Differential Impact of Lipoxygenase 2 and Jasmonates on Natural and Stress-Induced Senescence in Arabidopsis1[W]

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Jasmonic acid and related oxylipins are controversially discussed to be involved in regulating the initiation and progression of leaf senescence. To this end, we analyzed profiles of free and esterified oxylipins during natural senescence and upon induction of senescence-like phenotypes by dark treatment and flotation on sorbitol in Arabidopsis (Arabidopsis thaliana). Jasmonic acid and free 12-oxo-phytodienoic acid increased during all three processes, with the strongest increase of jasmonic acid after dark treatment. Arabidopside content only increased considerably in response to sorbitol treatment. Mono- and digalactosyldiacylglycerols decreased during these treatments and aging. Lipoxygenase 2-RNA interference (RNAi) plants were generated, which constitutively produce jasmonic acid and 12-oxo-phytodienoic acid but do not exhibit accumulation during natural senescence or upon stress treatment. Chlorophyll loss during aging and upon dark incubation was not altered, suggesting that these oxylipins are not involved in these processes. In contrast, lipoxygenase 2-RNAi lines and the allene oxid synthase-deficient mutant dde2 were less sensitive to sorbitol than the wild type, indicating that oxylipins contribute to the response to sorbitol stress.

Senescence is an important, highly regulated process at the end of development. Senescence is characterized by breakdown of organelles and molecules, export and transport of these nutrients to other organs/parts of the organism, and finally programmed cell death of the senescing organ.

The process of senescence has been intensively studied in leaves, and morphological as well as molecular changes in senescing leaves have been described. Yellowing as a consequence of chlorophyll and chloroplast degradation is the most obvious process during natural leaf senescence. In addition, gene expression changes dramatically during senescence. Some senescence-associated genes (SAG, SEN) have been reported that are induced during this process, and several of the encoded proteins function in macromolecule degradation, detoxification and defense metabolism, or signal transduction (Gepstein et al., 2003). Based on the degradation of chloroplasts and macromolecules, leaf metabolism changes from carbon assimilation to catabolism (Lim et al., 2007).

The initiation and progression of senescence is regulated by endogenous as well as exogenous factors.

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Among the endogenous factors, the developmental status of the organ and of the whole plant (e.g. age and progress in flowering and seed production) has a great impact on the process of senescence. Different stress factors such as pathogen attack, drought, osmotic stress, heat, cold, ozone, UV light, and shading can induce or accelerate senescence (Quirino et al., 2000). Phytohormones are very important regulators that integrate information about the developmental status and the environmental factors. Cytokinins are antagonistic signals and delay senescence. Endogenous levels of cytokinins decrease during senescence, and exogenous application and transgenic approaches, enhancing endogenous levels of these compounds, lead to delayed senescence (Gan and Amasino, 1995). There are also several indications that abscisic acid modulates senescence (van der Graaff et al., 2006). In contrast, the gaseous phytohormone ethylene is known to induce and accelerate senescence (John et al., 1995). There are also several indications that abscisic acid modulates senescence (van der Graaff et al., 2006).

The roles of other phytohormones/signaling compounds such as auxin, salicylic acid, and jasmonates are less clear (Lim et al., 2007). Jasmonates are oxylipin signaling molecules derived from linolenic acid. The term jasmonates comprises 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), and derivatives such as the methyl ester and amino acid conjugates of JA. One of the first biological activities described for these compounds was the promotion of senescence in oat (Avena sativa) leaves by methyl jasmonate (MeJa) isolated from Artemisia absinthium (Ueda and Kato, 1980). Later on, the induction of senescence-like phenotypes by exogenous application of MeJa was also found in other plant
Function of Oxylipins in Senescence

species (Ueda and Kato, 1980; Weidhase et al., 1987a; He et al., 2002). On the molecular level, this senescence-promoting effect of MeJA is accompanied by chlorophyll loss and decreases in Rubisco and photosynthesis (Weidhase et al., 1987a, 1987b). In addition, expression of some senescence-up-regulated genes is also responsive to JA; examples are SEN1, SEN4, SEN5, SAG12, SAG14, and SAG15 (Park et al., 1998; Schenk et al., 2000; He et al., 2002). Due to the results described above, jasmonates have been described for decades as compounds with senescence-promoting activities, while the function of these compounds in natural senescence in planta was critically discussed (Parthier, 1990; Sembdner and Parthier, 1993; Creelman and Mullet, 1997; Wasternack, 2007; Balbi and Devoto, 2008; Reinothe et al., 2009). Additional indications for a role of jasmonates in regulating senescence are the transient up-regulation of expression of some enzymes involved in JA biosynthesis, such as allene oxide synthase (AOS) and OPDA reductase 3 (OPR3), and the increase in JA levels during natural senescence (He et al., 2002). Furthermore, alterations in natural and induced senescence have been reported for some mutants with defects in the JA pathway. The mutant coi1, which is impaired in JA signaling, exhibited delayed chlorophyll loss upon dark incubation of detached leaves (Castillo and Leon, 2008). Plants with reduced expression of the 3-ketoacyl-CoA-thiolase KAT2, which is involved in β-oxidation and JA production, showed delayed yellowing during natural senescence and upon dark incubation of detached leaves (Castillo and Leon, 2008).

