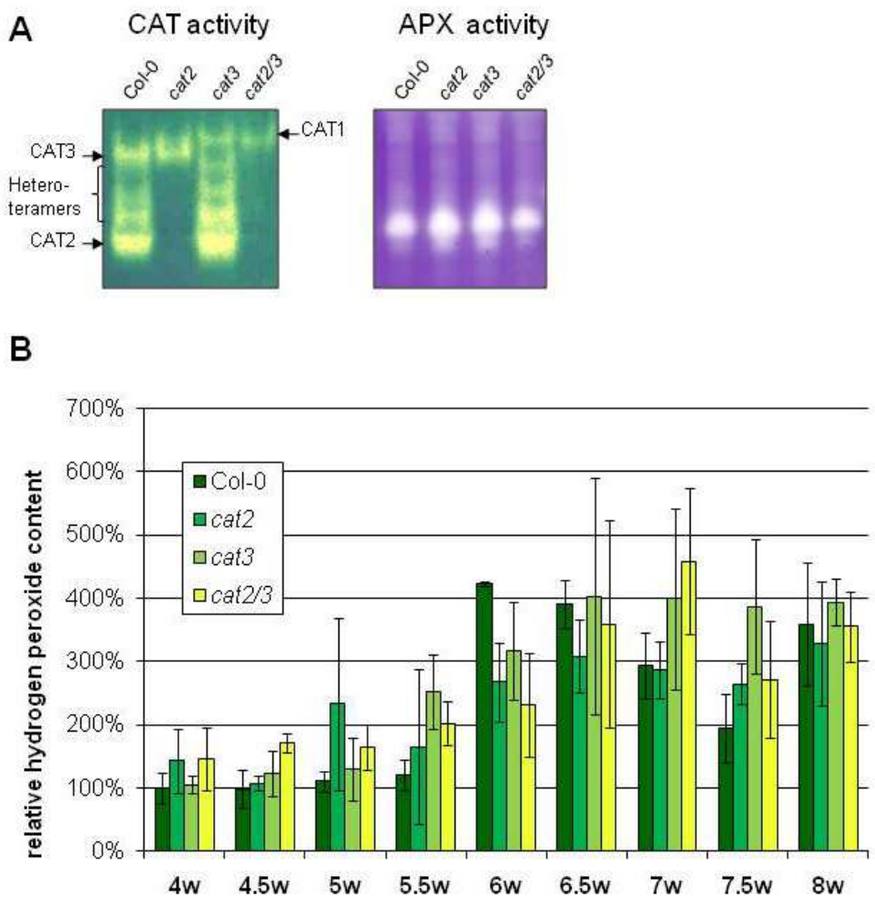

Fig. 6. *Physiological analyses of catalase mutants*
A) Catalase activity, **B)** Ascorbate peroxidase activity. **C)** Hydrogen peroxide content of wild type (Col-0), cat2, cat3, and cat2/3 mutant plants. The value of the 4–week-old wildtype plants was referred to as 100%. The error bars indicate the standard deviation of 4 independent experiments.

However, the hydrogen peroxide content between different leaves and plants varied remarkably so that the standard deviation was quite high in the measurements of the mutants. How the CAT2 or CAT3 activity losses were compensated is not yet clear but APX activity appeared to be not elevated (Fig 6B); in contrast, it appeared to be even slightly reduced in the double mutant. Hydrogen peroxide levels in these plants clearly indicated that the loss of the CAT activity must have been compensated. This is consistent with the finding of Rizhky and co-workers (2002), who claimed that there appears to be a sensitive balance between the antioxidant enzymes with compensating mechanisms, since they observed that double antisense plants for CAT or APX are more tolerant to oxidative stress than single antisense plants (Rizhsky et al., 2002). A slight activation of CAT1 can be observed in all our catalase mutants, especially in the *cat3* and *cat2/3* mutant plants. This is also indicated by the heterodimer formation between CAT2 and CAT1 in the *cat3* mutant. However, the activity of this isoform appears to be only low compared to the loss of catalase activity which would be present in wild type plants (Fig. 6B). Remarkably, glutathione levels are increased and shifted towards the more oxidized form in *cat2* plants under long day conditions (Queval et al., 2007). Taken together, the ROS levels appear to be very tightly regulated on many levels with the possibility of compensation if one detoxifying system fails.

