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Review

Apocarotenoid biosynthesis in arbuscular mycorrhizal roots: Contributions from methylerythritol phosphate pathway isogenes and tools for its manipulation

Michael H. Walter *, Daniela S. Floß, Joachim Hans [1], Thomas Fester, Dieter Strack

Leibniz Institute of Plant Biochemistry, Department of Secondary Metabolism, Weinberg 3, D-06120 Halle (Saale), Germany

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Abstract

During colonization by arbuscular mycorrhizal (AM) fungi plant roots frequently accumulate two types of apocarotenoids (carotenoid cleavage products). Both compounds, C_{14} mycorradicin and C_{13} cyclohexenone derivatives, are predicted to originate from a common C_{40} carotenoid precursor. Mycorradicin is the chromophore of the "yellow pigment" responsible for the long-known yellow discoloration of colonized roots. The biosynthesis of apocarotenoids has been investigated with a focus on the two first steps of the methylerythritol phosphate (MEP) pathway catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR). In *Medicago truncatula* and other plants the *DXS2* isogene appears to be specifically involved in the AM-mediated accumulation of apocarotenoids, whereas in the case of DXR a single gene contributes to both housekeeping and mycorrhizal (apo)carotenoid biosynthesis. Immunolocalization of DXR in mycorrhizal maize roots indicated an arbuscule-associated protein deposition, which occurs late in arbuscule development and accompanies arbuscule degeneration and breakdown. The *DXS2* isogene is being developed as a tool to knock-down apocarotenoid biosynthesis in mycorrhizal roots by an RNAi strategy. Preliminary results from this approach provide starting points to suggest a new kind of function for apocarotenoids in mycorrhizal roots.

Keywords: *Medicago truncatula*; Leguminosae; Arbuscular mycorrhiza; Biosynthesis; Suppression; Apocarotenoids; 1-Deoxy-D-xylulose 5-phosphate synthase; 1-Deoxy-D-xylulose 5-phosphate reductoisomerase; Mycorradicin; Cyclohexenone derivatives

Contents

* Corresponding author. Tel.: +49 345 5582 1520; fax: +49 345 5582 1509.
E-mail address: mhwalter@ipb-halle.de (M.H. Walter).
[1] Present address: Institute of Pharmaceutical Biology, Philipps-University Marburg, D-35037 Marburg, Germany.

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1. Introduction

Apocarotenoids, sometimes also called carotenoid cleavage products (CCPs), have recently become exciting molecules in mycorrhiza research and elsewhere with excellent prospects for novel functions (Akiyama et al., 2005; Bouvier et al., 2005). The plant factor in root exudates to induce hyphal branching of AM fungi hyphae as one of the first steps in plant-fungus communication (strigolactone, Akiyama et al., 2005) is an apocarotenoid (Matúšová et al., 2005). Another, still elusive novel apocarotenoid is produced by the action of two consecutive carotenoid cleavage enzymes in plant roots. It acts in minute amounts as a novel phytohormone-like compound to dramatically affect the branching of shoots and its lack causes aberrant shoot branching (Auldridge et al., 2006). Trisporic acid derivatives have important functions in fungal zygomycete communication as pheromone mating factors (Schachtschabel et al., 2005). Furthermore, apocarotenoids are now increasingly recognized as developmentally-regulated key aroma components of fruits and vegetables (Simkin et al., 2004). They are also produced by various organism as a response to abiotic and biotic stresses (Bouvier et al., 2005).

The connection between the arbuscular mycorrhizal symbiosis and apocarotenoids is much older than the very recent discovery of strigolactone action. Most mycorrhiza researchers agree on inquiry that they have at least occasionally encountered a yellow coloration of roots colonized by arbuscular mycorrhizal fungi. The phenomenon is thus very wide-spread and well-known as the so-called "yellow pigment". It was first described as early as 1924 for a legume plant (Jones, 1924), but is most pronounced in cereal roots. It took more than 70 years from the first description to the elucidation of the chemical structure of the chromophore as a linear C_{14} carotenoid cleavage product named mycorradicin by the group of H. Bothe (Klingner et al., 1995). However, despite many research efforts, the relevance and function of this pigment and of other cyclic apocarotenoids accumulating concomitantly with mycorradicin is still not understood. This review summarizes results on mycorrhiza-mediated apocarotenoid biosynthesis obtained during the SPP 1084 funding period and will offer recent tools and perspectives towards finally elucidating a function for linear and/or cyclic apocarotenoids in the arbuscular mycorrhizal symbiosis.

