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The biosynthesis of hydroxycinnamoyl quinate esters and their role in the storage of cocaine in *Erythroxylum coca*

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Dedicated to the memory of Meinhart H. Zenk.

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ABSTRACT

Complexation of alkaloids is an important strategy plants utilize to facilitate storage in vacuoles and avoid autotoxicity. Previous studies have implicated hydroxycinnamoyl quinate esters in the complexation of purine alkaloids in *Coffea arabica*. The goal of this study was to determine if *Erythroxylum coca* uses similar complexation agents to store abundant tropane alkaloids, such as cocaine and cinnamoyl cocaine. Metabolite analysis of various *E. coca* organs established a close correlation between levels of coca alkaloids and those of two hydroxycinnamoyl esters of quinic acid, chlorogenic acid and 4-coumaroyl quinate. The BAHD acyltransferase catalyzing the final step in hydroxycinnamoyl quinate biosynthesis was isolated and characterized, and its gene expression found to correlate with tropane alkaloid accumulation. A physical interaction between chlorogenic acid and cocaine was observed and quantified in vitro using UV and NMR spectroscopic methods yielding similar values to those reported for a caffeine chlorogenate complex in *C. arabica*. These results suggest that storage of cocaine and other coca alkaloids in large quantities in *E. coca* involves hydroxycinnamoyl quinate esters as complexation partners.

1. Introduction

Alkaloids are one of the largest groups of secondary metabolites in plants (Otani et al., 2005). Their abundance, together with their important biological activities, has stimulated great interest in the way alkaloids are transported and stored in planta (Ferreira et al., 1998; Shitan et al., 2009; Sirikantaramas et al., 2008; Yazaki et al., 2008). They are often stored in specialized compartments, such as the vacuole which can take up to 90% of the total cell volume (Kutchan, 2005; Roytrakul and Verpoorte, 2007; Yazaki et al., 2008). The late Meinhart H. Zenk devoted considerable effort to investigating alkaloid transport into vacuoles, evaluating the validity of different transport system models, such as the ion-trap mechanism and specific carrier-mediated transport (Deus-Neumann and Zenk, 1984, 1986).

Tropane alkaloids represent a medicinally important class of alkaloids most commonly found in the Solanaceae, although they also appear in the Convolvulaceae, Erythroxylaceae, Proteaceae, and Rhizophoraceae (Asano et al., 2001). Of the various tropane alkaloids, cocaine (**1**) (methylecgonine benzoyl ester) (Fig. 1) is the most prominent due to its social and economical importance throughout human history. It is found in the genus *Erythroxylum*, accumulating in quantities of up to 1% dry weight in the mature leaves of two of the 230 known species, *Erythroxylum coca* and *Erythroxylum novogranatense* (Bieri et al., 2006; Plowman and Hensold, 2004). Cocaine (**1**) appears to serve as a toxin deterring the attack of herbivores and pathogens (Nathanson et al., 1993). In addition, it has been shown to be a genotoxic compound inducing micronuclei formation in mammalian cells (Harborne and Khan, 1993; Sonnante et al., 2010). Plants that synthesize cocaine (**1**) may avoid autotoxicity by sequestering this alkaloid in their vacuoles. Such localization has been shown via immunogold labeling in the mature leaves (Ferreira et al., 1998).

To keep alkaloids trapped within the vacuoles, some researchers have proposed that they are complexed with polyphenols. The most intensively studied interaction to date is that of the purine alkaloid caffeine with chlorogenic acid (**3**) (caffeoyl quinate) (Baumann et al., 1991; Waldhauser and Baumann, 1996; Zeller and Saleeb, 1997). In addition, hydroxycinnamoyl quinate esters have also been implicated in complex formation with other purine alkaloids (Baumann et al., 1991; Wink and Roberts, 1998).

Chlorogenic acid (**3**) is the most abundant hydroxycinnamoyl quinate ester found in plants (Sondheimer et al., 1961). Found in high concentrations in many plant species, it acts as a defense

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against pathogens as well as serving as a UV protectant (Rhodes and Wooltorton, 1976; Sander and Petersen, 2011). Chlorogenic acid (**3**) and other hydroxycinnamoyl quinate esters are found in the vacuoles and apoplasts where they aid peroxidases in hydrogen peroxide scavenging (Sakihama et al., 2002; Takahama et al., 1999). These substances are also thought to participate in lignin biosynthesis by serving as a source of caffeoyl and coumaroyl subunits that can be converted to one of the three phenylpropanoids that comprise the lignin biopolymer (Chabannes et al., 2001; Riboulet et al., 2009).

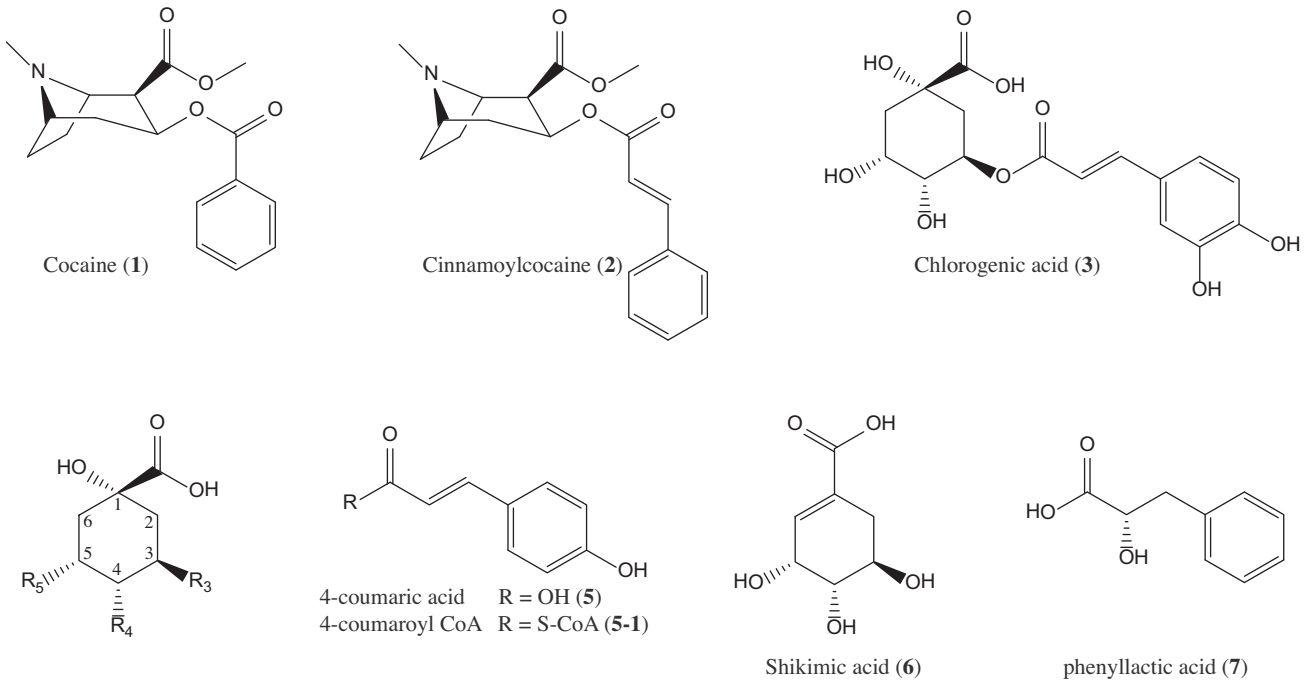
Chlorogenic acid (**3**) may be directly synthesized by the acylation of quinic acid (**4**) with caffeoyl-CoA. An indirect route proceeds via 4-coumaroyl quinate (**4-2**) ester which subsequently becomes hydroxylated via the action of a P450 monooxygenase (Lotfy et al., 1992; Niggeweg et al., 2004; Ulbrich and Zenk, 1979). In the direct route, the acylation reaction can be catalyzed by a hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase (HQT) (Ulbrich and Zenk, 1979). This type of acyltransferase belongs to the BAHD superfamily, a class of enzymes involved in the modification of a large variety of plant secondary metabolites (D'Auria, 2006).