3. Conclusion

Antimycin A treatment leads to the inhibition of the cytochrom *c* dependent electron transport lowering the production of hydrogen peroxide in mitochondria. Conversely, it is assumed that if stress occurs in a cellular compartment and increasing amounts of hydrogen peroxide are formed, these hydrogen peroxide molecules also can pass membranes and can be transported into the cytosol. This signal can then be transduced into the nucleus, where it induces the expression of many genes including *AOX*. As soon as the newly synthesized AOX protein is active, it minimizes the formation of ROS in the mitochondria by preventing the overreduction of the electron transport chain. Therefore, alternative oxidase might be regarded as mechanism to protect the plant from oxidative stress. Even though oxidative stress tolerance and longevity in Arabidopsis are tightly correlated (Kurepa et al. 1998, Woo et al., 2004) and hydrogen peroxide is discussed as signalling molecule to induce leaf senescence in Arabidopsis (Navabpour et al., 2003; Miao et al. 2004; Zimmermann et al., 2006), minimizing hydrogen peroxide production in the mitochondria by long-term antimycin A treatment did not delay senescence. In contrast, if down-regulation of *CAT2* expression and activity is abolished in *gbf1* mutants, the onset of senescence is delayed. On the other hand, if *CAT2* gene expression is prevented from early on in development in *cat2* T-DNA insertion lines, also no effect on senescence could be observed and hydrogen peroxide contents are not significantly altered. Therefore, we can assume that the intracellular origin but also the developmental time point of the hydrogen peroxide production might have an impact on its signalling function. In addition, the loss of one detoxifying system can be compensated by the cells and there seems to be a very sensitive balance between the different antioxidative protection systems. Remarkably, hydrogen peroxide plays a role in many different signal transduction pathways but how specificity is mediated is still an open question. Compartment-specific hydrogen peroxide fluorescent sensor molecules like roGFP or Hyper will help to clarify whether the intracellular origin of the hydrogen peroxide and changes during specific developmental time points might be important for its signalling function.

4. Experimental procedures

4.1 Plant material

Seeds from *Arabidopsis thaliana,* ecotype Columbia, were grown in a climatic chamber at 22°C under 16 h of illumination under low light conditions (60 µmol $s^{-1}m^{-2}$). Under these conditions plants developed flowers within 7 weeks, mature seeds could be harvested after 12 weeks. For long term treatment, plants were watered every second day with 5 ml of 40 µM antimycin A or 0.8 % ethanol as a control in addition to normal watering.

Suspension cells of *Arabidopsis thaliana*, ecotype Landsberg erecta, were grown under constant light on a rotary shaker (120 rpm) at 20°C and were subcultured every 7 days by 30-fold dilution in fresh growth medium (100 ml culture in 250 ml flasks). The Murashige and Skoog growth medium contains 3 % (w/v) sucrose, 0.5 mg/l α-naphthaleneacetic acid and 0.05 mg/l kinetin; pH was adjusted to 5.8 with KOH. Cell cultures with a density of about 100 mg/ml medium were treated with 5 µM antimycin A (Sigma) or 5 mM hydrogen peroxide.

The full length cDNA of AOX1a (At3g22370) was amplified by PCR form reverse transcripted poly A^+ RNA isolated from mature leaf material. The cDNA was cloned into the vector PY01 adjacent to a CaMV35S promoter. The construct was verified by sequencing. Arabidopsis transformation was performed by the vacuum infiltration procedure (Bechthold & Pelletier, 1998). The seeds of the transgenic plants were selected by spraying with 0.1% Basta. *WRKY53* overexpressing plants were constructed as described before (Miao et al., 2004)

T-DNA insertion lines in *CAT2* (SALK_057998) and *CAT3* (SALK_092911) were obtained from the Nottingham Arabidopsis Stock Centre (NASC). Homozygous lines were characterized by PCR using gene specific and T-DNA left border primers (LBb1 5'GCGTGG ACC GCT TGC TGC AAC T 3'; CAT2-LP2 5' TCG CAT GAC TGT GGT TGG TTC 3'; CAT2-RP2 5' ACC ACC AAC TCT GGT GCT CCT 3'; CAT3-LP 5' CAC CTG AGT AAT CAA ATC TAC ACG 3'; CAT3-RP 5' TCA GGG ATC CTC TCT CTG GTG AA 3'). Homozygous plants were crossed and homozygous double knock-out lines were selected by PCR screening using the same primers. Knock-out was verified by native PAGE and subsequent CAT activity staining. Since *CAT2* and *CAT3* are under circadian regulation, leaves were always harvested 3 h after the beginning of illumination. Leaves were pooled in all experiments.

