Chapter 3

Early leaf senescence of old13 is partially dependent on salicylic acid and associated with increased oxidative stress and altered water balance

Jos H.M. Schippers¹, Renate Ellens¹, Jacques Hille¹ and Paul P. Dijkwel¹

¹Molecular Biology of Plants, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN, Haren, The Netherlands
Abstract
Programmed cell death (PCD) manifests itself during the final stage of leaf development by leaf senescence. However, leaf senescence can occur prematurely as response to environmental stress and pathogen attack. The highly coordinated regulation of leaf senescence can increase plant fitness and survival. Here we report that the onset of leaf death 13 mutant displays enhanced ethylene-induced senescence and lesions formation in the absence of pathogen, suggesting that OLD13 is involved in stress response signaling and cell death regulation. The mutant has increased oxidative stress as determined by NBT staining and expression of ROS markers. Double mutant analysis reveals that the lesion formation requires salicylic acid. The ethylene-induced senescence phenotype was suppressed by a mutation in EIN2 but not by mutations in PAD4 and ABI4. The mutant has an increased anion content suggesting an altered water balance. Taken together we suggest that OLD13 functions as a modulator of leaf senescence upon environmental stress such as drought, salinity and pathogen attack.

Introduction
In the early sixties of the last century interest in the active onset of leaf senescence was emerging as depicted by the following citation: The signal for mass hari-kari, so to speak, for all members of these enormous populations is such a fantastically dramatic physiological event that it seems most singular that plant physiologists have not given more attention to the matter (Leopold, 1961). It is argued that senescence is beneficial for plant fitness and survival and suggests the existence of a mobilizing force that actively drives the senescence process. Since that time plant programmed cell death (PCD) has been implicated in the recycling of nutrients by senescence, the development of the plant body, self-elimination of cells invaded by pathogens and as a response to (a)-biotic stress (Jones, 2001). PCD is not a passive process resulting in death but requires the active regulation of thousands of genes which prepare the end phase of a cell. PCD in leaf senescence has some unique features when compared to other PCD processes. Leaf senescence occurs at the whole organ or whole plant level while other PCD processes occur highly localized (Pennel and Lamb, 1997). Next to that senescence is a slow progressing PCD especially compared to the hypersensitive-response (HR) to pathogen attack (Lamb and Dixon, 1997). The orderly dismantling of a leaf results
in efficient remobilization of nutrients to maintain development of other parts of the plant (Himelbau and Amasino, 2001). In *Arabidopsis* the execution of the senescence program is depending on the developmental age of the leaf (Bleecker, 1998; Jing et al., 2003). However, leaf senescence is also influenced by diverse internal and environmental signals that are integrated with the age to determine the onset (Lim et al., 2003). This integrated senescence response allows plants to obtain optimal fitness by incorporating the environmental and endogenous status of plants in a given ecological setting by fine-tuning the initiation timing, progression rate, and nature of leaf senescence (Lim et al., 2007). Unfavorable environmental factors like drought (Munné-Bosch and Alegre, 2004), nutrient limitation (Wingler et al., 2006), temperature (Page et al., 2001), oxidative stress by UV or ozone (Miller et al., 1999), darkness (Fujiki et al., 2001) and pathogen attack (Tang et al., 2005) can prematurely induce the senescence program.

The response of plants to environmental factors involves the transcriptional activation or repression of genes (Zhu, 2002). Several studies on improving stress resistance have revealed cross-talk and overlap between the different stress pathways and their activation. Overexpression of the dehydration responsive element binding (DREB) 1A results in plants more tolerant to drought, salt and cold stress but at the expense of growth and productivity (Kasuga et al., 1999). However, expression of DREB1A under the control of a stress-inducible promoter improves stress tolerance without affecting plant health and thus demonstrating the need for a targeted response. In another example, overexpression of heat shock transcription factor (Hsf) A2 confers higher tolerance to temperature, salt and osmotic stress but also results in growth retardation (Ogawa et al., 2007). Taken together, stress tolerance can delay the timing of senescence induced by stress and is thus an important factor in controlling onset of senescence. Interestingly, stress responses and senescence share overlapping signaling pathways as more than two-thirds of the transcription factors induced during senescence are also induced after various stress treatments (Chen et al. 2002).