2. Massive apocarotenoid accumulation in roots: an AM-specific phenomenon

The term mycorradicin, generated by Klingner et al. (1995) already implies a mycorrhiza-specific accumulation. Indeed, a yellow coloration and accumulation of related apocarotenoids (see below) has not been observed in cereal roots challenged by pathogens or abiotic stresses (Maier et al., 1997). Moreover, no reports of yellow coloration or mycorradicin accumulation exist for ectomycorrhizal or nodulated roots. Fester et al. (2002a) found small amounts of mycorradicin in non-mycorrhizal roots of a few plant species but in all these cases mycorradicin content of the corresponding mycorrhizal samples were markedly higher. Again, none of the other treatments applied (phytohormones, supply of mineral nutrients) did induce mycorradicin accumulation in mycorrhizal maize roots supporting earlier conclusions that, apart from minimal basal levels in rare cases, mycorradicin accumulation is a mycorrhiza-specific phenomenon. This result also strongly argues for a plant and not fungal origin of mycorradicin, which has been questioned in some of the early papers. The "yellow pigment" can be extracted and quantitatively determined, which has been used to assess the degree of arbuscular mycorrhizal colonization (Schmitz et al., 1991). However, the method is restricted to extensively colonized samples and can hardly be used to quantify early stages of colonization or low infection rates (Bothe et al., 1994).

At about the same time when the chemical structure of mycorradicin was elucidated the mycorrhiza-mediated accumulation of a cyclohexenone terpenoid glycoside (blumenin) was reported, which initially appeared to be unrelated to the yellow root phenomenon (Maier et al., 1995). The mycorrhiza-specific nature of the accumulation of this and other structurally related sesquiterpenoid-like glycosides was shown by treatments of roots with pathogens, heat, cold, drought and heavy metals, none of which did result in elevated levels of these compounds. Only by the year 2000 the cyclohexenone aglycons were recognized as another type of apocarotenoid called C_{13} cyclohexenone derivatives and the biosynthesis of both mycorradicin and cyclohexenone derivatives from a common precursor was proposed (Fig. 1, Walter et al., 2000). This result then raised the question, whether either the precursors (a still elusive C_{40} carotenoid or xanthophyll) or any of the two types of cleavage products or both were of functional relevance for the symbiosis. One initially proposed potential function for the precursors as a determinant of periarbuscular membrane properties was based on reports that xanthophylls can be rigidifying components of plant membranes owing to their dipolar nature (Walter et al., 2000). However, extensive efforts to isolate and identify significant amounts of carotenoid precursors from mycorrhizal roots were unsuccessful and did only show the presence of tiny amounts of ζ-carotene in colonized roots of *Zea mays* and *Medicago truncatula* (Fester et al., 2002b). This result argues for an uninterrupted flux to apocarotenoids and for a function of the cleavage products rather than the precursors. The accumulation of cyclohexenone derivatives is not dependent on a particular AM fungal species. Three different AM fungi (*Glomus intraradices*, *G. mosseae* and *Gigaspora rosea*) were tested on three cereals (barley, wheat and maize). There were slight differences between the magnitude and the ratio of the accumulating cyclohexenone derivatives but the general response was

very similar (Vierheilig et al., 2000). Inhibiting carotenoid biosynthesis by treatment with norflurazon, a specific phytoene desaturase inhibitor, revealed a more pronounced increase of phytoene in mycorrhizal compared to non-mycorrhizal roots (Fester et al., 2002b). The inhibitor approach was later extended to additional species, in which no mycorradicin had been found after AM colonization. These species all showed an increase in phytoene in mycorrhizal roots upon adding the inhibitor (Fester et al., 2005). The stimulation of carotenoid metabolism in mycorrhizal roots thus appears to be a general phenomenon, even when apocarotenoid end-products are undetectable.

3. Apocarotenoid biosynthesis in mycorrhizal roots: focus on the methylerythritol phosphate (MEP) pathway

Apocarotenoid biosynthesis can be subdivided into three pathways (Fig. 1): (i) synthesis of the central intermediates of isoprenoid biosynthesis, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP); (ii) the subsequent assembly of isoprene units and their modification to tetraterpene carotenoids; and (iii) the carotenoid cleavage step and further modifications of the primary cleavage products. This review covers results mainly on the first part catalyzed by enzymes of the methylerythritol phosphate (MEP) pathway. Of the specific steps to carotenoids (reviewed by Römer and Fraser, 2005) only phytoene desaturase has been investigated in the context of mycorrhiza-mediated apocarotenoid biosynthesis and shown to be AM-regulated (Fester et al., 2002b). Recent work by us on carotenoid cleavage enzymes will be addressed below.