4.2 RNA isolation and Northern and dot blot analyses

Total RNA was isolated from leaves according to the protocol of PURESCRIPT RNA isolation kit (Gentra). Total RNA was either denatured 15 min at 55°C and spotted on nylon membranes or separated on MOPS-formaldehyde (6.2 %) agarose gels (1.5 %) and transferred to nylon membranes using 10 x SSC as transfer buffer. The membranes were hybridized at 65°C, washed twice at room temperature for 20 min with 2 x SSPE, 0.1 % SDS and once at 65°C for 30 min with 0.2 x SSPE, 0.1 % SDS. A fragment of the 5′ UTR of the *AOX1a* gene or of the 3´UTR of the *SAG12* gene (At5g45890) was used as radioactive labeled hybridization probe.

4.3 Measurement and detection of hydrogen peroxide

Hydrogen peroxide was measured according to the method described by Kuźniak and others (1999). Ten leaf discs (diameter 1 cm) or pelleted suspension cells (approx. 100 mg)

were incubated for 2 h in 2 ml reagent mixture containing 50 mM potassium phosphate buffer pH 7.0, 0.05 % guaiacol (Sigma) and horseradish peroxidase (2.5 u/ml, Serva) at room temperature in the dark. Four moles of hydrogen peroxide are required to form 1 mole of tetraguaiacol, which has an extinction coefficient of $\varepsilon = 26.6\ cm^{-1}mM^{-1}$ at 470 nm. The absorbance in the reaction mixture was measured immediately at 470 nm.

4.4 Chlorophyll and total protein content

Leaf discs were homogenized in 0.2 ml 25 mM potassium phosphate buffer, pH 7.0, containing 2 mM EDTA. Subsequently, 0.8 ml acetone was added, and the samples were shaken vigorously for 1 h at room temperature. After centrifugation at 14000 g for 30 min at room temperature, the total chlorophyll content of the supernatant was measured and calculated following the method described by Arnon (1949). To determine total protein content, leaf discs were ground in 100 mM potassium phosphate buffer, pH 7.0, containing 1 mM EDTA at 4°C. After centrifugation at 14000 g for 30 min at 4°C, the supernatant was directly used for protein quantification according to the method of Bradford (1976) using BSA as standard.

4.5 CAT and APX acitivities

For analyses of APX isozymes, crude protein extracts were separated on 10% native polyacrylamide gels (0.375 M Tris-HCl, pH 8.8, as gel buffer) with a 5% stacking gel (0.125 M Tris-HCl, pH 6.8, as gel buffer) for 16 h (120V) at 4°C using 2 mM ascorbate, 250 mM glycine, and 25 mM Tris-HCl, pH 8.3, as electrophoresis buffer. After electrophoresis, the gels were soaked in 50 mM potassium phosphate buffer, pH 7.0, containing 2 mM ascorbate for 10 min (3x) and, subsequently, in 50 mM potassium phosphate buffer, pH 7.0, containing 4 mM ascorbate, and 1 mM H_2O_2 for 20 min. After rinsing in water, the gels were stained in 50 mM potassium phosphate buffer, pH 7.8, containing 14 mM TEMED (*N,N,N´,N´*-tetramethylethylenediamine) and 2.45 mM NBT (nitro blue tetrazolium) for 10-30 min. For the analyses of CAT isozymes the protein extracts were separated on 7.5% native polyacrylamide gels (0.375 M Tris-HCl, pH 8.8, as gel buffer) with a 3.5% stacking gel (0.125 M Tris-HCl, pH 6.8, as gel buffer) for 16 h (70-80V) at 18°C using 250 mM glycine and 25 mM Tris-HCl, pH 8.3, as electrophoresis buffer. Subsequently, the gels were stained for the activity of catalases as follows: The gels were soaked in 0.01% of hydrogen peroxide solution for 5 min, washed twice in water and incubated for 5 min in 1% $FeCl_3$ and 1% $K_3[Fe(CN)_6]$. After staining, the gels were washed once more in water.

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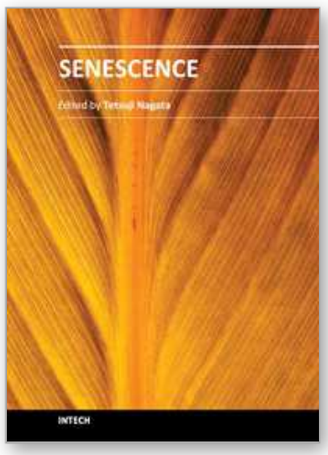

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The book "Senescence" is aimed to describe all the phenomena related to aging and senescence of all forms of life on Earth, i.e. plants, animals and the human beings. The book contains 36 carefully reviewed chapters written by different authors, aiming to describe the aging and senescent changes of living creatures, i.e. plants and animals.

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