In this study, the role of polyphenolic compounds in mediating the storage of tropane alkaloids in *E. coca* was investigated. First, a close correlation between tropane alkaloid and hydroxycinnamoyl quinate ester levels was established in various organs of the plant. Next, the BAHD acyltransferase responsible for producing hydroxycinnamoyl quinate esters was characterized and expression localized for the corresponding gene. Finally, experiments were conducted to demonstrate the association of tropane alkaloids and hydroxycinnamoyl quinate esters in vitro which suggests the likelihood of complexation in vivo.

2. Results and discussion

2.1. Content of tropane alkaloids and hydroxycinnamoyl quinate esters are correlated throughout leaf and organ development in coca

There are two major tropane alkaloids in coca (*E. coca*), cocaine (**1**) and cinnamoylcocaine (**2**). Previous work showed that these are present together in high concentrations in leaves ranging from 0.5% of dry weight in mature leaves to over 10% of dry weight in the youngest leaves (Docimo et al., 2012). Any compound that is involved in the complexation of these alkaloids in storage should be present in comparably high concentrations in the same organs and growth stages. Since alkaloids have been previously reported to be complexed with polyphenolic compounds, several developmental stages of *E. coca* leaves were screened, as well as the stems and the roots for the presence of polyphenolic compounds using LC–MS. The only compounds that had a significant correlation with cocaine (**1**) and cinnamoylcocaine (**2**) were the hydroxycinnamoyl esters of quinic acid (HQA) (Fig. 2). The major HQA constituents in *E. coca* were found to be chlorogenic acid (**3**) (caffeoyl quinate) and 4-coumaroyl quinate (**4-3**) which contribute 63% and 18% of the total HQAs present in stage one (young rolled) leaves, respectively. Similar to previous reports, the highest levels of cocaine (**1**) and cinnamoylcocaine (**2**) were found in the youngest leaves (stage 1) with an average quantity of 349 μmol g^{-1} dry wt (Docimo et al., 2012). The total amounts of HQAs in the youngest leaves were 243 μmol g^{-1} dry wt. In mature (stage 3) leaves, the average quantity of tropane alkaloids dropped to 50 μmol g^{-1} dry wt while the total HQAs were down to 27 μmol g^{-1} dry wt with the ratio of 4-coumaroyl quinate (**4-3**) to chlorogenic acid (**3**) at an average of 2:1. The roots, which do not contain tropane alkaloids at all, were also devoid of HQAs. Such close correlation of alkaloid and



Name	R_3	R_4	R_5	Number
Quinic Acid	OH	OH	OH	(**4**)
3-*O*-4-coumaroylquinic acid	(**5**)	OH	OH	(**4-1**)
4-*O*-4-coumaroylquinic acid	OH	(**5**)	OH	(**4-2**)
5-*O*-4-coumaroylquinic acid	OH	OH	(**5**)	(**4-3**)

Fig. 1. Structures of the main compounds utilized in this study.

hydroxycinnamate ester content has also been reported for both *Coffea arabica* (coffee) and *Atropa acuminata* plants (Aerts and Baumann, 1994; Harborne and Khan, 1993). In coffee plants, the correlation of alkaloids and HQAs can also be observed in the roots.

2.2. Isolation and characterization of *E. coca* hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase (HQT)

Since tropane alkaloid levels correlate with HQA content in *E. coca* plants, the enzyme(s) responsible for HQA biosynthesis were examined. Several plant HQT enzymes have been previously isolated and characterized, and all shown to belong to the BAHD class of acyltransferases (Lepelley et al., 2007; Lotfy et al., 1992; Niggeweg et al., 2004). Since the highest levels of HQAs were found in the young leaves, an *E. coca* young leaf λZAPII cDNA library was utilized to screen for candidate hydroxycinnamoyl quinate transferase enzymes. Out of the original 8000 sequences obtained from the library, 6 BAHD-like sequences were identified based upon their gene ontology and similarity with other previously identified BAHD acyltransferases.

Although it has proven to be very difficult to assign function of BAHD acyltransferases based on primary structure alone, the phylogenetic placement of related sequences provide hints as to their possible biochemical activities. All six EcBAHD protein sequences were thus aligned with 56 previously characterized BAHDs from other plant species using the ClustalX program. The corresponding alignment was subjected to Bayesian analysis which resulted in a phylogenetic tree (Fig. 3). Of the six EcBAHD candidates, only EcBAHD3 and EcBAHD4 cluster within the vicinity of several other biochemically characterized hydroxycinnamoyl transferases which utilize either shikimate and/ or quinate as the acyl acceptor. The most closely related sequences include enzymes from *Cynara cardunculus*, *Nicotiana tabacum* and *Coffea canephora* with amino acid identities of 60%, 59% and 58% respectively.

To determine which if any of the six *EcBAHD* candidate genes encoded an active hydroxycinnamoyl quinate transferase, the open reading frames of all six genes were heterologously expressed in *Escherichia coli* BL21 cells, and the recombinant proteins purified using Ni-chelate chromatography and subjected to enzyme assay. Only one of the candidates, EcBAHD4, was capable of converting 4-coumaroyl Coenzyme A (**5-1**) and quinic acid (**4**) to form the product 4-coumaroyl quinic acid (**4-3**) (Fig. 4), and was named EcHQT (hydroxycinnamoyl quinate transferase). A total of three peaks corresponding to the mass of 4-coumaroyl quinic acid ($[M-H]^-$: *m*/*z* 337) were visible in the assays with EcHQT. Using LC–MS^n techniques (Clifford et al., 2003, 2008), it was possible to ascertain that the minor peak 1 at a retention time of 10.4 min was the 3-*O*-ester of trans-4-coumaroyl quinic acid (**4-1**) (MS^2 of *m*/*z* 337: base peak *m*/*z* 163) and the large peak at a retention time of 14.0 min (peak 2 in Fig. 3) contained a mixture of both 5-*O* (**4-3**) (MS^2 of *m*/*z* 337: base peak *m*/*z* 191) and 4-*O* (**4-2**) (MS^2 of *m*/*z* 337: base peak *m*/*z* 173) esters of trans 4-coumaroyl quinic acid respectively. Peak 3 (retention time 15.5 min) was determined to be the 5-*O*-ester (MS^2 of *m*/*z* 337: base peak *m*/*z* 191) of cis-4-coumaroyl quinic acid (Clifford et al., 2008). None of the other EcBAHD proteins could carry out this reaction including EcBAHD3 (Fig. 4) which shares 48% identity with EcBAHD4 (EcHQT) on the amino acid level.