The integration of age and environmental signals involves, next to transcriptional factors, plant hormones which act as endogenous factors controlling growth and development including leaf senescence (Schippers et al., 2007). Ethylene, jasmonic acid (JA) and salicylic acid (SA) have all been implicated in the onset of senescence (Jing et al., 2002; Buchanan-Wollaston et al., 2005; Miao et al., 2007) and responses
to various stresses (Chen et al., 2002). The ENHANCED DISEASE RESISTANCE 1 (edr1) mutant shows enhanced resistance to pathogens, early ethylene-induced senescence and spontaneous lesion formation during drought stress. EDR1 functions both in ethylene and SA signaling pathways and regulates senescence and cell death (Tang et al., 2005). Another mutant that regulates the cross-talk between several hormones is the onset of leaf death 1 (old1) which shows early leaf senescence, constitutive expression of pathogenesis related proteins and hypersensitivity to sugars (Jing et al., 2007). Thus the response to various stresses is regulated by stress signaling pathways that are interconnected at several levels by shared genetic factors between the pathways (Knight and Knight, 2001). One common class of signaling molecules are the reactive oxygen species (ROS) which have been implicated in the regulation of development and stress response pathways (Pitzschke et al., 2006). Among the different ROS species only hydrogen peroxide can cross membranes and therefore function as a cell to cell signaling molecule. Hydrogen peroxide is involved in many plant processes including ABA-dependent stomatal closure (McAinsh et al., 1996), signaling via the ETR1 receptor (Desikan et al., 2005) and oxidative burst during pathogen attack and subsequent induction of systemic immunity (Alvarez et al., 1998). While during plant defense accumulation of ROS is required, ROS is scavenged during abiotic stress to counteract the accumulation (Pitzschke et al., 2006). The response of the plant to ROS is determined by the location of the signal in the cell where they are produced or accumulate. ROS have also been implicated in the onset of leaf senescence as demonstrated by treatment of Arabidopsis leaves with the herbicide 3-AT which inhibits catalase activity and causes H$_2$O$_2$ stress resulting in the expression of SAG genes (Navapbour et al., 2003). Next to that the senescence-specific transcription factor WRKY53 is induced by H$_2$O$_2$ (Miao et al., 2004; Miao and Zentgraf, 2007). Taken together the integration of signals from the environment with the age of the leaf involves a complex signaling network of overlapping and interacting pathways which together determine the onset of leaf senescence.

To gain insight into the pathways and mechanisms underlying leaf senescence the previously identified class II early leaf senescence mutant old13 (Chapter 2) was characterized in detail. Here we present evidence for the involvement of OLD13 in regulating stress induced senescence and lesion formation.
Results

Characterization of old13 mutant under standard conditions

Previously, we reported that the old13 mutant is phenotypically normal when grown under optimal conditions but displays early senescence upon ethylene treatment (Jing et al., 2005). To characterize the old13-mediated senescence response we examined plants grown under standard conditions and after stress treatment in detail. The development of the first leaf pair of old13 was followed and compared to wild type from day 21 till day 33. old13 plants are identical in size when compared to wild type, however, the initiation of the primary inflorescence starts 2-4 days earlier than the wild type (data not shown). A primary marker for early senescence is leaf yellowing caused by the loss of chlorophyll (Oh et al., 1997). From day 21 till day 33 no chlorophyll degradation and visual senescence is observed for the first leaf pair of both air-grown wild type and mutant old13 (Figure 1A and B). Next to that the photochemical efficiency of photosystem II stays at a similar level as wild type during development (Figure 1C). Thus old13 is a typical class II mutant that has no early senescence symptoms when grown under optimal conditions (Jing et al., 2002; Jing et al., 2005).

Figure 1. Characteristics of air-grown old13 mutants. (A) The chlorophyll contents of soil-grown old13 (open circles) and wild type (closed circles) were quantified every 3 days from day 21 till day 33. (B) Representative first leaves of old13 and wild type were selected and photographed at day 24. (C) The photochemical efficiency was determined as described in methods and is expressed as Fv/Fm. Error bars indicate SD; results are mean of 3 replicates.
A backcross of the *old13* mutant revealed that it segregates as a recessive trait both in the original mutant accession Landsberg (*Ler*) and the mapping background Columbia (*Col*). 1800 F$_2$ plants of the mapping population were phenotypically selected for positional cloning. The position of the mutated locus was narrowed down to a region on chromosome V, covering 101kb of sequence on BAC K19B1 and MRG21. The mapped region contains 32 annotated candidate genes which are listed in Table 1.

