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Lipid metabolism in arbuscular mycorrhizal roots of *Medicago truncatula*

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Abstract

The peroxidation of polyunsaturated fatty acids, common to all eukaryotes, is mostly catalyzed by members of the lipoxygenase enzyme family of non-heme iron containing dioxygenases. Lipoxygenase products can be metabolized further in the oxylipin pathway by several groups of *CYP74* enzymes. One prominent oxylipin is jasmonic acid (JA), a product of the 13-allene oxide synthase branch of the pathway and known as signaling substance that plays a role in vegetative and propagative plant development as well as in plant responses to wounding and pathogen attack. In barley roots, JA level increases upon colonization by arbuscular mycorrhizal fungi. Apart from this first result regarding JA, no information is available on the relevance of lipidperoxide metabolism in arbuscular mycorrhizal symbiosis. Thus we analyzed fatty acid and lipidperoxide patterns in roots of *Medicago truncatula* during mycorrhizal colonization. Levels of fungus-specific fatty acids as well as palmitic acid (16:0) and oleic acid (18:1 $n-9$) were increased in mycorrhizal roots. Thus the degree of arbuscular mycorrhizal colonization of roots can be estimated via analysis of fungal specific esterified fatty acids. Otherwise, no significant changes were found in the profiles of esterified and free fatty acids. The 9- and 13-LOX products of linoleic and α-linolenic acid were present in all root samples, but did not show significant differences between mycorrhizal and non-mycorrhizal roots, except JA which showed elevated levels in mycorrhizal roots. In both types of roots levels of 13-LOX products were higher than those of 9-LOX products. In addition, three cDNAs encoding *CYP74* enzymes, two 9/13-hydroperoxide lyases and a 13-allene oxide synthase, were isolated and characterized. The transcript accumulation of these three genes, however, was not increased in mycorrhizal roots of *M. truncatula*.

Keywords: *Medicago truncatula*; *Glomus intraradices*; Allene oxide synthase; Hydroperoxide lyase; Fatty acid methyl ester profiles; 16:1ω5; Jasmonic acid

1. Introduction

Peroxidation of polyunsaturated fatty acids (PUFAs), mostly catalyzed by members of the lipoxygenase (LOX) enzyme family of non-heme iron containing dioxygenases, is common to all eukaryotes (Brash, 1999). The LOX-derived hydroperoxy PUFAs are substrates of at least seven different enzyme families whose action leads to the synthesis of a diverse group of metabolites collectively called oxylipins, which includes

Abbreviations: AOS, allene oxide synthase; DES, divinylether synthase; 13-H(P)OTE, (13*S*)-hydro(pero)xy linolenic acid; 9-H(P)ODE, (9*S*)-hydro(pero)xy linoleic acid; HPL, hydroperoxide lyase; JA, jasmonic acid; KOD, keto linoleic acid; KOT, keto linolenic acid; LOX, lipoxygenase; OPDA, 12-oxo-phytodienoic acid; PUFAs, polyunsaturated fatty acids; TAG, triacylglycerols.

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signaling compounds such as jasmonic acid (JA), antimicrobial compounds such as divinyl ethers, and a plant-specific blend of volatiles including leaf aldehydes and alcohols (Blée, 1998; Farmer et al., 2003). The metabolism of PUFAs via LOXs and the subsequent reactions are collectively named LOX pathway (Fig. 1), which is involved in plant development as well as in plant reaction to diverse environmental stresses including pathogen attack (Feussner and Wasternack, 2002).

In plants, there are two different types of LOXs, those that oxygenate linoleic acid or α-linolenic acid at C-9 of the hydrocarbon backbone (9-LOXs), leading to the formation of the (9*S*)-hydroperoxy derivatives (9-HPOD and 9-HPOT) and those that oxygenate at C-13 (13-LOXs), leading to the formation of the (13*S*)-hydroperoxy derivatives (13-HPOD and 13-HPOT). LOX enzymes without a transit peptide for transport into chloroplasts are designated type 1 LOXs; this type includes 9-LOXs and 13-LOXs which can be soluble (cytosolic or vacuolar) or associated with lipid body, microsomal or plasma membranes. Up to now, all LOXs characterized that contain a plastidic transit peptide (type 2 LOXs) are 13-LOXs (Feussner and Wasternack, 2002).

Out of the seven routes, five major ones for the metabolization of LOX reaction products have been characterized in plants (Fig. 1). Three of them involve enzymes belonging to the same P450-containing monooxygenase family, the *CYP74* family, namely allene oxide synthase (AOS), hydroperoxide lyase (HPL) and divinylether synthase (DES) (Feussner and Wasternack, 2002). The reaction mechanisms of AOS, HPL and DES are similar in so far that they all share an intermediate epoxy allylic carbocation formed from the acyl hydroperoxide (Noordermeer et al., 2001). Afterwards, however, AOS and DES deprotonate the carbocation at different positions leading to stable derivatives, whereas HPLs catalyze a rearrangement of the positive charge leading to a fragmentation of

