Running head: stress-induced oxylipin synthesis in roots

corresponding author: Susanne Berger

Julius-von-Sachs-Institute for Biosciences, Pharm. Biology, Biocenter, University of Wuerzburg, Julius-von-Sachs-Platz 2, 97082 Wuerzburg, Germany

phone: 49 931 318 6170
FAX: 49 931 318 6182

berger@biozentrum.uni-wuerzburg.de

Research area: Signaling and Response
Lipoxygenase 6-dependent Oxylipin Synthesis in Roots is required for abiotic and biotic Stress Resistance of Arabidopsis thaliana

Wiebke Grebner, Nadja E. Stingl, Ayla Oenel, Martin J. Mueller, and Susanne Berger

Julius-von-Sachs-Institute for Biosciences, Pharm. Biology,
Biocenter, University of Wuerzburg, Julius-von-Sachs-Platz 2, 97082 Wuerzburg, Germany
Footnotes:

This work was supported by the GK 1342 and the SFB 567.

The authors responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors are: Susanne Berger (berger@biozentrum.uni-wuerzburg.de)

corresponding author, e-mail berger@biozentrum.uni-wuerzburg.de
ABSTRACT
Jasmonates are oxylipin signals which play important roles in the development of fertile flowers and in defense against pathogens and herbivores in leaves. The aim of this work was to understand the synthesis and function of jasmonates in roots. Grafting experiments with a jasmonate-deficient mutant demonstrated that roots produce jasmonates independently of leaves despite low expression of biosynthetic enzymes. Levels of 12-oxo-phytodienoic acid, jasmonic acid, and its isoleucine derivative increased in roots upon osmotic and drought stress. Wounding resulted in a decrease of preformed 12-oxo-phytodienoic acid concomitant with an increase of jasmonic acid and jasmonoyl-isoleucine. 13-lipoxygenases catalyse the first step of lipid oxidation leading to jasmonate production. Analysis of 13-lipoxygenase deficient mutant lines showed that only one of the four 13-lipoxygenases, LOX6, is responsible and essential for stress-induced jasmonate accumulation in roots. In addition, LOX6 was required for production of basal 12-oxo-phytodienoic acid in leaves and roots. Loss of function mutants of LOX6 were more attractive to a detritivorous crustacean and more sensitive to drought indicating that LOX6 derived oxylipins are important for the responses to abiotic and biotic factors.
INTRODUCTION

Oxylipins are ubiquitous signaling molecules which are derived from polyunsaturated fatty acids by enzymatic and non-enzymatic processes. In plants, the biosynthesis and function of oxylipins of the jasmonate family in above-ground tissues has been investigated in detail. Jasmonates comprise 12-oxo-phytodienoic acid (OPDA), jasmonic acid (JA), and derivatives of JA. In leaves, jasmonates accumulate in response to abiotic factors such as wounding, drought, osmotic stress, darkness, and ozone and during interactions with organism such as herbivores, pathogens, and mutualistic organisms (reviewed in Wasternack, 2007). The relevance of jasmonates in the wound response, ozone tolerance, and the defense against herbivores and necrotrophic pathogens in leaves has been well investigated using mutants in JA-biosynthesis and –signaling (reviewed in Browse, 2009a). In addition, jasmonates play an important role in flower development and Arabidopsis mutants in the JA pathway are male sterile (Browse, 2009b). The first step in jasmonate biosynthesis is catalysed by 13-lipoxygenases (LOX). The resulting 13(S)-hydroperoxyoctadecatrienoic acid (13-HPOTE) is converted by allene oxide synthase (AOS) and allene oxide cyclase (AOC) to OPDA (Wasternack, 2007). These enzymatic steps are located in plastids. OPDA is transported to peroxisomes and converted to JA. JA can be further metabolized to different derivatives which takes mainly place in the cytosol. The conjugation of JA with isoleucine is an important step because jasmonoyl-isoleucine (JA-Ile) has been identified as a biologically active jasmonate (Staswick and Tiryaki, 2004). OPDA is also biologically active without conversion to JA-derivatives. In contrast to all other jasmonates, the OPDA structure contains an electrophilic α,β-unsaturated carbonyl group which renders OPDA more reactive than JA. Therefore, OPDA is classified as reactive electrophile species with unique signaling properties different from other jasmonates (Farmer and Davoine, 2007).

Of the six LOX genes present in Arabidopsis four genes encode 13-LOX. For the respective enzymes, LOX2, LOX3, LOX4, and LOX6 it was shown that linolenic acid is the preferred substrate and that 13(S)-HPOTE is formed in vitro (Bannenberg et al., 2009). All four enzymes are proposed to be located in plastids. LOX2 is highly expressed in leaves; expression is up-regulated by jasmonates and stress treatments such as wounding and osmotic stress (Bell and Mullet, 1993; Seltmann et al., 2010a). LOX2 was shown to contribute the majority of jasmonate synthesis upon wounding, osmotic stress, and during senescence in leaves (Bell et al., 1995; Glauser et al., 2009). LOX2 is also responsible for the accumulation of arabidopsides which are galactolipids containing esterified OPDA in plastids by direct
oxidation of galactolipids (Zoeller et al., 2012). LOX3 and LOX4 are required for the
development of fertile flowers (Caldelari et al., 2011). LOX6 shows overall low expression
(Bannenberg et al., 2009). Recently, it was reported that LOX6 contributes to the fast
accumulation of JA and JA-Ile in wounded leaves and is required for the fast increase of JA
and JA-Ile in distal leaves after wounding (Chauvin et al., 2013).

In contrast to leaves and flowers, little is known on jasmonate biosynthesis and function in
roots. Expression of the plastid localized enzymes of jasmonate synthesis LOX2, AOS, and
AOC2 is very low in roots (Zimmermann et al., 2004). In contrast, enzymes such as 9-LOX
and α-dioxygenase 1 are strongly expressed in roots. These enzymes are involved in the
biosynthesis of oxylipins different from jasmonates and 9-LOX products have been shown to
regulate lateral root development since mutants in LOX1 and LOX5 produce more lateral
roots (Vellosillo et al., 2007). However, jasmonate function in roots is still obscure. Here we
analysed jasmonate accumulation in roots upon different stress treatments and show that
mutants defective in LOX6 are impaired in stress-induced jasmonate synthesis and are more
susceptible to drought and detritivore feeding.

