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Identification of Bacterial Carotenoid Cleavage Dioxygenase Homologues That Cleave the Interphenyl α,β Double Bond of Stilbene Derivatives via a Monooxygenase Reaction

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Carotenoid cleavage oxygenases (CCOs), which are also referred to as carotenoid cleavage dioxygenases (CCDs) are a new class of nonheme iron-type enzymes that oxidatively cleave double bonds in the conjugated carbon chain of carotenoids. The oxidative cleavage mechanism of these enzymes is not clear, and both monooxygenase and dioxygenase mechanisms have been proposed for different carotenoid cleavage enzymes. CCOs have been described from plants, animals, fungi, and cyanobacteria, but little is known about their distribution and activities in bacteria other than cyanobacteria. We surveyed bacterial genome sequences for CCO homologues and report the characterization of CCO homologues that were identified in Novosphingobium aromaticivorans *DSM 12444 (NOV1 and NOV2) and in* Bradyrhizobium *sp. (BRA-J and BRA-S). In vitro and in vivo assays with carotenoid and stilbene compounds were used to investigate the cleavage activities of the recombinant enzymes. The NOV enzymes cleaved the interphenyl α-β double bond of stilbenes that had an oxygen functional group at the 4′ carbon atom (e.g., resveratrol, piceatannol, and rhaponticin) to the corresponding aldehyde products. Carotenoids and apocarotenoids were not substrates for these enzymes. The two homologous enzymes from* Bradyrhizobium *sp. did not possess carotenoid or stilbene cleavage oxygenase activities, but showed activity with farnesol. To investigate whether the oxidative cleavage of stilbenes proceeds via a monooxygenase or dioxygenase reaction, oxygen-labeling studies were conducted with NOV2. Our labeling studies show that the double-bond cleavage of stilbenes occurs via a monooxygenase reaction mechanism.*

Introduction

Mono- and dioxygenases play important roles in the oxidative modification and cleavage of metabolic compounds. Recently, a new nonheme iron family of oxygenases has been described that catalyzes the cleavage of double bonds in the conjugated carbon chain of carotenoids to produce apocarotenoids.[1,2] Carotenoid cleavage products have important biological functions as signal molecules, hormones, and attractants for pollinators (reviewed in ref. [1]), and are also of considerable interest for medical and agricultural applications (reviewed in refs. [1–3]).

Carotenoid cleavage oxygenases (CCOs), which are also referred to as carotenoid cleavage dioxygenases (CCDs), have now been identified in all taxa.[4–9] In higher plants, cleavage enzymes have been identified that produce signaling molecules to regulate growth and development, influence fruit color, and affect aroma (reviewed in ref. [8]). Carotenoid oxygenases also play important metabolic and signaling roles in metazoans.[7,9] The symmetric cleavage of β,β-carotene to retinal via carotenoid oxygenase activity, for example, was an important medical discovery (reviewed in ref. [3]). The function of the oxygenase enzymes in microorganisms is far less clear, although recent studies on the cloning and characterization of CCOs from cyanobacteria are beginning to address these questions.[4,10,11]

Cloning and characterization of a number of mostly plant-derived CCOs showed that these enzymes exhibit different cleavage site and substrate specificities.[6,12–18] However, very little is known about the mechanism by which these enzymes catalyze the oxidative cleavage of double bonds to form two aldehyde cleavage products. Currently only one crystal structure is available for an apocarotenoid-specific CCO from the cyanobacteria *Synechocystis* sp. PCC6803.[19] The structure shows that the enzyme contains a Fe^{2+} that is coordinated to four His residues in the active site, which is embedded in a seven-bladed β-propeller chain arrangement that is topped by a dome; the dome is comprised of six large loops. Whether this enzyme catalyzes oxidative cleavage via a mono- or dioxygenase mechanism however, cannot be deduced from the structure. Labeling studies from plants that produce abscisic acid suggested a dioxygenase mechanism.[20] These data were supported by labeling studies that examined the production of the aroma compound β-ionone from *Arabidopsis thaliana* CCD1 (*At*CCD1);[21] however, researchers studying vitamin A biosynthesis have suggested at different times with different

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enzyme examples, both a dioxygenase mechanism and a monooxygenase-like mechanism through a postulated epoxy intermediate.[22] Consequently, we chose to use the term CCO (for carotenoid-cleavage oxygenase) to describe this class of enzymes.

We have recently begun to characterize the putative CCO homologues that have been identified from genome sequences of cyanobacterial strains to gain a better understanding of their functions in photosynthetic bacteria.[4] Our analysis of bacterial genome sequences for new members of the CCO family also identified several putative CCO homologues in carotenogenic and noncarotenogenic bacteria; this indicates that at least some of these enzymes probably cleave substrates other than carotenoids. In the early 1990s, enzymes that cleave the interphenyl α,β double bond of *trans*-stilbenes have been described from the soil bacterium *Sphinogomonas paucimobilis* TMY1009 (four isoforms SPA1–4).[23,24] These enzymes are believed to have a catabolic function by cleaving stilbene-type compounds that are derived from lignin degradation, and have therefore been named lignostilbene-α,β-dioxygenases (LSD, EC 1.13.11.43); although dioxygen incorporation has never been experimentally established for these enzymes. The identification of CCOs several years later showed that the *Sphingomonas* enzymes are the CCO homologues that are most closely related to 9-*cis*-epoxycarotenoid dioxygenases (NCEDs), which generate the precursor of the plant hormone abscisic acid.[16,25]

In this study we survey bacterial genomes for other CCO homologues and describe the characterization of two CCO paralogues, NOV1 and NOV2, which were identified in the noncarotenogenic *Novosphingobium aromaticivorans* DSM12444, and two CCO homologues from the noncarotenogenic *Bradyrhizobium japonicum* USDA110 and carotenogenic *Bradyrhizobium* sp. BTAi1 (BRA-J and BRA-S respectively). In addition, isotopic oxygen labeling experiments show that NOV2 is a monooxygenase, which is in contrast to a recent study that suggested a dioxygenase mechanism for the *Arabidopsis thaliana* CCD1 enzyme.[21]

Results and Discussion

Bacterial CCO homologues

We previously surveyed cyanobacterial genome sequences for putative CCO enzymes and characterized the cleavage activities of several recombinant enzymes.[4] As in plants, we expected to also find mostly CCO enzymes that cleave (apo)carotenoids in cyanobacteria based on their presumed function in general carotenoid breakdown and synthesis of apocarotenoids for light-sensing (retinal, rhodopsin) and/or other signaling functions. In fact, cloned putative cyanobacterial CCOs cleaved (apo)carotenoids with different selectivities and cleavage specificities (9, 10, 9′,10′; 15,15′; apo-9,10), although several of these CCO sequences, for example, from *Nostoc punctiforme* and *Nostoc* sp. PCC7120, were annotated as lignostilbene α,β-dioxygenases.[4] A BLAST analysis of published bacterial genome sequences with sequences of experimentally characterized CCOs identified a number of CCO homologues in bacteria with and without annotated carotenoid pathways in their genomes (Figure 1 and Table S1 in the Supporting Information). The absence of carotenoid biosynthetic pathways in some bacteria suggests the existence of catalytic activities other than carotenoid cleavage. We selected four sequences from carotenogenic and noncarotenogenic proteobacteria for further characterization: two paralogues from *Novosphingobium aromaticivorans* DSM12444 (NOV1 and NOV2), one each from *Bradyrhizobium japonicum* USDA110 (BRA-J) and *Bradyrhizobium* sp. BTAi1 (BRA-S). *Novosphingobium* does not have characterized carotenoid biosynthetic pathways, although there are several carotenoid-associated genes in the genome, according to the KEGG database.[26] *Novosphingobium* is well known for its ability to degrade phenolic structures.[27] *Bradyrhizobium* strains are nitrogen-fixing symbionts of legumes.[28] Whereas *B. japonicum* USDA110 is nonphotosynthetic and does not synthesize carotenoids, *Bradyrhizobium* sp. BTAi1 is photosynthetic and therefore makes carotenoids.

The phylogenetic tree in Figure 1 shows that BRA protein sequences are most closely related to putative CCO homologues in *Ralstonia* and *Rhodopseudomonas*, whereas NOV sequences cluster with two known LSDs from *Sphingomonas paucimobilis* SPA1 (protein accession number AAC60447) and SPA3 (protein accession number AAB35856).[29] All four putative CCO sequences (NOV and BRA) are more closely related to cyanobacterial enzymes than to plant CCOs. Alignments show that NOV1 and the SPA1 and SPA3 proteins from *Sphingomonas paucimobilis* share the highest degree of amino acid sequence identity (55% and 56% respectively) between genera (Figure S1). This is considerably different than the similarity between SPA enzymes and NOV2, which are 37–38%. The SPA enzymes are more alike one another (68% identity) than are the NOV enzymes (36%). The two BRA proteins share 32–37% identity to both the SPA enzymes and the NOV enzymes (with 80% similarity between BRA-J and BRA-S).