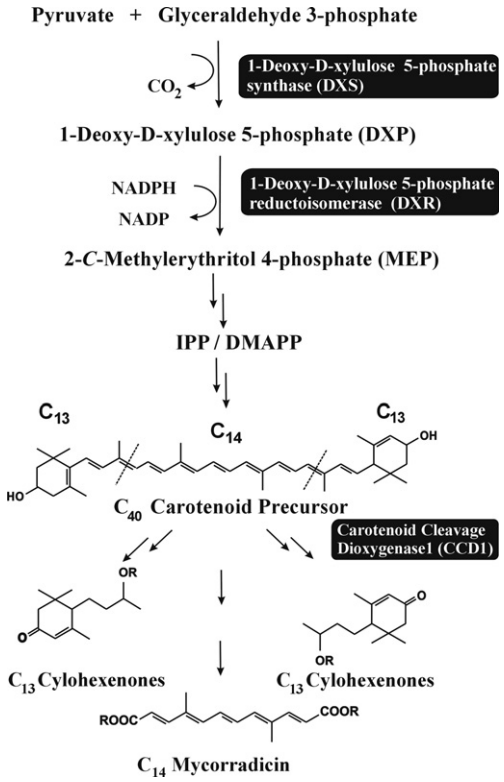

Fig. 1. Proposed biogenesis of C_{13} cyclohexenone derivatives and C_{14} mycorradicin from a common carotenoid precursor synthesized via the MEP pathway. Three enzymatic steps investigated are highlighted by black panels: the two first steps of the MEP pathway catalyzed by 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR), as well as a carotenoid cleavage enzyme with the predicted cleavage specificity (carotenoid cleavage dioxygenase 1, CCD1).

In the early-1990s a second pathway leading to IPP and DMAPP was discovered for certain bacteria and plants (Rohmer et al., 1993; Arigoni et al., 1997; Rodríguez-Concepción and Boronat, 2002). In contrast to the well-known cytosolic mevalonate pathway, the newly discovered pathway resides in the plastid compartment of plants and provides the isopentenyl diphosphate precursors for plastidial isoprenoids including carotenoids. It is now known as the MEP pathway. Initial experiments on the biosynthetic route to mycorrhizal C_{13} cyclohexenone structures by ^{13}C-labelling and NMR analysis indicated an involvement of the MEP pathway and delivered early arguments for an apocarotenoid nature of these compounds (Maier et al., 1998; Strack et al., 2003).

Since very little was known on the regulation of the MEP pathway in plants, we started working on mycorrhiza-mediated regulation of MEP pathway genes by the year 1999. Initially, heterologous probes from rice for the first biosynthetic genes of this pathway encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS) and 1-deoxy-D-xylulose 5-phosphate reductoisomerase (DXR) were used for Northern-type RNA blot analyses of wheat RNA samples. These experiment clearly demonstrated strongly elevated transcript levels of both *DXS* and *DXR* genes in mycorrhizal vs. non-mycorrhizal root samples (Walter et al., 2000). This increase in steady-state levels of transcripts nicely correlated with the accumulation of both cyclohexenone derivatives and mycorradicin as well as with the development of mycorrhizal structures (hyphae, arbuscules, vesicles and spores). The mycorrhiza-mediated induction of *DXS* and *DXR* transcript levels in roots was also shown for maize, barley and rice. The maize experiment gave some indications of several differentially regulated *DXS* transcript species. One such species was particularly abundant in yellow roots while another species did not show much variation between yellow and colorless roots (Walter et al., 2000).

4. A specific *DXS* isogene for apocarotenoid biosynthesis: regulatory and promoter features

The potential existence of different *DXS* isogenes was further investigated in the legume *M. truncatula*, a model system that was adopted upon transfer of the project into the SPP1084 consortium. Mycorrhizal roots of *M. truncatula* exhibit an orange-brown coloration instead of the bright yellow color of many cereal roots (Fig. 2). Their

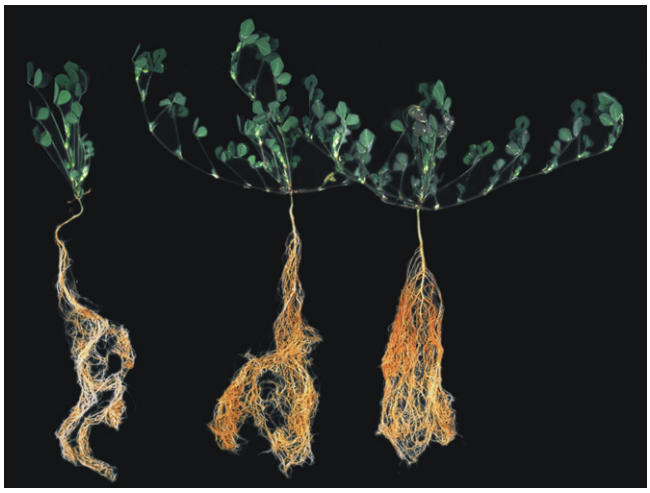

Fig. 2. Orange-brown coloration of mycorrhizal roots of *Medicago truncatula* colonized for 6 weeks by *Glomus intradices* (center) or *Glomus mosseae* (right) vs. the appearance of non-mycorrhizal roots (control, left).

mycorradicin content is also usually much lower than that obtained for cereals. A 13-hydroxy-blumenol C glucoside has been tentatively identified as a major cyclohexenone glycoside in this species (Fester et al., 2005).