However, there are also several reports that cast doubt on an important function of JA in senescence. For most mutants in JA biosynthesis or signaling, no differences in natural senescence are apparent (He et al., 2002; Schommer et al., 2008). In addition, mutants defective in the expression of AOS or OPR3 do not show altered senescence-like phenotypes upon dark treatment (Schommer et al., 2008; Kunz et al., 2009). It has to be taken into consideration that the knockout in these mutants has pleiotropic effects during whole plant development. For example, the leaves of plants with reduced expression of the lipase DGL or of OPR3 are larger (Hyun et al., 2008). In addition, several knockout mutants defective in JA biosynthesis or signaling do not produce fertile flowers (Feys et al., 1994; McConn and Browse, 1996; Sanders et al., 2000; Stintzi and Browse, 2000; Ishiguro et al., 2001; von Malek et al., 2002). These changes in development might affect other developmental processes such as senescence.

To investigate the function of jasmonates in senescence in more detail, we compared the oxylipin profile of wild-type leaves during natural senescence and upon stress induction of senescence-like phenotypes. The analysis of lipoxygenase 2 (LOX2)-RNA interference (RNAi) plants, which produce low basal levels of oxylipins but are impaired in the accumulation of OPDA and JA during senescence or in response to stress, indicates that 13-LOX products are not necessary for natural senescence or dark-induced chlorophyll loss but are involved in the response to sorbitol.

RESULTS

Oxylipin Profiles during Natural and Stress-Induced Senescence

For the analysis of natural leaf senescence, plants were grown in short-day conditions, and leaves 9 to 15 were marked and harvested after 6, 8, and 10 weeks. Chlorophyll levels were determined to monitor the progression of senescence. The chlorophyll content of leaves of 6-week-old plants was similar to younger plants (data not shown). At 8 weeks and later time points, a steady decrease in chlorophyll content was observed, which reached 78% and 60% after 8 and 10 weeks, respectively, relative to the level of 6-week-old plants (Fig. 1A). Concurrently, expression of the senescence marker SAG12 increased more than 1,000-fold from 6 to 10 weeks (Supplemental Fig. S1A). These changes in chlorophyll content and SAG12 expression correlated with an accumulation of JA and free OPDA (Fig. 1B). Levels of JA and OPDA increased from 6 and 20 ng g⁻¹ fresh weight for JA and OPDA to 298 and 112 ng g⁻¹ fresh weight at 10 weeks, respectively. OPDA is not only present in Arabidopsis (Arabidopsis thaliana) as free OPDA but also esterified in galactolipids termed arabinosides (Stelmach et al., 2001). It has been reported that arabinoside A promotes senescence in Arabidopsis (Hisamatsu et al., 2006). Levels of all arabinosides only showed subtle changes during this time period (Fig. 1C). This result indicates that increases in the levels of JA and OPDA are not necessarily correlated with an increase in arabinoside levels. However, in other situations, such as wounding and expression of avirulence proteins, a correlation of JA, OPDA, and arabinoside levels was reported (Buseman et al., 2006; Kourtchenko et al., 2007). In addition to OPDA-containing galactolipids, also the amounts of monogalactosyldiacylglycerols and digalactosyldiacylglycerols (MGDGs and DGDGs) with 18:3 and 16:3 as fatty acid components in at least one position were determined. In Figure 1D, the levels of the two most abundant MGDGs and DGDGs are depicted. A slight decrease in the levels of MGDG was detected. Since these substances are major components of plastid membranes, this decrease probably reflects the degradation of chloroplasts. However, no decline of the most prominent DGDG was observed during the time span analyzed. It was reported that predominantly MGDG levels drop during senescence (Yang and Ohlrogge, 2009).

Yellowing can be induced by incubation of leaves in the dark, and this “dark-induced senescence” is a frequently used model system (Weaver and Amasino, 2001; van der Graaff et al., 2006). For investigation of dark-induced senescence-like phenotypes, detached leaves of wild-type plants, plants with reduced expression of the lipase DGL or of OPR3, and SEN5 plants, which produce low basal levels of oxylipins but are impaired in the accumulation of OPDA and JA during senescence or in response to stress, was incubated in the dark, and this “dark-induced senescence” is a frequently used model system (Weaver and Amasino, 2001; van der Graaff et al., 2006). For investigation of dark-induced senescence-like phenotypes, detached leaves of wild-type plants, plants with reduced expression of the lipase DGL or of OPR3, and SEN5 plants, which produce low basal levels of oxylipins but are impaired in the accumulation of OPDA and JA during senescence or in response to stress, was incubated in the dark. The results indicated that 13-LOX products are not necessary for natural senescence or dark-induced chlorophyll loss but are involved in the response to sorbitol.
leaves of 6-week-old plants were incubated in the dark. Chlorophyll content decreased to 61% after 3 d and to 8% after 7 d of dark treatment relative to the chlorophyll content of leaves before treatment (Fig. 1E). This indicates a dramatic chlorophyll breakdown during 1 week. Also in this system, the decline in chlorophyll content correlated with accumulation of free oxylipins. Dark incubation resulted in a dramatic increase in JA, reaching levels of more than 4,000 ng g\(^{-1}\) fresh weight (Fig. 1F). There was also a strong increase in OPDA from 6 to 513 ng g\(^{-1}\) fresh weight, but OPDA levels did not reach the enormous amounts of JA. Levels of all arabidopsides showed a moderate increase at 3 d of dark incubation (5- to 10-fold higher levels relative to levels before treatment) and a subsequent drop at 7 d (arabidopsides A, B, and D) or an additional 2-fold increase (arabidopsides C, E, and G; Fig. 1G). The content of MGDG and DGDG had strongly declined at 7 d of dark treatment (13%–57% of levels before treatment), correlating with the strong decline in chlorophyll (Fig. 1H). In contrast to natural senescence, not only the content of MGDG but also of DGDG was reduced. Expression of SAG12 was upregulated upon dark treatment but did not show the strong increase typical for natural senescence (Supplementary Fig. S1B), which is in agreement with the gene expression data of Noh and Amasino (1999).