Survey of cleavage activities in carotenoid or stilbene synthesizing *E. coli* strains

A CCO enzyme from *Synechocystis* has been shown to be membrane associated;[11] membrane association is one contributing factor to the difficulty of developing optimal in vitro assay conditions for CCO enzymes (other difficulties are described in ref. [30]). As a result, in vivo detection of carotenoid cleavage activity through coexpression of the CCO enzyme in question with carotenoid biosynthetic pathways has become the standard approach for identifying active enzymes. To determine the cleavage activity of NOV and BRA enzymes, genes were amplified from genomic DNA and cloned into the constitutive *E. coli* expression vector pUCmod.[31] For a survey of stilbene or carotenoid cleavage activities, genes were expressed in recombinant *E. coli* that produced β-carotene, zeaxanthin, torulene, or different stilbene compounds.

The in vivo cleavage of carotenoid structures that were produced in *E. coli* was investigated essentially as described previously for the characterization of cyanobacterial CCOs.[4] Briefly,

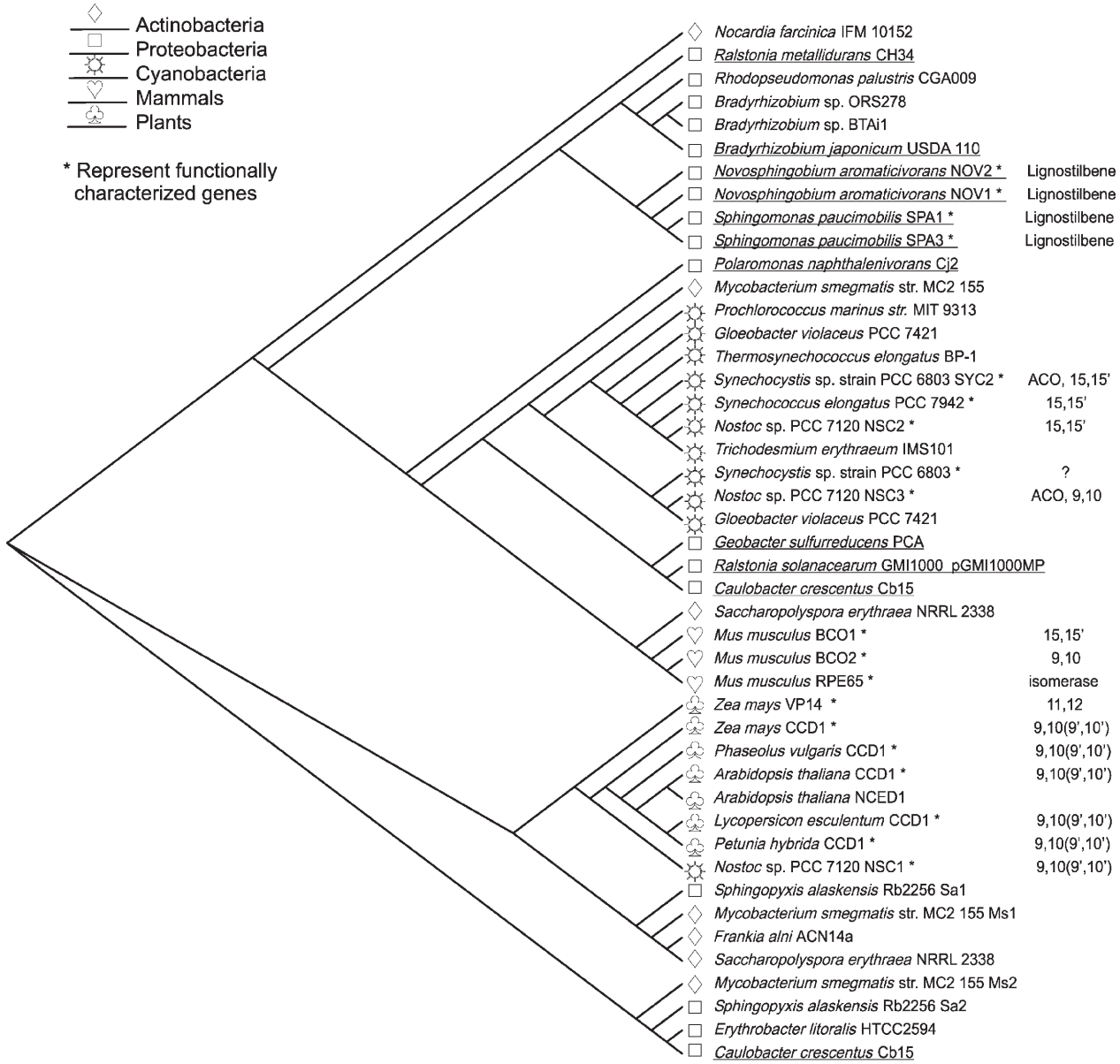


Figure 1. Phylogenetic tree (average distance by percent identity) from the NOV1 and NOV2 amino acid sequences with additional representatives from sequenced microbial genomes (BLAST hits with high identity) and other characterized carotenoid oxygenase family representatives. Lignostilbene oxygenase activity: NOV1 (accession no. YP_496081), NOV2 (accession no. YP_498079; this study), *Sphingomonas paucimobilis* isoform 1 (SPA1, accession no. AAC60447),[23] and isoform 3 (SPA3, accession no. AAB35856).[24] Apocarotenoid cleavage activity: *Synechocystis* PCC6803 (SYC2, accession no. S76169),[10] *Nostoc* sp. PCC7120 9,10 (NSC3, accession no. ZP_00112423).[4] 15,15′-Carotenoid cleavage activity: *Synechococcus elongatus* PCC7942 (SYO, accession no. ZP_00351210; unpublished), *Nostoc* sp. PCC7120 (NSC2, accession no. AE2341),[4] mouse 15,15′-dioxygenase (MmBCO1, accession no. Q9JJS6).[41] Unknown activity: *Bradyrhizobium japonicum* USDA110 (BRA-J, accession no. NP_772430; this study), *Bradyrhizobium* sp. Btai1 (BRA-S, accession no. ZP_008636652; this study), *Synechocystis* PCC6803 (SYC1, accession no. S76206).[10] 9,10-9′,10′-Carotenoid cleavage activity: mouse 9,10-9′,10′-dioxygenase (MmBCO2, accession no. Q99NF1),[9] *Zea mays* (ZmCCD1, ABF8565B), *Phaseolus vulgaris* (PvCCD1, Q94IR2),[6] *Arabidopsis thaliana* (AtCCD1, accession no. NP_191911.1),[6] *Lycopersicon esculentum* (LeCCD1, accession no. AAT68187),[17] *Petunia x hybrida* (PhCCD1, accession no. AAT68189),[18] *Nostoc* sp. PCC7120 (NSC1, accession no. BAB73063).[4] Isomerase activity: mouse RPE protein (MmRPE65, accession no. Q91ZQ5);[42] 9-*cis*-epoxycarotenoid 11,12 cleavage activity: *Zea mays* (VP14, accession no. AAB-621811.1),[16] *Arabidopsis thaliana* (AtNCED1, accession no. AAN17413). Additional enzymes and accession numbers for putative oxygenases can be found in Table S1. Underlined genomes do not contain carotenoid biosynthesis gene homologues, according to the KEGG database.

CCO homologues in pUCmod were coexpressed with a carotenoid pathway that was expressed from pACmod. Based on previous results that showed that the bicyclic carotenoid β-carotene is a substrate for many CCOs,[6,9,22] β-carotene produced by genes that are encoded by plasmid pAC-crtE-crtB-crtI14-crtY was chosen as the model carotenoid for this study.[31] Two additional carotenoids with other structural features were also tested: torulene, which is a monocyclic carotenoid with one β-ionone end group and a linear end, and zeaxanthin, which is a bicyclic carotenoid with hydroxylated β-ionone end groups. Cleavage of carotenoids in *E. coli* destroys the chromophore, which causes a loss of cell color (also referred to as bleaching)

that can be visually detected when compared to control cells. None of the tested four enzymes however, caused bleaching of the cell color of carotenoid-producing *E. coli*; this suggests that carotenoids are not likely to be a substrate of NOV and BRA enzymes.

