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Differential spatio-temporal expression of carotenoid cleavage dioxygenases regulates apocarotenoid fluxes during AM symbiosis

Juan A. López-Ráez [a], Iván Fernández [a,b], Juan M. García [a], Estefanía Berrio [a], Paola Bonfante [c], Michael H. Walter [d], María J. Pozo [a]

a Department of Soil Microbiology and Symbiotic Systems, Estación Experimental del Zaidín (CSIC), Granada, Spain

b Plant–Microbe Interactions, Department of Biology, Faculty of Science, Utrecht University, Utrecht, Netherlands

c Department of Life Sciences and Systems Biology, University of Torino, Viale Mattioli, Torino, Italy

d Leibniz Institute of Plant Biochemistry, Department of Cell & Metabolic Biology, D-06120 Halle (Saale), Germany

Highlights

•SL production is promoted by the AM fungus at the early stages of the interaction.

•AM symbiosis alters apocarotenoid fluxes along the plant–fungus interaction.

•Strigolactone and C13/C14 derivatives production is spatially and temporally regulated.

•This regulation occurs at the transcriptional level by changes in the CCD genes.

•CCD7 is a key enzyme acting as a hub in the regulation of the AM symbiosis.

Abstract

Apocarotenoids are a class of compounds that play important roles in nature. In recent years, a prominent role for these compounds in arbuscular mycorrhizal (AM) symbiosis has been shown. They are derived from carotenoids by the action of the carotenoid cleavage dioxygenase (CCD) enzyme family. In the present study, using tomato as a model, the spatio-temporal expression pattern of the CCD genes during AM symbiosis establishment and functioning was investigated. In addition, the levels of the apocarotenoids strigolactones (SLs), C13 α-ionol and C14 mycorradicin (C13/C14) derivatives were analyzed. The results suggest an increase in SLs promoted by the presence of the AM fungus at the early stages of the interaction, which correlated with an induction of the SL biosynthesis gene SlCCD7. At later stages, induction of SlCCD7 and SlCCD1 expression in arbusculated cells promoted the production of C13/C14 apocarotenoid derivatives. We show here that the biosynthesis of apocarotenoids during AM symbiosis is finely regulated throughout the entire process at the gene expression level, and that CCD7 constitutes a key player in this regulation. Once the symbiosis is established, apocarotenoid flux would be turned towards the production of C13/C14 derivatives, thus reducing SL biosynthesis and maintaining a functional symbiosis.

Keywords

Apocarotenoids; α-Ionols; Arbuscular mycorrhiza; Carotenoid cleavage dioxygenases; Mycorradicin; Strigolactones

1. Introduction

Arbuscular mycorrhizas (AM) are one of the most widespread and studied plant associations with beneficial microorganisms [1] and [2]. About 80% of all terrestrial plants, including most agricultural and horticultural crop species, are able to establish mutualistic associations with soil fungi from the phylum Glomeromycota. This association is considered to be older than 400 million years and a key step in the evolution of terrestrial plants [2]. Arbuscular mycorrhizal fungi (AMF) are obligate biotrophs that colonize the root cortex of the host plant, forming specialized and highly branched tree-like structures called arbuscules [2], [3] and [4]. In the AM symbiosis, the fungus obtains photosynthates from the plant and helps the plant in the acquisition of water and mineral nutrients, mainly phosphorous (P). The symbiosis does not only influence plant nutrition, but it also impacts the plant ability to overcome biotic and abiotic stresses [5], [6] and [7].

AM symbiosis is a very dynamic and asynchronous system, whose establishment and functioning requires a high degree of coordination between the two partners. The success of the association is based on a finely regulated molecular dialogue that orchestrates complex symbiotic programmes [4], [8] and [9]. Plant-AMF communication begins with the production and exudation of strigolactones (SLs) by the host plant [10] and [11]. SLs are multifunctional molecules acting both as rhizosphere cues and as phytohormones regulating plant architecture [12] and [13]. SL perception by the AMF engages its metabolism inducing the so-called pre-symbiotic stage. This stage is characterized by a profuse hyphal growth and branching of the fungus, thus increasing the probability of contact with the root and facilitating symbiosis establishment [10] and [14]. According to their role as pre-symbiotic signals during symbiosis establishment, SL biosynthesis is promoted under P deficient conditions [15] and [16]. Upon recognition of the fungal partner, the plant actively accommodates the fungus in the root tissue [3] and [4] and controls its proliferation [17]. While the importance of SLs in the initiation of AM symbiosis is well accepted, it is not clear whether they also play a role in subsequent steps of the symbiosis.

SLs are derived from carotenoids through sequential cleavage by two dioxygenases (CCD7 and CCD8) (Fig. 1), thus belonging to the class of the apocarotenoids [15] and [18]. It has been shown in several plant species that mutants or transgenic lines altered in CCD7 or CCD8 are less colonized by AMF than their corresponding wild-types [19], [20], [21] and [22]. The vast majority of known apocarotenoids are thought to originate from defined cleavage (“tailoring”) of C40 carotenoids by the carotenoid cleavage dioxygenase (CCD) enzyme family [23], [24] and [25]. This family displays a high degree of regio- and stereo-specificity, and can act in a single or sequential way on carotenoids, giving rise to a variety of different products with a multitude of biological functions not only in plants, but also in animals [26] and [27]. In Arabidopsis, the CCD family is made up of nine members forming the basis for CCD classification in plants. Five of them are designated as 9-cis-epoxycarotenoid dioxygenases (NCEDs) and are isoforms involved in the biosynthesis of the phytohormone abscisic acid (ABA) [23], [26] and [28] (Fig. 1). The other four members of the family (AtCCD1, AtCCD4, AtCCD7, and AtCCD8) cleave a variety of carotenoid substrates at specific double bonds [23]. In tomato, only six members (LeNCED1, LeCCD1a, LeCCD1b, SlCCD4, SlCCD7 and SlCCD8) have been described [20], [22], [29] and [30]. The aforementioned CCD7 and CCD8 enzymes are involved in the biosynthesis of SLs. The role of CCD4 is less well defined. It has been proposed that it might be involved in the biosynthesis of the volatile compound β-ionone, important in flower scent and fruit aroma [27] and [30]. Several reports link CCD4 activity with organ colour and carotenoid homeostasis in tubers, petals and seeds [31], [32] and [33].