As a third system for senescence-like phenotypes, sorbitol stress was chosen because it has been reported that this treatment leads to yellowing and accumulation of JA (Bohlmann et al., 1998). In agreement with the previous report, floating detached leaves on 500 mM sorbitol resulted in a dramatic increase in JA, reaching levels of more than 4,000 ng g\(^{-1}\) fresh weight (Fig. 1F). There was also a strong increase in OPDA from 6 to 513 ng g\(^{-1}\) fresh weight, but OPDA levels did not reach the enormous amounts of JA. Levels of all arabidopsides showed a moderate increase at 3 d of dark incubation (5- to 10-fold higher levels relative to levels before treatment) and a subsequent drop at 7 d (arabidopsides A, B, and D) or an additional 2-fold increase (arabidopsides C, E, and G; Fig. 1G). The content of MGDG and DGDG had strongly declined at 7 d of dark treatment (13%–57% of levels before treatment), correlating with the strong decline in chlorophyll (Fig. 1H). In contrast to natural senescence, not only the content of MGDG but also of DGDG was reduced. Expression of SAG12 was upregulated upon dark treatment but did not show the strong increase typical for natural senescence (Supplementary Fig. S1B), which is in agreement with the gene expression data of Noh and Amasino (1999).
mM sorbitol but not on water as a control treatment resulted in a strong decrease in chlorophyll content and a strong increase in free JA and OPDA. After 24 and 48 h, 49% and 14% of the chlorophyll content of control leaves remained (Fig. 1I). Oxylipin levels were highest at 24 h of sorbitol (Fig. 1J, 256 ng JA g\(^{-1}\) fresh weight, 462 ng OPDA g\(^{-1}\) fresh weight) and showed a slight decrease thereafter. Floating on water did not change levels of JA and led to a minor increase in free OPDA (3-fold higher levels at 48 h; Fig. 1N). All arabidopsides accumulated upon sorbitol treatment. Similar to free OPDA, maximum levels were reached at 24 h. There was a 2.5-fold to 9-fold increase of arabidopsides A to D and a stronger increase in arabidopsides E and G (Fig. 1K). The accumulation at 48 h was in the same range as 24 h of sorbitol. The content of MGDG and DGDG decreased upon sorbitol treatment to levels at 48 h between 30% and 57% relative to the levels at 0 h (Fig. 1L). No decline was observed in the water-floated control leaves; most MGDG/DGDG compounds showed even a small increase at 24 h (Fig. 1P). Expression of SAG12 showed some up-regulation by sorbitol treatment but did not reach the high expression detected during natural senescence (Supplemental Fig. S1C).

**Effects of Reduced Oxylipin Levels on Senescence-Like Phenotypes**

**Reduced Oxylipin Levels Do Not Alter Chlorophyll Loss during Natural Senescence**

In order to elucidate the functions of JA and OPDA in senescence, we aimed to generate plants that produce enough JA and OPDA for normal growth and fertile flowers but that do not show increased levels of JA and OPDA during natural senescence, dark incubation, and sorbitol treatment. To this end, RNAi approaches targeting the expression of the JA biosynthetic enzymes LOX2 and AOS were used. Expression of RNAi constructs was driven by the promoter of the senescence-induced gene SAG13. Analysis of mRNA levels of the respective target genes showed that RNA levels were indeed lower in senescing leaves (Supplemental Figs. S2A and S3A). However, there was no clear reduction in oxylipin levels in the transgenic lines (Supplemental Figs. S2, B and C, and S3, B and C). A possible explanation of this lack of strong effects on oxylipin levels is that these enzymes are not limiting JA biosynthesis or that these proteins are rather stable, so that during the time of the experiments no depletion of these enzymes might occur. Therefore, the stronger and constitutive 35S promoter was chosen to drive RNAi of LOX2 and different lines were analyzed. Two lines with a single insertion of the transgene and strongly reduced LOX2 expression (Supplemental Fig. S4) were selected for further experiments. These lines produce constitutive detectable but low levels of JA and OPDA, which do not considerably increase during natural senescence, dark incubation, and sorbitol treatment. No altered developmental phenotype of these lines regarding leaf size, flowering, and seed production was obvious.

