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ORIGINAL ARTICLE

Cloning and characterisation of a maize carotenoid cleavage dioxygenase (*ZmCCD1*) and its involvement in the biosynthesis of apocarotenoids with various roles in mutualistic and parasitic interactions

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Abstract Colonisation of maize roots by arbuscular mycorrhizal (AM) fungi leads to the accumulation of apocarotenoids (cyclohexenone and mycorradicin derivatives). Other root apocarotenoids (strigolactones) are involved in signalling during early steps of the AM symbiosis but also in stimulation of germination of parasitic plant seeds. Both apocarotenoid classes are predicted to originate from cleavage of a carotenoid substrate by a carotenoid cleavage dioxygenase (CCD), but the precursors and cleavage enzymes are unknown. A *Zea mays* CCD (*ZmCCD1*) was cloned by RT-PCR and characterised by expression in carotenoid accumulating *E. coli* strains and analysis of cleavage products using GC–MS. ZmCCD1 efficiently cleaves carotenoids at the 9, 10 position and displays 78% amino acid identity to *Arabidopsis thaliana* CCD1 having similar properties. *ZmCCD1* transcript levels were shown to be elevated upon root colonisation by AM fungi. Mycorrhization led to a decrease in seed germination of the parasitic plant *Striga hermonthica* as examined in a bioassay. *ZmCCD1* is proposed to be involved in cyclohexenone and mycorradicin formation in mycorrhizal maize roots but not in strigolactone formation.

Keywords Apocarotenoids · Arbuscular mycorrhizal fungi · Carotenoid cleavage dioxygenase · Germination stimulants · Maize · *Striga* spp.

Abbreviations

ABA	Abscisic acid
AM fungi	Arbuscular mycorrhizal fungi
DXR	Deoxyxylulose-5-phosphate reductoisomerase
NCED	9-*Cis*-epoxycarotenoid dioxygenase
ZmCCD1	*Zea mays* carotenoid cleavage dioxygenase 1

Introduction

Carotenoid cleavage dioxygenases (CCDs) and 9-*cis*-epoxycarotenoid dioxygenases (NCEDs) constitute a family of enzymes that catalyse the cleavage of carotenoids at specific double bonds. The cleavage products are collectively called apocarotenoids (Schwartz et al. 2001; Auldridge et al. 2006b). The first carotenoid cleaving enzyme (*Vp14*) was isolated from the abscisic acid (ABA) deficient viviparous maize mutant. Vp14 is an NCED and catalyses the rate-limiting step in ABA (also an apocarotenoid) biosynthesis, the cleavage of the 9-*cis*-isomer of neoxanthin or violaxanthin at the 11,12 position (Schwartz et al. 1997). Based on the sequence homology to *Vp14*, nine *CCDs* have been identified in the *Arabidopsis thaliana* genome. Among them, five are *Vp14*-like (*NCED2*, *NCED3*, *NCED5*, *NCED6* and *NCED9*) and are supposed to be involved in ABA biosynthesis (Tan et al. 2003). The other four have been given the generic designation carotenoid cleavage

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dioxygenase (*CCD1*, *CCD4*, *CCD7* and *CCD8*) (Auldridge et al. 2006a). *Arabidopsis* CCD1 (AtCCD1) symmetrically cleaves multiple *trans*-carotenoid substrates (*β*-carotene, lutein, zeaxanthin, trans-violaxanthin) at the 9, 10 and 9′, 10′ double bonds producing a C_{14} dialdehyde and two C_{13} products that vary depending on the carotenoid substrate (Schwartz et al. 2001). Recently, Klee et al. showed that AtCCD1 can also cleave lycopene at the 5, 6 and/or 5′, 6′ double bond leading to the formation of 6-methyl-5-hepten-2-one (Vogel et al. 2008). Although AtCCD7 cleaves at the same position as AtCCD1, AtCCD7 was shown to cleave *β*-carotene asymmetrically and therefore produces a C_{13} and a C_{27} product (Schwartz et al. 2004). This C_{27} apocarotenoid can be further catabolised by AtCCD8 yielding a C_{18} apocarotenoid (Schwartz et al. 2004). The function and enzymatic activities of AtCCD4 so far remain unknown but the ortholog in chrysanthemum (*Chrysanthemum morifolium* Ramat.), *CmCCD4a*, showed specific expression only in white petals which led to the conclusion that CmCCD4a cleaves carotenoids into, as yet unidentified, colourless compounds (Ohmiya et al. 2006).

Orthologous enzymes of AtCCDs have been reported in many other plant species and usually have the same cleavage activity as their *Arabidopsis* counterpart. For example, orthologous enzymes of AtCCD1 have been found in *Crocus sativus*, *Lycopersicon esculentum*, *Petunia hybrida*, *Vitis vinifera* and *Cucumis melo* and all cleave several carotenoid substrates at the 9, 10 and 9′, 10′ double bonds (Bouvier et al. 2003; Simkin et al. 2004a, b; Mathieu et al. 2005; Ibdah et al. 2006). However, from some plant species CCDs were cloned that cleave carotenoid substrates at positions different from the *Arabidopsis* CCDs. For example, Bouvier et al. cloned *CsZCD* from *C. sativus* that catalyses cleavage of zeaxanthin at the 7, 8 position (Bouvier et al. 2003).

Apocarotenoids resulting from the oxidative cleavage of carotenoids serve as important signalling molecules in a variety of biological processes. The plant hormone ABA has long been known to be involved in regulating plant responses to various environmental stresses, especially drought and salinity and also in long-distance signalling within the plant (Davies et al. 2005). Furthermore, a novel, unidentified, apocarotenoid phytohormone that regulates plant lateral shoot branching was recently postulated to be produced from an as yet unidentified carotenoid substrate by sequential cleavage by CCD7 and CCD8 (Booker et al. 2004; Schwartz et al. 2004). In addition to being plant hormones, some of the apocarotenoids (such as *β*-ionone, *β*-cyclocitral, geranial, geranial acetone, theaspirone, *α*-damascenone and *β*-damascenone) contribute to the flavour and/or aroma of flowers or fruits of a variety of agricultural products (Auldridge et al. 2006b). For example, AtCCD1 can cleave *β*-carotene to produce the C_{13} derivative *β*-ionone, an important fragrance compound in the flowers of many plant species (Schwartz et al. 2001).

The derivatives of the apocarotenoids mycorradicin (an acyclic C_{14} polyene) and C_{13} cyclohexenones accumulate during colonisation of roots by arbuscular mycorrhizal (AM) fungi, with the former causing the yellow colour of maize roots that are colonised by AM fungi (Klingner et al. 1995; Fester et al. 2002). These apocarotenoids are predicted to originate from an unknown C_{40} carotenoid precursor by cleavage at positions 9, 10 and 9′, 10′ by an unknown carotenoid cleavage enzyme (Walter et al. 2000; Fester et al. 2002; Strack and Fester 2006) (Fig. 1). During AM colonisation, carotenoid biosynthesis is upregulated in the roots of several plant species suggesting that the apocarotenoids play some important role in the symbiosis (Fester et al. 2002; Strack and Fester 2006; Walter et al. 2007).

Another class of interesting apocarotenoids are the strigolactones (Fig. 1), which form a separate group of structurally closely related molecules (Bouwmeester et al. 2003). Strigolactones are germination stimulants of the root parasitic *Striga* spp. and *Orobanche* spp., obligate parasitic plants that can only survive and reproduce when attached to the root of a host plant from which they obtain water, nutrients and assimilates. The seeds of these parasitic plants will only germinate in the presence of these germination stimulants (Bouwmeester et al. 2003). Interestingly, these strigolactones also induce branching in germinating AM fungal spores, a process required for host root colonisation (Akiyama et al. 2005; Besserer et al. 2006). Recently, we have demonstrated that the strigolactones are derived from the carotenoid pathway probably with the involvement of a carotenoid cleaving enzyme (Matusova et al. 2005). In addition to this common signalling molecule, AM fungi and parasitic plants have another relationship. In pot and field trials it was demonstrated that enhanced colonisation with AM fungi can reduce *Striga* infection in maize and sorghum (Gworgwor and Weber 2003; Lendzemo et al. 2007).