RESULTS

Oxylipin profiles in roots and shoots

The plastid localization and low expression in roots of several enzymes of the first steps of JA
biosynthesis prompted us to investigate whether the oxylipin profile mirrors the expression of
oxylipin biosynthetic enzymes. To enable gentle recovery of root material, plants were grown
in a hydroponic system. In this system, plant parts below the hypocotyl were cultivated in
darkness. Levels of hydroxyoctadecatrienoic acids (HOTEs) and jasmonates in leaves and
roots were analysed. OPDA levels were around 1 nmol g⁻¹ dw both in roots and shoots (Fig.
1). 9- and 13-HOTE can be synthesized non-enzymatically as well as enzymatically via 9- and
13-LOXs, respectively. In agreement with published data (Grun et al., 2007; Triantaphylides
et al., 2008), 13-HOTE (0.28 nmol g⁻¹ dw) constituted the highest amount of free HOTEs in
leaves followed by 9-HOTE (0.16 nmol g⁻¹ dw). The high amount of 13-HOTE is in
accordance with the high expression of 13-lipoxygenases in leaves. In roots, levels of 13- and
9-HOTE were similar (0.67 and 0.64 nmol g⁻¹ dw, respectively) and higher in comparison to
leaves. Amounts of OPDA (0.8 nmol g⁻¹ dw) were in roots only slightly lower than in the
shoot (1 nmol g⁻¹ dw) indicating that roots contain 13-LOX-products and especially
jasmonates despite the low expression of LOX2 and AOS. Basal levels of JA and JA-Ile were
very low and at the detection limit in roots as well as in leaves. In addition to free jasmonates
also levels of arabidopsides were analysed. The presence of arabidopsides in leaves has been reported (Stelmach et al., 2001; Hisamatsu et al., 2005; Kourtchenko et al., 2007). In roots, levels of all arabidopsides were below the detection limit (Supplemental Fig. S1).

**Synthesis of jasmonates is induced upon wounding in roots**

Leaves respond to wounding with an accumulation of arabidopsides, OPDA, JA, and JA-Ile (Buseman et al., 2006; Koo et al., 2009). Therefore we tested if also in roots levels of these oxylipins increase. To avoid exchange of signals between shoots and roots, both parts of the plant were separated, wounded, and oxylipin levels in leaves and roots were determined after incubation of 30 min, 2 h, and 24 h. As expected from published data, in leaves all oxylipins accumulated (Fig. 2, Supplemental Fig. S1). Levels of OPDA increased from 1 to 8 nmol g⁻¹ dw and remained high. JA increased from the limit of detection to around 11 nmol g⁻¹ dw at 0.5 and 2 h and dropped to 1 nmol g⁻¹ dw at 24 h. JA-Ile showed the most transient kinetic with a maximum of 2.7 nmol g⁻¹ dw at 30 min. In roots levels of arabidosides were also after wounding below or slightly above the detection limit (Supplemental Fig. S1). The course of JA and JA-Ile accumulation in roots was similar to leaves (Fig. 2). JA levels increased at 30 min to 5 nmol g⁻¹ dw and started to decline with 4 nmol g⁻¹ dw at 2 h. JA-Ile accumulated to 1.7 nmol g⁻¹ dw at 30 min and declined to 0.6 nmol g⁻¹ dw at 2 h. Interestingly, OPDA levels decreased from 0.8 nmol g⁻¹ dw to 0.3 nmol g⁻¹ dw at 30 min and showed a further decrease at later time points. This might be due to conversion of the OPDA present in the root to JA and JA-Ile. This raises the question if the roots are able to produce OPDA on their own or if OPDA is synthesized in plastids in the shoot and transported into the root where it is further metabolized to JA and JA-Ile upon stress stimuli.

**Roots synthesize OPDA independent from the shoot**

To test the possibility of OPDA import from the shoot into the root grafting experiments with the dde2 mutant were performed. This mutant is deficient in expression of AOS and therefore does not produce OPDA, JA, or JA-Ile. Both grafting combinations with wild type and dde2 were performed and for control experiments wild type shoot was grafted on wild type root. Grafting was done at seedling stage and plants were grown for additional 7 weeks before wounding experiments were performed. The 30 min time point was chosen for analysis because strongest changes were seen at this time point (Fig. 2). Basal and wound-induced oxylipin levels in the control grafts were similar to non-grafted wild type plants (Fig. 3). In the first combination dde2 shoot was grafted on Col-0 root stock. As expected, in the dde2
mutant leaves OPDA, JA, and JA-Ile were barely detectable both under basal and wound-induced conditions while the wild type roots contained normal basal OPDA levels indicating that this organ produces OPDA without the need for a shoot with JA-biosynthetic competence (Fig. 3). After wounding of roots, JA and JA-Ile increased similarly as in non-grafted plants. In the second combination Col-0 shoot was grafted on dde2 root stock. As expected OPDA, JA, and JA-Ile levels strongly increased in leaves upon wound treatment (Fig. 3). All three oxylipins were at the detection limit in dde2 roots before and after wounding. This indicates that there is no significant transport of oxylipins from the shoot to the root.

**LOX6 is required for maintaining basal OPDA levels in shoots and roots**

To investigate the contribution of the different 13-lipoxygenases on oxylipin production in roots, expression of the four 13-LOXs and oxylipin levels in the single mutants _lox2_ and _lox6_ as well as in the _lox3/4_ double mutant defective in the expression of the redundant _LOX3_ and _LOX4_ were determined in roots and leaves of non-treated plants and 30 min after wounding. In wild type leaves and roots, OPDA is the major jasmonate under basal conditions (Fig. 2). The _lox2_ and _lox3/4_ mutant lines displayed similar or even elevated OPDA levels (_lox2_ 1.3 and 1, _lox3/4_ 4 and 5.2 nmol g⁻¹ dw in roots and leaves, respectively) while OPDA levels in the _lox6_ mutant were at the detection limit under basal conditions both in leaves and roots (Fig. 4B). A second independent _lox6_ mutant showed similar effects (Supplemental Fig. S2). Hence, LOX6 appears to be essential for maintaining basal OPDA levels in both organs. In roots this is consistent with the expression profile since _LOX6_ exhibited the highest basal transcript level among the four LOXs (Fig. 4A). In leaves this result was rather unexpected because _LOX6_ showed only low expression while _LOX2_ is strongly expressed. Levels of JA and JA-Ile were in all LOX-mutants as low as in the wild type.

