The final steps of cocaine biosynthesis in the Erythroxylaceae provide insight into the biochemistry, physiology and evolution of tropane alkaloid biosynthesis

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1 Introduction

1.1 Early aspects of secondary metabolite research

A vast diversity of chemical compounds is found in nature. Plants in particular are responsible for a large share of this production. Many plant compounds have been used by mankind throughout history for various purposes including their medicinal properties (Schultes et al., 2001). For example, the opium poppy plant is used for its analgesic and euphoric properties. The medicinally active compound in the psychoactive latex of poppy was unknown for centuries until morphine was isolated 200 years ago and its action was investigated by the German apothecary Friedrich Wilhelm Sertuerner (Sertuerner, 1817; Schultes et al., 2001). The isolation of morphine initiated what is now designated as research on plant secondary metabolites (Hartmann, 2007). At the end of the 19th century, the German plant physiologist and later Nobel Prize awardee Albrecht Kossel proposed to focus research efforts on essential molecules which he associated with basic metabolism. He called these substances primary metabolites. Accordingly, the remaining metabolites were considered secondary, but this differentiation was more phenomenological then functional (Kossel, 1891; Jenke-Kodama et al., 2008). Nowadays, secondary metabolites are also referred to as natural products, phytochemicals, defense chemicals, defense metabolites, ecochemicals or specialized metabolites to accent their importance for the plants producing them (Pichersky and Lewinsohn, 2011; Kliebenstein, 2013). The absence of a clear biological function for some of these plant compounds even led to them being classified as by-products or waste-products in the early 20th century (Hartmann, 2007). The waste-product idea was derived from the fact that plants do not seem to produce large amounts of nitrogen-containing wastes like urea and uric acid of mammals. Thus nitrogen-containing plant secondary metabolites, such as alkaloids, were thought to be waste products (Wink, 1985). Since then, increased ecological awareness has shown that secondary metabolites are very important for plant interactions with biotic and abiotic stresses. Plants are sessile organisms and therefore need to cope directly with their environment in order to

deter or poison herbivores and inhibit the growth and development of bacteria, fungi and even viruses (Wink, 2008a). There is also competition for water and nutrients between plants, where secondary metabolites serve as allelopathic mediators. Secondary metabolites are also necessary for attracting pollinators and seed distributing animals and for the interaction between symbiotic bacteria/fungi and their plant hosts (Wink, 2008a). In fact, it was shown that plants with secondary metabolites have a better survival rate in the wild, growing with abiotic and biotic stresses and competitors around, compared to plants without secondary metabolites (Wink, 1985; Brown et al., 2005; Wink, 2008a). Many secondary metabolites in crop plants were lost or altered during thousands of years of domestication (Hyams, 1971). The human selection process for less toxins and better taste resulted in crop plants unable to cope with natural enemies like herbivores and pathogens (Wink, 1988). This is often the reason for the use of synthetic chemicals in food plant protection.

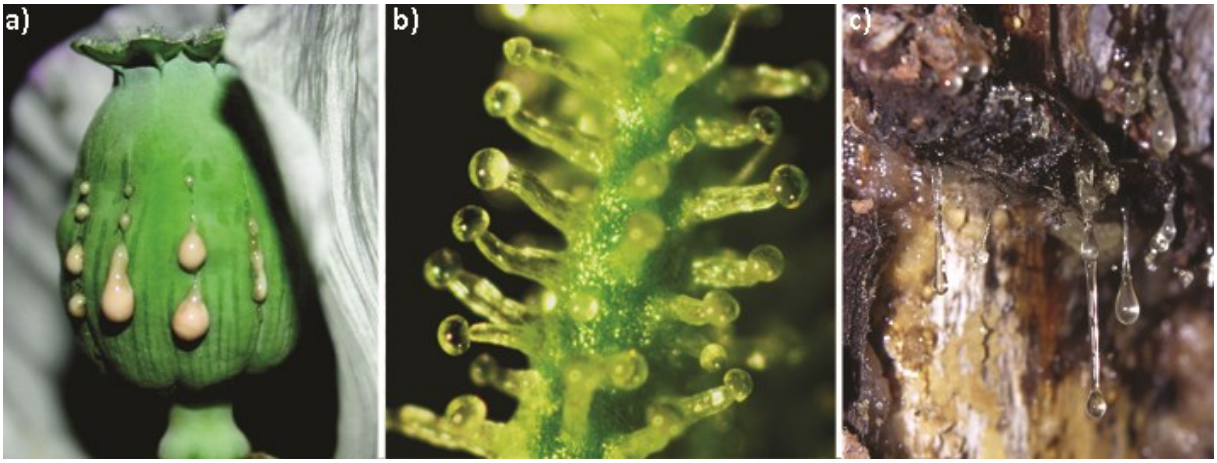

Figure 1 | Secondary metabolism is located in specialized cell types. a) Opium poppy capsule containing morphinan alkaloids from laticifers. **b)** Glandular trichomes of female flower of marijuana synthesize and store cannabinoids. **c)** Resin containing mainly of a mixture of terpenes is secreted from resin ducts of a conifer. Figure is modified from (Schilmiller et al., 2012; Jirschitzka et al., 2013) with permission from Elsevier (license number: 3277200689678 and 3304690200804).

1.2 Groups of secondary metabolites and localization in plants

According to a rough estimate, the diversity of plant secondary metabolites includes around 200,000 compounds (Dixon and Strack, 2003). However, of the approximately 350,000 plant species known to mankind, only 20-30% of these plants have been phytochemically examined in detail (Wink, 2008b). This means that the real number of secondary metabolites is very likely higher (Wink, 2008b; Pichersky and Lewinsohn, 2011). Generally, all these compounds arise from only five sets of precursors (acetyl-CoA, active isoprene units, shikimic acid metabolites, glycolysis products and citric acid cycle metabolites) and can be categorized into two groups: nitrogen containing and non-nitrogen containing secondary metabolites, which are then further sub-grouped (Wink, 2008b). Nitrogen containing secondary metabolites are namely non-protein amino acids (NPAAs), amines, cyanogenic glycosides, glucosinolates, alkylamines, lectins, peptides, polypeptides and alkaloids, with alkaloids the largest group of this category including 21,000 compounds. The group of secondary metabolites which do not contain nitrogen includes flavonoids, tannins, phenylpropanoids, lignin, coumarins, lignans, polyacetylenes, fatty acids, waxes, polyketides, carbohydrates, simple acids and the largest group terpenoides (monoterpenes, sesquiterpenes, diterpenes, triterpenes, steroids, saponins, tetraterpenes) which alone exceeds 22,000 compounds (Wink, 2008b). This wide array of secondary metabolites is necessary because plants must be able to cope with multiple environmental conditions. A specific secondary metabolite may become essential for survival when the appropriate challenge is present like herbivores or pathogens (Kliebenstein, 2013).

In addition to benefits, the biosynthesis of plant secondary metabolites is also associated with energetic costs. Therefore, plants restrict the production of secondary metabolites either in time (only produced in distinct life stages or after herbivore or pathogen challenge) or in space (special sites of synthesis and storage (see **Fig. 1**) (Kliebenstein, 2013). On the cellular level, specialized compartments like the cell wall, vacuole and cuticular wax layer often store secondary metabolites. On the tissue or

organ level, there are specialized storage structures like various types of glandular trichomes which produce and store terpenoids and other potential defense compounds. Terpenoids are also found to be stored in resin ducts. Other specialized structures for the accumulation of secondary metabolites are laticifers and S-cells, which contain high levels of glucosinolates (only in Brassicaceae) (Gershenzon, 1994). Both specialized structures are phloem associated, suggesting that these tissues derived from a vascular developmental module (Koroleva et al., 2010). A similar phloem structure, the extra fascicular phloem, is found in cucurbits only and is involved in defensive compound storage and transport. It seems that all these specialized structures evolved from already existing primary structures in parallel to the evolution of secondary metabolites they harbor (Kliebenstein, 2013).

The enzymes responsible for making these secondary metabolites are often associated with the specialized structures or are restricted to a special tissue within the plants. One of the first secondary metabolism enzymes, shown to be localized in a specific manner, was hyoscyamine 6-β-hydrolase (H6H), involved in tropane alkaloid biosynthesis in Solanaceae. H6H is located in the pericycle of cultured *Hyoscyamus niger* roots (Hashimoto et al., 1991). Another example for secondary metabolite pathway enzymes located in specific structures are the monoterpenoid biosynthetic enzymes found in glandular trichomes in peppermint (Turner and Croteau, 2004). The enzymes involved in benzylisoquinoline alkaloid biosynthesis in poppy plants are localized to different cell types within the laticifer structures (Ziegler and Facchini, 2008). The association of secondary metabolite producing enzymes with special structures or certain tissues has been utilized by researchers in order to identify these enzymes through targeted sequencing approaches. Since the enzyme abundance is higher in these specialized structures or certain tissues, the chance of finding the corresponding gene is also higher. In trichome containing plants like mint, hops, tomato or cannabis, glandular trichomes have been used for the creation of cDNA libraries following gene discovery (Lange et al., 2000; Nagel et al., 2008; Schilmiller et al., 2009; Stout et al., 2012). But not every plant has obvious specialized structures for

secondary metabolite synthesis and storage like trichomes. Therefore, large sequencing projects of medicinal plants like the Medicinal Plant Genomics Resource are using cDNA libraries made from other tissues. Various plant parts or elicitor-treated plants are typically used to cover the most possible expression profiles of genes throughout the plant (http://medicinalplantgenomics.msu.edu).

1.3 Evolution of plant secondary metabolism

Just like primary metabolites, secondary metabolites have explicit stereochemistries and are synthesized by specific enzymes. Often, these specific enzymes have evolved from enzymes already present in the plant (Wink, 2008a). A gene coding a new enzyme for secondary metabolism is most likely to arise from a gene which is already present in the tissue where the secondary metabolite is localized (Pichersky and Gang, 2000). Acetyl-CoA-benzylalcohol acetyltransferase (BEAT) which is involved in floral scent biosynthesis and anthocyanin 5-aromatic acyltransferase (GAT4) are examples of genes involved in secondary metabolism which arose from genes already present in the same tissue (Pichersky and Gang, 2000; St-Pierre and De Luca, 2000). For secondary metabolite genes that have evolved from genes of primary metabolism, the number of potential progenitors are rather limited (Wink, 2008a).

To explain the origins of secondary metabolite pathways, the following scenarios should be considered. One theory is that secondary metabolite pathways are either a recent acquisition of present-day plants or that these secondary metabolite pathways are an old acquisition from the time when the first land plants evolved (Wink, 2008a). If secondary metabolite pathways are an old acquisition, then genes of these pathways should be present in genomes of most if not all present-day plants. This theory can explain the wide distribution of secondary metabolites like flavonoids. However, for the "old acquisition" idea to explain the scattered distribution of alkaloids or the taxonomically restricted presence of glucosinolates, it must be

assumed that the alkaloid biosynthesis pathways and glucosinolate biosynthesis pathways have been lost or somehow silenced in the present-day plants which do not produce them (Wink et al., 2010). Another theory is that plants obtained their secondary metabolite pathway genes from primary metabolism genes through duplication, leaving one copy of the original gene and mutation events altered the gene copy resulting in new functions to the corresponding enzyme (Pichersky, 1990; Ober, 2010). These genes then may have been somehow beneficial for the plant and positive selection occurred. The third possible scenario is that secondary metabolite pathways were acquired from other life forms through transfer of the genes (Wink, 2008a). These life forms may have lived symbiotically with plants. Possible symbiotic candidates are *Actinomycetes* and *Streptomyces*, cyanobacteria and fungi (endophytes, ectomycorrhiza) which are known to produce secondary metabolites. Interestingly, symbiotic relationships between fungi and plants seem instrumental for the colonization of the land (Simon et al., 1993). The oldest plant-fungi symbiosis found to occur can be dated back 400 million years (Remy et al., 1994). To date, approximately 80% of plants live in a symbiotic relationship with another organism (Wink, 2008a). Secondary metabolites are often produced by the symbiont in exchange for nutrients and water. For example, some fungal symbionts produce ergot alkaloids for their host grasses (notably rye) as defensive compounds (Wink, 2008a).

1.3.1 Horizontal gene transfer in plant secondary metabolism

The basic theoretical mechanisms explaining the 500 million-year process of secondary metabolite evolution are referred to as divergent evolution, convergent evolution and horizontal gene transfer (Wink et al., 2010). Horizontal gene transfer (HGT), also termed lateral gene transfer (LGT), describes the observation that genetic material can be transferred in an asexual manner. This can happen within or across species boundaries. For bacteria, such mechanisms are known as transfection (uptake of free DNA), conjugation (plasmid mediated transfer) and transduction (bacterial

virus mediated transfer) (Syvanen, 2012). The exchange of genetic material between distinct organisms is known for bacteria and plants. For example, present-day *Agrobacterium* can insert genetic material through a conjugation based mechanism into plants, illustrating the possibility of contemporary gene transfer (Suzuki et al., 2009). Therefore, in evolutionary terms, an early HGT event is one scenario for the evolution of key enzymes in secondary metabolism in plants (Wink et al., 2010). Bacteria, which also contain secondary metabolite genes, have developed into mitochondria and chloroplasts and genes were transferred over time into the genome located in the nucleus (Bock and Timmis, 2008). This could explain the occurrence of flavonoids, which are one of the most widespread secondary metabolites throughout the plant kingdom (Wink et al., 2010). Two prominent enzymes of the flavonoid pathway, phenylalanine ammonia lyase (PAL) and chalcone synthase (CHS) have been found in spore-forming plants as well as in seed plants (Wink, 2008a). The same is true for terpenoids which are found in mosses and other lineages leading up to and including higher plants. Land plant ancestors should have had the pathways leading to active isoprenes, the basic subunits in terpenoid biosynthesis (Wink, 2008a). An even more ancient HGT event is given by the examples of berberine bridge enzyme (BBE) and strictosidine synthase (STS), involved in specific steps in protoberberine and indole / isoquinoline alkaloid biosynthesis, respectively. For both enzymes (BBE and STS) gene and protein homologues are found distributed in higher plants, but also in bacteria and fungi (BBE only) or animals (STS only) (Wink, 2008a). However, not all organisms which were found to have BBE or STS sequences make the corresponding alkaloids. Protoberberine and indole / isoquinoline alkaloid biosynthesis is restricted to certain angiosperm lineages (Wink, 2008a). If the ancient HGT event hypothesis is true, then the protoberberine and indole / isoquinoline alkaloid biosynthesis pathway or parts of it got lost or deactivated, resulting in the absence of production capabilities for the corresponding alkaloids, but leftover gene sequences (like BBE and STS) are still present in the genome. There are exceptions for HGT as an explanation for the occurrence of secondary metabolites. Phylogenetic comparison of polyketide synthase

type III enzymes (PKS III) (chalcone and stilbene synthase) revealed that bacterial PKS III enzymes are more similar to FabH type β-ketoacyl synthase ancestors in plants and bacteria. But plant FabH type β-ketoacyl synthases and plant PKS III are very distant from each other (Gross et al., 2006). Currently biochemically characterized bacterial PKS III enzymes exhibit higher substrate diversity than plant PKS III and only a subgroup of bacterial PKS III clusters with plant PKS III. Furthermore, PKS III enzymes are found in all major bacterial subgroups, but only minorities of these are plant associated (Gross et al., 2006). Taken all together, an early HGT event should be excluded as possible scenario for the PKS III occurrence in bacteria, fungi and plants. Thus, HGT is not an explanation for the scattered distribution of all plant secondary metabolites, HGT should not be considered first (Jenke-Kodama et al., 2008).

1.3.2 Divergent evolution in plant secondary metabolism

Divergent evolution is the acquisition of new enzymes with new functions from related genes. At least for primary metabolism, this is achieved via gene duplication followed by sequence divergence (Ohno, 1970; Pichersky, 1990). One gene keeps its function and the copy, which is not under any selection pressure, can accumulate mutations until it acquires a new functionality and becomes fixed in the population (Pichersky and Gang, 2000). Short segmental and single gene duplications or duplications of complete chromosomes or genomes are common processes leading to gene duplications (Ober, 2010). Within these duplications, unequal crossing-over and retroposition are the most important mechanisms to explain sequence divergence (Ober, 2010). The new gene becomes divergent from the original gene when it encodes an enzyme performing a different chemical reaction either by using another substrate or forming another product as the originally encoded enzyme (Pichersky and Gang, 2000).

In plant secondary metabolism, there is a wide array of genes that originated from gene duplication followed by diversification of both copies (Ober, 2010). Examples of gene duplication are known from the following enzyme families (Ober, 2010): terpene synthases (Tholl, 2006), cytochrome P450-dependent monooxygenases (Bak et al., 2006), chalcone synthases (Durbin et al., 2000) and glucosidases (Kliebenstein et al., 2005). A more specific example of gene duplication and diversification is putrescine *N*-methyltransferase involved in nicotine and tropane alkaloid biosynthesis, which was derived from spermidine synthase with which it shares common motifs as well as 65% similarity on the amino acid level (Biastoff et al., 2009). Both enzymes use putrescine as a substrate resulting in the production of *N*-methyl putrescine and spermidine, respectively. Another interesting example can be found among the enzymes involved in benzoxazinoid production. Multiple independent duplication events following diversification, especially among the cytochrome P450s, gave rise to various enzymes of the early steps in benzoxazinoid biosynthesis (Ober, 2010). A third example is found in the biosynthesis of pyrrolizidine alkaloids, which are scattered among unrelated angiosperm plant families (Ober, 2010). The first specific enzyme in this pathway is homospermidine synthase (HSS), which transfers the aminobutyl moiety from spermidine to putrescine and forms homospermidine. HSS evolved through a duplication event from deoxyhypusine synthase (DHS), which is involved in the post-translational modification of a eukaryotic initiation factor 5A (eIF5A) by transferring the aminobutyl moiety from spermidine onto eIF5A (Ober and Hartmann, 1999). DHS is able to catalyze the HSS reaction, but HSS is unable to perform the DHS reaction, providing an example of gene duplication and loss of function. Remarkably, this gene duplication and loss of function event has been detected in more than one plant family (Asteraceae, Boraginaceae, Convolvulaceae and the monocots) producing pyrrolizidine alkaloids (Kaltenegger et al., 2013).

1.3.3 Convergent and repeated evolution in plant secondary metabolism

Convergent evolution occurs when a biological function evolves independently in different species (Pichersky and Lewinsohn, 2011). On the molecular level, there are several possibilities. Different plant lineages could evolve the biosynthesis of different compounds fulfilling the same function by using similar or different substrates. They could also synthesize the same compounds by using different pathways. One obvious example for the production of different compounds used for a similar purpose is the occurrence of various pigments produced by plants (anthocyanins, betalains, xanthophylls, chalcones and aurones) (Pichersky and Lewinsohn, 2011). For some plant families it is speculated, that they lost the ability to produce anthocyanins (through loss of dihydroflavanol 4-reductase and anthocyanin synthase leaving them only dihydroflavonols) and therefore evolved betalain-type pigments (betacyanins) to replace the function of anthocyanins (Shimada et al., 2005). An example for the utilization of different enzymes using the same substrate is the flavone apigenin. Apigenin is synthesized by the enzyme flavone synthase (an oxoglutarate-dependent dioxygenase family) in the Apiaceae, whereas in most other plant families that reaction is performed by a cytochrome P450 family enzyme (Tanaka et al., 2008). Another case where the same compound is produced through different pathways is the production of methyl anthranilate in different plant species either through a BAHD acyl transferase using the substrate anthraniloyl-CoA or through a SABATH methyl transferase using the substrate anthranilic acid (Wang and De Luca, 2005; Köllner et al., 2010; Pichersky and Lewinsohn, 2011).

A special case of convergent evolution is termed repeated evolution. Here a similar function arises from related sequences (Pichersky and Lewinsohn, 2011). The term itself was first introduced by describing the evolution of enzymes in terpenoid biosynthesis (Cseke et al., 1998). The monoterpene linalool which is found in several different terpene-producing plant species is synthesized by linalool synthase. Interestingly, in each plant species, linalool synthase is more similar to other monoterpene synthases from within that plant species then it is to other linalool

synthases found in other plant species (Dudareva et al., 1996; Crowell et al., 2002; Chen et al., 2003; Pichersky and Lewinsohn, 2011). This observation is also true for other terpene synthases. Within a plant species, terpene synthases often evolve through divergent evolution, but across plant species the rise of a specific terpene synthase activity like linalool synthase could be described by repeated evolution (Pichersky and Lewinsohn, 2011). A similar example is given by the scattered distribution of stilbenes. The important enzyme stilbene synthase possesses a similar reaction mechanism to the enzyme chalcone synthase (CHS) which is involved in flavonoid biosynthesis. Sequence analysis reveals that stilbene synthase is more similar to CHS from its descending plant species then it is to stilbene synthases from other plant species (Tropf et al., 1994). Another example can be found in caffeine biosynthesis. In all different plant species producing caffeine, the enzymes to methylate the purine intermediates all belong to the SABATH carboxyl methyl transferase family, but evolved from different branches therein (Yoneyama et al., 2006; Ashihara et al., 2008). Hence, the biosynthesis of caffeine has evolved independently at least twice (Pichersky and Lewinsohn, 2011).

1.4 Plant secondary metabolism evolution cannot be explained by one model alone

One example for secondary metabolite evolution, where more than one model can be used to explain the evolutionary origins is homospermidine synthase (HSS). The evolution of HSS includes a duplication event (divergent evolution) followed by repeated evolution, giving rise to the same function multiple times. HSS evolved at least five times independently from deoxyhypusine synthase (DHS) in different plant families (Reimann et al., 2004; Kaltenegger et al., 2013). Gene duplication is not always required in secondary metabolite enzyme evolution. Orthologous genes encoding enzymes of secondary metabolism can diverge in different species without prior duplication (Pichersky and Gang, 2000; Schilmiller et al., 2009). However, regardless of whether gene duplication has occurred or not, variation through random mutation

changing the plant`s fitness is what drives natural selection for secondary metabolites (Pichersky and Lewinsohn, 2011).

Our actual knowledge of the enzymes of plant secondary metabolism is extremely incomplete and therefore their evolutionary origins remain for the large part a matter of conjecture (Pichersky and Lewinsohn, 2011). But the fact that individual evolutionary occurrences can be used as examples for multiple mechanisms shows that all these theories and evolutionary mechanisms should not be considered strictly alone, but rather in combinations. With every newly obtained piece of information on enzymes involved in plant secondary metabolism, our understanding of evolutionary processes behind enzyme and secondary metabolite development will increase. In addition, more knowledge about genes and enzymes of secondary metabolite biosynthesis also provides more targets to scientists for engineering secondary metabolite pathways towards medicinal and agricultural uses (Hartmann, 2007).

1.5 Questions addressed in this thesis

Today several consortiums have been formed in order to achieve the large scale production of high-value plant secondary metabolites (alkaloids, terpenoids and polyketides) through engineering pathways in yeast (Facchini et al., 2012). Usually, alkaloids are among the secondary metabolites of highest interest, since many of them have medicinal properties and have proven to be effective painkillers, anti-cancer agents and strong hallucinogens (Evans, 2009). Surprisingly, the pathway to one of the major groups of alkaloids, the tropane alkaloids is poorly understood despite intense biochemical investigation. Although scattered amongst several major lineages of angiosperms, the tropane alkaloid biosynthesis pathway has only been studied in members of the Solanaceae (Brock et al., 2008). In order to understand tropane alkaloid biosynthesis as a whole, more plant families need to be examined. Every plant family may have the same core pathway for producing a certain class of

compounds, but not every plant family contains the same set of compounds and therefore the same set of enzymes involved in the biosynthesis (Facchini and De Luca, 2008). The occurrence of tropinone in most tropane alkaloid containing plants while methylecgonone seems exclusive to members of the Erythroxylaceae, is such an example. The difference between the two molecules is the carbomethoxy function at the C-2 position of the tropane ring (see **Fig. 2**). The enzymes for forming the tropane ring might be the same, but in the case of tropinone, the carboxyl function is lost at the C-2 position through decarboxylation and in case of methylecgonone, the carboxyl function is stabilized through methylation (Dewick, 2009). The tropinone / methylecgonone example shows the importance of investigating more than one plant family. The methylation step is not performed in Solanaceae (see **Fig. 3**), but belongs to the overall tropane alkaloid pathway in plants.

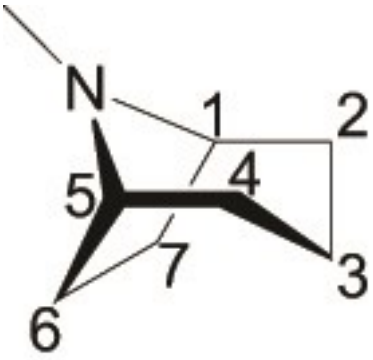

Figure 2 | Numbered tropane skeleton

The goal of my thesis was to elucidate the biochemical steps needed to produce tropane alkaloids in a non-solanaceous species. The model chosen for this study was the coca plant (*Erythroxylum coca*) a member of the Erythroxylaceae (see **Fig. 4**). My focus was on the latter steps of the pathway which included the search for the enzyme responsible for the reduction of methylecgonone and to characterize the enzyme which performs the subsequent esterification of methylecgonine with benzoic acid forming cocaine. A comprehensive overview of tropane alkaloid biosynthesis as it currently stands is presented in the review chapter "Increasing the pace of new discoveries in tropane alkaloid biosynthesis".

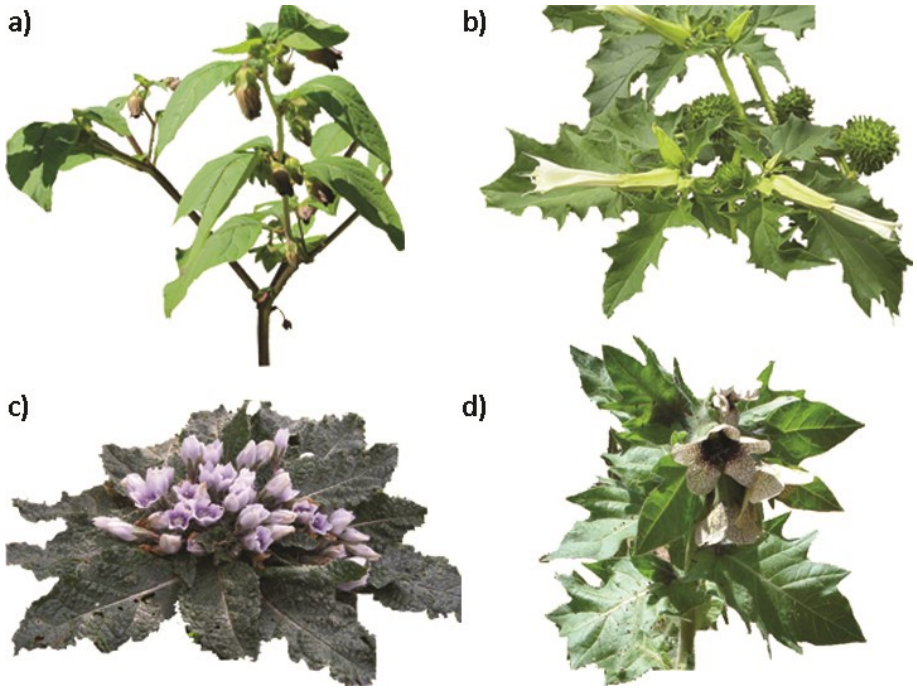

Figure 3 | Selected members of the Solanaceae: a) belladonna (*Atropa belladonna*), **b)** datura (*Datura stramonium*), **c)** mandrake (*Mandragora officinalis*), **d)** henbane (*Hyoscyamus niger*). All images are from http://commons.wikimedia.org licensed through a Creative Commons Attribution License. Images are modified by background erasure.

In order to be able to produce high value tropane alkaloids synthetically, the plant biochemical pathway needs to be more fully elucidated. Currently, the medicinally valued tropane alkaloids are atropine and scopolamine which are C-3α-esterified tropanes. Cocaine in contrast is a C-3β-esterified tropane alkaloid with less medicinal value, but high value as a drug of abuse. To understand the enzyme structure-function relationship in the production of tropane alkaloids, the enzymes responsible need to be investigated. Medicinally-useful semi-synthetic C-3α-esterified tropanes have modifications at the C-2 position (Singh, 2000). However, the identified enzymes of the Solanaceae are not capable of producing any modification on the C-2 position of the tropane ring or accept a C-2 modified compound as a substrate. Yet this task can be performed by enzymes from the Erythroxylaceae. Analysis of the solanaceous and erythroxylaceous enzymes involved in tropane alkaloid biosynthesis can reveal what features allow them to produce the C-2 modification or accept a

compound with this substituent as a substrate. This information can be used for engineering tropane alkaloid biosynthesis enzymes to produce C-3α-esterified tropanes with modifications on the C-2 position. Therefore my elucidation of enzymes involved in the final steps of tropane alkaloid biosynthesis in Erythroxylaceae is not necessarily the beginning of synthetic cocaine production, but the start for understanding the tropane alkaloid biosynthesis pathway in plants, and opens up new possibilities for plant metabolic engineers.

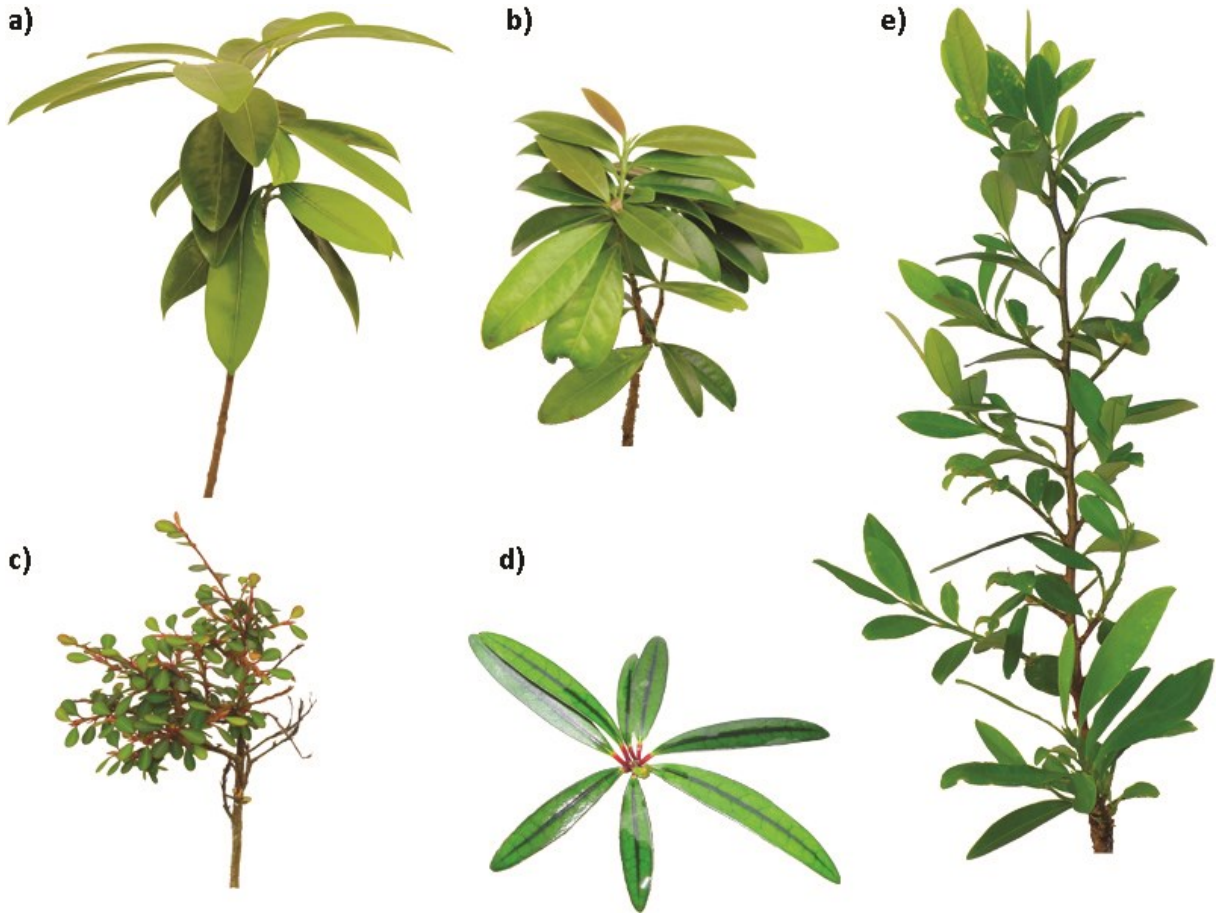

Figure 4 | Selected members of the Erythroxylaceae: a) *Erythroxylum fischeri* and **b)** *Erythroxylum emarginatum* both from Africa, **c)** *Erythroxylum hypericifolium* and **d)** *Erythroxylum laurifolium* both from Mauritius, **e)** the coca plant *Erythroxylum coca* from South America. Pictures taken by Jan Jirschitzka.

2 Overview of the Manuscripts

Manuscript I: **Increasing the pace of new discoveries in tropane alkaloid biosynthesis**

Jan Jirschitzka, Franziska Dolke, John Charles D'Auria

This review on tropane alkaloids is published in the scientific book series *Advances in Botanical Research* vol. 68, New Light on Alkaloid Biosynthesis and Future Prospects, 2013. The reuse in this dissertation is with permission from Elsevier (license number: 3281430586080).

Author contributions

J.J. performed literature search and made figures. F.D. helped with literature search and initial writing. J.J. and J.C.D. co-wrote the manuscript.

Summary

In this book chapter, the current state of research on tropane alkaloids is reviewed. It covers the angiosperm families producing tropane alkaloids, ethnobotanical aspects, historical information, ecological importance as well as the theoretical biosynthesis pathway of tropane alkaloids in plants. Metabolic engineering approaches for increase of valued tropane alkaloids are also summarized.

Manuscript II: Plant tropane alkaloid biosynthesis evolved independently in the Solanaceae and Erythroxylaceae

Jan Jirschitzka, Gregor Wolfgang Schmidt, Michael Reichelt, Bernd Schneider, Jonathan Gershenzon, John Charles D'Auria

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Author contributions

J.J. and J.C.D. designed research; J.J., G.W.S., B.S., and J.C.D. performed research; M.R. contributed new reagents/analytic tools; J.J., G.W.S., M.R., B.S., J.G., and J.C.D. analyzed data; and J.J., J.G., and J.C.D. wrote the paper.

Summary

In this article the biochemical characterization of the enzyme responsible for the reduction of methylecgonone (2-carbomethoxy-3-tropinone) in the coca plant (*Erythroxylum coca*) is described. Methylecgonone is the penultimate intermediate in tropane alkaloid biosynthesis in *E. coca* (Erythroxylaceae) and is very similar to tropinone. Tropinone reduction is well characterized in Solanaceae, but unknown in Erythroxylaceae. A homology based approach using tropinone reductases did not yield active reductase enzymes. Therefore a classical biochemical approach was used to identify the enzyme responsible for the reduction of methylecgonone. Methylecgonone reductase was not found in the below ground plant tissues and was most active in the leaves. This enzyme belongs to a different reductase enzyme family than the tropinone reductases. This discovery is an indication that tropane alkaloid biosynthesis evolved more than once throughout the angiosperms.