In recent years, large progress was made with the characterisation of plant CCD enzymes and their apocarotenoid products (Auldridge et al. 2006b). However, the biosynthetic origin of some biologically important apocarotenoids is still unknown. For example, although it is likely that the "yellow pigment" formed in AM colonised roots is derived from carotenoids by oxidative cleavage, neither the carotenoid precursor nor the cleavage enzyme are known. The same holds for the strigolactones. In this study, we have cloned and characterised a maize *CCD1* cDNA (*ZmCCD1*) and have analysed the recombinant protein it encodes. We provide arguments for involvement of ZmCCD1 in the formation of the "yellow pigment" apocarotenoids and discuss its possible relation to the formation of strigolactones.

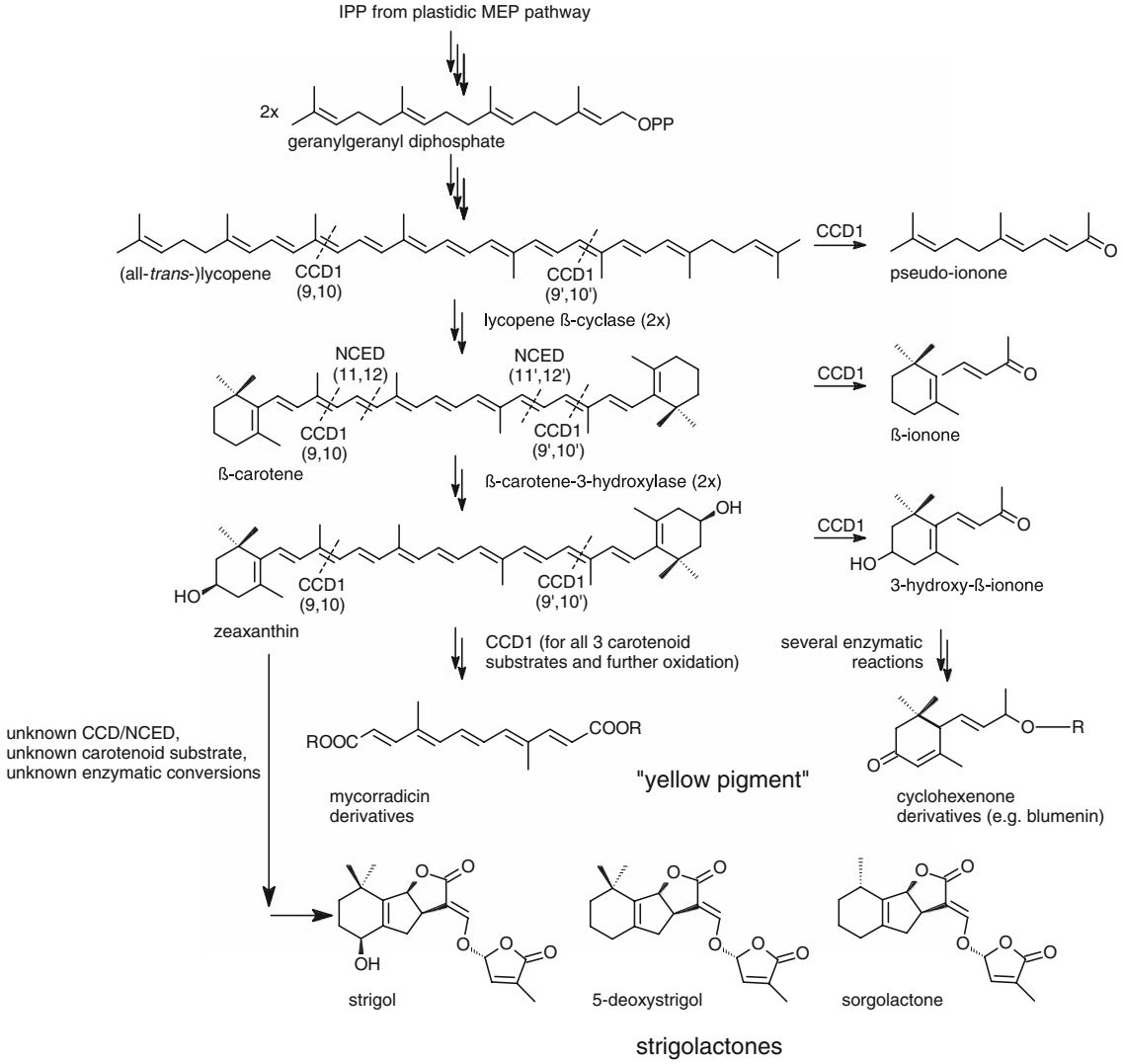


Fig. 1 Schematic overview of reactions catalysed by ZmCCD1. The three carotenoid substrates tested (lycopene, β-carotene, and zeaxanthin) were cleaved by ZmCCD1 yielding: pseudo-ionone (6,10-dimethyl-3,5,9-undecatrien-2-one), β-ionone (9-apo-β-caroten-9-one) and 3-hydroxy-β-ionone (3-hydroxy-9-apo-β-caroten-9-one), respectively. In mycorrhizal roots ZmCCD1 is predicted to be involved in the formation of the "yellow pigment", consisting of cyclohexenone and mycorradicin derivatives, from an as yet unknown carotenoid precursor (Strack and Fester 2006). Also strigolactones, of which three examples are shown, are derived from carotenoids by an unknown route (Matusova et al. 2005)

Materials and methods

Plant materials and chemicals

Maize (*Zea mays* L.) seeds of cultivar MBS 847 (Dent type; obtained from J. C. Robinson Seeds, Ottersum, The Netherlands) were washed with 70% ethanol for 10 min (all chemicals from Sigma–Aldrich, Zwijndrecht, The Netherlands, unless specified otherwise). Subsequently, the seeds were washed with 25 mL of 2% sodium hypochlorite with 0.02% (v/v) Tween-20 for 30 min. Subsequently, the seeds were washed four times using sterilized tap water. Finally, the seeds were pre-germinated for 2 days in a dark climate room at 25°C in a Petri dish with moist filter paper. Maize plants were then grown in 1-L plastic containers containing perlite under greenhouse conditions with a day/night temperature of 25°C (16 h)/18°C (8 h). The plants were only given tap water. After 2 weeks, roots and leaves were harvested separately and ground under liquid nitrogen for total RNA extraction.