**LOX6 is essential for JA signal production in response to wounding in roots**

Since OPDA is a precursor of JA and JA-Ile, deficiency in basal OPDA accumulation in the _lox6_ mutant might affect stress-induced accumulation of JA and JA-Ile. In wild type roots, as shown in Fig. 2, OPDA levels drop after wounding while JA and JA-Ile transiently accumulate. This suggests that preformed as well as _de novo_ formed OPDA is rapidly converted to JA and JA-Ile. In wounded roots of the _lox6_ mutant levels of JA and JA-Ile were dramatically lower than in the wild type (Fig. 4B, Supplemental Fig. S2). This indicates that LOX6 is not only essential for basal OPDA but also for wound-induced accumulation of JA and JA-Ile in roots. Although _LOX3_ and _LOX4_ are more highly expressed in wounded roots
(Fig. 4A), these LOXs appear not to be involved in root jasmonate production since wound-induced levels of all three jasmonates are not lower in lox3/4 roots in comparison to wild type (Fig. 4B). Also jasmonate amounts in lox2 roots were similar or slightly higher than in wild type roots.

Unlike roots, leaves of the lox6 mutant exhibited a strong increase of OPDA, JA, and JA-Ile after wounding. However, the JA level was significantly lower in wounded lox6 leaves suggesting that LOX6 is involved but not essential for wound-induced accumulation of jasmonates in leaves. In the lox3/4 mutant wound-induced levels of OPDA, JA, and JA-Ile in leaves were similar to the wild type (Fig. 4B) despite a strong up-regulation of both genes by wounding (Fig. 4A). JA accumulated to lower levels in leaves of the lox2 mutant upon wounding. The effect on OPDA levels was even more dramatic since OPDA did not increase in lox2 leaves upon wounding but showed a clear decrease (Fig. 4B). This is in agreement with LOX2 being a major contributor to wound-induced oxylipin production in leaves.

LOX6 is involved in oxylipin formation in roots in response to osmotic and drought stress

Jasmonates accumulate in leaves also in response to stresses such as osmotic stress and drought (Creelman and Mullet, 1995; Seltmann et al., 2010b; de Ollas et al., 2012). Therefore we investigated if LOX6 also contributes to oxylipin synthesis in response to these stress factors. Roots of intact plants grown in the hydroponic system were incubated in 500 mM sorbitol and oxylipin accumulation was analysed after 24 h.

In wild type roots, OPDA levels increased about 3.6-fold after sorbitol treatment (Fig. 5). Also JA and JA-Ile levels were clearly elevated. The increase in JA and JA-Ile in lox2 roots was similar to the wild type while the rise in OPDA was lower. Roots of lox3/4 showed upon osmotic stress wild type levels of all jasmonates. In line with the results on wound stress, lox6 roots exhibited strong differences in oxylipin production to the wild type. lox6 roots did not accumulate OPDA, JA, or JA-Ile after sorbitol treatment. This indicates that LOX6 is also required for oxylipin accumulation in roots in response to osmotic stress.

In leaves of the wild type OPDA levels rose about 3-fold (Fig. 5). JA and JA-Ile strongly accumulated with especially JA showing a dramatic increase up to 52 nmol g⁻¹ dw. No clear elevation of these oxylipins was detected in lox2 leaves consistent with a major role of LOX2 in the response of leaves to osmotic stress. In leaves of lox3/4 all oxylipins rose but to a smaller extend than in the wild type. No differences were obvious in the levels of OPDA and
JA while JA-Ile levels were even slightly higher in leaves of the lox6 mutant after osmotic stress treatment in comparison to wild type. To investigate the contribution of the LOXs to drought stress the liquid medium was removed from plants grown in the hydroponic system and roots were exposed to air. In leaves, JA-Ile levels were at the detection limit and JA levels remained below 0.1 nmol g\(^{-1}\) dw after 48 h drought treatment (Fig. 6). Similarly, no strong increase in OPDA levels was detectable. In contrast, roots responded with an accumulation of OPDA as well as JA. In comparison to osmotic stress, increases were moderate reaching about 7 and 0.3 nmol g\(^{-1}\) dw for OPDA and JA, respectively. JA-Ile levels were at the detection limit but nevertheless tend to rise (Supplemental Fig. S3). Also in lox2 and lox3/4 mutants levels of JA and OPDA increased. In contrast, in lox6 plants OPDA and JA were basal and after drought treatment below the detection limit. This suggests that LOX6 is also responsible for drought-induced accumulation of jasmonates in roots.

**Defect in LOX6 expression results in increased sensitivity to drought**

Drought stress eventually results in wilting and death of the plant. To assess whether the lack of LOX6 leads to altered tolerance to water limitation, plants were grown in soil and at the age of 4 weeks watering was stopped. Wilting symptoms were documented, plants were re-watered and the survival rate was determined. The lox6 mutant showed earlier wilting symptoms in comparison to the wild type. Enhanced sensitivity of lox6 to water limitation was supported by a lower survival rate of plants after re-watering (Fig. 7A,B). Only 14 % of lox6 plants in comparison to 78 % of wild type plants survived the treatment. The altered drought tolerance might be due to lower levels of jasmonates or other 13-LOX products. To distinguish between these possibilities dde2 plants were tested in addition to the lox6 mutant. Interestingly, dde2 plants showed not the wilting and survival phenotype of lox6 but were similar to wild type plants (Fig. 7A,B). This indicates that mechanisms other than lack of OPDA and derivatives of JA are responsible for enhanced drought sensitivity of lox6.