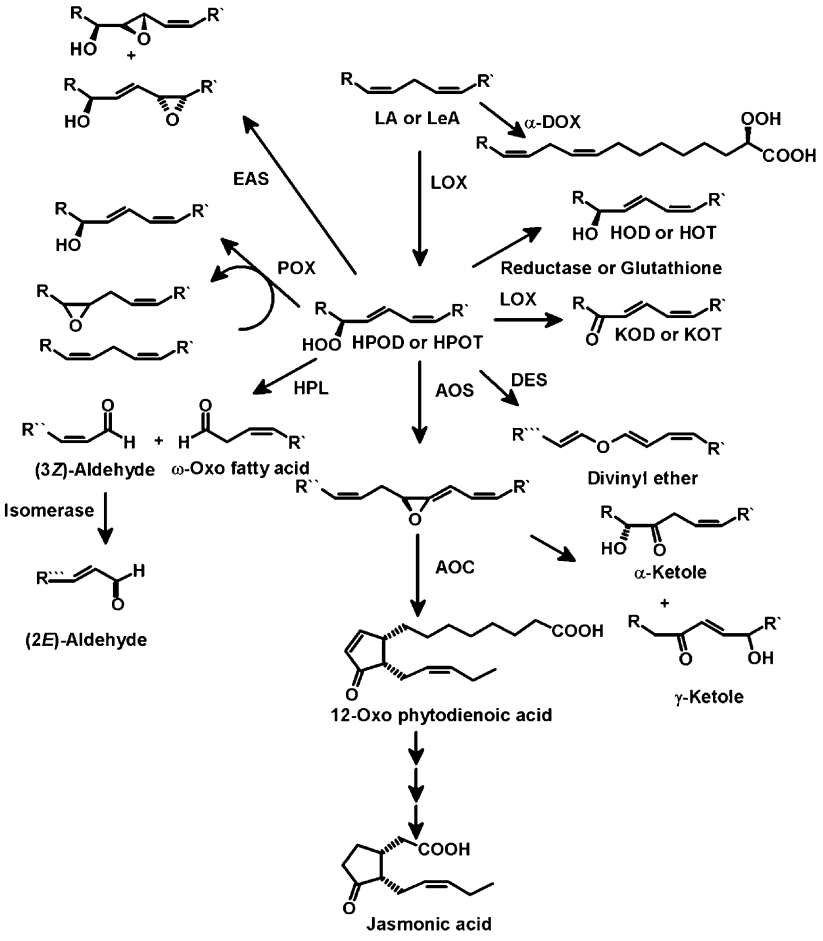

Fig. 1. Oxylipin pathway. Peroxidation of PUFAs by LOXs leads to 9-LOX-derived and 13-LOX-derived hydro(pero)xy PUFAs that are the substrates of allene oxide synthase (AOS), divinyl ether synthase (DES), hydroperoxide lyase (HPL), α-dioxygenase (DOX), epoxy alcohol synthase (EAS), and peroxygenase (POX). LA, linoleic acid; LeA, linolenic acid.

the molecule into an aldehyde and an ω-oxo-fatty acid (Noordermeer et al., 2001). Many *CYP74* enzymes known so far were localized in the envelope of plastids based on the fact that they contain plastidic signal sequences. However, there are exceptions, e.g., AOS from guayule which is assumed to be located in the cytoplasm (Pan et al., 1995). Substrate specificity in the *CYP74* family is variable; there are 9-AOSs (Itoh et al., 2002) as well as 13-HPLs and 13-AOSs (Howe and Schilmiller, 2002) as well as HPLs or AOSs with indeterminate substrate specificity (Matsui et al., 2000; Maucher et al., 2000), although no 9-HPL has been isolated to date. From DES, up to now only 9-DESs have been characterized (Itoh and Howe, 2001; Stumpe et al., 2001). However, since it is still unknown whether LOX-reaction products can be exchanged between compartments, the role of the different *CYP74* enzymes in the synthesis of different oxylipins is not yet clear.

JA, its methyl ester and precursor 12-oxo phytodienoic acid (OPDA) as well as its alternative precursor dinor-OPDA, a product of roughanic acid (16:3) oxidation, are signaling compounds that play a role in vegetative and propagative plant development. Levels of JA have been shown to increase in response to wounding and microbial attack (Howe and Schilmiller, 2002; Wasternack and Hause, 2002). However, JA has also been linked to symbiotic plant–microbe interactions; increased JA (but not OPDA) contents were shown for arbuscular mycorrhizal roots of barley in comparison with non-mycorrhizal roots (Hause et al., 2002).

Arbuscular mycorrhizal symbioses are established between the roots of more than 80% of terrestrial plant species and fungi of the order Glomales. Fungal hyphae enter the root and subsequently form highly branched structures, the arbuscules, within root cortical cells. The plant supplies the microsymbiont with carbohydrates, while the fungus provides the plant with phosphate and other minerals; nutrient exchange takes place in arbuscules and intraradical hyphae (Harrison and Baldwin, 2004). In barley, expression of a *9/13-AOS* gene, as well as the expression of a JA-induced gene, *JIP23*, is induced in arbuscule-containing root cortical cells, indicating that arbuscule formation is accompanied by JA production (Hause et al., 2002).

Apart from these first results regarding JA, no information is available on the relevance of oxylipin metabolism for the development of an arbuscular mycorrhizal symbiosis. Furthermore, up to now oxylipin profiling and studies on the expression of genes encoding LOX pathway enzymes have mostly been restricted to aboveground organs. The aim of this study was to obtain a profile of oxylipins in roots and of the changes in oxylipin metabolism during mycorrhizal colonization of plant roots. For this purpose, *Medicago truncatula* plants were grown in expanded clay with and without *Glomus intraradices*, and root material was harvested once per week in order to obtain a time course of changes in oxylipin profiles. Further, three cDNAs representing genes encoding *CYP74* enzymes were isolated from *M. truncatula*, and their expression patterns and the specificities of the encoded enzymes were analyzed.

2. Results

Lipid profiles were determined in three independent time course experiments involving mycorrhizal plants grown in substrate containing *G. intraradices* and non-mycorrhizal plants grown in sterilized substrate. Before harvest, the degree of mycorrhizal colonization was determined in at least five inoculated root systems after staining according to Vierheilig et al. (1998). The results were consistent in most respects except that absolute JA contents varied between experiments.