Striga hermonthica (Del.) Benth. seeds used in the experiments were collected from a *S. hermonthica* population growing on maize in Kibos, Kenya in 1994 (kindly provided by Vicky Child of Long Ashton Research Station, Bristol, UK). To assess the effect of mycorrhizal colonisation of maize on the germination of *Striga*, maize plants of cv MBS 847 (Dent type) were grown under the same conditions as described above but in expanded clay

(Lecaton, 2–5 μm particle size; Fibo Exclay, Pinneberg, Germany). The expanded clay consisted of one part expanded clay on which leek plants (*Allium porrum* L.) inoculated with the AM fungus *Glomus intraradices* had been growing, and two parts of clean expanded clay. Plants were watered with half-strength Hoagland's solution containing onetenth of the normal phosphate concentration. Four plants for each treatment were carefully removed from the expanded clay at 14, 21, 28 and 34 days after inoculation and root exudates were collected from each single plant separately for 24 h in demineralised water. Rates of colonisation by AM fungi were estimated by staining roots with trypan blue (Maier et al. 1995). The exudates were diluted to the same concentration of gram root fresh weight per millilitre root exudate and induction of *S. hermonthica* germination was assessed. Before the germination bioassay, the *S. hermonthica* seeds were preconditioned. Hereto, seeds were surface-sterilized in 2% sodium hypochlorite containing 0.1% Tween 20 for 5 min. Then seeds were rinsed three times with sterile demineralised water, and excess water was removed by filtration through a Büchner funnel. The sterile seeds were allowed to air dry for 2 h and subsequently approximately 50–100 seeds were placed on 9-mm diameter glass filter paper (Sartorius, Germany) discs. Twelve discs were placed in 9-cm diameter Petridishes with a filter paper (Whatman, UK) moistened with 3 mL demineralised water. The Petri dishes were sealed with parafilm and wrapped in aluminium foil and placed in a growth chamber at 30°C for 10 days. Before applying root exudates, the discs of the seeds were dried on sterile filter paper for 3 min and transferred to a new Petri dish with a 1-cm wide filter paper ring (outer diameter of 9 cm), moistened with 1 mL sterile demi water to keep a moist environment inside the Petri dish. Fifty microlitre of the root exudates to be tested were applied to triplicate discs. The synthetic strigolactone analogue GR24 (0.1 mg L^{-1}) (kindly provided by Prof. B. Zwanenburg, Radboud University, Nijmegen, The Netherlands) was used as a positive control and sterile demineralised water as a negative control in each germination assay. Seeds were then incubated at 30°C in darkness for 2 days. After 2 days, germination was assessed using a binocular microscope. Seeds were considered to be germinated if the radicle protruded through the seed coat. Although germination of *Striga* can be induced by several different chemical compounds in vitro, the evidence is accumulating that in plant root exudates the strigolactones are the major factor responsible (Bouwmeester et al. 2003, 2007).

For *ZmCCD1* transcript analysis, maize (cv dwarf-1) was grown in expanded clay in 250-mL plastic pots under a 16-h light/8-h dark regime in a growth chamber at 25°C. Plants were fertilised once a week using Long Ashton nutrient solution with onetenth of the original phosphate content. Inoculation with AM fungi was done as described before (Hans et al. 2004). Roots were collected and frozen in liquid nitrogen either entirely or after separation into white and yellow segments and stored at −80°C until used. Sampling was done from at least three independent root systems for each treatment, which were combined for analysis. The collection of white and yellow roots was similarly done from three root systems each.

Statistical analysis

Germination data were transformed by taking the arcsine of the square root of the proportion of germinated seeds prior to analysis of variance (ANOVA).

Cloning and characterisation of ZmCCD1

Total RNA was extracted from maize roots and leaves using Tri Reagent and quantified using the NanoDrop ND-1000 spectrophotometer (Isogen Life Science, IJsselstein, The Netherlands). RT-PCR was performed in a 20 μL volume, with 10 μL (1 μg) of total RNA as the template, 1 μL of Primer Oligo dT21 (25 ng μL^{-1}), 2 μL of DTT (0.1 M), 2 μL dNTP (10 mM), 4 μL of 5× RT buffer and 1 μL Superscript II Reverse Transcriptase (200 units μL^{-1}) (all from Invitrogen, Breda, The Netherlands) according to the manufacturer's instructions. A full-length fragment was amplified using 35 cycles and the following nested specific primers: forward primer 5′-CTTCGCTACAAGTCATC TCG-3′, reverse primer 5′-AGTGAAGATACGGCACC TGC-3′; and nested forward primer 5′-CAAGTCATCT CGCCGCAACC-3′, nested reverse primer 5′-GCAGGA CGTGTATTCGAACC-3′. Primers were designed according to a TC sequence from maize (TC220599 TIGR), which is highly similar to the *Arabidopsis* CCD1 and obtained from Biolegio, Nijmegen, The Netherlands. The PCR fragments were cloned into the pGEM®-T Easy vector using the TA-cloning kit (Promega, Leiden, The Netherlands) and sequenced on a DNA sequencer model 3730X DNA Analyzer (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands).

The obtained *ZmCCD1* sequence showed two possible start codons in the same reading frame. Therefore, we amplified *ZmCCD1A* (long) by PCR using the forward primer 5′-CGCAGGATCCATGGGGACGGAGG-3′, and the reverse primer 5′-ATATGAATTCGCAGGTGCCGT ATCTTCAC-3′, and *ZmCCD1B* (short) using the forward primer 5′-GGATCCATGGACAGCCACCG-3′ and the reverse primer 5′-GCCACCGCTGAGCAATAACTA-3′. The resulting PCR products were cloned into the *Bam*HI and *Eco*RI sites in pRSETA (Invitrogen Breda, The Netherlands). Plasmid pRSETA-*ZmCCD1A*, plasmid pRSETA-*ZmCCD1B,* plasmid pRSETA-*AtCCD1* as positive control

and pRSETA (empty vector) as a negative control were transformed to *E. coli* BL21 (DE3) pLysS carrying expression plasmids for lycopene, β-carotene, and zeaxanthin biosynthesis (Cunningham et al. 1996). The transformed *E. coli* were grown overnight at 37°C on LB solid medium containing 50 μg mL^{-1} of ampicillin, 35 μg mL^{-1} chloramphenicol and 1% glucose (ampicillin and chloramphenicol from Duchefa, Haarlem, The Netherlands). Selected colonies were streaked on the same LB solid medium and incubated at 21°C for 4–7 days in darkness for expression. In this system carotenoid cleavage activity is visualised by the absence of accumulating carotenoids, hence the absence of the yellow to orange colour. Enzyme activity was further analysed using GC–MS. Briefly, a 1 mL aliquot of a culture of each construct grown overnight at 30°C was used to inoculate 25 mL of LB medium containing 50 μg mL^{-1} ampicillin and 35 μg mL^{-1} chloramphenicol in a 250 mL conical flask. Cultures were grown overnight in darkness at 22°C with shaking at 250 rpm. Then the 25 mL liquid cultures were mixed with 5 mL of pentane:diethylether (1:4, v/v) (from Biosolve, Valkenswaard, The Netherlands) and the phases separated in a separation funnel after thorough mixing. The organic phase was transferred into a glass centrifugation tube and centrifuged at 1,200*g* for 5 min to further separate the organic phase from the water. The organic phase was passed over a short column containing anhydrous Na_2SO_4 into a new vial and concentrated under a flow of N_2 until about 1 mL. Of this 1 mL, 2 μL were injected into the injection port of a gas chromatograph coupled to a mass spectrometer (5890 series II, Hewlett-Packard GMI, USA) with a Zebron ZB-5ms column (30 m, 0.25 mm I.D., 0.25 μm film thickness) (Phenomenex, USA). The oven was programmed at an initial temperature of 45°C for 1 min, with a ramp of 10°C per min to 280°C, and final time of 5.5 min. The injection temperature was 250°C, and the detection temperature was 290°C. Products were identified by comparison to reference standards.