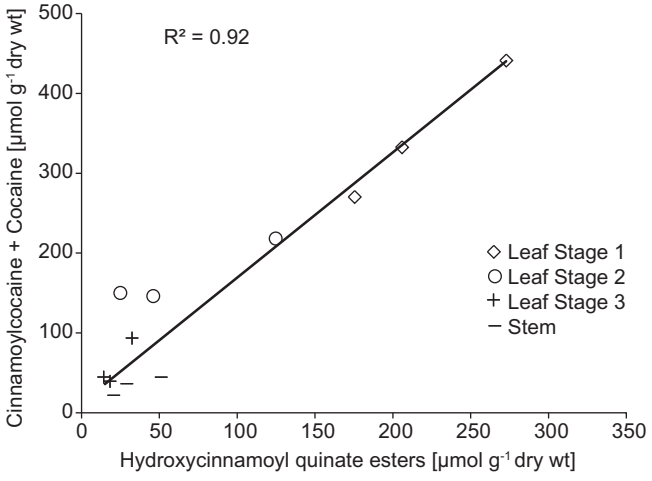


Fig. 2. Correlation of tropane alkaloid content in *E. coca* with that of hydroxycinnamoyl quinate esters (HQAs) in different organs and developmental stages. The two major tropane alkaloids are cocaine and cinnamoylcocaine (**2**), while the two major HQAs are caffeoyl quinate (**3**) (chlorogenic acid) and 4-coumaroyl quinate (**4-2**). Leaf stage 1 (◇) represents the youngest leaves which are still rolled. Leaf stage 2 (○) includes slightly older leaves that are almost completely unrolled but still expanding. Leaf stage 3 (+) are mature and fully expanded. Stems are represented by (–). Each dot represents one biological replicate. The relationship between tropane alkaloids and HQAs is linear with an r^2 of 0.92 and a $P < 0.001$.

In order to understand how EcHQT contributes to HQA levels and a possible interaction between HQAs and tropane alkaloids in coca, its biochemical properties were further investigated using larger amounts of active enzyme obtained by heterologous expression in the yeast *Pichia pastoris*. To facilitate purification, the EcHQT recombinant protein used for characterization experiments contained an extra 8 amino acids at the N-terminus corresponding to the introduction of a Strep II tag. The pH optimum of the EcHQT enzyme using phosphate buffer was found to be pH 7.5 with activity reduced to 50% and 57% at pH 5.7 and 8.3, respectively. The use of bis-tris propane and Tris–HCl buffers at pH 7.5 reduced the activity to 78% and 50%, respectively, compared to that with phosphate buffer at pH 7.5. Such a reduction of activity in Tris based buffers has been reported for other HQT enzymes (Lotfy et al., 1992; Ulbrich and Zenk, 1979). The addition of various monovalent or divalent metal ions did not enhance the activity significantly, but Cu^{2+} as well as Fe^{2+} almost completely inhibited the enzyme activity at 5 mM. Zn^{2+} also inhibited the activity to 78% of the maximal activity when used at a concentration of 5 mM. To assess temperature stability, EcHQT was incubated for 30 min at 4, 10, 25, 35, 45 and 55 °C before being added to the enzyme assay. The enzyme was stable at 4 and 10 °C, but activity was reduced to 65% and 31% of maximal activity when incubated at 25 and 35 °C, respectively. No activity was observed after incubation at higher temperatures.

Since *E. coca* contains a mixture of hydroxycinnamate quinate esters, the ability of EcHQT to utilize a wide range of CoA thioesters as well as acyl acceptors was investigated. When quinic acid (**4**) is substituted with shikimic (**6**) or phenyllactic (**7**) acid, the relative activities with 4-coumaroyl-CoA (**5-1**) are reduced to 3.8% and 0.7%, respectively (Table 1). Substitution of 4-coumaroyl-CoA (**5-1**) with caffeoyl-CoA led to a reduction in relative activity to 22%. No activity was detected using acetyl-CoA, cinnamoyl-CoA, hexanoyl-CoA or malonyl-CoA. Kinetic parameters were determined for the best substrates, quinic acid (**4**) and 4-coumaroyl-CoA (**5-1**) (Table 2). The K_m value for quinic acid (**4**) was 537.4 µM with a turnover number of 1.69 s^{-1}. For 4-coumaroyl-CoA (**5-1**), substrate inhibition was observed with an inhibition constant of 3.28 mM. The K_m value for 4-coumaroyl-CoA (**5-1**) was 18.5 µM with a turnover number of 1.73 s^{-1}.

Previously characterized HQT enzymes show similar kinetic traits (Table 2). For example, HQT from *N. tabacum* has a K_m value of 727 µM for quinate (**4**) using 4-coumaroyl-CoA (**5-1**) as a substrate (Niggeweg et al., 2004). Other HQT enzymes biochemically characterized include those from *C. cardunculus* and *Arabidopsis thaliana* which have similar substrate affinities to those reported here for EcHQT (Hoffmann et al., 2003; Sonnante et al., 2010). It