2.1. Fatty acid profiles of non-mycorrhizal roots and of roots colonized by G. intraradices

A comparison between esterified and free fatty acids in mycorrhizal versus non-mycorrhizal roots showed that the esterified fraction contained fungus-specific fatty acids: (11*Z*)-hexadecenoic acid (16:1 $n-5$), (11*Z*)-eicosenoic acid (20:1 $n-9$), dihomo-γ-linolenic acid (20:3 $n-6$), arachidonic acid (20:4 $n-6$) and eicosapentaenoic acid (20:5 $n-3$) that occur only in mycorrhizal, but not in non-mycorrhizal roots (Fig. 2(a)). Their levels increased with the degree of mycorrhization. All fatty acids were identified by comparison with authentic standards. In addition, the fatty acid methyl esters were converted into their 4,4-dimethyloxazoline derivatives and the exact positions of the double bonds were confirmed by the corresponding series of characteristic fragment ions (data not shown). Levels of palmitic acid (16:0) and oleic acid (18:1 $n-9$) were increased in mycorrhizal roots. Otherwise, no significant changes were found in the profiles of esterified fatty acids.

Among the free fatty acids (Fig. 2(b)), oleic acid was found only in mycorrhizal roots. Levels of palmitic acid were occasionally – but not in all experiments – increased in mycorrhizal roots. Hence, the composition of free fatty acids did not reflect the composition of esterified fatty acids.

Among the mycorrhiza-specific esterified fatty acids, 16:1 ($n-5$) was present at the highest levels. Our data show that the total amount of esterified 16:1 ($n-5$) as well as the sum of 16:0 and 16:1 ($n-5$) is well correlated with the degree of mycorrhizal colonization of roots by

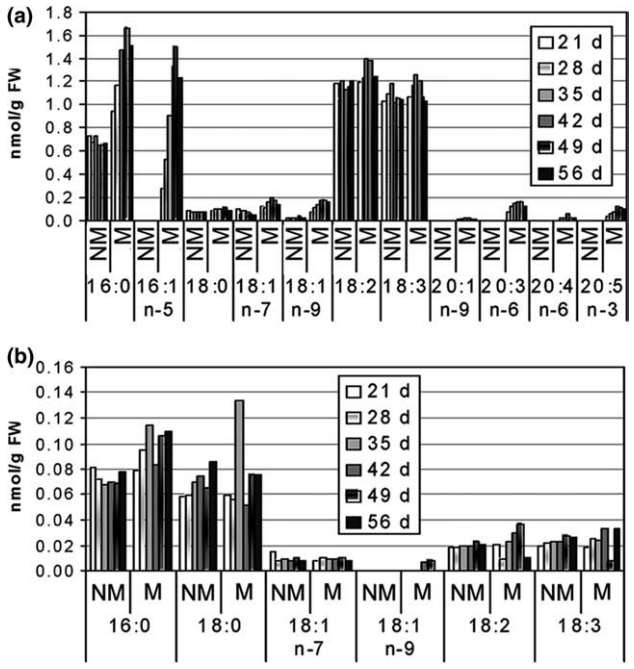

Fig. 2. Fatty acid profiles of mycorrhizal and control roots harvested at different time points. (a) Esterified fatty acids; (b) free fatty acids. Levels are given in nmol/g fresh weight. One representative time course experiment is shown; the time points are given in days after transfer into pots without (NM) or with fungal propagules (M). The degrees of mycorrhization were 35% at 21 days, 69% at 35 days, 55% at 35 days, 57% at 42 days and 91% at 49 days after planting.

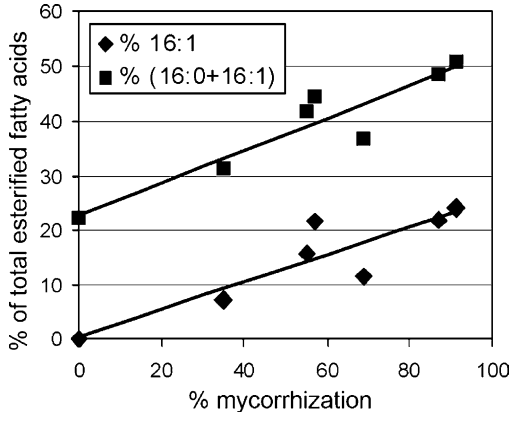

Fig. 3. Correlation of percentage of fungal fatty acids (16:1 $n-5$ or 16:0 and 16:1 $n-5$, respectively) and degree of root mycorrhization. The x-axis denotes the percentage of root colonization as determined according to McGonigle et al. (1990), the y-axis the contribution of 16:1 $n-5$ or the sum of 16:0 and 16:1 $n-5$ to the total content of esterified fatty acids in %. By this calculation, errors due to different yields of total fatty acids in single preparations were avoided. Regression lines have been added. Note that higher precision is achieved when the percentage of the sum of 16:0 and 16:1 $n-5$ is used then when the calculation based on the 16:1 $n-5$ content alone.

G. intraradices as determined by cytological means according to McGonigle et al. (1990) and Trouvelot et al. (1986) (Fig. 3).

2.2. Oxylipin profiles of non-mycorrhizal roots and of roots colonized by G. intraradices

The 9- and 13-LOX products of linoleic and α-linolenic acid were present in all root samples examined. No significant differences were found between levels in mycorrhizal and non-mycorrhizal roots. In both types of roots, however, levels of 13-LOX products were higher than those of 9-LOX products (Fig. 4(a)). Levels of (13*S*)-hydroxy linoleic acid (13-HOD) were always