In leaves of 6-week-old plants of both RNAi lines, levels of JA and OPDA were similar to those in the wild type. In contrast to the wild type, no increase during natural senescence until 10 weeks was detected (Fig. 2, A and B); levels remained between 4 and 12 ng g\(^{-1}\) fresh weight. In addition, levels of arabidopsides were

![Figure 2. Comparison of oxylipin levels and chlorophyll content of leaves of the wild type (WT) and LOX2-RNAi lines during natural senescence. Leaves were harvested at 6, 8, and 10 weeks of plant age, and levels of JA (A), OPDA (B), and chlorophyll (C) were determined. Data represent means of at least three biological replicates ± so. FW, Fresh weight.](image-url)
very low in the RNAi lines, mostly at the detection limit (Supplemental Fig. S5C). However, despite the lack of accumulation of these oxylipins in the transgenic lines, there was no obvious phenotypic difference from the wild type. Although chlorophyll levels at 6, 8, and 10 weeks showed in most experiments a tendency to be higher in both RNAi lines, there was no significant difference from wild-type levels (Fig. 2C). Also, SAG12 expression was similar in wild-type and RNAi lines (Supplemental Fig. S1A). This indicates that increases in JA and OPDA are not necessary for normal progression of chlorophyll loss and SAG12 expression during natural senescence and that this process is also independent of arabidopside. Levels of MGDG/DGDG were similar in wild-type and RNAi lines (Supplemental Fig. S5, A and B).

Reduced Oxylipin Levels Do Not Alter Senescence-Like Phenotypes Induced by Dark Treatment

JA levels in the RNAi lines showed a minor rise (from 4 and 3 ng g
\textsuperscript{-1} fresh weight to 22 and 13 ng g
\textsuperscript{-1} fresh weight) upon dark incubation, which is negligible in comparison with the dramatic increase up to more than 4,000 ng g
\textsuperscript{-1} fresh weight in the wild type (Fig. 3A). OPDA levels did not change upon dark treatment in both RNAi lines (Fig. 3B). Similar to natural senescence, no phenotypic differences between the RNAi lines and the wild type were obvious. Chlorophyll contents decreased similarly in the RNAi lines and the wild type despite the strong differences in JA and OPDA accumulation (Fig. 3C). This indicates that the increase in OPDA and the very high JA amounts are not required for regulating yellowing phenotypes in response to dark incubation. As for natural senescence, arabidopside levels in the LOX2-RNAi lines were at the detection limit and levels of MGDG/DGDG were similar in wild type and RNAi lines (Supplemental Fig. S6).

Reduced Oxylipin Levels Alter the Response to Sorbitol

Sorbitol treatment induced little accumulation of JA up to 10 and 9 ng g
\textsuperscript{-1} fresh weight in the RNAi lines, while 257 ng g
\textsuperscript{-1} fresh weight was found in the wild type at 24 h (Fig. 4A). OPDA levels showed a slight increase to 37 and 26 ng g
\textsuperscript{-1} fresh weight in the RNAi lines at 24 h of sorbitol compared with the stronger increase up to 462 ng g
\textsuperscript{-1} fresh weight in the wild type (Fig. 4B). Arabidopside levels in the LOX2-RNAi lines were at the detection limit (Supplemental Fig. S7). In contrast to natural senescence and dark incubation, leaves of the RNAi lines showed obvious differences from the wild type in their response to sorbitol treatment. Leaves stayed greener and remained in a stronger constitution than wild-type leaves (Fig. 4C). At 48 h, chlorophyll content of the RNAi lines was 166 and 186 ng g
\textsuperscript{-1} fresh weight, significantly but not drastically higher than the wild type level of 105 ng g
\textsuperscript{-1} fresh weight (Fig. 4D). The reduced sensitivity of the RNAi lines was also reflected by the fact that levels of MGDG and DGDG showed less decrease upon sorbitol flotation in the RNAi lines than in the wild type. Especially levels of MGDG 18:3-18:3 were 67% to 79% higher in the RNAi lines relative to the wild type (Fig. 4E). This suggests that the reduced levels of 13-LOX products lead to a delay in yellowing of leaves upon sorbitol flotation. Also, expression of the senescence marker genes SAG13 and SEN1 was tested, since SAG12 was not highly expressed upon sorbitol treatment. Transcript levels of SAG13 and SEN1 showed a stronger increase in the wild type at 24 h in comparison with the RNAi lines (Fig. 4F), further supporting a delay in sorbitol-induced senescence-like phenotypes.

Reduced Sensitivity to Sorbitol Is Due to AOS Products

The product of LOX2, 13-hydroperoxyoctadecatrienoic acid, is further metabolized by different pathways, leading to a multitude of different compounds (Feussner and Wasternack, 2002). One metabolic route is the AOS pathway leading to OPDA and JA. To elucidate if products of the AOS pathway are responsible for the phenotypic differences between the RNAi lines and the wild type, the dde2 mutant, which is defective in AOS (von Malek et al., 2002), was analyzed. Also, leaves of the dde2 mutant remained greener upon sorbitol treatment than wild-type leaves. Chlorophyll content at 48 h of sorbitol treatment was
217.7 ± 34.2 μg chlorophyll g⁻¹ fresh weight, about twice the content of wild-type leaves (105.5 ± 14.7 μg chlorophyll g⁻¹ fresh weight) and even higher than in the RNAi lines. No JA, OPDA, or arabidopsides were detectable, as expected.

To investigate if the reduced sensitivity to sorbitol is specific for the yellowing phenotype in the leaf-floatation assay or if this is a more general phenomenon, seedlings were germinated on agar plates containing 500 mM sorbitol. Germination of the wild type was strongly inhibited. After 4 d, only 33% of the seeds had germinated, which increased to 40% after 10 d in comparison with almost 100% on agar plates without sorbitol (Fig. 5). In contrast, the germination rate of dde2 was 85% and increased to 92% at day 10. This indicates a more general reduced sensitivity of this AOS-deficient mutant against sorbitol.