We applied the same in vivo experimental approach to test the activity of the NOV and BRA enzymes against stilbene substrates. In previous research, we created recombinant *E. coli* cells that coexpress stilbene synthase (STS) and 4-coumaroyl CoA-ligase (4CL); this enabled the synthesis of stilbene compounds from phenylpropionic acid precursor compounds, which were fed to recombinant cells.[32] Biotranformation of the phenylpropionic acid precursors coumaric acid, cinnamic acid, or caffeic acid resulted in the synthesis of resveratrol (3,5,4′-trihydroxy-*trans*-stilbene), pinosylvin (5-(2-phenylvinyl)-1,3-benzenediol), and piceatannol (3,3′.4,5-tetrahydroxy-*trans*-stilbene), respectively (Figure 2). *E. coli* cells that expressed the stilbene pathway were cotransformed with NOV and BRA genes, and the enzymatic cleavage of resveratrol, pinosylvin, and piceatannol produced by the recombinant cultures was investigated. In addition, *E. coli* cells that coexpressed only 4CL and putative CCO enzymes were fed phenylpropionic acid precursors, and the resulting compounds were analyzed to rule out the cleavage of CoA-activated phenylpropionic acids by NOV and BRA enzymes.

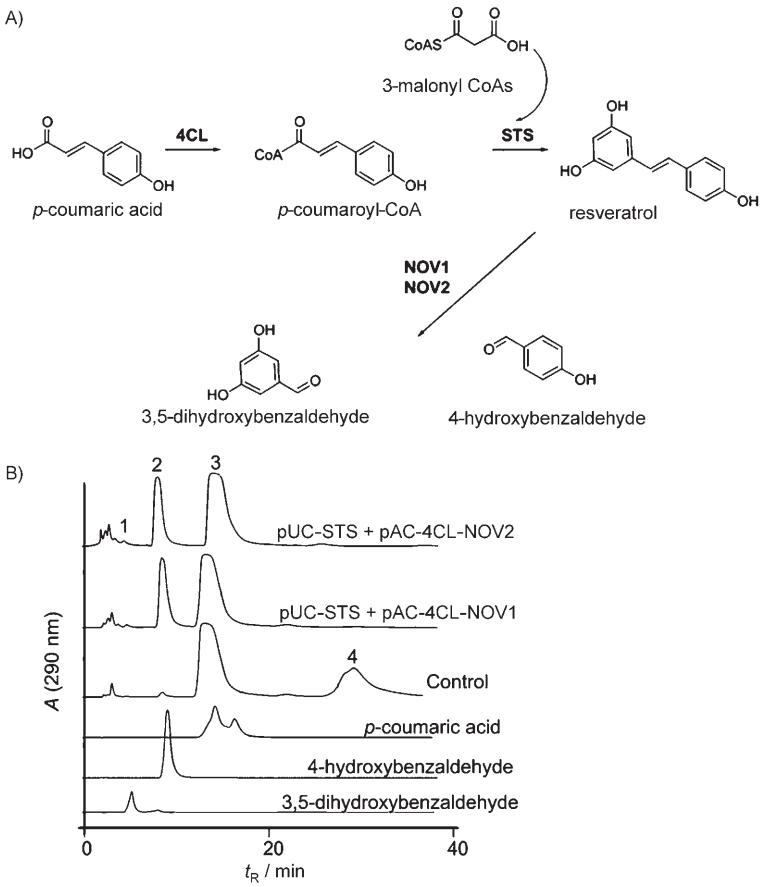


Figure 2. In vivo cleavage of resveratrol by NOV1 and NOV2. A) Engineered pathway in *E. coli* for resveratrol biosynthesis from fed coumaric acid, and cleavage of synthesized resveratrol in *E. coli* by coexpressed NOV enzymes. The enzymes that are shown are: 4-coumaroyl-CoA ligase (4CL; EC 6.2.1.12), stilbene synthase (STS; EC 2.3.1.95), and NOV oxygenases (NOV1, NOV2). B) HPLC analysis of extracts from coumaric-acid-fed recombinant *E. coli* cultures that coexpressed the stilbene biosynthetic genes and NOV1 (pUC-STS + pAC-4CL + NOV1) or NOV2 (pUC-STS + pAC-4CL + NOV2). The control culture contained only stilbene biosynthesis genes (pUC-STS + pAC-4CL). HPLC traces of culture extracts and of authentic standard compounds are shown. Peaks are: 1) 3,5-dihydroxybenzaldehyde (*m/z* 137.0), 2) 4-hydroxybenzaldehyde (*m/z* 121.0), 3) *p*-coumaric acid (*m/z* 163.2), 4) resveratrol (*m/z* 227.1). Control cultures converted coumaric acid to resveratrol. Addition of the oxygenase enzymes NOV1 and NOV1 resulted in a decrease in resveratrol and the appearance of 4-hydroxybenzaldeyde and 3,5-dihydroxybenaldehyde. The products were confirmed by standards and mass spectral analysis. 3,5-Dihydroxybenzaldehyde was not extracted from the medium in stoichiometric amounts.

NOV1 and NOV2 both efficiently cleaved resveratrol, which resulted in the complete degradation of resveratrol by recombinant *E. coli* strains after 16 h incubation and in the accumulation of two new products (Figure 2). The two new compounds were structurally identified as 4-hydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde by comparison of retention time and mass spectra with those from authentic compounds. The dihydroxy product, 3,5-dihydroxybenzaldehyde was not extracted from the medium in stoichiometric amounts; this suggests that it was further degraded by *E. coli* enzymes or it formed Schiff base adducts. Piceatannol, the stilbene compound that is produced from caffeic acid, was also cleaved by NOV1 and NOV2 into the corresponding aldehyde products 3,4-dihydroxybenzaldehyde and 3,5-dihydroxybenzaldehyde (data not shown). Because the conversion of the caffeic acid that was fed to the corresponding stilbene piceatannol by recombinant *E. coli* cells was much slower than the production of resveratrol from 4-coumaric acid,[32] the amounts of piceatannol cleavage products that were detected in the cultures were correspondingly lower. Cleavage of pinosylvin, which is the stilbene product from cinnamic acid, by NOV1 or NOV2 was not observed. Both NOV1 and NOV2 were specific for stilbene cleavage, and no cleavage products of fed phenylpropionic acids or CoA-activated phenylpropionic acids were detected in culture extracts (data not shown).

Surprisingly, no stilbene cleavage products were detected in recombinant *E. coli* cultures that expressed BRA-J or BRA-S despite their sequence similarity to the NOV and SPA enzymes. Previously characterized cyanobacterial CCOs, such as NSC1, NSC2, and SYC2,[4,10] were also expressed in stilbene-producing *E. coli* cells and were found to not cleave stilbenes.

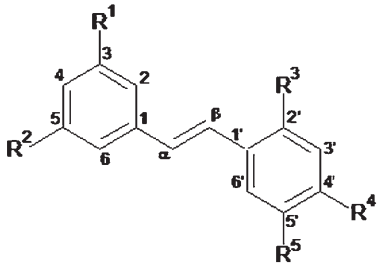
Characterization of in vitro cleavage activities

Assays with purified protein and/or whole cell protein extracts were conducted to confirm the cleavage results that were obtained in recombinant *E. coli*, and to test additional substrates. NOV and BRA genes were overexpressed from a pET expression vector in a recombinant *E. coli* strain that also expressed the GroEL and GroES chaperones to aid in the production of solu-

ble protein. Overexpressed histidine-tagged proteins were purified by immobilized metal affinity chromatography and were used in in vitro assays. However, as with other reports from this family of enzymes,[21,30] purified enzymes were far less active than enzymes in protein extracts from whole-cell lysates (more than 75% of their activity was lost during purification). As a consequence, protein extracts from whole-cell lysates are frequently used in assays with CCO enzymes.[21] We used both purified proteins and protein extracts from whole-cell lysates for in vitro assays with NOV and BRA enzymes and observed similar cleavage specificities for both preparations; but ca. fivefold lower cleavage rates were obtained with purified proteins. A series of cofactors and reducing agents that were tested with the enzyme (NAD, FAD, NADH, FADH, ascorbate, excess Fe^{2+}, glutathione, dithiothreitol) did not improve enzyme activity. Protein extracts from whole-cell lysates were prepared from *E. coli* cells that overexpressed the NOV and BRA genes from the constitutive expression vector pUCmod (Figure S2).