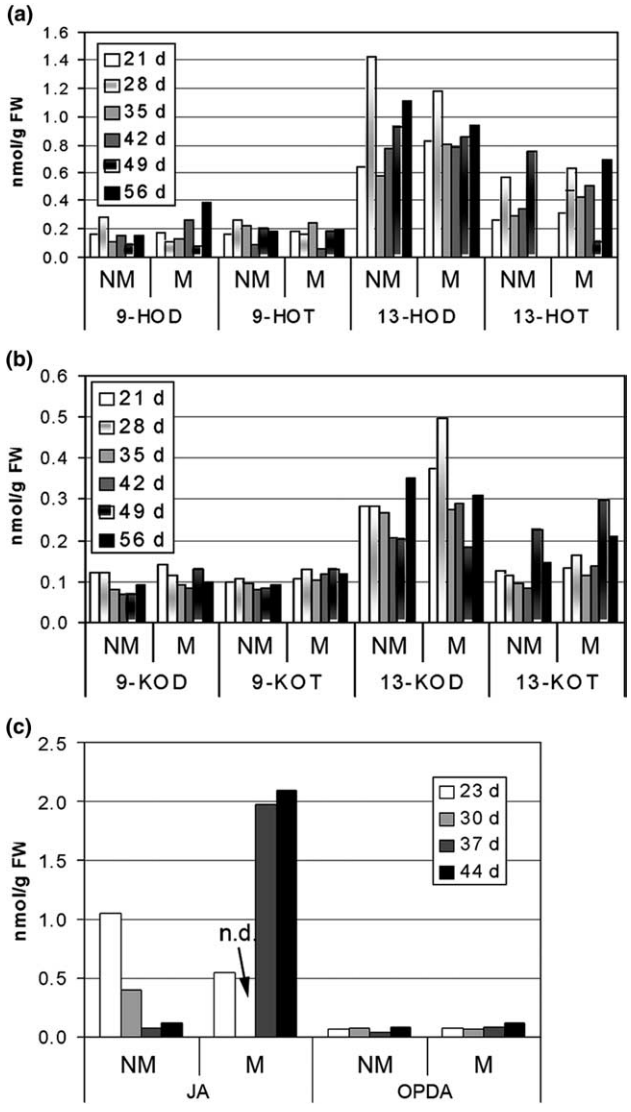

Fig. 4. Oxylipin profiles of mycorrhizal and control roots harvested at different time points. (a) Free oxygenated fatty acids, (b) free ketodienes, (c) jasmonates. Levels are given in nmol/g fresh weight. The result of one representative time course experiment is shown, the time points are given in days after transfer into pots without (NM) or with fungal propagules (M). The degrees of mycorrhization were 35% at 21 days, 69% at 35 days, 55% at 35 days, 57% at 42 days and 91% at 49 days after planting for (a, b) and 20% at 23 days, 30% at 30 days, 50% at 37 days and 70% at 44 days after planting for (c). n.d. – not detected.

higher than those of 13-hydroxy linolenic acid (13-HOT), but 9-HOD and 9-HOT were present at similar levels. Analysis of the ketodienes derived either from linoleic acid (KODs) and linolenic acid (KOTs), which are formed by LOXs under oxygen limitation (Kühn et al., 1991), revealed always higher levels of 13-KODs and 13-KOTs than those of 9-KODs and 9-KOTs (Fig. 4(b)). Moreover, 13-KOD was always present at higher levels than all other ketodienes including 13-KOT, while similar amounts of 9-KOD and 9-KOT were found.

With regard to aldehydes, only (2*E*)-hexenal and (2*E*, 6*Z*)-nonadienal could be identified, although in previous experiments using a different growth system, hexanal and (2*E*)-nonenal have been found in roots of *M. truncatula* (Stumpe et al., 2003). Aldehyde levels did not differ significantly between mycorrhizal and non-mycorrhizal roots and were rather constant over the time course. However, relative levels of aldehydes could differ between experiments by nearly one order of magnitude.

JA and OPDA were found in all samples (Fig. 4(c)), while dinor-OPDA was not detectable. JA determinations were difficult since root systems could be shown to produce JA rapidly in response to mechanical stress (B. Hause, unpublished). Thus differences in handling the root material could cause differences in JA contents. Accordingly, JA levels differed strongly between experiments. From the comparison of seven different time course experiments, we conclude that JA levels are indeed increased in mycorrhizal roots, but to variable levels. Water stress seemed not be responsible, since water-stressed *M. truncatula* roots did not show increased JA levels (B. Hause, unpublished). In contrast to JA, levels of OPDA did not vary much between different samplings.

2.3. Cloning of three CYP74 enzymes encoding cDNAs from M. truncatula

To understand oxylipin patterns in roots, expression levels of genes encoding enzymes catalyzing rate-limiting steps in the different branches of the pathway should be examined. Analysis of the currently available ESTs from *M. truncatula* shows contigs coding for at least 19 different LOXs. Therefore, we decided to concentrate first on those enzymes metabolizing LOX products. *M. truncatula* contains at least four different *HPL* genes and two different *AOS* genes. Only one *HPL* and one *AOS* gene, respectively, are represented by ESTs from belowground organs (TC77257 and TC86521).

cDNA fragments representing partial cDNAs of one AOS and two HPL enzymes, respectively, were obtained by RT-PCR based on RNA isolated from JA-methyl ester-treated *M. truncatula* leaves. The PCR products were used to screen a cDNA library prepared from RNA from mycorrhizal roots of *M. truncatula* (van Buuren et al., 1999) leading to the isolation of three full length cDNAs. Sequencing of these cDNAs and analysis of the encoded amino acid sequences revealed that two of them encode HPLs (GenBank Accession Nos. CAC86898 and CAC86899, termed *MtHPL1* and *MtHPL2*, respectively), while the third encodes an AOS (GenBank Accession No. CAC86897, termed *MtAOS1*). *MtHPL1* and *MtAOS1* represent TC77257 and TC86521, respectively.