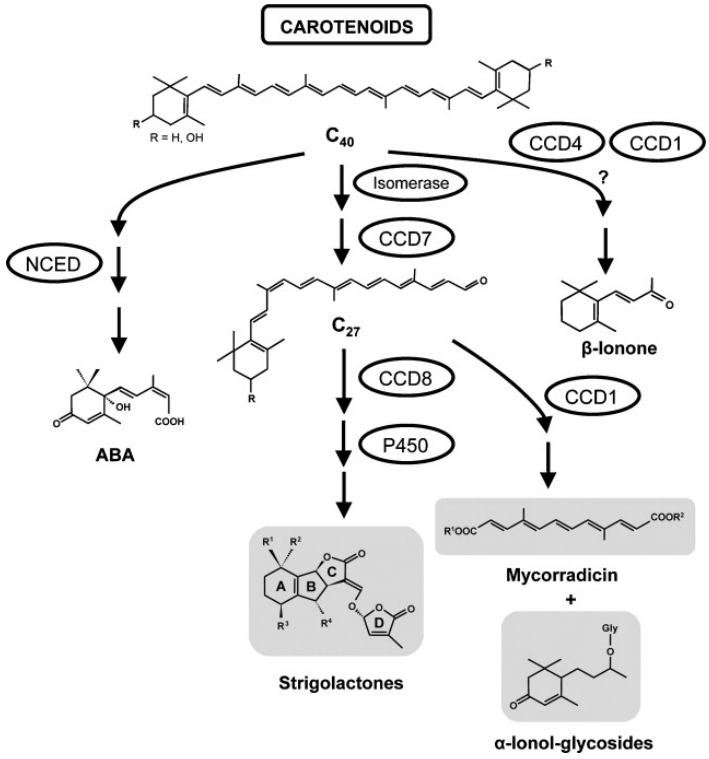

Fig. 1.

Apocarotenoid biosynthetic pathways in plants. NCED, 9-cis-epoxycarotenoid dioxygenase; CCD1, CCD4, CCD7 and CCD8 represent carotenoid cleavage dioxygenases 1, 4, 7 and 8, respectively. P450 represents the cytochrome P450 MAX1, involved in SL biosynthesis. Boxes indicate the end-products (SLs, C13 α-ionol glycoside and C14 mycorradicin derivatives) analyzed in this study.

The remaining CCD enzyme – CCD1 – is, next to NCEDs, the best-studied carotenoid dioxygenase enzyme. It is involved in the biosynthesis of β-ionone, but can also produce minor side products [30], [34] and [35] (Fig. 1). More recently, a role of CCD1 in the production of certain apocarotenoids specifically accumulated during AM symbiosis in roots was described [36] and [37]. Subsequently, it was shown that CCD1 can also cleave C27 apocarotenoid derivatives produced by CCD7, which is also involved in SL biosynthesis, catalyzing the second of two sequential cleavage steps towards the end-products [22] and [27] (Fig. 1). These AM-induced apocarotenoids consist of derivatives of a cyclic C13 α-ionol (formerly called cyclohexenone) and a linear C14 mycorradicin type [38] and [39] (Fig. 1). They form part of the so-called apocarotenoid glycoside-containing 'yellow pigment complex', giving rise to the typical yellow colouration observed in certain mycorrhizal roots [27], [40] and [41]. The production and accumulation of C13 α-ionol and C14 mycorradicin derivatives starts at later stages of the symbiosis, after arbuscule formation has commenced and increases steadily during colonization. Therefore, their accumulation in and next to cells harbouring arbuscules has been associated with a well established and functional symbiosis [28] and [36]. It was shown in Medicago that RNAi-mediated repression of CCD1 resulted in a reduction in C13 α-ionol and C14 mycorradicin derivatives that correlated with more degenerating arbuscules. Thus, a hypothetical role

for these compounds in maintaining the functionality of the AM symbiosis by regulating the degradation and turnover of arbuscules was proposed [28]. Recently, the current status of the discussion on the different roles of apocarotenoids throughout the process of mycorrhization, including SLs, ABA and the AM-induced α-ionols/mycorradicins has been reviewed [28]. However, their precise role in the symbiosis, as well as how their production is regulated is still unclear. Understanding the molecular basis of AM symbiosis establishment and functioning is an ongoing challenge. In the present study, by using an agricultural and economical important crop such as tomato, we have carried out an integrative analysis of the metabolic and transcriptional changes related to the apocarotenoids that take place during AM symbiosis. The results provide insights into the regulation of the biosynthesis of these compounds along the symbiosis and the importance of the CCDs in this regulation.

2. Materials and methods

2.1. Plant growth and AM inoculation

The AMF Rhizophagus irregularis (BEG 121) (formerly Glomus intraradices) and Funneliformis mosseae (BEG12) (formerly Glomus mosseae) were maintained as a soil-sand based inoculum. Tomato seeds (Solanum lycopersicum L. cv. MoneyMaker) were surfaced-sterilized in 4% sodium hypochlorite containing 0.02% (v/v) Tween 20, rinsed thoroughly with sterile water and germinated for 2 days in a plate on moistened filter paper at 25 °C in darkness. Subsequently, tomato seedlings were grown hydroponically in 3 L plastic containers with Long Ashton nutrient solution and constant aeration. The nutrient solution was replaced once a week. After 2 weeks, split-root systems were established for individual plants. The root system of each tomato plant was divided in two parts, and separately transferred to 0.45 L pots with a sterile sand:soil:vermiculite (3:1:1) mixture. Soil was collected from Granada province (Spain). It had a pH of 7.2, 1.6% organic mater and nutrient concentrations (μg kg−1) were: N, 2.1; P, 1.7; and K, 0.8. One side of the split-roots was inoculated by adding 10% (v:v) R. irregularis inoculum (roots with local effects), while the other remained uninoculated (roots with systemic effects). The same amount of soil:sand mix but free from AMF was added to control plants. All plants received an aliquot of a filtrate (<20 μm) of AM inoculum to homogenize the microbial populations. For each treatment a total of 9 plants were used. Plants were randomly distributed and grown in a greenhouse at 24/16 °C with 16/8 h photoperiod and 70% humidity and watered three times a week with Long Ashton nutrient solution [42] containing 25% of the standard P concentration. Plants were harvested after 1, 2, 4 and 6 weeks of growth. Shoot and root fresh weight were determined, and immediately frozen in liquid nitrogen and stored at −80 °C. An aliquot of each individual root system was reserved for mycorrhizal quantification.

2.1.1. In vitro assay

For pre-symbiotic analyses an in vitro system was used. Monoxenic cultures from R. irregularis, using Ri T-DNA (Agrobacterium rhizogenes)-transformed carrot (Daucus carota L. clone DC2) roots, were established according to St-Arnaud et al. [43]. Cultures were grown in compartmental 120 mm Petri dishes, one compartment containing mycorrhizal roots (mycorrhizal compartment) and the other only the extraradical

mycelium and spores (fungal compartment). Cultures were started by placing a mycorrhizal carrot root segment in a compartment containing M medium [44]. Petri dishes were incubated in the dark at 24 °C until the fungal compartment, containing M medium without sucrose, was profusely colonized by the fungus (approximately 20 weeks). Then, the mycorrhizal compartment was removed and the compartment containing the fungal mycelium was used for the experiment. A small hole was carefully made in the lid of the Petri dishes containing R. irregularis cultures. Three-week old tomato plants grown in hydroponics, as described above, were transferred to the Petri dishes extending the roots on the surface of the fungal compartment and the shoot extending beyond the hole, to keep them in open air conditions according to Voets et al. [45]. One plant per plate was used. For controls, plants were transferred to Petri dishes with medium but without fungal mycelium. Plates were covered with aluminium foil and plants were kept in a growth chamber at 24 °C, 16/8 photoperiod and 70% humidity for 4, 24 and 96 h. Three independent replicates were used per time point. After harvesting, the roots were frozen in liquid nitrogen and stored at −80 °C.