Attempts to isolate clones encoding DXS from a mycorrhizal root cDNA library of *M. truncatula* resulted in the identification of three different classes of cDNA clones. The longest representatives of each class were sequenced. Class 1 (*MtDXS1*) was shown to be constitutively expressed in all above-ground tissues (leaves, stems, flowers) but not or only at a very low level in non-mycorrhizal and mycorrhizal roots. In contrast, *MtDXS2* transcripts levels were very low in the former tissues and in non-mycorrhizal roots, but were strongly elevated in mycorrhizal roots colonized by either *Glomus intraradices* or *Glomus mosseae* (Walter et al., 2002). Induction of *DXS2*-type isogenes by mycorrhization was also shown for maize, tomato and tobacco. It can thus be concluded that a specific *DXS* isogene (*DXS2*) is involved in the AM-mediated biosynthesis of root apocarotenoids. In an independent study, a class 2-*DXS* gene of rice was recently classified among genes exclusively responsive to mycorrhization but not to root pathogens or phosphate addition in a whole-transcriptome analysis approach (Güimil et al., 2005).

Apart from the specific case of mycorrhizal apocarotenoid biosynthesis the *DXS2* isogenes appear to be generally involved in many other instances of secondary isoprenoid formation (monoterperpenes, diterpenes, etc.), whereas the *DXS1* isogene seems to represent a housekeeping isogene, responsible among other tasks for carotenoid formation in photosynthetic tissues (Walter et al., 2002). Recombinant MtDXS1 and MtDXS2 were both shown to exhibit DXS activity upon expression in *Escherichia coli*. The two proteins share only about 70% amino acid sequence similarity in the mature proteins. The similarity at the nucleotide level is 64% (Walter et al., 2002). A third class of *DXS*-like clones, designated *DXS3*, was not regulated by mycorrhization. The identity of this latter class as a true *DXS* is still uncertain. The occurrence of *DXS2*-type genes is very widespread and almost universal as deduced from many ESTs available. Interestingly, the only confirmed example of a genome, which does not contain a *DXS2*-type gene, is the AM-nonhost plant *Arabidopsis thaliana*.

The genomic organization of *MtDXS2* in the *M. truncatula* genome was further investigated by analyzing clones from a genomic library. Unfortunately, genomic information on *MtDXS2* is still not available from the genome sequencing project. Sequence analysis of a first genomic clone indicated a noticeable difference to the cDNA (1–2%) despite the fact that both libraries were derived from the A17 genotype. This could not be explained by sequencing mistakes and prompted the isolation of further genomic clones. It turned out that this clone encoded a second, almost identical copy of *MtDXS2* (*MtDXS2-2*, M.H. Walter, unpublished). The gene corresponding to the cDNA (*MtDXS2-1*) was also identified. It resides immediately upstream of the second copy in the *M. truncatula* genome (M.H. Walter, unpublished). Both genes are thus organized as a tandem repeat with only about 3 kb distance between the coding regions. The second copy is almost identical in coding and proximal promoter regions and also highly similar in intron sequences indicating an origin from a recent gene duplication event. It is therefore classified as a *MtDXS2* paralogue. *MtDXS2-2* is also regulated by mycorrhization but its transcript levels were always considerably lower than those of *MtDXS2-1*. Several ESTs for *MtDXS2-2* were found in libraries from nodulated roots leading to the speculation that *MtDXS2-2* could play a role in nodulation. We therefore initiated RT-PCR experiments to verify its potential upregulation during nodulation. However, in several experiments using nodulated roots we only observed minor changes in *MtDXS2-2* transcript levels in such roots, which did not follow a reproducible pattern. There is thus no evidence for a *Rhizobium*-related regulation of the MEP pathway, which is in agreement with other information on an AM-specific nature of apocarotenoid induction in roots.

The *MtDXS2-1* promoter contained on one of the genomic clones was chosen for a closer analysis of its mycorrhiza-inducible activity. The full promoter comprising 2500 bp upstream of the presumed transcriptional start site was fused to a *uidA*(GUSint) reporter gene and analyzed in a hairy root expression system after *Agrobacterium rhizogenes* transfer of constructs into *M. truncatula* hypocotyls as described (Vieweg et al., 2004). The −2500 promoter-*uidA*-(GUSint) construct conferred GUS activity to colonized regions of transformed roots but not to non-mycorrhizal roots or non-colonized regions of mycorrhizal roots. GUS activity conferred by the *MtDXS2-1* promoter was clearly associated with arbuscules (D.S. Floß and M.H. Walter, unpublished). Promoter deletion constructs are

currently being analyzed to delineate promoter regions responsible for the mycorrhiza-mediated inducibility and finally define specific elements involved in AM-related regulation. Such elements will be compared with elements defined by other groups within the SPP1084 working on unrelated, but mycorrhiza-induced genes. It will be interesting to learn, if one or many "mycorrhiza-boxes" exist within AM-regulated promoters. From a simple comparison of available promoter sequences no such boxes are as yet discernable.