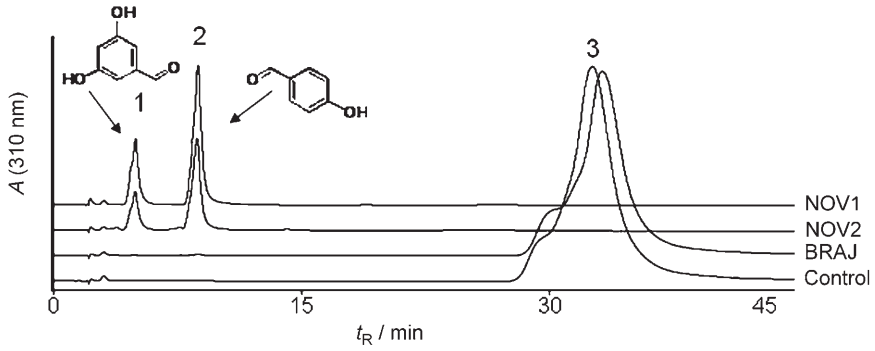
A series of stilbene substrates (rhapontigenin, resveratrol, rhaponticin, piceatannol and pinosylvin) with different hydroxyl and methoxy functional groups were tested in in vitro assays with NOV and BRA enzymes (Figure 3). NOV1 and NOV2 cleaved stilbene compounds that have a hydroxy or methoxy group at the 4′ position at the central double bond. As observed in the in vivo cleavage survey, pinosylvin, which carries no substitution at the 4′ position was not a substrate for NOV enzymes. Resveratrol was the preferred substrate for NOV1 and NOV2, followed by piceatannol and the 4′-methoxy-group-bearing stilbenes rhapontigenin and rhaponticin. In vitro assays with equal amounts of protein lysates resulted in complete cleavage of 1 mM (68.5 μg) resveratrol in 20 min, whereas complete cleavage of 1 mM (73.3 μg) piceatannol required 60 min of incubation (Figure 3). Cleavage of 1 mM (77.4 μg) rhapontigenin and its glucosylated derivative rhaponticin by NOV1 and NOV2 was much slower, and only 20% of the substrates were cleaved after 60 min (data not shown).

A)



Compound	R^1	R^2	R^3	R^4	R^5	BRA J	BRA S	NOV1	NOV2
rhapontigenin	OH	OH	H	OCH_3	OH	-	nd	+	+
resveratrol	OH	OH	H	OH	H	-	-	+	+
rhaponticin	*O*-glucose	OH	H	OCH_3	OH	-	nd	+	+
piceatannol	OH	OH	H	OH	OH	-	nd	+	+
pinosylvin	OH	OH	H	H	H	-	nd	-	-

B)



C)

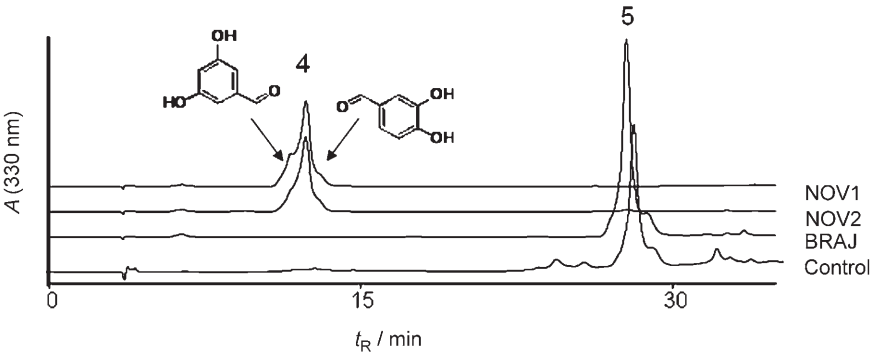


Figure 3. In vitro cleavage of stilbene compounds. A) Stilbene compounds with different functional groups were chosen as substrates for in vitro assays with NOV1, NOV2, BRA-J, and BRA-S protein lysates. Cleavage of the substrates is indicated by a "+" in the table based on product identification by HPLC and LC–MS; none of the enzymes cleaved pinosylvin. B) HPLC analysis of in vitro assays with resveratrol as substrate. Synthesis of 3,5-dihydroxybenzaldehyde (peak 1) and 4-hydroxybenzaldehyde cleavage products (2) from 1 mM resveratrol (3) with NOV1 and NOV2 protein extracts. Residual resveratrol was not detected by HPLC or LC–MS. C) HPLC trace of in vitro assay with piceatannol as substrate. Synthesis of 3,5-dihydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde cleavage products (4) from 1 mM piceatannol (5) with NOV1 and NOV2 enzymes protein extracts. Small amounts of residual piceatannol could be detected for NOV2. BRA-J protein extracts did not produce cleavage products with either resveratrol or piceatannol. Protein extracts from *E. coli* served as control.

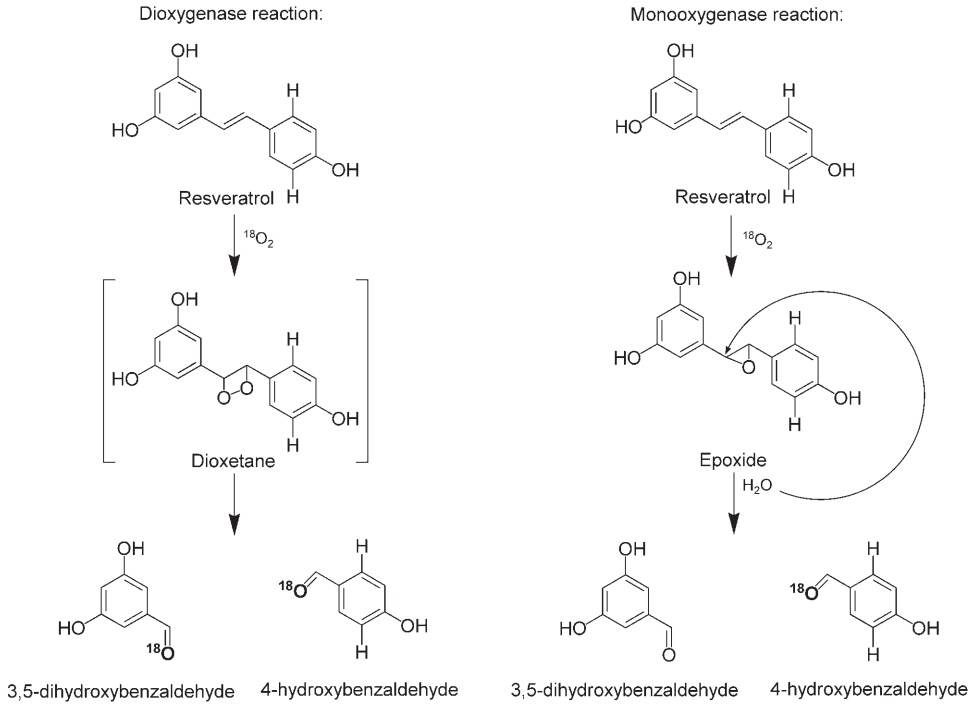
To confirm the in vivo results that showed that none of the four tested enzymes cleaved carotenoids, NOV and BRA enzymes were tested against the apocarotenoid substrate β-apo-8′-carotenal. β-Apo-8′-carotenal was chosen as a substrate instead of β,β-carotene because of its greater solubility; this results in higher activities of carotenoid-cleaving enzymes with apocarotenoid substrates compared to full-length carotenoids.[21] Moreover, some carotenoid cleavage enzymes are specific for apocarotenoids,[10] but no cleavage products of β-apo-8′-carotenal were detected in in vitro assays with the NOV and BRA enzymes.

The BRA-J and BRA-S enzymes were also assayed with several stilbene compounds (Figure 3), but cleavage activity of stilbene compounds was not detected; this confirms the results that were obtained with stilbene-producing *E. coli* cells. *Bradyrhizobium* strain USDA110 has been shown to produce the plant phytohormone abscisic acid via an unknown pathway.[33] We confirmed abscisic acid biosynthesis for strain *B. japonicum* USDA110 and *Bradyrhizobium* sp. BTAi1 (Figure S4). Because these *Bradyrhizobium* strains produce either no carotenoids at all (USDA110) or no epoxy-carotenoids (BTAi1), abscisic acid biosynthesis in *Bradyrhizobium* must occur via a different route than the plant pathway, which involves oxidative cleavage of epoxy-carotenoids by a CCO (NCEDs).[16] In filamentous fungi, abscisic acid is synthesized from farnesol via a partially described pathway. The recent identification of an abscisic acid gene cluster in *Botrytis cinerea* suggests the involvement of several oxidative steps in the conversion of farnesol to abscisic acid.[34] Therefore, we tested whether the BRA CCO homologues have activity against farnesol. GC–MS analysis of in vitro assays with farnesol showed the conversion of farnesol into a new compound by BRA-J and BRA-S, but not by the NOV enzymes or in the control reaction (Figure S5). The parent ion detected for this new product had *m/z* 290, which is consistent with a methanol adduct [*M*+32] of a farnesol derivative that contains two additional oxygen groups *m/z* 258 $[M]^+$. However, the structure of this compound can not be deduced from the MS data alone.