2.2. Determination of mycorrhizal colonization

Roots were stained with trypan blue [46] and examined using a Nikon Eclipse 50i microscope and bright-field conditions. Root colonization by the AMF was determined as described by Trouvelot et al. [47], using the MYCOCALC software (http://www.dijon.inra.fr/mychintec/Mycocal-prg/download.html). The parameters measured were the frequency (F%) and intensity (M%) of root cortex colonization, arbuscular (A%) and vesicle (V%) abundance in the whole root system, and the relative colonization intensity (m%) in the mycorrhizal roots. At least five slides, containing 30 root pieces of 1 cm length, were analyzed per biological replicate. Five replicates were analyzed per treatment.

2.3. Extraction and detection of α-ionols and mycorradicin by HPLC

Homogenized and lyophilized root material (50 mg) was extracted three times with 400 µl of 80% MeOH after the addition of 50 µl ribitol (2 mg ml−1 H_2O) as internal standard to yield the polar fraction, as previously described [22] and [36]. For the detection of mycorradicin derivatives, 300 µl of the supernatant was adjusted with KOH to a final 0.5 M concentration. Samples were further treated according to the method described by Fester et al. [48], and analytical HPLC was performed according to the method described by Schliemann et al. [49].

2.4. Extraction and indirect quantification of strigolactones from roots

SLs are germination stimulants of root parasitic plant seeds [11] and [50]. Because of this germinating activity, although other compounds might be also involved, bioassays based on seed germination of root

parasitic plants using a specific fraction of the host plant extract or exudates can be used as a reliable indirect way to quantify the levels of SLs produced by plant roots [15], [18] and [51].

For SL analysis in root extracts, 0.3 g of roots from each treatment and harvest were ground in a mortar with liquid nitrogen. Root material was extracted with 0.5 mL of 40% acetone. Tubes were vortexed for 2 min and centrifuged at 4 °C for 5 min at 8000 × g in a table top centrifuge. This fraction of the root extracts was discarded. Then, the roots were extracted twice with 0.5 mL of 50% acetone. This organic phase, containing the main tomato SLs [15], was carefully transferred to new 2 mL glass vials and stored at −20 °C until use. Before germination bioassays were performed, the acetone was removed from the samples by first adding a corresponding volume of demineralised water and then evaporating the solvent in vacuo in a SpeedVacuum SC100 (Savant Instruments). Germination bioassays with P. ramosa seeds were performed as described in López-Ráez et al [15]. Briefly, seeds of P. ramosa were preconditioned for 12 days at 21 °C. Then, aliquots of 50 µl of root extracts were added to triplicate discs bearing approximately 100 preconditioned seeds and incubated at 25 °C. The synthetic germination stimulant GR24 and demineralised water were included as positive and negative controls, respectively, in each bioassay. After 7 days, the germinated and non-germinated seeds were counted using a binocular microscope. To avoid saturation of the germination response, a series of dilutions of the extracts (1:20, 1:40, 1:80) with demineralised water were used.

2.5. RNA isolation and gene expression analysis by quantitative real time RT-PCR (qPCR)

Total RNA was extracted using Tri-Reagent (Sigma–Aldrich) according to the manufacturer's instructions. The RNA was treated with RQ1 DNase (Promega), purified through a silica column using the NucleoSpin RNA Clean-up kit (Macherey-Nagel). RNA was quantified using a Nanodrop (Thermo Scientific), its integrity checked by gel electrophoresis and stored at −80 °C until use. The first strand cDNA was synthesized with 1 µg of purified total RNA using the iScript cDNA Synthesis kit (Bio-Rad) according to the manufacturer's instructions. Real time quantitative RT-PCR (qPCR) was performed using the iCycler iQ5 system (Bio-Rad) and gene specific primers (Table S1). In tomato, two different CCD1 isoforms – CCD1a and CCD1b – are described [30]. In mycorrhizal roots, an increase in SlCCD1a expression was previously shown, while the transcript levels of SlCCD1b were not altered [52]. Therefore, in the present study only expression of SlCCD1a was analyzed. Three independent biological replicates were analyzed per treatment. Relative quantification of specific mRNA levels was performed using the comparative $2^{-\Delta(\Delta Ct)}$ method [53]. Expression values were normalized using the housekeeping gene SlEF-1, which encodes for the tomato elongation factor-1 α.

2.6. Laser microdissection (LMD)

Roots from 8 weeks-old tomato plants inoculated with the AMF F. mosseae were dissected into 0.5–1 cm length pieces. The root pieces were placed in acetone under vacuum for 15 min, and then kept at 4 °C overnight. The next day they were gradually dehydrated in a graded series of acetone: Neoclear (Merck) (3:1, 1:1 and 1:3) followed by Neoclear 100% (twice) with each step being carried out on ice for 1 h. The

Neoclear was gradually replaced with paraffin (Paraplast Plus; Sigma–Aldrich). The embedding step was as described in Balestrini et al [55]. Laser microdissection to isolate cells from the tissue sections was performed using a Leica AS laser Microdissection system (Leica Microsystem, Inc.). After collection, RNA was extracted with the Pico Pure kit (Arcturus Engineering), as described by Balestrini et al [55]. RNA was quantified using a NanoDrop 1000 (Thermo Scientific) spectrophotometer (Table S2). Absence of DNA contaminations was confirmed by PCR assays in samples without retrotranscription (RT). RT and PCR amplification were carried out using the One Step RT-PCR kit (Qiagen). The samples were incubated for 30 min at 50 °C, followed by 15 min of incubation at 95 °C. Amplification reactions were run in a thermocycler Flexcyler (Analytik Jena) for 40 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s. For the genes SlUbiquitin and SlCCD8 35 cycles of amplification were used.

2.7. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA) using the software SPSS Statistics v. 20 for Windows. When appropriate, the Duncan's multiple range test was applied. Percentage values were arcsine [squareroot(x)] transformed before statistical analysis.