ZmCCD1 transcript analysis

Total RNA was extracted from maize roots or root segments with and without AM fungi and used for various analyses. Northern-blot analysis was done as previously described (Hans et al. 2004). Briefly, hybridisations were done in 7% SDS (w/v), 250 mM NaPi, pH 7.0, 250 mM NaCl and 1 mM EDTA at 60°C overnight using an [α-32P] dATP-labeled *ZmCCD1* cDNA fragment as probe. Final washes were in 0.5 SSC, 0.1% (w/v) SDS at 65°C. For RT-PCR analysis a *ZmCCD1* fragment was specifically amplified by using primers ZmCCD1f (GACGGGATGATTCATG CCATGC) and ZmCCD1r (CAAGGCGGCAGGTAATG AGAACAA). For normalisation primer pairs for elongation factor 1α, EF1αf (AGAAGGAAGCTGCTGAGATGAAC) and EF1αr (TGACTGTGCAGTAGTACTTGGTG) were used. For assessment of mycorrhizal colonisation, expression of an AM-induced phosphate transporter gene (*ZmPht1-6*) (Nagy et al. 2006) was assessed using primers ZmPht1.6f (CAGGTACCTGATCCAGCTCATC) and ZmPht1-6r (GTTCGAGGCGTGATCACATGGA). Real-time RT-PCR was performed on a Strategene MxPro Mx3005p qPCR system using SYBR green dye and an assay from Applied Biosystems (Warrington, UK) using 5 ng reverse-transcribed total RNA and 100 ng primers. Primers for *ZmCCD1* were RtZmCCD1f (CTGCTGTGG ATTTTCCTCGTG) and RtZmCCD1r (TATGATGCC AGTCACCTTCGC). For normalisation again elongation factor EF1α was used with primers RtEF1αf (GCTT GGGAAGTGCCAGTGAT) and RtEF1αr (GCCCTGT GGAAGTTCGAGAC) and for assessment of mycorrhizal colonisation *ZmPht1.6* using primers RtZmPht1.6f (AAA CGCCCTCAAGGAGGTGTT) and RtZmPht1.6r (CCT-GCCCATTTTGTCGATGA). Differences in relative expression levels of *ZmCCD1* were calculated from $E^{-\Delta Ct}$ values after normalisation of *ZmCCD1* data to EF1α. All analyses were performed using three technical replicates.

Results

Cloning of ZmCCD1

To find the *Z. mays* orthologue of *AtCCD1*, we blasted *AtCCD1* against the maize DFCI Gene Index EST database http://compbio.dfci.harvard.edu/tgi/. This yielded one contig, TC220599, with 64% identity to *AtCCD1*. According to the TC220599 sequence, specific nested primers were designed and used to amplify the full-length cDNA by PCR using maize cv. MBS 847 root cDNA as a template. This yielded one clear band of the expected size (~1,600 bp) after agarose gel electrophoresis. This band was purified, subcloned in pGEM-T Easy and sequenced. The maize *CCD1* had two open-reading frames starting with an ATG, one of 1,650 bp (*CCD1A*) and another of 1,623 bp (*CCD1B*), encoding proteins of 550 and 541 amino acids, respectively, with calculated molecular weight of about 61 kD (Fig. 2). Based on the alignment with other plant CCD1s (Fig. 2) and expression in *E. coli* (see below) we assume that the shorter version (CCD1B) represents the natural protein. The predicted maize CCD1 protein has a high similarity (77–78% identity) to CCD proteins of *Crocus* (CsCCD1), petunia (PhCCD1), tomato (LeCCD1A), grape (VvCCD1) and *Arabidopsis* (AtCCD1) that all catalyse the symmetrical 9, 10 (9′, 10′) cleavage of several linear and bicyclic carotenoids (Schwartz et al. 2001; Bouvier et al. 2003; Simkin et al. 2004a, b; Mathieu et al. 2005) (Fig. 2). Because of its high identity to these proteins the maize CCD1 was designated as

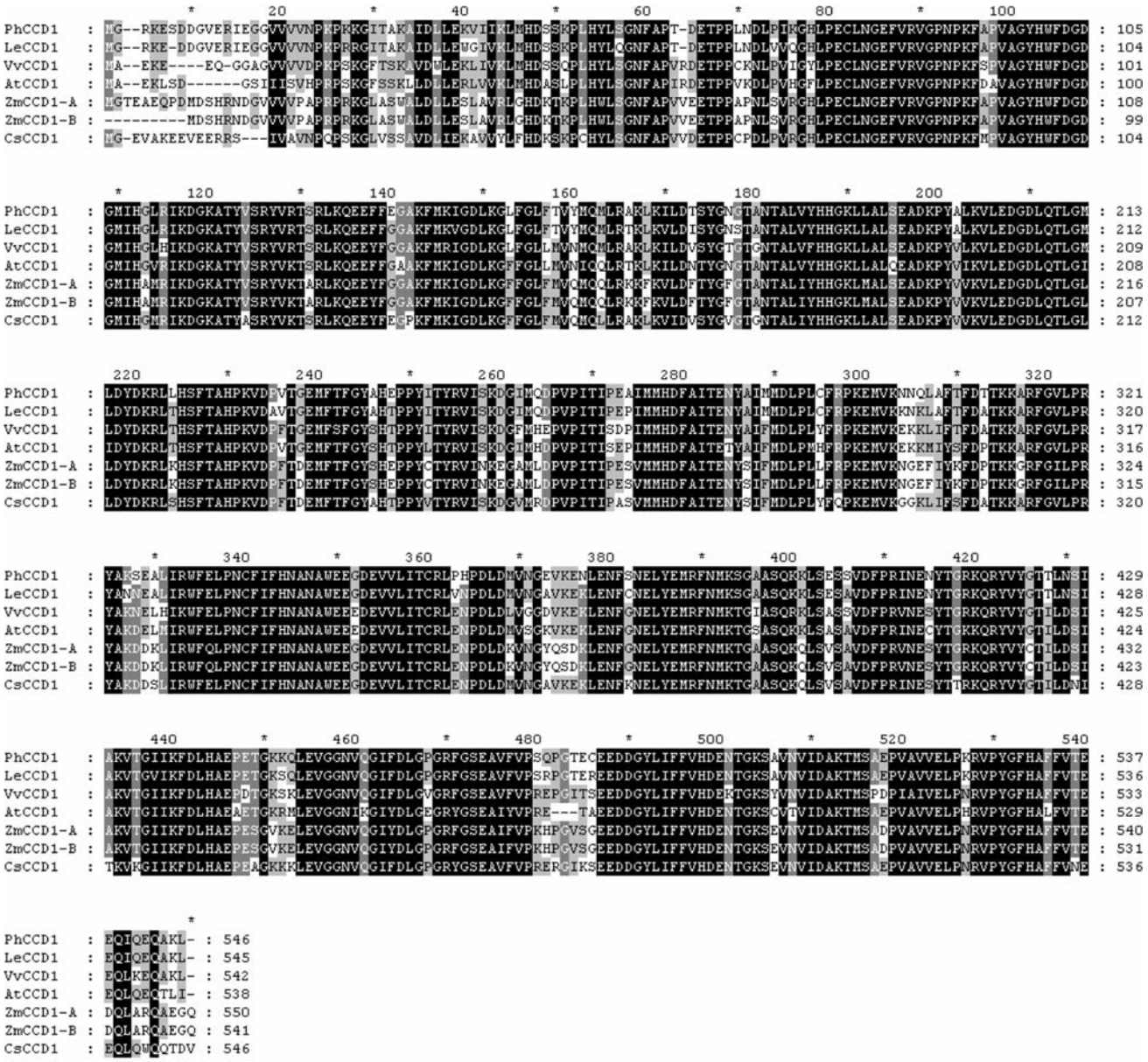


Fig. 2 Alignment of the deduced amino acid sequences of maize CCD1A (ZmCCD1-A) and CCD1B (ZmCCD1-B; DQ539625) with CCD1 from petunia (PhCCD1; AY576003) (Simkin et al. 2004b), tomato (LeCCD1; AY576001) (Simkin et al. 2004a), grape (VvCCD1; AY856353) (Mathieu et al. 2005), *Crocus* (CsCCD1; AJ132927) (Bouvier et al. 2003) and *Arabidopsis* (AtCCD1; AJ005813) (Neill et al. 1998)

ZmCCD1 (Genbank accession number DQ539625). A second variant of *ZmCCD1* was isolated from a maize cv dwarf-1 cDNA library. The nucleotide sequence obtained was 99% identical to the *ZmCCD1* amplified by PCR, resulting in only one conserved amino acid change (K160E) (Genbank accession number AY773278).