**Table 1.** Genes annotated for the mapped region that contains the *old13* mutation. The 32 genes listed are shown with a putative function and description according to TAIR annotation. Asterisks indicate that gene has been cloned and sequenced but no mutation was detected.

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Ethylene and detachment induce early leaf senescence in old13

In Arabidopsis leaves, diverse internal and environmental signals are integrated with the age to modulate the onset of leaf senescence (Grbic and Bleecker, 1995; Jing et al., 2003; Lim et al., 2003). Ethylene, detachment and drought are known to induce premature senescence (Lim et al., 2007). old13 plants were grown under standard growth conditions for 3 weeks and subsequently treated with ethylene or subjected to a detachment experiment, to follow the progression of leaf senescence. Mutant leaves show reduced chlorophyll levels when compared to wild type after 3 days of ethylene treatment (Figure 2A). Noteworthy, ethylene treatment results in a more pronounced early flowering phenotype in the mutant, as compared to growth under standard conditions (results not shown). For the detachment experiment the first leaf pair was followed for 12 days during incubation in the dark (Figure 2B).

Figure 2. The senescence syndrome of old13 leaves. (A) The chlorophyll contents of old13 (open circles) and wild type (closed circles) were quantified during ethylene treatment of 3 days. Content is shown in mean percentage ±SD of total content at day 21. (B) Detachment induced senescence of the wild type and old mutants was photographed after 9 days of treatment. WT, wild type; 5, old5; 9, old9; 11, old11; 13, old13; 14, old14. (C) Chlorophyll content of old13 (open circles) and wild type (closed circles) during the detachment experiment are represented as the mean± SD of three replicates of 6 leaves. (D) Nitrogen, carbon and sulfur content at 21 and 24 days of growth in air and after 3 days of ethylene treatment for wild type (black bars) and old13 leaves (gray bars). Content is represented as mean percentage ±SD of total content at day 21.
Detachment of *old13* leaves results in a slightly enhanced breakdown of chlorophyll when compared to the wild type (Figure 2C). As a control we tested the effect of detachment on leaves of other *old* mutants and demonstrate that the class II mutants *old9* and *old11* appear wild type while the class I mutants *old5* and *old14* senesce early upon detachment and more severe than *old13* (Figure 2B). We observed that *old13* plants are more sensitive than wild-type plants to limited water availability, resulting in early leaf senescence (data not shown). Taken together these results suggest that early senescence in *old13* plants can be induced by different stress signals.

We examined the ethylene-induced leaf senescence in more detail by examining nutrient remobilization and expression of senescence-associated genes (SAGs). A characteristic feature of leaf senescence is the relocalization of nutrients to other parts of the plant (Himelbau and Amasino, 2001). Remobilization starts with the transport of stored compounds, once these reserves have been drained, proteins and polymers will start to be catabolized (Thomas et al., 2002). Up to 95% of the stored nitrogen in leaves is relocated during senescence which mainly arises from the degradation of the chloroplast (Hortensteiner and Feller, 2002). By determining the elemental composition of dried leaves we estimated the relocalization of nitrogen, carbon and sulfur. Interestingly, both the wild type and the mutant show a strong decline in nitrogen (Figure 2D). The significant decrease in the mutant when compared to the wild type correlates with the start of chlorophyll degradation after 3 days of ethylene. The level of carbon remains at a similar level while sulfur declines both during air-growth as during ethylene treatment. Thus ethylene treatment induces remobilization of nitrogen containing compounds in wild type and *old13*, although significantly more in the mutant. Analyses of the expression profiles of senescence-associated genes after 1 day of ethylene treatment showed that *old13* mutants exhibit higher transcription levels of SAG12, SAG13, and SAG21 in comparison with wild-type plants (Figure 3A). Next to that the expression of CAB was approximately 5-fold reduced after ethylene treatment. This result, the increased expression of SAG12 and SAG13 (Weaver et al., 1998) demonstrates that *old13* enhances the ethylene-dependent senescence process not only on physiological level but also at the molecular level.
Figure 3. SAGs, Hypersensitive response-like lesions, and oxidative stress. (A) Marker transcripts levels after ethylene treatment. SAG12, SAG13, SAG21 and CAB transcript levels after 1 day of ethylene treatment in the wild type (black bars) and old13 (gray bars). The relative expression shown is the mean of three repeats ± SD (indicated by error bars). (B) Tryphan blue stain of ethylene treated leaves of old13 and a representative photograph of a first leaf after 3 days of ethylene (arrow indicates lesions). (C) Staining for superoxide by NBT of 24 day old leaves. (D) Marker transcript levels during development. DEFL, PAD3 and PR2 relative transcript abundance after 21 day of growth on soil for the wild type (black bars) and old13 (gray bars).