3. Results

3.1. Analysis of mycorrhizal colonization by R. irregularis

As mentioned above, AM symbiosis is a highly dynamic process. In order to analyze the changes produced in the roots during AM colonization over time, both locally at the colonization sites and systemically, a split-root system experiment was carried out. Half of the roots were inoculated with the AMF R. irregularis (local effects, AMF+) and the other half remained non-inoculated (systemic effects, AMF−) (Fig. 2A). The development of the AM symbiosis, as well as the formation of different fungal structures within the roots was followed in a time course series. As expected, no fungal colonization was observed in the non-inoculated compartment. In the inoculated one, both frequency (F%) and intensity (M%) of root colonization significantly ($P < 0.05$) increased over time, reaching the maximum levels at the latest time point, 51 and 18%, respectively (Table 1). The number of vesicles and arbuscules followed a similar pattern. Both were first detected after 4 weeks, with a maximum at 6 weeks of the interaction (Table 1).

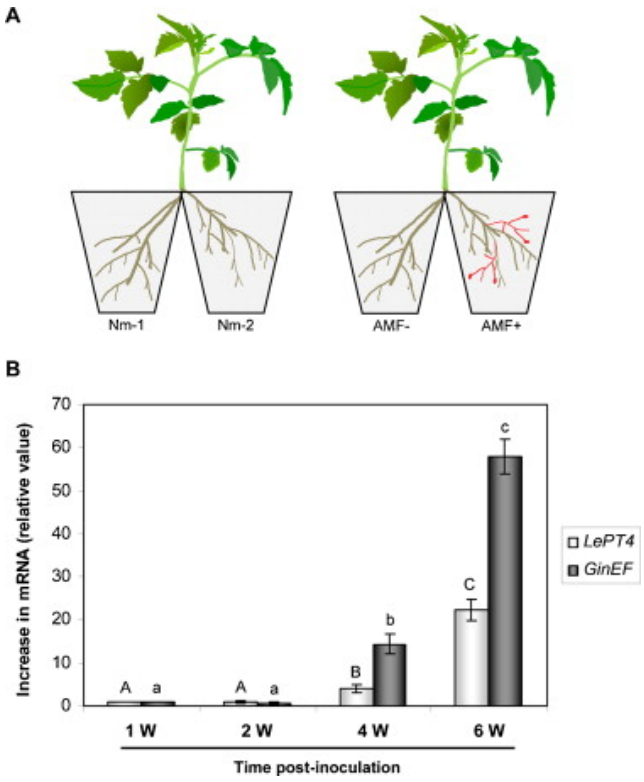

Fig. 2.

Effect of AM symbiosis on local and systemic roots from split-root tomato plants over time. (A), Diagram showing the split-root experimental set-up. (B), Gene expression analysis by real time qPCR for the mycorrhizal marker genes LePT4 (white bars) and GinEF (black bars) in local roots (AMF+) of plants colonized by R. irregularis 1, 2, 4 and 6 weeks after inoculation. Expression of each gene after 1 week inoculation was used as reference. Systemic roots (AMF−) exhibited undetectable levels in all cases (data not shown). GinEF, elongation factor from R. irregularis; LePT4, tomato phosphate transporter 4. Data points represent the means of three replicates (±SE). Bars with different letters are significantly ($P < 0.05$) different according to Duncan's multiple range test.

	Weeks			
	1	2	4	6
F%	8.3 ± 4.8a	18.9 ± 5.9a	48.3 ± 2.4b	51.1 ± 7.8b
M%	0.2 ± 0.1a	0.3 ± 0.1a	9.4 ± 3.8b	18.4 ± 5.8c
m%	0.9 ± 0.4a	1.6 ± 0.4b	19.1 ± 7.4c	34.4 ± 5.3d
V%	0.0 ± 0.0a	0.0 ± 0.0a	0.5 ± 0.4b	1.2 ± 0.6c
A%	0.0 ± 0.0a	0.0 ± 0.0a	0.8 ± 0.4b	1.7 ± 0.6c

Table 1.

Mycorrhizal colonization of tomato roots inoculated with R. irregularis over a period of six weeks evaluated microscopically from trypan-blue-stained samples. Scores according to the method of Trouvelot et al. are given for frequency (F%), intensity (M%) and relative intensity (m%) of mycorrhizal colonization. Scores for the total number of

vesicles (V%) and arbuscules (A%) are also given. Data represent the means of 5 replicates (±SE). Data not sharing a letter in common differ significantly ($P < 0.05$).

In addition to the histochemical analyses, the presence of the AMF in mycorrhizal roots was quantified at the molecular level. Transcripts of the constitutive gene GinEF, encoding an elongation factor from R. irregularis [54], increased along the time of experimentation, showing the same pattern as that determined histochemically (Fig. 2B). Moreover, the functionality of the symbiosis was also analyzed by molecular methods. The tomato gene LePT4 encodes a phosphate transporter specifically associated to AM symbiosis. This gene is expressed in arbusculated cells and considered a marker for a functional symbiosis [55]. LePT4 expression increased over time in mycorrhizal roots, with a maximum at 6 weeks after inoculation (Fig. 2B), confirming a well established and a functional symbiosis. In agreement with the lack of fungal colonization determined by microscopy, no expression of GinEF or LePT4 was observed in the non-mycorrhizal roots (AMF−).

3.2. Impact of AM symbiosis on strigolactone production

To assess the influence of AM symbiosis on SL production over time, a germination bioassay with P. ramosa seeds using the 50% acetone fraction of extracts from roots of the split-root system experiment was carried out. This fraction was previously shown to contain the main tomato SLs [15]. The synthetic SL analogue GR24 (10−9 and 10−10 M), used as a positive control, always induced germination of pre-preconditioned P. ramosa seeds (Fig. 3). Water alone, used as a negative control, did not induce any germination.

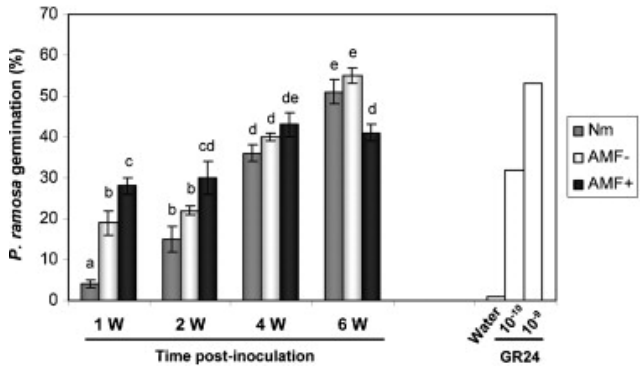

Fig. 3.

Influence of mycorrhizal colonization on root-extractable strigolactones quantified by a germination bioassay. Germination of P. ramosa seeds induced by extracts from non-mycorrhizal plants (grey bars, Nm), and systemic (light grey bars, AMF−) and local (dark grey bars, AMF+) roots from tomato mycorrhizal plants 1, 2, 4 and 6 weeks after inoculation. GR24 (10−9 and 10−10 M) and demineralized water were used as positive and negative controls, respectively. Bars represent the means of five independent replicates (±SE). Bars with different letters are significantly ($P < 0.05$) different according to Duncan's multiple range test.