**DISCUSSION AND CONCLUSION**

Natural senescence is a developmental process that is regulated by a multitude of internal and external factors. Jasmonates are signaling compounds that have been reported to be substantially involved in regulating developmental processes such as germination, growth, and flowering (Wasternack, 2007). In addition, there are reports suggesting an involvement of jasmonates in senescence progression, while other reports lead to contrary conclusions (see introduction).
To elucidate the function of oxylipins in senescence, we used LOX2-RNAi plants, which are able to produce low basal levels of 13-LOX products and jasmonates but that are impaired in the accumulation of higher levels during senescence. The lack of increase in JA and OPDA in these plants suggests that LOX2 is involved in the generation of oxylipins during senescence. No differences in senescence initiation and progression based on the determination of chlorophyll loss and SAG12 expression between the LOX2-RNAi plants and the wild type were observed. These data indicate that the increase in jasmonates that takes place during aging is not necessary for promoting senescence in Arabidopsis. This conclusion is in agreement with the fact that no difference in natural senescence has been observed with knockout mutants defective in JA biosynthesis or signaling. However, senescence is a complex process, and it is possible that jasmonates are involved in regulating more subtle changes related to senescence that do not strongly impact chlorophyll loss and SAG12 expression, which were used as indicators of senescence in this study. Furthermore, it cannot be excluded that jasmonates are important for senescence when the environmental conditions such as light and nutrient availability are different. In addition, other oxidized lipids might contribute to the regulation of senescence, since expression of a 9-LOX increases during aging and also nonenzymatic lipid peroxidation occurs (Berger et al., 2001; van der Graaff et al., 2006).

Oxylipin application and different stress factors induce senescence-like phenotypes such as yellowing of leaves. For quite some time, effects of these treatments have been categorized as senescence (see introduction and “Results”). Based on differences in gene expression, the question has already been raised how close stress and MeJa treatment resemble naturally occurring senescence processes (Parthier, 1990; van der Graaff et al., 2006). Here, oxylipin profiles during natural senescence were compared with profiles upon dark and sorbitol treatments in the wild type, revealing obvious differences. During natural senescence and upon sorbitol treatment, moderate increases in JA and free OPDA occur (to levels between 110 and 460 ng g⁻¹ fresh weight), with JA exhibiting a stronger increase in natural senescence and OPDA upon sorbitol, respectively. In contrast, in response to dark incubation, JA levels increase dramatically to levels (around 4,000 ng g⁻¹ fresh weight) that are not observed by other treatments, such as wounding or pathogens. Remarkably, this strong JA accumulation upon dark incubation does not correlate with enhanced expression of JA biosynthetic enzymes such as OPR3, AOS, and LOX2 (van der Graaff et al., 2006). In comparison, the increase of the levels of free OPDA was low. The strongest accumulation of OPDA-containing galactolipids was detected after sorbitol treatment. However, the induction was lower compared with wounded leaves (Buseman et al., 2006). From the fact that levels of JA and free OPDA increase during natural senescence without accumulation of esterified OPDA, several conclusions can be drawn. First, this indicates that levels of these oxylipins are not strictly correlated. This is supported by the finding that in distal leaves of wounded plants, levels of JA and JA-Ile increase, OPDA first declines and later increases, and arabidopside levels do not change (Koo et al., 2009). Similarly, Glauser et al. (2009) found only a small increase of arabidopsides in distal leaves of wounded plants. This suggests that the accumulation of different oxylipins in response to a particular stimulus is regulated by different signals. Second, this indicates that arabidopside accumulation might be related to local stress. Third, it argues against an involvement of arabidopsides in the promotion of natural senescence. In addition, the very low levels of arabidopsides in the RNAi lines suggest that LOX2 is substantially involved in the generation of arabidopsides. This is similar to the situation during the wound response. Comparing the impact of knockdown or knockout of LOX2 on the accumulation of JA, OPDA, and arabidopsides, Glauser et al. (2009) reported the strongest effect on arabidopside levels.

In summary, the results of this work show that natural senescence and stress-induced senescence-like phenotypes are fundamentally different regarding the oxylipin profiles, even though some of the resulting
symptoms have some overlap, such as yellowing. Additionally, our data further support that the effect of exogenous application of biologically active compounds is not necessarily related to the effect and function of endogenous compounds. The senescence-like phenotype of MeJa treatment might rather result from the induction of stress by high amounts of this compound than from effects resembling physiologically relevant conditions.

In addition to their function in development, oxylipins accumulate upon stress stimuli and have been shown to signal stress responses. Therefore, we investigated the function of oxylipins in regulating the senescence-like phenotypes upon incubation in the dark or treatment with sorbitol. Both treatments resulted in strong increases in JA and OPDA in the wild type. The LOX2-RNAi plants showed no or only weak accumulation of JA and OPDA. This suggests that LOX2 is involved in the generation of oxylipins upon dark and sorbitol stress. The chlorophyll loss upon dark treatment in the transgenic lines was similar to the wild type, despite the lack of increase in jasmonates. This indicates that LOX2 products are not important for the chlorophyll loss upon dark incubation. This is remarkable, because JA is produced in such huge amounts under these conditions. Upon sorbitol flotation, the LOX2-RNAi lines showed differences from the wild type. Chlorophyll loss in leaves of the LOX2-RNAi lines was lower than in the wild type. This indicates that 13-LOX products are involved in the response of leaves to sorbitol stress. Also, leaves and seedlings of the dde2 mutant were less sensitive to sorbitol, suggesting that the deficiency in jasmonates (and not other 13-LOX products) is responsible for the altered sensitivity to sorbitol.