Oxygen labeling studies with NOV2

Catalytic cleavage of the central double bond of stilbenes by CCO homologues from *Novosphingobium* identified in this study and the previously reported *Sphingomonas* LSDs[35] is similar to the carotenoid cleavage reaction that is observed with NCED enzymes in the production of abscisic acid,[16] and the central double bond cleavage that is observed by CCOs responsible for the production of retinal from β-carotene.[36] An enzyme mechanism that uses molecular oxygen and ferrous iron is thought to be similar among the different types of carotenoid or stilbene-cleaving oxygenases.[25] Incorporation of one or two molecules of oxygen from atmospheric oxygen during catalysis by these enzymes is still controversial, and both mono- and dioxygenase mechanisms have been suggested for carotenoid cleaving oxygenases (Scheme 1).[20–22] Poor activities of CCOs in in vitro assays and cleavage of water-insoluble substrates might largely be responsible for the lack of rigorous mechanistic studies of this new class of nonheme-iron oxygenases. Despite a few reports of characterized purified recombinant CCOs[10,36] many studies rely on protein lysates.[21] However, compared to previously studied CCOs,[4,21] cleavage reactions with NOV enzymes are fast and stilbene substrates are much more soluble than carotenoids and can be analyzed by GC–MS. Both properties allow short assay times and a quick analysis of the reaction products, which minimizes unspecific label exchange during oxygen labeling studies. We therefore sought to perform oxygen labeling studies with NOV2 to determine whether this class of oxygenases uses a mono- or dioxygenase mechanism.

First, resveratrol cleavage by NOV2 was assayed in an atmosphere of labeled oxygen $^{18}O_2$. The reaction was stopped after 15 min and the labeled cleavage products were analyzed by



Scheme 1. Possible mechanisms for the oxidative cleavage of resveratrol. Two proposed mechanisms are shown for oxidative cleavage with CCO enzymes. A dioxygenase mechanism results in both aldehyde cleavage products being labeled with isotopic ^{18}O when the reactions are performed in an ^{18}O atmosphere. The monooxygenase mechanism results in a single isotopic ^{18}O-labeled cleavage product when reactions are performed in an ^{18}O atmosphere.

GC–MS. Only one of the expected cleavage products, 4-hydroxybenzaldehyde, was found to carry a significant amount of the ^{18}O label (over 69% labeled; Figure 4). In contrast, the 3,5-dihydroxybenzaldehyde product did not contain an equivalent amount of the heavy oxygen label; this suggests that its aldehyde oxygen must come from unlabeled water in the reaction.

To confirm the oxygen labeling results, a second assay was performed with labeled ^{18}O-water. As expected, a reverse labeling pattern of the cleavage products was observed (Table 1). Now, the 3,5-dihydroxybenzaldehyde carried the ^{18}O label (*m/z* 282/284 = 7.1:92.9) and the 4-hydroxybenzaldehyde cleavage product was predominately unlabeled (*m/z* 194/196 = 93.3:6.7; Figure 4). The ratio was determined from the extracted ion chromatograms [*M*+H] by using the ratio of heavy to total 4-hydroxybenzaldehyde (i.e., 196/194 + 196) as in Schmidt et al.[21] The ratio of labeled to unlabeled aldehyde cleavage products were higher in this ^{18}O-water labeling experiment compared to the corresponding atmospheric oxygen labeling experiment. One reason for the apparent imbalance might be due to nonenzymatic oxygen exchange of the hydroxyl groups in the labeled water, which might lead to an over representation of the labeled 3,5-dihydroxybenzaldehyde product. A time course (1, 5, 15, 30 min) was performed to monitor oxygen exchange during the enzyme assay and in control reactions that contained authentic 4-hydroxybenzaldehye and 3,5-dihydroxybenzaldehyde without the NOV2 protein (Figure S3, Table S2). Exchange of the oxygen label in the enzyme assays occurred more rapidly with the dihydroxy cleavage product, 3,5-dihydroxylbenzaldehyde, than with the mono-substituted 4-hydroxybenzaldehyde (Figure S3). After 15 min the labeling pattern in the enzyme reaction clearly showed heavy label mainly on one product, 3,5-dihydroxylbenzaldehyde, and only a small fraction of the 4-hydroxybenzaldehyde was labeled. Control reactions showed that some nonenzymatic label ex-

Table 1. Summary of labeling pattern from isotope experiments.

	Product mass	$^{18}O_2$	Product mass	^{18}O-water
3,5-dihydroxybenzaldehyde	*m/z* 282	unlabeled ~65%	*m/z* 284	labeled ~92%
4-hydroxybenzaldehyde	*m/z* 196	labeled ~69%	*m/z* 194	unlabeled ~90%

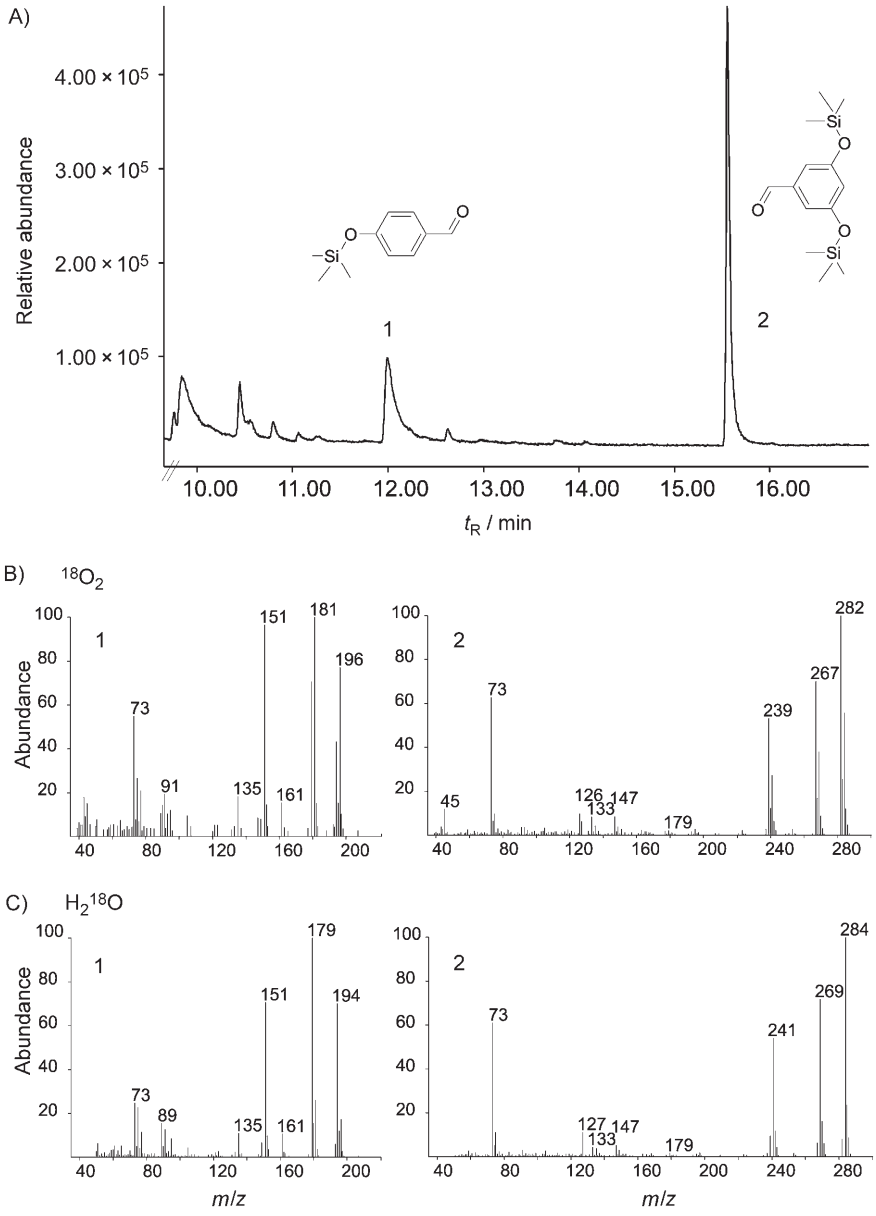


Figure 4. GC–MS analysis of oxygen-labeled cleavage products of resveratrol synthesized by NOV2. A) Silylated esters of cleavage products 4-hydroxybenzaldehyde (peak 1) and 3,5-dihydroxybenzaldehyde (2). B) Incorporation of molecular oxygen into 4-hydroxybenzaldehyde. Mass spectra of 4-hydroxybenzaldehyde (left spectrum) and 3,5-dihydroxybenzaldehyde (right spectrum) in an $^{18}O_2$-atmosphere showing labeled 4-hydroxybenzaldehyde (*m/z* 196) and unlabeled 3,5-dihydroxybenzaldehyde (*m/z* 282). C) Mass spectral data from incorporation of oxygen from ^{18}O-water. Incorporation of label into 3,5-dihydroxybenzaldehyde (*m/z* 284); 4-hydroxybenzaldehyde remained unlabeled (*m/z* 194). Note that the main fragments in panel B and C (*m/z* 181 and *m/z* 179) result from the loss of a [-CH_3] group from 4-hydroxybenzaldehyde.

change occurred predominantly with the dihydroxy cleavage product. Together these results suggest that NOV2 cleaves resveratrol via a monooxygenase mechanism and might have a stereopreference for oxygen attack.