As expected, no significant differences in germination inducing activity were detected between the two halves of the root system from non-mycorrhizal plants – Nm-1 and Nm-2. Therefore, hereafter only the data corresponding to one of the two halves will be presented (named as Nm). A significant ($P < 0.05$) increase in germination inducing activity of P. ramosa was detected along the time of experimentation, with an initial activity of 4% and a maximum of about 55% at 6 weeks (Fig. 3, Table S3). Phosphate starvation promotes SL biosynthesis [15] and [16]. Since a certain P limitation was applied in our system, the increase in SLs observed in Nm roots could be a consequence of the P deficiency. A similar pattern as for Nm was observed in AMF-roots, but with a higher germination activity after one week inoculation. Here, an 80% higher activity was observed in AMF− compared with Nm roots (Fig. 3, Table S3). A similar behaviour was also observed in fungal-inoculated roots (AMF+), with about 90% higher SL production than in Nm roots one week post-inoculation. Interestingly, at this time point germination activity in AMF+ roots was also significantly higher than that observed in AMF− roots (Fig. 3). This pattern was maintained up to 4 weeks, where no differences in SLs between the three types of roots were detected. The maximum levels of SLs in Nm and AMF− roots were detected at 6 weeks. However, unlike in Nm and AMF− roots, no further increase was detected in AMF+ at this time point (Fig. 3, Table S3). Here, a difference of about 20% activity between AMF+ and AMF− mycorrhizal roots was detected, suggesting that AM symbiosis induces a reduction in SL production and that this reduction depends on the presence of fungal structures.

3.3. Accumulation of C13 and C14 apocarotenoids is induced locally by AM colonization

The accumulation of C13 α-ionol glycoside and C14 mycorradicin derivatives constitutes a reliable metabolic marker for a functional AM symbiosis, since their production depends on arbuscule occurrence [28] and [36]. Two α-ionol derivatives have previously been described from tomato, of which a monoglucoside of 6-(9-hydroxybutyl)-5-hydroxymethyl-1,1-dimethyl-4-cyclohexen-3-one was the most abundant [56]. Mycorradicin has also been detected previously exclusively in mycorrhizal roots of this species [40]. HPLC was used to monitor the α-ionol glucoside and mycorradicin levels in local AMF+ and systemic AMF− roots during mycorrhizal colonization of split-root plants. Neither C13 α-ionol glucoside nor C14 mycorradicin were detected in AMF− roots at any time point (Table 2), confirming the dependency on mycorrhizal structures for the production of these compounds. Both types of metabolites were identified, based on retention times and spectral properties, exclusively in AMF+ roots. However, their presence was only detectable 6 weeks after inoculation (Table 2), coincident with the highest colonization levels and the highest number of arbuscules (Table 1).

Inoculation timing	α-ionols		Mycorradicins	
	AMF−	AMF+	AMF−	AMF+
1 week	n.d	n.d	n.d	n.d
2 weeks	n.d	n.d	n.d	n.d
4 weeks	n.d	n.d	n.d	n.d
6 weeks	n.d	++	n.d	+++

Table 2.

Evaluation of abundance of an α-ionol glycoside and of mycorradicin derivatives in mycorrhizal and non-mycorrhizal roots by HPLC analysis of root extracts. Peak areas of compounds identified by retention times and UV spectral properties were compared from local (AMF+) and systemic (AMF−) roots colonized by R. irregularis in a split-root set-up over six weeks of experimentation. n.d, not-detectable; ++ and +++ specify different levels of metabolite abundance. Five individual plants were analyzed per time point.

3.4. Root colonization differentially affects CCD transcript levels

Existing knowledge indicates that CCD7 has a dual role in SL and C13/C14 apocarotenoid biosynthesis [22], whereas CCD8 acts only as a second cleavage enzyme in SL formation [57]. CCD1 is involved in mycorrhizal C13/C14 apocarotenoid biogenesis only [28] and [36] (Fig. 1), although it has various other roles in above-ground tissues. In order to study the influence of mycorrhizal colonization on their expression in a time course, the transcript levels of these genes were analyzed in roots from the split-root experiment by qPCR. In non-mycorrhizal Nm roots an increase in the tomato SlCCD7 transcript levels of 22-fold was detected after 4 weeks, which continued at 6 weeks (Fig. 4A). A similar pattern was observed in AMF− roots, with an increase of about 50 times at 6 weeks after inoculation compared to the one week Nm reference sample (Fig. 4A). However, unlike in Nm the induction in SlCCD7 expression in AMF− roots was detected after one week inoculation (about 3 times up). As for Nm and AMF− roots, an induction of SlCCD7 transcripts was detected over time in AMF+ roots. In this case, an increase of more than 4 times was observed one week after inoculation. At 6 weeks post-inoculation, transcript levels in AMF+ roots were very high and even more abundant than in AMF− and Nm roots (Fig. 4A). No significant ($P < 0.05$) differences in SlCCD8 transcript levels were observed neither in mycorrhizal nor non-mycorrhizal roots at any time point tested (Fig. 4B). However, looking at absolute rather than relative values, the transcript levels of SlCCD8 were higher than those of SlCCD7 (data not shown), suggesting that due to a constitutively high expression of this gene there is no requirement for up-regulation to maintain SL biosynthesis. When analyzing the expression of SlCCD1a, no differences were detected in the Nm roots (Fig. 4C). In the case of mycorrhizal plants, no changes in transcript levels were detected at earlier time points. However, a significant induction of about 4 and 6 times was observed in AMF+ roots 4 and 6 weeks after inoculation, respectively (Fig. 4C). Remarkably, in contrast to SlCCD7, no changes in SlCCD1a were observed in AMF− roots at these time points (Fig. 4C). As for SlCCD1a, no differences in expression levels were observed for SlCCD4, the other CCD gene so far described in tomato (data not shown).

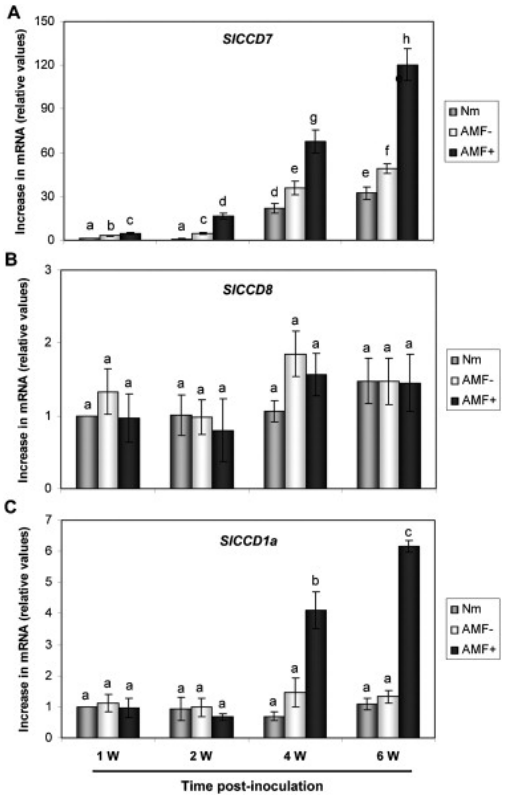

Fig. 4.