**Defect in LOX6 expression renders roots more attractive to detritivores**

Jasmonates are important signals in defense responses against herbivores and detritivores. In particular it was shown that freshly detached leaves of dde2 plants are consumed by woodlice which usually prefer dead tissue (Farmer and Dubugnon, 2009). Woodlice are soil-living organisms which are in frequent contact with plant roots. Therefore this organism provides a suitable bioassay to test if the lower levels of jasmonates in roots of lox6 result in less defense
against detritivores and render them more attractive as potential food. Leaves and roots of hydroponically grown plants were provided to rough woodlice (*Porcellio scaber*) which were starved for 2 d prior to the experiment. Pictures were taken at different time points. No clear differences were detectable in the consumption of leaves of *lox6* and wild type plants (Fig. 8B). However, in agreement with published data (Farmer and Dubugnon, 2009), *dde2* leaves were consumed faster than wild type leaves (Supplemental Fig. S4B) confirming that jasmonate deficiency of leaves results in enhanced attractiveness. Compared to leaves, roots were eaten considerably slower. Roots of *lox6* plants were consumed more rapidly than roots of wild type plants (Fig. 8A). After 8 d 5% of the original root material was left in *lox6* compared to 36% in wild type, respectively. This indicates that consistent with the result of wound and osmotic stress LOX6 is important to restrain crustacean feeding in roots but not in leaves. Roots of *dde2* plants were also preferred over wild type roots (Supplemental Fig. S4A) suggesting that jasmonates are responsible for the root defense against woodlice.

**DISCUSSON AND CONCLUSION**

**Differential impact of 13-LOXs on oxylipins biosynthesis in roots and leaves**

In this work, the accumulation of jasmonates in leaves and roots after different stress treatments was compared and the LOX isoform involved in root jasmonate synthesis was identified. Roots accumulated JA/JA-Ile in response to wounding, osmotic stress and, to a smaller extend, drought. OPDA levels increased upon osmotic stress and drought but decreased upon wounding. Roots synthesize jasmonates independently of the shoot despite the low expression of several JA-biosynthetic enzymes in Arabidopsis roots. This was concluded from wounding experiments with grafted *dde2* mutant and wild type plants. LOX6 was identified as the main enzyme involved in the production of jasmonates in roots constitutively and upon wound, osmotic and drought stress. The effects were very clear and none of the mutations in the other LOX genes showed a strong effect on jasmonate production in roots although LOX3 and LOX4 are expressed in roots and show induction by wounding (Fig. 4). This indicates that LOX2, 3, and 4 cannot substitute a loss of function of LOX6 in roots even though all four LOXs have been shown to catalyse the formation of 13-HPOTE from linolenate *in vitro*.

Wounding of leaves or osmotic stress applied to roots resulted in accumulation of OPDA, JA, and JA-Ile in leaves which is in agreement with published data (Koo et al., 2009; Zoeller et al., 2012). In leaves, strongest effects on jasmonate levels were observed in *lox2* mutants in which wound- and sorbitol-induced jasmonate accumulation was significantly lower
compared to wild type. This correlates with the very high expression of LOX2 in leaves (Fig. 4, Zimmermann et al., 2004) and indicates that LOX2 is the main enzyme responsible for jasmonate accumulation in leaves after both stresses. LOX2 is involved in the accumulation of the bulk of free jasmonates after wounding, osmotic stress, and during senescence but not after pathogen attack (Bell et al., 1995; Glauser et al., 2009; Seltmann et al., 2010b; Zoeller et al., 2012). Lower JA accumulation compared to wild type leaves was also detectable in \textit{lox6} mutant leaves after wounding and in \textit{lox3/4} mutant leaves after osmotic stress. This suggests that LOX3, 4, and 6 contribute to stress-induced JA-biosynthesis in leaves and that the contribution depends on the specific stress-induced condition. This is in agreement with a recent report that all 13-LOXs contribute to the increase of JA in wounded leaves at a very early time point (190 s) (Chauvin et al., 2013). Unexpectedly, we found that synthesis of basal OPDA was completely prevented in the \textit{lox6} mutant. This indicates that LOX6 is important for OPDA formation in leaves although basal expression of \textit{LOX6} is very low (Fig. 4, Zimmermann et al., 2004; Vellosillo et al., 2007). Since LOX2 is highly expressed constitutively in leaves but cannot ensure basal production of OPDA, oxylipin biosynthesis is regulated on other levels than transcription of biosynthetic genes.

**How is the stress-induced synthesis of jasmonates regulated?**

As discussed above expression of 13-LOXs did not always correlate with accumulation of jasmonates. In addition, changes of JA were not necessarily concomitant with OPDA. For instance, JA and JA-Ile levels increased in \textit{lox6} leaves despite very low basal levels of OPDA and vice versa increase of JA was reduced in the \textit{lox2} mutant even though basal levels of the precursor OPDA were available. Different mechanisms might account for these phenomena. Firstly, enzymatic activity might be regulated posttranslationally. The requirement for regulation of the activity of enzymes which are already present is also obvious from the very fast accumulation of jasmonates upon wounding (Glauser et al., 2009). Regarding the regulation mechanism of LOX2 it was reported that the ion channel FOU2 regulates activity of LOX2 in response to wounding (Bonaventure et al., 2007; Beyhl et al., 2009). Secondly, different LOXs may generate separate pools of 13-HPOTE with different metabolic fates. 13-HPOTE might be used for partially competing metabolic routes eventually leading to the accumulation of arabidopside, OPDA, JA, and its metabolites or channeled into other oxylipin pathways such as the hydroperoxy lyase or peroxygenase pathways (Feussner and Wasternack, 2002). For example in roots, only LOX6 products are channeled into the jasmonate pathway while LOX3 and LOX4 products might be metabolized to
oxylipins other than jasmonates and serve other functions. Thirdly, the conversion of OPDA to JA might be regulated. In roots OPDA levels decrease in response to wounding concomitant with an increase in JA. This suggests that OPDA constitutively present in roots is used for JA synthesis. This is in accordance with a lack of JA accumulation in roots of lox6 mutants where the basal OPDA level is at the detection limit. However, the decrease in OPDA (less than 0.7 nmol g\(^{-1}\) dw at 30 min) is not sufficient to account for the increase in JA (more than 3 nmol g\(^{-1}\) dw, Fig. 2 and 4) suggesting that de novo synthesis of OPDA is necessary. Changes in oxylipin levels similar to the changes in wounded roots have been demonstrated in unwounded, distal leaves of wounded plants. Also in this system, accumulation of JA and JA-Ile in distal leaves correlated with a decrease in OPDA (Koo et al., 2009). This indicates that the regulated step of JA production in both scenarios is the conversion from OPDA to JA. In Arabidopsis OPR3 is the first enzyme of this pathway catalysing the reduction of OPDA. OPR3 activity has been discussed to be regulated posttranslationally by homodimerization (Breithaupt et al., 2006). In wounded roots as well as distal leaves of wounded plants OPDA is present and OPR3 is expressed which is consistent with a regulation of JA production by affecting OPR3 activity on a posttranslational level. In the case of osmotic and drought stress the process of jasmonate production seems to be regulated by different mechanisms because here OPDA levels increase to similar (osmotic) or even higher (drought) levels than JA. It has to be taken into account that in contrast to the early time point measured after wounding, oxylipin levels were detected after 24 h of sorbitol or 48 h after drought treatment, respectively. Therefore, timing, oxylipin profiles and regulation of oxylipin synthesis of the responses to these different stresses are obviously different.