Manuscript III: A BAHD acyltransferase catalyzes the final step in cocaine biosynthesis

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Author contributions

G.W.S. and J.J. contributed equally to this work. J.C.D. screened coca for BAHD enzymes. G.W.S cloned BAHD enzymes. G.W.S. and J.J. sub-cloned BAHD enzymes and expressed BAHD enzymes. G.W.S. and J.J. developed the immunolocalization procedure. G.W.S., J.J. and J.C.P.T. performed immunolocalization. G.W.S., J.J., F.D. and K.L. performed and analyzed enzyme assays. F.D. supplied CoA esters. K.L. performed qPCR experiments. G.W.S. and J.J. performed immunoprecipitation and relative activity assays. M.R. contributed analytical tools. T.P., E.V. and G.H. performed MALDI experiments and wrote the draft for MALDI section. E.V. and G.H. final edited the MALDI section. G.W.S. and J.J. prepared figures. J.J. and J.C.D. co-wrote the manuscript. J.G. edited the final manuscript.

Summary

In this article the identification and characterization of the enzyme cocaine synthase, the last enzyme in tropane alkaloid biosynthesis in *Erythroxylum coca*, is described. Cocaine synthase belongs to the BAHD acyltransferase superfamily and is the first BAHD reported to be involved in tropane alkaloid biosynthesis. Like the enzyme methylecgonone reductase, cocaine synthase is not found in the below ground tissues and is most active in the leaves. Furthermore, cocaine was co-localized with cocaine synthase. MALDI imaging experiments showed a difference in the abundance of cocaine and cinnamoyl cocaine in adaxial and abaxial leaf surfaces.

Manuscript IV: Learning from nature: new approaches to the metabolic engineering of plant defense pathways

Jan Jirschitzka, Derek Joseph Mattern, Jonathan Gershenzon, John Charles D'Auria

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Author contributions

J.G. initially drafted the manuscript. J.J. and D.J.M. performed literature search, made figures/tables and drafted the manuscript. J.J., J.G. and J.C.D. edited the manuscript.

Summary

This review focuses on the current state of research on plant metabolic engineering in the context of plant defense pathways. Several examples of increased plant defense through engineered plants are included. We conclude that further information on the natural defense regulation mechanisms through transcription factors, multiple gene clusters or tissue specific expression can improve future metabolic engineering of plant defense pathways.

3.1 Manuscript I

Increasing the pace of new discoveries in tropane alkaloid biosynthesis

CHAPTER TWO

Increasing the Pace of New Discoveries in Tropane Alkaloid Biosynthesis

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Abstract

Tropane alkaloids (TAs) are plant-derived natural products that have been exploited throughout history for their pharmaceutical properties. TAs are characterised by the presence of a tropane ring and are present in a variety of plant families including the Solanaceae, Convolvulaceae, Proteaceae, Rhizophoraceae, Brassicaceae and Erythroxylaceae. The structural genes and enzymes involved in TA biosynthesis have been characterised primarily in the Solanaceae, but several key steps remain unresolved. The scattered distribution of TAs in different angiosperm families suggests that there may be several alternative pathways for TA biosynthesis, and TA biosynthesis appears to have a polyphyletic origin. Molecular techniques are providing an ever-increasing amount of information on the nature and evolution of TA biosynthesis pathways and are aiding the development of techniques for increasing the production of useful tropanes through metabolic engineering.

ABBREVIATIONS

H6H hyoscyamine 6β-hydroxylase
MecgoR methylecgonone reductase
MeJA methyl jasmonate
MPO *N*-methylputrescine oxidase

ODC ornithine decarboxylase
PMT putrescine *N*-methyltransferase
SA salicylic acid
SAM *S*-adenosylmethionine
SDR short-chain dehydrogenase/reductase
TA(s) tropane alkaloid(s)
TR tropinone reductase

1. INTRODUCTION

Tropane alkaloids (TAs) are a large class of plant-derived secondary metabolites and are defined by their core structure, the 8-azabicyclo [3.2.1]octane nucleus (Fig. 2.1). There have been more than 200 structures reported in the literature (Lounasmaa & Tamminen, 1993), and many TAs are being identified in a range of plant families (de Oliveira et al., 2011; Eich, 2008; El Bazaoui, Bellimam, & Soulaymani, 2011; Griffin & Lin, 2000; Queiroz et al., 2009; Razzakov & Aripova, 2004; Sena-Filho et al., 2011). Like other alkaloids, TAs possess many potent pharmacological activities, including acting as cholinergic agents, deliriants and narcotic analgesics. Humans have exploited the pharmacological properties of TAs since ancient times.

One of the first domesticated plant species used exclusively for its medicinal properties is *Erythroxylum coca*, which produces the well-known TA cocaine (**42**). Recent archaeological evidence has found coca leaves in Peruvian house floors, dating the use of this species back at least 8000 years (Dillehay et al., 2010). Coca leaves are obtained from the two closely related species *E. coca* and *E. novogranatense* (Plowman, 1982). During the Incan empire (thirteenth to sixteenth century), coca leaves were used as a sacrificial offering and were additionally used by the aristocracy and religious elite for its medicinal properties (Naranjo, 1981). Interestingly, the practice of

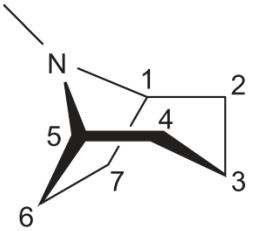

Figure 2.1 The tropane core structure. The basic tropane skeleton with the accepted carbon numbering system is depicted. Metabolites missing the methyl modification on the nitrogen are referred to as nortropanes.

chewing the leaves by the ruling classes was restricted to *E. novogranatense* because it contains high levels of methyl salicylate and thus is perceived to have a minty taste (Naranjo, 1981; Plowman & Rivier, 1983). In the Spanish colonial era, coca leaves were originally described as the "Devil's leaf" (Parkerson, 1983). However, conquistadores later recognised that chewing coca leaves increased stamina in addition to reducing thirst. Due to these observations, the coca leaf began to be used to increase the productivity of natives working as slaves in the mines (Plowman, 1984). In 1860, Albert Niemann pioneered the modern method for the isolation of cocaine (**42**) as a pure substance (Niemann, 1860). Pure cocaine (**42**) in its crystallised form then began to be consumed in large quantities for its euphoric and analgesic effects. The drug's rise to fame was partly due to its use and endorsement by famous individuals such as Sigmund Freud (Freud, 1884) and its inclusion as an ingredient in drinks such as Vin Mariani (wine) (Wink, 1998c) and Coca Cola (Plowman, 1981). The discovery of cocaine's (**42**) narcotic properties in the twentieth century quickly changed this "magic drug" into an illegal substance (Wink, 1998c). The modern abuse of the pure form of cocaine (**42**) has led to worldwide socioeconomic and health-related issues (Streatfeild, 2001).

Other well-known TAs that have been utilised throughout history include atropine (**1**) (Fig. 2.2) and scopolamine (**44**) (Fig. 2.7), which are

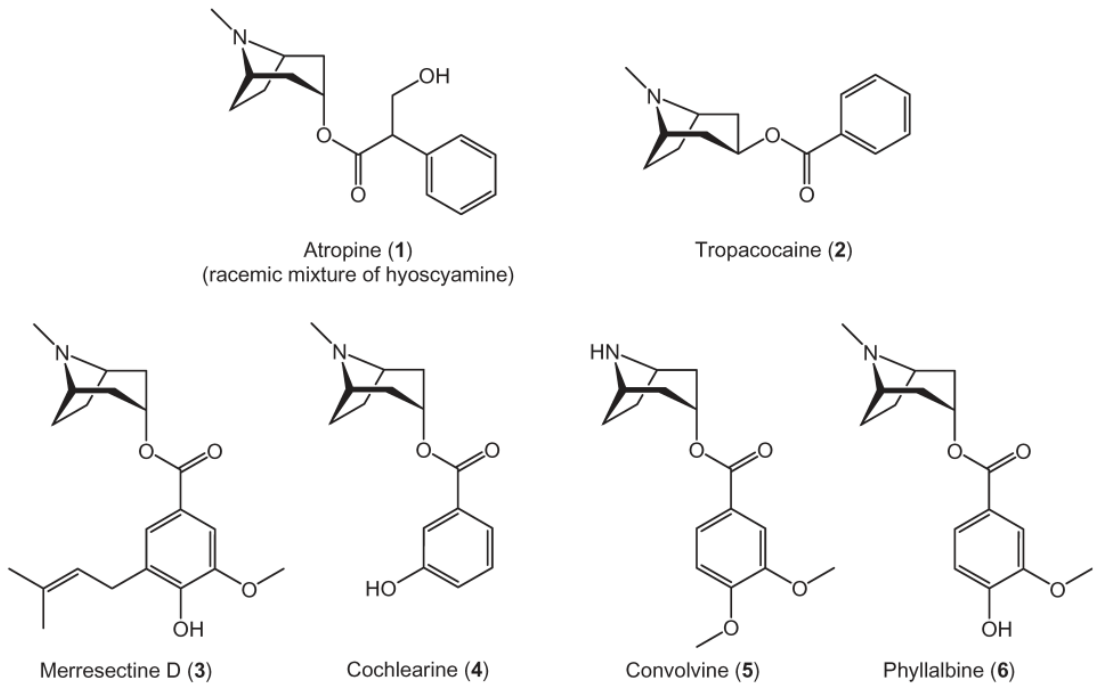

Figure 2.2 Examples of tropane alkaloids found in plants of the Solanaceae, Convolvulaceae, Brassicaceae and Phyllanthaceae.

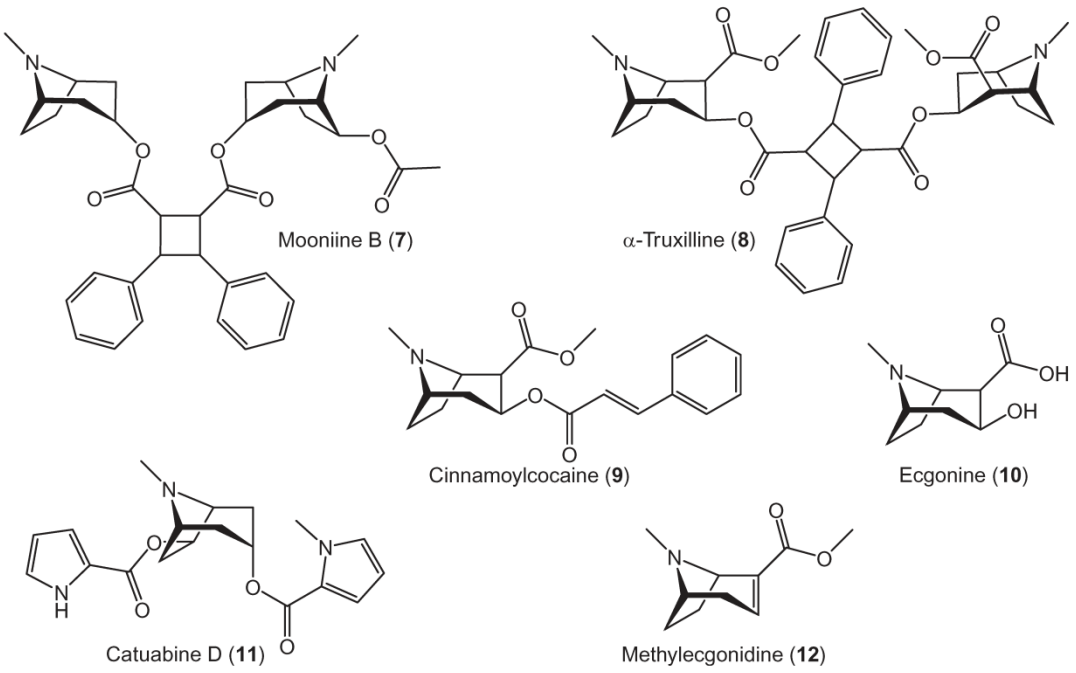

Figure 2.3 Examples of tropane alkaloids found in the Erythroxylaceae.

found in several genera of the Solanaceae. *Datura* species, for example, were described in the Ebers papyrus in about 1550 BC and were also used in Ayurvedic medicine in about 900 BC (Wink, 1998c). The plants were used to treat mental illness, fever, tumours, eczema and infections (Wink, 1998c). In medieval Europe, TAs from solanaceous plants were important in medicine, witchcraft and divination. The four solanaceous plants thorn apple (*Datura stramonium*), mandrake (*Mandragora officinarum*), henbane (*Hyoscyamus niger*) and belladonna (*Atropa belladonna*) were used as sedatives, sleep-inducing agents (henbane), aphrodisiacs and panaceas (mandrake). These plants were often ingredients added to witches' brews, and the association between witches and brooms may have its origin in the use of brooms to apply alkaloid-containing salves (Mann, 1992). The administration of plant salves was found to be particularly rapid when absorbed through the mucous membranes, including those of the female genitalia (Mann, 1992). Belladonna (Italian for beautiful woman) was used to enhance feminine beauty and sensuality by dropping plant extracts into the eyes of women and dilating their pupils (Schultes, 1976). In China, *Datura metel*, *D. stramonium* and *Anisodus tanguticus* (known as *Scopolia tanguticus*) are among the 50 essential Chinese medicinal plants (Schnorrenberger, 1978). Atropine (**1**) was first isolated in 1833 from the roots and leaves of *A. belladonna* while the first isolation of scopolamine (**44**) was performed using *Scopolia japonica* in 1888 (Geiger & Hesse, 1833; Mein, 1833; Schmidt & Henschke, 1888).

Solanaceous plants are often used for their hallucinogenic properties and their use in shamanistic and religious rituals. The majority of these plants contain high quantities of scopolamine (**44**), such as the red-flowered Peruvian plant known as Tonga, Huacacachu or grave plant (*Brugmansia sanguinea*). Tonga is reported to be used as an aid in contacting deceased ancestors (Schultes, 1976). Similarly, the main ingredient of Wysoccon, a drug used for the manhood initiation rituals carried out by Algonquin Indians that leads to the user "losing all memory of ever having been boys," is *D. stramonium* (Schultes, Hofmann, & Rätsch, 2001).

In contrast, plants of the Erythroxylaceae are more often valued for their medicinal properties. The leaves of Olokuto, a wild *Erythroxylum species* known as *Erythroxylum dekindtii*, are used to reduce fever in Angola. Olokuto contains ecgonine (**10**), methylecgonine (**38**), tropacocaine (**2**) and pseudotropine (**35**) (Campos Neves & Campos Neves, 1966). It is also very common for tropane alkaloid-producing plants to be used as a panacea. One example of this is *Erythroxylum emarginatum* from South and East Africa. This plant contains large quantities of methylecgonidine (**12**) as the main TA (Nishiyama et al., 2007) and its boiled leaf extracts are drunk to treat asthma, kidney problems, arthritis, child bearing problems and influenza (De Wet, 2011). In addition, the roots and leaves of *E. emarginatum* are used for pain relief, to increase alertness and also as an aphrodisiac (Nishiyama et al., 2007). Many other TA-containing plants are commonly used by different native tribes as aphrodisiacs. One of the most well-known plants in this category is *E. vacciniifolium*, also known as Catuaba. In Brazil, the bark and leaves of Catuaba are sold as a "natural Viagra." Catuabines (**11**) are the TA associated with many of these preparations (Graf & Lude, 1977; Kletter et al., 2004; Queiroz et al., 2009). Lastly, *E. moonii* from Sri Lanka does not fall into the traditional medicinal categories assigned to TA-containing plants. The leaves of this plant, which contain large amounts of mooniines (**7**), dimeric tropane alkaloids, are boiled and used as an anthelmintic for the treatment of parasitic roundworms (Atta-Ur-Rahman et al., 1998).

In contrast to the pharmacology of TAs, the natural role of these compounds in plants is poorly understood. While it is commonly assumed that TAs serve to defend plants against herbivores and pathogens, there is a paucity of studies available on this topic. Scopolamine (**44**) and hyoscyamine (**41**) appear to be utilised by solanaceous plants to mediate a wide array of ecological interactions with many types of insects. Scopolamine (**44**) has been implicated as a phagorepellent to adult lepidoptera including the silk moth (*Bombyx mori*) and the cabbage butterfly (*Pieris brassicae*) (Levinson,

1976). Further, scopolamine (**44**) has also been classified as a feeding deterrent for insects such as *Syntomismoga dorensis* and the honey bee (*Apis mellifera*) (Wink, 1998a). Besides serving as a deterrent, TAs can also function as toxic compounds. Cocaine (**42**) in concentrations similar to those found in coca leaves can serve as a natural insecticide for the tobacco hornworm (*Manduca sexta*) when sprayed on tomato leaves (Nathanson, Hunnicutt, Kantham, & Scavone, 1993). In addition, several studies have shown that scopolamine (**44**) increases mortality in the worm *Tubifex tubifex* (Wink, 1998b) and the insect *Spodoptera frugiperda* (Alves, Sartoratto, & Trigo, 2007). There is evidence that scopolamine (**44**) can also serve as an allelopathic compound, inhibiting the growth of the roots of the plant *Lepidium sativum* (Wink, 1998b).

Sequestration of plant-derived TAs in insects for use as defensive or toxic substances has also been observed in several instances. The winter cherry bug (*Acanthocoris sordidus*) is capable of absorbing scopolamine (**44**) produced by *Duboisia leichhardtii* and transforming it into atropine (**1**) via enzymatic de-epoxidisation (Kitamura, Tominaga, & Ikenaga, 2004). The aposematic butterfly *Placidula euryanassa* utilises scopolamine (**44**) obtained from *Brugmansia suaveolens* as a toxic compound to protect developing larvae from vertebrate predators (Freitas et al., 1996). The specialist lymantriid *Eloria noyesi* feeds exclusively on coca plants, sequesters cocaine (**42**) during the larval stage and retains cocaine (**42**) in the adult moths (Blum, Rivier, & Plowman, 1981). Polyhydroxylated TAs known as calystegines (**37**) are sequestered by several lepidopteran species and render the lepidopterans indigestible by inhibiting the glycosidases of potential predators (Nash & Watson, 1995).

2. TROPANE ALKALOIDS IN PLANTS: FROM HERBS TO TREES

In order to better understand the biological roles of TAs, it is critical to also understand the distribution of these metabolites across the plant kingdom. They are present in five major lineages of dicotyledons: the peripheral Eudicots (Proteaceae), the Malvid (Brassicaceae) and Fabid (Elaeocarpaceae, Erythroxylaceae, Moraceae, Phyllanthaceae, Rhizophoraceae) clusters of the Rosid lineage, the peripheral Asterids (Olacaceae) and the Lamid cluster of the Asterid lineage (Solanaceae, Convolvulaceae) (The Angiosperm Phylogeny Group, 2009). This includes seven different orders, which contain in total ten different families (Fig. 2.4). The scattered distribution pattern of TA-producing plant families raises the question of whether or not biosynthesis

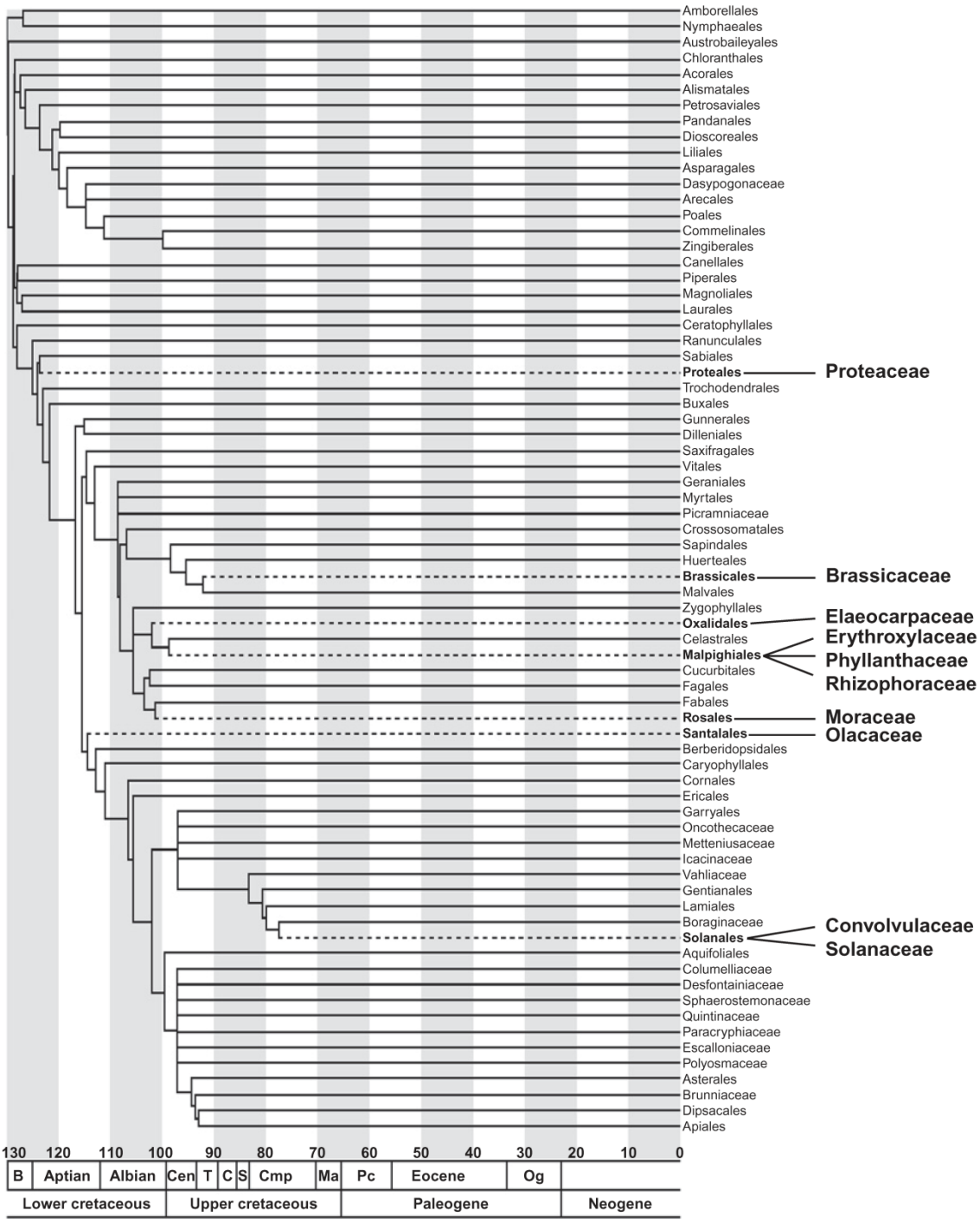

Figure 2.4 The scattered distribution of tropane producing among the angiosperms. Plant families that have been shown to produce tropane alkaloids are highlighted and the orders they belong to are displayed using a dashed line. The scale bar below represents millions of years. This phylogenetic tree has been modified and republished with permission of the Botanical Society of America, from Angiosperm diversification through time, Susana Magallon and Amanda Castillo, 96, 1, 2009; permission conveyed through Copyright Clearance Center, Inc.

of TAs is monophyletic or polyphyletic. The Solanaceae and Convolvulaceae, two closely related families of the Solanales, are separated from other major tropane alkaloid-containing families such as the Erythroxylaceae and Proteaceae by at least 120 million years (Fig. 2.4). Until recently, molecular data for genes and enzymes involved in TA biosynthesis were available only for members within the Solanaceae. However, new data on TA biosynthesis in *E. coca* suggest that TA biosynthesis originated at least twice during the course of angiosperm diversification (Jirschitzka et al., 2012).

The largest single family whose members are known to make TAs is the Solanaceae. There are 29 genera in this family with the ability to produce TAs. Some examples of the most common TAs found in the Solanaceae are shown in Fig. 2.2. In general, TAs present in the Solanaceae are esterified at the C-3 hydroxyl position, and the stereochemistry of this substituent is most often α. Atropine (**1**) and scopolamine (**44**), the most prominent representatives of TAs in this family, are both esterified with a tropic acid moiety. The major difference between these two metabolites is the epoxy group linking position C-6 and C-7 of the tropane skeleton. Both atropine (**1**) and scopolamine (**44**) are included in the World Health Organization's (WHO) essential drugs list (WHO, 2011). These alkaloids completely inhibit the action of the acetylcholine receptors of postganglionic parasympathetic nerves (Reas & Tsai, 1966). These receptors are involved in the constriction of the pupil, vasodilation and moderation of the heartbeat (Henderson & Roepke, 1937). As a result, these compounds are used in a wide variety of treatments including those for motion sickness, in ophthalmic surgery and as a treatment for bradycardia (Ebert, Siepmann, Oertel, Wesnes, & Kirch, 1998; Honkavaara & Pyykkö, 1999; Schwartz, de Roetth, & Papper, 1957). Recent studies have revealed that anisodamine (**43**), the biosynthetic intermediate between hyoscyamine (**41**) and scopolamine (**44**), is less toxic to the central nervous system than scopolamine. This led to the discovery that high doses of anisodamine (**43**) can ameliorate cognitive disorders and, therefore, it has been suggested as a novel treatment for Alzheimer's disease (Zhang et al., 2008).

The Convolvulaceae, also known as the morning glory family, contains 25 genera reported to make TAs. Like the TAs found in its sister family, the Solanaceae, the dominant TAs are tropine (3α-hydroxy) esters (Fig. 2.2). Convolvine (**5**), a dimethoxy benzoic acid ester of nortropine, was found in *Convolvulus pseudocantabricus* and was the first compound to be described from this family (Orechoff & Konowalowa, 1933). Convolvine (**5**) blocks the M-receptors of the heart and intestine while raising the sensitivity of

the M-receptors of the salivary gland and the central nervous system. It has been suggested that this compound and its related substances maybe used as sedatives and nootropic agents (Mirzaev & Aripova, 1998). Some additional compounds found in this family are unique because they contain modifications that occur in the aromatic ester moiety. These compounds include merresectines (**3**) (Fig. 2.2) from the genus *Merrima,* which can be prenylated as well as glycosylated (Jenett-Siems et al., 2005).

A subclass of polyhydroxylated tropane alkaloids known as calystegines (**37**) were first discovered in the roots of *Calystegia sepium* (Convolvulaceae) (Goldmann et al., 1990; Tepfer et al., 1988). Unlike the other TAs described thus far, the calystegines (**37**) tend to be distributed across all plant tissue types. These compounds are proposed to function as glycosidase inhibitors like other monosaccharide-mimicking alkaloids (Asano, Nash, Molyneux, & Fleet, 2000). However, no concrete evidence for their use as a defensive compound in plants has been reported. Calystegines (**37**) have been found in nearly all families that make TAs, but the Moraceae is the only family that contains only calystegines (**37**) and no other type of TA (Asano, Oseki, Tomioka, Kizu, & Matsui, 1994; Asano, Tomioka, Kizu, & Matsui, 1994).

Cocaine (**42**) is one of the most well-known TAs and is exclusively found in members of the Erythroxylaceae. Since its isolation by Albert Niemann (Niemann, 1860), it has become infamous for its abuse as an illegal narcotic. This is due to the euphoria induced by its activity as a dopamine reuptake inhibitor (Galloway, 1988). Cocaine (**42**) has been found in 23 of the approximately 230 species in the genus *Erythroxylum* (Bieri, Brachet, Veuthey, & Christen, 2006). However, the difference in cocaine (**42**) concentration between wild and cultivated species can be more than 100-fold (Aynilian, Duke, Gentner, & Farnswor, 1974). It is believed that cocaine (**42**) is stored as a chlorogenic acid complex in the vacuoles found in the palisade parenchyma in the leaves (Ferreira, Duke, & Vaughn, 1998; Pardo Torre et al., 2013). One of the distinguishing characteristics of TAs found in the Erythroxylaceae is the common occurrence of a carbomethoxy group on the C-2 position of the tropane ring (Fig. 2.3). Like the TAs found in the Solanaceae, many of the TAs in the Erythoxylaceae are esterified at the C-3 position. However, the configuration at this position is predominantly β. Other common TAs found in the Erythroxylaceae include cinnamoylcocaine (**9**) and its dimeric derivatives, the truxillines (**8**) (Fig. 2.3). Truxillines (**8**) are thought to be the result of dimerisation due to UV radiation (Lydon et al., 2009). Despite the negative associations with cocaine (**42**), its derivatives have the potential to be legitimate medicines.

For example, modification of dopamine transporter function may help to alleviate some symptoms associated with Alzheimer's disease, Parkinson's disease, attention-deficit hyperactivity disorder, ageing and depression (Runyon & Carroll, 2006; Singh, 2000). The synthetic compound fluorotropacocaine can be used as a local anaesthetic and its ^{18}F-labelled derivative has been used as a muscarinic acetylcholine ligand for PET imaging (Kavanagh et al., 2012).

The model plant *Arabidopsis thaliana* has never been shown to contain TAs (Brock, Herzfeld, Paschke, Koch, & Drager, 2006), but TAs have been isolated from other members of the Brassicaceae. An alkaloid isolated from *Cochlearia arctica* (now *C. groenlandica*) and *Cochlearia officinalis*, cochlearine (**4**), has been shown to be a TA (Liebisch, Bernasch, & Schütte, 1973; Platonova & Kuzovkov, 1963). Since this discovery, a further 12 genera in the Brassicaceae have been reported to contain TAs (particularly calystegines (**37**); see above).

The Proteaceae represents the oldest tropane-producing family among the angiosperms (Fig. 2.4). There are five genera reported to contain tropanes and they are geographically limited to Australia and New Caledonia (Bick et al., 1981; Butler et al., 2000). Both 3α and 3β esters of tropine and pseudotropine, respectively, have been reported in the literature. The esters consist mainly of aromatic and aliphatic acids. Pyranotropanes contain a γ-pyrano group attached to the C-3 and C-4 position of the tropane ring (Fig. 2.5). Examples of these compounds include strobamine (**14**) and bellendine (**13**). In addition, the compounds ferruginine (**15**) and ferugine (**17**) found in the genus *Darlingia* have also been described and contain unique modifications at the C-4 position of the tropane ring. Both ferruginine (**15**) and ferugine (**17**) are nicotinic receptor antagonists and have been suggested to be used as a potential treatment for Alzheimer's disease (Lazny, Sienkiewicz, Olenski, Urbanczyk-Lipkowska, & Kalicki, 2012).

Very little data exist on the remaining TA-containing plant families. In fact, for some of these families, only one report has ever been published. *Bruguiera*, *Crossostylis* and *Pellacalyx*, three genera from the Rhizophoraceae, contain common tropanes such as tropinone (**30**), tropine (**34**) and various aromatic tropine esters (Arbain, Wiryani, & Sargent, 1991; Kato, 1975; Loder & Russell, 1966, 1969; Media, Pusset, Pusset, & Husson, 1983). Brugine (**16**) (Fig. 2.5), a unique dithiolane tropane alkaloid, has been identified from members belonging to the Rhizophoraceae. There is some controversy surrounding the description of TAs in *Peripentadenia mearsii*, a member of the family Elaeocarpaceae. The original study reported

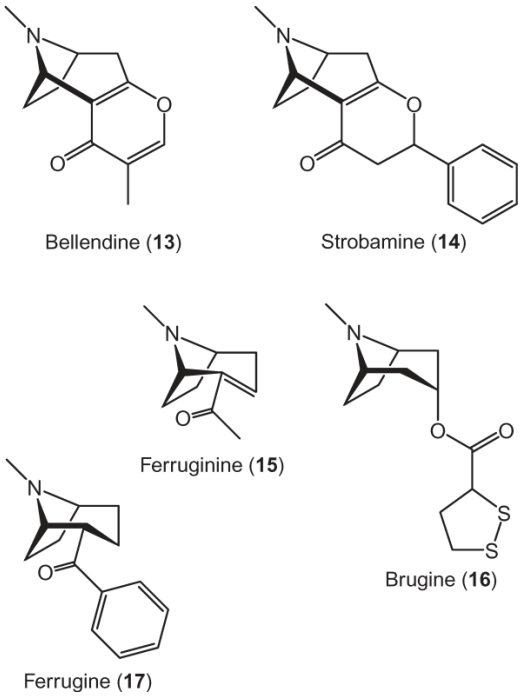

Figure 2.5 Examples of tropane alkaloids found in plants of the Proteaceae and Rhizophoraceae.

the discovery of 3α-acetoxy-6β-hydroxytropane, 2α-benzoyloxy-3β-hydroxynortropane, and 3β-benzoyloxytropane (Johns, Lamberto, & Sioumis, 1971). However, a year later, the same authors reported that they could not reproduce their original results (Johns, 1972). Therefore, a more detailed study of this species with a larger sample size is necessary to conclusively determine whether *P. mearsii* has the ability to synthesise TAs. Originally considered as a member of the Euphorbiaceae, the tropical plant *Phyllanthus discoides*, now a member of the family Phyllanthaceae (Nahar, Sarker, & Delazar, 2011), has been described to contain phyllalbine (**6**) (Parello, Longevialle, Vetter, & McCloskey, 1963). Scopolamine (**44**) was also found in *Heisteria olivae* (Olacaceae), which grows above 1000 m in the Andean highlands (Valera, De Budowski, Delle Monache, & Marini-Bettolo, 1977). This report should be taken with caution as there is no other description of tropanes in the Olacaceae, and scopolamine (**44**) has otherwise been reported only from plants in the Solanaceae.

3. BIOSYNTHESIS OF TROPANE ALKALOIDS

The biosynthesis of TAs has been a subject of study for nearly 200 years. The early studies were interested in the crystallisation of pharmaceutically important TAs such as atropine (**1**), scopolamine (**44**) and cocaine

(**42**) (Geiger & Hesse, 1833; Mein, 1833; Niemann, 1860; Schmidt & Henschke, 1888). Richard Willstätter's synthesis of ecgonine (**10**) in the beginning of the twentieth century established the first true foundations for applying chemical and other analytical tools to our understanding of how tropane alkaloids are made (Willstätter & Hollander, 1903). The predominant methods used to elucidate both structure and potential biosynthetic steps in the pathway have been radiolabelled feeding studies followed by chemical degradation analysis. Based on this type of investigation, a biosynthetic model has been established that can be attributed principally to Leete (1990). His predictions about the enzymatic steps in the biosynthesis in both cocaine (**42**) and other TAs have provided direction to biochemists and molecular biologists alike (Bjorklund & Leete, 1992). Because of the commercial interest in TAs of the Solanaceae and the genomic tools available for selected species, studies of the genes and enzymes involved in TA biosynthesis have focused on members of this family. As previously mentioned, the scattered distribution of TAs throughout the angiosperms suggests that their biosynthetic origins may be polyphyletic. Therefore, the current state of knowledge regarding the enzymes involved in TA production is heavily biased to a single family, and alternative pathways may be identified in other families.