Transcript level analysis of CCD genes in tomato roots from a split-root system over time. Relative gene expression analysis by real time qPCR for the genes SlCCD7 (A), SlCCD8 (B) and SlCCD1a (C), in roots from non-mycorrhizal plants (Nm, grey bars), and roots with systemic (AMF−, light grey bars) or local effects (AMF+, dark grey bars) from tomato mycorrhizal plants 1, 2, 4 and 6 weeks after inoculation. Expression of each gene in Nm roots after 1 week inoculation was used as reference. Data points represent the means of three replicates (±SE). Bars with different letters are significantly ($P < 0.05$) different according to Duncan's multiple range test.

n addition to SLs and C13/C14 apocarotenoids, an essential role for ABA in AM symbiosis establishment was shown [58]. When the expression of the ABA biosynthesis gene LeNCED1 was checked in the split-root system experiment, no significant changes were detected for this gene in any root sample at any time point analyzed (data not shown). In agreement with this, we previously found no differences in ABA content in mycorrhizal tomato roots [59].

3.5. CCD expression during the early AM fungus-host plant interaction

To get a further insight about how the expression of the CCD genes is affected during the early stages of the interaction between the host plant and the AMF, even before intraradical colonization occurs, a time course experiment was conducted with monoxenic cultures of R. irregularis and tomato. No differences in expression were observed in control plants for any of the tested genes (Fig. 5). In contrast, a strong increase in SlCCD7 expression was observed along the time course in roots in the presence of the fungus. This induction started 24 h after contact (about 3-fold), reaching the highest levels at 96 h with an increase

of 5.5 times compared to the time 0 h (Fig. 5A). No significant ($P < 0.05$) changes were detected for SlCCD8 and SlCCD1a at any of the time points analyzed (Figs. 5B and C), but as at the later time points, absolute transcript levels of SlCCD8 were high exceeding those of SlCCD7.

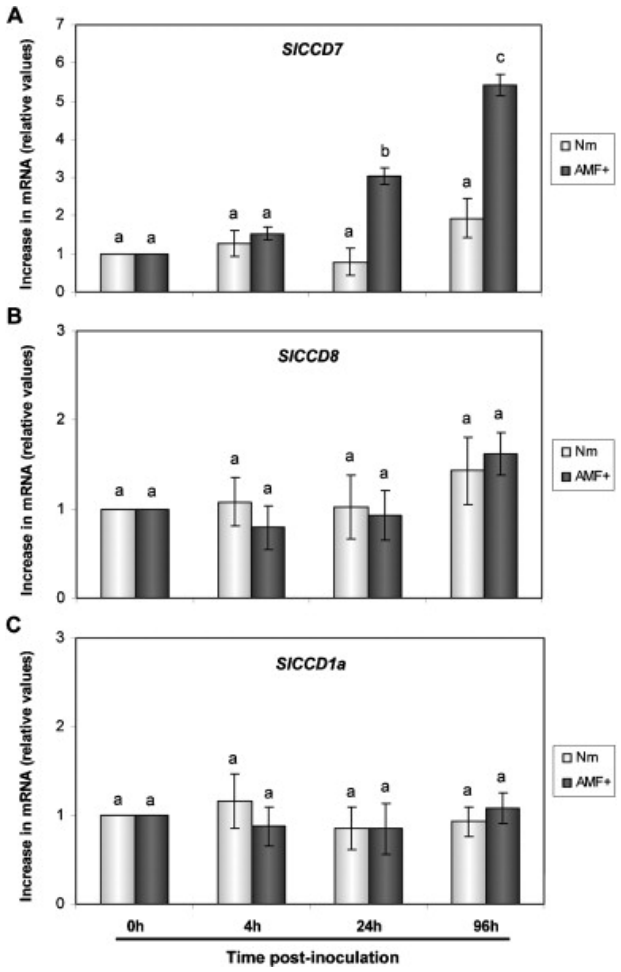

Fig. 5.

Analysis of transcript levels of tomato CCD genes during the mycorrhizal pre-symbiotic stage. Relative gene expression analysis by real time qPCR for the genes SlCCD7 (A), SlCCD8 (B) and SlCCD1a (C), in tomato roots after 0, 4, 24 and 96 h after contact with R. irregularis (dark grey bars, AMF+) and corresponding controls without fungus (light grey bars, Nm) in an in vitro system. Expression of each gene after 0 h contact in Nm roots was used as reference. SlCCD7, SlCCD8 and SlCCD1a, tomato carotenoid cleavage dioxygenases 7, 8 and 1a, respectively. Data points represent the means of three replicates (±SE). Bars with different letters are significantly ($P < 0.05$) different according to Duncan's multiple range test.

3.6. Cell specific analysis of gene expression

Laser microdissection (LMD) offers an effective way to check gene expression in individual cells [60] and [61]. This is particularly important considering the asynchronous character of the AM symbiosis. Although

LMD is not a quantitative technique, it was used to monitor the expression of the different CCD genes at the cellular level in the root cortex of tomato plants upon a well established colonization by the AMF F. mosseae 8 weeks after inoculation. Arbuscule containing cells (AMF+) and cortical cells from F. mosseae colonized roots without arbuscules (AMF−) were obtained, and compared with cortical cells from non-mycorrhizal plants (Nm) (Fig. 6A). Absence of DNA contamination was confirmed by PCR in RNA samples (data not shown). Equal RNA loading was assessed by analysis of transcript levels of the tomato housekeeping gene SlUbiquitin (Fig. 6B). Expression of the AM fungal specific gene Fm18S rRNA was only detected in cells from mycorrhizal roots, and mainly in AMF+ cells (Fig. 6B). The faint band detected in AMF− cells was probably due to the presence of intercellular AM hyphae. Expression of the mycorrhizal marker LePT4 was only detected in AMF+ cells (Fig. 6B), confirming the correct discrimination between arbusculated and non-arbusculated cells. When analyzing the expression of the CCD genes, SlCCD7 was detected in all three types of cells. However, its transcript levels seemed to be higher in the arbusculated AMF+ cells (Fig. 6B). SlCCD8 transcripts were detected in all cell types, although no differences in band intensity were observed (Fig. 6B). Remarkably, transcript levels of SlCCD8 were higher than those of SlCCD7. Unlike SlCCD7 and SlCCD8, SlCCD1a expression was only detected in arbusculated AMF+ cells, and at a high level. No expression was observed in either Nm or Fm-cells (Fig. 6B).