Ethylene and drought induce hypersensitive response-like lesions in old13 which are associated with oxidative stress

In addition to early leaf senescence, old13 plants also developed spontaneous yellow-brown necrotic lesions under drought conditions or after ethylene treatment (Figure 3A). The necrotic spots resemble phenotypically the hypersensitive-response (HR) which is commonly observed during pathogen attack (Mur et al., 2007). Staining of ethylene treated mutant leaves with tryphan blue reveals the appearance
of randomly localized dead cells after two days of ethylene treatment (Figure 3B). Interestingly, staining of air-grown old13 plants with NBT for superoxide reveals that the mutant has an increased level of cellular ROS during unchallenged conditions (Figure 3C). Moreover, old13 plants have a 6-fold increased expression of a defensin-like (DEFL) gene which is expressed upon 7 different ROS-inducing agents (Gadjev et al., 2006) (Figure 3D). Thus old13 plants have increased oxidative stress when compared to the wild type and develop spontaneous lesions during ethylene treatment and drought stress.

old13 functions both in biotic and a-biotic plant response pathways

During the hypersensitive response, an oxidative burst through rapid accumulation of ROS is essential for the local cell death and the activation of a signaling cascade to induce systemic acquired resistance (Alvarez et al., 1998). Next to that a-biotic factors that cause salt, osmotic and drought stress all result in increased oxidative stress (Knight and Knight, 2001). We found that a salicylic acid dependent defense gene, PAD3 (Glazebrook and Ausebel, 1994) and the PR2 gene which is related to pathogenesis but also leaf senescence (Gaffney et al., 1993) are 5 to 9 fold increased in expression in the mutant when compared to the wild type (Figure 3D).

Figure 4. Anion content of wild type and old13 leaves during development. Chloride, nitrate and sulfate content were measured every 6 days starting from day 21 until day 33. Wild type is represented as black bars and mutant as gray bars. Content is represented as content ±SD of mean.

We noticed that the timing of watering before, at the start or during the ethylene treatment could influence the senescence response of old13. Therefore we anticipated that the mutant might have a defect in its water balance. To test this we determined the anion concentration during the development of the first leaf pair. Anions play a role both in the regulation of the membrane potential and in the
osmolarity of the cell (Tyerman, 1992). The determination of the anion content by HPLC revealed that the mutant exhibited elevated levels of Cl\(^-\) especially at young age when the levels are 6-fold increased (Figure 4). Next to that the concentration of nitrate is elevated at 21 days but decreases below wild type levels at 33 days. Sulfate levels are at day 21 and 27 two-fold increased in comparison to the wild type but restore to wild type levels at day 33. Taken together, the results suggest that old13 mutant plants grow under constitutive water stress.

**HR-like lesions and senescence in old13 are dependent on intact hormone signaling pathways**

We showed before that the early induction of senescence in old13 is not a consequence of increased sensitivity to ethylene (Chapter 2). To test the role of hormone signaling pathways in adult plants we constructed double mutants between

![Images of plants showing senescence phenotypes](A) Senescence phenotypes of old13 double mutants. (A) Plants were grown in air until 21 days and subsequently treated with ethylene for 3 days after which representative plants were selected and photographed. Double mutant abbreviations: o13a4, old13/abi4-1; o13e2, old13/ein2-1; o13p4, old13/pad4-1. Bars indicate 5 mm. (B) Leaf yellowing after