5. Arbuscule-associated localization of DXR deposition in maize: timed late in arbuscule development

The work on AM-mediated DXR induction was continued in the maize cereal root system, since many prerequisites and tools generated in the beginning were designed for maize. Moreover, as mentioned above, apocarotenoid accumulation and arbuscule formation is usually stronger in the cereal systems. A maize *DXR* cDNA was isolated from a library generated from mycorrhizal roots of the *Zea mays* dwarf1 cultivar, which exhibits a particularly bright yellow color (see Walter et al., 2000). In contrast to *DXS* only a single class of clones was identified. It encodes a 473-amino acid protein with a calculated mass of 51.2 kDa including a transit peptide of 48 amino acids for import into plastids. The mature protein has a calculated mass of 45.8 kDa. The ZmDXR protein is highly similar to other DXR from rice (91% identity), barley (88% identity) and peppermint (74% identity). The recombinant ZmDXR protein obtained from expression in *E. coli* did exhibit DXR activity (Hans et al., 2004). Inspection of EST databases from maize and other cereals did not reveal the existence of additional *DXR* isogenes as in the case of *DXS*. A blot analysis of RNA from different maize tissues did show a considerable increase of transcripts in mycorrhizal vs. non-mycorrhizal roots. However, the strongest signal was obtained with leaf samples. Both results support the notion that, in contrast to DXS isoenzymes, a single DXR protein is involved in both the specific AM-mediated apocarotenoid biosynthesis and the housekeeping tasks.

The main focus of the DXR project was on the protein level and on the cell- and potential arbusculated cell-specific localization of the protein. The ZmDXR recombinant protein obtained from *E. coli* was used to generate specific antibodies in rabbits. To improve specificity, the antibodies were affinity-purified on immobilized antigen resulting in the recognition of a single band with the correct Mr in Western blot analysis (Hans et al., 2004). The purified antibodies were used to show an induction of DXR in mycorrhizal roots at the protein level. Subsequently, immunolocalization studies with maize root sections were performed and analyzed by confocal microscopy. In non-colonized root cells DXR was detected in plastid aggregations around the nucleus and in single distinct plastids in the cytoplasm (Hans et al., 2004). In arbusculated cells the DXR signal was strongly increased. DXR containing plastids were tightly associated with fungal structures. A change in plastid morphology was also noticeable leading to the formation of so-called stromules (stroma-filled tubules), which interconnect single plastids. This results in a plastid network surrounding the arbuscule branches (Fig. 3, Hans et al., 2004).

To recognize possible preferences of DXR protein accumulation in specific steps of arbuscule development, the different stages were defined by arbuscule morphology after staining with wheat germ agglutinin-tetramethyl rhodamine isothiocyanate (WGA-TRITC). The early stages are characterized by few relatively thick unbranched hyphae. Subsequently, the distal hyphae start to branch intensely. The arbuscular branches extend further until almost the whole cell is filled by the arbuscule. This is the mature stage and probably the one with the highest physiological activity. The beginning degeneration is characterized by a collapse of distal branches, followed by a more extended collapse of branches finally leading to a clumpy appearance of a degenerated arbuscule. DXR deposition starts to increase at the intermediate stage of maturation and is already strong at the mature stage. However, the strongest DXR signal is associated with the early stages of degeneration concomitant with a condensation of arbuscule branches (Fig. 3, Hans et al., 2004). It appears that the maximum of DXR accumulation is somewhat shifted to the later stages as compared to the mycorrhiza-specific phosphate transporter protein MtPT4 in *M. truncatula*, a marker protein and gene often used to characterize physiologically active arbuscules (Harrison et al., 2002). Recent use of the DXR antibodies on mycorrhizal roots of *M. truncatula* confirms a preferential association of DXR deposition with late stages of arbuscule development (Lohse, 2006; Fester et al., 2006, this issue).

The late appearance in arbuscule development of an enzyme of apocarotenoid biosynthesis is in agreement with results obtained for the accumulation of the apocarotenoids themselves. Yellow droplets and increased amounts of extractable mycorradicin were not observed at early stages of root colonization. Accumulation of mycorradicin only starts about a week later, which roughly corresponds to the reported total life span of an arbuscule of 7–10 days. The average period to maturity for an individual arbuscule is only about 3–4 days (Alexander et al., 1988). Electron microscopy studies did not allow a definitive localization of the yellow pigment, but did show the accumulation of hydrophobic osmiophilic droplets probably representing apocarotenoids particularly close to disintegrating arbuscules (Fester et al., 2002a). These droplets appear within the cytoplasm at the onset and the peak of arbuscule disintegration. In cells with nearly fully collapsed arbuscules these droplets accumulate in the vacuole (Fester et al., 2002a). Taken together, biosynthesis and massive accumulation of apocarotenoids accompany the early and intermediate stages of arbuscule degeneration and collapse, and a potential function may therefore be related to this process.