2.4. Analysis of substrate specificity and expression analysis of MtAOS1, MtHPL1 and MtHPL2

For biochemical characterization, all three cDNAs were cloned in the *Escherichia coli* expression vector pQE30 and transformed into *E. coli* SG13009. Extracts of induced *E. coli* cultures were examined photometrically by recording the absorption at 234 nm for conversion of the hydroperoxides 13-HPOT and 9-HPOD. While both HPLs converted 13-HPOT as well as 9-HPOD, the AOS only converted 13-HPOT at significant activities. The analysis of the enzyme products with radioactively labeled 13-HPOT was published elsewhere (Stumpe et al., 2003). The main product obtained after the conversion of 13-HPOT by MtAOS1 was isolated, derivatized with methoxamine and subjected to GC/MS. The mass spectrum was identical to that of the corresponding standard 13-hydroxy-12-oxo-octadecadienoic acid. When the reaction products of MtHPL1 and MtHPL2, both supplied with a mixture of 13-HPOT and 9-HPOD, were derivatized with 2,4-dinitrophenylhydrazine and subjected to HPLC, they co-eluted with the standards for (2*E*)-hexenal and (2*E*)-nonenal (Stumpe et al., 2003). Altogether, the identification of the enzymes, based on amino acid sequences as HPLs and AOS, was confirmed by analysis of their reaction products. The substrate specificities are in accordance with the amino acid sequence characteristics for 9/13-HPLs and 13-AOSs as depicted in the analysis of *CYP74* enzymes where *MtHPL1*, *MtHPL2* and *MtAOS1* are included (Feussner and Wasternack, 2002).

The expression levels of the three genes encoding *CYP74* enzymes in non-mycorrhizal and mycorrhizal roots were compared using RNA gel blot hybridization with full length cDNA probes (Fig. 5). None of the genes was found to be induced in mycorrhizal in comparison with control roots.

3. Discussion

To analyze lipid patterns in roots and their changes during arbuscular mycorrhizal colonization, *M. truncatula* plants were grown with and without *G. intraradices*,

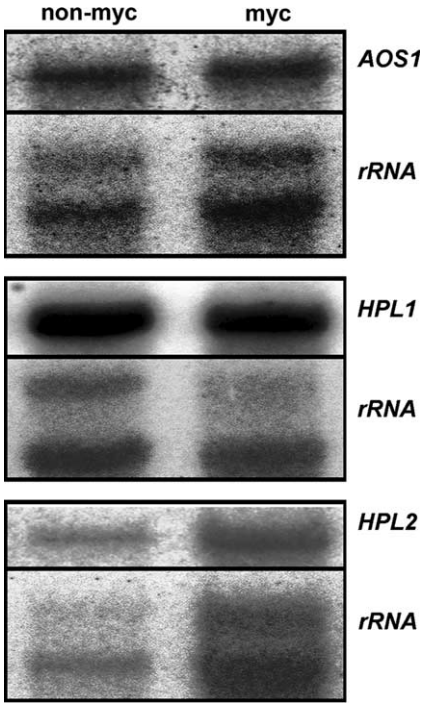

Fig. 5. RNA gel blot hybridization. Total RNA was isolated from non-mycorrhizal (non-myc) and mycorrhizal (myc) roots (harvested after 35 days, 70% mycorrhization), separated on a formaldehyde–agarose gel, transferred onto Hybond XL membrane and hybridized with ^{32}P-labeled full length cDNAs of *MtAOS1*, *MtHPL1* and *MtHPL2*. Afterwards, blots were stripped and re-hybridized with a plant rDNA probe.

and fatty acids as well as oxylipins were prepared from root material harvested at regular intervals over a period of six to eight weeks. To understand the biochemical basis of the oxylipin profiles, the expression patterns of three cDNAs representing genes encoding *CYP74* enzymes and the specificities of the encoded enzymes were analyzed.

3.1. Fatty acids

The use of fatty acid methyl ester profiles has already been proposed for the identification of arbuscular mycorrhizal fungi (Bentivenga and Morton, 1996; Madan et al., 2002) and for estimating the biomass of their spores in soil (Madan et al., 2002). While esterified palmitic acid and 16:1 ($n-5$) were found in all members of the Gigasporaceae (Bentivenga and Morton, 1996; Madan et al., 2002), there is a strain-specific occurrence of 20:1 ($n-9$), 20:3 ($n-6$) 20:4 ($n-6$) and 20:5 ($n-3$). The presented analyses revealed that among the esterified mycorrhiza-specific fatty acids, 16:1 ($n-5$) was present at the highest levels. Moreover, levels of esterified palmitic acid (16:0) and oleic acid (18:1 $n-9$) were increased in mycorrhizal roots compared to controls. This indicates a strong contribution of the plastidic pathway to fatty acid biosynthesis in mycorrhizal roots, probably due to the proliferation of plastids in arbuscule-containing cells (Fester et al., 2001).

As signature fatty acid of Glomelean fungi, 16:1 ($n-5$) has already been identified (McGonigle et al., 1990; Olsson, 1999) and *G. intraradices* has been shown to be particularly rich in 16:1 ($n-5$). 16:1 ($n-5$) is present in both membrane and storage lipids (van Aarle and Olsson, 2003) and its relative amounts in phospholipids (denoting arbuscular colonization) versus neutral lipids (denoting vesicle formation) has been applied to estimate the physiological state of the fungus in mutualistic symbiosis (Olsson et al., 1997; van Aarle and Olsson, 2003). Here, we show that the total amount of esterified 16:1 ($n-5$) as well as the sum of 16:0 and 16:1 ($n-5$) are well correlated with the degree of mycorrhizal colonization of roots by *G. intraradices* (Fig. 3).

3.2. LOX products

Only the products of 13-LOXs are precursors of jasmonates, while the products of both 9-LOXs and 13-LOXs can be precursors of volatile aldehydes. 13-LOXs are found exclusively in plastids except for legumes and cucurbits where 9- as well as 13-LOXs are found in the cytoplasm (Feussner and Wasternack, 2002). By EST analyses in www.tigr.org using the active site signature to distinguish plant 9- and 13-LOXs (Hornung et al., 1999), 18 cytoplasmic 13-LOXs and a single cytoplasmic 9-LOX were found in *M. truncatula*, but no plastid-located LOX could be anotated. That plastidic LOXs are underrepresented in the genome is not uncommon, as described, e.g., for potato (Rosahl and Feussner, 2005). Moreover, this is consistent with immunohistochemical analyses of uninoculated *M. truncatula* roots using a polyclonal antibody raised against the lipid body LOX from cucumber. The antibody recognizes plastidic and cytoplasmic LOXs and led to immunolabeling in the cytoplasm, but not in the plastids (K. Demchenko, I. Feussner and K. Pawlowski, unpublished results). Whether in *M. truncatula* roots the amounts of plastid-located 13-LOX are below the detection limit remains to be elucidated. At least the enzymes following LOX in JA-biosynthesis, AOS and AOC, are both found in plastids (Wasternack and Hause, 2002).