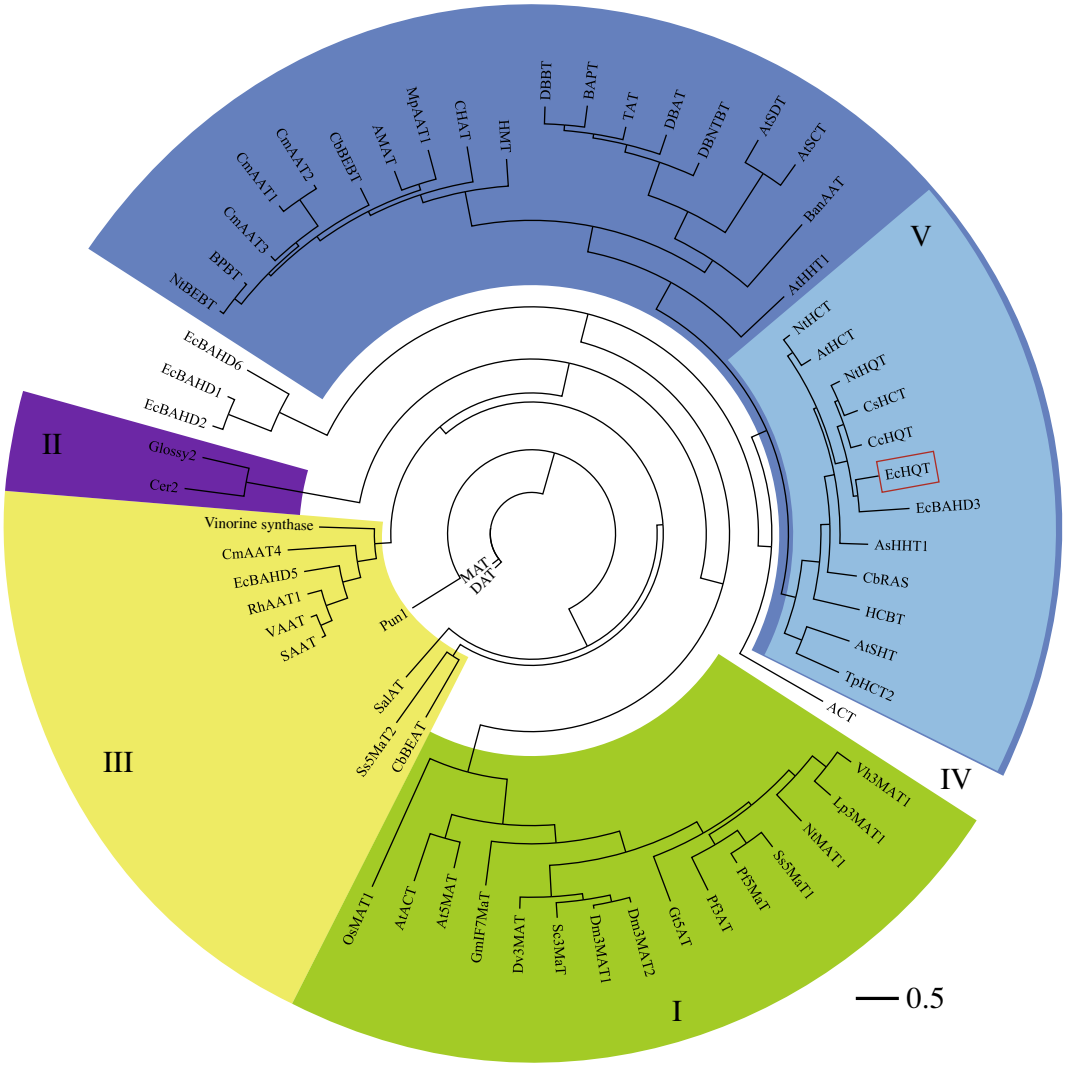


Fig. 3. A phylogenetic tree of the BAHD family of acyltransferases updated from D'Auria (2006) illustrating the affinities of the six *E. coca* sequences isolated in this study. Enzymes included in this tree have been functionally characterized by either genetic mutant screening or biochemical assay (accession numbers are listed in Section 4.6 and in more detail in Supplementary Table 1). Two of the *E. coca* sequences, EcBAHD3 and EcBAHD4 (later characterized as EcHQT and highlighted in red) cluster within the vicinity of other hydroxycinnamoyl transferases. The color scheme used represents clades I through V according to the scheme reported by D'Auria (2006) with the addition of a lighter shade in clade V representing HQT-like enzymes. The distance bar represents the number of amino acid changes per site.

is not possible to predict whether an HQT-like enzyme is capable of using either shikimate (**6**) or quinate (**4**) based on sequence similarity alone. In fact, in some plant species separate enzymes carry out the two reactions separately, while in other plants the reactions can be catalyzed by the same enzyme (Petersen et al., 2009). In coca however, no shikimate esters have been found to date. Additionally, the relative activity of EcHQT with shikimate (**6**) suggests that this enzyme is most likely a true HQT.

Substrate inhibition via hydroxycinnamoyl-CoA has been reported once before for a member of the BAHD family, a rosmarinic acid synthase from *Coleus blumei* (Sander and Petersen, 2011). This property may play a role in regulating the amount and proportions of HQAs in plants, as well as the rate of lignin formation and its composition, since HQAs are associated with lignin deposition and their biosynthesis consumes hydroxycinnamoyl-CoAs which are intermediates to the major lignin monomers. Measurements of the internal pool sizes of hydroxycinnamoyl-CoA esters in different organs and tissues should help to understand the significance of substrate inhibition. Although three possible pathways for the biosynthesis of chlorogenic acid (**3**) have been proposed, the substrate specificity of *E. coca* HQT supports the idea that it is synthesized via a coumaroyl quinic acid intermediate in this species (Niggeweg et al., 2004; Stöckigt and Zenk, 1974; Ulbrich and Zenk, 1979).

2.3. EcHQT gene expression

Given the possible role of HQAs in tropane alkaloid complexation, the localization of *EcHQT* transcript in *E. coca* organs was investigated (Fig. 5). The highest *EcHQT* mRNA levels were found in stage 1 leaves with a 13-fold higher expression compared to stage 3 leaves. This pattern correlated well with metabolite analysis in which hydroxycinnamate quinate esters in the stage 1 leaves were found to be approximately 10 times more abundant than in stage 3 leaves. A close correlation between *EcHQT* transcripts and HQA metabolite levels was not seen in stems. Maturing stems are known to be active in lignin deposition (Grabber, 2005) which consumes large amounts of chlorogenic acid (**3**) and its precursor 4-coumaroyl quinate (**4-3**). Neither HQA metabolites nor *EcHQT* transcripts could be detected in coca root tissue, which coincides with the absence of cocaine and other tropane alkaloids in coca roots. Interestingly, plants producing and storing alkaloids in their

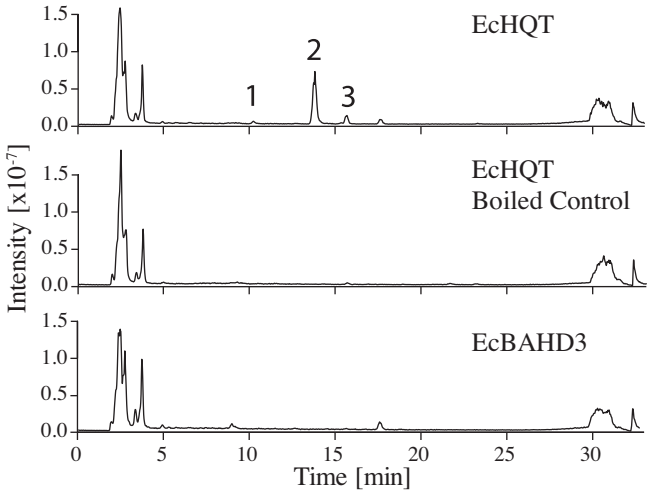


Fig. 4. HPLC-MS analysis of acyltransferase assays with recombinant protein using coumaroyl-CoA and quinic acid as substrates. EcHQT (EcBAHD4) was demonstrated to catalyze this reaction, but assays of boiled EcHQT and EcBAHD3 protein showed no activity. Total ion chromatograms obtained in the negative mode are depicted. Peak 1: 3-*O*-ester of trans-4-coumaroyl quinic acid (**4-1**), peak 2: a mixture of both 5-*O* and 4-*O* esters of trans 4-coumaroyl quinic acid (**4-2**) and (**4-3**), peak 3: 5-*O*-ester of cis-4-coumaroyl quinic acid.

Table 1
Catalytic activity of EcHQT with various substrates relative to activity with quinic acid and 4-coumaroyl-CoA, which had a specific activity of 8.54 nkat (mg protein)$^{-1}$.

Substrate 1	Substrate 2	Relative activity (%)
Quinic acid (**4**)	4-Coumaroyl-CoA (**5-1**)	100
Shikimic acid (**6**)	4-Coumaroyl-CoA (**5-1**)	3.8
Phenyllactic acid (**7**)	4-Coumaroyl-CoA (**5-1**)	0.7
Quinic acid (**4**)	Acetyl-CoA	0
Quinic acid (**4**)	Cinnamoyl-CoA	0
Quinic acid (**4**)	Caffeoyl-CoA	22
Quinic acid (**4**)	Hexanoyl-CoA	0
Quinic acid (**4**)	Malonyl-CoA	0

root tissues, such as *N. tabacum* and *C. arabica*, are reported to accumulate both HQAs and transcripts encoding their respective hydroxycinnamoyl transferases in the roots (Lepelley et al., 2007; Niggeweg et al., 2004).