**Function of oxylipins in roots**

There are several indications for a role of oxylipins in root responses to biotic and abiotic factors. Accumulation of jasmonates has been observed in Medicago in the mutualistic interactions with soil-born microorganisms. Using AOC-RNAi approaches it was demonstrated that jasmonate synthesis is important for the development of arbuscular mycorrhizal symbiosis but not for nodule formation (Isayenkov et al., 2005; Zdyb et al., 2011). In addition, it was shown that jasmonate synthesis is important for defense in Arabidopsis and maize against Pythium species which are soil-borne oomycetes. In Arabidopsis fad3/7/8 and jar1 mutants and in maize an opr7/8 double mutant are more susceptible to Pythium (Staswick et al., 1998; Vijayan et al., 1998; Yan et al., 2012). The jar1 mutant also develops more disease symptoms after challenge with *Phytophthora parasitica*
Furthermore, altering the expression of LOX3 in maize renders plants more susceptible to root-knot nematodes (Gao et al., 2008). Even though it was not explicitly shown that jasmonate production in the root is relevant, the fact that these organisms originated from soil and that in the experiments the microorganism/nematodes were applied to roots suggests that root-derived jasmonates are important in these interactions.

The identification of LOX6 as the one LOX responsible for stress-induced oxylipin accumulation in roots enabled the investigation of the function of oxylipins specifically in roots using the lox6 mutant. The experiments show that oxylipins in roots are involved in detritivore defense and drought tolerance. Detritivorous crustacean feed faster on roots of lox6 and dde2 mutant plants (Fig. 8, Supplemental Fig. S4) and on leaves of dde2 (Farmer and Dubugnon, 2009, Supplemental Fig. S4). The fact that lox6 and dde2 mutations have similar effects indicates that jasmonates are responsible to deter crustacean feeding in the wild type. Restraining detritivore attack might be even more important in roots than in leaves because roots are more accessible to this soil organism. The molecular basis of lower attractiveness of wild type roots in comparison to dde2 and lox6 roots is not clear. For leaves of different plant species it was demonstrated that jasmonates are important to induce secondary metabolism and expression of defense proteins (Howe et al., 1996; Reymond et al., 2000), reviewed in Browse and Howe (2008). Comparison of the metabolite profile of wild type and lox6 roots will contribute to understand the mechanisms involved.

In contrast to animal feeding, drought tolerance was impaired in lox6 but not in dde2. This suggests that oxylipins different from jasmonates are important for drought tolerance. The product of 13-LOX, 13-HPOTE can be converted to a variety of oxylipins such as ketones, aldehydes, epoxides, hydroxides, and divinyl ethers (Feussner and Wasternack, 2002). Several of these compounds have been shown to be biologically active for instance in regulating root growth and gene expression (Vellosillo et al., 2007). It will be interesting to elucidate which and how products of LOX6 regulate drought tolerance. Possibilities are the formation of a long distance signal in the root which signals water limitation to the leaves. The involvement of oxylipins in root to shoot communication has also been suggested in response to wounding (Hasegawa et al., 2011). Alternatively, LOX6 derived products might directly play a role in regulating stomatal opening. Oxylipins such as coronatine, JA, and its methyl ester are discussed to regulate stomatal closure (Suhita et al., 2004; Melotto et al., 2006). Interestingly, LOX6 has been described to be expressed in guard cells (Leonhardt et al., 2004) which points
to a role in stoma function. It is a challenge for the future to identify the oxylipins and mechanisms responsible for the impact of LOX6 on drought tolerance.

MATERIALS AND METHODS

Plant material
Arabidopsis thaliana wild type ecotype Columbia (Col-0), dde2, and LOX mutant lines lox2, lox3/4, lox6 were used. The lox2 (Glauser et al., 2009), lox3/4, and both lox6 mutants (Caldelari et al., 2011; Chauvin et al., 2013) were kindly provided by E. Farmer. The lox6 mutant analysed throughout the manuscript corresponds to the line SALK_138907. Absence of LOX6 expression is shown in Supplemental Fig. S5. The lox6 line used in Supplemental Fig. S2 corresponds to SALK_083650. The identity of both lox6 mutants was confirmed by PCR analysis using primers suggested by the iSect tool (http://signal.salk.edu/tdnaprimers.2.html). The dde2-2 mutant (Malek et al., 2002) was kindly provided by B. Keller. For the dde2 and lox3/4 mutant the male sterile phenotype was monitored.

For investigations plants were grown in soil at 22°C under a 9 h photoperiod (100 µmol photons m⁻² s⁻¹) in a climate chamber or hydroponically in a plant cabinet.

Hydroponic cultivation
The hydroponic system was set up according to Tocquin (2003). Seeds were surface sterilized and sown on 0.5 ml micro tubes filled with the standard nutrient solution described in Tocquin (2003) with 1% Phyto Agar (Duchefa) and cultivated in a sterile box which was placed in a plant cabinet for 14 d at 20°C under a 9 h photoperiod (80 µmol photons m⁻² s⁻¹). For further cultivation 2 mm of the micro tube bottom was removed and the tube placed in a sterile 50 ml Falcon containing sterile liquid standard nutrient solution. To avoid the exposure of roots to light the Falcons were placed in a light-tight box (internal dimensions: 152x104x102 mm) with the micro tube placed through a hole in the lid of the box. The boxes, each containing 11 plants, were cultivated for further 4 weeks at 20°C under a 9 h photoperiod (80 µmol photons m⁻² s⁻¹) in a plant cabinet. One week before harvest the standard nutrient solution was refilled.
Grafting of Arabidopsis plants