LOX2 is the most prominent of the 13-LOX enzymes located in the chloroplast. The function of the three additional 13-LOX genes is far less understood. For LOX2, an involvement in JA and arabinodipside biosynthesis upon wounding has been shown (Bell et al., 1995; Schommer et al., 2008; Glauser et al., 2009). Some factors regulating LOX2 expression and activity are already known. LOX2 expression is induced by jasmonates, wounding, and other stress factors, and this up-regulation is dependent on COI1. Besides the regulation of transcription, also regulation of LOX2 activity by the Ca$^{2+}$-dependent cation channel FOU2/TPC1 has been shown (Bonaventure et al., 2007). The enhanced JA and OPDA generation after wounding in the gain-of-function mutant fou2 in comparison with the wild type further supports the role of LOX2 in stress responses. LOX2 is also involved in the generation of JA during development. LOX2 expression during development but not upon wounding is dependent on the transcription factor TCP4 (Schommer et al., 2008). Plants overexpressing TCP4 exhibited accelerated senescence. The mutant jawD, which has reduced expression of several TCP factors, strongly reduced LOX2 expression, and reduced JA levels, shows enhanced leaf production before flowering and delayed senescence (Schommer et al., 2008). The phenotype of our RNAi lines is complementary to the jawD phenotype. While jawD exhibits changes in development but not stress response, our LOX2-RNAi lines are altered in stress response but not development. It is somewhat contrasting that OPDA and JA levels increase during natural senescence and dark treatment, even though LOX2 mRNA levels are not strongly up-regulated during this event but rather down-regulated (Berger et al., 2001; van der Graaff et al., 2006). This increase in jasmonates is impaired in the LOX2-RNAi lines controlled by the 35S promoter but not by the SAG13 promoter. A possible explanation is that the LOX2 protein that is responsible for this increase is already present before senescence is initiated.

Why are jasmonates accumulating during senescence and dark incubation if they do not have obvious functions in regulating this process? There are different possibilities. (1) These compounds are just produced by degradation of membrane lipids. During natural senescence (from 6 to 10 weeks of plant age), around 10% of the linolenic acid esterified in galactolipids is metabolized. In comparison, the reduction of the amount of esterified linolenic acid upon dark treatment (from day 0 to day 7) is around 84%. In the process of degradation of plastid membranes, linolenic acid might be released from membranes by lipases and converted to JA and other derivatives. (2) These compounds function to signal changes in plant metabolism that have not been studied here, for example in the reallocation of nutrients. It might be plausible that the increase results from membrane breakdown and that by the same time the accumulation of these compounds is used to signal further breakdown of macromolecules and adjust further aspects of metabolism to the developmental status or the environmental conditions. Interestingly, endogenous JA accumulation does not always result in the same response. A typical marker gene for JA is VSP. Expression of VSP is induced by exogenous application of JA and MeJa and by sorbitol stress (data not shown). In contrast, VSP expression does not increase during aging and old leaves do not respond to MeJa with VSP induction (Matthes et al., 2008). Also, VSP is not induced upon dark incubation, despite the high increase in endogenous JA (He et al., 2002). This indicates that sorbitol treatment is more similar to typical stress and JA responses than dark incubation and aging, supporting the function of JA in sorbitol response indicated by our results. Furthermore, it underlines that networking of different signaling pathways determines the resulting physiological changes and stress responses in plants.

**MATERIALS AND METHODS**

**Plant Material**

*Arabidopsis (Arabidopsis thaliana)* wild-type ecotype Columbia (Col-0), dde2, and transgenic lines were used. The dde2 mutant (von Malek et al., 2002) was kindly provided by B. Keller.
Plants were grown on soil at 22°C under a 9-h photoperiod (100 μmol photons m⁻² s⁻¹) in a climate chamber or plant cabinet. For experiments in natural leaf senescence, rosette leaves 9 to 15 (counting from bottom to top without cotyledons) were marked by colored yarn and harvested at 6, 8, and 10 weeks after sowing. These leaves were chosen because they are consistently present at all time points analyzed, showed the most constant leaf size, and did not directly contact the soil. For analysis of natural senescence, each replicate consisted of leaves of at least four plants.

**Construction of Gene-Silencing Plasmids**

For RNAi constructs targeted against LOX2 (At3g45140), a 455-bp fragment was cloned by PCR from full-length cDNA using the oligonucleotides LOX2fwd (5′ - TCTCGACTGAGATGGACGGAGCTTAGGAC-3′) and LOX2rev (5′ - ATCACTGGTCAGGAGATTAGTGCAGAC-3′). For cloning a 465-bp fragment of AOS (At5g26580) cDNA, the oligonucleotides AOSfwd (5′- TCTAGAATCCGACGTTCACTAATCTGAGGACC-3′) and AOSSrev (5′-ATCGATGAGTACCGCTGCTGGTGGACC-3′) were used. The primer contained the additional 5′ end restriction sites (underlined) Xhol and Clal, respectively. The derived fragments were ligated into pGEM T-Easy vector (Promega) and transformed into Escherichia coli strain DH5α. Fragments were subcloned in the sense and antisense directions into pHANNIBAL vector (Hale and Lesley, 2001), using either Xhol-Clal or XhoI-KpnI sites.