2.4. UV–Vis and NMR spectral studies demonstrate the interaction of cocaine and chlorogenic acid

Earlier work had reported evidence for a physical interaction between caffeine and chlorogenic acid (**3**) which was thought to be important for the storage of caffeine in the vacuole (Kappeler et al., 1987). A similar approach was used herein in *E. coca* to look for a physical interaction between cocaine (**1**) and chlorogenic acid (**3**) starting by measuring the UV spectrum of chlorogenic acid (**3**) when incubated in the presence of cocaine (**1**). Fig. 6A shows the absorbance shift induced by incubating both cocaine (**1**) and chlorogenic acid (**3**) together. To understand which function on the cocaine (**1**) molecule has the greatest ability to interact with chlorogenic acid (**3**), tropine and benzoic acid were tested separately. While the addition of tropine has a negligible effect on the spectrum of chlorogenic acid (**3**) (Fig. 6B), the addition of benzoic acid induces a strong absorbance shift (Fig. 6C). These results strongly suggest that the interaction between cocaine (**1**) and chlorogenic acid (**3**) is based on the benzoic acid moiety of cocaine (**1**).

Table 2
Kinetic parameters of EcHQT and related enzymes from *Nicotiana tabacum* and *Cynara cardunculus*. The substrate used in a constant concentration is indicated in parentheses.

	K_M (μM)	k_{cat} (s^{-1})	k_{cat}/K_M ($\mu M^{-1}\ s^{-1}$)	K_I (mM)
EcHQT				
Quinic acid (**4**) (4-coumaroyl-CoA)	537.4 ± 122	1.69	0.003	–
4-Coumaroyl-CoA (**5-1**) (quinic acid)	18.5 ± 1.9	1.73	0.093	3.28 ± 1.01
NtHQT				
Quinic acid (**4**) (4-coumaroyl-CoA)	727 ± 150	4.62[a]	0.006[a]	–
CcHQT1				
Quinic acid (**4**) (4-coumaroyl-CoA)	216 ± 76	16.93[a]	0.078[a]	–
4-Coumaroyl-CoA (**5-1**) (quinic acid)	1034 ± 77	11.68[a]	0.011[a]	–
CcHQT2				
Quinic acid (**4**) (4-coumaroyl-CoA)	59 ± 4	21.82[a]	0.370[a]	–
4-Coumaroyl-CoA (**5-1**) (quinic acid)	1065 ± 70	5.73[a]	0.005[a]	–

[a] Calculated from data in the original publications: NtHQT (Niggeweg et al., 2004), CcHQT1 and CcHQT2 (Sonnante et al., 2010).

By measuring the spectral shift of chlorogenic acid (**3**) with increasing concentrations of cocaine (**1**), it is possible to calculate the strength and stoichiometry of the interaction from the Beer–Lambert Law and the equilibrium constant (Kappeler et al., 1987; Sondheimer et al., 1961). Using six different concentrations of cocaine (**1**) and a wavelength of 364 nm, the molar absorptivity (the molar extinction coefficient, ε_C) was calculated and plotted vs. the inverse of the equilibrium constant (K_C^{-1}) (Fig. 7B). Based on the mean of the intersection points, the equilibrium constant and the molar absorptivity were determined to be 9.1 ± 1.4 mol L^{-1} and 1901 ± 87 L mol^{-1} cm^{-1}, respectively. The identification of 15 valid intersection points suggests that the model used is accurate and that cocaine (**1**) and chlorogenic acid (**3**) associate in a 1:1 ratio (Fig. 7B). The equilibrium constant determined previously for the caffeine-chlorogenate complex, which was also shown to associate

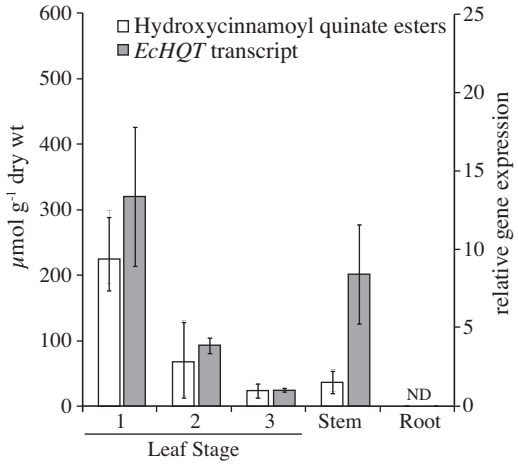


Fig. 5. Transcript levels of *EcHQT* correlate with levels of hydroxycinnamoyl quinate esters in most organs of four month old *E. coca* plants. The values represent the average of three biological replicates. Transcript measurements are normalized to leaf stage 3. Error bars represent standard deviations. Neither *EcHQT* transcript nor hydroxycinnamoyl quinate esters were detected in the roots.

in a 1:1 ratio, was 47.3 ± 9.5 L mol^{-1} (Kappeler et al., 1987). This corresponds to a ΔG value for the association reaction of −9.56 kJ mol^{-1} for the caffeine-chlorogenate complex and −5.47 kJ mol^{-1} for the cocaine-chlorogenate complex.

Further evidence for the interaction between cocaine (**1**) and chlorogenic acid (**3**) was obtained from ^{1}H NMR experiments. Chemical shift differences ($\Delta\delta$) were measured in the proton spectrum of chlorogenic acid (**3**) in the presence of excess amounts of cocaine (**1**) and in the proton spectrum of cocaine (**1**) in the presence of excess amounts of chlorogenic acid (**3**) (Fig. 8). ^{1}H,^{1}H COSY experiments were performed to accurately assign all of the overlapping signals (see Supplemental Fig. 1). The highest $\Delta\delta$ values were generally observed for those protons near to or attached to the aromatic rings of both molecules. However, the two highest shifts corresponded to protons at C2 (80.4 Hz) and C3 (85.3 Hz) of the tropane ring. All chemical shift differences detected were towards higher field, which means greater nuclear shielding caused by increased magnetic anisotropy due to the induced field from the overlap of the π orbitals of the two compounds, as proposed for the caffeine-chlorogenate complex (D'Amelio et al., 2009; Horman and Viani, 1972). The structural similarity of 4-coumaroyl quinate (**4-3**) to chlorogenic acid (**3**) should allow the complexation of 4-coumaroyl quinate (**4-3**) with cocaine (**1**) in a corresponding manner, especially since the hydroxyl groups responsible for the difference between the two HQAs were not found to be significantly involved in these interactions. Similarly, complexation between cinnamoylcocaine (**2**) and chlorogenic acid (**3**) would also be likely.

3. Conclusions

In the present study, three lines of evidence were obtained suggesting that hydroxycinnamate esters of quinate complex with tropane alkaloids in *E. coca* and thus facilitate storage in vacuoles. First, there was a strong correlation between the content of the hydroxycinnamate quinate esters (HQAs), chlorogenic acid (**3**) and 4-coumaroyl quinate (**4-3**), and the tropane alkaloids, cocaine (**1**) and cinnamoylcocaine (**2**), in various organs of *E. coca*. In addition, in vitro complexation of chlorogenic acid and cocaine was demonstrated independently by shifts in UV absorption maxima and ^{1}H NMR signals. The enzyme responsible for quinate ester formation from hydroxycinnamoyl-CoA and quinic acid, EcHQT, was also isolated and shown to belong to the BAHD family of acyltransferases (D'Auria, 2006). The expression of the gene encoding EcHQT was found to correlate with tropane alkaloid accumulation.