In general, alkaloid biosynthesis begins with the recruitment of a nitrogen-containing metabolite of central metabolism. In many cases, amino acids serve as the initiating intermediate (Zulak, Liscombe, Ashihara, & Facchini, 2007). As early as 1954, the amino acids ornithine and arginine were predicted to be the starting substrates in the biosynthesis of TAs (Leete, Marion, & Spenser, 1954). van Soeren (1962), ^{14}C-proline fed to the roots of *A. belladonna*, showed that proline (**18**) could also be incorporated into the tropane ring. Several other studies using *D. metel* and *D. stramonium* also reported the incorporation of proline (**18**) into the compounds tropine (**34**) and scopolamine (**44**) (Liebisch & Schütte, 1967). Arginine (**20**), ornithine (**21**) and proline (**18**) are readily interconvertible via the shared intermediate pyrroline-5-carboxylate (**19**) (Fig. 2.6) (Delauney & Verma, 1993). Therefore, interpretation of the results following amino acid feeding has made it difficult in determining whether one or a combination of amino acids are truly responsible for entry into the TA pathway. Labelling studies on several different TA-producing plant species using [2-^{14}C]-ornithine have produced conflicting results: a symmetrical incorporation at positions C-1 and C-5 of the tropane ring has been reported for *Hyoscyamus albus* and *E. coca*, while an asymmetrical labelling (at C-5 only)

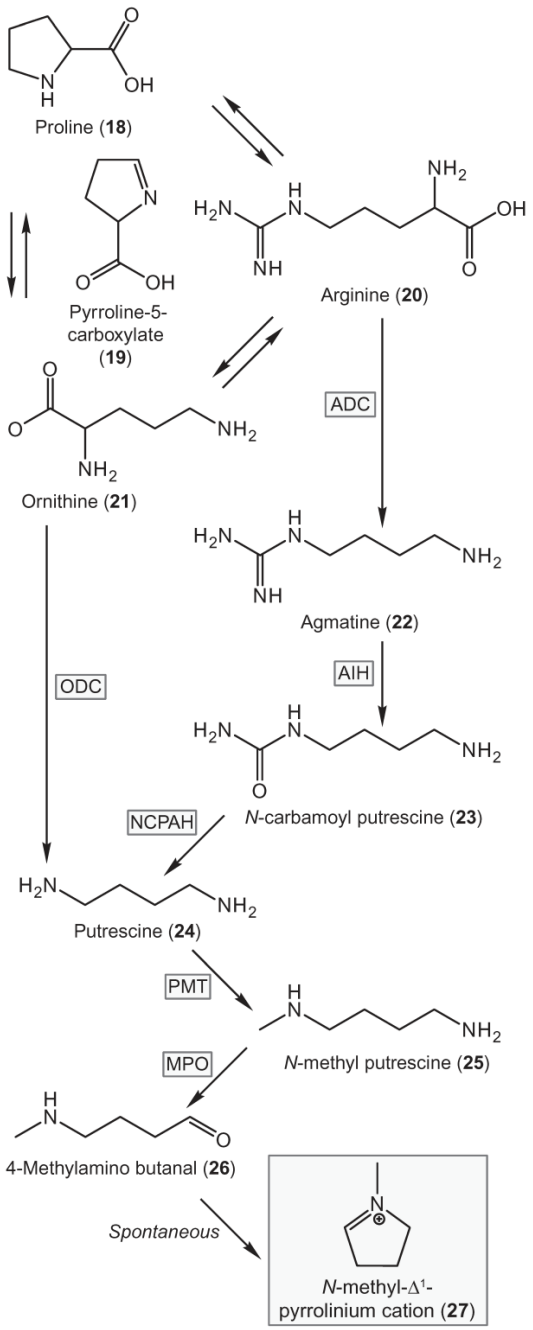

Figure 2.6 The initial steps of tropane biosynthesis leading to the formation of the *N*-methyl-Δ^1-pyrrolidinium cation. The amino acids proline, arginine and ornithine have all been implicated in the biosynthesis of tropane alkaloids. Their shared biosynthetic intermediate pyrroline-5-carboxylate has complicated the determination of which amino acid is the direct precursor. The production of putrescine can be directly formed via the decarboxylation of ornithine or indirectly from arginine. Following methylation, *N*-methylputrescine is oxidised to the intermediate 4-methylamino butanal, which spontaneously cyclizes to yield the *N*-methyl-Δ^1-pyrrolidinium cation. Tropane alkaloid biosynthetic enzymes that have been isolated and biochemically characterised appear in the shaded boxes. The abbreviations for these enzymes are as follows: ODC, ornithine decarboxylase; ADC, arginine decarboxylase; AIH, agmatine imino hydrolase; NCPAH, *N*-carbamoylputrescine amido hydrolase; PMT, putrescine *N*-methyltransferase; MPO, methyl putrescine oxidase.

was reported in *D. stramonium* and *D. metel* (Hashimoto, Yukimune, & Yamada, 1989; Leete, 1962, 1964, 1982; Liebisch, Ramin, Schoffin, & Schütte, 1965). It was suggested that selective methylation of ornithine (**21**) could explain the asymmetrical pattern observed in *Datura*. This hypothesis was tested by Ahmad and Leete by feeding DL-α-*N*-methyl-[2-^{14}C]-ornithine or DL-δ-*N*-methyl-[2-^{14}C]-ornithine to *D. stramonium* (Ahmad & Leete, 1970). Incorporation at a very low level was observed only in the case of feeding with DL-δ-*N*-methyl-[2-^{14}C]-ornithine providing evidence for the theory of selective methylation of ornithine.

It is possible to reach a symmetrical intermediate in one enzymatic step by conversion of ornithine (**21**) to putrescine (**24**), one of the simplest polyamines. This reaction is catalysed by ornithine decarboxylase (ODC), an enzyme isolated from several TA-producing plant species (Fig. 2.7) (Docimo et al., 2012; Imanishi et al., 1998; Michael, Furze, Rhodes, & Burtin, 1996). ODC is a pyridoxal phosphate-dependent decarboxylase. It is predicted to be a cytosolic enzyme (Sandmeier, Hale, & Christen, 1994) but appears to accumulate in the nucleus (Schipper, Cuijpers, de Groot, Thio, & Verhofstad, 2004).

An indirect route to putrescine (**24**) via arginine (**20**) has also been demonstrated. The first enzymatic step involves decarboxylation of arginine (**20**) by arginine decarboxylase (Fig. 2.6) (ADC). The decarboxylated product, agmatine (**22**), is then converted to *N*-carbamoyl putrescine (**23**) via the enzyme agmatine imino hydrolase. *N*-carbamoyl-putrescine amido hydrolase catalyses the final step resulting in the formation of putrescine (**24**). Biochemical studies performed by Malmberg revealed that putrescine (**24**) production via ODC is important for the supply of polyamines for primary metabolic processes such as cellular division, differentiation and development (Malmberg, Watson, Galloway, & Yu, 1998). In contrast, putrescine (**24**) supplied via the ADC-route is thought to be required for responses related to environmental stress (Malmberg et al., 1998).

The first committed step in TA biosynthesis is the formation of *N*-methyl putrescine (**25**). The methyl group attached to the nitrogen in the tropane skeleton is derived from methionine via the common methyl donor *S*-adenosylmethionine (SAM). The SAM-dependent methyl transferase responsible for this reaction is referred to as putrescine *N*-methyltransferase (Fig. 2.6) (PMT). The first PMT sequence isolated from plants was from tobacco (Hibi, Higashiguchi, Hashimoto, & Yamada, 1994). The biosynthesis of the pyrrolidine ring in both nicotine and TAs is thought to have the same origins within plants of the Solanaceae. Thus

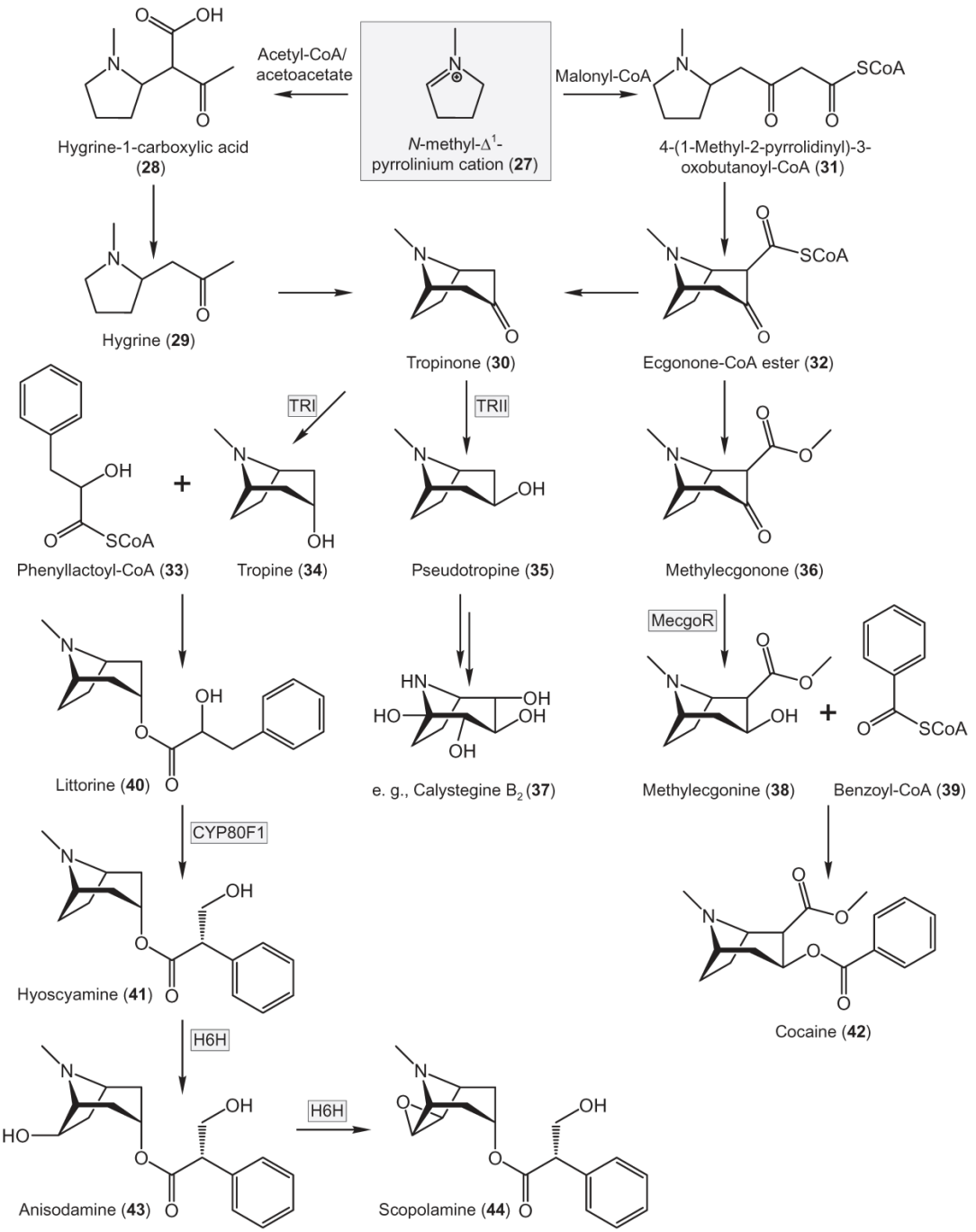

Figure 2.7 The mid- and late-biosynthetic reactions in tropane alkaloid production. Depicted here are two possible models for the condensation of the tropane ring. The utilisation of acetyl-CoA directly or indirectly via acetoacetate can yield hygrine-1-carboxylate. Alternatively, 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoyl-CoA can be formed via the decarboxylative condensation of two malonyl-CoA subunits. Once the ring is formed, the keto function at the C-3 position is reduced and the corresponding alcohol is esterified using an acyl-CoA substrate. The epoxidation of hyoscayamine is catalysed from one enzyme in a two-step process. Tropane alkaloid biosynthetic enzymes that have been isolated and biochemically characterised appear in the shaded boxes. The abbreviations for these enzymes are as follows: TR I, tropinone reductase I; TR II, tropinone reductase II, MecgoR, methylecgonone reductase; H6H, 6β-hydroxy hyoscyamine epoxidase.

far, PMTs from several TA-producing members of the Solanaceae and one member of the Convolvulaceae have been isolated (Kai, Zhang, et al., 2009; Liu, Zhu, Cheng, Meng, & Zhu, 2005; Teuber et al., 2007). PMT belongs to a large family of enzymes which are involved in the production of polyamines. Other members of this family include the spermidine and spermine synthases. There is evidence to suggest that PMT function evolved from an ancestral spermine or spermidine synthase as these enzymes are essential for supplying polyamines used in primary metabolism (Hashimoto, Tamaki, Suzuki, & Yamada, 1998). Immunolocalisation of both PMT and spermidine synthase demonstrated that both enzymes are present in the below-ground portions of the plant. This is consistent with the observation that TA biosynthesis in members of the Solanaceae occurs mainly in the roots (Nakajima & Hashimoto, 1999; Ziegler & Facchini, 2008). Although much attention has been given to *N*-methyl putrescine (**25**), at least one alternative intermediate has been identified. Leete (1985) discovered that *N*-methylspermidine can serve as an indirect precursor for the formation of the pyrrolidine ring of nicotine in *Nicotiana glutinosa*.

In order to convert *N*-methylputrescine (**25**) into the cyclic pyrrolidine ring, an oxidative deamination is required. The resulting compound, 4-methylaminobutanal (**26**), spontaneously cyclises to yield the *N*-methyl-Δ^1-pyrrolidinium cation (**27**) (Fig. 2.6) (Leete, 1990). Labelled 4-methylaminobutanal (**26**) was detected in *D. stramonium* plants following feeding with [2-^{14}C]-ornithine. Enzyme activities for this oxidation reaction have been described for several TA-producing members of the Solanaceae (Feth, Wray, & Wagner, 1985; Hashimoto, Mitani, & Yamada, 1990; Mizusaki, Tanabe, Noguchi, & Tamaki, 1973). The enzyme *N*-methylputrescine oxidase (MPO) was first characterised from *Nicotiana tabacum* and the corresponding gene has been isolated (Heim et al., 2007; Katoh, Shoji, & Hashimoto, 2007). MPO belongs to a class of copper-dependent diamine oxidases. The copper is required to oxidise a conserved tyrosine residue into a topaquinone, which is essential for enzyme catalysis (Matsuzaki, Fukui, Sato, Ozaki, & Tanizawa, 1994). Evidence suggests that MPO associates with other important enzymes involved in the biosynthesis of nicotine. This has led to the hypothesis that a metabolic channel exists in which a multi-enzyme complex is active. Although no MPO has yet been reported to exist in *E. coca*, a recent study using the remote isotope method has established that the oxidation of *N*-methylputrescine (**25**) in this species is stereoselective. The *pro*-S hydrogen atom is selectively removed in a ratio of 6–10:1 (Hoye, Bjorklund, Koltun, & Renner, 2000). A similar ratio has

been reported for *N. tabacum* and *N. glutinosa,* which strongly suggests that an MPO homolog is present in *E. coca* (Wigle, Mestichelli, & Spenser, 1982).

The specific details of how the second ring in the tropane skeleton is formed are not yet known. It is clear, however, that some type of reaction has to occur with the *N*-methyl-Δ^1-pyrrolidinium cation (**27**). Evidence for the incorporation of *N*-methyl-Δ^1-pyrrolidinium (**27**) into the tropane core structure was observed by feeding [2-^{13}C,^{15}N]-*N*-methylpyrrolinium chloride to coca plants and analysing its incorporation into methylecgonine (**38**) (Leete, Bjorklund, Couladis, & Kim, 1991; Leete, Kim, & Rana, 1988). Several substrates for the condensation have been proposed (Fig. 2.7). Acetic acid, most likely in the form of acetyl-CoA, was observed to be incorporated into carbons C-2, C-3 and C-4 in hyoscyamine (**41**) and into carbons C-3 and C-9 of cocaine (**42**) (Kaczkowski, Schütte, & Mothes, 1961; Leete, 1983a; Liebisch, Peisker, Radwan, & Schütte, 1972). It was suggested at the time that two molecules of acetyl-CoA would first condense to yield acetoacetyl-CoA. The resulting condensation product, hygrine-1-carboxylic acid (**28**), a β-keto acid would have to spontaneously decarboxylate forming the alkaloid hygrine (**29**). An additional oxidation at the C-2 position of hygrine (**29**) would then be required for the final ring closure and subsequent formation of tropinone (**30**). This hypothesis lost favour because it was shown that hygrine (**29**) formation from the condensation of *N*-methyl-Δ^1-pyrrolidinium cation (**27**) with acetoacetyl-CoA can occur non-enzymatically (Endo, Hamaguchi, Hashimoto, & Yamada, 1988). In addition, feeding studies using stable isotopically labelled acetate in *D. stramonium* reported label incorporation only into position C-6 and C-7 of the pyrrolidine ring, instead of the previously reported positions C-2 and C-4 (Duran-Patron, O'Hagan, Hamilton, & Wong, 2000).

A more plausible hypothesis for the second ring closure involves the condensation of malonyl-CoA with the *N*-methyl-Δ^1-pyrrolidinium cation (**27**) (Fig. 2.7). Two successive rounds of decarboxylative condensation would yield the intermediate 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoyl-CoA (**31**). The evidence supporting this hypothesis comes from the feeding of racemic ethyl [2,3-$^{13}C_2$]-4-(*N*-methyl-2-pyrrolidinyl)-3-oxobutanoate to *Datura innoxia* (Abraham & Leete, 1995; Robins, Abraham, Parr, Eagles, & Walton, 1997). Further support for this hypothesis comes from the feeding of methyl (RS)-[1,2-$^{13}C_2$,1-^{14}C]-4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate to leaves of *E. coca* (Leete et al., 1991). This led to the labelling of incorporation in both cocaine (**42**) and methylecgonine (**38**). In plants, these types of condensation reactions are commonly catalysed by type III polyketide

synthases (PKSs). However, stereotypical reactions catalysed by type III PKSs use a CoA-containing starter molecule. No reports in the literature exist for a PKS that would use a substrate such as the *N*-methyl-Δ^1-pyrrolidinium cation (**27**). The formation of hygrine (**29**) in this model can be explained by the spontaneous decarboxylation of 4-(1-methyl-2-pyrrolidinyl)-3-oxobutanoate. In addition, this model also explains the presence of the carbomethoxy group commonly found at the C-2 position in cocaine (**42**) and other TAs found in the Erythroxylaceae. Protection of the carboxylic acid found in the ecgonone-CoA ester (**32**) by the formation of the methyl ester would prevent spontaneous decarboxylation from occurring. The lack of this function in TAs found in the Solanaceae can be explained by the absence of the esterification reaction. Regardless of this functional group, the product formed following the second ring closure will contain a keto group at the C-3 position.

The reduction of the keto group found in tropinone (**30**) and methylecgonone (**36**) has been the subject of intense biochemical research. The first evidence for the stereospecific reduction of the keto function was observed by feeding [9-^{13}C,^{14}C,*O*-methyl-^{3}H]-2-carbomethoxy-3-tropinone to coca plants (Leete, 1983b). At the same time, an enzyme activity specific for the reduction of tropinone (**30**) into tropine (**34**) was purified from the roots of *D. stramonium* (Fig. 2.7) (Koelen & Gross, 1982). It was discovered that the enzyme requires NADPH as a cofactor. A second enzyme-producing pseudotropine (**35**) from the reduction of tropinone was purified from the roots of *H. niger* (Dräger, Hashimoto, & Yamada, 1988). At this time, it was discovered that pseudotropine (**35**) does not spontaneously isomerise to tropine (**34**) (Yamada et al., 1990).

Two genes encoding tropinone reductase I (TRI) and tropinone reductase II (TRII) specific for producing tropine (**34**) and pseudotropine (**35**), respectively, were isolated from the roots of *H. niger* (Hashimoto, Nakajima, Ongena, & Yamada, 1992). Both TRI and TRII belong to the short-chain dehydrogenase/reductase superfamily (SDR), members of which produce a wide variety of both primary and secondary metabolites. Enzymes of the SDR family share a common tertiary structure consisting of the "Rossmann" fold, a conserved motif consisting of six parallel β-sheets and two pairs of α-helices, have a dinucleotide cofactor-binding motif and an active site catalytical motif YxxxK (Moummou, Kallberg, Tonfack, Persson, & van der Rest, 2012). Since their initial isolation, many genes encoding TRI and TRII have been isolated from solanaceous species (Dräger & Schaal, 1994; Kai, Li, et al., 2009; Keiner, Kaiser, Nakajima,

Hashimoto, & Dräger, 2002; Portsteffen, Dräger, & Nahrstedt, 1994). Overall, TRI and TRII share amino acid sequence similarity of more than 50% and are assumed to have evolved from a common ancestor (Dräger, 2006). Although there is significant amino acid sequence differences between the two forms of tropinone reductases, a change of as little as five amino acids is required to change the stereospecificity of the reaction product (Nakajima, Kato, Oda, Yamada, & Hashimoto, 1999). Immunolocalisation experiments performed on potatoes revealed that TRs are localised to the tuber and roots (Kaiser et al., 2006). Recently, a tropinone reductase-like enzyme was discovered in *C. officinalis* (Brassicaceae), which reduces tropinone (**34**). Unlike the stereospecific tropinone reductases from the Solanaceae, this reductase is capable of producing both tropine (**34**) and pseudotropine (**35**) in equal ratios. Designated CoTR, the reductase from *C. officinalis* accepts a much broader range of substrates than other tropinone reductases (Brock, Brandt, & Drager, 2008). Further, phylogenetic analysis reveals that CoTR is more related to other members of the SDR family in the Brassicaceae than it is to either the TRIs or TRIIs found in the Solanaceae.

The first evidence for a polyphyletic origin for TA biosynthesis in plants came from the discovery of an alternate reductase enzyme for the reduction of methylecgonone (**36**) in *E. coca*. A homology-based approach using TR sequences from the Solanaceae identified several TR homologs within the coca transcriptome. However, all these enzymes failed to exhibit methylecgonone-reducing activity when heterologously expressed in *E. coli*. Using classical biochemical techniques, methylecgonone reductase (MecgoR) was purified from crude protein extracts from coca leaves (Jirschitzka et al., 2012). Unlike solanaceous TRs, MecgoR belongs to the aldo-keto reductase (AKR) superfamily of enzymes. MecgoR shares some similarity with chalcone reductase, responsible for the formation of deoxychalcones, as well as codeinone reductase, an enzyme involved in benzylisoquinoline biosynthesis in *Papaver somniferum* (opium poppy). In addition, MecgoR protein has been localised to the palisade parenchyma of young developing leaves. This is in contrast to the root localisation of TRs, catalysing the equivalent step in the roots of solanaceous plants. MecgoR is stereospecific for the production of the 3β-hydroxy-containing metabolite methylecgonine (**38**) (Fig. 2.7). MecgoR is also capable of using tropinone (**30**) as a substrate, however, it produces pseudotropine (**35**) exclusively. This is consistent with the presence of only 3β-hydroxy esters in *E. coca*.

The most common forms of TAs are esterified with either aromatic or aliphatic acids. The stereochemistry of the hydroxyl group is determined by the respective reductase. In *D. stramonium*, the accumulation of 3-acetyl-tropine occurs in cultured roots. Biochemical separation of this acyltransferase activity was performed, revealing that the enzymes responsible for the reaction utilise acetyl-CoA as the activated acid (Robins, Bachmann, Robinson, Rhodes, & Yamada, 1991). Further biochemical studies using *D. stramonium* successfully purified an acyltransferase, which utilised tigloyl-CoA to esterify the 3β-hydroxy group of pseudotropine (**35**) (Rabot, Peerless, & Robins, 1995). This 65-kDa enzyme was active in its monomeric form and could accept a wide variety of different acyl-CoA thioesters when using pseudotropine (**35**). However, attempts to use tropine (3α-hydroxytropane) (**34**) were unsuccessful.

Cocaine (**42**) is a benzoic acid ester of methylecgonine (**38**) (Fig. 2.7). Feeding studies using *trans*-[3-^{13}C,^{14}C]-cinnamic acid and the *N*-acetylcysteamine thioester of [3-^{13}C,^{14}C]-*trans*-cinnamic acid led to the prediction that the acyltransferase in *E. coca* utilises benzoyl-CoA (**39**) as the activated acid (Bjorklund & Leete, 1992). Although no gene sequences encoding acyltransferases involved in tropane ester formation have been reported, it is likely that these enzymes will be members of the BAHD family (D'Auria, 2006). This family of enzymes is responsible for the esterification of a wide range of plant-specific metabolites and uses CoA thioesters as co-substrates. In addition, BAHD-type enzymes are monomeric and similar in size to tigloyl-CoA:pseudotropine acyl transferase.

One step in TA side chain biosynthesis that has drawn particular interest is the rearrangement of the hydroxyl group of the phenyllactic acid moiety of littorine (**40**), which occurs in atropine (**1**) and scopolamine (**44**) formation (Fig. 2.7). In this process, a branched-chain residue, tropic acid, is formed from the straight chain phenyllactic acid. Many radiolabelled feeding studies have been performed to understand the mechanisms involved in this reaction (Ansarin & Woolley, 1994; Chesters, O'Hagan, & Robins, 1995; Leete, Kowanko, & Newmark, 1975; Robins, Bachmann, & Woolley, 1994; Robins et al., 1995). The pervading hypothesis for the conversion of the littorine (**40**) precursor into the hyoscyamine (**41**) product predicts that a cytochrome p450 coupled with an alcohol dehydrogenase is involved. These predictions are based on both feeding study results as well as quantum chemistry calculations (Sandala, Smith, & Radom, 2008). Using virus-induced gene silencing techniques, Li et al. (2006) were able to suppress the expression of the cytochrome p450 CYP80F1. This reduced the levels

of hyoscyamine (**41**) and encouraged the accumulation of littorine (**40**). The involvement of a p450 was successfully probed by performing enzyme assays with synthetic deutero and arylfluoro analogues of littorine (Nasomjai et al., 2009).

The conversion of hyoscyamine (**41**) into the epoxide scopolamine (**44**) is catalysed by hyoscyamine 6β-hydroxylase (Fig. 2.7) (H6H). This enzyme was purified from *H. niger* and was shown to be a 2-oxoglutarate-dependent dioxygenase (Hashimoto, Matsuda, & Yamada, 1993; Hashimoto & Yamada, 1986). H6H catalyses a two-step reaction in which the hydroxylation at the C-6 position of hyoscyamine (**41**) is followed by epoxidation of the corresponding intermediate, anisodamine (6β-hydroxy hyoscyamine) (**43**). Further, the enzyme was determined to be localised exclusively to the pericycle of roots (Hashimoto et al., 1991). Information about the enzymes involved in the polyhydroxylation of the tropane ring skeleton resulting in the formation of calystegines (**37**) has not yet been determined. However, homology-based approaches using H6H as well as co-expression analysis with cytochrome p450s may be beneficial.

4. METABOLIC ENGINEERING OF TROPANE ALKALOIDS

In recent years, the majority of research on tropane alkaloid biosynthesis has focused on the engineering of increased levels of commercially important metabolites such as scopolamine (**44**) and atropine (**1**). These compounds are traditionally difficult to produce via chemical synthesis. Extensive reviews about this subject have been written by Zhang et al. (2005) and Palazon, Navarro-Ocana, Hernandez-Vazquez and Mirjalili (2008). All of these studies have used hairy root cultures rather than intact plants because TA biosynthesis in solanaceous species occurs in below-ground tissues (Zhang et al., 2005). This approach assumes that all the enzymatic machinery needed to supply important starting substrates is also present in these tissues. *Agrobacterium rhizogenes* is used to ensure that the roots can grow rapidly in hormone-free media and can also be used in genetic transformation (Chandra & Chandra, 2011). Because of our limited knowledge about the structural genes involved in TA biosynthesis, the choice of candidates has been restricted to ODC, PMT, TR and H6H. In an attempt to increase polyamine starter substrates to the pathway, an ODC gene isolated from mouse was overexpressed under the control of the CaMV 35S promoter in *D. innoxia*. The production of scopolamine (**44**) increased as much as six times over that observed in the controls.

The use of a non-plant-derived ODC may have avoided the common problem of transgene silencing that often occurs in genetically modified plants (Singh et al., 2011).

Transformation of a multigene construct for the overproduction of TAs was first reported by Zhang et al. (2004). Since the publication of the successful results of this attempt, several other groups have repeated this method in other solanaceous root cultures. Combining the overexpression of both PMT and H6H in *A. belladonna* has resulted in approximately a 2.5-fold increase of scopolamine above wild-type levels (Liu et al., 2010). A separate experiment, again using this same combination of genes, was able to increase hyoscyamine (**41**) levels to more than 24 times that found in wild-type *A. belladonna* (Yang et al., 2011). Using the plant *Scopolia parviflora*, a similar construct using PMT and H6H was used to make transgenic hairy roots. Increases in both hyoscyamine (**41**) and scopolamine (**44**) were observed in addition to an increase in the growth rate of the roots when compared with the no-insert transgenic controls. This growth increase could be attributed to the role of polyamines in the processes of growth and cell differentiation (Kang et al., 2011). A different combination of genes encoding PMT and TRI to make transgenic hairy root cultures of *Anisodus acutangulus* resulted in an overall increase in TAs that was 3–8 times higher than in the control lines (Kai et al., 2011). The same group, using a new construct consisting of the genes TRI and H6H, was able to observe a fivefold increase in total TA levels. The same construct also led to an improvement in the accumulation of anisodamine (**43**) when compared to both wild-type root cultures and transgenic root cultures using the PMT/TRI construct (Kai, Zhang, et al., 2012).

The induction of plant defense responses can also be beneficial to metabolic engineers wishing to increase the quantity of their targeted metabolites. For example, the treatment of hairy root cultures of *H. niger* with the phytohormone methyl jasmonate (MeJA) resulted in a fivefold increase in scopolamine (**44**) levels (Zhang et al., 2007). Table 2.1 summarises the known organic and inorganic elicitors used to increase TA production. Many other elicitors of plant defense metabolism have also been successful in increasing TA levels in root cultures. Besides MeJA, both yeast elicitor and abscisic acid treatment were capable of increasing TAs in *A. acutangulus* (Luo et al., 2012). Salicylic acid (SA), another common plant defense hormone, is often antagonistic to MeJA-induced responses (Niki, Mitsuhara, Seo, Ohtsubo, & Ohashi, 1998). While SA was not effective in increasing scopolamine levels in transgenic hairy root cultures of *Atropa*

Table 2.1 A summary of the biotic and abiotic elicitors used as treatments for increasing tropane alkaloid content in plants

Plant	Elicitor	Effect	Target	Reference
Atropa baetica (hairy root culture)	MeJa	Increase	Hyoscyamine	el Jaber-Vazdekis, Barres, Ravelo, and Zarate (2008)
	ASA	Increase	Anisodamine	
	SA	None	Scopolamine	
Datura metel (hairy root culture)	SA	Increase	Hyoscyamine	Ajungla, Patil, Barmukh, and Nikam (2009)
	Fungal extract	Increase	Scopolamine	
	Yeast extract	Increase		
	Aluminium chloride	Increase		
Hyoscyamus niger (hairy root culture)	Chitosan	Decrease	Hyoscyamine	Hong, Bhatt, Ping, and Keng (2012)
	Casein	None	Scopolamine	
	Yeast extract	Increase		
	D-sorbitol	None		
Anisodus acutangulus (hairy root culture)	MeJA	Increase	Hyoscyamine	Kai, Yang, et al. (2012)
	Silver nitrate	Increase	Anisodamine	
	Ethanol	Increase	Scopolamine	
	SA/ethanol	Decrease except anisodine	Anisodine	
Datura stramonium (hairy root culture)	Nitrate	Increase	Hyoscyamine	Amdoun et al. (2009)
	Phosphate	Increase		
	Calcium	Increase		
	Nitrate/ calcium	Increase		
	Nitrate/ phosphate	Decrease, none		
	Phosphate/ calcium	Decrease, none		

Continued

Table 2.1 A summary of the biotic and abiotic elicitors used as treatments for increasing tropane alkaloid content in plants—cont'd

Plant	Elicitor	Effect	Target	Reference
Datura innoxia (cultured plantlets)	Aluminium chloride	Increase	Hyoscyamine	Karimi and Khataee (2012)
			Scopolamine	
Anisodus acutangulus (whole plant)	MeJA	Increase	Hyoscyamine	Luo et al. (2012)
	Yeast extract	Increase	Scopolamine	
	ABA	Increase except scopolamine	Anisodine	
Brugmansia suaveolens (whole plant)	MeJA	Increase	Scopolamine	Alves et al. (2007)

MeJA, methyl jasmonate; SA, salicylic acid; ASA, acetylsalicylic acid; ABA, abscisic acid.

baetica, acetyl salicylic acid (ASA) significantly increased gene transcript levels for several TA structural genes (el Jaber-Vazdekis et al., 2008). The use of heavy metal elicitors has also been shown to successfully increase the production of TAs. For example, both trivalent chromium [Cr(III)] and aluminium are capable of increasing the hyoscyamine (**41**) and scopolamine (**44**) content in *A. belladonna* and *D. innoxia* (respectively) (Karimi & Khataee, 2012; Vakili, Karimi, Sharifi, & Behmanesh, 2012). However, caution must be used when working with these types of elicitors because of their negative effects on plant growth and development.

Scaling-up of hairy root cultures will be required for any large-scale production of TAs. Bioreactors are therefore of particular interest, and both bubble column and stirred tank types have successfully been employed for the task of overproduction of TAs (Cardillo et al., 2010; Min et al., 2007). Optimisation of the media used in root culture must be performed in order to increase TA production while at the same time considering the growth rate of the culture. For example, increasing nitrate concentrations in *A. belladonna* hairy root cultures yielded higher amounts of TAs, at the same time reducing the overall growth of the culture (Chashmi, Sharifi, Karimi, & Rahnama, 2010). Other techniques that may prove helpful in the future for commercial production of TAs in culture include exploiting the process of exudation. This process has been documented

to be successful for several other hairy root culture systems and includes the alteration of membrane permeability and development of constant extraction and removal of the metabolites of interest from the medium (Cai, Kastell, Knorr, & Smetanska, 2012). Other approaches that may be useful in increasing TA yield in culture include the use of specific transcription factors for the pathway and the employment of promoters appropriate for hairy roots (Jirschitzka, Mattern, Gershenzon, & D'Auria, 2013).

5. CONCLUSIONS

In contrast to other alkaloid classes such as the benzyl isoquinoline and terpene indole alkaloids, the knowledge base available for both the biosynthesis and molecular biology of tropane alkaloids is relatively small. Future prospects to increase this knowledge base will require a broadening of the model systems currently being used, especially those outside of the Solanaceae. The polyphyletic origin of TAs in plants, in conjunction with the multiple possibilities for starter substrates and the ring closure steps, strongly suggests that different enzyme classes have been recruited during the diversification of TA-producing plant lineages. The increasing ease of gene discovery through the constantly decreasing costs of high-throughput sequencing should facilitate detailed biochemical investigations into members of these other families.