Characterisation of recombinant ZmCCD1 catalytic activity

To investigate the catalytical function of ZmCCD1, the cDNA cloned from maize cv. MBS 847 was cloned into the *E. coli* expression vector pRSETA, which was transformed to *E. coli* strains engineered to accumulate lycopene, β-carotene and zeaxanthin (Cunningham et al. 1996). Colonies of these three carotenoid accumulating *E. coli* strains develop a reddish, orange or yellowish colour, respectively. Loss of colour upon introduction of a putative carotenoid cleaving enzyme encoding cDNA indicates that the carotenoids are metabolised to colourless compounds. When the short version of *ZmCCD1* (*ZmCCD1B*) was expressed in cells producing lycopene (Fig. 3a), β-carotene (Fig. 3b), and zeaxanthin (Fig. 3c), colonies indeed failed to develop the yellow to orange colour that did show up in the empty

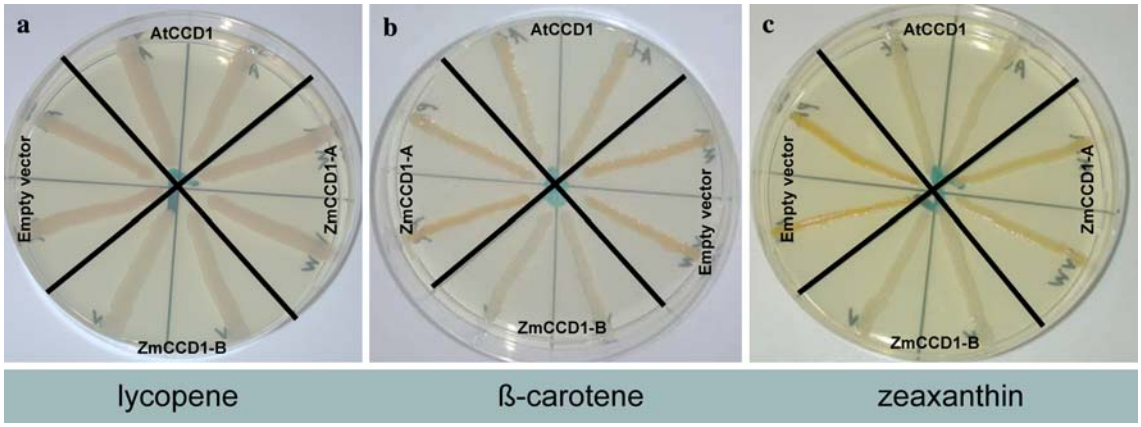


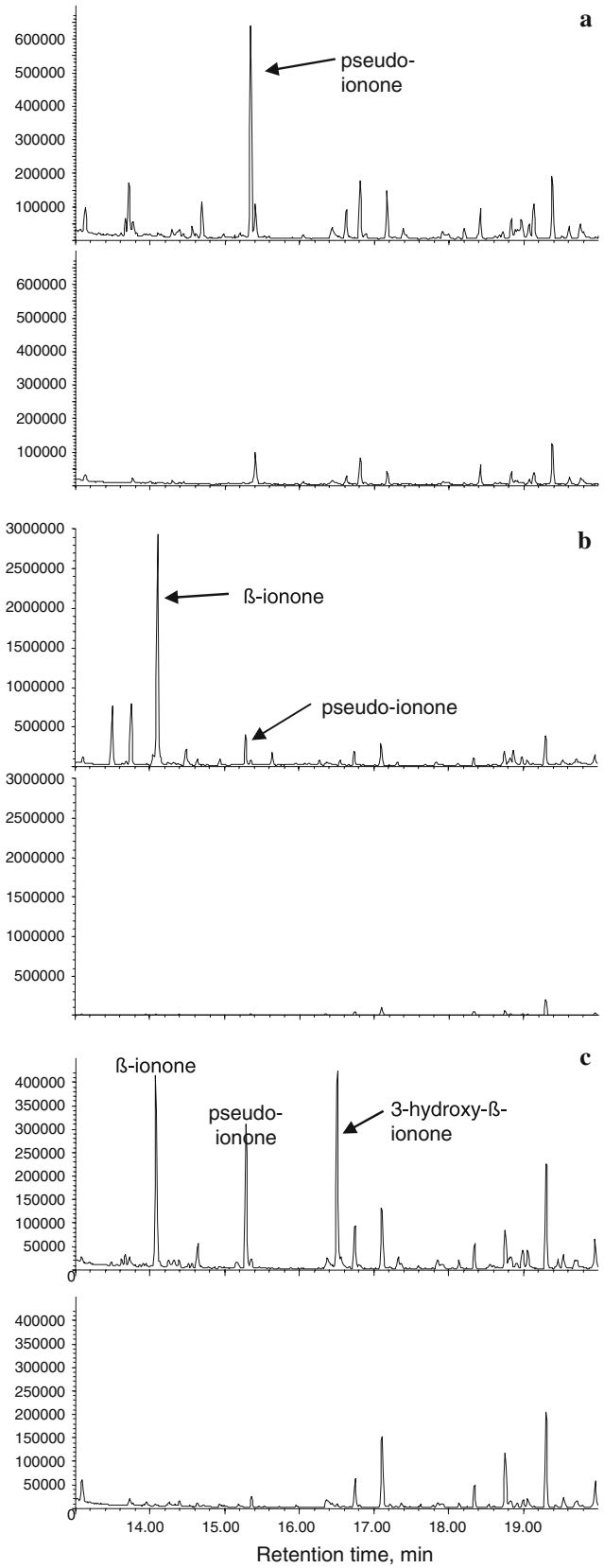
Fig. 3 Expression of *ZmCCD1* in *E. coli* strains accumulating different carotenoid substrates: **a** lycopene (pACLYC), **b** *β*-carotene (pACBETA), **c** zeaxanthin (pACZEAX) (Cunningham et al. 1996). *Arabidopsis thaliana CCD1* (AtCCD1) was used as positive, an empty vector as negative control; *ZmCCD1-A* is a variant of *ZmCCD1-B* having a 27-bp 5′ extension starting with an ATG in the same reading frame as *ZmCCD1-B*. Each treatment was streaked twice within the same quarter section indicated by the *bold lines*

vector control, showing that ZmCCD1B cleaves the carotenoids produced by the *E. coli* strains (Fig. 3). This decolouration did not occur with the long version of *ZmCCD1* (*ZmCCD1A*), which suggests that expression of the extended open reading frame version leads to an inactive recombinant ZmCCD1 protein. Using the ZmCCD1B construct the colonies lost colour more effectively when *β*-carotene (Fig. 3b) and zeaxanthin (Fig. 3c) were the substrate than with lycopene (Fig. 3a). ZmCCD1B more efficiently prevented carotenoid accumulation than AtCCD1, particularly for lycopene and *β*-carotene (Fig. 3). The two enzymes seemed equally efficient for zeaxanthin cleavage. Whether these differences indeed reflect differences in the activities or specificities of the recombinant CCDs remains to be investigated.

To characterise the cleavage products, the *E. coli* strains were grown overnight in liquid medium which was subsequently extracted with pentane-diethylether. GC–MS analysis revealed the presence of pseudo-ionone in the culture of lycopene accumulating *E. coli* (Fig. 4a), pseudo-ionone and *β*-ionone in the culture of *β*-carotene accumulating *E. coli* (Fig. 4b) and pseudo-ionone, *β*-ionone and 3-hydroxy-*β*-ionone in the culture of zeaxanthin accumulating *E. coli* (Fig. 4c). All cleavage products were absent in the empty vector controls (Fig. 4). The positive control, *AtCCD1*, appeared to be slightly less active than *ZmCCD1*, but the product patterns were similar (data not shown). We did not detect any other cleavage products but assume that ZmCCD1, analogous to other CCD1s characterised, is also cleaving symmetrically by 9, 10 and 9′, 10′ cleavage (Fig. 1) and hence also yields the C_{14} dialdehyde. This product is not volatile and prone to further modifications and will thus not show up in GC–MS analysis.