The biological roles of HQA-tropane alkaloid complex formation remain to be ascertained. However, previous work on coffee plants indicated that complexation between chlorogenic acid (**3**) and caffeine increases the apparent solubility of caffeine (Zeller and Saleeb, 1997). The highest levels of caffeine in coffee plants are approximately 2% dry weight in the cotyledons (Zheng and Ashihara, 2004). Since the levels of tropane alkaloids in *E. coca* can be even higher, over 10% dry weight, a similar mechanism may be required to achieve vacuolar storage. The formation of a complex between tropane alkaloids and HQAs may aid in vacuolar storage by withdrawing alkaloids from the transport equilibrium. Another role for complex formation may be to prevent the leakage of tropane alkaloids from the vacuole by changing their partition coefficients in a similar fashion to what was shown in studies using caffeine (Waldhauser and Baumann, 1996). In fact, the authors of this study suggest that complex formation may be the driving force behind transporting purine alkaloids into the vacuole. Testing the biological importance of HQA-tropane alkaloid complexation in vivo will require independent manipulation of tropane alkaloid or HQA content.

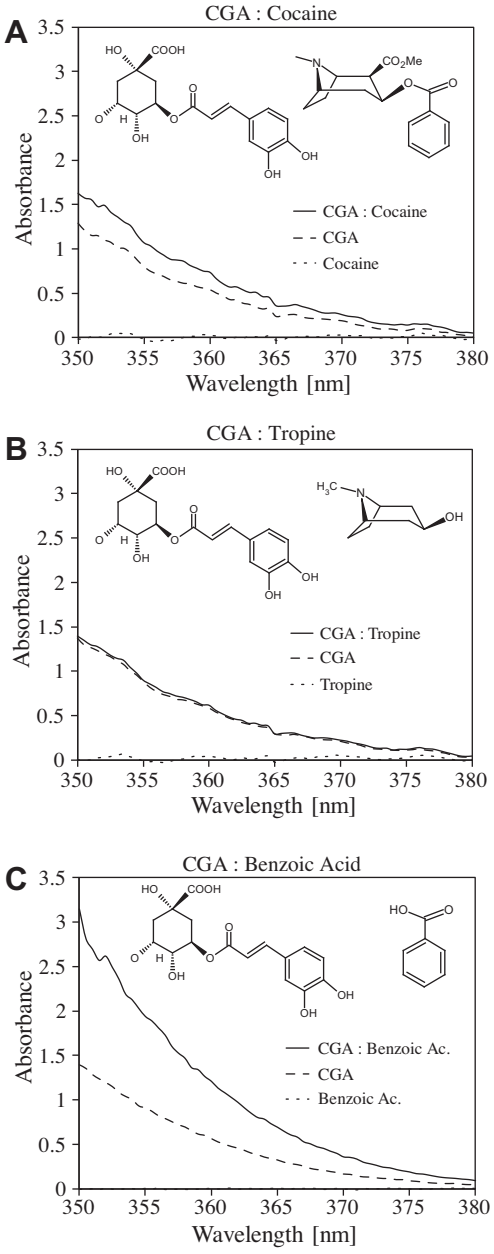


Fig. 6. UV–Vis spectrophotometry: absorbance shift measurements in the spectrum of chlorogenic acid (0.2 mM) in the presence of (A) cocaine (**1**) (4 mM), (B) tropine (4 mM) and (C) benzoic acid (4 mM). Solid lines represent measurements obtained from mixed samples, whereas dotted and dashed lines represent single compound spectra. No absorbance shift was detected when chlorogenic acid was incubated with tropine.

4. Experimental

4.1. General experimental procedures

LC–MS was performed using a HPLC 1100 series chromatograph system (Agilent Technologies, Böblingen, Germany) equipped with a diode array detector and coupled to an Esquire 6000 ESI-Ion electrospray ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany) for cocaine (**1**) and chlorogenic acid derivatives. For the EcHQT assay, an API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbo spray ion source was used. ^{1}H NMR spectra were measured on a DRX 500 NMR spectrometer (Bruker-BioSpin, Rheinstetten, Germany) equipped with an inverse probe (5 mm).

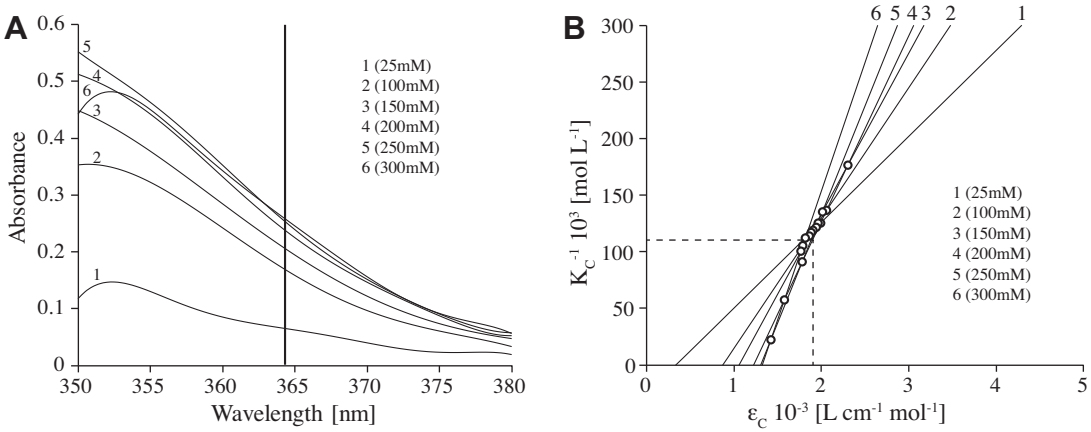


Fig. 7. (A) Bathochromic shift measurements in the spectrum of chlorogenic acid (**3**) (0.2 mM) in the presence of increasing concentrations of cocaine (**1**) (lines 1–6). Quantification of the interaction between cocaine (**1**) and chlorogenic acid (**3**) was performed at 364 nm, represented by the vertical line in panel A. (B) Curve molar absorptivity (ε_C) vs. inverse equilibrium constant (K_C^{-1}) plots. The data was based on the values recorded from panel A (lines 1–6). ε_C and K_C were calculated from the mean of the intersection points (○) of lines 1 through 6 using the equations described by Kappeler et al. (1987). ε_C and K_C were determined to be 1901 ± 87 L mol^{-1} cm^{-1} and 9.1 ± 1.4 L mol^{-1}, respectively (dotted lines).