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3.2 Manuscript II

Plant tropane alkaloid biosynthesis evolved independently in the Solanaceae and Erythroxylaceae

Plant tropane alkaloid biosynthesis evolved independently in the Solanaceae and Erythroxylaceae

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The pharmacologically important tropane alkaloids have a scattered distribution among angiosperm families, like many other groups of secondary metabolites. To determine whether tropane alkaloids have evolved repeatedly in different lineages or arise from an ancestral pathway that has been lost in most lines, we investigated the tropinone-reduction step of their biosynthesis. In species of the Solanaceae, which produce compounds such as atropine and scopolamine, this reaction is known to be catalyzed by enzymes of the short-chain dehydrogenase/reductase family. However, in *Erythroxylum coca* (Erythroxylaceae), which accumulates cocaine and other tropane alkaloids, no proteins of the short-chain dehydrogenase/reductase family were found that could catalyze this reaction. Instead, purification of *E. coca* tropinone-reduction activity and cloning of the corresponding gene revealed that a protein of the aldo-keto reductase family carries out this reaction in *E. coca*. This protein, designated methylecgonone reductase, converts methylecgonone to methylecgonine, the penultimate step in cocaine biosynthesis. The protein has highest sequence similarity to other aldo-keto reductases, such as chalcone reductase, an enzyme of flavonoid biosynthesis, and codeinone reductase, an enzyme of morphine alkaloid biosynthesis. Methylecgonone reductase reduces methylecgonone (2-carbomethoxy-3-tropinone) stereospecifically to 2-carbomethoxy-3β-tropine (methylecgonine), and has its highest activity, protein level, and gene transcript level in young, expanding leaves of *E. coca*. This enzyme is not found at all in root tissues, which are the site of tropane alkaloid biosynthesis in the Solanaceae. This evidence supports the theory that the ability to produce tropane alkaloids has arisen more than once during the evolution of the angiosperms.

convergent evolution | pseudotropine | immunoprecipitation | immunolocalization

Tropane alkaloids consist of over 200 known compounds with a tropane ring in their structures, such as the anticholinergic drugs atropine and scopolamine and the stimulant cocaine (1). Like other plant secondary metabolites, tropane alkaloids have a scattered distribution in the angiosperms, being reported from seven families, including the Proteaceae, Convolvulaceae, Brassicaceae, Euphorbiaceae, Rhizophoraceae, Solanaceae, and Erythroxylaceae (2), many of which are taxonomically distant from one another. For example, the Erythroxylaceae and Solanaceae are members of completely different clades, and their last common ancestor is believed to have lived ~120 million y ago (3). The uneven distribution of tropane alkaloids in the angiosperms and the evolutionary distances between those families producing them suggest two alternative hypotheses: Either the ability to produce tropane alkaloids has arisen independently in multiple plant lineages, or the tropane alkaloid biosynthetic pathway was present in an ancestor basal to much of the angiosperms and was subsequently lost in most lineages. Determining which hypothesis is correct may help explain why many other groups of secondary metabolites also have scattered distributions among the angiosperms.

Studies of the biosynthesis of tropane alkaloids have been predominantly performed with members of the Solanaceae and to a lesser extent with cultivated species of the Erythroxylaceae. The majority of these studies used in vivo feeding of radiolabeled precursors (4–6) to elucidate the outlines of a general tropane alkaloid biosynthetic pathway (7, 8). Biosynthesis is initiated from the polyamine putrescine, which is derived from the amino acids ornithine or arginine (Fig. S1). Putrescine becomes *N*-methylated via the action of putrescine methyltransferase in what is generally considered to be the first committed step in tropane alkaloid production (9). This compound is then oxidized to yield 4-(methyl-1-amino)butanal, which under normal physiological conditions is thought to spontaneously rearrange into the five-membered-ring compound *N*-methyl-Δ^1-pyrrolinium. The formation of the second ring in tropane alkaloid biosynthesis is a subject of debate. One hypothesis is that *N*-methyl-Δ^1-pyrrolinium condenses with acetoacetate and the ring closure occurs following oxidation and another round of aldol condensation (10). Another hypothesis is that the *N*-methyl-Δ^1-pyrrolinium acts as a starter unit for two rounds of polyketide-type extension with malonyl-CoA (11). In both schemes, the resulting second ring contains a keto function at the 3 position. Reduction at this position is required for subsequent ester formation.

In members of the Solanaceae, reduction of the keto group in the tropane ring is catalyzed by enzymes known as tropinone reductases (TRs) (12). These enzymes belong to the short-chain dehydrogenase/reductase (SDR) enzyme family, which are NAD(P)(H)-dependent monomeric oxidoreductases with low sequence identities and a catalytic Asn-Ser-Tyr-Lys tetrad (13). There are two separate tropinone reductases (TRI and TRII) in the Solanaceae, and they lead to a significant bifurcation of tropane alkaloid biosynthesis in this family. TRI converts the 3-keto function exclusively to a product with a 3α-configuration, producing a tropine (3α-tropanol), which is the precursor to a variety of esterified tropane alkaloids, such as atropine. On the other hand, TRII produces exclusively an alcohol with a 3β-configuration, referred to as pseudotropine (3β-tropanol), which is then converted to various nonesterified tropane alkaloids called calystegines. The occurrence of these two separate TRs in the Solanaceae has been attributed to a gene-duplication event (14). TRs are characteristic of tropane alkaloid biosynthesis, and are even proposed to be limited to tropane alkaloid-producing plants (15). However, it is unclear whether

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all tropane alkaloid biosynthesis relies on TRs for catalysis. Besides the TRs of the short-chain dehydrogenase/reductase family, there are several other major groups of plant proteins that contain enzymes that could supply reductase activity for tropane alkaloid biosynthesis, including the medium-chain dehydrogenase/reductases, the aldehyde dehydrogenases, and the aldo-keto reductases.

To determine whether tropane alkaloid formation in distant angiosperm lineages has a common evolutionary origin, we investigated the tropinone-reduction step in *Erythroxylum coca* (Erythroxylaceae), a species that accumulates cocaine and other tropane alkaloids in abundance (16) and is taxonomically very remote from the Solanaceae. For the tropane alkaloids of *E. coca*, tropinone reduction involves the conversion of 2β-carbomethoxy-3-tropinone (methylecgonone) to 2β-carbomethoxy-3β-tropine (methylecgonine). Interestingly, tropinone reductases from the Solanaceae have not been shown to have significant methylecgonone reducing activity (15, 17), suggesting the existence of a different type of catalyst in the Erythroxylaceae.

In this study, we report the biochemical and molecular characterization of the tropinone-reduction step of tropane alkaloid biosynthesis in *E. coca*, which converts methylecgonone to methylecgonine, the penultimate step in cocaine biosynthesis. Our initial approach was based on the assumption that this reduction step is carried out by a TR-like member of the SDR family of reductases. However, homology-based cloning and heterologous expression of TR-like sequences from organs involved in tropane alkaloid biosynthesis failed to yield an enzyme with the requisite activity. We therefore purified the tropinone reductase activity from *E. coca* leaves, isolated the corresponding gene, and found it to encode a member of the aldo-keto reductase protein family. The result demonstrates that a major enzyme of tropane alkaloid biosynthesis has been independently recruited in the Solanaceae and the Erythroxylaceae, and suggests that the entire pathway has evolved more than once in the evolution of the angiosperms.

Results

Homology-Based Attempt to Isolate a Methylecgonone Reductase from *E. coca* Was Not Successful. The tropane alkaloid biosynthetic pathway in the Erythroxylaceae is thought to be similar to that in the Solanaceae (7, 18). Therefore, enzymes such as the tropinone reductases identified in the Solanaceae that reduce tropinone to tropines should exist as homologs in the Erythroxylaceae with the ability to reduce methylecgonone (2-carbomethoxy-3-tropinone) to methylecgonine (2-carbomethoxy-3β-tropine) (Fig. 1 and Fig. S1). Starting with this hypothesis, known TR protein sequences (GenBank accession nos. AAA33281, AAA33282, ACG34080, CAC34420, CAD20555, BAA85844, AAB09776, and CAB52307) were blasted individually with "tblastn" against an *E. coca* EST library (19). Three sequences were identified that encoded for polypeptides ranging between 258 and 275 amino acids (GenBank accession nos. JQ015102, JQ015103, and JQ015104). The full-length ORFs of these tropinone reductase-like genes were cloned and expressed in *Escherichia coli*, and the crude supernatants were tested for TR activity. To verify the appropriateness of the assay conditions, *Solanum tuberosum* tropinone reductase II (GenBank accession no. CAB52307) was used as a positive control. The expressed empty pET-28a vector served as a negative control. Tropinone reductase activity was detected in the positive control and the crude plant extract, but not for any of the heterologously expressed tropinone reductase-like enzymes from *E. coca*.

Methylecgonone Reductase Activity Was Detected in Various Above-Ground Organs of *E. coca*. Although none of the proteins encoded by tropinone reductase-like genes from *E. coca* exhibited any detectable TR activity, it was possible to detect this activity in crude plant extracts. Assays of desalted plant extracts from various organs showed the ability to reduce 2-carbomethoxy-3-tropinone (methylecgonone) to 2-carbomethoxy-3β-tropine (methylecgonine), the expected reaction in the biosynthesis of cocaine. Methylecgonine was initially identified by comparison of chromatographic retention time and mass spectral fragmentation patterns with those reported in the literature for an authentic standard and later confirmed by ^{1}H NMR comparison (see below). The highest

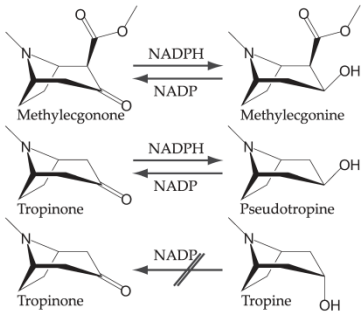

Fig. 1. MecgoR catalyzes the reduction of methylecgonone (2-carbomethoxy-3-tropinone) to methylecgonine (2-carbomethoxy-3β-tropine) and tropinone to 3β-tropine (pseudotropine). The oxidation in the reverse direction is possible, but stereoselective: The 3β-isomers are converted, but not the 3α-isomers.

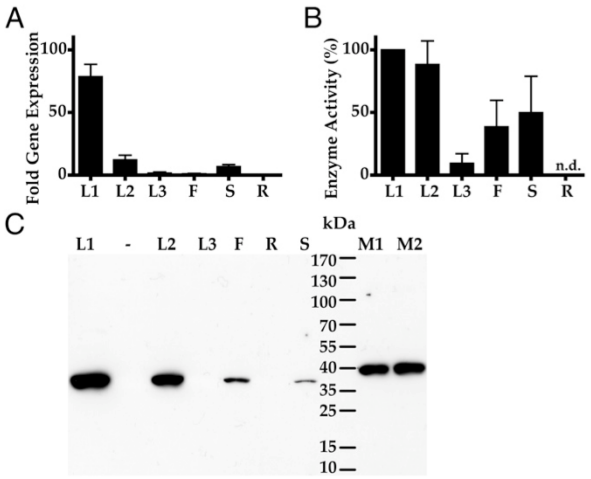

Fig. 2. Comparison of methylecgonone reductase gene expression, enzyme activity, and protein levels in different *E. coca* organs and development stages: leaf stage 1 (L1), leaf stage 2 (L2), leaf stage 3 (L3), flowers (F), stems (S), and roots (R). n.d., not detected. (*A*) Relative transcript levels of *MecgoR* in 4-mo-old plants normalized to internal reference genes. Transcript levels in the flowers were set to a value of 1. Values displayed are means ± SD of three technical replicates from each of three biological replicates. DNA blot analysis and other information (Fig. S7) suggest that *MecgoR* is a single-copy gene. (*B*) Enzyme activity. Depicted are the means of activity obtained from desalted protein extracts from three replicates of each organ and developmental stage. Highest activity was detected in stage 1 leaves at 249.4 pkat (mg protein)$^{-1}$. The reaction was run in the reducing direction, converting methylecgonone to methylecgonine. (*C*) Protein levels were determined by immunoblotting. Samples consisting of 50 μg of protein extracted from each organ as well as 250 ng (M1) and 500 ng (M2) 9× His-tagged MecgoR (40.4 kDa) were run on SDS/PAGE and gels were blotted onto filters. The filters were first probed with anti-MecgoR antibodies, followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Polyclonal antibodies are specific for MecgoR protein (Fig. S8). Bands were visualized with chemiluminescence.

methylecgonone reductase (MecgoR) activity was found in the youngest leaves (stage 1; Fig. S2) at 249.4 pkat (mg protein)$^{-1}$ (Fig. 2*B*), and the next-highest activity in stage 2 leaves. Stem and flower extracts exhibited less than 20% of the activity of the youngest leaves. No methylecgonone reducing activity was detected in either the stage 3 (mature) leaves or the roots.

Localization of MecgoR activity is consistent with the previously reported localization of alkaloid (cocaine and cinnamoyl cocaine) content in *E. coca* plants (20). No alkaloids were detected in roots, with only low cocaine and cinnamoyl cocaine levels found in the stem. The highest alkaloid levels (cinnamoyl cocaine) were present in the early leaf developmental stages, the same stages that show high MecgoR activity in the present study.

MecgoR Activity Was Purified from *E. coca* and the Corresponding Gene Was Isolated. Because we were able to readily detect MecgoR activity in young leaves, this activity was purified from young leaf extracts (Table S1). After ammonium sulfate fractionation and purification of the 40–80% ammonium sulfate pellet via hydrophobic-interaction chromatography (phenyl Sepharose), a >100-fold purification was achieved. Purity was increased over fourfold further by dye-interaction chromatography on a column with the Cibacron blue F3G-A dye, which has been previously shown to bind proteins that use nucleotide cofactors (21), such as the NADPH used in the reduction of methylecgonone. Additional purification was achieved by anion-exchange chromatography, although there was a drop in the specific activity, most likely due to the interference of high salt concentrations in the enzyme assay. The fraction containing the highest activity was subsequently subjected to gel filtration to determine a mass of 36.37 kDa for the active protein.

A preparative 2D gel of the active fraction from the anion-exchange column revealed over 30 spots following staining with colloidal Coomassie blue. All visually identified proteins were then eluted for de novo sequencing via protein mass spectrometry, and the resulting peptide fragment sequences corresponding to the isolated proteins were blasted against the *E. coca* EST library (22). Three full-length sequences coding for polypeptides of 253–364 amino acid residues were isolated and cloned into the expression vector pH9GW for heterologous expression in *E. coli* (GenBank accession nos. GU562618, JQ804916, and JQ804917) (Fig. S3). One polypeptide consisting of 327 amino acid residues exhibited reducing activity with both tropinone and methylecgonone, and was designated MecgoR.

Immunoprecipitation Confirmed That the Protein Encoded by the *MecgoR* Gene Is Responsible for the Corresponding Activity *in Planta*. Polyclonal antibodies were produced against the heterologously expressed MecgoR protein. These reduced the methylecgonone reductase activity of the young leaf extract to 6% of that of a control extract without antibodies. Preimmune serum reduced enzyme activity to 60% of the control. The immunoprecipitated proteins were separated on a protein gel and sequencing identified MecgoR within the precipitate formed by the anti-MecgoR antibodies, but this protein was not detected in the precipitate formed by the preimmune serum.

MecgoR Is Not Related to the TRs of the Solanaceae, but Is a Member of the Aldo-Keto Reductase Protein Family. Amino acid analysis of MecgoR revealed that the enzyme is most similar to three proteins from the aldo-keto reductase (AKR) enzyme family: chalcone reductase from *Sesbania rostrata*, codeinone reductase from *Papaver somniferum*, and deoxymugineic acid synthase from *Zea mays*. An alignment with these three sequences revealed that MecgoR shares 48%, 50%, and 58% identity at the amino acid level with deoxymugineic acid synthase, codeinone reductase, and chalcone reductase, respectively. MecgoR and other proteins of the AKR family are distinct from the tropinone reductases of the Solanaceae, which are assigned to the short-chain dehydrogenase/reductase family. The overall identity of MecgoR with any TRI or TRII is less than 10% at the amino acid level. MecgoR is also not closely related to members of the two other large protein families containing reductases, the medium-chain dehydrogenases/reductases and the aldehyde dehydrogenases (Fig. 3).

MecgoR Shows Considerable Specificity for Substrate, Cofactor, and Other Properties. To obtain enough pure protein for biochemical characterization, the coding region of MecgoR was cloned into a vector that introduced an N-terminal strep tag and was used for heterologous expression in the yeast *Pichia pastoris*. The recombinant protein increased in size from 36.9 kDa to 39.8 kDa with the N-terminal strep tag, and was purified to homogeneity following StrepTrap HP and HiTrap Blue affinity chromatography. The pH optimum for the reduction of methylecgonone to methylecgonine was determined to be 6.8, and 9.8 for the reverse reaction. When preincubated at 4, 25, 37, 50, and 65 °C for 30 min, MecgoR activity was reduced over 30% at 25 °C and over 70% at 37 °C compared with the 4 °C sample and was undetectable at higher temperatures. MecgoR activity was not stimulated by the addition of monovalent or divalent metal cations. Cu^{2+} and Zn^{2+} had a strong inhibitory effect on enzyme performance when tested at concentrations of 5 mM, reducing activity to 8% and 58%. However, both Co^{2+} and Fe^{2+} only inhibited MecgoR by less than 30%.

Of all of the compounds tested as substrates besides methylecgonone, only 6-hydroxytropinone, tropinone, and nortropinone were reduced to their corresponding alcohols with 45%, 36%, and 6%, respectively, of the activity with methylecgonone as substrate (Fig. S4). No activity was detected for 8-thiabicyclo [3.2.1]octan-3-one (TBON), cyclohexanone, cyclooctanone, and

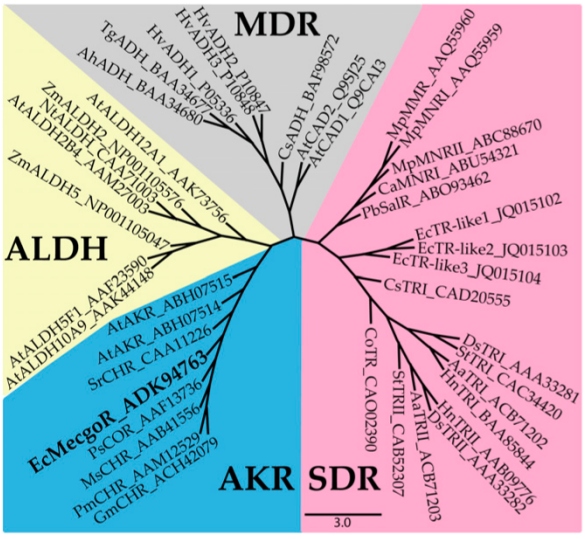

Fig. 3. Phylogenetic relationships of reductases of plant secondary metabolism. Selected reductases of four reductase superfamilies were aligned using the CLUSTAL X program with standard settings for protein alignment. The phylogenetic tree was built by the Bayesian method using the MRBAYES program. The four reductase superfamilies shown are ALDH (aldehyde dehydrogenases), SDR (short-chain dehydrogenases/reductases), MDR (medium-chain dehydrogenases/reductases), and AKR (aldo-keto reductases). MecgoR, which belongs to the AKR family, is depicted in bold. The tropinone reductases carrying out similar reactions in the biosynthesis of tropane alkaloids in the Solanaceae belong to the SDR family. The scale bar represents 3.0 amino acid substitutions per site. Please refer to Table S2 for an explanation of the abbreviated names.

N-methyl-4-piperidone. The Michaelis–Menten constant (K_m) for methylecgonone and NADPH were 69.6 ± 14.1 μM and 5.1 ± 0.9 μM, respectively. For the reverse reaction in the oxidative direction, K_m values of 151.2 ± 16.8 μM and 29.6 ± 5.3 μM were determined for methylecgonine and NADP, respectively (Table 1). Substrate inhibition was also observed, and the inhibitory constant (K_i) was 1,136.0 ± 257.9 μM for methylecgonone, 2,480.0 ± 1,149.0 μM for NADPH, 2,673.0 ± 484.6 μM for methylecgonine, and 506.3 ± 97.3 μM for NADP. NADH can successfully substitute for NADPH in the reduction of methylecgonone, but the activity is decreased to 14% of the value with NADPH. No activity was detected in the absence of a nucleotide cofactor.

The stereochemistry of the MecgoR reaction was first studied with tropinone as substrate. The configuration of the product was determined by comparing its ^{1}H NMR spectrum to those of tropine (3α-tropanol) and pseudotropine (3β-tropanol) (Fig. S5). The product of the reduction of tropinone was found to be pseudotropine, without any trace of tropine detected. To confirm this, the reverse assay was performed with both tropine and pseudotropine as substrates, but only pseudotropine was converted to tropinone (Fig. 1). When the native substrate methylecgonone was used, the ^{1}H NMR spectrum of the product was compared with that of a methylecgonine standard (Fig. S5). Although 3α-methylecgonine was not available for comparison, the spectrum of the product of the reduction of methylecgonone was found to match that of the commercial methylecgonine standard, including the expected pattern of signals for the H-3 position. Thus, the reduction of methylecgonone to methylecgonine is stereospecific, with the product C3-alcohol having exclusively a β-configuration.

***MecgoR* Gene Expression Was Highest in the Youngest Leaf Stage.** To compare the MecgoR enzymatic activity obtained from crude plant extracts of various organs and developmental stages with the expression of the *MecgoR* gene, quantitative real-time PCR was performed with transcript levels normalized to those in the flowers, the organ exhibiting the lowest detectable amounts of transcript (Fig. 2*A*). *MecgoR* expression was highest in stage 1 leaves, with an expression of 78-fold relative to the flowers. Transcript levels dropped to a value of 11-fold for stage 2 leaves, followed by 1.5-fold for stage 3 leaves. The stems had average transcript levels of 6.7-fold compared with flowers. *MecgoR* transcripts were not detected in the roots at a level above that in reactions with no-RNA controls.

MecgoR Protein Levels Were also Highest in Young Leaves. The polyclonal antibodies raised against MecgoR were used to detect protein in different plant organs and developmental stages. Equal amounts of extracted protein from different organs were separated by a protein gel, transferred, and subjected to immunoblotting. MecgoR was detected as a single band with a size of ~37 kDa in stage 1 and 2 leaves, flowers, and stems, in decreasing order of abundance. No MecgoR protein was detected in either the stage 3 leaves or in the roots.

The heterologously expressed His-tagged MecgoR was used as a control on the gel and appears slightly bigger due to the addition of 31 amino acids from the tag (Fig. 2*C*). The co-occurrence of the highest amount of MecgoR protein with the highest amount of gene transcript and the highest enzyme activity in stage 1 leaves suggests that MecgoR activity is controlled at the transcriptional level.

MecgoR Is Localized in the Leaf Mesophyll. Based on the immunoblot results of various organs, young leaves and flowers were used for subsequent tissue-level immunolocalization experiments. Cross-sections of young leaves and longitudinal sections of flowers are depicted in Fig. 4 and Fig. S6, respectively. Preimmune serum was used as a negative control, and the fluorescence signal obtained from these samples was considered unspecific. Whereas there was only little fluorescence detected in the preimmune images of all three analyzed organs, a strong signal was detected after the application of anti-MecgoR antibodies. The overlay of transmitted light and fluorescent images localized the detected signal to the palisade and spongy mesophyll tissue of leaves and sepals. No MecgoR signal was detected in the upper or lower epidermis tissue or cuticle of these organs, and none in any tissue in the stems, roots, and mature leaves (Fig. 4).

Discussion

Tropane alkaloids, like other major categories of plant secondary metabolites, are scattered among the major clades of angiosperms. They are present in four major lineages of dicotyledons: the peripheral eudicots (Proteaceae), the malvid (Brassicaceae) and fabid (Erythroxylaceae, Rhizophoraceae, Euphorbiaceae) clusters of the rosid lineage, and the lamid cluster of the asterid lineage (Solanaceae, Convolvulaceae) (23). To determine whether this distribution is a result of independent evolution or a shared ancestral angiopserm pathway that was selectively lost in most lineages, we compared a key step of tropane biosynthesis between the Solanaceae and the Erythroxylaceae. The reduction of tropinone to tropine or pseudotropine in Solanaceae species is catalyzed by the well-described tropinone reductase enzymes of the SDR family (24). Screening of an *E. coca* young leaf EST database yielded three distinct TR-like genes; however, the corresponding proteins exhibited no reducing activity either with the substrate tropinone, typical of the Solanaceae, or the substrate methylecgonone, typical of the Erythroxylaceae. The methylecgonone reductase of *E. coca*, identified by purification of the enzymatic activity from an extract of young leaves and sequencing of the resulting protein, proved to be a member not of the short-chain dehydrogenase/reductase family but rather of the aldo-keto reductase family.

Confirmation of MecgoR's role in tropane alkaloid biosynthesis came from immunoprecipitation experiments. In addition, the stereospecificity of the expressed protein (all products had a β-configuration at C-3) perfectly matched the C-3–configuration of the tropane alkaloids reported from *E. coca* (25). Both 3α- and 3β-tropane alkaloids are known in nature. In the Solanaceae, one group of TRs forms 3α-configured products and

Table 1. Kinetic constants for *E. coca* MecgoR reaction measured in forward (reductive) and reverse (oxidative) directions

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($M^{-1}s^{-1}$)	K_i (μM)
Methylecgonone	69.6 ± 14.1	0.240 ± 0.023	3,443.7	1,136.0 ± 257.9
NADPH	5.1 ± 0.9	0.121 ± 0.005	24,052.1	2,480.0 ± 1,149.0
Methylecgonine	151.2 ± 16.8	5.538 ± 0.329	36,627.0	2,673.0 ± 484.6
NADP	29.6 ± 5.3	5.238 ± 0.418	176,704.8	506.3 ± 97.3

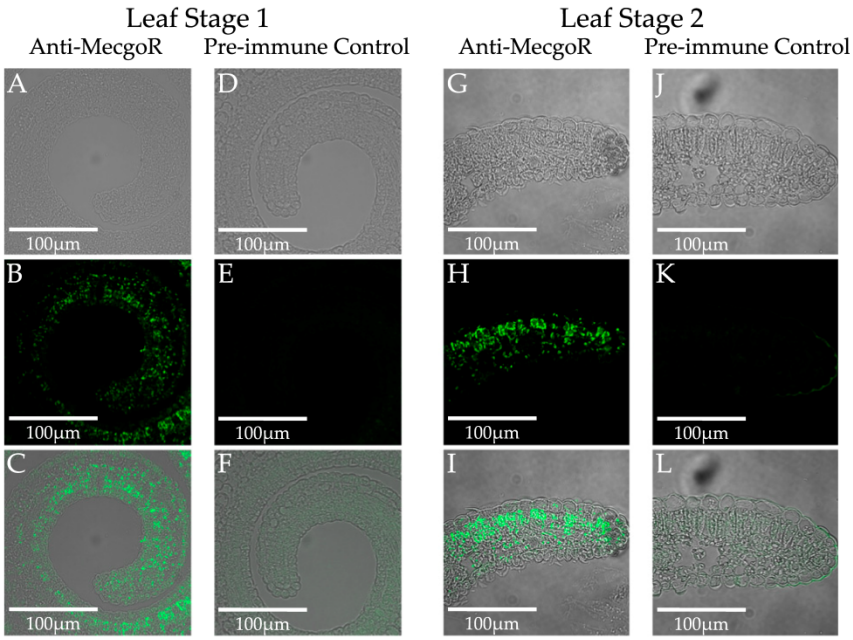

Fig. 4. Immunolabeling of MecgoR in cross-sections of *E. coca* leaf stage 1 (*A–F*) and leaf stage 2 (*G–L*). The samples were labeled with either anti-MecgoR antibodies (*A–C* and *G–I*) or preimmune serum (*D–F* and *J–L*). (*Top*) Transmitted light (*A*, *D*, *G*, and *J*). (*Middle*) Fluorescent images (*B*, *E*, *H*, and *K*). (*Bottom*) Overlays of transmitted light and fluorescent images (*C*, *F*, *I*, and *L*). Single sections were probed with primary antibody (anti-MecgoR or preimmune serum) and secondary antibody (anti-rabbit conjugated to horseradish peroxidase assayed with chemiluminescent substrate). Fluorescence excitation was at 543 nm and detection used a BP 585–615 filter.

the other group forms products with a 3β-configuration (12), with several 3α- and 3β-configured products becoming esterified (1).

E. coca MecgoR belongs to the AKR protein family, a large group of enzymes not restricted to plants but also described from mammals, amphibians, yeast, protozoa, and bacteria (26). Previously characterized AKR proteins are very diverse in their biological roles, ranging from participating in reduction reactions in carbohydrate and steroid metabolism to various detoxification reactions (27). To date, all plant AKR enzymes described belong to one group (family 4) that shares a minimum of 40% amino acid identity (28). This group includes other enzymes of alkaloid metabolism (codeinone reductase), an enzyme of flavonoid biosynthesis (chalcone reductase), and a protein catalyzing a step in siderophore formation in graminaceous plants (deoxymugineic acid synthase). There are at least 21 members of the AKR family represented in the whole genome of *Arabidopsis thaliana*, although very few of the encoded enzymes have been characterized (29). All AKRs characterized so far share a common α/β-barrel motif that uses either NADH or NADPH as cofactors (26), and activity is mediated through the conserved catalytic AKR tetrad His, Lys, Tyr, and Asp.

The fact that species of the Solanaceae and Erythroxylaceae (*E. coca*) have recruited enzymes from two completely different protein groups to carry out the same reaction in tropane alkaloid biosynthesis strongly suggests that other steps of this pathway have also evolved independently in the two plant families. The distinct location of tropane alkaloid formation in the two families also supports this hypothesis. In the Solanaceae, tropane alkaloids are produced in the roots and transported to the leaves and reproductive organs. All biosynthetic enzymes, including the TRs, have been localized in root tissue (30, 31). However, in the Erythroxylaceae, tropane alkaloids are found in shoots. Moreover, we showed that the site of biosynthesis of these compounds in *E. coca* is the young leaves, based on the incorporation of $^{13}CO_2$ in these compounds (20). In the present paper, MecgoR enzyme activity was found to be highest in young leaf tissue (Fig. 2), with lower levels in stems and flowers and no activity in roots. A similar pattern was found for the accumulation of MecgoR protein and transcript (Fig. 2). Within the leaf, the majority of MecgoR protein was localized to the palisade parenchyma and spongy mesophyll (Fig. 4).

Many other types of plant secondary metabolites have scattered distributions in the plant kingdom, and for these an independent origin of biosynthesis in different lineages is also likely. For example, the pyrrolizidine alkaloids are found in such diverse families as the Orchidaceae, Fabaceae, Boraginaceae, and Asteraceae. Analysis of the first biosynthetic step, homospermidine synthase, indicated that this enzyme has been recruited from a primary metabolic enzyme, deoxyhypusine synthase, at least four times over the course of evolution (32). Besides alkaloids, groups such as the cardenolides, a type of glycosylated steroid found in the Liliaceae, Ranunculaceae, Brassicaceae, and Apocynaceae, among other families, have also been proposed to have originated polyphyletically (33). Thus, the great diversity of secondary metabolites in plants is likely a product of the continual gain (and loss) of biosynthetic pathways throughout evolution.

The independent recruitment of novel enzymes into biochemical pathways often takes advantage of their broad substrate specificity (34), which becomes narrower under the influence of natural selection. *E. coca* MecgoR is different from the tropinone reductases of the Solanaceae in a variety of properties. Whereas MecgoR can use the Solanaceae TRII substrate tropinone, the TRII enzymes have no activity with the MecgoR substrate methylecgonone (15, 17). In addition, MecgoR is capable of catalyzing the reverse reaction whereas the TRII enzymes are not (35–37), although the reverse reaction has an optimal pH of 9.8 and is thus unlikely to be physiologically relevant for an enzyme that is thought to be localized in the cytosol. Other differences

between the Solanaceae TRII enzymes and *E. coca* MecgoR are that the TRIIs are not able to substitute NADPH with NADH, as we have shown for MecgoR (35–37). Furthermore, the relative activity data presented in Fig. 4 suggest that MecgoR contains an active site that is optimized for the recognition of the carbomethoxy group of methylecgonone. In addition, we have determined that MecgoR can be inhibited by its substrate, which has not been reported for any of the previously characterized TRs or closely related members of the AKR family. Thus, MecgoR has not only been recruited to tropinone reduction separately from the tropinone reductases of the Solanaceae but has a number of very different biochemical characteristics.

In summary, we have shown that the reduction of the 3-keto function of tropane alkaloids in *E. coca* is catalyzed by MecgoR, which belongs to a class of enzymes that is very different from that of the Solanaceae enzymes catalyzing a similar reaction. This finding provides a strong indication that tropane alkaloid biosynthesis has evolved more than once in different plant lineages. There are several other lines of evidence suggesting that the pathway of tropane alkaloid biosynthesis in the Erythroxylaceae has an independent origin from tropane alkaloid formation in the Solanaceae and other plant families. For example, the Erythroxylaceae tropane alkaloids possess a carbomethoxy function on the tropane ring that is not found in tropane alkaloids of the Solanaceae. In addition, tropane alkaloids with 3β-aromatic ester functions, which are dominant in the Erythroxylaceae, are rarely found in the Solanaceae. There are also prominent spatial and temporal differences in tropane alkaloid biosynthesis between the Erythroxylaceae and the Solanaceae. To gain further insight into the evolution of tropane alkaloids in the Erythroxylaceae, focus on the early steps of the pathway is required.

Materials and Methods

A crude enzyme extract from young expanding *E. coca* leaves was purified over several chromatographic steps using an ÄKTApurifier preparative protein liquid chromatography system (GE Healthcare). Fractions were assayed for tropinone reducing activity, and both substrate and products were analyzed by LC-MS. A high-activity fraction from the final ion-exchange column was subjected to 2D gel electrophoresis, and selected spots were picked for de novo peptide sequence identification. Peptide sequences were used to screen a young leaf *E. coca* EST library. For biochemical characterization, heterologous expression was performed in *P. pastoris* KM71. The sequence of MecgoR was registered at GenBank (GenBank accession no. GU562618) as well as on the AKR website (http://www.med.upenn.edu/akr; accession no. AKR4B10). Polyclonal rabbit antibodies, produced from purified recombinant MecgoR protein, were used for immunoprecipitation and immunolocalization. Details of plant material, reagents, protein purification/sequencing, LC-MS, GC-MS, NMR, localization experiments, quantitative RT-PCR, phylogenetic analysis, and all other methods used in this study are described in *SI Materials and Methods*.

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Supporting Information

Jirschitzka et al. 10.1073/pnas.1200473109

SI Materials and Methods

Chemicals and Plant Material. Nortropinone and nortropine were purchased from Toronto Research Chemicals. 6-Hydroxytropinone was purchased from Extrasynthèse S.A.S. and reduced with sodium borohydride/ethanol to yield 6-hydroxytropine. The 8-thiabicyclo[3.2.1]octan-3-one (TBON) was kindly provided by Birgit Dräger (Martin Luther University, Halle, Germany) (1). The 2β-carbomethoxy-3-tropinone (methylecgonone) was synthesized as described, and purity was confirmed using ORD, NMR, and LC-MS/MS (2). Pseudotropine was purchased from AK Scientific.

All other chemicals were purchased from Sigma-Aldrich, Carl Roth, or Merck and had the highest available quality. Water was supplied by a Milli-Q Synthesis System (Millipore).

Seeds of *Erythroxylum coca* were obtained from the Bonn Botanical Garden. These seeds were removed from the surrounding pulp and germinated in pots with Perlite and fertilized once a week with Ferty 3 (15-10-15) and Wuxal Top N (Planta). Plants were grown at 22 °C under a photoperiod of 12 h light/12 h dark, with a humidity of 65% and 70%, respectively. A voucher specimen of the plants used for this study was deposited at the Herbarium Haussknecht, Friedrich Schiller University, Jena, Germany.