ZmCCD1 transcripts are up-regulated in mycorrhizal maize roots

Because ZmCCD1 cleaves carotenoids at 9, 10 and 9′, 10′ positions, it might be involved in cyclohexenone/mycorradicin formation in maize (Fig. 1). We therefore analysed *ZmCCD1* expression in maize roots colonised by AM fungi. The full-length sequence of *ZmCCD1* was used as a probe in a Northern-blot analysis with total RNA isolated from non-mycorrhizal and mycorrhizal maize roots. The probe recognised a single band of about 1,600 nt. *ZmCCD1* transcript accumulation was increased about 1.5 to 2-fold upon mycorrhizal colonisation by *G. mossae* and *G. intraradices*, respectively (Fig. 5a, b). Using the *G. intradices*-colonised samples and control samples this result was confirmed by RT-PCR (Fig. 5c). RT-PCR analysis on white or distinctly yellow roots—both taken from mycorrhizal roots—revealed a stronger signal for *ZmCCD1* transcript in the yellow root fraction than in the white roots (Fig. 5c). A strong signal was obtained for the AM-inducible maize phosphate transporter gene (*ZmPht1.6*, Nagy et al. 2006)—used as a molecular marker for AM colonisation—in all mycorrhizal samples but not in the non-mycorrhizal controls (Fig. 5c). *ZmPht1.6* transcript levels were higher in mycorrhizal yellow roots than in mycorrhizal white roots. The results were further confirmed and quantified by realtime RT-PCR analysis (Fig. 5d). Transcript levels of *ZmCCD1* in non-separated colonised roots were 2.1-fold higher than in non-mycorrhizal control roots. In yellow segments of colonised roots *ZmCCD1* transcript levels were 4.1-fold higher than in the white segments of the same mycorrhizal root system. The phosphate transporter *ZmPht1.6* was strongly upregulated in colonised total roots (128-fold) compared with



non-colonised total roots, but it was expressed only tenfold higher in yellow sections than in white sections of colonised roots (data not shown).

◀ **Fig. 4** GC–MS analysis of ZmCCD1 cleavage products. *ZmCCD1* was expressed in *E. coli* strains accumulating different carotenoid substrates: **a** lycopene (pACLYC), **b** *β*-carotene (pACBETA), **c** zeaxanthin (pACZEAX) (Cunningham et al. 1996). Each panel shows a chromatogram for *ZmCCD1* (*upper chromatogram*) and the corresponding empty vector control (*lower chromatogram*). Cultures of these *E. coli* were grown overnight in liquid medium and the medium was extracted with pentane-diethylether. The identity of the cleavage products (*peaks marked*) was confirmed using authentic standards

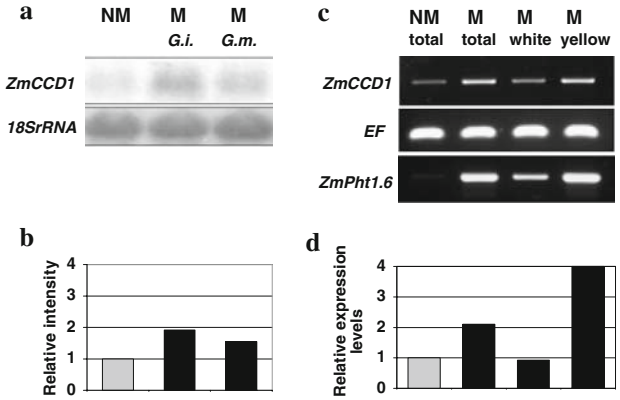


Fig. 5 Upregulation of *ZmCCD1* transcript levels in maize roots upon mycorrhizal colonisation. Results were obtained with several independent biological materials showing a similar trend. Maize plants (cv dwarf-1) were grown without inoculum (non-mycorrhizal, *NM*) or with inoculum (mycorrhizal, *M*) of two arbuscular mycorrhizal fungi, *Glomus intraradices* (*G.i.*) or *Glomus mosseae* (*G. m.*). In addition, white or faintly coloured roots were compared to yellow roots within the same mycorrhizal root system (*M*). RNA was isolated from roots 6 weeks after inoculation (comparison NM to M) or 5 weeks after inoculation (comparison of differently coloured mycorrhizal roots) in separate experiments. The latter comparison was performed with *G.i.*-colonised roots only. **a** Northern blot analysis using 5 µg RNA per *lane*. A radiolabelled full length cDNA of *ZmCCD1* was used for hybridisation. Equal loading was verified by hydridisation with an 18S rRNA probe. **b** Quantification of the autoradiograph signals shown in **a** by a phosphor imager and normalisation of *ZmCCD1* results to the 18S rRNA loading control. Transcript levels in M roots are shown relative to NM control roots set at 1. **c** RT-PCR analysis of RNA from non-mycorrhizal or mycorrhizal root system or a mycorrhizal root system separated into white and yellow roots as indicated. Elongation factor 1*α* (EF) amplification was used to show equal efficiencies of cDNA synthesis and PCR. Transcript levels of an AM-induced phosphate transporter gene (*ZmPht1.6*) are used as molecular markers for development and activity of mycorrhizal structures. **d** Quantification of *ZmCCD1* transcript levels by real-time RT-PCR analysis normalised to equal levels of EF 1*α* transcripts. Relative expression levels were calculated from $E^{-\Delta Ct}$ values. The same RNA samples were used as in **c** but different primer sets were employed (see Materials and methods)

Mycorrhizal colonisation of maize roots results in decreased *Striga* germination

Since it has been shown that maize and sorghum colonised by AM fungi are infected to a lesser extent by *Striga* than non-colonised control plants, we decided to study whether this reduction of infection is caused by the decreased

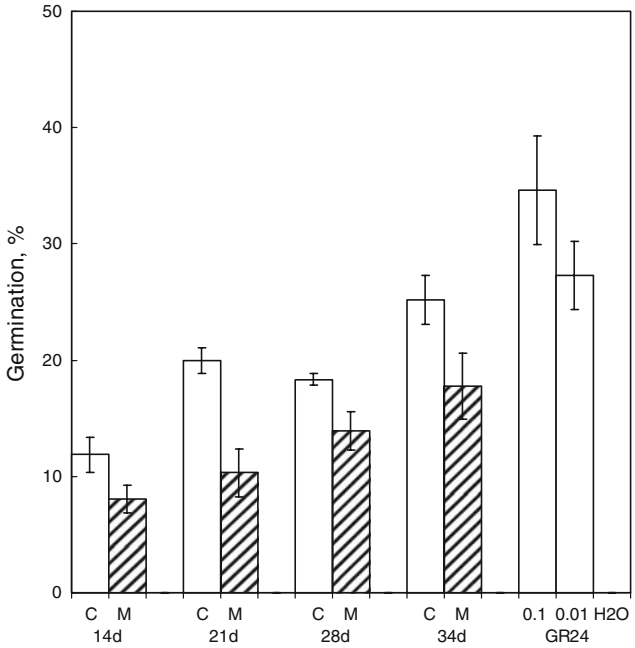


Fig. 6 Effect of colonisation by *Glomus intraradices* of maize roots on the induction of *Striga hermonthica* seed germination. Root exudates from four plants per treatment (*C*, non-colonised control plants; *M*, plants colonised by *G. intraradices*) were collected separately for 24 h in demineralised water at 14, 21, 28 and 34 days after inoculation. The exudate of each plant was diluted to the same concentration of g root freshweight per millilitre of root exudate and induction of *S. hermonthica* germination was assessed. GR24 was used as positive control and demineralised water as negative control. *Error bars* indicate standard error ($n = 4$)

production of germination stimulants by AM maize roots. Maize roots were inoculated with *G. intraradices* and progress of fungal colonisation of roots was inspected at regular intervals. A colonisation rate of about 11% of total root length was observed after 14 days. This percentage increased to 47% after 34 days, when hyphae, vesicles and arbuscules were clearly visible. Exudates of mycorrhizal and control roots were collected and applied to preconditioned seeds of *S. hermonthica*. The germination inducing capacity of the exudates increased over time throughout the experimental period of 34 days. At all time points the root exudates of maize plants that were colonised by *G. intraradices* induced significantly ($P < 0.01$) lower germination than the root exudates of control plants (Fig. 6).