Conclusion

In this work we describe the cloning and partial characterization of four bacterial enzymes that belong to a recently described class of nonheme-iron oxygenases that so far mostly includes carotenoid-cleaving enzymes from plants, mammals, and cyanobacteria. Two enzymes (SPA1 and SPA3) from *Sphingomonas paucimobilis,* which are known to cleave substrates other than carotenoids, have been named lignostilbene dioxygenases (LSDs; for reviews see ref. [3]). This study expands the number of noncarotenoid-cleaving family members with the finding that two enzymes from *Novosphingomonas aromaticivorans* DSM12444 are stilbene-cleaving oxygenases. We show in vivo and in vitro that the enzymes NOV1 and NOV2 cleave the central double bond of *trans*-stilbene derivatives, but do not cleave bicyclic, monocyclic, or hydroxylated model carotenoid substrates. In contrast, the putative CCOs from *Bradyrhizobium japonicum* USDA110 and *Bradyrhizobium* sp. BTAi1 were not active against stilbenes or carotenoids, but showed activity with farnesol. In the course of these studies, we also tested known carotenoid cleavage enzymes for their ability to cleave stilbenes and we did not find stilbene cleavage activity with enzymes from the cyanobacteria *Nostoc punctiforme, Nostoc* sp. PCC7120, or *Synechocystis* sp. PCC6803.[4,10,11]

NOV1 and NOV2 are, to our knowledge, the second reported examples of stilbene-cleaving oxygenases. Studies of LSD isoforms from *Sphingomonas paucimobilis* differ from this report in a few significant respects.[23,24,29,35,37] In our assays, the recombinant enzymes were tested in vivo and in vitro against natural substrates from plants such as resveratrol and piceatannol. Previous reports identified the 4′-hydroxyl group of stilbenes as a key structural feature for cleavage.[35] We found that the recombinant *Novosphingobium* enzymes cleaved compounds with 4′-hydroxyl groups or 4′-methoxy functional groups in vitro; this suggests that an oxygen functional group (not specifically a hydroxyl) in that position might be important for substrate binding. NOV1 and NOV2 did not cleave pinosylvin, which is a substrate that lacks oxygen on the 4′ carbon. The NOV enzymes in this study displayed similar substrate preferences (e.g., they cleaved resveratrol better than the other substrates) as opposed to the *Sphingomonas* enzymes, which all had different substrate specificities.[29] Finally, we performed labeling studies to determine the oxygenase cleavage mechanism of these enzymes.

The current controversy over the oxygenase mechanism of this family of nonheme-iron enzymes stems from contradictory findings from previous labeling studies, a lack of rigorous biophysical studies, and the difficulties associated with assays that use purified carotenoid cleavage enzymes.[21,22] The stilbene cleavage reaction has several advantages over carotenoid cleavage; this makes it a good candidate for mechanistic studies. First, the reaction, when carried out with protein extracts from whole-cell lysates, is fast (complete resveratrol cleavage under 20 min with 5 μg of protein). Second, the stilbene substrate resveratrol is more soluble in aqueous systems than the lipophilic carotenoids and the cleavage products can be easily worked up in organic solvents; this limits water exchange. Third, stilbene cleavage products can be readily identified by GC–MS after derivatization; this limits the amount of oxygen exchange of the carbonyl group. In contrast, the aqueous and acidic HPLC conditions that were used for the analysis of carotenoid cleavage products in previous labeling studies led to oxygen exchange, which rendered the interpretation of the results difficult.[20,21]

Assays performed in an $^{18}O_2$ environment with NOV2 and resveratrol as substrate resulted in predominant labeling (~69%) of one product, 4-hydroxybenzaldehdye. In the converse experiment with ^{18}O-water in an unlabeled O_2 environment, the aldehyde group of the other cleavage product, 3,5-dihydroxybenzaldehyde, was almost completely labeled. Control experiments with authentic aldehyde cleavage products and no added enzyme showed some unspecific label exchange with the more reactive 3,5-dihydroxybenzaldehedye product and ^{18}O-water (Table S2 and Figure S3). Despite the observed label exchange with the 3,5-dihydroxybenzaldehyde product, data from these assays indicate that recombinant NOV2 stilbene oxygenase uses a monooxygenase reaction mechanism, and that the atmospheric oxygen is preferentially added to the 4-hydroxybenzaldehyde cleavage product.

Our results contradict findings from an abscisic acid labeling study in plants as well as a recent in vitro oxygen labeling study that was conducted with the recombinant CCO from *Arabidopsis* (*At*CCD1) that suggested a dioxygenase mechanism for this enzyme family.[20,21] The abscisic acid study has been criticized for examining only one cleavage product and describing the reaction as a dioxygenase mechanism;[21] we examined both cleavage products and found regioselectivity, which could explain the abundant label found on the one cleavage product that was analyzed in the abscisic acid study. In vitro labeling studies with a 15,15′ carotenoid cleavage oxygenase from the chicken also suggest a monooxygenase mechanism, which is similar to our findings.[22] However, Leuenberger et al. used a coupled enzyme reaction in which the formed aldehyde cleavage products were converted in situ to the corresponding less reactive alcohol products to reduce label exchange and facilitate GC–MS analysis of derivatized alcohols. It is possible that different members of this enzyme family catalyze similar reactions by a different oxygenase mechanism, which might even vary depending on the cleaved substrate. Rieske oxygenase family members, for example, have been shown to have mono- or dioxygenase activity based on different substrates[38–42] and enzymes.[43] Examinations of Rieske-type oxygenases show that subtle changes in the active site can alter the enzyme mechanism.[43] Perturbations in the active site might create an environment that is advantageous for a monoxygenase-like mechanism rather than a dioxygenase mechanism or vice versa.

Exact mechanistic details have not been determined for this new class of nonheme-iron oxygenases because of the poor

activity of in vitro assays. Assays frequently use a reductant such as ascorbate, DTT, TCEP, or excess Fe^{2+} to preserve the ferrous iron, but no other cofactors, iron–sulfur proteins or reductases have been identified as required to balance the electron flow. It has been suggested that all the electrons in the product can come exclusively from the substrate and oxygen.[2] Unfortunately, there is only one crystal structure of a carotenoid cleavage enzyme (*Synechocystis* sp. PCC6803)[19] available, and the stilbene oxygenases from *Novosphingobium* model poorly onto the solved structure (Figure S6). The β-strands that form the propellers are conserved, but there is large variation in the amino acid residues that form the dome and entrance loop. Descriptions of the *Synechocystis* protein structure state that ring structures will not fit through the active-site tunnel.[29] Cleavage of stilbene structures, however, requires positioning of at least one phenol ring in the active site of the NOV enzyme; this illustrates that there might be important differences in the structure and function among the members of this enzyme family. NOV2 residues that surround the tunnel entrance loops (residues Leu239–Lsy243 and Phe106–Pro110) are structurally different in the model than in the *Synechocystis* structure. More labeling and mechanistic studies along with structural investigations are required to begin to understand the catalysis of these enzymes.

This study also showed that analysis of sequence information is not sufficient to predict carotenoid or stilbene activity, and that the substrate specificity needs to be determined empirically for all new examples of these oxygenases. The two *Bradyrhizobium* enzymes share a similar degree of sequence identity to *Sphingomonas* enzymes as the NOV2 enzyme does (~35%). However, the two enzymes from *Bradyrhizobium* did not cleave stilbenes or carotenoids and instead showed activity with farnesol. Although the mass fragmentation pattern of the farnesol reaction product does not allow structural assignment, the fragment at *m/z* 259 $[M]^+$ that arises from the loss of methanol from the methanol adduct parent (*m/z* 290) suggests the addition of two oxygen groups to farnesol (and likely bond rearrangement in order to arrive at *m/z* 258) rather than oxidative cleavage of farnesol by BRA enzymes, which would result in products with lower molecular weight and shorter retention times. Farnesol has been identified as the precursor of abscisic acid in filamentous fungi.[34] It is postulated that conversion of farnesol to abscisic acid involves several oxidative steps.[34] Knockout studies in *Botrytis cinerea* have identified two P450 monooxygenases that likely catalyze two of the postulated oxidation reactions.[38] Biosynthesis of the phytohormone abscisic acid in the *Bradyrhizobium* must also occur through a different route than the epoxy-carotenoid cleavage pathway in plants because these bacteria are either noncarotenogenic or do not produce epoxy carotenoids. Additional studies that involve the creation of gene knockouts will be necessary to investigate whether BRA-J and BRA-S are involved in abscisic acid biosynthesis in the plant symbiont *Bradyrhizobium*. Interestingly, a BLAST search of the *Botrytis cinerea* genome sequence (Broad Institute) with the BRA protein sequences identifies two putative CCO homologues. Deletion of these putative CCO genes could establish whether one of them catalyzes yet unknown steps in abscisic acid biosynthesis in this ascomycete.