As shown in Figs. 4(a) and (b), the amounts of 13-hydroperoxides in non-mycorrhizal and mycorrhizal roots were consistently higher than those of 9-hydroperoxides, with 13-HPOT showing the highest levels. No striking changes in any hydroperoxide levels took place during the mycorrhization time course. Similarly, the relative levels of ketodienes did neither differ significantly between mycorrhizal and non-mycorrhizal roots nor change over the time course, and 13-KOD was always present at significantly higher levels than the other ketodienes (Fig. 4(b)). Therefore, it can be concluded

that in roots of *M. truncatula* 13-LOXs display significantly higher activities than 9-LOXs. This is supported by the results of the EST analysis although it is not possible to predict an organ-specific distribution of these LOXs based on the data available.

A comparison of the amounts of 9-/13-HOD *versus* 9-/13-KOD and 9-/13-HOT *versus* 9-/13-KOT shows that the levels of the hydroxides are about two times as high as those of the ketodienes. This is an unusually high ratio of KOD/T to HOD/T as compared to aboveground plant organs (Miersch et al., 2004). Studies on LOXs done with animal systems, such as reticulocytes, and pea have shown that under oxygen limitation, LOXs themselves catalyze the formation of ketodienes by an intrinsic hydroperoxidase reaction (Kühn et al., 1986, 1991). It is likely that the quite high contribution of ketodienes to root oxylipins of *M. truncatula* is due to oxygen limitations in roots caused by the growth system.

3.3. Products of CYP74 enzymes

While *AOS* and *HPL* were identified in *M. truncatula*, no DES-encoding gene could be found, which is consistent with the fact that no divinyl ethers were detected in *M. truncatula* roots. According to EST analysis in www.tigr.org, *M. truncatula* contains two AOS genes, only one of which – *MtAOS1*, described in this manuscript – is represented by ESTs from belowground organs. Hence, it is likely that *MtAOS1* transcription may not be limiting for JA production in *M. truncatula* roots. Indeed, in spite of the increased JA levels found in mycorrhizal roots of *M. truncatula*, there is no transcriptional induction of *AOS* in such roots. A positive feedback loop as described for the induction of *AOS* transcription preceded by increased JA-levels during herbivore feeding on *Nicotiana attenuata* (Ziegler et al., 2001) could be undetectable due to the long-term effects of mycorrhization. It is more likely, however, that AOS activity can be regulated at the protein level or by substrate availability as shown for AOS in *Arabidopsis* (Laudert and Weiler, 1998) and for AOC in the wound response of tomato (Stenzel et al., 2003).

Upon mycorrhization of barley and *M. truncatula*, JA levels increased, whereas OPDA levels remained consistently low (Hause et al., 2002; Fig. 4(c)). These data may indicate that OPDA reductase (OPR3) activities in roots are high, so that OPDA is metabolized very efficiently and does not accumulate. This is in contrast to leaves of other plants, which in absence of induction exhibit higher levels of OPDA than of JA (Laudert and Weiler, 1998; Strassner et al., 2002). Dinor-OPDA, the product of roughanic acid oxidation (Weber et al., 1997), was not detectable, which is likely to be due to the lack of roughanic acid. Legumes are 18:3 plants that do not contain 16:3 fatty acids in their plastidial galactolipids, in contrast, e.g., to members of the Brassicaceae (Heinz, 1977).

Only two types of aldehydes were found in non-mycorrhizal and mycorrhizal roots of *M. truncatula*, (2*Z*,6*E*)-nonadienal (product of 9-HPOT) and (2*E*)-hexenal (product of 13-HPOT). Their relative levels varied between experiments, although in all cases, amounts of 13-HPOT were significantly higher than those of 9-HPOT.

3.4. Mycorrhization and JA

Mycorrhization led to an increase in JA levels in *M. truncatula* roots, but not to a significant shift in the levels of any other product of the oxylipin pathway. As already mentioned above, only long-term effects were examined; no short-term changes could be detected in oxylipin patterns as may play a role during mycorrhiza establishment. JA levels were two to three times higher in mycorrhizal than in non-mycorrhizal roots, a relatively low increase in JA levels compared to the up to fivefold increase found in mycorrhizal barley (Hause et al., 2002).

JA levels in non-mycorrhizal barley roots were between 1.0 and 1.5 nmol/g fresh weight (Hause et al., 2002), while for *M. truncatula* values between 0.07 and 1.27 nmol/g fresh weight JA were found within a single time course experiment. Whether root JA levels are more variable in *M. truncatula* in general or whether *M. truncatula* was sustaining more stress in the growth system used for mycorrhization than barley did, is not clear. Moreover, a dislocation of JA to the shoots in *M. truncatula* cannot be excluded. As shown for the wound response of tomato, JA may be a systemic signal and can be dislocated throughout the plant (Li et al., 2002). It is tempting to speculate that the difference between barley and *M. truncatula* might be due to different functions of jasmonates in a monocotyledonous and a dicotyledonous plant as previously deduced from different JA-perception sites shown for barley and tomato (Bücking et al., 2004). Furthermore, the increase in JA levels may depend on the species of plant and fungus used, with *G. intraradices* being a more efficient symbiont for barley than for *M. truncatula*.