Discussion

Characterisation of maize CCD1 cDNAs, transcripts and recombinant proteins

In this paper, we have shown that a recombinant maize carotenoid cleavage dioxygenase, ZmCCD1, cleaves carotenoid substrates (lycopene, β-carotene and zeaxanthin) at the 9, 10 (and 9′, 10′) positions leading to the formation of C_{13} apocarotenoids, that vary according to the substrate, and most likely a C_{14}-dialdehyde (not detected in our GC–MS analysis) (Figs. 1, 4). These results are consistent with previously reported in vitro results for the orthologous enzyme AtCCD1, which cleaves carotenoids (β-carotene, lutein, zeaxanthin, trans-violaxanthin) at the same position as ZmCCD1 (Schwartz et al. 2001) and other enzymes orthologous to AtCCD1 (Bouvier et al. 2003; Simkin et al. 2004a, b; Mathieu et al. 2005). Recently, Klee et al. demonstrated that a (non-disclosed) ZmCCD1 and the tomato and *Arabidopsis* CCD1s—in addition to 9, 10 and/or 9′, 10′ cleavage—also cleave lycopene, but not bicyclic carotenoids, at the 5, 5 and/or 5′, 6′ position leading to the formation of 6-methyl-5-hepten-2-one. In our assays we did not detect this product perhaps as a result of different assay conditions or because we have a different ZmCCD1 variant. When using β-carotene and zeaxanthin accumulating *E. coli* strains we also detected the cleavage products of the carotenoid intermediates (pseudo-ionone in β-carotene accumulating *E. coli* resulting from lycopene cleavage; pseudo-ionone and β-ionone in zeaxanthin accumulating *E. coli* resulting from lycopene and β-carotene cleavage, respectively) (Fig. 4b, c). All these data show that the recombinant CCD1 enzymes have a broad substrate specificity but high regioselectivity—with cyclic carotenoids—for cleavage at the 9, 10 and/or 9′, 10′ position.

We did not detect the C_{14} dialdehyde cleavage product in the *E. coli* cell extracts, presumably because it is not volatile enough for GC–MS analysis. In plant roots the C_{14} dialdehyde is converted to dicarboxylic acid derivatives (mycorradicins), which are yellow and cause the yellow colour of mycorrhizal roots (Klingner et al. 1995; Fester et al. 2002; Walter et al. 2000). Why the bacterial colonies do not turn yellow (Fig. 3) is unknown. The C_{14} dialdehyde might be converted to the colourless C_{14} dialcohol (rosafluene), which has been reported as a byproduct of C_{13} apocarotenoid scent volatile production in rose petals (Eugster and Märki-Fischer 1991) or be further catabolised as suggested (Schwartz et al. 2001; Vogel et al. 2008). Otherwise not much is known about the metabolic fate of the primary or secondary carotenoid cleavage products in plants. Only in mycorrhizal roots their metabolic conversion to a mixture of oxidised, esterified, and glycosylated apocarotenoids including yellow mycorradicin derivatives, deposited partly in the vacuole, has been described in some detail (Schliemann et al. 2006; Strack and Fester 2006).

The carotenoid precursor of cyclohexenone and mycorradicin derivatives in AM maize roots that is possibly cleaved by ZmCCD1 is still unknown. It was postulated that cyclohexenone and mycorradicin biosynthesis could involve zeaxanthin cleavage at the 9, 10 and 9′, 10′

position by a CCD-like enzyme (Fester et al. 2002). Indeed, we showed that ZmCCD1 can cleave zeaxanthin (Fig. 3). Two other candidates are lutein and lactucaxanthin (Siefermann-Harms et al. 1981). Especially the latter one shows ionone motives structurally closely related to the accumulating cyclohexenone derivatives in AM-colonised roots but this carotenoid has not been reported in plant roots so far (Strack and Fester 2006). Generally, the carotenoid composition of plant roots has hardly been studied but it is well-documented that roots do contain carotenoids, for example β-carotene, α-carotene, lutein and violaxanthin (Maudinas and Lematre 1979; Baranska et al. 2006).

Carotenoid cleavage is commonly assumed to occur in plastids, after which the cleavage products are exported to the cytosol (Cutler and Krochko 1999; Laule et al. 2003). However, several studies have suggested that the CCD1 enzymes reside in the cytosol. Indeed, AtCCD1 is the only *Arabidopsis* carotenoid cleaving enzyme which is not localised in plastids but in the cytosol (Auldridge et al. 2006a) and immunolocalisation indicated that the *Crocus* CCD1 protein is also localised in the cytoplasm (Bouvier et al. 2003). Prediction algorithms (SignaIP 3.0) clearly suggest that all CCD1 enzymes are devoid of a plastid targeting signal. This also applies to ZmCCD1 suggesting that its site of action is the cytosol. Nothing is known about how carotenoids produced in plastids come into contact with a cleavage enzyme in the cytosol. Transport of carotenoids across the plastidial membrane or degradation of plastids, which would lead to the release of the carotenoids, may be possible explanations. All this shows that the roles of CCD1 and its substrate(s) in planta are still largely unknown and may not necessarily be identical with its action in the artificial *E. coli* system.

Possible biological functions of ZmCCD1

At present CCD1 enzymes are best known for their involvement in the biosynthesis of apocarotenoid flavour, aroma and scent volatiles in leaves, flowers and fruits. *CCD1* genes have been shown to be constitutively expressed in these tissues. For example, two variants of tomato CCD1 can cleave several carotenoid substrates and some of the cleavage products are present in or are emitted from tomato fruits, such as 6-methyl-5-hepten-2-one, β-ionone and geranylacetone, which play a key role in tomato flavour (Simkin et al. 2004a; Vogel et al. 2008). Similarly, a petunia CCD1 is leading to β-ionone biosynthesis in the flowers when β-carotene is cleaved and this volatile is possibly involved in the attraction of pollinating insects (Simkin et al. 2004b). Other potential biological roles of apocarotenoids including various signalling functions have recently been reviewed (Auldridge et al. 2006b).

We have demonstrated here that *ZmCCD1* transcript levels are up-regulated in mycorrhizal maize roots upon colonisation by AM fungi (Fig. 5). Elevation to a comparable extent (2–3 fold) of *CCD1* transcript levels in mycorrhizal roots has also been found in *Medicago truncatula* (Lohse et al. 2005; Walter et al. 2007). Moreover, the approximately twofold upregulation of *ZmCCD1* in total extracts of mycorrhizal maize roots (Fig. 5) is similar to the upregulation of transcripts of *1-deoxy*-d-*xylulose-5-phosphate reductoisomerase* (*DXR*) upon mycorrhizal colonisation in the same experimental system (Hans et al. 2004). The enzyme encoded by *DXR* catalyses an early step of apocarotenoid biosynthesis in the methylerythritol phosphate pathway. Immunolocalisation of DXR protein in mycorrhizal maize roots showed strong accumulation of DXR in root cells harbouring fungal arbuscules but not in other root cells clearly indicating that apocarotenoid biosynthesis is a highly localised event related to the occurrence of fungal arbuscules (Hans et al. 2004). This result is in agreement with results from studies on the localisation of the apocarotenoid products (Fester et al. 2002). A strong upregulation of gene expression in relatively few arbusculated cells can therefore disappear in the background of non-arbusculated and therefore non-responsive root cells or at least be underestimated. This view is supported by the result obtained for *ZmCCD1* transcript levels after enriching for responsive root cells by separating white from yellow root segments within mycorrhizal roots (Fig. 5c, d). *ZmCCD1* expression was about fourfold higher in the yellow root segments whereas the white root segments displayed about the same level of *ZmCCD1* transcripts as the non-mycorrhizal control roots (Fig. 5d). *ZmCCD1* upregulation in mycorrhizal roots is therefore likely to be a local and not a systemic response and may be linked to as yet unknown arbuscule functions in the AM symbiosis. Although there are no immunolocalisation or in situ hybridisation experimental results yet available for CCD1 enzymes or transcripts in mycorrhizal roots the present data are compatible with a view of ZmCCD1 participating in a local biosynthesis of cyclohexenone and mycorradicin apocarotenoids in arbusculated cells.