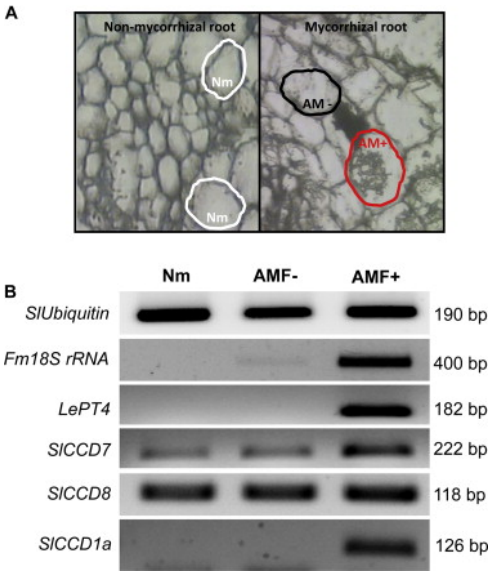

Fig. 6.

RT-PCR analysis of marker genes from tomato roots obtained by Laser Microdissection (LMD). Microscopic images showing borders of excision of non-mycorrhizal cortical cells (Nm, white lines), non-arbusculated cells in root cortex colonized by F. mosseae (AMF−, black lines) and of arbuscule-containing cortical cells (AMF+, grey lines) (A). Gel image after electrophoresis and staining with RedSafe of amplification products as indicated (B). Corresponding amplicon sizes are indicated on the right. SlUbiquitin, tomato ubiquitin; Fm18S rRNA, F. mosseae 18S ribosomal RNA; LePT4, tomato phosphate transporter 4; SlCCD7, SlCCD8 and SlCCD1a, tomato carotenoid cleavage dioxygenases 7, 8 and 1a, respectively.

4. Discussion

AM symbiosis is a complex and dynamic system, which must be precisely controlled by the host plant to avoid over-colonization and maintain the symbiosis as mutualistic [9] and [62]. Proteins, microRNAs and small signalling molecules, including phytohormones and secondary metabolites, play an important role in this plant control [4], [8] and [9]. Apocarotenoids are a class of carotenoid cleavage products some of which exert important biological functions in both animals and plants. In recent years, a prominent role for certain apocarotenoids in AM symbiosis has been proposed [11] and [28]. Indeed, a general stimulation of the carotenoid pathway has been described in mycorrhizal roots [52] and [59]. Moreover, it was shown that blocking the first step of the biosynthetic pathway, catalyzed by the enzyme DXS2, negatively affected mycorrhizal colonization [63]. In the present study, we show that the biosynthesis of apocarotenoids is finely regulated at the transcriptional level during AM symbiosis. An important role for CCDs in this regulation is proposed, being CCD7 a key player.

The best studied apocarotenoids related to AM symbiosis are the SLs. They act preferentially during the pre-symbiotic stage, acting as a 'cry for help' plant signal under nutritional stresses, thus favouring symbiosis establishment [10] and [11]. Accordingly, SL-deficient plants are less mycorrhizal, but still contain mycorrhizal structures without obvious morphological changes [19], [20], [21], [64], [65] and [66]. In agreement with their role at the early steps of root colonization, we observed an increase in the germination inducing activity of P. ramosa, an indirect quantification of SLs, already after one week of inoculation with the AMF R. irregularis in our split-root experiment. Here, an increase of more than 80% activity was detected between mycorrhizal and non-mycorrhizal (Nm) plants, suggesting an increase in SL production. This tendency was maintained until two weeks, reaching a similar level of activity at 4 weeks inoculation (Fig. 3). An induction of SL biosynthesis was previously shown by P and nitrogen deficiency [15] and [16]. In the present study, we show for the first time a P-unrelated increase in SLs, which is promoted by the presence of the AMF prior to intraradical colonization. The increase correlated with an induction of the SL biosynthesis gene SlCCD7, while the expression of the other biosynthetic gene – SlCCD8 – did not change significantly. A similar behaviour was observed in an independent in vitro experiment, where only transcripts of SlCCD7 were increased during the early stages of the plant-AMF interaction. Again, no changes in SlCCD8 expression were detected at these stages. The results suggest that, although essential for SL biosynthesis, SlCCD8 might not be a transcriptional regulatory step in the biosynthesis of SLs during the early stages of AM establishment. Since transcript levels of SlCCD8 in roots are normally higher that those of SlCCD7, it might be that an up-regulation of SlCCD8 is not required to maintain SL biosynthesis. On the other hand, a post-transcriptional regulation for this enzyme cannot be discarded. Indeed, post-transcriptional regulation of CCD8 by auxin was previously proposed in Arabidopsis [67]. As for SlCCD8, no differences in transcript levels at these early stages were detected for SlCCD1a, suggesting that increased levels of this enzyme are not required at these initial stages of the interaction.

Interestingly, differences in the germination inducing activity were also detected within the mycorrhizal plants at the early stages of mycorrhization. Inoculated (AMF+) roots showed about 20% more activity than non-inoculated (AMF−) roots, differences that were maintained up to two weeks after inoculation. These results indicate that the plant senses the presence of the fungus, especially at the early time points, being even able to distinguish between mycorrhizal and non-mycorrhizal roots. Consequently, it actively reacts by increasing SL production favouring fungal development, thus increasing the probability of contact and symbiosis establishment. In this sense, it has been shown that the metabolically active AMF produce

diffusible molecules – Myc factors – which are perceived by the host root, inducing the molecular responses required for a successful colonization [68] and [69]. Therefore, these Myc factors would act as a cue indicating the presence of the fungus in the vicinity of the host root, and inducing the production of SLs by the plant in order to stimulate their own growth during the pre-symbiotic interaction. Interestingly, it has been recently demonstrated that the addition of GR24 elicits the production of these Myc factors in R. irregularis [68], indicating that both partners mutually sense its presence and that they respond accordingly. On the other hand, in addition to their role as signalling molecules in the rhizosphere, SLs are phytohormones regulating root hair and lateral root formation [70] and [71]. Since lateral roots are the preferred sites for AM hyphal colonization, this initial induction of SLs could also modify root architecture to favour colonization by increasing the number of potential entry points for the fungus.

A different behaviour in SL production was observed at the later stages of the inoculation timing, which correlated with the highest levels of mycorrhization. At these stages, a continuous increase in the germination activity up to the end of the experiment was detected in non-mycorrhizal roots, both Nm and AMF−. Conversely, no further increase was observed in mycorrhizal AMF+ roots after 4 weeks inoculation. Indeed, a reduction of about 30% was detected in AMF+ compared with Nm and AMF− roots at the latter time point (Fig. 3). A reduction in SLs by AM symbiosis has been previously described in several plant species such as pea, lettuce and tomato [51], [72] and [73], although a well established mycorrhization is required for such reduction [51]. In agreement with the reduced levels of SLs in AM plants, a reduced expression of the gene PDR1, encoding a SL transporter, was shown at later stages of colonization of petunia roots by R. irregularis [21]. In this scenario, plants would reduce SL biosynthesis to avoid an over-colonization, which could be metabolically costly, a mechanism known as autoregulation [74]. In the present study, only moderate levels of colonization were reached after 6 weeks of inoculation, which may explain why the reduction in SLs was subtle. As for the early stages, the transcript levels of SlCCD8 in roots were not altered at the later stages of colonization. No changes in CCD8 expression in a well established symbiosis were also reported in petunia [75]. As far as we know, CCD8 regulation by AM symbiosis has not been reported in any plant. Similarly, in the SL-deficient tomato mutant Sl-ORT1, which showed a reduced mycorrhization, SlCCD8 expression was not altered, while SlCCD7 levels were reduced [65].