Different reasons are possible to be discussed for the increased JA levels in mycorrhizal roots. As assumed for mycorrhizal barley roots, increased JA levels could be linked to osmotic stress caused by increased sugar influx due to an increased sink strength of mycorrhizal compared to non-mycorrhizal roots (Hause et al., 2002). Alternatively, the increased JA levels of mycorrhizal roots might be linked to the enhanced defensive capacity conferred by mycorrhization (see Hause and Fester, 2005 and references therein). In roots colonized by AM fungi various elements of induced systemic resistance have been observed, which seems – in contrast

to systemic acquired resistance – to be dependent on JA (van Loon et al., 1998; Pozo et al., 2005). Another option is that arbuscular mycorrhization affects JA levels via N-metabolism. Jasmonates have been implicated in the control of N-partitioning, specifically the control of accumulation and mobilization of N reserves in roots (Goulas et al., 2003; Meuriot et al., 2004; Rossato et al., 2002). The increased phosphate supply due to arbuscular mycorrhization increases the N-status of plants (Jia et al., 2004), which may result in increased biosynthesis of storage protein in roots, modulated by jasmonates.

3.5. Conclusions

The profiling of fatty acids presented here shows that in mycorrhizal roots levels of fungus-specific fatty acids as well as palmitic acid (16:0) and oleic acid (18:1 $n-9$) were increased in dependence on the percentage of root colonization. Hence, the degree of arbuscular mycorrhizal colonization can be estimated via analysis of esterified fatty acids. Currently, mycorrhizal colonization can be determined cytologically or via quantification of fungus-specific DNA by PCR (Filion et al., 2003) or of fungus-specific RNA by quantitative RT-PCR (Isayenkov et al., 2004). Comparing to those methods, the analysis of fatty acid methyl ester profiles is faster and less labor-intensive. Furthermore, it was shown that 9- and 13-LOX products of linoleic and α-linolenic acid were present in all root samples and levels of 13-LOX products were higher than those of 9-LOX products. Regarding long-term effects on oxylipin metabolites, mycorrhization mainly increases JA levels. Further research is needed to understand the function(s) of elevated JA synthesis in mycorrhizal symbiosis.

4. Experimental

4.1. Plant and fungal growth conditions

Plants (*M. truncatula* L. Gaertn. "Jemalong") were grown in a greenhouse in pots filled with expanded clay (Lecaton, 2–5 mm particle size; Fibo Exclay, Pinneberg, Deutschland). Seven-day-old seedlings (5 plants per 10 cm diameter pot) were inoculated with the arbuscular mycorrhizal fungus *G. intraradices* Schenck & Smith by the application of propagates in expanded clay (isolate 49, provided by H. von Alten, University Hannover, Germany). Further details of plant growth conditions have been described previously (Maier et al., 1995). All experiments were performed in triplicate.

4.2. Molecular methods

The sequences of the ESTs AW267966 (*AOS*), AW559526 (*HPL1*) and AW573758 (*HPL2*) (all from NCBI database) were used to deduce primers for amplifying *MtAOS*- and *MtHPL*-specific fragments from JAME-treated *M. truncatula* leaves. With these, a cDNA expression library from roots of *M. truncatula* (genotype A17) infected with *G. versiforme* (kindly provided by M. Harrison) was screened for full-length cDNAs coding for the respective genes. For heterologous expression, truncated sequences starting from amino acid 60, thus lacking the putative chloroplast target sequence, were subcloned into pQE30. pQE30 with or without insert was transformed into the host strain *E. coli* M15 (Qiagen, Hilden, Germany).

RNA was isolated from mycorrhizal and control roots using the TRIzol method (Invitrogen, Carlsbad, CA) and separated on formaldehyde–agarose gels as described by Sambrook et al. (1989). RNA was transferred to Hybond XL membranes, cross-linked using UV-light and hybridized with ^{32}P-labeled full size cDNA probes according to the instructions of the manufacturer. Membranes were washed at 65 °C in 2× SSC, 0.1% SDS for 2×20 min and in 0.5× SSC, 0.1% SDS for 20 min and evaluated using a phosphoimager (Fuji FLA-3000, Raytest, Sprockhövel). For control of the amount of RNA transferred to the membrane, blots were re-hybridized with an rDNA probe from wheat (pTA71, 18S-5.8S-25S rDNA; Gerlach and Bedbrook, 1979). In this case, washing was performed at 65 °C in 2× SSC, 0.1% SDS for 3×20 min. Hybridization intensity was quantified using the AIDA software package (Raytest, Sprockhövel, Germany).

4.3. Fatty acid and oxylipin profiling

LOX-derived products were analyzed as described before, but with some modifications (Göbel et al., 2003; Weichert et al., 2002). 1 g of frozen plant material was added to 20 ml of extraction medium (hexane:2-propanol, 3:2 (v/v), with 0.0025% (w/v) butylated hydroxytoluene) and was immediately homogenized with an Ultra Turrax for 45 s under a stream of argon on ice. The extract was shaken for 10 min and centrifuged at 3200 *g* at 4 °C for 15 min. The clear upper phase was collected, and a 6.7% (w/v) solution of potassium sulfate was added up to a volume of 32.5 ml. After vigorous shaking and centrifugation at 3200 *g* at 4 °C for 10 min the upper hexane-rich layer which potentially contained oxylipins was subsequently dried under a nitrogen stream. The remaining lipids were re-dissolved in 0.2 ml of methanol and stored under an argon atmosphere at −20 °C until use.