The activity of the BRA enzymes indicates that there might be many new activities to be discovered for other putative microbial CCO homologues (Figure 1). Of equal interest are investigations that are aimed at identifying the biological functions of these enzymes in bacteria and fungi. Carotenoid cleavage in plants and mammals has functions that extend beyond pigment degradation and synthesis of visual pigments as more and more roles of carotenoid cleavage compounds in signaling are being discovered.[8] It can be assumed that the bacterial and fungal representatives of the CCO family have similarly diverse functions beyond simple degradation.

Experimental Section

Chemicals and materials: Caffeic acid, ferulic acid, piceatannol, rhaponticin, β-apo-8′-carotenal, and bis(trimethylsilyl)trifluoroacetamide (BSTFA) were purchased from Sigma–Aldrich. 4-Coumaric acid was purchased from ICN (Aurora, OH, USA), and resveratrol was from Calbiochem. The 95% ^{18}O-water was from Cambridge Isotope Laboratories (Andover, MA, USA). All solvents were of HPLC grade and were purchased through Fisher Scientific. HPLC grade water was purchased from Malllinckrodt Chemicals (Phillipsburg, NJ, USA). Vent DNA polymerase, T4 DNA ligase and restriction enzymes were from New England Biolabs. Restriction buffers were SuRE/Cut buffers from Roche.

Gene cloning: Homology searches were performed by using NCBI BLAST software based on the *Sphingomonas paucimobilis* lignostilbene oxygenase proteins, SPA1 (isoform I) AAC60447, SPA3 (isoform III) AAB35856,[24,29] and the previously characterized cyanobacterial CCOs.[4,10] CCO homologues were identified in published complete genome sequences from NCBI and Joint Genome Institute (Figure 1 and Table S1). Putative CCO homologues from *Novosphingobium aromaticivorans* DSM 12444 (NOV1 (YP_496081); NOV2 (YP_498079)), *Bradyrhizobium japonicum* USDA110 (BRA-J (NP_772430) and *Bradyrhizobium sp.* BTAi (BRA-S) were selected for cloning and functional characterization. The putative CCO genes were amplified from genomic DNA by PCR with Vent polymerase by using gene-specific primers with added restriction sites. NOV1 and NOV2 were cloned into the BglII and NotI sites of the constitutive expression vector pUCmod[31] to give pUCmod-NOV1 and pUCmod-NOV2. BRA-J and BRA-S were cloned into the NdeI and XhoI sites of pUCmod to yield pUCmod-BRA-J and pUCmod-BRA-S.

For the expression of larger amounts of protein for purification of NOV1 and NOV2, genes were subcloned into the NdeI and XhoI sites of the inducible expression vector pET28b+ (Invitrogen) to give plasmids pET-NOV1 and pET-NOV2. The stop codon was eliminated from the sequences for in-frame fusion with a C-terminal 6× histidine tag that was encoded in the pET28b+ vector to facilitate protein purification. BRA-J and BRA-S were subcloned in a similar fashion into the inducible expression vector pET24b+ (Invitrogen) to yield plasmids pET-BRA-J and pET-BRA-S.

For coexpression of the CCO homologues NOV1, NOV2, BRA-J, and BRA-S (in the following collectively referred to as CCOs) with stilbene biosynthetic genes in *E. coli*, the entire CCO expression cassettes in pUCmod-CCO (including the constitutive *lac* promoter and gene coding region[31]) were amplified by PCR by using sequence-specific primers with added restriction sites. The products were subcloned into the XbaI site of plasmid pAC-4CL,[32] which

contained the gene for 4-coumaroyl ligase (4CL) from *Arabidopsis thaliana* under the control of a constitutive *lac* promoter. The resulting plasmid was called pAC-4CL-CCO.

All cloning and DNA manipulation were carried out in *E. coli* strain JM109 by following standard techniques described elsewhere.[32] Cloned gene sequences were verified by sequencing.

Culture conditions and strains: Unless otherwise indicated, *E. coli* cultures were grown in Luria–Bertani (LB) medium that was supplemented with ampicillin (100 $\mu g\,mL^{-1}$) and chloramphenicol (50 $\mu g\,mL^{-1}$) at 30 °C. *E. coli* strains JM109 and BL21(DE3) were used for gene expression from pUCmod and pET-plasmids, respectively.

E. coli strain BW27784[40] was used for in vivo analysis of stilbene cleavage by CCO homologues. A modified M9 medium that contained yeast extract (1.25 $g\,L^{-1}$), glycerol (0.5% *v/v*), and the appropriate antibiotics was used for stilbene biosynthesis as described previously.[32]

Protein expression and purification: Both pET-CCO and pUCmod-CCO plasmids were used for protein overexpression. For expression of genes from pUCmod-CCO, recombinant *E. coli* JM109 overnight cultures (4 mL) were used to inoculate 1:100 LB medium (400 mL) that contained the appropriate antibiotics. Cultures were grown for 16 h at 30 °C and the cells were harvested by centrifugation and stored at −20 °C until used. Cells that were collected from the culture (50 mL) were lysed with BugBuster® protein extraction reagent (2 mL; Novagen). Cell debris was cleared by centrifugation (14000 rpm, 5 min, 4 °C), and the cleared protein extract was used in in vitro activity assays. Protein levels were estimated by SDS gel electrophoresis, and the concentrations were adjusted so that comparable levels were added to assays.

The pET-CCO plasmids were used for the overexpression of CCO proteins for protein purification. As previously observed with other CCOs,[4,21] these proteins are prone to inclusion body formation when expressed at high levels (for example, from the strong T7-promoter present in pET plasmids). To facilitate the expression of soluble proteins, pET-CCO plasmids were transformed into *E. coli* BL21(DE3) that harbored the plasmid pGRO7 (Takara, Madison, Wisconsin, USA), which expresses the GroEL and GroES chaperones (see also ref. [4]). *E. coli* BL21 cotransformed with putative CCO homologue and groES–groEL were grown, overnight, at 30 °C in LB media (4 mL). This culture was used to inoculate (1:100) LB (400 mL), and chaperone expression was induced with arabinose (0.5 $mg\,mL^{-1}$). Cells were grown at 30 °C until an OD_{600} of 0.6. Then the cultures were cooled on ice and induced with isopropyl-β-D-thiogalactopyranoside (IPTG; 1 mM) before incubation was continued, overnight, at 18 °C; the cells were then harvested by centrifugation and stored at −20 °C until use. Cells that were collected from culture (50 mL) were lysed with BugBuster® protein extraction reagent (2 mL; Novagen). Cell debris was cleared by centrifugation (14000 rpm, 5 min, 4 °C). Aliquots of the cleared protein extract were saved for in vitro assays and the remainder was used to purify CCO proteins by metal affinity chromatography. Soluble protein was loaded onto a Talon Resin immobilized metal affinity chromatography (IMAC; Invitrogen) column and eluted in phosphate buffer (50 mM, pH 7.2) with imidazole (300 mM) after three washing steps. The CCO proteins were eluted (4 mL) and the fraction was concentrated by using an Amicon ultracentrifuge concentrator with a 10 kDa molecular weight cut-off. The Amicon concentrator was used to desalt the protein by exchanging the buffer 4 times against phosphate buffer (50 mM pH 7.2). The protein was subjected to iron center reconstitution by incubation with $FeSO_4$ (100 mM) under argon gas for 30 min to insure incorporation of Fe^{2+} into the active site. Protein concentrations were determined by using the Bradford assay (BioRad).