Homology-Based Search for Tropinone Reductase Analogs in *E. coca*. Stage 1 and 2 *E. coca* leaves (Fig. S2) were used to construct an enriched cDNA library. Library construction and sequencing of the pBluescript vectors have been previously described (3). For sequence similarity searches, the MS-BLAST server at the Max Planck Institute for Chemical Ecology was used (4). Known tropinone reductase (TR) sequences were downloaded (GenBank accession nos. AAA33281, AAA33282, ACG34080, CAC34420, CAD20555, BAA85844, AAB09776, and CAB52307) and blasted with "tblastn" against sequences from the *E. coca* EST library using the blosum62 matrix. The top hits were taken (E value < e^{-05}) from every TR blast. EST clones were only chosen if they had a full ORF and poly(A) tail. Proteins were expressed in the pH9GW vector described below.

Purification of Methylecgonone Reductase from *E. coca* Extract. Leaf stage 1 and 2 *E. coca* leaves were harvested and ground in a mortar and pestle precooled with liquid nitrogen. The plant powder was mixed in a ratio of 1:5 with extraction buffer [0.1 M KH_2PO_4, pH 7.8, 10% (vol/vol) glycerol, 2% (wt/vol) polyvinyl polypyrrolidone (PVPP), 50 mM $Na_2S_2O_5$, 5 mM DTT, 1 mM PMSF]. The resulting emulsion was mixed and incubated for 10 min on ice followed by centrifugation at 15,000 × *g* at 4 °C for 30 min. The supernatant is referred to as the "crude" plant extract. For ammonium sulfate precipitation, solid $(NH_4)_2SO_4$ was added to the crude plant extract to reach 20% saturation and stirred until the salt was dissolved, followed by a 30-min centrifugation at 3,600 × *g* and 4 °C. The pellet was resuspended in 50 mM bis-Tris (pH 8.0), 10% (vol/vol) glycerol, and 5 mM DTT and called the "20%" pellet. The remaining supernatant was used for further precipitations in the same manner to obtain a 40% pellet, an 80% pellet, and an 80% supernatant. Protein concentration was measured with the Bradford Protein Assay (Bio-Rad) according to manufacturer's manual.

Active fractions were further purified on an ÄKTApurifier preparative protein liquid chromatography system (GE Healthcare) composed of the Box-900, pH/C-900, UV-900, P-900, and fraction collector Frac-950 modules. All columns and calibration kits for protein purification were purchased from GE Healthcare. Ammonium sulfate precipitation was followed by hydrophobic-interaction chromatography. The sample buffer was adjusted to contain 50 mM bis-Tris (pH 8.0), 10% (vol/vol) glycerol, 5 mM DTT, and 1 M $(NH_4)_2SO_4$. The sample was loaded on a HiTrap Phenyl FF (high) 5-mL column (phenyl Sepharose). The flow rate was set to 2.5 mL/min, and unbound sample was eluted by washing with 4 column volumes (CV) of sample buffer. Next, a linear gradient was run starting with the sample buffer containing 1 M $(NH_4)_2SO_4$ and ending with buffer lacking $(NH_4)_2SO_4$ in 20 CV. Fractions of 10 mL were collected and tested for enzyme activity. Fractions with activity were pooled for further purification.

Pooled fractions were desalted into 50 mM bis-Tris (pH 8.0), 10% (vol/vol) glycerol, and 5 mM DTT using a HiPrep 26/10 desalting column at a flow rate of 3 mL/min to enable loading on a HiTrap Blue HP 1-mL column (blue Sepharose). Unbound sample was removed by washing with sample buffer for 10 CV. The elution buffer contained 50 mM bis-Tris (pH 8.0), 10% (vol/vol) glycerol, 5 mM DTT, and 2 M KCl, and a flow rate of 1 mL/min was maintained. The protein was eluted using a linear gradient of 10 CV collecting 5-mL fractions. Fractions were tested for activity and desalted.

The active fraction was subjected to ion-exchange chromatography using a Resource Q strong anion-exchange column. The running buffer consisted of 50 mM bis-Tris (pH 8.0), 10% (vol/vol) glycerol, and 5 mM DTT. The elution buffer consisted of 50 mM bis-Tris (pH 8.0), 10% (vol/vol) glycerol, 5 mM DTT, and 2 M KCl. The flow rate was 2 mL/min, the equilibration volume was 5 CV, and the wash volume was 5 CV with fraction size 3.75 mL. A linear elution gradient was used with 20 CV while collecting 2-mL fractions, followed by a step gradient of 50–100% elution buffer. Fractions were collected and tested for activity. To reduce the sample volume, Microsep 10k Omega UF Spin Filter columns (Pall) were used. Protein size was determined using a HiLoad 16/60 Superdex 200 prep-grade column. The running buffer consisted of 50 mM bis-Tris (pH 8.0) with 150 mM NaCl. The column was calibrated using the Gel Filtration LMW Calibration Kit as described in the manual. Samples were loaded at a flow rate of 0.5 mL/min. Protein was eluted at a flow rate of 1 mL/min for 1.5 CV while collecting fractions of 6 mL.

All fractions were tested for enzyme activity. The assay buffer consisted of 100 mM phosphate buffer (pH 6.4) with 10% (vol/vol) glycerol, 0.1% (vol/vol) Tween 20, 10 mM DTT, 1 mM NADPH, 10 mM tropinone, and 10 μL protein extract in a total volume of 100 μL. The assay was stopped after 2 h with 10 μL 1 N HCl. Chromatographic analysis was carried out on HPLC 1100 Series equipment (Agilent) coupled to an Esquire 6000 ion trap mass spectrometer (Bruker Daltonics), electrospray ionization operated in positive mode, mass range *m/z* 60–500; capillary voltage, −4,000 V; nebulizer pressure, 35 psi; drying gas, 11 L/min; gas temperature, 330 °C. Separation was accomplished using a Nucleodur Sphinx RP 5-μm column (25 cm × 4.6 mm; Macherey-Nagel) with a gradient of 20 mM ammonium acetate (pH 6.5) (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min at 25 °C as follows: 5–37% (vol/vol) B (8 min), 37–100% (vol/vol) B (10 s), 100% B (3 min), 100–5% (vol/vol) B (10 s), 5% (vol/vol) B (4 min). Flow coming from the column was diverted in a ratio of 4:1 before reaching the electrospray unit. Methylecgonine or tropine masses (*m/z* of 200 and 142, respectively) were monitored, and the area under the product peak was used for activity comparison.

Two-Dimensional Gel Electrophoresis and Protein Sequencing of Purified Methylecgonone Reductase Fraction. The concentrated ion-exchange fraction was subjected to isoelectric focusing with the Ettan IPGphor II Isoelectric Focusing System (GE Healthcare) according to the manufacturer's instructions. The Immobiline DryStrip pH 3–10, 18 cm (GE Healthcare), was chosen, and the protein sample was cleaned up with the 2-D Clean-Up Kit. The pellet was dissolved in 350 μL rehydration buffer with 1.75 μL ampholyte buffer 3–10. Rehydration time was 20 h. A standard program for the 18-cm IEF strip 3–10 was chosen. The IEF strip was equilibrated with SDS buffer plus DTT for 15 min followed by a 15-min equilibration with SDS buffer plus iodoacetamide. Immediately after equilibration, the IEF strip was applied to a preparative 12% (wt/vol) SDS gel. The IEF strip was fixed with 0.5% (wt/vol) agarose. The gel was run for ~24 h at 44 V, and stained with colloidal Coomassie blue (Roti-Blue; Carl Roth) according to manufacturer's manual.

Gel spots were picked with a pipette tip cut to 3 mm with a blunt inlet hole. Gel pieces were transferred with water to a 96-well microtiter plate. All of the liquid around the gel plug was removed, and the gel piece was covered with 20 μL reduction buffer (10 mM DTT, 25 mM NH_4HCO_3) and reduced for 1 h at 56 °C. After cooling back to room temperature, the solution was replaced by 20 μL alkylation buffer (55 mM iodoacetamide, 25 mM NH_4HCO_3), and incubated for 45 min in the dark at room temperature with occasional vortexing. The gel pieces were then washed with 100 μL 25 mM NH_4HCO_3 for 5 min with occasional vortexing. Destaining, digestion, and peptide extraction were done automatically on an Ettan TA Digester (GE Healthcare). Gel pieces were destained four times with 70 μL 50 mM ammonium bicarbonate/50% acetonitrile for 20 min. The gel pieces were then rinsed with 70 μL 70% (vol/vol) acetonitrile for 20 min. Subsequently, the gel pieces were air-dried for 1 h and overlaid with 10 μL 50 mM ammonium bicarbonate containing 70 ng sequencing-grade modified trypsin (Promega). The microtiter plates were subsequently covered with aluminum foil and the proteins were digested overnight at 37 °C. The resulting peptides were extracted from the gel pieces twice by adding 50 μL 50% (vol/vol) acetonitrile in 0.1% (vol/vol) trifluoroacetic acid for 20 min. The extracts were collected in an extraction microtiter plate and vacuum-dried to remove any remaining liquid and ammonium bicarbonate.

Samples (*ca.* 4.5 μL) were separated on a nanoACQUITY UPLC system (Waters). Mobile phase A (0.1% (vol/vol) formic acid at 15 μL/min for 1 min) was used to concentrate and desalt the samples on a 20 × 0.18 mm Symmetry C18 5-μm particle precolumn (Waters). The samples were then eluted on a 100 mm × 100 μm i.d., 1.7-μm BEH nanoACQUITY C18 column (Waters). Phases A and B (100% acetonitrile, 0.1% (vol/vol) formic acid) were linearly mixed in a gradient to 5% (vol/vol) phase B at 0.33 min, increased to 40% (vol/vol) B at 10 min, and finally increased to 85% (vol/vol) B at 10.5 min, holding at 85% (vol/vol) B to 11 min and decreasing to 1% (vol/vol) B at 11.1 min of the run. The eluted peptides were transferred to the nanoelectrospray source of a Synapt HDMS tandem mass spectrometer (Waters) equipped with metal-coated nanoelectrospray tips (PicoTip), 50 × 0.36 mm, 10 μm i.d. (Waters). The source temperature was set to 80 °C, the cone gas flow was 20 L/h, and the nanoelectrospray voltage was 3.2 kV. The TOF analyzer was used in reflection mode. The MS/MS spectra were collected at 1-s intervals (50–1,700 *m*/*z*). A 650 fmol/μL human Glu-Fibrinopeptide B dissolved in 0.1% formic acid/acetonitrile (1:1 vol:vol) was infused at a flow rate of 0.5 μL/min through the reference NanoLockSpray source every 30th scan to compensate for mass shifts in the MS and MS/MS fragmentation mode.

The data were collected by MassLynx version 4.1 software (Waters). ProteinLynx Global Server Browser version 2.3 software (Waters) was used for baseline subtraction and smoothing, deisotoping, and de novo peptide sequence identification. Three most likely sequence versions were generated with a fragment ion accuracy of 20 ppm and 0.05 Da mass deviation of precursors. For sequence similarity searches, the MS-BLAST Server program at the Max Planck Institute for Chemical Ecology (5) was used. The fragment spectra were searched against sequences from the *E. coca* EST library obtained as described above. The protein identification from MS/MS fragment spectra in the database used the following parameters and considerations: peptide mass tolerance 15 ppm and a minimum of two peptides found, estimated calibration error 0.005 Da, one possible missed cleavage, fixed carbamidomethylation of cysteines, and possible oxidation of methionine and deamidation of asparagine and glutamine. Sequences of the best hits were chosen for cloning and expression.

Cloning, Heterologous Expression, and Purification of Methylecgonone Reductase. For cloning, the forward primer 5′-CTG GTT CCG CGT GGT TCC ATG GAG AGA CAA GTC CCT CGA GT-3′ and the reverse primer 5′-CAA GAA AGC TGG GTC ACG AGG ATG CAA CCA TCA CAA A-3′ were used. For analyzing initial activity, the encoded protein was expressed using *Escherichia coli* BL21 (DE3) cells (Invitrogen) with the vector pH9GW, a derivative of the pET-T7 (28a) vector (Merck), that has an N-terminal His tag and is Gateway-compatible (6). Expression was performed with Overnight Express Media (Novagen) at 28 °C. For obtaining sufficient protein for biochemical characterization, expression was performed in *Pichia pastoris* using a modified pPICHOLI vector (MoBiTec) with an N-terminal StrepTagII that is Gateway-compatible, with *P. pastoris* KM71 cells according to the manufacturer's manual. Cell rupture was achieved using sonication for *E. coli* or an SPCH-10 Pressure Cell Homogenizer (Stansted Fluid Power) for *P. pastoris*. The lysate was centrifuged at 15,000 × *g* and 4 °C for 15 min. For purification of the recombinant protein either HisTrap HP or StrepTrap HP columns (GE Healthcare) were used. The purified fractions were desalted into 50 mM NaH_2PO_4 (pH 7), with 10% (vol/vol) glycerol and 5 mM DTT, using a HiPrep 26/10 desalting column. The purity of recombinant protein was evaluated by SDS/PAGE gel electrophoresis followed by silver staining (SilverXpress Silver Staining Kit; Invitrogen). For cleavage of the affinity tag the Thrombin Cleavage Capture Kit (Merck) was used, and cleavage was checked by Western blotting using the anti-His antibody provided. No differences between cleaved and noncleaved samples were recognized, and therefore no tag was removed. The *P. pastoris*-expressed protein was used for enzyme kinetics. The sequence of methylecgonone reductase (MecgoR) was registered at GenBank (accession no. GU562618) as well as on the AKR website (http://www.med.upenn.edu/akr; accession no. AKR4B10).

MecgoR Assays and Characterization. Enzyme concentration and incubation parameters were chosen so that the reaction velocity was linear during the incubation time for all kinetic measurements. Biochemical properties were determined as previously described (7), except for the following changes. For determination of the pH optimum, phosphate-citrate buffer covered the range from 2.6 to 7, bis-Tris/propane buffer covered the range from 6.22 to 9.6, and glycine-NaOH buffer covered the range from 8.6 to 10.6 (8). Standard assays contained 1 mg/mL BSA, 100 μM NADP(H), and 200 μM each substrate unless stated otherwise. Phosphate-citrate buffer (pH 6.8) was used for enzyme assays in the reductive direction and glycine-NaOH buffer (pH 9.8) for assays in the oxidative direction. Enzyme and substrate concentrations were varied during characterization progress. For kinetic assays, the cosubstrate concentration was held constant. Reactions (100 μL) were either stopped with 10 μL 1 N HCl and used for LC-MS/MS analysis or stopped with 10 μL 1 N NaOH and extracted with ethyl acetate, and the extract was analyzed using GC-MS. Calculations were performed with GraphPad Prism version 5.04 using the built-in enzyme kinetics module.

LC-MS/MS Method for MecgoR Assay Product Determination. Chromatography was performed on a 1200 HPLC System (Agilent). Separation was achieved on a ZORBAX RRHT Eclipse XDB-C18 column (4.6 × 50 mm, 1.8 μm) (Agilent). Formic acid (0.05% (vol/vol)) in either water or methanol was used as mobile phases A and B, respectively. Elution was with a flow rate of 800 μL/min and a profile as follows: 0–0.2 min, 5% (vol/vol) B; 0.2–1 min, 5–100% (vol/vol) B; 1–2.5 min, 100% B; 2.5–2.6 min, 100–5% (vol/vol) B; 2.6–6 min, 5% (vol/vol) B. The column temperature was maintained at 20 °C. The tray temperature was 25 °C. An API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbo spray ion source was operated in positive ionization mode. The instrument parameters were optimized by infusion experiments with pure standards. The ion spray voltage was maintained at 5,500 eV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 20 psi, heating gas at 60 psi, and collision gas at 6 psi. Scheduled multiple-reaction monitoring was used to monitor analyte parent ion-to-product ion formation: tropinone *m/z* 140.2→98.2 [collision energy (CE): 28 V, declustering potential (DP): 55 V, entrance potential (EP): 11 V, collision cell entrance potential (CEP): 13.34 V, collision cell exit potential (CXP): 6 V); tropine *m/z* 142.15→98.2 (CE: 31 V, DP: 51 V, EP: 10.5 V, CEP: 13.4 V, CXP: 4 V); methylecgonone *m/z* 198.2→166.3 (CE: 21 V, DP: 31 V, EP: 3 V, CEP: 15.25 V, CXP: 4 V); methylecgonine *m/z* 200.2→182.3 (CE: −23 V, DP: 31 V, EP: 5 V, CEP: 15.32 V, CXP: 4 V); nortropinone *m/z* 126.14→84.2 (CE: 23 V, DP: 36 V, EP: 8 V, CEP: 8 V, CXP: 4 V); nortropine *m/z* 128.15→84.2 (CE: 27 V, DP: 46 V, EP: 7.5 V, CEP: 8 V, CXP: 4 V); 6-hydroxytropinone *m/z* 156.14→98.2 (CE: 23 V, DP: 41 V, EP: 4 V, CEP: 12 V, CXP: 4 V); 6-hydroxytropine *m/z* 158.14→140.14 (CE: 23V, DP: 35 V, EP: 7 V, CEP: 11 V, CXP: 4 V). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies was verified by analyzing an enzyme dilution series. External calibration curves for tropine, methylecgonine, nortropine, and 6-hydroxytropine were used for kinetic calculations.

GC-MS Method for MecgoR Assay Product Determination. This method was used for the analyses of enzyme assays using the substrates TBON, cyclooctanone, cyclohexanone, and *N*-methyl-4-piperidone. Gas chromatography was performed on an Agilent 6890 GC System coupled to an Agilent 5973 Mass Detector on an SLB-5ms column (30 m × 0.25 mm and 0.25-μm film thickness; Sigma-Aldrich) with the carrier gas helium at 1 mL/min and splitless injection (injection temperature, 250 °C; injection volume, 1 μL). A temperature gradient of 5 °C/min from 40 °C (2-min hold) to 200 °C, followed by a steep ramp to 250 °C (1-min hold), was applied. Parameters for electron impact ionization in the quadrupole mass detector were as follows: 30 V repeller, 34.6 μA emission, 70 eV electron energy, 150 °C quadrupole temperature, 230 °C source temperature, 250 °C transfer line temperature. The mass spectrometer was run in the scan mode with a mass range *m/z* of 40–350. Standards were used for peak assignment.

NMR Analysis of MecgoR Assay Product. An enzyme assay was performed using heterologously expressed MecgoR and tropinone or methylecgonone as described above. The assay was stopped with 1 N NaOH and extracted with $CHCl_3$. The $CHCl_3$ extract was evaporated to dryness, dissolved in 150 μL MeOH-d_4 (99.96%; Deutero GmbH), and transferred to a 2.5-mm-diameter NMR tube for analysis. Samples of authentic standards were prepared by dissolving tropine, pseudotropine and methylecgonine in MeOH-d_4. ^{1}H NMR spectra were recorded at 297 °K using an Avance NMR spectrometer operating at a proton resonance frequency of 500.13 MHz (Bruker BioSpin). The spectrometer was equipped with a Bruker TCI cryoprobe (5-mm). The residual water signal in the spectra of the assay samples was suppressed using the PURGE sequence (9). The residual signal of MeOH-d_4 at δ_{1H} 3.31 was used as a chemical shift reference.

MecgoR Antibody Production. Purified recombinant MecgoR protein from *E. coli* was used to produce polyclonal antibodies in rabbits. Antibodies were affinity-purified using epoxy-activated Sepharose conjugated to the same recombinant protein that was used for immunization (Davids Biotechnologie).

MecgoR Localization in *E. coca* Organs. For immunoblot analysis, crude protein from different plant organs was extracted using a 3:1 ratio of extraction buffer:plant material as described above. Equal amounts (50 μg) of desalted crude plant extract were separated by SDS/PAGE under reducing conditions and transferred to a nitrocellulose membrane (Whatman). Membranes were blocked with TTBS (20 mM Tris, pH 7.5, 150 mM NaCl, 0.1% (vol/vol) Tween 20) and 50 g/L milk powder followed by incubation with 1:500 anti-MecgoR and 1:2,000 anti-rabbit HRP-conjugated antibodies (Sigma-Aldrich) in blocking solution (TTBS and milk powder) and subsequent TTBS washing. Signal detection was achieved using West Pico Chemiluminescent Substrate (Thermo Scientific) and Amersham Hyperfilm ECL (GE Healthcare).

For immunohistochemistry, selected organs were harvested and fixed overnight at 4 °C in 63% (vol/vol) ethanol, 5% (vol/vol) acetic acid, 2% (vol/vol) formaldehyde, and 1% (wt/vol) tannic acid with vacuum infiltration. After fixation, organs were dehydrated in an ascending series of ethanol (70% (vol/vol) and 90% (vol/vol) at 1 h each and 100% overnight) followed by an ascending series in Roti-Histol (Carl Roth), 25% (vol/vol), 50% (vol/vol), 75% (vol/vol), and 100% at 1 h each. Continued processing was performed at 60 °C. Liquid Paraplast X-Tra (Carl Roth) was added dropwise, and solvent composition was slowly switched to this solvent over 2 d by changing Paraplast X-Tra daily. Each organ was embedded in custom aluminum molds, and 10-μm slices were cut on a rotary microtome and incubated at 42 °C on polylysine slides (Thermo Scientific) overnight.

To prepare for antibody treatment, slides were dewaxed in Roticlear (Carl Roth), rehydrated by a descending ethanol series (100%, 90% (vol/vol), 70% (vol/vol), 50% (vol/vol), 30% (vol/vol), 10% (vol/vol) H_2O at 2 min each), and treated against endogenous peroxidase with 0.3% (vol/vol) H_2O_2 (in methanol) for 30 min. Slides were washed with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4) and incubated for 15 min with 1% (wt/vol) BSA in PBS. Slides were incubated with 1:50 anti-MecgoR antibody in 1% (wt/vol) BSA in PBT (PBS, 0.1% (vol/vol) Tween 20) overnight at 4 °C in a humid chamber. After washing slides with PBT, incubation was performed with 1:100 anti-rabbit HRP-conjugated antibody (Sigma-Aldrich) in 1% (wt/vol) BSA in PBT for 30 min. TSA Kit 24 dye (Invitrogen) was used according to the manufacturer's manual with 25-min developing time. Slides were examined at 543 nm by confocal laser-scanning microscopy using a Zeiss LSM510 confocal microscope equipped with an HeNe/Ar laser module and a 40× objective lens (C-Apochromat; Carl Zeiss) using a BP 585–615 filter. Pictures (1,936 × 1,936 pixels) were taken with the contrast of the transmitted light picture adjusted for all rolled leaf images using LSM Image Browser (Carl Zeiss) and transferred to Illustrator (Adobe Systems) without further manipulation.

Immunoprecipitation of MecgoR. A crude protein extract from stage 1 leaves was prepared as described earlier and desalted into 50 mM NaH_2PO_4 (pH 7.0) with 10% (vol/vol) glycerol and 5 mM DTT using Illustra NAP columns (GE Healthcare) according to the manufacturer's instructions. Protein A Sepharose beads

(Immunoprecipitation Starter Pack; GE Healthcare) were equilibrated with 50 mM NaH_2PO_4, 100 mM NaCl, 0.5% (vol/vol) Nonidet P-40, and 5 mM DTT as a lysis buffer. Desalted plant extract (1 μg) was incubated with 5 μg anti-MecgoR antibodies for 1 h at 4 °C with gentle agitation. Protein Sepharose beads (50 μL) were added and incubated again for 1 h. The beads were separated from the supernatant by centrifugation at 16,000 × *g* for 2 min, and enzyme assays were performed as described above. For protein sequencing, this procedure was scaled up by using 300 μg plant extract, 500 μg antibodies, and 200 μL Sepharose beads. The bead-bound immunoprecipitate was eluted by incubation for 10 min at 100 °C with protein-loading buffer (50 mM Tris·HCl, pH 6.8, 2% (vol/vol) SDS, 10% (vol/vol) glycerol, 1% (vol/vol) β-mercaptoethanol, 12.5 mM EDTA, 0.02% (wt/vol) bromophenol blue) and subjected to SDS/PAGE. The gel was stained with Coomassie blue, and protein bands were picked for sequencing as described above.

Quantitative Real-Time PCR Analysis. Experiments were performed as described (3). Primers targeting the MecgoR transcript were designed (forward: 5′-CAACTATACCTCCTGCCGTCAATC-3′; reverse: 5′-GCTCAATCTTTCGCCCGTCTTC-3′), and standard curve analysis showed a PCR efficiency of 95% and R^2 of 0.998. Expression of MecgoR was normalized to gene 6409 and gene 10131 (3) expression using qBase version 1.3.5 (10).

Phylogenetic Analysis. Sequences of the aldo-keto reductase, aldehyde dehydrogenase, medium-chain dehydrogenase/reductase, and short-chain dehydrogenase/reductase enzyme superfamilies were aligned using the CLUSTAL X program with standard settings (11). The phylogenetic tree was made using the MRBAYES program (12) with a Poisson matrix and CoTR_CAO02390 as the outgroup. Branch lengths were unconstrained. The final tree picture was exported to Illustrator CS4 (Adobe Systems).

Predictions Made for MecgoR Amino Acid Sequence. The amino acid sequence of MecgoR (GenBank accession no. ADK94763) was submitted to YinOYang 1.2 (13), NetPhos 2.0 (14), and PSORT (15) to predict posttranslational modifications and localization within the cell.

Southern Blot Analysis. *E. coca* genomic DNA (20 μg) was digested under standard conditions with different restriction enzymes (New England BioLabs) and ethanol-precipitated. Separation occurred on a 1% (wt/vol) agarose gel. Hybond-N (Amersham) was used as a blotting membrane with 10× SSC (1.5 M NaCl, 0.15 M sodium citrate) as transfer buffer, followed by cross-linking (Stratalinker UV Crosslinker; Stratagene). The probe was labeled with the Rediprime II DNA Labeling System (Amersham) according to the manual. Hybridization was carried out in 5× SSC, 5× Denhardt's (0.1% (wt/vol) BSA, 0.1% (wt/vol) Ficoll 400, 0.1% (wt/vol) PVP 360), 0.1% (wt/vol) SDS, 1 mM EDTA, and 20 μg/mL boiled salmon sperm DNA at 65 °C. Blots were washed at 65 °C twice with 2× SSC, 0.1% (wt/vol) SDS, then 1× SSC, 0.1% (wt/vol) SDS, and finally (if necessary) with 0.1× SSC, 0.1% (wt/vol) SDS. The blot was autoradiographed using phosphor screens and Phosphorimager Storm 840 (Molecular Dynamics).

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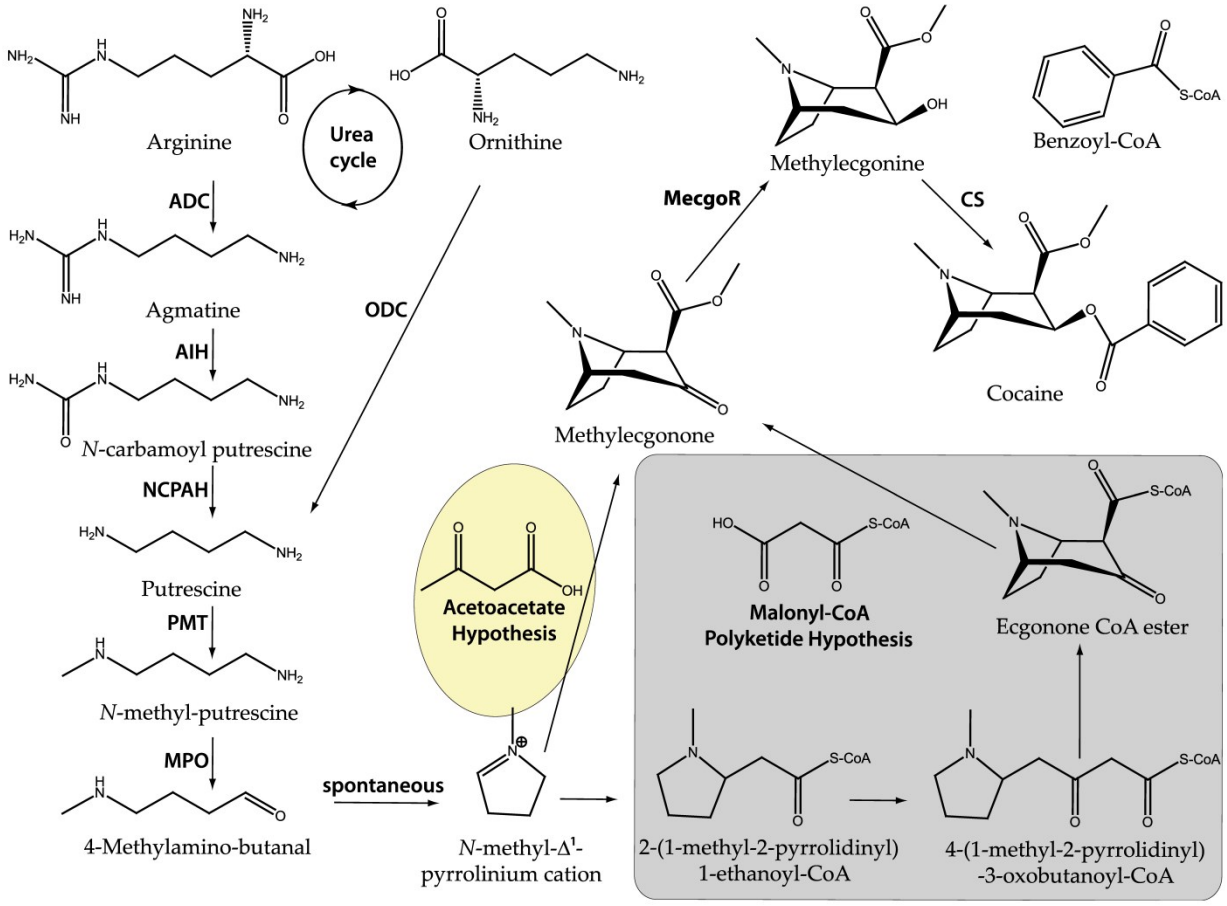

Fig. S1. Hypothetical pathway of the biosynthesis of cocaine. ADC, arginine decarboxylase; AIH, agmatine deiminase; CS, cocaine synthase; MPO, *N*-methylputrescine oxidase; NCPAH, *N*-carbamoylputrescine amidohydrolase; ODC, ornithine decarboxylase; PMT, putrescine *N*-methyltransferase.

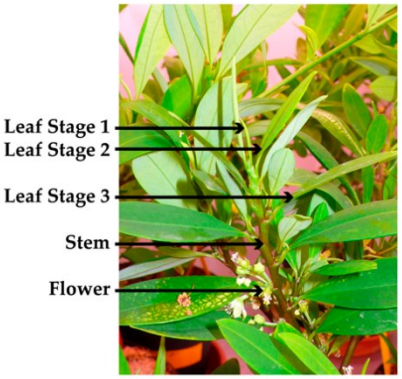

Fig. S2. Upper shoot of an *E. coca* plant illustrating the plant parts used. Developmental leaf stages are labeled from 1 to 3. Leaf stage 1 corresponds to expanding leaves that are still rolled after emerging from the bud. Leaf stage 2 corresponds to a later stage in which leaves are still expanding but have unrolled. Leaf stage 3 corresponds to mature leaves that are no longer increasing in size.

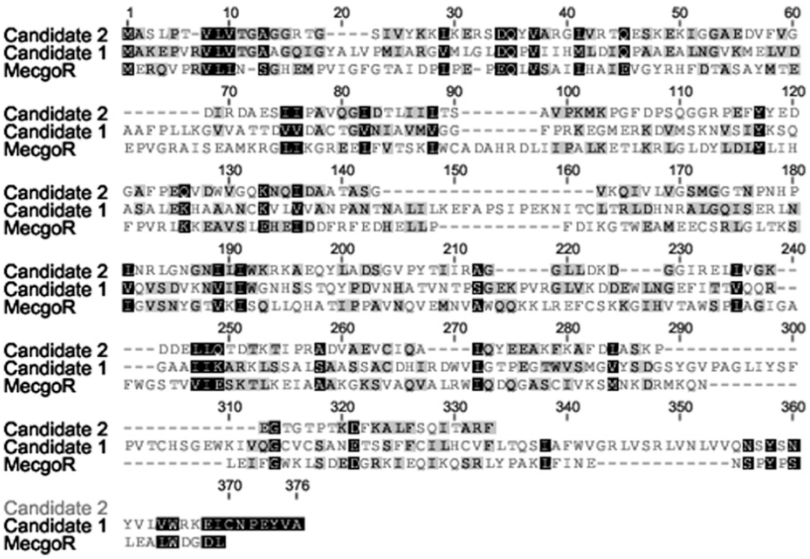

Fig. S3. Alignment of three candidate genes obtained after blast search of de novo sequenced proteins obtained from MecgoR purification against an *E. coca* EST library. Only the third candidate was shown to encode a protein exhibiting MecgoR activity, and is here designated "MecgoR." The identity matrix shows that MecgoR (GenBank accession no. GU562618) shares only 8% identity with candidate 1 (GenBank accession no. JQ804916) and 12% identity with candidate 2 (GenBank accession no. JQ804917).

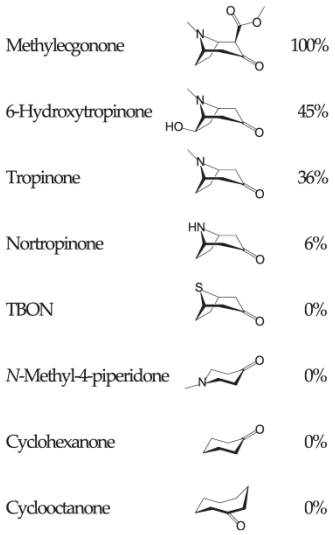

Fig. S4. Relative activity of *P. pastoris* KM71-expressed and purified MecgoR with a variety of tropinone derivatives and other cyclic ketones. All assays were performed with NADPH, and activities are shown as the percentage of activity with methylecgonone. The specific activity of MecgoR with methylecgonone (2-carbomethoxy-3-tropinone) as substrate was 36.4 pkat (mg protein)$^{-1}$. Substrates that yielded no detectable products are labeled as 0% relative activity.