Subsequently, esterified fatty acids were transmethylated with sodium methoxide and free fatty acids were methylated with EDAC (op den Camp et al., 2003). The analysis of the corresponding fatty acid methyl esters was performed with a Agilent (Waldbronn, Germany) 6890 gas chromatograph fitted with a capillary

DB-23 column (30 m × 0.25 mm; 0.25 μm coating thickness; J&W Scientific, Agilent). Helium was used as carrier gas (1 ml/min). The temperature gradient was 150 °C for 1 min, 150 – 200 °C at 8 K/min, 200–250 °C at 25 K/min and 250 °C for 6 min. As internal standard for the quantification of esterified fatty acids, triheptadecanoate was used. As internal standard for the quantification of free fatty acids, heptadecanoic acid was used. For the determination the position of double bound positions in fatty acids not present in the standard mixture (16:1 ($n-5$), 20:1 ($n-9$), 20:3 ($n-6$) 20:4 ($n-6$) and 20:5 ($n-3$)), fatty acid methyl esters were converted into their 4,4-dimethyloxazoline derivatives as described (Sperling et al., 2000) and analyzed mass-spectrometrically according to Christie (1998), using the 6890 Gas Chromatograph/5973 Mass Selective Detector system (Agilent).

For analysis of free oxylipins, samples were directly subjected to HPLC analysis. First, oxylipins were purified by reverse-phase HPLC. This was performed on an ET250/2 Nucleosil 120-5 C18 column (250 × 2.1 mm, 5 μm particle size; Macherey & Nagel, Düren, Germany) with a methanol:water:acetic acid (85:15:0.1, v/v/v) solvent system at a flow rate of 0.18 ml/min. Straight-phase HPLC for separation of hydroperoxy fatty acids, hydroxy fatty acids as well as keto fatty acids was performed on a Zorbax Rx-SIL column (150 × 2.1 mm, 5 μm particle size, Agilent) with *n*-hexane:2-propanol:acetic acid (100:1:0.1, v/v/v) as a solvent system at a flow rate of 0.2 ml/min. For detection of the hydroperoxy fatty acids and hydroxy fatty acids, the absorbance at 234 nm indicating the conjugated diene system was recorded. For detection of keto fatty acids, the absorbance at 270 nm was recorded. The enantiomer composition of the hydroperoxy fatty acids as well as hydroxy fatty acids was analyzed by chiral-phase HPLC on a Chiral OD-H column (150 × 2.1 mm, 5 μm particle size; Baker, Griesheim, Germany) with *n*-hexane:2-propanol:acetic acid (100:5:0.1, v/v/v) as a solvent system at a flow rate of 0.1 ml/min. For quantification of these oxylipins, (6*Z*,9*Z*,11*E*,13*S*)-13-hydroxy-6,9,11-octadecatrienoic acid was used as the internal standard. For detection of JA, OPDA, and dinor-OPDA, these compounds were converted to their pentafluorobenzyl esters after purification by RP-HPLC according to Müller and Brodschelm (1994). The analysis was carried out using a ThermoFinnigan (Austin, Texas, USA) Polaris Q mass selective detector connected to ThermoFinnigan Trace gas chromatograph equipped with a capillary Rtx-5MS column (15 m × 0.25 mm, 0.25 μm coating thickness; Resteck, Bad Homburg, Germany). Helium was used as carrier gas (1 ml/min). The temperature gradient was 60 °C for 1 min, 60–180 °C at 25 K/min, 180–270 °C at 5 K/min, 270 °C for 1 min, 270–300 °C at 10 K/min, 300 °C for 20 min. The pentafluorobenzyl esters were detected by negative chemical ionization with ammonium as ionization gas. Under these conditions, the retention times of the pentafluorobenzyl esters of JA, OPDA, and dinor-OPDA were 11.7, 20.9, and 18.1 min, respectively. For quantification, the ions *m/z* 215 (D_6-JA), 209 (JA), 296 (D_5-oDPA), 291 (OPDA), and 263 (dinor-OPDA) were used, respectively.

Aldehydes were extracted and analyzed as described (Kohlmann et al., 1999).

4.4. Expression of AOS and HPL in E. coli and identification of reaction products

AOS or HPL expressing cells were grown at 37 °C in LB medium, induced with 1 mM IPTG and cultivated for 24 h at 16 °C. Cells were harvested by centrifugation and disrupted by sonification in 100 mM sodium phosphate buffer (pH 8). The lysate was centrifuged and the supernatant served as protein extract for the activity tests. The activities of the recombinant enzymes were measured photometrically by monitoring the rate of decrease in absorbance at 234 nm resulting from disruption of the conjugated diene bond of the substrate (Zimmerman and Vick, 1970). For the product analysis, the protein extract was diluted 1:6 with 100 mM sodium phosphate buffer (pH 6.5) and incubated with 100 nmol of 13-HPOT for 20 min at room temperature. The reaction was stopped by adding 40 μl of acetic acid and extracted with methanol/chloroform. The organic phase was evaporated under a stream of nitrogen and reaction products were reconstituted in 100 μl HPLC solvent (acetonitrile:water:acetic acid, 50:50:0.1, v/v/v). The HPLC analysis was performed using a Lichrospher (Merck, Darmstadt, Germany) 100-RP-18 (5 μm) column (4 × 125 mm) with a solvent mixture of acetonitril:water:acetic acid (50:50:0.1; v/v/v) at 1 ml/min for about 25 min followed by the same system at 80:20:0.1 (v/v/v) to complete the run. For further product analysis, non-labeled 13-HPOT was incubated with MtAOS1 and the main reaction product, known from the assay using radiolabeled substrate, was purified by HPLC. The purified reaction product was methylated and derivatized over night at room temperature by dissolving in 500 μl pyridine containing 2% methoxyamin. The solution was dried under a nitrogen stream and extracted twice with *n*-hexane. The GC/MS analysis was performed as described (Stumpe et al., 2001).

To analyze the formation of aldehydes, the lysate was incubated with 13-HPOTE and 9-HPOD for 20 min. The reaction mixture was acidified to pH 3 with HCl and incubated with 2.5 ml of 0.1% 2,4-dinitrophenylhydrazin for 1 h at room temperature. The hydrazones were extracted twice with hexane, evaporated under a stream of nitrogen and subjected to HPLC analysis (see above).

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