Parasitic plant seed germination and effects of mycorrhization

In a number of studies with the parasitic plant *S. hermonthica* it was demonstrated that maize and sorghum have a 30–50% reduction in the number of *S. hermonthica* shoots after inoculation with AM fungi, while also displaying the yellow root colour known from other plants (Gworgwor and Weber 2003; Lendzemo et al. 2007). AM fungi may confer resistance to other biotic stresses as well. For example, there are a number of reports showing that plants colonised

by AM fungi are protected against subsequent infection with nematodes and plant pathogenic fungi (Borowicz 2001; Johansson et al. 2004). This protection has been suggested to be due to improved nutritional status of the host but there is ample evidence that this cannot be the (only) explanation (Johansson et al. 2004; Harrison 2005). Several studies have shown that during mycorrhizal symbiosis defence-related genes are induced (Pozo et al. 2002; Kuster et al. 2004). Increased defence gene expression could possibly also explain why sorghum and maize that are colonised by AM fungi are infected to a lesser extent by *Striga* as defence gene expression could play a role in *Striga* resistance (Gowda et al. 1999).

However, improved defence is not the only possible explanation for the lower infection of mycorrhizal sorghum and maize by *Striga*. We have shown here that the exudates of maize roots, colonized by AM fungi, induce less germination of *Striga* seeds than control root exudates (Fig. 6). Control experiments in which the synthetic strigolactone analog GR24 was mixed with exudates of AM colonised maize showed that this effect was not due to the presence of inhibitors. A similar and even more convincing result was obtained with sorghum where germination of *Striga* seeds induced by root exudates of plants colonised by AM fungi was dramatically reduced (Lendzemo et al. 2007). This all suggests that the reduction of *Striga* infection of sorghum and maize, when colonised by AM fungi, is caused at least partly by a decrease in the formation or secretion of strigolactone germination stimulants. In contrast, in another study a positive effect of AM fungal colonisation on parasitic plants has been reported. Mycorrhizal colonisation of *Trifolium pratense* improved growth of the host as well as the attached parasitic plant *Rhinanthus serotinus* (Salonen et al. 2001) arguing against an effect of AM fungi on the defence capacity of plants against parasitic plants. However, the facultative parasite *R. serotinus* does not require a strigolactone apocarotenoid germination signal (Matthies 1995). Therefore, a reduction in strigolactone formation in *T. pratense* upon mycorrhizal colonisation is not expected to reduce *R. serotinus* germination and hence mycorrhizal colonisation will not reduce infection with this facultative parasite. Also in a study with cucumber, it was shown that the exudate of AM-colonised cucumber is less stimulatory to AM fungi than the exudate of control plants (Pinior et al. 1999). In retrospect the authors now also assume this is due to a lower secretion of strigolactones (Steinkellner et al. 2007). Nevertheless, we cannot completely exclude the presence of *Striga*-inhibitory compounds in or in the vicinity of mycorrhizal roots produced by the AM fungi, the plant itself in response to the AM fungi, or by microorganisms in an altered rhizosphere (Bais et al. 2004; Lendzemo et al. 2007).

ZmCCD1 might affect strigolactone precursor availability

Strigolactones are apocarotenoid host-signalling compounds for AM fungi in an ancient symbiotic relationship, which are apparently abused by parasitic plants to also detect the presence of a plant host (Matusova et al. 2005; Bouwmeester et al. 2007). While strigolactones are involved in early recognition processes of the AM symbiosis in very low concentrations (Akiyama et al. 2005) other apocarotenoids (cyclohexenone and mycorradicin derivatives) accumulate to high concentrations in later stages of the symbiosis (Maier et al. 1995; Walter et al. 2000). Any potential functional relationship between these different apocarotenoids or their carotenoid precursors is unknown at present. Different carotenoid cleavage enzymes may be involved in the formation of the different kinds of apocarotenoids but these enzymes could act on the same carotenoid precursor. Cyclohexenone and mycorradicin formation is preceded by the up-regulation of many genes of the carotenoid biosynthetic pathway such as deoxyxylulose-5-phosphate synthase 2 (*DXS2*), *DXR*, and phytoene desaturase (*PDS*) (Walter et al. 2000, 2007) probably leading to a considerable increase in carotenoid precursor pools in the roots of plants colonised by AM fungi. Why then would there be a reduction in strigolactone formation as judged from the reduction in *Striga* germination (Fig. 6) despite this increased pool of root carotenoids? A possible explanation could be the efficient depletion of the carotenoid precursor pools by ZmCCD1 or other CCDs involved in cyclohexenone and mycorradicin formation. This possibly depletes not only the AM-induced carotenoid precursors but also the basal levels of root carotenoids normally available for strigolactone formation in non-mycorrhizal plants.

Despite many attempts to identify the AM-induced carotenoid precursor of the mycorrhizal cyclohexenone and mycorradicin apocarotenoids only tiny amounts of potential parent carotenoids could be detected, indicating that the AM-induced carotenoids are immediately cleaved into apocarotenoids (Fester et al. 2002). Strigolactone formation does not benefit from a high activity of CCD1-type enzymes in maize roots but rather seems to be reduced instead (Figs. 5, 6). It is therefore unlikely that ZmCCD1 contributes to strigolactone formation. This is in line with the previous proposal that strigolactone biosynthesis proceeds by carotenoid cleavage at the 11, 12/11′, 12′ position (Matusova et al. 2005). As a result of high ZmCCD1 activity carotenoid precursor availability may become limiting to strigolactone biosynthesis ultimately reducing its steady state levels. In addition, it is possible that—through an unknown signalling mechanism—mycorrhizal colonisation has a direct down-regulating effect on the strigolactone biosynthetic pathway. A direct effect of existing AM fungal colonisation on the further production/secretion of a recognition

and branching factor for newcomer AM fungi is not unlikely as it would result in auto-regulation of host roots already colonised by AM fungi. Work is in progress to further underpin this hypothesis.

Concluding remarks

We have cloned a carotenoid cleavage enzyme (*ZmCCD1*) from maize roots that cleaves carotenoids at the 9, 10/9′, 10′ position and may be involved in the formation of the yellow pigment (cyclohexenone and mycorradicin derivatives) in mycorrhizal maize roots. Mycorrhizal maize roots display enhanced *ZmCCD1* expression and at the same time induce lower germination of *Striga* possibly via depleted carotenoid pools for strigolactone formation. Our future work will be to overexpress and knockout *ZmCCD1* in maize—or orthologs in other plant species—to study the importance of the cyclohexenone and mycorradicin derivatives for the symbiotic interaction of plants with AM fungi and to study whether root-directed *CCD1* overexpression without mycorrhizal colonisation also leads to reduced strigolactone formation. If this is true, it could potentially be used to develop crop varieties with improved *Striga* resistance through a lower production of germination stimulants.

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