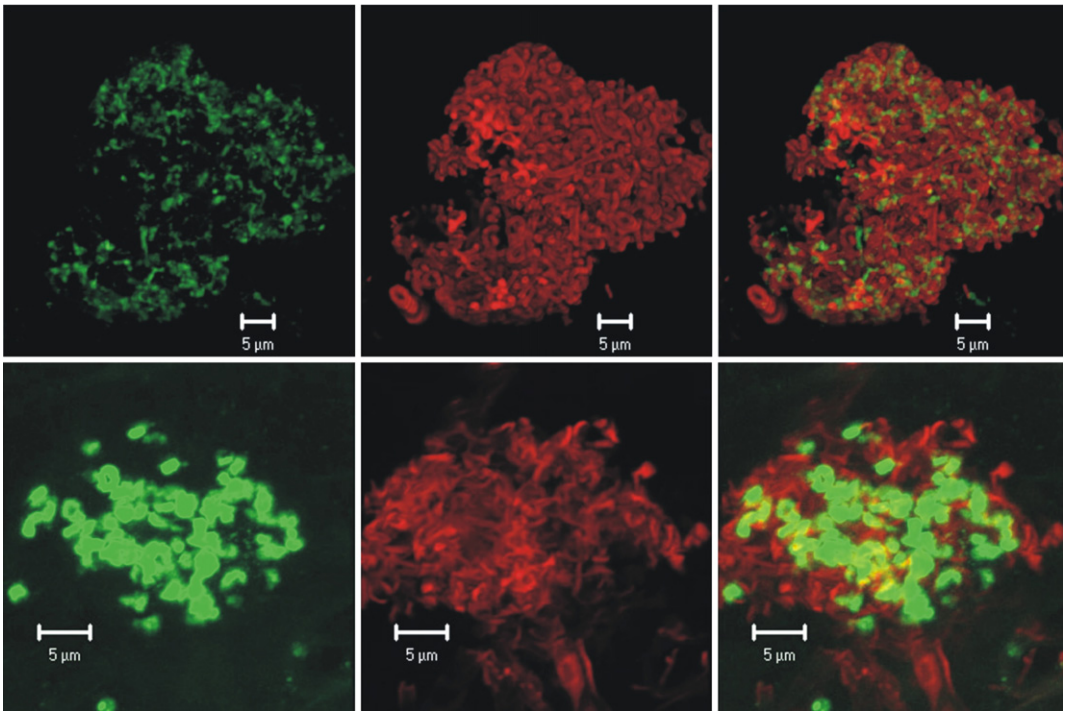

Fig. 3. DXR accumulation in two different stages of arbuscule development. Confocal laser scanning micrographs show an highly branched arbuscule at or close to maturity (upper panels) and an arbuscule in an early degeneration stage characterized by collapsing branches becoming thicker and clumpy (lower panels). The panels on the left show DXR staining detected by anti-6x-His-ZmDXR/anti-rabbit IgG-Alexa488 (green channel). Next to it the fungal structures are visualized by WGA-TRITC staining (red channel). On the right overlays of the two channels are shown.

6. A tool for specific manipulation of apocarotenoid biosynthesis: the *DXS2* isogene

Functional identification of proteins and metabolites can often only be achieved if a mutant with a lack-of-function is available. However, such mutants can be lethal and the objective can therefore not be reached. A valuable tool towards functional identification is therefore the specific downregulation (but not knock-out) of the target by antisense or RNAi technologies (knock-down approach).

A particularly attractive target to downregulate apocarotenoid biosynthesis is the *DXS2* isogene. As judged by the rather strict division of labour between *DXS1* (housekeeping) and *DXS2* (special tasks, secondary metabolism) becoming evident from the comparison of expression profiles, it should be possible to interfere with secondary metabolism (carotenoid and apocarotenoid biosynthesis) without affecting primary functions (primary carotenoid formation, e.g. in photosynthesis). Such a divergence of isogene commitment is not known for any of the later steps of carotenoid and apocarotenoid biosynthesis.