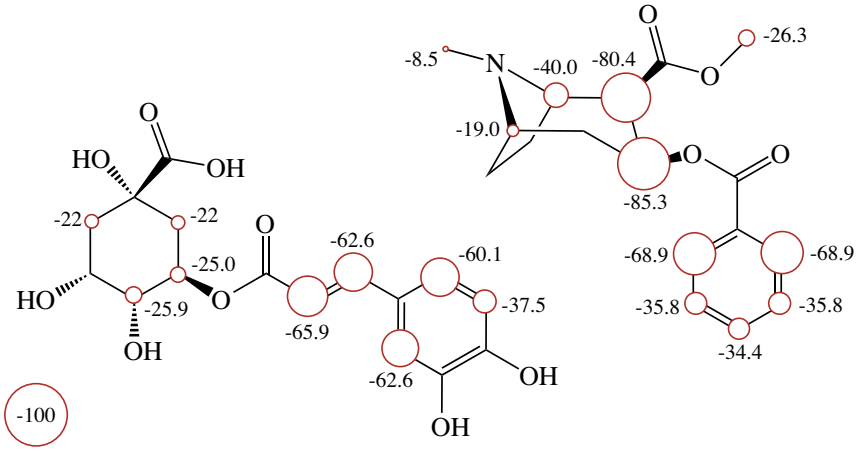


Fig. 8. Complex formation between chlorogenic acid (**3**) and cocaine (**1**) is indicated by chemical shift differences ($\Delta\delta$) shifts observed in the ^{1}H NMR spectra of each molecule in the presence of the other as compared to a reference compound measured in pure form (left structure: chlorogenic acid (**3**); right structure: cocaine (**1**)). Proton shifts are represented by circles at the attached carbon atom that differ in size according to the magnitude of the shift. The exact values are shown in Hz next to the circle. All $\Delta\delta$ values are given in Hz and are negative because the shifts were always towards high field (lower δ values). Some protons could not be assigned due to overlap. A circle representing 100 Hz is included for scale.

4.2. Plant growth conditions and extraction

E. coca plants were grown as reported previously (Docimo et al., 2012). Leaves, roots and stems from *E. coca* plants were frozen in N_2, ground and extracted in 1 mL of 5% MeOH, 0.1% HCO_2H in H_2O.

4.3. Chromatographic analysis of plant extracts

Qualitative LC–MS analysis was carried out on a Agilent 1100 HPLC (Agilent Technologies, Böblingen, Germany) equipped with a Nucleodur Sphinx RP 5 µm column (25 cm × 4.6 mm, 5 µm, Macherey–Nagel, Düren, Germany) coupled to an Esquire 6000 mass spectrometer, operated in alternating ion polarity mode in the range m/z 60–1000 with a target mass m/z 300; nebulizer pressure, 40 psi; drying gas flow, 11 L/min; gas temperature, 330 °C; capillary voltage, 113.5 V). The mobile phase gradient used consisted of 0.2% HCO_2H (A) and CH_3CN (B) at a flow rate of 1 mL/min as follows: 90–69% A (21 min), 69–45% A (6 min), 45–0% A (0.1 min), 0–90% A (2 min), 90% A (3.9 min), the injection volume was 20 µL. Quantitative chemical analysis of plant tissue extracts was carried out on a Agilent 1100 HPLC with diode array detector (Agilent Technologies, Böblingen, Germany) with UV detection at 235, 280 and 320 nm for cocaine (**1**), cinnamoylcocaine and HQAs, respectively. The column and chromatographic conditions were the same as in the qualitative analysis, using 0.05% CF_3CO_2H instead of HCO_2H, and an injection volume of 40 µL. Compounds were quantified through external standard calibration curves recorded with authentic standards for cocaine and chlorogenic acid. 4-Coumaroyl quinate (**4**-(**1-3**)) was quantified using chlorogenic acid (**3**) as an external standard using a molar response factor of 0.79, which was experimentally determined as follows: standard curves were created for caffeic acid and chlorogenic acid (**3**) on a molar basis at 320 nm resulting in the same slope for both regression lines. Thus it was concluded that a response factor of 1 can be used between the hydroxycinnamic acid and its quinate ester. In a second step, standard curves on a molar basis were created for caffeic acid and 4-coumaric acid (**5**) at 320 nm. The slopes for the two compounds were different (slope

caffeic acid: 4523, slope 4-coumaric acid: 5736), resulting in a response factor of 0.79. As the response factor between the hydroxycinnamic acid and its quinate ester is 1, it was deduced a response factor at 320 nm of 0.79 for 4-coumaroylquinic acid (**4-**(**1-3**)) in relation to chlorogenic acid (**3**). Cinnamoylcocaine (**2**) was quantified using cinnamic acid as external standard using a molar response factor of 1.

4.4. Chemicals

4-Coumaroyl-CoA (**5-1**) was synthesized as previously described (Wang et al., 2000), with the following exceptions. The activated succinimide ester of 4-coumaric acid was first purified using preparative TLC, employing $CHCl_3$/MeOH (20:1) as the mobile phase before proceeding to the reaction with Coenzyme A. Following synthesis, the 4-coumaroyl-CoA (**5-1**) was purified using solid-phase extraction cartridges (500 mg Chromabond C 18 ec, Macherey–Nagel) preconditioned with consecutive washes of methanol, dH_2O, and 20 mM (NH_4)OAc solution (5 column vol each). After evaporation of the organic solvent, (NH_4)OAc was added to the water phase to a final concentration of 20 mM, and the mixture was loaded onto the SPE cartridge. The column was rinsed with 5 column volumes of 20 mM (NH_4)OAc solution, to elute unreacted Coenzyme A. The 4-coumaroyl-CoA (**5-1**) was recovered by elution with distilled H_2O. Pure fractions containing only 4-coumaroyl-CoA (**5-1**) were identified by HPLC-MS and HPLC-UV, and lyophilized overnight.

Quinic acid (**4**) was purchased from Alfa Aesar (Karlsruhe, Germany). The rest of the chemicals and solvents were purchased from Sigma–Aldrich (Munich, Germany), Carl Roth (Karlsruhe, Germany), Merck (Darmstadt, Germany) or VWR (Leuven, Belgium).

4.5. Identification of EcHQT

Six sequences from an *E. coca* young leaf cDNA library (Docimo et al., 2012), identified as putative BAHD acyltransferases genes, were cloned into the Gateway expression vector system and expressed in *E. coli* BL21 (DE3) cells (Invitrogen), using the pH9GW vector, a modified pET-T7 (28a) vector (Merck) with a N-terminal His-Tag which is Gateway compatible (Yu and Liu, 2006). The forward primer TAA TAC GAC TCA CTA TAG GG and the reverse primer CCC AAG GGG TTA TGC TAG TT were used for the cloning. Heterologous expression of the proteins and purification via Ni-chelate chromatography was performed as previously described (D'Auria et al., 2007).