In vivo analysis of carotenoid cleavage activity: To investigate in vivo carotenoid cleavage, CCO enzymes on pUCmod were coexpressed with β,β-carotene biosynthetic genes expressed from pAC-crtE-crtB-crtI14-crtY,[31] pAC-crtE-crtB-crtI14-crtY-crtX, pAC-crtE-crtB-crtI14-crtY2 in *E. coli* JM109 as described previously for cyanobacterial CCO.[4] Briefly, single colonies of *E. coli* JM109 transformants that harbored the carotenoid and CCO plasmids to be tested were grown, overnight, for 48 h in LB media (50 mL) that was supplemented with ampicillin and chloramphenicol at 30 °C. The color intensities of the resulting cell pellets from six replicate cultures were then compared by visual comparison with control cells that harbored the corresponding carotenoid plasmid and empty pUCmod plasmid.

In vivo analysis of stilbene cleavage activity: CCO enzymes in plasmid pAC-4CL-CCO, which also contained 4-coumaroyl CoA-ligase 4CL, were coexpressed with stilbene synthase (STS) from *Arachis hypogaea* (peanut) in the constitutive expression vector pUC-STS. The construction of stilbene and flavonoid biosynthetic pathways has been described previously.[31,32]

To investigate the in vivo stilbene cleavage by CCOs, single colonies of *E. coli* BW27784 transformants that harbored plasmids pUC-STS and pAC-4CL-CCO, or only pAC-4CL-CCO (control for cleavage of CoA-activated phenylpropionic acids), were grown, overnight, in modified M9 medium (4 mL) at 30 °C, and then used to inoculate (1:100) a larger volume of modified M9 medium (50 mL). Cultures were grown to an OD of 0.1 at 30 °C when phenyl propionic acid precursor compounds (1 mM, 200 μL of 4-coumaric acid, caffeic acid or ferulic acid in DMSO) were added to the cultures to initiate their biotransformation into stilbene compounds by the recombinant *E. coli* pathway (STS and 4CL). Following an additional 16 h incubation at 30 °C, the culture supernatant was extracted and analyzed for product formation essentially as described previously.[32] In brief, the culture (1 mL) was centrifuged at maximum speed to pellet cells. The media was decanted to a fresh 1.5 mL microfuge tube, and the pH was adjusted by addition of 1 N HCl (50 μL). Then the media was extracted twice with EtOAc (500 μL) and the extracts were combined and dried under N_2. The dried residue was resuspended in MeOH (100 μL) and all samples were stored at −20 °C prior to HPLC and LC–MS analysis (see below).

In vitro assays: Assays were either performed with purified protein (100–250 μg) or protein extracts (50 μL) in 300 μL reactions that contained phosphate buffer (50 mM, pH 7.2), NaCl (300 mM), sodium ascorbate (10 mM), and $FeSO_4$ (0.5 mM). After 5 min of equilibration, stilbene substrates (1 mM from 1 M resveratrol, piceatannol or rhapotinigenin dissolved in DMSO) or carotenoid substrate (0.27 mM from 2 mM β-apo-8′-carotenal dissolved in 1% Tween 40[4]) were added. In vitro reactions were carried out at 30 °C for the prescribed amount of time (5 min to 12 h) before being stopped with 1 N HCl (50 μL) and extracted three times with EtOAc (500 μL). β-Apo-8′-carotenal assays were extracted with Et_2O. The organic fractions were combined, dried under N_2, and stored until HPLC or GC analysis.

Isotope labeling: For labeling experiments with ^{18}O-water, the protein extracts were freeze-dried to remove all water, and the residues were resuspended in of ^{18}O-water (100 μL). No buffer, NaCl or $FeSO_4$ was added to avoid contamination with unlabeled H_2O. Reactions were started by adding resveratrol (1 mM in DMSO). After 15 min incubation at 30 °C, assays were extracted two times with

EtOAc (500 μL), dried under N_2, and immediately derivatized to silyl ethers for GC–MS analysis as described below.

Labeling experiments with $^{18}O_2$ were performed in screw-capped glass vessels (2 mL) with a gas-tight Teflon septum by using reaction conditions described above for standard assays with resveratrol. The vials were flushed three times with $^{18}O_2$ and protein extract was added with an airtight Hamilton syringe. The reaction mixture was allowed to equilibrate for 5 min before resveratrol (1 mM) was injected into the vial. The reaction was stirred for 15 min before being extracted twice with EtOAc (500 μL). Samples were dried and immediately derivatized to silyl ethers for GC–MS analysis as described below.

HPLC and LC/MS analysis: HPLC analysis was performed by using an Agilent 1100 HP system with a quaternary pump and a photodiode array detector (Palo Alto, CA). Several HPLC conditions were used to analyze possible β-apo-8′-carotenal cleavage products, as described.[4] Briefly, cleavage of β-apo-8′-carotenal at the 15,15′ position to retinal was analyzed by applying sample (50 μL) to an Adsorbosil C18 column (4.6×250 mm, 5 μm; Alltech, Deerfield, IL). The gradient program was modified from Ruch et al.[10] by using a solvent system of MeOH/*tert*-butylmethyl ether/H_2O (120:4:40, *v*/*v*/*v*; B) and MeOH/*tert*-butylmethyl ether (500:500, *v*/*v*; A). The gradient conditions were solvent B (100%) to solvent B (43%) over 45 min, solvent B (43%) to solvent B (0%) for 11 min, solvent B (0%) for 14 min with a flow rate of 1 mL min^{-1}. Dialdehyde cleavage products were determined by applying sample (100 μL) to a Zorbax RX-C18 column (4.6×250 mm, 5 μm; Agilent Technologies). The solvent system was MeOH/water (70:30, *v*/*v*) with 0.1% NH_4OAc (B) and MeOH (A). The gradient conditions were solvent B (100%) to solvent B (0%) over 16 min, solvent B (0%) until 26 min, and then return to A (100%) with a flow rate of 0.8 mL min^{-1}.

Stilbene cleavage products were detected by using conditions that were modified from the HPLC methods described by Watts et al.[32] for the analysis of stilbene compounds. Sample (20 μL) was applied to a reversed-phase Eclipse XDB-C8 column (4.6×150 mm, 5 μm; Alltech, Deerfield, IL) and analyzed with an isocratic program by using a solvent system of H_2O/trifluoroacetic acid (99.9:0.1, *v*/*v*; A) and MeOH/trifluoroacetic acid (99.9:0.1, *v*/*v*; B) in a ratio of 73:27 with a flow rate of 0.8 mL min^{-1}. To achieve better resolution of piceatannol cleavage products, a gradient program was used with a flow rate of 0.5 mL min^{-1} and the following conditions: from 0–10 min A/B (75:25), followed by a gradient from A/B (75:25) to A:B (50:50) in 15 min, followed by 5 min A/B (50:50. Stilbenes and cleavage compounds were identified by comparisons of retention times and UV/Vis spectra of standard compounds (resveratrol, piceatannol, rhaptonin, ferulic acid, coumaric acid, caffeic acid, 4-hydroxybenzaldehyde, 3,5-dihydroxybenzladehyde, 3,4-dihydroxybenzaldehyde) and mass spectrometry. For quantification of products, standard curves were constructed by plotting peak areas of known quantities of standards.

Mass fragmentation spectra were monitored in a mass range of *m*/*z* 50–500 on a LCQ mass spectrophotometer that was equipped with electrospray chemical ionization interface (Thermo Finnigan). Mass fragmentation spectra of standard compounds and the extracted compounds were monitored with a negative electron spray ionization (ESI) interface. Negative ion values for standard compounds were as follows: 4-coumaric acid (*m*/*z* 163.1), caffeic acid (*m*/*z* 179.1), ferulic acid (*m*/*z* 193.1), resveratrol (*m*/*z* 227.1), piceatannol (*m*/*z* 243.1), 4-hydroxybenzaldehyde (*m*/*z* 121.0), 3,5-dihydroxybenzaldhyde (*m*/*z* 137.0). The chromatography conditions were identical to the HPLC conditions described above with the exception that trifluoroacetic acid was excluded.

GC–MS analysis: Dried samples were derivatized to silyl ethers by addition of bis(trimethylsilyl)acetamide (BSTFA; 50 μL) reagent. GC–MS analyses were performed with a HP6890 Series GC coupled to a HP5973 mass-selective detector. GC conditions consisted of an HP-5 column (30 m by 0.25 mm ID by 1.5 μm coated with 5% phenyl methyl silicone) and a split injector (1:20) set to a temperature of 250 °C. The temperature started at 60 °C and increased to 280 °C at 8 °C/minute intervals with a helium flow rate of 1 mL min^{-1}. The EI-MS ionization voltage was 70 eV (electron impact ionization) and the ion source and interface temperature were both 250 °C. Mass spectra were scanned in a range of *m*/*z* 40–500 at 1 s intervals.

Abbreviations: CCO, carotenoid cleavage oxygenase; NCED, 9-*cis*-epoxydioxygenase; LSD, lignostilbene-α,β-dioxygenases; STS, stilbene synthase; 4CL, 4-coumaroyl ligase.

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