Arabidopsis Col-0 wild type plants and dde 2 plants were grafted using 7 d old seedlings based on a micrografting technique described by Turnbull et al. (2002). Briefly, surface sterilized seeds were sown in petri dishes on Murashige and Skoog medium containing 1.5 % sucrose. Seeds were stratified for 2 d at 4°C in the dark and then petri dishes with seeds were transferred to a growth cabinet with 24°C and constant light (40 µmol photons m\(^{-2}\) s\(^{-1}\)) and placed in a vertical orientation. After 5 d light intensity was reduced to 25 µmol photons m\(^{-2}\) s\(^{-1}\). For grafting seedlings were placed under a binocular and cut at the hypocotyl with a razor blade. Shoots and roots were placed in intimate contact on new petri dishes with Murashige and Skoog medium containing 0.5 % sucrose. After 7 d growing on vertically oriented plates in a growth cabinet at 24°C under constant light (25 µmol photons m\(^{-2}\) s\(^{-1}\)) all successful grafted plants were transferred to petri dishes with Murashige and Skoog medium containing no sucrose and grown for further 14 d at 20°C under a 9 h photoperiod (80 µmol photons m\(^{-2}\) s\(^{-1}\)) in a plant cabinet. All surviving plants were then transferred to the hydroponic system and cultivated until they were 8 weeks old.

Wounding and sorbitol treatment of plants

For wounding experiments 6 to 8 weeks old, hydroponically cultivated plants were used. First the plants were cut above and below the micro tubes, in which they had been sown for germination, to obtain the separated shoot and root. The part of the hypocotyl was removed. The shoots were squeezed with a forceps once across every leaf blade and the roots were squeezed five times along its full length. Wounded shoots were incubated in the light, roots in the dark until shock freezing in liquid nitrogen and stored at -80°C for further analysis. Each replicate consisted of shoots/roots of at least three to four plants.

For sorbitol treatment the standard liquid media of 6 week old hydroponically cultivated plants were replaced by 0.5 M sorbitol solution or water (control). After 24 h incubation the roots were washed with water and shoots and roots were harvested as described above, shock frozen in liquid nitrogen, and stored at -80°C for further analysis. Each replicate consisted of shoots/roots of at least three to four plants.

RNA-isolation and quantitative RT-PCR

Total RNA was extracted from ground plant material using TriFast™ reagent (PEQLAB, Erlangen, Germany) according to the manufacturer’s protocol. RNA concentration was determined spectrophotometically. Remaining DNA was removed using RNase-free DNase I
(Fermentas, St. Leon-Rot, Germany) according to the manufacturer’s protocol. First-strand

cDNA and real time PCR were performed as described previously (Szyroki et al., 2001) using

SYBR-Green Capillary Mix (ThermoFisher Scientific, Hamburg, Germany) and a CFX 96™

Real-Time System C1000™ Thermal Cycler (Bio-Rad, California, USA). Primers used (TIB

MOLBIOL, Berlin, Germany) were:

LOX2 (At3g45140): fwd 5’-GCCATTGAGTTGACTTGTCC-3’,

rev 5’-CACTTAGTTGTCTATTTGCCGC -3’;

LOX3 (At1g17420): fwd 5’-TCCCTGCCATCTAA-3’,

rev 5’-GTTTGGGACGTAAGCCA-3’

LOX4 (At1g72520) : fwd 5’-GCTTGCTTAGATACGACACT -3’,

rev 5’-ATG TGCTTCCCGTGAGAGC-3’

LOX6 (At1g67560) : fwd 5’-AAGACTGTTACTGCGTGTG -3’,

rev 5’-GGCTGTGAATACGAGGTATC -3’

The number of transcripts was normalized to AtSAND (At2g28390) cDNA fragments

(Czechowski, 2005) amplified by AtSANDfwd (5’-AACTCTATGCAGCATT-3’) and

AtSANDrev (5’-GGTGGGTACTACGACACA-3’) primers.

Analysis of JA, JA-Ile, and OPDA in A. thaliana

For analysis of oxylipins fresh (shock frozen) plant material was ground by mortar and pestle

and afterwards freeze dried. 25 mg shoots or 18 mg roots of freeze-dried material were

extracted with 950 µl of ethyl acetate/formic acid (99:1, v/v). DihydroJA (dhJA, 50 ng), JA-
norvaline (JA-Nval, 50 ng), and [18O2] OPDA (50 ng) were added as internal standards.

Extraction samples were homogenized with a ball mill for 3 min at 20 Hz. After

centrifugation, the supernatant was dried in a vacuum concentrator and the extraction step was

repeated with 1 ml ethyl acetate/formic acid (99:1, v/v). After evaporation of the ethyl acetate,

samples were dissolved in 40 µl acetonitrile/H2O (50:50, v/v) for LC-MS/MS analysis.

Ultra high performance liquid chromatography (UPLC)/tandem mass spectrometry (MS/MS)

analyses were performed on a Waters Quattro Premier XE triple-quadrupole mass

spectrometer with an electrospray interface (ESI) coupled to a Waters Aquity UPLC (Milford,

MA, USA). Chromatographic separations were carried out using a Aquity UPLC BEH C18

column (2.1 x 50 mm, 1.7 μm particle size with a BEH C18 guard column equipped with

prefilter, Waters, Milford, MA, USA) with the following solvent system: solvent A = 0.1 %

formic acid in water, solvent B = acetonitrile. A gradient elution was performed at a flow rate

of 0.25 ml min⁻¹ at 40°C: 97 % A, followed by 0 % A in 7 min.
The ESI source was operated in negative ionization mode with a capillary voltage of 3.0 kV at 120°C. Quantification was performed using multiple reaction-monitoring (MRM) with a scan time of 0.025 s per transition (see Supplemental Table 1). Cone voltage and collision energy was set 20 eV, the desolvation temperature was 400°C. Nitrogen was used as the desolvation and cone gas with a flow rate of 800 and 50 L h⁻¹, respectively. Argon was used as the collision gas at a pressure of approximately 3.10 × 10⁻³ bar.