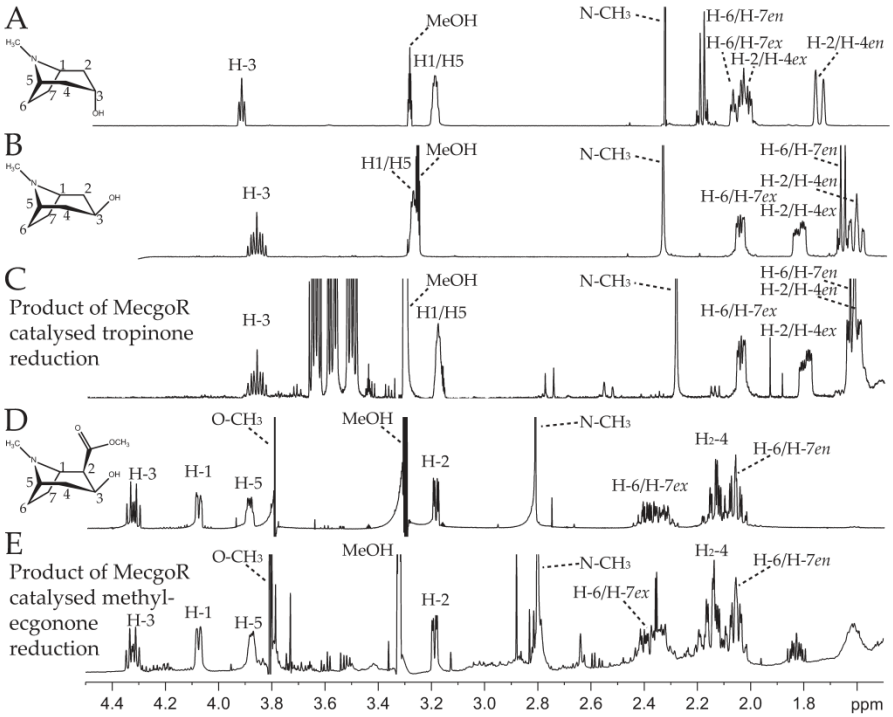

Fig. S5. Stereospecificity of MecgoR-catalyzed reduction demonstrated by ^{1}H NMR spectroscopy. The spectrum of the product of tropinone reduction (*C*) is compared with spectra of authentic samples of 3α-tropine (tropine) (*A*) and 3β-tropine (pseudotropine) (*B*). Chemical shifts (ppm) and multiplicity of ^{1}H NMR signals (*C*) match closely with corresponding signals of the 3β-product but not the 3α-product. The spectrum of the product of methylecgonone reduction (*E*) is compared with the spectrum of authentic methylecgonine (*D*). Again, chemical shifts (ppm) and multiplicity of ^{1}H NMR signals match corresponding signals of the 3β-product. ^{1}H NMR spectra were recorded at 500 MHz in MeOH-d_4 in 2-mm capillary tubes using a TCI cryoprobe (Bruker BioSpin). The assay samples (*C* and *E*) were recorded with 160 scans, and tropine, pseudotropine, and methylecgonine were recorded with 16 scans.

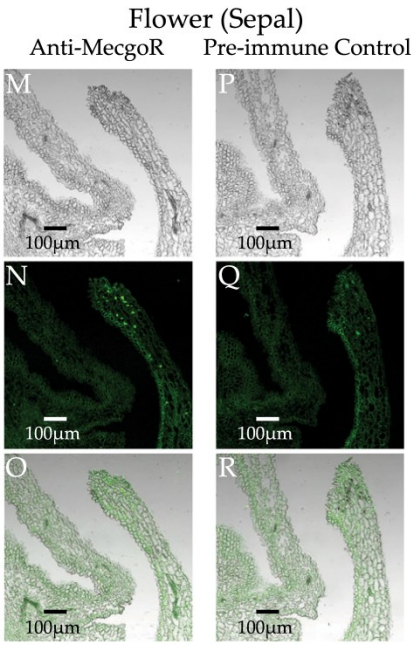

Fig. S6. Immunolabeling of MecgoR in longitudinal sections of *E. coca* flowers. The samples were labeled with either anti-MecgoR antibodies (*M*–*O*) or preimmune serum (*P*–*R*). (*Top*) Transmitted light (*M* and *P*). (*Middle*) Fluorescent images (*N* and *Q*). (*Bottom*) Overlays of transmitted light and fluorescent images (*O* and *R*). Single sections were probed with primary antibody (anti-MecgoR or preimmune serum) and secondary antibody (anti-rabbit conjugated to horseradish peroxidase assayed with chemiluminescent substrate). Fluorescence excitation was at 543 nm and detection used a BP 585–615 filter.

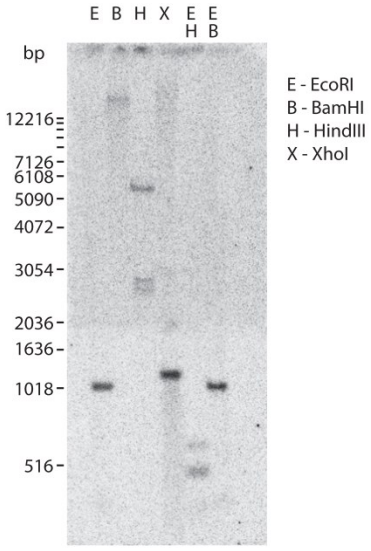

Fig. S7. Southern blot of genomic *E. coca* DNA digested with the restriction enzymes EcoRI (E), BamHI (B), HindIII (H), and XhoI (X). The autoradiographed blot shows four lanes with a single restriction enzyme cutting the genomic DNA and two lanes where two restriction enzymes digested the genomic DNA. These data suggest that MecgoR is a single-copy gene, a conclusion supported by the fact that sequencing of quantitative PCR products did not detect alternate *MecgoR* gene sequences.

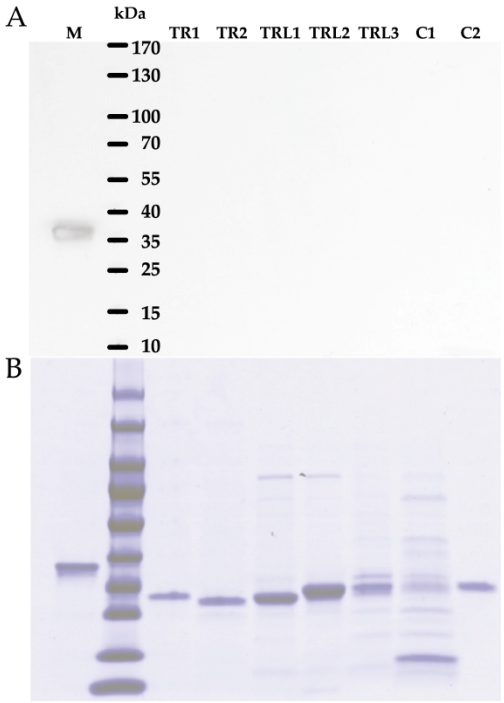

Fig. S8. Immunoblot to assess the potential cross-reactivity of the polyclonal anti-MecgoR antibody with other MecgoR-like proteins from *E. coca* and tropinone reductase proteins from *Solanum tuberosum* and *E. coca*. Depicted are (*A*) an immunoblot with anti-MecgoR antibody and (*B*) a corresponding Coomassie blue-stained protein gel of the heterologously expressed purified proteins. M, *E. coca* MecgoR (GenBank accession no. GU562618); TR1, *S. tuberosum* tropinone reductase 1 (accession no. CAC34420); TR2, *S. tuberosum* tropinone reductase 2 (accession no. CAB52307); TRL1, *E. coca* tropinone reductase-like protein 1 (GenBank accession no. JQ015102); TRL2, *E. coca* tropinone reductase-like protein 2 (GenBank accession no. JQ015103); TRL3, *E. coca* tropinone reductase-like protein 3 (GenBank accession no. JQ015104); C1, *E. coca* candidate 1 (GenBank accession no. JQ804916); C2, *E. coca* candidate 2 (GenBank accession no. JQ804917).

Table S1. Purification of MecgoR from crude extract of *E. coca* young leaves

	Volume (mL)	Activity (pkat/mL)	Total activity (pkat)	Total protein (mg)	Specific activity (pkat/mg)	Purification factor
Crude extract	79	0.04	3.37	354.58	0.01	1.0
Ammonium sulfate precipitation	45	0.53	23.76	154.19	0.15	16.2
Phenyl Sepharose	144	0.08	12.22	11.34	1.08	113.5
Blue Sepharose	19.6	0.52	10.23	2.21	4.63	487.8
Strong anion exchanger	2	0.54	1.09	1.30	0.84	88.3

Table S2. Names of abbreviated enzymes used in Fig. 3

Abbreviated enzyme	Full name
CoTR	Cochlearia officinalis tropinone reductase
StTRII	Solanum tuberosum tropinone reductase II
AaTRII	Anisodus acutangulus tropinone reductase II
DsTRII	Datura stramonium tropinone reductase II
HnTRII	Hyoscyamus niger tropinone reductase II
HnTRI	Hyoscyamus niger tropinone reductase I
AaTRI	Anisodus acutangulus tropinone reductase I
StTRI	Solanum tuberosum tropinone reductase I
DsTRI	Datura stramonium tropinone reductase I
CsTRI	Calystegia sepium tropinone reductase I
EcTR-like 3	putative Erythroxylum coca tropinone reductase 3
EcTR-like 2	putative Erythroxylum coca tropinone reductase 2
EcTR-like 1	putative Erythroxylum coca tropinone reductase 1
PbSalR	Papaver bracteatum salutaridine reductase
CaMNRI	Capsicum annuum menthone:neomenthol reductase 1
MpMNRII	Mentha x piperita menthone:neomenthone reductase II
MpMNRI	Mentha x piperita menthone:neomenthone reductase I
MpMMR	Mentha x piperita menthol dehydrogenase
AtCAD1	Arabidopsis thaliana cinnamyl alcohol dehydrogenase 1
AtCAD2	Arabidopsis thaliana cinnamyl alcohol dehydrogenase 2
CsADH	Calystegia soldanella alcohol dehydrogenase
HvADH2	Hordeum vulgare alcohol dehydrogenase 2
HvADH3	Hordeum vulgare alcohol dehydrogenase 3
HvADH1	Hordeum vulgare alcohol dehydrogenase 1
TgADH	Turritis glabra alcohol dehydrogenase
AhADH	Arabis hirsuta alcohol dehydrogenase
AtALDH12A1	Arabidopsis thaliana aldehyde dehydrogenase 12A1
ZmALDH2	Zea mays aldehyde dehydrogenase 2
NtALDH	Nicotiana tabacum aldehyde dehydrogenase
AtALDH2B4	Arabidopsis thaliana aldehyde dehydrogenase 2B4
ZmALDH5	Zea mays aldehyde dehydrogenase 5
AtALDH5F1	Arabidopsis thaliana aldehyde dehydrogenase 5F1
AtALDH10A9	Arabidopsis thaliana aldehyde dehydrogenase 10A9
AtAKR	Arabidopsis thaliana aldo-keto reductase
SrCHR	Sesbania rostrata chalcone reductase
EcMecgoR	Erythroxylum coca methylecgonone reductase
PsCOR	Papaver somniferum codeinone reductase
MsCHR	Medicago sativa chalcone reductase
PmCHR	Pueraria montana chalcone reductase
GmCHR	Glycine max chalcone reductase

3.3 Manuscript III

A BAHD acyltransferase catalyzes the final step in cocaine biosynthesis

A BAHD acyltransferase catalyzes the final step in cocaine biosynthesis

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Keywords:

Cocaine, Cinnamoylcocaine, Tropane alkaloids, MALDI-MSI, Erythroxylum coca, BAHD acyltransferase

Abstract

The esterification of 2-carbomethoxy-3β-tropine with benzoic acid is the final step in the biosynthetic pathway leading to the production of cocaine in *Erythoxylum coca*. Here we report the identification and characterization of cocaine synthase, a member of the BAHD family of plant acyltransferases. Cocaine synthase is capable of producing both cocaine and cinnamoylcocaine via the activated benzoyl- or cinnamoyl-CoA thioesters, respectively. Cocaine synthase activity is highest in the young developing leaf tissues and accumulates in the palisade parenchyma and spongy mesophyll. This data correlates with the homogenous distribution pattern of cocaine and cinnamoylcocaine as visualized using MALDI-MSI. Our findings provide further evidence that tropane alkaloid biosynthesis in Erythroxylaceae occurs in the above ground portions of the plant and contrasts with what is known about tropane alkaloid biosynthesis in Solanaceae.

Introduction

Erythroxylum coca has been cultivated for religious and medicinal purposes for more than 8000 years [1]. It is believed that this is one of the first plants to be exclusively cultivated based solely upon its pharmacological properties. The result of long term cultivation and selection for increasing alkaloid content has led to the existence of several cultivars containing cocaine in amounts of up to 1% dry weight in the plant leaves [2]. Cocaine (**2**), the benzoate ester of 2-carbomethoxy-3β-tropine (methylecgonine) (**10**), belongs to a class of alkaloids commonly referred to as tropane alkaloids. Tropane alkaloids are defined by a common chemical substructure, the azabicyclo[3.2.1]octane skeleton (**1**) or tropane nucleus (**Fig. 1**). Esterifications and hydroxylations of the tropane skeleton are common in nature, and more than 200 tropane alkaloids with drastically different pharmacological activities are known to exist [3]. The physiological interactions mediated by these compounds have been largely attributed to the modification and decorations made to the tropane skeleton. The methylated nitrogen atom in the tropane skeleton mimics acetylcholine and thereby inhibits the muscarinic acetylcholine receptors [4]. Furthermore, the stereochemical orientations at both the C-2 and C-3 positions are important for binding at the dopamine receptor [5,6], with the strongest affinity being found on compounds containing an aromatic ring connected directly or indirectly to the C-3β position [7]. This in turn explains why cocaine is used for both its anesthetic and euphorigenic properties.

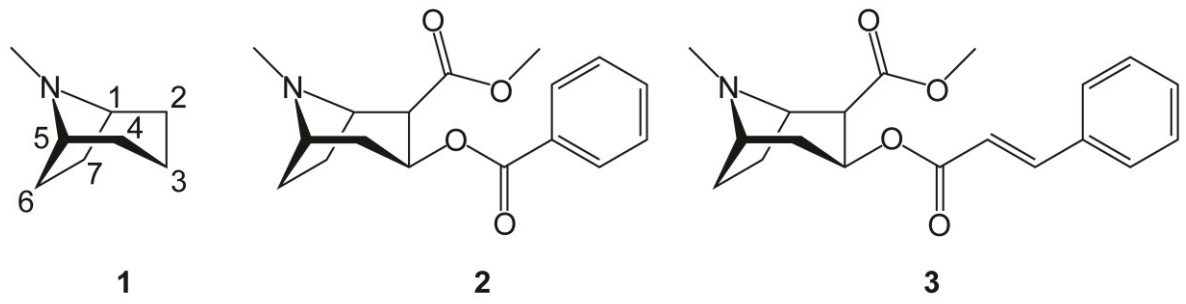

Figure 1 | Structures of selected tropane alkaloids. (**1**) numbered tropane nucleus, (**2**) cocaine and (**3**) cinnamoylcocaine.

Extensive research on tropane alkaloid biosynthesis using isotopically labeled compounds and chemical analyses has been carried out using *E. coca* in the past, establishing a theoretical biosynthetic pathway to cocaine (**Supplementary Fig. 1**) [8]. In short, the early biosynthetic steps go through ornithine or arginine to produce the committed polyamine known as *N*-methylputrescine (**5**). After oxidation of *N*-methylputrescine to 4-methylaminobutanal (**6**), which undergoes spontaneous cyclization to an *N*-methyl-Δ^1-pyrollinium cation (**7**), two acetyl units are added. The oxobutanoic acid intermediate (**8**) then cyclizes to form the tropane intermediate called methylecgonone (**9**). In the penultimate biosynthetic step to cocaine, methylecgonone is reduced to form methylecgonine (**10**). This reaction is catalyzed by the enzyme methylecgonone reductase (MecgoR) [9], which was the first structural gene of cocaine biosynthesis to be identified.

The last step in cocaine biosynthesis, esterification of methylecgonine, was hypothesized to utilize benzoyl coenzyme A as the activated acyl donor [10]. Shortly thereafter, the origin of this benzoyl moiety in cocaine was determined to be derived from cinnamic acid. However, this study could not determine whether the benzoic acid comes from benzoyl-CoA or via benzaldehyde [11]. In parallel, enzyme activities responsible for the acetylation of tropane alkaloids were purified, but no structural genes were ever isolated [12,13]. In plants, acylation reactions of secondary metabolites are performed by several acyltransferase families, namely the THT/SHT, BAHD and SCPL acyltransferases. However, of these three groups only the BAHD acyltransferases are known to utilize activated acyl coenzyme A thioesters [14]. In this study, we report the identification and characterization of the enzymes responsible for the last biosynthetic step in the formation of cocaine in *E. coca*. This particular step is of high biological importance since it converts a molecule with little physiological activity, methylecgonine, into the medicinally active end product of the pathway, cocaine [15].

Results

Exploration of cocaine synthase activity

To investigate whether cinnamoylcocaine and cocaine formation is dependent upon coenzyme A thioesters, enzyme assays were performed on crude plant *E. coca* extracts using methylecgonine and either cinnamoyl- or benzoyl-CoA. Ester forming activities were found in all tissues extracts with the exception of the roots (**Fig. 2a**). The highest enzyme activities are present in the early leaf stages (L1 and L2) with 313 ± 20 pkat / mg (fresh weight) for benzoyl-CoA and 1285 ± 97 pkat / mg (fresh weight) for cinnamoyl-CoA as substrate. Stem, flower and leaf stage 3 (L3) extracts were reduced by 20%, 40% and 60%, respectively when compared to leaf stages L1 and L2. Overall metabolite levels correlate with enzyme activities in all the tissues tested (**Fig. 2b**). Cocaine, cinnamoylcocaine and methylecgonine are highest in the early leaf stages (L1and L2) followed by leaf stage 3 stem and flower. None of these three metabolites were detected in the roots.

Cocaine synthase is a BAHD

Recently, six BAHD acyl transferases were reported from an *E. coca* λZAPII cDNA library [16] Additionally, the screening of a transcriptome database made from *E. coca* early leaf tissues (L1 and L2) yielded two more BAHD sequences designated EcBAHD7 and EcBAHD8. All eight EcBAHD´s were heterologously expressed in *Escherichia coli* and the resulting recombinant proteins were purified using Ni-chelate chromatography. Verification of protein expression for all eight EcBAHDs was achieved using immunoblot analysis with anti-His antibodies (**Supplementary Fig. 2a**). The recombinant purified proteins were tested in enzyme assays using the substrates methylecgonine and benzoyl-CoA. Of the eight individual proteins tested only EcBAHD7 and EcBAHD8 exhibited ester forming activity. A sequence alignment of EcBAHD7 and BAHD8 reveals that both enzymes share 77.3% identity positions at

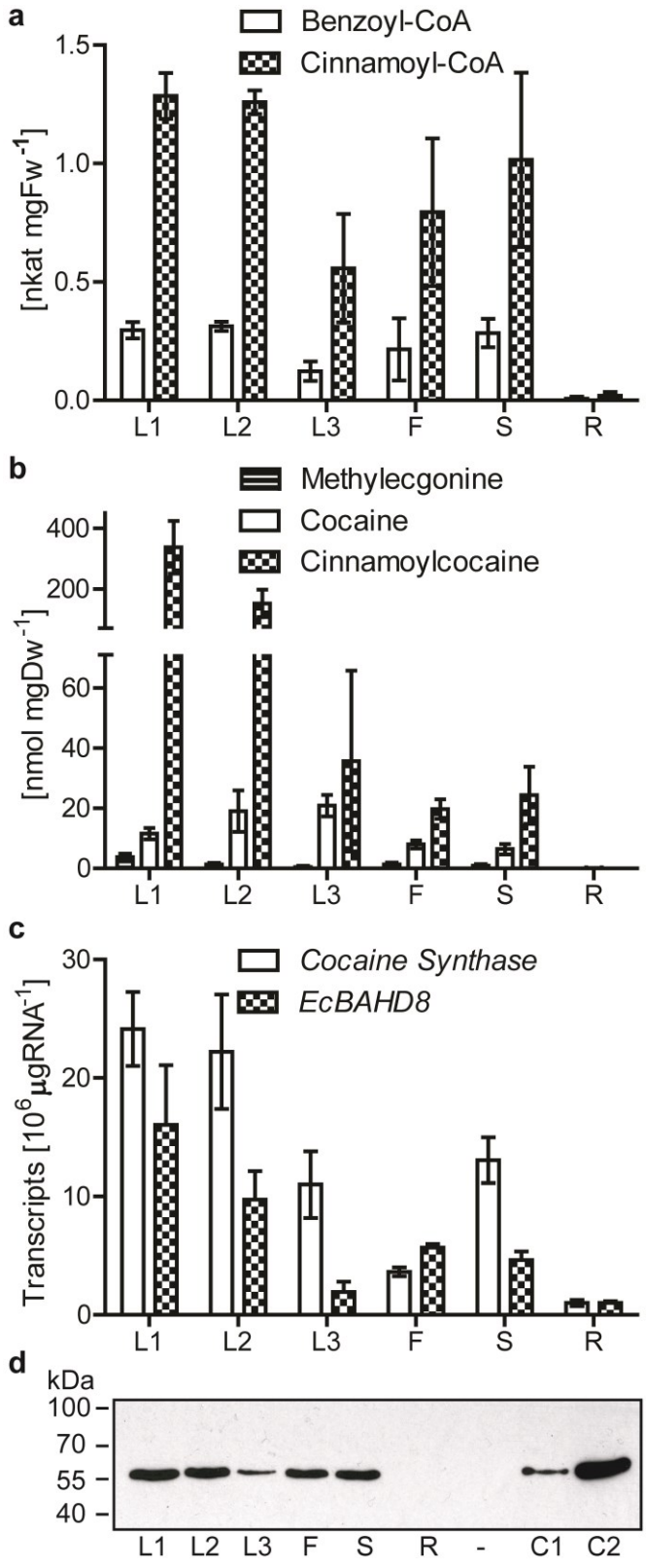

Figure 2 | Enzymatic activity and expression of cocaine synthase and EcBAHD8 compared to metabolite levels of compounds involved in the cocaine pathway, in different *E. coca* organs and development stages.

(**a**) Cocaine and cinnamoylcocaine forming enzyme activity in desalted *E. coca* protein preparations. Enzymatic assays were performed using methylecgonine with either benzoyl- or cinnamoyl-CoA, and detecting cocaine or cinnamoylcocaine as the reaction products, respectively. Values displayed are means ± SD of three technical replicates from each of three biological replicates. (**b**) Quantification of methylecgonine, cocaine and cinnamoylcocaine in different organs and development stages of *E. coca*. Values displayed are means ± SD of at least three biological replicates. (**c**) Absolute quantification of RNA transcripts of cocaine synthase and EcBAHD8 in *E. coca*. Values displayed are means ± SD of three technical replicates from each of three biological replicates. (**d**) Protein levels were determined by immunoblotting. Samples consisting of 15 µg of protein extracted from each organ as well as 75 ng (C1) and 150 ng (C2) recombinant Strep-tagged cocaine synthase (50.7 kDa) were run on SDS/PAGE and gels were blotted onto filters. The filters were first probed with anti-cocaine synthase antibodies, followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Polyclonal antibodies recognize cocaine synthase as well as EcBAHD8 (**Supplementary Fig. 2b**). Bands were visualized by chemiluminescence. Leaf stage 1 (L1), Leaf stage 2 (L2), Leaf stage 3 (L3), Flower (F), Stem (S), Root (R).

the amino acid level. Both EcBAHD7 and EcBAHD8 contain recognizable BAHD motifs including the DFGWG motif found near the C-terminus as well as the HxxxD motif which is critical for a catalytic function [14]. Phylogenetic analysis reveals that EcBAHD7 and EcBAHD8 belong to the clade III of the BAHD superfamily (**Fig. 3**). Within this clade EcBAHD7 and EcBAHD8 cluster with three other BAHDs involved in alkaloid biosynthesis. Two of these enzymes, DAT and MAT from Madagascar periwinkle (*Catharanthus roseus*) are involved in the formation of vindoline and minovincine, respectively, both monoterpenoide indole alkaloids, whereas Pun1 has shown to be involved in capsaicin formation in pepper [17].

Enzymatic characterization reveals BAHD7 as true cocaine synthase (CS)

Heterologously expression for EcBAHD7 and EcBAHD8 for biochemical characterization was carried out by *Saccharomyces cerevisiae* as host organism. Although abundant protein could be attained from expression in *E. coli*, overall enzyme activities were determined to be optimal in yeast. In addition, a codon optimized version of EcBAHD7 was developed to further enhance protein expression levels for biochemical characterization (**Supplementary Fig. 3**). The native EcBAHD7 protein with the addition of StrepTagII (10 amino acids added) was analyzed via gel sizing chromatography and yielded a single peak corresponding to a size of 43.9 kDa. This data suggests that EcBAHD7, like other characterized acyl transferases, is monomeric [14]. The pH optimum of the heterologously expressed EcBAHD7 protein was determined to be 9.4 when catalyzing the esterification of methylecgonine with benzoyl-CoA as the acyl donor. The enzyme activities were reduced to 39% and 44% of maximal activity when using a pH of 9.1 or 10.4, respectively. Similar pH optima were also obtained for the heterologously expressed EcBAHD8.

Temperature stability experiments revealed that the enzymatic activity of EcBAHD7 when incubated for 30 min at 25 °C and 37 °C were reduced by 45 % and 29% respectively when compared to 0 °C control. Higher incubation temperatures led

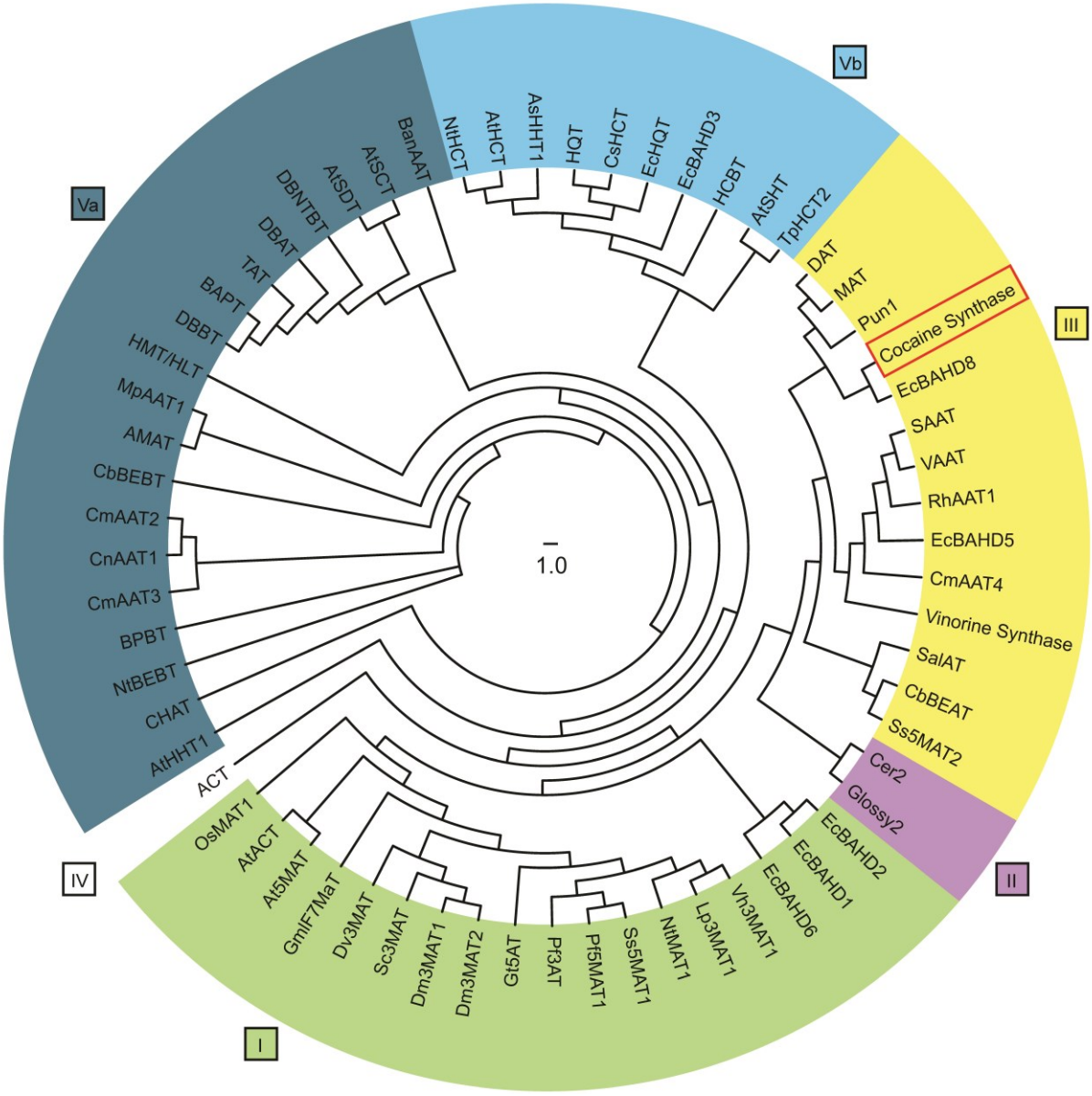

Figure 3 | Phylogenetic relationships of BAHD acyltransferases of plant secondary metabolism. Selected BAHD acyltransferases were aligned using the CLUSTAL X program with standard settings for protein alignment. The phylogenetic tree was built by the Bayesian method using the MRBAYES program. The different clades of BAHD acyltransferases are depicted according to [14]. The scale bar represents 1.0 amino acid substitutions per site. Please refer to **Supplementary Table 1** for an explanation of the abbreviated names. Accession number: cocaine synthase (KC140149) and EcBAHD8 (KC140150).

to complete loss of ester forming activity. In contrast, EcBAHD8 did not exhibit any reduction in enzymatic activity when incubated for 30 min at 20 °C and activity levels remained at 96% after incubation at 37 °C. However, higher incubation temperatures

also led to complete loss of ester forming activity. Neither, EcBAHD7 or EcBAHD8 were capable of being stimulated by the inclusion of monovalent or divalent cations. A marginal reduction in the ester forming capabilities of both enzymes was observed by performing enzyme assays with the inclusion of 5 mM Na^+ and K^+. Complete inhibition of enzyme activity was achieved for EcBAHD7 with Cu^{2+} and Zn^{2+}. Strong inhibition (less than 10% maximal activity) was also observed with the addition of Mg^{2+}, Co^{2+}, Fe^{2+} and EDTA when compared to no added metal cation control. A similar pattern of inhibition was observed for EcBAHD8.

Table 1 | Kinetic parameters of EcCS and EcBAHD8.

		K_m [μM]	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1}s^{-1}$]	K_i [mM]
EcCS	methylecgonine	369 ± 26	10 ± 0	26340	n. a.
	benzoyl-CoA	93 ± 7	8 ± 0	81192	n. a.
	methylecgonine	62 ± 11	19 ± 1	301485	n. a.
	cinnamoyl-CoA	103 ± 8	46 ± 0	446522	n. a.
EcBAHD8	methylecgonine	843 ± 64	0.02 ± 0.00	24	n. a.
	benzoyl-CoA	7 ± 1	0.02 ± 0.00	2447	5 ± 1
	methylecgonine	57 ± 6	0.003 ± 0.000	54	n. a.
	cinnamoyl-CoA	93 ± 7	0.005 ± 0.000	23	14 ± 3

Kinetic values were obtained for EcBAHD7 and EcBAHD8 using methylecgonine, benzoyl-CoA and cinnamoyl-CoA as substrates (**Table 1**). The EcBAHD7 Michaelis-Menten constant (K_m) for methylecgonine and benzoyl-CoA is 369 ± 26 μM and 93 ± 7 μM, respectively. When using methylecgonine and cinnamoyl-CoA as substrates, K_m values of 62 ± 11 μM for methylecgonine and 103 ± 8 μM for cinnamoyl-CoA were obtained. K_m values of 843 ± 64 μM and 7 ± 1 μM were calculated for EcBAHD8 when given the substrates methylecgonine and benzoyl-CoA,

respectively. Enzyme assays using methylecgonine and cinnamoyl-CoA with EcBAHD8 yielded K_m values of 57 ± 6 μM and 23 ± 2 μM, respectively. Furthermore, substrate inhibition was observed for the CoA thioesters in EcBAHD8 kinetic assays. K_i values of 5 ± 1 mM and 14 ± 3 mM were observed for benzoyl- and cinnamoyl-CoA, respectively. Comparison of catalytic efficiencies (k_{cat} / K_m) revealed that EcBAHD7 is more than 1,000 fold higher when using methylecgonine and benzoyl-CoA as substrates. The difference increases to 5,000 fold when comparing the catalytic efficiencies of these two enzymes using methylecgonine and cinnamoyl-CoA as substrates. Because EcBAHD8 is far less efficient at catalyzing the production of cocaine or cinnamoylcocaine when compared to the activity of EcBAHD7, we designated EcBAHD7 as a true cocaine synthase (CS).

Relative Activity assays performed using cocaine synthase yielded ester forming activities only when using pseudotropine or methylecgonine as the alcohol containing substrate. No esterified compounds were detected when using tropine or nortropine as substrates. The specific activity of CS for the reaction of methylecgonine and benzoyl-CoA was 4.03 pkat/mg. Reductions of 73%, 81% and 99% were observed when using cinnamoyl-CoA, hexanoyl-CoA or coumaroyl-CoA.

Polyclonal antibodies were produce against the heterologously expressed CS protein. The specificity of the polyclonal antibodies as well as the identity of the cocaine forming activity *in planta* was evaluated by immunoprecipitation. The cocaine forming activity in *E. coca* leaf stage 2 (L2) protein extracts was measured at 3.41 pkat / mg and was subsequently reduced to 1.23 pkat / mg. Pre-immune serum did not reduce enzyme activity. Immunoprecipitated proteins were separated on a protein gel and subsequent protein sequencing analysis identified EcCS within the sample. No EcCS was detected in the precipitate formed by the pre-immune serum. Furthermore, we were unable to detect EcBAHD8 in any of these samples.

Cocaine synthase and BAHD8 are localized to above ground tissue

Both *cocaine synthase* (*CS*) and *EcBAHD8* gene expression is highest in the early leaf stages (L1 and L2) (**Fig. 2c**). Overall, cocaine synthase transcript levels were at least a 2 fold higher in all the tissues tested with the exception of the flowers. In this case, there was no difference in transcript levels. Transcript abundance for *CS* and *EcBAHD8* were reduced by 50% in the mature leaves (L3) and stems when compared to the early leaf stages. While transcripts could be detected for both genes in the roots they, exhibited more than a 25 fold reduction when compared to L2 stage leaves.

Protein levels in different plant organs were evaluated for cocaine synthase using immunoblot analysis. The results revealed that CS protein is detected in all green tissues including the leaves, flowers and stems. However, no cocaine synthase protein was detected in the roots (**Fig. 2d**).

Cocaine synthase is localized to the palisade parenchyma

Based on the immunoblot results obtained from various organs, green tissues including flowers and stems were used for subsequent tissue level immunolocalization experiments. In addition, anti-cocaine antibodies were used to localize cocaine in the same tissues. **Fig. 4** shows cross sections for stage 1 leaves and stems as well as longitudinal sections made from the flowers. Preimmune serum was employed as negative control and the fluorescence signal obtained from these samples is considered unspecific. All panels in **Fig. 4** are overlays of fluorophore and autofluorescence channels. Strong fluorescence signal was observed following the use of anti-cocaine synthase and anti-cocaine antibodies. Cocaine is localized between the upper and lower epidermis with fluorescence signal detection strongest in the palisade and spongy mesophyll. A similar pattern can be observed with the CS protein. The strongest signals appear in the palisade with a diffused distribution observable in the spongy mesophyll. In the flower, CS protein accumulates in the

tapetum cells of the anther. In addition, the cocaine synthase protein was observed to accumulate in the ground tissue of green stems. The upper or lower epidermal tissues including the cuticle were devoid of fluorescence signal attributed to anti-cocaine antibodies.