To knock-down apocarotenoid biosynthesis without affecting primary carotenoid formation the *MtDXS2* isogene has been selected for an RNAi approach. We have by now generated several data sets from *M. tuncatula* plants with transgenic hairy roots harboring silencing constructs (D.S. Floß and M.H. Walter, unpublished results). These plants and empty vector control plants were mycorrhized for 4–9 weeks. In all experiments the transcript levels of *MtDXS2* as determined by real-time RT-PCR and the levels of mycorradicin and cyclohexenone derivatives as determined by HPLC analysis were typically reduced to about 10% residual levels as compared to transformed plants harbouring the empty vector, with even lower values in a few cases. The summary of a typical experimental result is shown in Fig. 4. This indicates that targeting a

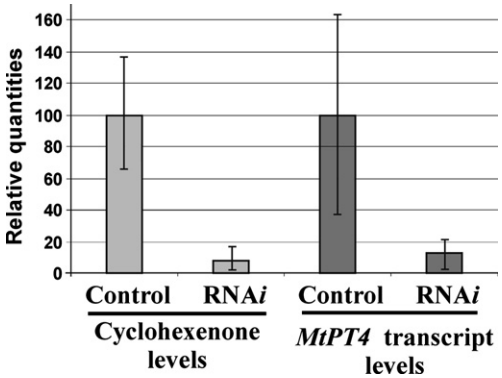

Fig. 4. Summary of results from a 9-week *Glomus intraradices* colonization experiment of empty vector ($n = 5$) and *MtDXS2*-RNAi ($n = 9$) *M. truncatula* plants. *Agrobacterium rhizogenes*-mediated transformation and the pRedRoot vector were used to generate transgenic roots on composite plants according to Limpens et al. (2004) and Vieweg et al. (2004). Cyclohexenone levels were determined from root extracts by HPLC. *MtPT4* transcript levels were analyzed by real-time RT-PCR.

secondary metabolism-specific isogene (*DXS2*) of an early step of carotenoid and apocarotenoid biosynthesis in the MEP pathway can result in a significant knock-down of specific end-products.

7. Alterations of mycorrhizal parameters in apocarotenoid-suppressed plants: starting points to suggest a new kind of function

Regarding the consequences of apocarotenoid suppression on mycorrhizal colonization and the development of fungal structures the results are currently not as clear-cut as the knock-down data (D.S. Floß and M.H. Walter, unpublished results). Colonization of transgenic plants for 9 weeks led to a strong suppression of apocarotenoid accumulation in plants expressing the RNAi constructs (Fig. 4). The intensity of total mycorrhizal structures estimated from stained roots was markedly reduced as was arbuscule abundance. The estimation of metabolically active fungus by determination of *β-tubulin* transcript levels showed an even further decrease. The parameter most strongly affected in this experiment was the molecular marker for functional arbuscules, the mycorrhiza-specific phosphate transporter MtPT4 (Harrison et al., 2002). It was reduced to about 14% of control plants on average (Fig. 4), and in more than half of the RNAi plants the *MtPT4* transcripts were hardly detectable. This result implies an important function of apocarotenoids in late stages of the symbiosis. For both the total mycorrhizal structures and the arbuscule abundance the relative values for the parameters specifying functionality (*β-tubulin* and *MtPT4* transcript levels) were strikingly lower than those for the total structures. This may indicate that apocarotenoids somehow support a functional mycorrhiza and that particularly the functionality of arbuscules suffers in their absence under the conditions of this experiment.

However, after both a 4-week and a 7-week colonization period the impact of apocarotenoid suppression on mycorrhization was different. The intensity of total stainable mycorrhizal structures was not significantly changed in RNAi plants, yet their metabolic activity (fungal *β-tubulin* transcript levels) was lower. Surprisingly, the total arbuscule count from stained roots did exhibit a slight upwards trend in the RNAi plants, yet the molecular marker for functional arbuscules (*MtPT4* transcripts) was slightly lower but far from the strong reduction in the 9 weeks experiment. While the results from the late stage and the two earlier time points are thus somewhat conflicting, a commonality can be recognized in the stronger decrease or lower figures, respectively, in functional structures compared to the total stainable structures particularly for the arbuscules.

In summary, at this point we conclude that apocarotenoids can exert a strong effect particularly on functional arbuscules in the late stages of the symbiosis and that their presence appears to contribute to the preservation of functional arbuscules. Apocarotenoids thus appear to give a boost to the efficiency of the AM symbiosis and might be an optional feature of the plant macrosymbiont to act on the microsymbiont fungal partner to improve its performance in delivering phosphorus and other nutrients by increasing the number of functional arbuscules. Such a non-essential, but optional AM-performance-optimizing function could also explain the strong variability in the levels of apocarotenoids found in different plant families and their absence in some well-colonized roots (Fester et al., 2002a; Fester et al., 2006, this issue).

8. New strategies to elucidate the function of apocarotenoids in mycorrhizal roots: which one of the two is it?

The conclusions drawn above are based on the striking correlation of reduced apocarotenoid levels and the mycorrhizal phenotypes described. However, we cannot exclude at present that other metabolites appearing or disappearing as direct or indirect consequence of the interruption of the flux in the secondary isoprenoid pathway are responsible for the observed alterations or contribute to it.