![Bar graphs showing leaf yellowing and leaves with lesions](B and C)
ethylene treatment, yellowing is presented as the mean ± SD of 30 plants. (C) Number of leaves with lesions after ethylene treatment as mean ± SD of 30 plants.

old13 and mutants impaired in the sensing and/or signaling of ethylene, salicylic acid and abscisic acid. The double mutants were selected by PCR based identification of the mutant alleles. Subsequently the double mutants were grown till 21 days in air and treated for 3 days with ethylene (Figure 5A). The abi4-1 mutation (Finkelstein et al., 1998) did not affect the old13 senescence symptoms or appearance of lesions after ethylene treatment (Figure 5B and C). Crossing the old13 mutation with ethylene insensitive mutant ein2-1 (Guzman and Ecker, 1990) results in absence of leaf senescence and lesions during ethylene treatment. Interestingly, a cross between old13 and pad4-1 (Jirage et al., 1999) results in the loss of the lesion phenotype, while the effect on leaf yellowing was limited.

These data suggest that the old13-mediated ethylene-induced senescence phenotype is independent of SA signaling, while the lesion phenotype is dependent on the salicylic acid pathway.

Discussion

Senescence is a developmental event that results in the death of a cell, an organ, or an organism upon aging. In plants senescence has evolved as a beneficial trait which increases the survival of the species (Bleecker, 1998).

While the onset of leaf senescence in old13 mutant is similar to wild type under optimal growth conditions, we observed that old13 mutants senesce early and display spontaneous lesions formation after ethylene treatment or drought stress. This suggests that old13 mutants are more sensitive to environmental conditions than wild type and that OLD13 might be involved in stress-induced growth, senescence and cell death.

One effect of the old13 mutation is the increased anion content, which might suggest a defect in water balance. Most strongly increased is the level of chloride, which is 6-fold higher at day 21. An increase in chloride content has been observed before during N limitation experiments in which chloride replaces nitrate as an osmoticum (White and Broadley, 2001). However, we measured an increased availability of nitrate in leaves of old13 which argues against the possibility that nitrogen deficiency plays a role in the mutant. Drought and salt stress have been shown before to induce senescence. In rice it was shown that NaCl increases the rate of developmental...
senescence (Lutts et al., 1996) while in Arabidopsis several SAG genes including ERD1, SAG13, SAG14, SAG21 respond to drought-induced senescence (Weaver et al., 1998). High saline environments cause cytosolic accumulation of calcium which is a potent signal for stress responses that can result in either adaptation or death (Hasegawa et al., 2000). Recently a calcium-dependent protein kinase has been characterized which plays a role in salt and drought tolerance (Ma and Wu, 2007). One response to accumulation of Cl⁻ is restoration of the osmotic balance by the accumulation of a non-harmful compatible solute like sucrose, fructose, glycerol, trehalose or proline (Hasegawa et al., 2000). Levels of ROS increase rapidly during salt stress and can result into an oxidative burst (Apel and Hirt, 2004). Tryphan blue staining of old13 together with the increased expression of a defensin-like gene indicates increased ROS levels in the mutant. Next to that it was shown for Arabidopsis that increased H₂O₂ levels promote reproductive growth (Zimmermann et al., 2006), which might explain the early flowering of old13. A possible explanation for the increased ROS is that during water deficit photorespiration is inhibited due to a reduced availability of CO₂ which results in excess excitation energy and subsequent increased oxidative stress (Smirnoff, 1993). Interestingly, the assimilation of nitrate depends on the rate of photorespiration (Rachmilevitch et al., 2004). This observation which might explain the measured increase in nitrate, although it was only observed for young leaves. Increased ROS production causes lipid peroxidation and can affect the integrity of the membrane resulting in permeability to electrolytes and finally changes in the cellular ionic homeostasis (Kourie, 1998). ROS scavenging is an important factor in adapting to salt stress as shown by ascorbate-deficient Arabidopsis plants which have an increased sensitivity to salt-stress (Huang et al., 2005). The photoautotrophic salt tolerate 1 (pst1) mutant has increased superoxide dismutase (SOD) and ascorbate peroxidase (APX) activity resulting in increased salt and drought tolerance (Tsugane et al., 1999). Thus, the rapid detoxification of ROS during salt or drought stress is an important factor in determining tolerance. Detoxification of ROS can also be achieved by increasing the level of non-enzymatic antioxidant compounds. Synthesis of the sulfur-containing anti-oxidant glutathione (GSH) is upregulated during oxidative stress (Noctor and Foyer, 1998). Ozone is a potent inducer of oxidative stress which results in accumulation of glutathione and up-regulation of sulfur assimilation (Bick et al. 2001). Therefore the increased sulfate content in old13 might be the direct consequence of oxidative stress. However, the
observed changes in anion content can be explained as a general change in ion-transport (Frachisse et al., 1999) and thus may not be related to salt stress. Excess nitrate and chloride are stored by the same mechanism in the vacuole and therefore the *OLD13* gene product might function directly in ion transport (Harada et al., 2004). Although the mechanism might relate to storage or transport of anions, in *old13* the increased anion content might cause the drought sensitive phenotype. Next to increased cellular ROS and anion concentration the *old13* mutant also displays lesion formation. The physiology of *old13* resembles that of the *edr1* mutation which results in early ethylene-induced senescence, spontaneous lesion formation during drought stress and enhanced resistance to pathogens. Although, pathogen response of *old13* has not been tested we did find that the pathogenesis-related transcripts of PR2 and PAD3 accumulate in the mutant. EDR1 encodes for a MAPKKK suggesting that different stress factors might initiate the same signaling cascade (Tang and Innes, 2005). By double mutant analysis it was shown that the drought-induced phenotype of *edr1* is depending on SA while the senescence phenotype requires ethylene signaling. Our double mutant analysis shows that lesion formation in *old13* is also dependent on SA-signaling pathway while *ein2* blocks the senescence phenotype. In contrast to the role of PAD4 during developmental senescence in which it modulates the switch to PCD during the final phase (Morris et al., 2000) it is necessary for the onset of senescence during pathogen attack (Pegadaraju et al., 2005). This suggests that only a part of the *old13* senescence response is related to defense since the lesion and senescence phenotype are regulated by different pathways. Interestingly crossing the late senescence mutant *ore9* with *edr1* results in suppression of ethylene and drought induced senescence but not plant defense responses (Tang et al., 2005), confirming that the different phenotypes can be separated in the *edr1* mutant as well and are the result of different signaling pathways. Previously, the early leaf senescence mutant *old1* has been implicated as an integration node of different stimuli including pathogen response, ethylene-induced senescence and sugar signaling (Jing et al., 2007). Taken together, our analysis suggests that OLD13 might represent another OLD protein that regulates plant development and senescence by monitoring the environment and endogenous status of plants and adjusts development accordingly.