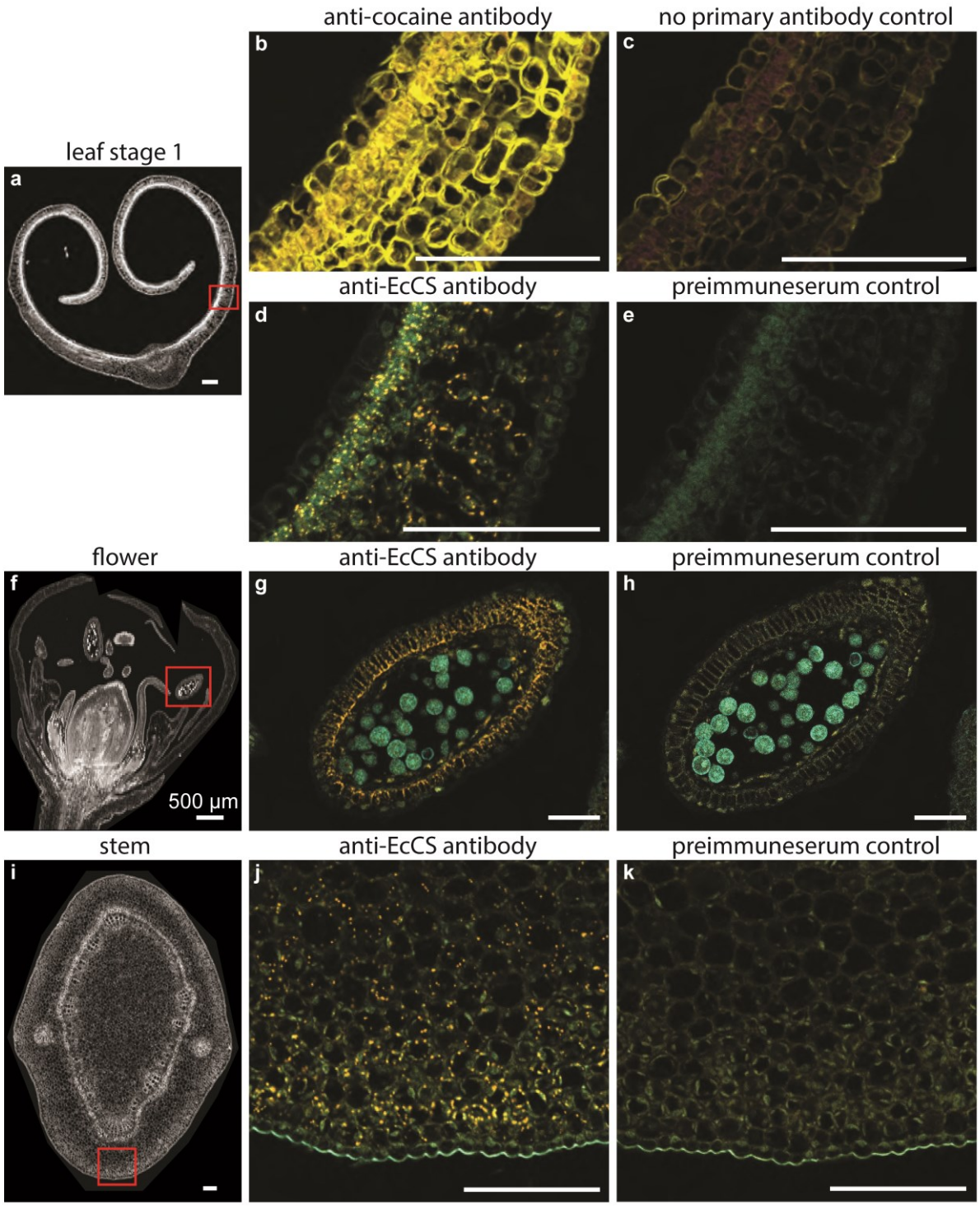

Figure 4 | Immunolocalization of cocaine and cocaine synthase. Fluorescence micrographs of immunolabelled cross-section of different *E. coca* organs. All scale bars represent 100 µm

unless otherwise indicated. (**a**) Overview of leaf stage 1 cross-section with the region of interest marked by a red rectangle. (**b**) and (**c**) Leaf stage 1 cross-section immunolabelled with monoclonal anti-cocaine antibodies and no primary antibody, respectively. Fluorescence signal of secondary antibody shown in yellow. Background autofluorescence shown in purple. (**d**) and (**e**) Leaf stage 1 cross-section immunolabelled with polyclonal anti-cocaine synthase antibodies and preimmuneserum, respectively. Fluorescence signal of secondary antibody shown in orange. Background autofluorescence shown in cyan. (**f**) Overview of flower cross-section with the region of interest marked by a red rectangle. (**g**) and (**h**) Flower cross-section immunolabelled with polyclonal anti-cocaine synthase antibodies and preimmuneserum, respectively. Fluorescence signal of secondary antibody shown in orange. Background autofluorescence shown in cyan. (**i**) Overview of stem cross-section with the region of interest marked by a red rectangle. (**j**) and (**k**) Stem cross-section immunolabelled with polyclonal anti-Cocaine Synthase antibodies and preimmuneserum, respectively. Fluorescence signal of secondary antibody shown in orange. Background autofluorescence shown in cyan. Single sections were probed with primary antibody (anti-cocaine, anti-cocaine synthase or preimmune serum) and secondary antibody (anti-rabbit conjugated to horseradish peroxidase) and subsequently assayed with fluorescent tyramide substrate. Excitation of fluorophore for cocaine imaging was at 543 nm and detection using a BP 585–615 filter. Plant autofluorescence was excited at 488 nm and detected using LP 505nm filter. For imaging of cocaine synthase excitation of fluorophore was at 561 nm and detection using 585–614 nm lambda channels. Plant autofluorescence was excited at 488 nm and detected using 495-534 nm lambda channels. All pictures are overlays of fluorophore and autofluorescence channels.

Cocaine is evenly distributed within the whole leaf

In order to observe accumulation patterns for methylecgonine, cinnamoylcocaine and cocaine, MALDI imaging techniques were employed. These three compounds were detected by selected reaction monitoring (SRM) acquisition mode and their identities further confirmed by enhanced product ion MS/MS experiments. **Fig. 5** displays homogenous distribution for the three compounds in intact leaf samples of all leaf stages (L1, L2, L3) (**Supplementary Fig. 4**).The cocaine signal was the highest of all three metabolite signals followed by cinnamoylcocaine and methylecgonine. Furthermore, there is a difference in signal intensity between the adaxial and abaxial surfaces of the leaf stages 2 and 3 (L2 and L3). The cocaine signal intensity is highest in the abaxial surface of leaf stage 2 and 3 (L2 and L3) compared to

the adaxial leaf surface. In contrast, the signal intensity of cinnamoyl cocaine is higher in the adaxial surface of leaf stage 2 and 3 (L2 and L3). The methylecgonine signal intensity is overall very low, but is higher in the adaxial leaf surface of leaf stage 2 (L2) and the abaxial leaf surface of leafs stage 3 (L3) compared to the opposite leaf surface. An equal signal intensity distribution of all three metabolites can be observed in adaxial and abaxial leaf surfaces of leaf stage 1 (L1).

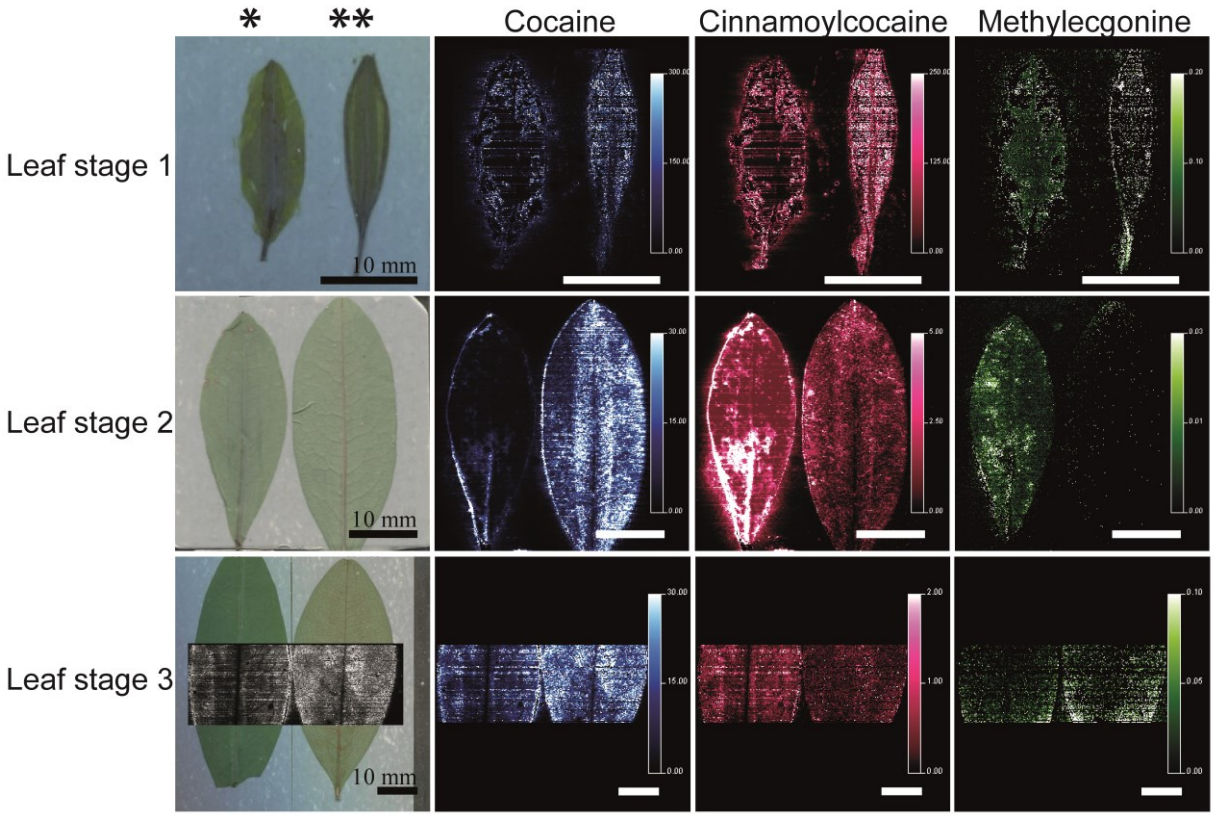

Figure 5 | MALDI-MSI of different developmental stages of *E. coca* leaves. MALDI-SRM/MS images based on the respective SRM traces for targeted analytes. Cocaine trace is shown in blue at *m/z* 304 > *m/z* 182, cinnamoylcocaine trace is shown in pink at *m/z* 330 > *m/z* 182 and methylecgonine trace is shown in green at *m/z* 200 > *m/z* 182 in leaf stage 1, 2 and 3 leaves. The images are displayed after normalization with the SRM trace of cocaine-d_3. The vertical color scale represents the respective SRM signal intensity ratio of the analyte over the reference compound cocaine-d_3: white pixels give the highest signal; dark pixels give the lowest signal. Horizontal bars represent 10 mm scale for each image. *adaxial leaf surface; **abaxial leaf surface. MS image resolution: pixels of 50x50 μm.

Discussion

Tropane alkaloids (TAs) are commonly modified via esterification of the hydroxyl function at the C-3 position of the tropane ring. The four major plant lineages known to contain TA producing plant species all exhibit examples in which this modification occurs [18]. Coenzyme A (CoA) activated thioesters were first suggested to be the actual substrates for the corresponding acylation reaction occurring in *Erythroxylum coca* [10]. Shortly thereafter, two CoA dependent acyltransferases involved in TA modification were purified from *Datura stramonium*, a member of the Solanaceae. However, the sequence of the proteins and their corresponding genes were never reported [13]. After confirming, that the cocaine synthase reaction in *E. coca* does indeed use benzoyl-CoA as a cosubstrate, we began to look for the most likely candidates responsible from a 454 transcriptome database obtained from young leaf tissues. Given the CoA dependent nature of the enzyme reaction as well as the physical properties reported for the tigloyl-CoA:pseudotropine acyl transferase from *D. stramonium* we hypothesized that cocaine synthase is most likely a member of the BAHD superfamily [12].

BAHD acyltransferases are well known for participating in the modification of secondary metabolites producing both esters and amides [14]. A total of eight BAHD-like acyltransferases were subsequently identified in our databases. From these eight, only two exhibited cocaine synthase activity. Another member from this group was recently reported to be EcHQT, responsible for making 4-coumaroylquinate, a compound that is likely to be involved in the storage of cocaine and cinnamoylcocaine *in planta* [16]. The activities of the remaining five BAHD-like enzymes remain to be determined. It is expected that the *E. coca* genome should contain more members of the BAHD acyltransferase family since species like *Arabidopsis thaliana* and *Populus trichocarpa* contain between 64 and 149 unique members, respectively [14,19].

Although cocaine synthase and EcBAHD8 share over 77% sequence identity at the amino acid level, cocaine synthase has a much higher efficiency for the production

of both cocaine and cinnamoylcocaine (1,000 fold and 5,000 fold, respectively). The two enzymes share similar Michaels-Menten constants for methylecgonine and either benzoyl- or cinnamoyl-CoA. Therefore the large catalytic efficiency differences are attributed to very low turnover numbers in respect to EcBAHD8. Moreover, the two purified TA acyltransferases from *D. stramonium* also show similar simple Michaels-Menten kinetics for their two respective substrates. We observed substrate inhibition kinetics only for EcBAHD8 in respect to the use of either benzoyl- or cinnamoyl-CoA. However the high K_i values of 5 mM and 14 mM, respectively, do not reflect the natural concentration of CoA thioesters reported in plants [20,21].

The pH optima for enzyme catalyzed reactions involving the acylation of TA is in the range of 9 to 9.5 [12,22]. This contrasts with pH optima reported for nearly all other BAHD members. In those cases the alcohol containing substrates are nearly always neutral in charge. At pH´s above 8 the nitrogen present in the tropane ring is neutral which may be important for substrate binding and the acid based catalyzed reaction mechanism of BAHD enzymes.

The ability of cocaine synthase to accept other CoA thioesters, albeit at very low rates may explain the trace amounts of other acylated methylecgonine derivatives found in *E. coca* such as hexanoylecgonine methyl ester [23,24]. In addition we determined that cocaine synthase is specific for catalyzing the esterification of only 3β-hydroxyl substrates. This specificity is reflected in the supply of methylecgonine via the enzyme methylecgonone reductase (MecgoR). MecgoR only supplies the 3β-tropane alcohol methylecgonine and is unable to supply the corresponding 3α-substrate [9]. Specificity for the alcohol containing substrate was also observed for the tropine:acyl-CoA transferase and the pseudotropine:acyl-CoA transferase purified from transformed roots of *D. stramonium* and *Brugmansia candida x aurea* hybrid [22]. Lastly, the role of cocaine synthase *in planta* was confirmed using immunoprecipitation followed by protein sequencing.

The distribution of different classes of plant derived alkaloids is depended upon key factors such as substrate availability, tissue specific gene expression, biosynthetic enzyme localization and transport. In many cases, the interaction of all these factors leads to a complex distribution pattern within the plant. For example, tropane alkaloids produced by members of the Solanaceae largely accumulate in the above ground tissues while the biosynthesis of core metabolites occurs in the roots [25]. In addition, other alkaloids such as the benzylisoquinoline derivatives in opium poppy accumulate mainly in specialized laticifers found in the vascular tissue while some biosynthetic enzymes are localized to nearby sieve elements [26]. In *E. coca* the accumulation and biosynthesis of tropane alkaloids occurs within the same tissues. Methylecgone reductase, the penultimate enzyme in cocaine biosynthesis, is localized to the palisade parenchyma and spongy mesophyll of the leaves [9]. In addition the highest accumulation of tropane alkaloids is also found in these tissues. Moreover the biosynthesis is not dependent upon root tissue [27]. In this study, we have confirmed the tissue localization for the storage of cocaine and other related metabolites in the palisade parenchyma and spongy mesophyll. Cocaine synthase, like MecgoR, is also localized to the same tissues. Cocaine synthase can also be found in the anther tapetum. In addition the enzyme 6β-hydroxylase (H6H) involved in tropane alkaloid biosynthesis from *Atropa belladonna* is also found in the anther tapetum suggesting a similar biological function of tropane alkaloids in this plant organ [28]. The anther tapetum is known to accumulate flavonoids as well as anthocyanins which are involved in pollen pigmentation. In order for this pathway to exist, hydroxycinnamoyl-CoA thioesters are required [29]. This could also supply the required CoA substrates for cocaine synthase. The defensive properties of cocaine have been investigated [30,31]. However detailed ecological studies on relevant wild relatives are required.

Cocaine synthase is responsible for the production of both cocaine and cinnamoylcocaine in *E. coca*. In the young leaves, cinnamoylcocaine is present in higher amounts and as the leaf matures cocaine levels increase. This is most likely due

to the difference in availability of cinnamoyl- and benzoyl-CoA during leaf development. Young coca leaves are high in flavonoids and thus rely upon large pools of hydroxycinnamoyl-CoA thioesters [32,33]. As the leaf develops, more of those thioesters can be diverted into the production of benzoyl-CoA [34].

Direct analysis followed by mass spectrometry detection has been found particularly attractive in many fields of applications since it tends to minimize the sample preparation and keeps intact the sample spatial integrity. Particularly, matrix-assisted laser desoption/ionization mass spectrometry imaging (MALDI-MSI) has been used to map plant metabolites within tissues [35-37] as well as to monitor specifically cocaine and its metabolites in single intact hair samples from humans for toxicological screening [38]. Mass spectrometry can detect individual tropane alkaloids based on their molecular weight as opposed to the less specific recognition abilities of the antibodies. To date, no other study using MALDI-MSI of plant leaf tissues has reported differences between the adaxial and abaxial leaf surfaces [39-42]. The adaxial surface of coca leaves has a thick waxy cuticle containing hentriacontane as one of the major lipid components [10]. Wax components in the cuticle of *A. thaliana* are known to decrease the abundance of ions corresponding to flavonoids present within the leaf [43]. In order to remove the wax for further study, the Arabidopsis leaves were dipped in chloroform. In our case, chloroform treatment of leaf stage 2 and 3 leaves did not change the abaxial and adaxial distribution pattern, but it should be noted that the amount of cuticle wax removed was not determined (**Supplementary Fig. 5**). Observed differences in MS signal ratio intensities between both sides of the intact or treated leaves do not take into account possible matrix effects (i.e. ionization suppression or analyte recovery) due to the different wax layer thickness or other endogenous compound present. Despite the additional experiments to remove surface wax layers by dipping the leaves into chloroform, tropane alkaloids could still be identified on both sides of the leaves, but no relative quantitation between abaxial or adaxial leaf surfaces could be achieved. Nevertheless the distribution pattern of tropane alkaloids remains evenly distributed across the leaf in *E. coca* which was also

confirmed by recording the MS/MS spectrum and by comparison with authentic standards. In contrast, the tropane alkaloids scopolamine and atropine are mainly found in the vascular tissues of *D. stramonium* leaves (Solanaceae) when analyzed by indirect desorption electrospray (DESI) [44].

In summary we have determined that the acylation of the 3β-hydroxyl function of tropane alkaloids in *E. coca* is catalyzed by cocaine synthase, a member of the BAHD acyltransferase family. This is the first report showing that a BAHD member is involved in tropane alkaloid production in plants. We have further been able to show that the distribution of the enzyme is consistent with the hypothesis that tropane alkaloid biosynthesis in *E. coca* occurs in the above ground tissues. This is in contrast to what is known about the tropane alkaloid biosynthetic pathway in members of the Solanaceae. We predict that the dominant esters of tropane alkaloids found in other plant families such as Proteaceae, Brassicaceae, Rhizophoraceae, Convolvulaceae and Solanaceae will be also formed by the action of a BAHD acyltransferase. Isolation of these acyltransferases will be required to determine whether they have been recruited more than once throughout evolutionary history of plants.

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Methods

General materials, methods and procedures. See **Supplementary Methods.**

Cloning, heterologous expression, and purification of cocaine synthase and EcBAHD8. For details see **Supplementary Methods.** Previously, six BAHD acyltransferases were identified from a *E. coca* young leaf λZAPII cDNA library 16. Using these six sequences, a Blast search was performed on an in-house 454 cDNA sequencing database of E .coca young leaf tissue yielding two additional sequences, respectively BAHD7 (designated EcCS) and BAHD8 27. The open reading frames of EcCS (Genbank Accession No. KC140149) and EcBAHD8 (Genbank Accession No. KC140150) were amplified from *E. coca* leaf stage 2 cDNA using primer pairs EcCS_EC_Fwd / EcCS _EC_Rev and EcBAHD8_EC_Fwd / EcBAHD8_EC_Rev (Supplemental Table 2), respectively and gateway cloned. For obtaining sufficient protein for biochemical characterization, the open reading frame of EcCS was synthesized as a codon-optimized version designated EcCSopt by GenScript (Piscataway, NJ, USA).

Extraction of protein from *E. coca* tissues. See **Supplementary Methods.**

Synthesis of cinnamoyl–CoA. The synthesis of the activated cinnamic acid was performed as described 45 with some modifications. See **Supplementary Methods.**

Measurement of methylecgonine, cinnamoylcocaine and cocaine in plant tissue. See **Supplementary Methods.** EcCS and EcBAHD8 localization in *E. coca* Organs. Protein from different plant organs was extracted as described above. Equal amounts (15 μg) of protein were immunoblotted as described previously 9 with the following exceptions. After blocking, the membranes were incubated with 1:1000 anti-EcCS and 1:5000 anti-rabbit HRP-conjugated antibodies (Sigma-Aldrich) in blocking solution. For immunohistochemistry details see **Supplementary Methods.**

For localization of EcCS slides were incubated with 1:100 anti-EcCS antibody. For localization of cocaine slides were incubated with 1:100 sheep anti-benzoylecgonine antibodies (RayBiotech, Norcross, USA). Anti-rabbit HRP-conjugated antibody (Sigma-Aldrich) or 1:100 anti-sheep HRP-conjugated antibody (RayBiotech, Norcross, USA) was used

as secondary antibody. For fluorescent staining, TSA Kit #24 (Invitrogen) was used according to the manufacturers' manual. For cocaine localization slides were imaged using a Zeiss LSM510 confocal microscope and a 20x objective lens (Plan-Apochromat 20x/0.8 M27, Carl Zeiss). TSA fluorescence was excited at 543 nm and detected using a BP 585–615 filter. Plant autofluorescence was excited at 488 nm and detected using LP 505nm filter. For EcCS localization slides were imaged using a Zeiss LSM710 confocal microscope and a 20x objective lens (EC Plan-Neofluar 20x/0.5 M27, Carl Zeiss). TSA fluorescence was excited at 561 nm and detected using 585–614 nm lambda channels. Plant auto fluorescence was excited at 488 nm and detected using 495-534 nm lambda channels. Overview pictures were obtained, by taking tile pictures of whole tissue sections. TSA fluorescence and plant auto fluorescence were imaged simultaneously, overlayed using ImageJ 46 and transferred to Illustrator CS6 (Adobe Systems) without further manipulation.

EcCS Antibody Production. See **Supplemental Methods.**

Immunoprecipitation. See **Supplementary Methods.**

Cocaine Synthase Substrate Specificity. See **Supplementary Methods.**

Size-exclusion chromatography. See **Supplementary Methods.**

Synthesis of $^{13}C_7$-Cocaine. ^{13}C-benzoyl coenzyme A was prepared as described [47] with the exception that benzoic acid was isotopical labeled. $^{13}C_7$ benzoic acid was obtained from Isotec (Miamisburg, OH, USA). For details see **Supplementary Methods.**

Enzyme Assays for Kinetic Analysis of Enzymes and Plant Activity Determination. Protein concentration and incubation parameters were chosen so that the reaction velocity was linear in respect to enzyme concentration and incubation time for all enzyme assays. Standard assays contained 50 mM glycine-NaOH pH 9.4, 1 mg/mL BSA, 1 mM TCEP, 1 mM methylecgonine, 1 mM of benzoyl- or cinnamoyl-CoA, and the enzyme preparation. All reactions were carried out at 20 °C in a PCR cycler Primus 96 plus (MWG Biotech, Ebersberg, Germany). For determination of the pH optimum 50 mM glycine-NaOH buffer covering the

range from pH 8.6 to 10.4 was used. Enzyme and substrate concentrations were varied during characterization process. For kinetic assays, the co-substrate concentration was held constant. Reactions (50 μL) were stopped after 10 min by adding 5 μL 1 N HCl and 5 μl of 2.5 μM $^{13}C_7$-cocaine was added as internal standard. 100 μl chloroform were added to the assays and shaken in a paint shaker for 3 min, to remove the protein from the assay. The aqueous phase was transferred into fresh HPLC vials and subjected to LC-MS/MS analysis. For details see **Supplementary Methods.**

Quantitative real-time PCR analysis. See **Supplementary Methods**.

Phylogenetic Analysis. Analysis of protein sequences (see **Supplemental Table 1** for accession numbers) was performed as previously described [16]. The following enzymes and their corresponding genbank accession numbers were added to the analysis EcCS (cocaine synthase) - KC140149 and EcBAHD8 - KC140150.

MALDI Imaging. For details see **Supplementary Methods**. *E. coca* leaf samples were collected at three different stages of maturity: leaf stage 1; leaf stage 2 and leaf stage 3. After being cut at the basis of the stem, samples were then placed in-between two aluminum foils (to maintain their flat shape), and immerged immediately in liquid nitrogen for flash freezing. Additionally, leaf stage 2 and leafs stage 3 leaves were plunged in chloroform for 5 seconds prior flash freezing in order to remove waxy cuticle. Frozen samples were then stored at -80°C prior to the analysis. Acquisitions were performed on a triple quadrupole linear ion trap mass spectrometer (AB Sciex, Concord, ON. Canada) equipped with a MALDI source and a frequency-tripled Nd:YAG laser 355 nm (elliptical beam shape of 100x200 μm). MS and MS/MS images were acquired in positive ionization mode. General operating conditions were: data acquisition mode = line scan in rastering mode, repetition rate laser = 1'000 Hz; laser energy = 60 μJ; MALDI source and q0 region pressures were of 1 Torr and 8 mTorr, respectively; vacuum gauge in q2 = 2.4 10-5 Torr (nitrogen); declustering potential = 70 V; entrance potential = 10 V; quadrupole resolution was set to unit for Q1 and Q3.

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Supplementary Information

A BAHD acyltransferase catalyzes the final step in cocaine biosynthesis

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Chemicals and plant material. Methylecgonine (2-carbomethoxy-3β-tropine) was purchased from LGC Standards GmbH (Wesel, Germany). Cocaine, cocaine-d_3, cinnamoylcocaine and methylecgonine for MALDI mass spectrometry imaging were provided by Lipomed (Arlesheim, Switzerland). All other chemicals were purchased from Sigma-Aldrich, Carl Roth, or Merck and had the highest available quality. Water was supplied by a Milli-Q Synthesis System (Millipore). Seeds of *Erythroxylum coca* were obtained from the Botanical Garden in Bonn (Germany). The seeds were removed from the surrounding pulp and germinated in Perlite. The plants were grown at 22 °C under a photoperiod of 12 h light/12 h dark, with a humidity of 65% and 70%, respectively and fertilized once a week with Ferty 3 (15-10-15) and Wuxal Top N (Planta). A voucher specimen was deposited at the Herbarium Haussknecht (JE), Friedrich Schiller University, Jena, Germany. Leaves for MALDI mass spectrometry imaging experiments were collected from *Erythroxylum coca* species at the Botanical Garden in Zurich (Switzerland).

Cloning, heterologous expression, and purification of cocaine synthase and EcBAHD8. Previously, six BAHD acyltransferases were identified from a *E. coca* young leaf λZAPII cDNA library [1]. Using these six sequences, a Blast search was performed on an in-house 454 cDNA sequencing database of *E .coca* young leaf tissue yielding two additional sequences, respectively BAHD7 (designated *EcCS*) and *BAHD8*. The open reading frames of *EcCS* (Genbank Accession No. KC140149) and *EcBAHD8* (Genbank Accession No. KC140150) were amplified from *E. coca* leaf stage 2 cDNA using primer pairs EcCS_EC_Fwd / EcCS _EC_Rev and EcBAHD8_EC_Fwd / EcBAHD8_EC_Rev (**Supplemental Table 2**), respectively and gateway cloned into the *Escherichia coli* expression vector pH9GW as previously described [2]. The expression vectors were introduced into *E. coli* BL21(DE3) (Invitrogen) and the bacterial culture was grown in LB medium supplemented with 50 μg/ml kanamycin at 37°C with shaking at 220 rpm until an OD_{600} of 0.4 - 0.5 was reached. Protein expression was induced by addition of 1 mM IPTG with further cultivation at 18 °C for 24 h. The cells were resuspended in 50 mM Bis-tris buffer (pH 8) supplemented with 10% glycerol and 5 mM DTT and disrupted by sonication. The lysate was centrifuged at 15,000 × g and 4 °C for 15 min and the soluble fractions were tested for enzymatic activity. The assay buffer consisted of 50 mM Bis-Tris propane buffer (pH 8.0) supplemented with 10% (vol/vol) glycerol, 5 mM Tris(2-carboxyethyl)phosphine hydrochloride (TCEP), 1 mM methylecgonine, 1 mM benzoyl-CoA, and 10 μL protein extract in a total volume of 100 μL. The assay was stopped after 2 h with 10 μL 1 N HCl.

The assays were analyzed by liquid chromatography- ion trap mass spectrometry on an 1100 series equipment HPLC (Agilent Technologies, Germany) coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Germany) operated in positive ionization mode in the range m/z 50-500. Skimmer voltage, 42.4 V; capillary exit voltage, 110 V; capillary voltage, -4,000 V; nebulizer pressure, 35 psi; drying gas, 9 L min^{-1}; gas temperature, 330 °C. Elution was accomplished using a Nucleodur Sphinx RP column (25 cm x 4.6 mm, 5 μm, Macherey-Nagel, Germany) with a gradient of 0.2% (vol/vol) formic acid (solvent A) and methanol (solvent B) at a flow rate of 1 mL/min at 25 °C as follows: 45-65% B (5 min), 65-100% B (0.1 min), 100% B (1.9 min), 100-45% B (0.1 min), 45 % B (3.9min). Flow coming from the column was diverted in a ratio 4:1 before reaching the ESI unit. Cocaine mass (m/z of 304) and

Cinnamoylcocaine (m/z of 330) was monitored, and the area under the product peak was used for activity comparison.

For obtaining sufficient protein for biochemical characterization, the open reading frame of *EcCS* was synthesized as a codon-optimized version designated *EcCSopt* by GenScript (Piscataway, NJ, USA) and supplied in pUC57 vector (GeneScript, USA). *EcCSopt* was amplified from the pUC57 vector using the primer pair EcCSopt_SC_Fwd / EcCSopt_SC_Rev (**Supplemental Table 2**) and gateway cloned into the *Saccharomyces cerevisieae* expression vector pYes-NStrep-GW (*EcBAHD8* was also gateway cloned into pYes-NStrep-GW, using the pDONR207 clone from the initial gateway cloning into pH9GW). The vector pYes-NStrep-GW is a modified pYes-DEST52 (Invitrogen) *S. cerevisieae* expression vector that was built in-house, to facilitate the production of recombinant proteins carrying an N-terminal StrepTagII and a thrombin cleavage site. Furthermore, *EcCSopt* and *EcBAHD8* were amplified using the primer pairs EcCSopt_SC_Fwd / EcCSopt_SC_C-Strep_Rev and EcBAHD8_SC_Fwd / EcBAHD8_SC_C-Strep_Rev (**Supplemental Table 2**) and gateway cloned into the *S. cerevisieae* expression vector pYes-DEST52 to facilitate the production of recombinant proteins carrying a C-terminal StrepTagII. The expression constructs were introduced into *S. cerevisieae* Inv-Sc1 (Invitrogen) and the protein was expressed as described in the manufacturers` manual. For protein purification the cells were resuspended in 100 mM Tris-HCl (pH 8) supplemented with 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5 mM TCEP and 1 mM phenylmethanesulfonyl fluoride (PMSF) and lysed using a pressure cell homogenizer (Stansted Fluid Power, UK). The lysate was centrifuged at 15,000 × g and 4 °C for 15 min and the soluble protein was purified using FPLC. After filtration of the sample using a 0.45 μm-pore-size filter (Millipore), the filtrate was directly loaded onto a StrepTrap HP column (GE Healthcare) using an FPLC machine (ÄKTApurifier, GE Healthcare). Recombinant protein was eluted using 100 mM Tris-HCl (pH 8) supplemented with 150 mM NaCl, 1 mM EDTA, 5 mM TCEP, 1 mM PMSF and 2.5 mM desthiobiotin. Glycerol was added to a final concentration of 10% (vol/vol), and the mix was aliquoted and stored at −20 °C and used directly for enzyme assays. The purity of recombinant protein was evaluated by SDS/PAGE gel electrophoresis followed by colloidal coomassie staining. Protein concentration was measured using the Bradford Protein Assay (Bio-Rad) according to manufacturers' manual. Equivalent amounts of N-terminal and C-terminal StrepTagII-tagged recombinant protein were tested for enzyme activity, but no significant difference was observed. N-terminal StrepTagII-tagged protein expressed in *S. cerevisieae* was used for enzyme kinetics.

Extraction of protein from *E. coca* tissues. Fresh *E. coca* tissues were harvested and ground using a mortar and pestle precooled with liquid nitrogen. The plant powder was mixed in a ratio of 1:5 with 100mM Tris-HCl pH 8 supplemented with 10% (vol/vol) glycerol, 2% (wt/vol) polyvinylpolypyrrolidone (PVPP), 50 mM $Na_2S_2O_5$, 5 mM DTT and 1 mM PMSF. The resulting emulsion was mixed and incubated for 10 min on ice followed by centrifugation at 16,000 × g at 4 °C for 10 min. The supernatant is referred to as the plant extract, and was used directly for immunoblotting analysis. For plant activity assays, the plant extracts were desalted into 100 mM TrisHCl pH 8 supplemented with 10% (vol/vol) glycerol and 5 mM TCEP using Ilustra Nap-5 columns (GE Healthcare) according to the manufacturers' instructions. Protein concentration was measured using the Bradford Protein Assay (Bio-Rad) according to the manufacturers' manual.

Synthesis of cinnamoyl–CoA. The synthesis of the activated cinnamic acid was performed as described [3] with some modifications. 50 mmol cinnamic acid was added to a solution of 120 mmol carbonyldiimidazole in tetrahydrofuran. The reaction mixture was stirred for 5 h at reflux. After cooling to room temperature any solids were filtered off. The solvent was removed at a rotary evaporator. The pale yellow solid was washed with 10 mL water and dried *in vacuo*. 0.16 mmol of activated cinnamic acid was dissolved in 700 µL 50mM $NaHCO_3$ and added slowly to a solution of 0.13 mmol CoA in 500 µL of 50mM $NaHCO_3$ at 3°C. Acetone was added until the precipitate dissolved completely. The mixture was stirred 24 h at 3°C. Afterwards the acetone was removed with N_2. The CoA ester was purified as described in [1] and fractions were analysed by HPLC UV. The absorbtion at 260 nm was used for quantification using coenzyme A tritium salt The fraction with with absorbtion at 260nm (6.3 min) was further analyzed by liquid chromatography - ion trap mass spectrometry on an 1100 series equipment HPLC (Agilent Technologies, Germany) coupled to an Esquire 6000 ESI-Ion Trap mass spectrometer (Bruker Daltonics, Germany) operated in negative ionization mode in the range m/z 100-1000. Skimmer voltage, 42.4 V; capillary exit voltage, 123.7 V; capillary voltage, -4,500 V; nebulizer pressure, 35 psi; drying gas, 10 L min^{-1}; gas temperature, 330 °C. Elution was accomplished using a Nucleodur Sphinx RP column (25 cm x 4.6 mm, 5 µm, Macherey-Nagel, Germany) with a gradient of 20 mM (vol/vol) ammonium acetate (solvent A) and acetonitrile (solvent B) at a flow rate of 1 mL/min at 25 °C as follows: 15-29% B (7 min), 29-90% B (1 min), 90% B (2 min), 90-15% B (0.1 min), 15 % B (4.9min). Flow coming from the column was diverted in a ratio 4:1 before reaching the ESI unit.