We are therefore about to extend the biosynthesis suppression strategy to a step, which is closer to the end-products. An attractive potential target in the downstream pathway is the carotenoid cleavage step (Fig. 1). It is predicted to be catalyzed by a carotenoid cleavage dioxygenase (CCD)1-type of enzyme, which cleaves the 9,10 (9′,10′) double bonds of C_{40} carotenoid precursors leading to C_{13} and C_{14} cleavage products (Schwartz et al., 2001). A cDNA homologous to CCD1 has been isolated from maize and its transcript levels were shown to be elevated in mycorrhizal maize roots (J. Hans and M.H. Walter, unpublished). Inspection of available extensive EST data argues for an upregulation of this step in *M. truncatula* roots as well (four EST from mycorrhizal vs. one EST from non-mycorrhizal root libraries). The mycorrhiza-mediated regulation of this step in roots provides a further argument for an uninterrupted metabolic flux to apocarotenoids and against a function for the precursors. However, it cannot be excluded that the CCD step imposes at least some fine-tuning control on substrate flux.

Leaves contain the highest *CCD1* transcript levels or EST numbers, respectively, both in *Zea mays* and *M. truncatula*. A single gene copy therefore seems to be involved in housekeeping and inducible biosynthetic tasks as in the case of *DXR* (Hans et al., 2004). The MtCCD1 protein deduced from the TC100912 sequence of the TIGR data base (assembled from 27 ESTs) is 87% identical to the CCD1 protein from *Phaseolus vulgaris* (accession **AY029525**, Schwartz et al., 2001) and 76% identical to the maize ZmCCD1 protein described by us (accession **AY773278**). The root EST libraries of *M. truncatula* do not provide any evidence for CCD1 sequence variants. In order not to interfere with shoot and leaf functions, the *MtCCD1* suppression is to be performed with the same approach as for the *MtDXS2* isogene.

While in this review a preference is given to a function for the apocarotenoids rather than for the carotenoid precursors, it remains still obscure whether mycorradicin or cyclohexenone derivatives or both are functional. Blumenin has been isolated from mycorrhizal roots and fed back to roots during colonization. Such exogenous blumenin can strongly inhibit mycorrhization and arbuscule formation in low concentrations, particularly when applied at early stages at which endogenous levels are still low (Fester et al., 1999). Mycorradicin might play a role in the detoxification of H_2O_2 accompanying arbuscule disintegration, as described earlier (Salzer et al., 1999). However, such a specific association of H_2O_2 with degenerating arbuscules has not been confirmed by recent results (Fester and Hause, 2005). Moreover, mycorradicin did not suppress an elicitor-induced oxidative burst as did the structurally related fungal molecule corticrocin (Schröder et al., 2001). Therefore, the currently available evidence argues rather for a major contribution from the cyclohexenones to apocarotenoid function than for one from mycorrradicin.

In this context it should be mentioned that the aglycones of cyclohexenone derivatives can have antifungal activities, as has been shown in a search for inhibitors of soil-borne plant pathogens in maize root exudates (Park et al., 2004), and has also been discussed elsewhere (Bouvier et al., 2005). However, the inhibitory activity of these compounds against fungi is rather weak as compared to the activity of cyclic hydroxamic acids such as 6-methoxybenzoxazolinone (MBOA) and 6,7-dimethoxybenzoxalinone (DIMBOA). These latter compounds are known pesticidal secondary metabolites of maize and are toxic at low concentration to a broad range of insects, bacteria and fungi (Park et al., 2004; Frey et al., 1997). Testing antifungal activity of blumenin in a well-established bioassay with the pathogenic fungus *Cladosporium cucumerinum* did not show fungitoxicity under the conditions applied (Fester et al., 1999). A fungitoxic or fungistatic activity of apocarotenoids, if any, is therefore likely to be of a subtle nature so as to treat and control a friend (symbiont) and not to kill a foe (pathogen).

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Michael H. Walter graduated as a chemist at the University of Karlsruhe in 1979. He moved into biology for his Ph.D. work on stress proteins in cultured parsley cells supervised by Klaus Hahlbrock at the University of Freiburg. From 1984 he spent two years as a postdoctoral fellow at the Salk Institute for Biological Studies in San Diego, USA, with Chris Lamb. He then held a position at the University Paul Sabatier in Toulouse, France, to work on lignin biosynthesis with Alain Boudet. In 1988 he returned to Germany moving to the University of Hohenheim in Stuttgart, where he completed his habilitation in 1995. He joined the IPB in Halle in 1997 and became senior scientist and group leader in 2000. His research group focuses on the biosynthesis and function of isoprenoids in the arbuscular mycorrhizal symbiosis. Special interests are directed to the organization, expression and evolution of MEP pathway genes.