4.6. Phylogenetic analysis of BAHD family members

Analysis of protein sequences (see Supplementary Table 1 for accession numbers) were aligned using ClustalX, and the resulting alignment was submitted to Bayesian analysis using Mr. Bayes v3.1.2 with two MCMC runs each of 10,14,311 generations (Huelsenbeck and Ronquist, 2001). The 50% consensus tree was visualized as an unrooted tree using FigTree v1.3.1 (http://tree.bio.ed.ac.uk/). The following enzymes and their corresponding Genbank accession numbers were used for the analysis (enzyme-accession number): DAT-AAC99311, MAT-AAO13736, CbBEAT-AAC18062, CbBEBT-AAN09796, NtBEBT-AAN09798, CHAT-AAN09797, BPBT-AAU06226, HCBT-CAB06430, SAAT-AAG13130, Vinorine synthase-CAD89104, SalAT-AAK73661, Ss5MaT2-AA R26385, Gt5AT-BAA74428, Dm3MAT2-AAQ63616, Dm3MAT1-AAQ63615, Dv3MAT-AAO12206, Vh3MAT1-AAS77402, Lp3MAT1-AAS77404, Ss5MaT1-AAL50566, Sc3MaT-AAO38058, Pf5MaT-AA L50565, Pf3AT-BAA93475, ACT-AAO73071, NtHCT-CAD47830, AtH-CT-NP_199704, AsHHT1-BAC78633, NtHQT-CAE46932, CmAAT (**1-4**)-CAA94432; AAL77060; AAW51125; AAW51126, Pun1-AAV66311, DBNTBT-AAM75818, BAPT-AAL92459, DBBT-Q9FPW3, TAT-AAF34254, DBAT-AAF27621, Cer2-AAM64817, Glossy2-CAA61258, AMAT-AAW22989, NtMAT1-BAD93691, MpAAT1-AAU14879, HMT/HLT-BAD89275, RhAAT1-AAW31948, VAAT-AF193790_1, BanAAT-AX025506, At5MAT-NM_113880, AtSHT-NP_179497, CsHCT-ACF37072, AtHHT1-NP_851111, Os-MAT1-NP_001046857, TpHCT2-ACI16631, AtACT-NP_200924, AtSDT-NP_179932, AtSCT-AAP81804, GmIF7MaT-NP_001237760, CbRAS-CAK55166, CcHQT-ABO77956, EcBAHD1-JQ413184, EcBAHD2-JQ413185, EcBAHD3-JQ413186, EcHQT-JQ413187, EcBAHD5-JQ413188, and EcBAHD6-JQ413189.

4.7. Expression and purification of EcHQT

The EcHQT open reading frame was ligated into a pPICHOLI vector (MoBiTec, Göttingen, Germany) modified with a Gateway-compatible N-terminal Strep Tag II. Introduction of the plasmid into *P. pastoris* strain KM71 via electroporation was performed according to the pPICHOLI manual. Expression, lysis, purification and SDS–PAGE electrophoresis analysis were performed as previously described (Jirschitzka et al., 2012) with the following exceptions. The forward primer GAC TGG TTC CAA TTG ACA AGC and the reverse primer GCA AAT GGC ATT CTG ACA TCC were used for cloning. The purified fraction of the StrepTrap HP column was run through a Resource Q 1 ml column (GE Healthcare, Freiburg, Germany) and was eluted in 20 column volumes of a NaCl (0–500 mM) linear gradient in Tris–HCl (100 mM), 10% (v/v) glycerol, pH 8.0 buffer.

4.8. Characterization and kinetics of EcHQT, chemical analysis of enzyme assays

Biochemical characterization and determination of the kinetic parameters was achieved as described (D'Auria et al., 2002) with the following exceptions. K_2HPO_4/KH_2PO_4 buffer, bis-tris propane buffer and Tris–HCl buffer were tested in the pH ranges 5.7–8.3, 6.7–9.5 and 7.3–9.1, respectively. The assays were stopped adding HCl (1 M) to pH 1.0 and the analysis was carried out on a Agilent 1200 series equipment (Agilent Technologies, Böblingen, Germany) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a turbo spray ion source, using a ZORBAX RRHT Eclipse XDB-C18 column (5 cm × 4.6 mm, 1.8 μm) (Agilent Technologies, Böblingen, Germany). Injection volume was 5 μL, separation was achieved within 8 min, at 20 °C and a flow rate of 800 μL/min, using HCO_2H 0.05% (A) and CH_3CN (B) as mobile phases as follows: 95% A (0.5 min), 95–40% A (3.5 min), 40–0% A (0.1 min), 0% A (0.9 min), 0–95% A (0.1 min), 95% A (2.9 min); injection volume was 5 μL. The mass spectrometer was operated in negative ionization mode; curtain gas, 25 psi; turbo heater temperature, 700 °C; nebulising gas, 70 psi; heating gas, 60 psi; collision gas, 6 psi; ion spray voltage, −4500 eV. Analytes were monitored by scheduled multiple reaction monitoring (MRM): chlorogenic acid (**3**) m/z 353.13 → 190.88; 4-coumaroyl quinic acid m/z 337.13 → 190.88; declustering potential (DP), −35 V; entry potential (EP), −4 V; collision cell entry potential (CEP), −36 V; collision energy (CE), −22 V; collision cell exit potential (CXP), −4 V. Quantification was based on a standard curve of authentic chlorogenic acid (**3**) (Sigma–Aldrich).

Enzyme assays where quinic acid (**4**) was substituted with shikimic (**6**) or phenyllactic acid (**7**) were analyzed according to Supplemental Table 2, while assays with caffeoyl-CoA, acetyl-CoA, cinnamoyl-CoA, hexanoyl-CoA, and malonyl-CoA were analyzed according to Supplemental Table 3.

4.9. Quantitative real-time PCR analysis

Experiments were performed as described (Docimo et al., 2012). Primers targeting the EcHQT transcript were designed (Fwd: 5′-CG ATGTGGAGGAGTCTGTCTTGG-3′, Rev: 5′-CCGTCTTGTTGTTTGGAGG TTGC-3′) and standard curve analysis showed a PCR efficiency of 77% and R^2 of 0.996. Expression of EcHQT was normalized to gene 6409 (Genbank Accession No. JN020150) and gene 10,131 (Genbank Accession No. JN020153) (Docimo et al., 2012) expression using qBASE v1.3.5 (Hellemans et al., 2007).

4.10. UV and NMR spectroscopy

The UV spectra of cocaine hydrochloride (**1**) (4 mM) and chlorogenic acid (**3**) (0.2 mM) were measured separately as well as together in solution in a Shimadzu UV-2501PC spectrometer (Duisburg, Germany) in quartz cuvettes QS 104.002B-QS (Hellma Analytics, Müllheim, Germany) in the range 350–380 nm. The same procedure was followed using tropine (4 mM) and benzoic acid (4 mM) instead of cocaine (**1**). The equilibrium constant, K_C, and the extinction coefficient (ε_C, molar absorptivity) at a chosen wavelength (364 nm) were obtained as a mean of the intersection points of different ε_C vs K_C^{-1} curves as described (Kappeler et al., 1987).

$$CGA + TA \overset{K_C}{\rightleftharpoons} C \tag{1}$$

$$\frac{1}{K_c} = \frac{[TA]_0[CGA]_0}{A(\lambda)} \cdot \varepsilon_0 - [TA]_0 - [GCA]_0 + \frac{A(\lambda)}{\varepsilon_0} \tag{2}$$

where CGA, chlorogenic acid (**3**); TA, tropane alkaloid (namely cocaine (**1**)); C, complex of both compounds.

Reference spectra of chlorogenic acid (**3**) and cocaine (**1**) (0.2 mm each) were measured as references against the external standard TMSP-d_4 prior to the complexation experiments.

The shift differences ($\Delta\delta$) in the ^{1}H NMR spectrum of chlorogenic acid (**3**) (0.2 mM) in the presence of cocaine (**1**) hydrochloride (50 mM) and vice versa were measured in deuterated phosphate buffer (10 mM, pH 7.5) at 27 °C. Chemical shift differences ($\Delta\delta$) in the ^{1}H NMR spectra were determined by comparing the chemical shifts of the non-complexed reference and the resulting chemical shifts after complexation (see Supplementary Material). Water suppression was achieved using the PURGE sequence as described (Simpson and Brown, 2005).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.09.009.

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