To restrict the RNAi effect on later stages of leaf age, in addition to construct the constitutive 35S promoter was replaced by cloned full-length promoter of SAG12 (At2g29350). This promoter sequence was cloned with the following oligonucleotides containing MutI and Xhol restriction sites (underlined), respectively: SAG12fwd (5′ - ACGGCTCATCTGTCCGATGC-3′) and SAG12srev (5′-CTCGAGGATCCGGTATAC-3′). The fragment was cloned into pGEM T-Easy vector (Promega) and transformed into E. coli strain DH5α. The 3S promoters of the above-prepared RNAi vectors were then replaced using MutI and XhoI restriction sites.

The resulting ihpRNAi constructs termed pHAN_35S::LOX2i, pHAN_35S::AOSi and pAHN_35S::LOX2i, pAHN_35S::AOSi were cut by restriction sides (underlined) Xhol, XhoI and Clal, respectively. The derived fragments were ligated into pGEM T-Easy vector (Promega) and transformed into Escherichia coli strain DH5α. Fragments were subcloned in the sense and antisense directions into pHANNIBAL vector (Hale and Lesley, 2001), using either Xhol-Clal or XhoI-KpnI sites.

**Plant Transformation**

The resulting ihpRNAi constructs were transformed by the floral dip method (Clough and Bent, 1998). Transformed plants were selected on Murashige and Skoog medium containing 2% Suc and 50 mg L⁻¹ kanamycin. Green plants were transferred to soil and grown in a climate chamber under short-day conditions (9-h day, 15-h night) until flowering. Seeds were harvested from each line and tested for segregation.

For further cultivation and experiments.

**Dark and Sorbitol Treatment of Plants**

For dark incubation, rosette leaves 5 to 15 of 6-week-old plants were detached and incubated on wet paper tissue in petri dishes for 3 and 7 d in the dark at 22°C. After incubation, leaves were immediately frozen in liquid nitrogen and stored at −80°C for further analysis. Each replicate consisted of leaves of at least three plants.

For sorbitol treatment, fully expanded green rosette leaves from 7- to 8-week-old plants were detached and floated on 500 mM sorbitol solution or water as a control at 22°C under continuous light (120 μmol photons m⁻² s⁻¹). After 24 and 48 h of incubation, leaves were rinsed with water and immediately frozen in liquid nitrogen. Each replicate consisted of leaves of at least two plants. Frozen plant material was freeze dried and stored at −20°C for further analysis. The fresh weight and dry weight of representative samples were measured. The dry weight was found to equal 6% of the fresh weight. Using this relation, fresh weight was calculated from dry weight.

**Seed Germination Assay**

Seeds were surface sterilized and sown on half-strength Murashige and Skoog medium containing 7.5% Suc (control) or 1.5% Suc with 500 mM sorbitol. Seeds were stratified for 2 d at 4°C in the dark and then transferred to a growth cabinet (22°C under a 9-h photoperiod [120 μmol photons m⁻² s⁻¹]). Germination was monitored every 2 d.

**RNA Isolation and Quantitative RT-PCR**

Total RNA was extracted from ground plant material using the Plant RNA Mini Kit (Omega) according to the manufacturer’s protocol. RNA concentration was determined spectrophotometrically. Remaining RNA was removed using RNase-free DNase I (Invitrogen) according to the manufacturer’s protocol. First-strand cDNA and real-time PCR were performed as described previously (Szyröki et al., 2001) using SYBR-Green Capillary Mix (Thermo Fisher Scientific) and a Mastercycler gradient PCR machine (Eppendorf).

Primers used (TIB Molbiol) were as follows: LOX2 (At2g45140) forward (5′-TGATCGTCTGACGATAACC-3′) and reverse (5′-TCTGCGACCTCATGAA-3′); AOS (At5g26580) forward (5′-CCGTGTACCCCTCAAG-3′) and reverse (5′-GATCCGTTAGTAGGCCC-3′); and SAG12 (At2g29350) forward (5′-TGATCCGTGTGATTGCGACC-3′) and reverse (5′-GATCCGTGGTTTGACC-3′). The number of transcripts was normalized to act2/8 cDNA fragments amplified by ACT2/8/act2/8 (An et al., 1996).

**Chlorophyll Quantification**

Chlorophyll extraction and calculation were performed according to Arnon (1949). Fifty milligrams of fresh plant material or 10 mg of freeze-dried plant material was dissolved in acetone:water (80:20, v/v) mix, incubated for several hours at 4°C in the dark, and centrifuged. The extinction of the supernatant was measured by spectral photometer at 664 and 647 nm.

**Analysis of Free Oxylinps and Galactolipids**

For analysis of oxylipins and galactolipids, fresh (shock-frozen) or freeze-dried plant material was ground by mortar and pestle.