The characterization of the *old13* mutation reveals a novel locus involved in modulating the developmental program of *Arabidopsis* during stress conditions. One
important concept is that during stress a plant has to decide if a certain organ can be sacrificed or not. Although most of the current research focuses on delayed senescence to improve productivity in several crops it is important to note that a rapid and localized senescence response is essential for stress tolerance and survival. Therefore, we would like to encourage future research that aims at senescence modulators that control plant fitness.

**Methods**

*Plant materials and growth conditions*

*Arabidopsis thaliana* ecotype *Landsberg erecta* (Ler-0) was used in this study. The *old9* mutant was obtained from an EMS mutagenized collection (Jing et al., 2005). Plants were grown on either soil or half-strength Murashige and Skoog medium at 23°C and 65% relative humidity with a day length of 16 h. The light intensity was set at 120 µmol·m⁻²·s⁻¹. An organic-rich γ-ray radiated soil was used (Hortimea Groep, Elst, The Netherlands). Plants for ethylene exposure were treated in a flow-through chamber at 20 °C and a humidity of 40% under continuous illumination. The ethylene dosage was set at 10 µl l⁻¹ as suggested by Chen and Bleecker (1995). Cotyledons or rosette leaves with over 5% yellow area of the leaf blade were judged as yellow as suggested by Lohman et al. (1994).

The mutant alleles and transgenic plants used for the double mutants were *ein2-1* (Guzman and Ecker, 1990), *pad4-1* (Jirage et al., 1999), and *abi4-1* (Finkelstein et al., 1998). The *old13* mutation was crossed into the mutant lines that have a Col-0 background, thus the *F₂* double mutant population contains a mixed Ler-0 and Col-0 background.