Conversely, the increase in SLs at these later stages correlated with the expression of SlCCD7 in non-mycorrhizal Nm and AMF− roots. However, after 6 weeks of inoculation no correlation between SLs and SlCCD7 levels was observed in AMF+ roots. At this time point, the germination activity was decreased while transcript levels of SlCCD7 continued to increase (Fig. 3 and Fig. 4). In addition to their role in SL biosynthesis, an involvement of CCD7 in the production of AM-induced C13/C14 apocarotenoids was proposed [76] and later shown explicitly [22]. These compounds appear preferentially at later stages of mycorrhization and their accumulation is dependent on arbuscule occurrence [36] and [77]. It has been recently proposed that they play a role in the active plant control of arbuscule turnover (reviewed in Walter [28]). Accordingly, we only detected C13 α-ionol glycoside and C14 mycorradicin derivatives in AMF+ roots 6 weeks after inoculation, when the maximum rate of colonization and number of arbuscules were observed (Table 1). Interestingly, an increase in the transcript levels of SlCCD7 was specifically detected by LMD analysis in arbusculated cells, the place where the α-ionols and mycorradicins are accumulated [40], compared with cells without arbuscules. A reduction in C13/C14 apocarotenoids in mycorrhizal SlCCD7 antisense lines compared with the corresponding wild-type was shown in tomato [22]. Therefore, our

results confirm the involvement of this enzyme in the production of these AM-induced apocarotenoids and link their production specifically to arbusculated cells.

As above mentioned, in addition to CCD7 and CCD8, CCD1 has been shown as an important player in AM symbiosis. Knock-down approaches in Medicago and mycorrhizal experiments in maize suggested that this enzyme is also involved in the biosynthesis of C13 α-ionol and C14 mycorradicin derivatives [36] and [37]. In this sense, it was proposed that CCD1 would act sequentially after CCD7 cleaving the C27 product into C13 and C14 apocarotenoids (Fig. 1) [77]. In agreement with the involvement of CCD1 in mycorrhizal colonization and C13/C14 apocarotenoid biosynthesis, we observed a clear increase in the transcript levels of SlCCD1a in AMF+ roots at the later stages of colonization, while no significant differences were detected in non-mycorrhizal roots. Moreover, its expression was locally induced in arbusculated cells (Fig. 6). Induction of CCD1 expression has been previously described in mycorrhizal maize, petunia and tomato roots [37], [52] and [78]. However, in the present study we show for the first time that the SlCCD1a transcripts are exclusively detected in cells containing arbuscules. The expression pattern of SlCCD1a at these later stages of colonization agreed with that observed for SlCCD7 in mycorrhizal roots, both in the split-root experiment and in LMD analysis (Fig. 3 and Fig. 4), and correlated with the detection of the C13/C14 apocarotenoids. Therefore, although the involvement of CCD4 cannot be discarded, our findings support the hypothesis of the joint involvement of CCD7 and CCD1 in the production of these compounds in arbusculated cells and suggest that they are exclusively produced in these cells.

Remarkably, the expression pattern of SlCCD1a in non-mycorrhizal roots at the later stages, where the reduction in SLs did not take place, differed from that observed for SlCCD7. Therefore, it seems clear that CCD7 plays a dual role during AM symbiosis. On one hand, it is involved in the biosynthesis of SLs during pre-symbiosis and early stages of the interaction to facilitate AM fungal colonization (Fig. 7), and possibly to induce lateral root formation as well. On the other hand, at later stages CCD7 is related to the production of C13/C14 apocarotenoids (Fig. 7), likely to maintain the functionality of the symbiosis. However, how this dual function of CCD7 is regulated during AM symbiosis is still unknown. Since SlCCD8 expression is not changing and SlCCD1a is specifically induced at the later stages of mycorrhization, it might be that the latter regulates the flux of the C27 precursor produced by CCD7 depending on the symbiotic stage. In this sense, the presence of arbuscules would increase CCD1 levels, which would reduce the amount of substrate available for CCD8.

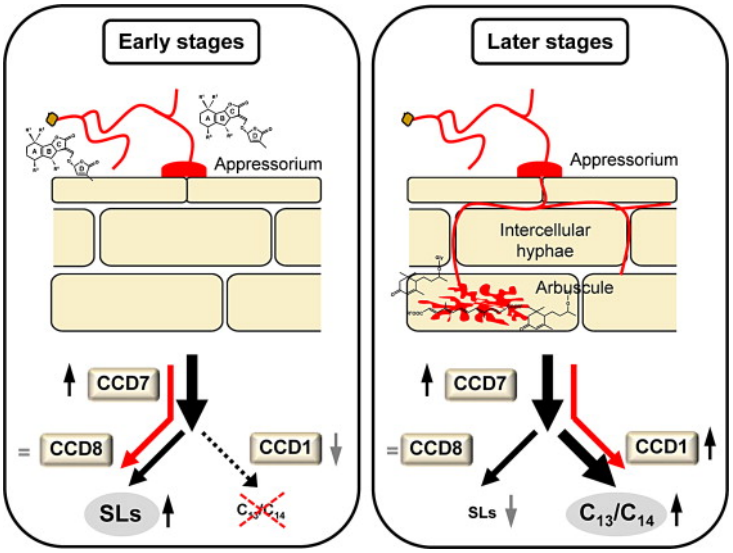

Fig. 7.

Schematic summary of the host root colonization by AMF during the early and later stages. Induction of CCD7 promotes SL biosynthesis to favour host plant-AMF contact during the pre-symbiotic stage. During a well established symbiosis, induction of both CCD7 and CCD1 derives the flux of apocarotenoids towards production of C13/C14 compounds, thus reducing SL production.

In conclusion, we show here that the biosynthesis of apocarotenoids during AM symbiosis establishment and functioning is tightly regulated at transcriptional level throughout the entire process, and that this regulation is cell specific. An initial induction of SL production upon AMF detection by the host, which is accompanied by an up-regulation of SlCCD7 expression, is shown for the first time. At later stages of the symbiosis, the tandem induction of SlCCD7 and SlCCD1 would turn the flux towards the production C13/C14 apocarotenoids, thus reducing SL production and avoiding an over-colonization. However, further research is required to precisely decipher how the plant regulates the apocarotenoid's flux in order to maintain colonization rates and symbiosis functionality at optimum levels.

Acknowledgments

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