**Plant leaves (100-250 mg of fresh or 15-25 mg of freeze-dried material) were extracted with 1 mL of methanol:acetic acid (99:1, v/v) for jasmonate analysis or 1 mL of boiling 2-propanol for galactolipid analysis, respectively.**

For jasmonate extraction, samples were homogenized with a ball mill for 5 min. After centrifugation, the supernatant was dripped in a vacuum concentrator. For quantification of JA and its precursor OPDA, 32 mg of δ[9]JJA and 88 mg of δ[9]JOPDA (both diluted in acetone) were added. After evaporation of the acetone, samples were dissolved in 50 μL acetone/1 mL acetonitrile. The solution was further extracted with 1 mL of chloroform:2-propanol (50:100, v/v) and 1 mL of methanol/chloroform (100:50, v/v). After each extraction, samples were centrifuged and the supernatants were combined. The pooled lipid extract was dried under a stream of nitrogen at 60°C and reconstituted in 100 μL of methanol containing 1 μmol ammonium acetate for liquid chromatography-tandem mass spectrometry (MS/MS) analysis.

For galactolipid analysis, 3 μg of MGDG 18:0-18:0 and 5 μg of DGDG 18:0-18:0 were added as internal standards into the 2-propanol extraction mixture, and the sample was incubated at 75°C for 15 min to inactivate lipases. After centrifugation, the supernatant was recovered and the residue was further extracted with 1 mL of chloroform:2-propanol (50:100, v/v) and 1 mL of methanol/chloroform (100:50, v/v). After each extraction, samples were centrifuged and the supernatants were combined. The pooled lipid extract was dried under a stream of nitrogen at 60°C and reconstituted in 100 μL of methanol containing 1 μmol ammonium acetate for liquid chromatography-MS/MS analysis.

For analysis of galactolipids, 3 μg of MGDG 18:0-18:0 and 5 μg of DGDG 18:0-18:0 were added as internal standards into the 2-propanol extraction mixture, and the sample was incubated at 75°C for 15 min to inactivate lipases. After centrifugation, the supernatant was recovered and the residue was further extracted with 1 mL of chloroform:2-propanol (50:100, v/v) and 1 mL of methanol/chloroform (100:50, v/v). After each extraction, samples were centrifuged and the supernatants were combined. The pooled lipid extract was dried under a stream of nitrogen at 60°C and reconstituted in 100 μL of methanol containing 1 μmol ammonium acetate for liquid chromatography-MS/MS analysis.

**Ultra-high-performance liquid chromatography-MS/MS analyses were performed on a Waters Quattro Premier XE triple-quadrupole mass spectrometer with an electrospray interface coupled to a Waters Acquity ultra-high-performance liquid chromatograph. Chromatographic separations were carried out using an Acquity BEH C18 column (2.1 × 30 mm, 1.7-μm particle size with a 2.1-×5-mm guard column; Waters) with the following solvent systems.**

(1) For jasmonate analyses, solvent A = 1 μmol aqueous ammonium acetate and solvent B = acetonitrile. A gradient elution was performed at a flow rate of 0.3 mL min⁻¹ at 27°C: 5% to 60% B in 3 min, followed by 100% B for 5 min, and reconditioning at 5% B for 3 min. (2) For galactolipid analyses, solvent A = 1 μmol aqueous ammonium acetate and solvent B = 1 μmol ammonium acetate in methanol. The linear step gradient (0.3 mL min⁻¹ flow rate, 30°C) consisted of 75% B for 1 min, followed by 75% to 100% B in 10 min, 100% B for 1 min, and 75% B for 4 min. The electrospray interface source was operated in negative ionization mode at a capillary voltage of 3.0 kV at 120°C. Quantification was performed using multiple reaction monitoring with a scan time of 0.025 s per transition (Supplemental Table S1). Cone voltage and collision energy was set at 26 eV. The optimal MS parameters obtained were as follows: capillary, 3.0 kV; source
temperature, 120°C; desolvation temperature, 400°C (jasmonates) or 450°C (galactolipids). Nitrogen was used as the desolvation and cone gas with flow rates of 800 and 100 l.h⁻¹, respectively. Argon was used as the collision gas at a pressure of approximately 3.10 × 10⁻³ bar.

Web Sites
Gene regulation analyses were done using the Web site www.genevestigator.ethz.ch (Zimmermann et al., 2004). Analysis of gene homology was performed using National Center for Biotechnology Information BLAST (www.ncbi.nlm.nih.gov).

Supplemental Data
The following materials are available in the online version of this article.

Supplemental Figure S1. Expression of SAG12 in 35S::LOX2-RNAi lines 2 and 9 in comparison with the wild type during natural senescence (A) and in response to dark incubation (B) and sorbitol floation (C).

Supplemental Figure S2. Expression of AOS and levels of JA and OPDA in SAG13::AOS-RNAi line 1 in comparison with the wild type.

Supplemental Figure S3. Expression of LOX2 and levels of JA and OPDA in SAG13::LOX2-RNAi line 6 in comparison with the wild type.

Supplemental Figure S4. Expression of LOX2 in 35S::LOX2-RNAi lines 2 and 9 in comparison with the wild type.

Supplemental Figure S5. Levels of MGDG, DGDG, and arabidopsides in the wild type and LOX2-RNAi at 6, 8, and 10 weeks of plant age.

Supplemental Figure S6. Levels of MGDG, DGDG, and arabidopsides in the wild type and LOX2-RNAi in dark-induced senescence-like phenotypes.

Supplemental Figure S7. Levels of MGDG, DGDG, and arabidopsides in the wild type and LOX2-RNAi in sorbitol-induced senescence-like phenotypes.

Supplemental Table S1. Mass-to-charge proportions of parent and daughter ions from analyzed molecules.

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