**Analysis of free hydroxy fatty acids**

Analysis of free hydroxy fatty acids was performed similarly as described in (Zoeller et al., 2012). Briefly, fresh (shock frozen) plant material was ground by mortar and pestle and afterwards freeze dried. For plant shoot 25 mg, for plant roots 18 mg of freeze-dried material were extracted with 800 µl isopropanol containing 0.5 mg triphenylphosphane and 0.75 mg butylated hydroxytoluene. After 20 min reduction 15-HEDE (150 ng) was added as internal standard. Samples were sonicated for 5 min and centrifuged. The supernatant was recovered and the residue was further extracted with 1.5 ml chloroform/isopropanol (1:2, v/v), and 1.5 ml chloroform/methanol (2:1, v/v). After each extraction, samples were centrifuged and the supernatants were combined. The combined lipid extract was dried under a stream of nitrogen at 60°C and reconstituted in 40 µl 1 mM ammonium acetate in water/acetonitrile (1:2, v/v) for LC-MS/MS analysis.

Ultra high performance liquid chromatography (UPLC)/tandem mass spectrometry (MS/MS) analyses were performed on a Waters Quattro Premier XE triple-quadrupole mass spectrometer with an electrospray interface (ESI) coupled to a Waters Aquity UPLC (Milford, MA, USA). Chromatographic separations were carried out using a Aquity UPLC BEH C18 column (2.1 x 50 mm, 1.7 µm particle size with a BEH C18 guard column equipped with prefilter, Waters, Milford, MA, USA) with the following solvent system: solvent A = 1 mM aqueous ammonium acetate, solvent B = acetonitrile. A gradient elution was performed at a flow rate of 0.25 ml min⁻¹ at 40°C: 65 % A, followed by 30 % A in 6 min.

The ESI 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 (MRM) with a scan time of 0.025 s per transition (see Supplemental Table 1). Cone voltage and collision energy was set 20 eV, desolvation temperature was 350°C. Nitrogen was used as the desolvation and cone gas with a flow rate of 800 and 50 L h⁻¹, respectively. Argon was used as the collision gas at a pressure of approximately 3.10 × 10⁻³ bar.
**Detritivore feeding**

The detritivores rough woodlouse (*Porcellio scaber*) were collected and cultivated as described in Farmer and Dubugnon (2009). For the feeding assay detached leaves and roots of 6 week old hydroponically cultivated plants were used. Approximately 15 isopods were fed with the detached plant organs. For quantification digital pictures were taken including a 5 cm² size marker. The area of leaves and roots was calculated with Photoshop7 (Adobe).

**Drought resistance experiments**

To investigate drought tolerance 4 week old plants grown in 30 ml soil (“Einheitserde P”, Klasmann-Deilmann, Geeste, Germany; pot dimensions: 5 cm height, 7 cm diameter at the top, 4.5 cm diameter at the bottom) at 22°C under a 9 h photoperiod (100 μmol photons m⁻² s⁻¹) in a climate chamber were watered for 16 h. The remaining water was removed and the plants were grown for 25 d without watering. When all plants showed wilting symptoms plants were re-watered and the survival rate was calculated after two days. For oxylipin analysis the liquid medium of 6 week old hydroponically grown plants was removed and the plants were further cultivated at 20°C under a 9 h photoperiod (80 μmol photons m⁻² s⁻¹) in a plant cabinet. After 48 h incubation the shoots and roots were harvested, shock frozen in liquid nitrogen and stored at -80°C.

**Statistical analysis**

For statistical analysis of differences between the wild type and several mutants one-way ANOVAs (Fisher's Least Significant Difference (LSD) test) were performed with SPSS statistics software (IBM).

**Accession numbers and websites**

In addition to the analyses performed by qPCR, information on gene regulation was obtained using the web site [www.genevestigator.ethz.ch](http://www.genevestigator.ethz.ch) (Zimmermann et al., 2004).

**ACKNOWLEDGMENTS**

We are grateful to E.E. Farmer for his help in setting up the woodlice system and providing lox mutants.
LITERATURE CITED


Browse J (2009a) The power of mutants for investigating jasmonate biosynthesis and signaling. Phytochemistry 70: 1539-1546


Figure legends

**Figure 1:** Oxylipin levels in roots and leaves. Roots and shoots of 6 week old hydroponically grown plants were harvested and free OPDA, JA, JA-Ile and hydroxy fatty acids were determined. Data represent the mean of at least 3 biological replicates ± sd. The experiment has been repeated with similar results.

**Figure 2:** Oxylipin accumulation in roots and leaves in response to wounding. Roots and shoots were separated, wounded and harvested after 30 min, 2 h and 24 h after wounding. 0 h indicates untreated plant material. Data represent the mean of at least 3 biological replicates ± sd. The experiment has been repeated with similar results.

**Figure 3:** Oxylipin accumulation in partially jasmonate-deficient plants. Levels of OPDA, JA, and JA-Ile in shoots and roots of dde2 shoot grafted on Col-0 root stock (left) and of Col-0 shoot grafted on dde2 root stock (middle) basal and 30 min after wounding. Control grafts were performed with Col-0 shoots on Col-0 roots (right). Black bars: non-wounded; grey bars: wounded. Data represent the mean of at least 3 biological replicates ± sd. The experiment has been repeated with similar results.

**Figure 4:** Involvement of 13-LOXs in the wound response in roots and leaves. Roots and shoots were separated, wounded and harvested after 30 min (grey bars). As control untreated plant samples were analysed (black bars). Transcript levels of LOX2, LOX3, LOX4, and LOX6 were determined by qRT-PCR (A). Levels of OPDA, JA, and JA-Ile were determined in Col-0 and different LOX mutants (B). Data represent the mean of at least 3 biological replicates ± sd. Stars indicate significant differences of the level in the mutant compared to the corresponding wild type sample (* p< 0.05; ** p<0.01). The experiment has been repeated with similar results.

**Figure 5:** Osmotic stress-induced oxylipin accumulation in roots and shoots of Col-0 and different LOX mutants. Levels of OPDA, JA, and JA-Ile were determined 24 h after replacing the medium with 500 mM sorbitol (grey bars). Medium of control plants was replaced with water. Data represent the mean of at least 3 biological replicates ± sd. Stars indicate significant differences of the level in the mutant compared to the corresponding wild type sample (* p< 0.05; ** p<0.01). The experiment has been repeated with similar results.

**Figure 6:** Drought-induced oxylipin accumulation in roots and shoots of Col-0 and different LOX mutants. Levels of OPDA and JA were determined in untreated plant samples (black bars) and 48 h after removing the liquid medium from the roots (grey bars). Data represent the mean of at least 3 biological replicates ± sd. Stars indicate significant differences of the level
in the mutant compared to the corresponding wild type sample (* p< 0.05; ** p<0.01). The experiment has been repeated with similar results.