*NBT staining*

The first leaf pair was harvested and placed in an aqueous solution of 0.05% nitroblue tetrazolium; blue color indicated superoxide (O₂⁻) generation (Flohe and Otting, 1984). After 2 hours leaves were fixed and decolorized in 96% ethanol overnight. Decolorized and cleared leaves were mounted in saturated chloral hydrate and analyzed under a light microscope. Subsequently, photographs were taken of representative leaves.
**Tryphan blue staining**

Appearance of cell death was studied in whole leaf mounts stained with lactophenol-tryphan blue (10 mL of lactic acid, 10 mL of glycerol, 10 g of phenol, 10 mg of tryphan blue, dissolved in 10 mL of distilled water) (based on Keogh et al., 1980). Whole leaves were boiled for 1 min in the staining solution and then overnight decolorized in chloral hydrate. Subsequently the leaves were mounted in chloral hydrate and viewed under a light microscope.

**Pigment determination and measurement of photochemical efficiency**

For extraction of chlorophyll and carotenoids samples were incubated overnight with N,N-dimethylformamide at 4°C in darkness. The chlorophyll content was quantified spectrophotometrically according to Wellburn (1994) at 647 and 664 nm.

Chlorophyll fluorescence emission was measured from the upper surface of the first leaf, at room temperature (23 °C) with a pulse-amplitude modulation portable fluorometer (PAM-2000; H. Walz, Effeltrich, Germany) according to Maxwell and Johnson, 2000. Plants were dark-adapted for 1 to 2 hr before measurements to ensure complete relaxation of the thylakoid pH gradient. An attached, fully expanded rosette leaf was placed in the leaf clip, allowing air to circulate freely on both sides of the leaf. At the start of each experiment, the leaf was exposed to 2 min of far-red illumination (2 to 4 µmol photons m-2 s-1) for determination of Fo (minimum fluorescence in the dark-adapted state). Saturating pulses of white light (8000 µmol photons m-2 s-1) were applied to determine Fm or Fm' values. PSII efficiency was calculated as (Fm - Fo)/Fm.

**RNA-isolation and RT-PCR**

Total RNA was isolated using TRIZOL reagent (Sigma) according to the manufacturer's protocol. Five hundred nanograms of RNA were used as template for first-strand cDNA synthesis using 200U of RevertAid H-minus MMuLV reverse transcriptase (Fermentas, USA) and an oligo(dT21) primer. Primer pairs for real-time PCR were designed with open-source PCR primer design program PerlPrimer v1.1.10 (Marshall, 2004). The primer sequences are available upon request. Briefly, real-time PCR amplification was performed with 50 µL of reaction solution, containing 2 µL of 10-fold–diluted cDNA, 0.5 µl of a 10 mM stock of each primer, 1 µl of 25mM stock MgCl2 (Fermentas), 5 µl PCR buffer +Mg (Roche), 1 µl of a 1000x diluted
SYBR-green stock (Sigma), 0.5 µl 100xBSA (New England Biolabs), and 1u of Roche Taq Polymerase. The PCR program was 2’ at 94, 40x (94-10”/60-10”/72-25”). Obtained data was analyzed with BioRad software.

*CNS measurements*

The first leaf pair of wild type and mutant was collected after treatment and oven dried at 80°C. Subsequently the tissue was weighted and grinded to a fine powder. For determination of the C, N, and S content we made use of a LECO CHNS 932 (USA) analyzer by combustion at 1200°C.

*Detachment-induced senescence*

Leaves were incubated in light on two layers of Whatman filter papers saturated with MES solution (pH 5.7) and collected after 0, 3, 6, 9 and 12 days for chlorophyll content measurement. Three replicates of 6 pairs of leaves were analyzed for each data point.

*Intracellular anion content*

For chloride, nitrate and sulfate analysis, fresh first leaf pairs were harvested and homogenized in demineralized water, with an Ultra Turrax (T25 Basic Ika Labortechnik, Staufen, Germany). The homogenate was incubated at 100 °C for 10 min, filtered and centrifuged at 30 000 g for 15 min. Chloride, nitrate and sulfate were separated by HPLC on an IonoSpher A anion exchange column (Varian/Chrompack Benelux, Bergen op Zoom, The Netherlands) and determined refractrometrically according to Buchner et al. (2004).

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References


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