**Figure 7: Wilting phenotype of Col-0, lox6 and dde2**

Drought-stressed plants were re-watered and pictures were taken 2 d after re-watering (A) and the surviving plants were scored (B). Data represent the percentage of survived relative to total plants. Shown is mean of at least 3 biological replicates ± sd. The experiment has been repeated with similar results.

**Figure 8: Feeding of P. scaber on roots of Col-0 and lox6**

Roots (A) and leaves (B) of wild type and lox6 plants were provided to *P. scaber* and pictures were taken at 0 h and after 4 d (leaves) and 8 d (roots). The area of roots (C) and leaves (D) was quantified at the beginning of the experiment and at the time points indicated. Data represent the area relative to the original area. The experiment has been repeated with similar results.
**Supplemental Material**

**Supplemental Table S1**: Mass to charge proportions of parent- and daughter ions from analysed molecules

**Supplemental Figure S1**: Basal and wound-induced arabidopside levels in Col-0 roots and shoots. Roots and shoots of 6 week old hydroponically grown plants were separated, wounded and harvested after 30 min (grey bars). As control untreated plant samples were analysed (black bars). Levels of arabidopside A, B, C, D, E, and G were determined. Data represent the mean of at least 3 biological replicates ± sd.

**Supplemental Figure S2**: Involvement of LOX6 in the wound response in roots and leaves. Roots and shoots of 6 week old hydroponically grown plants were separated, wounded and harvested after 30 min (grey bars). As control untreated plant samples were analysed (black bars). Levels of OPDA, JA, and JA-Ile were determined in Col-0 and lox6B mutant plants. Data represent the mean of at least 4 biological replicates ± sd.

**Supplemental Figure S3**: Drought-induced oxylipin accumulation in roots of Col-0 and different LOX mutants. Levels of JA-Ile were determined in untreated plant samples (black bars) and 48 h after removing the liquid medium from the roots (grey bars). Data represent the mean of at least 3 biological replicates ± sd. Stars indicate significant differences of the level in the mutant compared to the corresponding wild type sample (*p<0.05; **p<0.01). The experiment has been repeated with similar results.

**Supplemental Figure S4**: Feeding of *P. scaber* on *dde2* and Col-0

Roots (A) and leaves (B) of wild type and *dde2* were provided to *P. scaber* and pictures were taken at the beginning of the experiment and at the time points indicated. The experiment has been repeated with similar results.

**Supplemental Figure S5**: Transcript levels of *LOX6* in Col-0 and the *lox6* mutant plants.

Roots and shoots of 6 week old hydroponically grown plants were harvested and transcript levels were determined by qRT-PCR. Data represent the mean of at least 3 biological replicates ± sd. The experiment has been repeated with similar results.
**Figure 1:** Oxylipin levels in roots and leaves. Roots and shoots of 6 week old hydroponically grown plants were harvested and free OPDA, JA, JA-Ile and hydroxy fatty acids were determined. Data represent the mean of at least 3 biological replicates ± sd. The experiment has been repeated with similar results.
Figure 2: Oxylin accumulation in roots and leaves in response to wounding. Roots and shoots were separated, wounded and harvested after 30 min, 2 h and 24 h after wounding. 0 h indicates untreated plant material. Data represent the mean of at least 3 biological replicates ± sd. The experiment has been repeated with similar results.
Figure 3: Oxylipin accumulation in partially jasmonate-deficient plants. Levels of OPDA, JA, and JA-Ile in shoots and roots of dde2 shoot grafted on Col-0 root stock (left) and of Col-0 shoot grafted on dde2 root stock (middle) basal and 30 min after wounding. Control grafts were performed with Col-0 shoots on Col-0 roots (right). Black bars: non-wounded; grey bars: wounded. Data represent the mean of at least 3 biological replicates + sd. The experiment has been replicated with similar results.
Figure 4: Involvement of 13-LOXs in the wound response in roots and leaves. Roots and shoots were separated, wounded and harvested after 30 min (grey bars). As control untreated plant samples were analysed (black bars). Transcript levels of LOX2, LOX3, LOX4, and LOX6 were determined by qRT-PCR (A). Levels of OPDA, JA, and JA-Ile were determined in Col-0 and different LOX mutants (B). Data represent the mean of at least 3 biological replicates ± sd. Stars indicate significant differences of the level in the mutant compared to the corresponding wild type sample (* p< 0.05; ** p<0.01). The experiment has been repeated with similar results.
Figure 5: Osmotic stress-induced oxylipin accumulation in roots and shoots of Col-0 and different LOX mutants. Levels of OPDA, JA and JA-Ile were determined 24 h after replacing the medium with 500 mM sorbitol (grey bars). Medium of control plants was replaced with water. Data represent the mean of at least 3 biological replicates ± sd. Stars indicate significant differences of the level in the mutant compared to the corresponding wild type sample (* p< 0.05; ** p<0.01). The experiment has been repeated with similar results.
**Figure 6:** Drought-induced oxylipin accumulation in roots and shoots of Col-0 and different LOX mutants. Levels of OPDA and JA were determined in untreated plant samples (black bars) and 48 h after removing the liquid medium from the roots (grey bars). Data represent the mean of at least 3 biological replicates ± sd. Stars indicate significant differences of the level in the mutant compared to the corresponding wild type sample (* p< 0.05; ** p<0.01). The experiment has been repeated with similar results.
**Figure 7:** Wilting phenotype of Col-0, *lox6* and *dde2*

Drought-stressed plants were re-watered and pictures were taken 2 d after re-watering (A) and the surviving plants were scored (B). Data represent the percentage of survived relative to total plants. Shown is mean of at least 3 biological replicates ± sd. The experiment has been repeated with similar results.
Figure 8: Feeding of *P. scaber* on roots and leaves of Col-0 and lox6

Roots (A) and leaves (B) of wild type and *lox6* were provided to *P. scaber* and pictures were taken at 0 h and after 4 d (leaves) and 8 d (roots). The area of roots (C) and leaves (D) was quantified at the beginning of the experiment and at the time points indicated. Data represent the area relative to the original area. Shown is the mean of at least 3 biological replicates ± sd. The experiment has been repeated with similar results.