Measurement of methylecgonine, cinnamoylcocaine and cocaine in plant tissue. *E. coca* leaves of all three leaf stages (L1, L2, L3), flower, stem and roots were ground to a fine powder in liquid N2. Tropane alkaloids were extracted 1:10 (w/v) using 0.1% (v/v) formic acid, 30% (v/v) methanol at room temperature for 15 min. Samples were centrifuged at 4000 x g for 15 min. The supernatant was filtered using 0.45 µm syringe filters and adjusted to pH 8 with saturated sodium carbonate solution followed by reextraction with three times chloroform. Phase separation was achieved using Chromabond PTS columns (Macherey-Nagel, Germany). The organic phase was vacuum dried and solved in water for analysis. Measurements were taken for six replicates of each sample. Analysis was done as described in **Supplementary Methods** section **Enzyme assays for kinetic analysis of enzymes and plant activity determination** using authentic standards of methylecgonine, cocaine and cinnamoylcocaine.

EcCS and EcBAHD8 Localization in *E. coca* Organs. Protein from different plant organs was extracted as described above. Equal amounts (15 µg) of protein were immunoblotted as described previously with the following exceptions [2]. After blocking, the membranes were incubated with 1:1000 anti-EcCS and 1:5000 anti-rabbit HRP-conjugated antibodies (Sigma-Aldrich) in blocking solution.

For immunohistochemistry, selected fresh tissues were harvested and fixed for 4 h at room temperature in 50% (vol/vol) ethanol, 5% (vol/vol) acetic acid and 3.7% (vol/vol) formaldehyde using vacuum infiltration. Samples were rinsed 3 times for 10 min in PBS (2.7 mM KCl, 137 mM NaCl, 1.8 mM KH_2PO_4, 10 mM Na_2HPO_4, adjusted to pH 7.4 with 1N KOH). Samples were dehydrated in a ascending series of ethanol solutions (vol/vol) (10%, 30%, 50%, 70%, 90%) each time for 1h. Samples were left in 96% (vol/vol) ethanol overnight, followed by an ascending series in Roti-Histol (Carl Roth), 25% (vol/vol), 50% (vol/vol), 75% (vol/vol), and 100% for 1 h each. The following steps were performed at 60 °C. Roti-Histol (Carl Roth) was exchanged with liquid Paraplast X-Tra (Carl Roth) over a course of 4 d by replacing half of the solution with liquid Paraplast X-Tra every 12 h. Remaining Roti-Histol was allowed to evaporate in an open container at 60 °C for 5 h. Single plant organs were embedded in aluminum molds and left to settle at room temperature for 30 min, before being transferred to 4 °C for storage. Samples were cut on a rotary microtome to obtain 10 µm slices which were adhered to polylysine coated slides (Thermo Scientific) at 42 °C overnight. To prepare for antibody treatment, slides were incubated in Roticlear (Carl Roth) and rehydrated by a descending ethanol series (2x 100%, 95% (vol/vol), 70% (vol/vol), 50% (vol/vol), 30% (vol/vol), 15% (vol/vol) and 2x H_2O) for 2 min at each concentration. Endogenous peroxidases were blocked with 3% (vol/vol) H_2O_2 in PBS for 30 min. Slides were washed twice with PBS and once with PBT (PBS supplemented with 0.1% (vol/vol) Tween 20) for 5 minutes each, and blocked overnight with 1% (wt/vol) BSA in PBT at 4 °C.

For localization of EcCS slides were incubated with 1:100 anti-EcCS antibody in 1% (wt/vol) BSA in PBT overnight at 4 °C in a humid chamber. For localization of cocaine slides were incubated with 1:100 sheep anti-benzoylecgonine antibodies (RayBiotech, Norcross, USA) in 1% (wt/vol) BSA in PBT overnight at 4 °C in a humid chamber. After washing slides with PBT, incubation with 1:100 anti-rabbit HRP-conjugated antibody (Sigma-Aldrich) or 1:100 anti-sheep HRP-conjugated antibody (RayBiotech, Norcross, USA) in 1% (wt/vol) BSA in PBT for 1 h was performed, for localization of EcCS or cocaine respectively. For fluorescent staining, TSA Kit #24 (Invitrogen) was used according to the manufacturers' manual, with a developing time of 30 min. For cocaine localization slides were imaged using a Zeiss LSM510 confocal microscope and a 20x objective lens (Plan-Apochromat 20x/0.8 M27, Carl Zeiss). TSA fluorescence was excited at 543 nm and detected using a BP 585–615 filter. Plant autofluorescence was excited at 488 nm and detected using LP 505nm filter. For EcCS localization slides were imaged using a Zeiss LSM710 confocal microscope and a 20x objective lens (EC Plan-Neofluar 20x/0.5 M27, Carl Zeiss). TSA fluorescence was excited at 561 nm and detected using 585–614 nm lambda channels. Plant auto fluorescence was excited at 488 nm and detected using 495-534 nm lambda channels. Overview pictures were obtained, by taking tile pictures of whole tissue sections. TSA fluorescence and plant auto fluorescence were imaged simultaneously, overlayed using ImageJ (open source, National Institutes of Health, USA) and transferred to Illustrator CS6 (Adobe Systems) without further manipulation.

EcCS antibody production. Purified recombinant EcCS protein carrying an N-terminal StrepTagII produced in *S. cerevisieae* as described above, was used to produce polyclonal antibodies in rabbits. Antibodies were affinity-purified using epoxy-activated Sepharose conjugated to the same recombinant protein that was used for immunization (Davids Biotechnologie, Regenburg, Germany). Specificity of the purified antibodies to EcCS in comparison to other *E. coca* BAHD proteins was assessed by immunoblotting of recombinant

proteins expressed in *E. coli* using the pH9GW vector as described above (**Supplemental Fig. 2b**).

Immunoprecipitation. Protein from leaf stage 2 leaves of *E. coca* was extracted as described in above. Protein was desalted into immunoprecipitation buffer (50 mM BisTris pH 8 supplemented with 10% (vol/vol) glycerol, 1 mM TCEP, 100 mM NaCl, 1 mM PMSF and 0.5% (vol/vol) NP-40). Protein concentration was measured as described earler. 100 µl Protein A coupled agarose beads (GE Healthcare) were prepared as described in the manual. 500 ng protein were adjusted to 700 µl with immunoprecipitation buffer. 5 µg of anti-EcCS antibody and 20 µl of agarose bead slurry were added to the protein sample and incubated at 4 °C for one hour. The immunoprecipitate, represented by the agarose beads, was separated from the supernatant by centrifugation at 12000 x g for 20 s at 4 °C. The supernatant was kept as the IP supernatant sample. The immunoprecipitate was washed three times with 1ml of immunoprecipitation buffer, and the wash solutions were discarded. Enzyme assays on the immunoprecipitate and supernatant were analyzed on a HPLC 1200 series equipment (Agilent) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbo spray ion source, using a ZORBAX RRHT Eclipse XDB-C18 column (5 cm x 4.6 mm x 1.8 µm) (Agilent). Separation was achieved in 7 min, at 20 ºC and a flow rate of 800 µL/min, using formic acid 0.05% (A) and acetonitrile (B) as mobile phase as follows: 90% A (0.5 min), 90-30% A (3.5 min), 30-0% A (0.1 min), 0% A (0.7 min), 0-90% A (0.1 min), 90% A (2.1 min). The spectrometer operated in positive ionization mode; injection volume, 5 µL; curtain gas, 30 psi; turbo heater temperature, 700 ºC; nebulising gas, 60 psi; heating gas, 70 psi; collision gas, 6 psi; ion spray, 5000 eV. Analytes were monitored by multiple reaction monitoring (MRM): cocaine m/z 304.3 → 182.3 (collision energy (CE) 26 V; declustering potential (DP) 45 V). Quantification was based on a standard curve of authentic cocaine. Both Q1 and Q3 quadrupoles were maintained at unit resolution.

Cocaine synthase substrate specificity. Enzyme assays were performed as described in **Supplementary Methods** section **Enzyme assays for kinetic analysis of enzymes and plant activity determination** with the exception that various coenzyme A esters were used for the esterification reaction with methylecgonine. Besides benzoyl and cinnamoyl coenzyme A, acetyl, acetoacyl, coumaroyl, hexanoyl and malonyl coenzyme A were tested. In lack of access to the corresponding esters, the product ion was predicted and the peak area of the product peaks was used for comparison with the peak area of cocaine, assuming that ionization of the molecules as well as the response of the machine are similar for the ester products. Quantification was based on an authentic standard curve for cocaine. The relative activities compared to cocaine are only based upon peak areas. Atropine served as internal standard and enzyme assays (100 µl) were diluted with 900 µl of methanol, spiked with internal standard before injection.

Analysis was carried out on a HPLC 1200 series equipment (Agilent) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbo spray ion source, using a ZORBAX RRHT Eclipse XDB-C18 column (5 cm x 4.6 mm x 1.8 µm) (Agilent). Separation was achieved in 7 min, at 20 ºC and a flow rate of 800 µL/min, using formic acid

0.05% (A) and acetonitrile (B) as mobile phase as follows: 90% A (0.5 min), 90-30% A (3.5 min), 30-0% A (0.1 min), 0% A (0.7 min), 0-90% A (0.1 min), 90% A (2.1 min). The spectrometer operated in positive ionization mode; injection volume, 5 µL; curtain gas, 30 psi; turbo heater temperature, 700 ºC; nebulising gas, 60 psi; heating gas, 70 psi; collision gas, 6 psi; ion spray, 5000 eV. Analytes were monitored by multiple reaction monitoring (MRM): cocaine m/z 304.3 → 182.3 (collision energy (CE) 26 V; declustering potential (DP) 45 V); cinnamoylcocaine m/z 330.3 → 182.3 (CE 26V; DP 45V); methylecgonine m/z 200.2 → 182.3 (CE 23V; DP 31V); acetyl product m/z 242.3 → 182.3 (CE 26V; DP 45V); acetoacyl product m/z 284.3 → 182.3 (CE 26V; DP 45V); coumaroyl product m/z 346.3 → 182.3 (CE 26V; DP 45V); hexanoyl product m/z 298.3 → 182.3 (CE 26V; DP 45V);malonyl product m/z 286.3 → 182.3 (CE 26V; DP 45V); atropine m/z 290.1 → 124.1 (CE 31V; DP 51V) Quantification was based on a standard curve of authentic cocaine and cinnamoylcocaine, taking the signal from the internal standard into account. Both Q1 and Q3 quadrupoles were maintained at unit resolution.

Size-exclusion chromatography. Protein size of EcCS was determined using an ÄKTApurifier (GE Healthcare) equipped with a HiLoad 16/60 Superdex 200 prep-grade column (GE Healthcare). Recombinant protein was expressed in *S. cerevisieae* and purified as described earlier. The running buffer consisted of 50 mM BisTris pH 8.0 supplemented with 150 mM NaCl. The column was calibrated using the Gel Filtration LMW Calibration Kit (GE Healthcare) as described in the manufacturers' instructions. Samples were loaded at a flow rate of 0.5 mL/min. Protein was eluted at a flow rate of 1 mL/min for 1.5 CV while collecting fractions of 6 mL.

Synthesis of $^{13}C_7$-Cocaine. ^{13}C-benzoyl coenzyme A was prepared as described [4] with the exception that benzoic acid was isotopical labeled. $^{13}C_7$ benzoic acid was obtained from Isotec (Miamisburg, OH, USA). An enzymatic assay (200 µl) containing 50 mM potassium phosphate buffer pH 8 supplemented with 1 mM TCEP, 1 mg/mL BSA, 0.1 mM $^{13}C_7$-benzoyl-CoA, 0.5 mM methylecgonine and 8 µg purified EcCS was left to react overnight at room temperature. Another 8 µg purified EcCS was added and left to react at room temperature for 12 h. The solution was basified using 20 µl 1 N NaOH and extracted 3 times with 500 µl chloroform. The chloroform phase was dried down under nitrogen flow and resuspended in 200 µl 10% (vol/vol) ethanol supplemented with 0.1% (vol/vol) formic acid. To determine the concentration for use as internal standard, dilutions of the product were analyzed on an API 3200 tandem mass spectrometer (Applied Biosystems) as described below.

Enzyme assays for kinetic analysis of enzymes and plant activity determination. Protein concentration and incubation parameters were chosen so that the reaction velocity was linear in respect to enzyme concentration and incubation time for all enzyme assays. Standard assays contained 50 mM glycine-NaOH pH 9.4, 1 mg/mL BSA, 1 mM TCEP, 1 mM methylecgonine, 1 mM of benzoyl- or cinnamoyl-CoA, and the enzyme preparation. All reactions were carried out at 20 °C in a PCR cycler Primus 96 plus (MWG Biotech, Ebersberg, Germany). For determination of the pH optimum 50 mM glycine-NaOH buffer covering the range from pH 8.6 to 10.4 was used. Enzyme and substrate concentrations were varied during characterization process. For kinetic assays, the co-substrate concentration was held constant. Reactions (50 µL) were stopped after 10 min by adding 5 µL 1 N HCl and 5 µl of 2.5 µM $^{13}C_7$-cocaine was added as internal standard. 100 µl chloroform were added to the assays and

shaken in a paint shaker for 3 min, to remove the protein from the assay. The aqueous phase was transferred into fresh HPLC vials and subjected to LC-MS/MS analysis.
Analysis was carried out on a HPLC 1200 series equipment (Agilent) coupled to an API 3200 tandem mass spectrometer (Applied Biosystems) equipped with a turbo spray ion source, using a ZORBAX RRHT Eclipse XDB-C18 column (5 cm x 4.6 mm x 1.8 μm) (Agilent). Separation was achieved in 7 min, at 20 ºC and a flow rate of 800 μL/min, using formic acid 0.05% (A) and acetonitrile (B) as mobile phase as follows: 90% A (0.5 min), 90-30% A (3.5 min), 30-0% A (0.1 min), 0% A (0.7 min), 0-90% A (0.1 min), 90% A (2.1 min). The spectrometer operated in positive ionization mode; injection volume, 5 μL; curtain gas, 30 psi; turbo heater temperature, 700 ºC; nebulising gas, 60 psi; heating gas, 70 psi; collision gas, 6 psi; ion spray, 5000 eV. Analytes were monitored by multiple reaction monitoring (MRM): cocaine m/z 304.3 → 182.3 (collision energy (CE) 26 V; declustering potential (DP) 45 V); cinnamoylcocaine m/z 330.3 → 182.3 (CE 26V; DP 45V); methylecgonine m/z 200.2 → 182.3 (CE 23V; DP 31V); 13C7-cocaine m/z 311.3 → 182.3 (CE 26V; DP 45V). Quantification was based on a standard curve of authentic cocaine and cinnamoylcocaine, taking the signal from the internal standard into account. Both Q1 and Q3 quadrupoles were maintained at unit resolution. For kinetic analysis of EcBAHD8 with cinnamoyl-CoA and methylecgonine, the analysis was carried out as described above except for the following changes. An API5000 tandem mass spectrometer (Applied Biosystems) was used and all chromatographic and mass spectrometer parameters were the same, except for the declustering potential settings which were as follows: cocaine 75 V; cinnamoylcocaine 75V; methylecgonine 61V; $^{13}C_7$-cocaine 75V. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Calculations and fitting of kinetic curves was performed using Origin 8.5 (OriginLab, USA).

Quantitative real-time PCR analysis. For relative quantification experiments were performed as described [5]. Primer pairs targeting the *EcCS* and *EcBAHD8* transcripts EcCS_qPCR_Fwd / EcCS_qPCR_Rev and EcBAHD8_qPCR_Fwd / EcBAHD8_qPCR_Rev (**Supplemental Table 2**) were designed. Standard curve analysis showed a PCR efficiency of 95.5% and 88.3% and R^2 values of 0.9983 and 0.9992 for EcCS and EcBAHD8 primer pairs, respectively. Expression of the genes was normalized to gene 6409 and gene 10131 [5] expression using qBase version 1.3.5 [6].

For absolute quantification experiments, pDONR207 plasmids harbouring *EcCS* or *EcBAHD8* obtained during the cloning procedure, were quantified using NanoDrop 2000c (NanoDrop Technologies, Wilmington, USA). Standardcurves of plasmids ranging from 10 to 10^9 plasmids per assay were run in parallel to qPCR assays as described [5]. Standard curve analysis showed a PCR efficiency of 78.8% and 85.8% and R^2 values of 1.000 and 0.998 for EcCS and EcBAHD8 primer pairs, respectively. The data was normalized to the input amount of the total RNA to the original cDNA synthesis reaction.

MALDI Imaging. Standard and matrix solutions: α-cyano-4-hydroxycinnamic acid (CHCA) was dissolved at a concentration of 10 mg/mL in MeOH:H_2O:HCOOH (75:25:0.1, vol/vol/vol). Deuterated cocaine (cocaine-d_3) was added to the matrix solution (final concentration of 200 ng/mL) and sprayed simultaneously with the matrix. Matrix solutions were kept in amber glass bottles to prevent their degradation by UV and stored at 4°C between uses.

E. coca leaf samples were collected at three different stages of maturity: leaf stage 1; leaf stage 2 and leaf stage 3. After being cut at the basis of the stem, samples were then placed in-between two aluminum foils (to maintain their flat shape), and immerged immediately in liquid nitrogen for flash freezing. Additionally, leaf stage 2 and leafs stage 3 leaves were

plunged in chloroform for 5 seconds prior flash freezing in order to remove waxy cuticle. Frozen samples were then stored at -80°C prior to the analysis.

After drying in a desiccator under vacuum for 20 min at room temperature, intact leaf samples were mounted onto the stainless steel MALDI plate (OPTI TOF 384 well Insert - 123 x 81 mm or OPTI TOF 192 well insert – 44 x 44 mm, AB Sciex, Concord, ON, Canada) using a double-sided adhesive tape (Plasto, Chenove, France). Two intact leaves from the same maturity stage were placed on the MALDI plate in order to analyze both sides of the leaves in the same run. Approximately 12 mL of CHCA solution were manually sprayed using a Custom Micron CM-C Plus airbrush 0.18 mm (ANEST IWATA Corporation, Yokohama, Japan) held at a distance of 20 cm from the plate. N_2 was used as the nebulizer gas (purity > 99.995%, Messer, Switzerland) and operated at a constant pressure of 0.5 bars. For the semi-automated spraying, the airbrush was fixed on two axis-stages motors (models A-LSQ300B [horizontal axis] and A-LSQ150B [vertical axis], Zaber Technologies Inc., Vancouver, BC, Canada), to allow for controlling the sprayer motion and speed along a fixed axis. The sprayer was placed at a distance of 25 cm from the target plate and moved linearly at a constant speed of 15 mm/s. 30 layers of CHCA matrix solution containing the internal standard (solution of 200 ng/mL) were applied.

Acquisitions were performed on a triple quadrupole linear ion trap mass spectrometer (AB Sciex, Concord, ON. Canada) equipped with a MALDI source and a frequency-tripled Nd:YAG laser 355 nm (elliptical beam shape of 100x200 μm). MS and MS/MS images were acquired in positive ionization mode. General operating conditions were: data acquisition mode = line scan in rastering mode, repetition rate laser = 1'000 Hz; laser energy = 60 μJ; MALDI source and q_0 region pressures were of 1 Torr and 8 mTorr, respectively; vacuum gauge in q_2 = 2.4 10^{-5} Torr (nitrogen); declustering potential = 70 V; entrance potential = 10 V; quadrupole resolution was set to unit for Q_1 and Q_3.

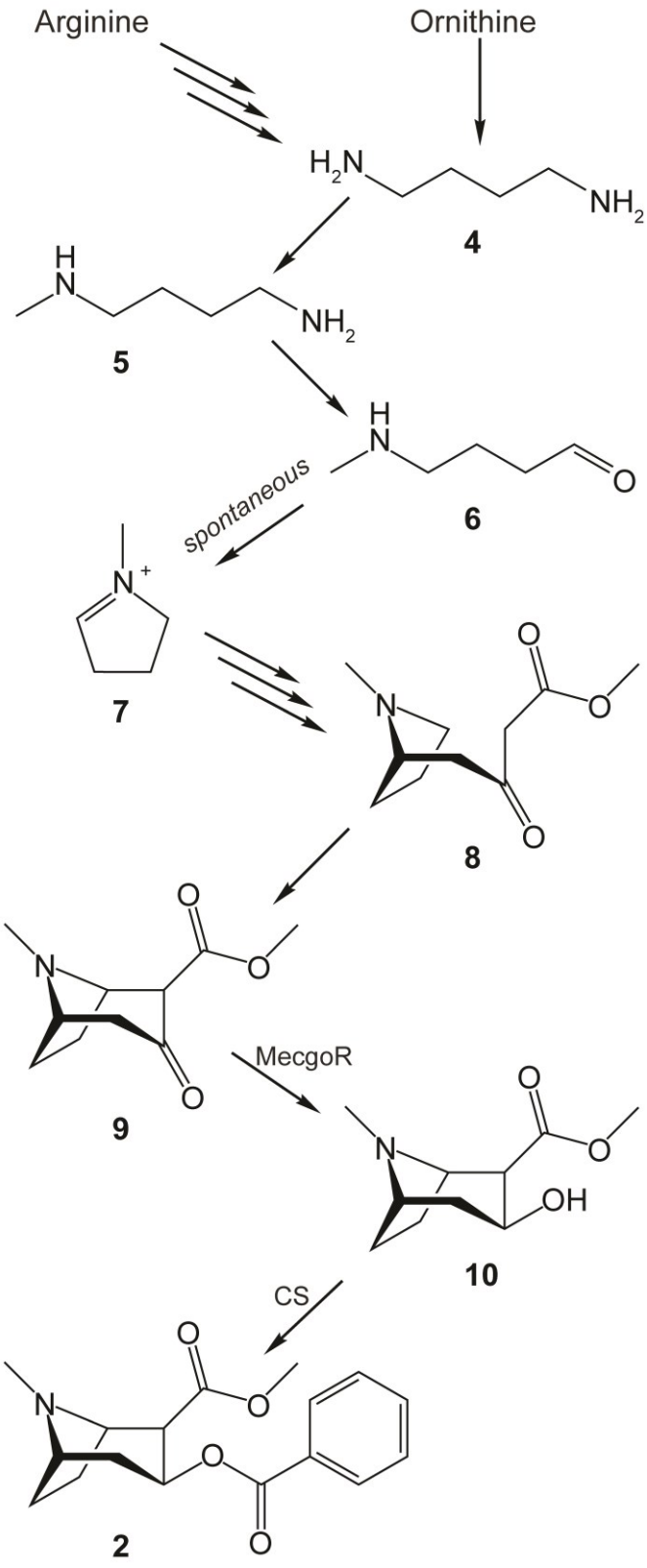
SRM data were acquired by monitoring simultaneously the following transitions: *m/z* 304 > *m/z* 182 for cocaine (CE = 35 eV), *m/z* 307 > *m/z* 185 for cocaine-d_3 (CE = 35 eV), *m/z* 330 > *m/z* 182 for cinnamoylcocaine (CE = 40 eV) and *m/z* 200 > *m/z* 182 for methylecgonine (CE = 40 eV). The dwell time was set to 500 ms (TST = 2.020 s) and the distance between two line scans was of 1 mm (rastering speed of 0.5 mm/s), resulting in MALDI-SRM/MS-based images with pixels of 1x1 mm for the four compounds. To generate MALDI-SRM/MS images at higher resolution (*i.e.* pixels of 50x50 μm^2), the plate speed was kept constant at 0.5 mm/s, the dwell time was decreased to 20 ms, and the distance between 2 line scans was set to 50 μm (oversampling technique, see results and discussion part).

MS/MS spectra in the enhanced product ion (EPI) scan mode were acquired at a plate speed of 1 mm/s and the distance between two line scans was of 2 mm (pixels of 2 x 2 mm^2). The conditions for each compounds were: i) for cocaine: precursor ion = *m/z* 304; CE = 35 eV, scan range = *m/z* 80 – 310, TST = 1.991 s ; ii) for cinnamoylcocaine: precursor ion = *m/z* 330; CE = 35 eV, scan range = *m/z* 80 – 340, TST = 2.051 s; iii) for methylecgonine: precursor ion = *m/z*

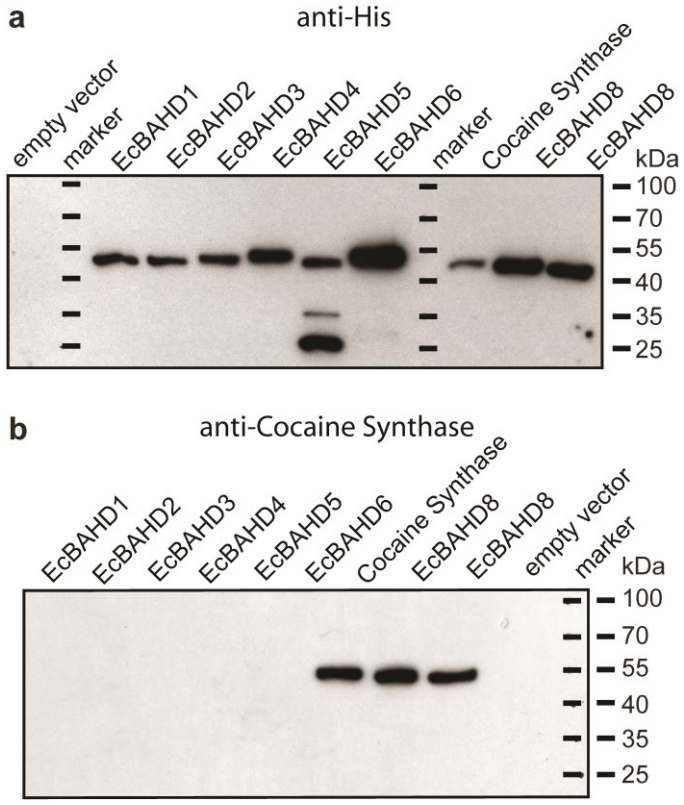
200; CE = 45 eV, scan range = *m*/*z* 80 – 210, TST = 1.991 s. Reference spectra were acquired from a MALDI spot where 1 µL of a standard solution with the 3 analytes at a concentration of 100 ng/mL mixed 1:1 (v:v) with CHCA (10 mg/mL) was spotted onto the target plate. Spectra for blank samples and for structural confirmation at the different maturation stages were generated by summing 6 vertical adjacent pixels over selected regions.

The MALDI source and its laser were controlled using a custom M3Q Server software based on a LabView platform (AB Sciex). Analyst 1.5 software (AB Sciex) was used for mass spectrometer control and for data collection. PeakView software (v.1.0.0.3, AB Sciex) was used for raw data processing. A dedicated script was provided by Eva Duchoslav (AB Sciex) to convert raw MS data files into an .img file that is compatible with the TissueView software (v.1.0, AB Sciex) for MS and MS/MS images generation and processing.

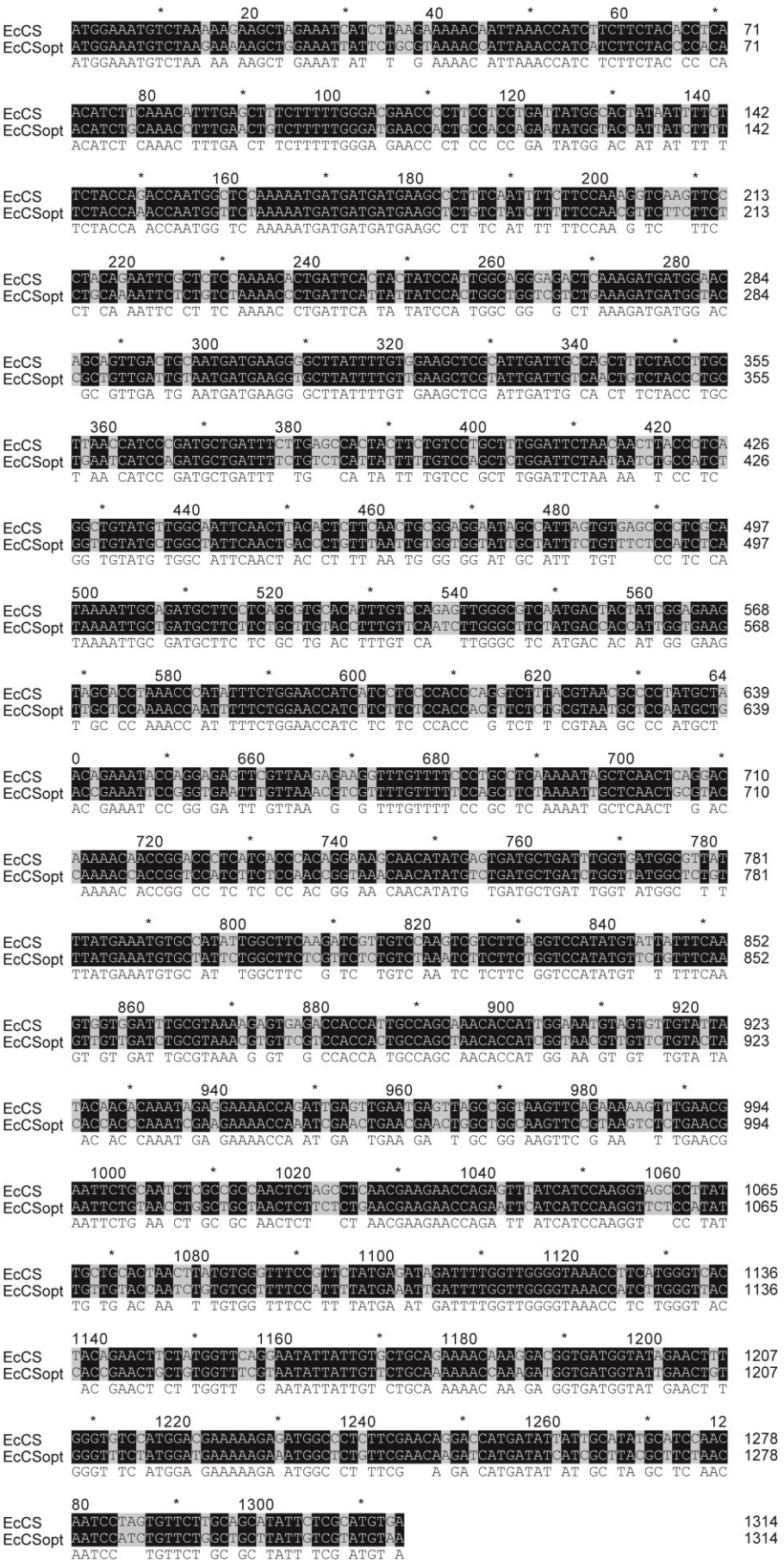
Supplementary figures



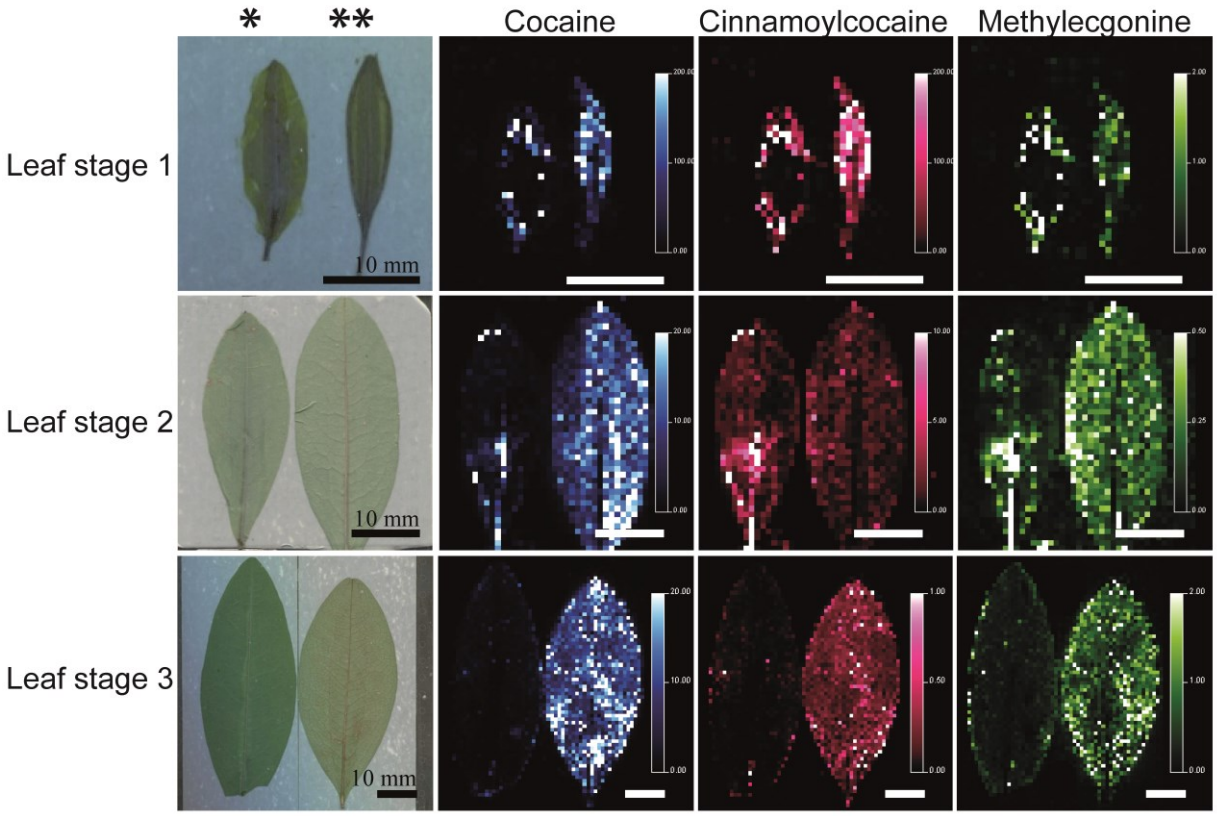
Supplemental Figure 1 | Hypothetical cocaine biosynthesis pathway. The pathway recruits the primary metabolites ornithine and arginine, to form putrescine (**4**) which is methylated to the first dedicated intermediate *N*-methylputrescine (**5**) which is subsequently oxidized to 4-methylaminobutanal (**6**). Spontaneous cyclisation of (**6**) leads the formation of the *N*-methyl-Δ^1-pyrollinium cation (**7**). Further biosynthetic steps proceed through an oxobutanoic acid intermediate (**8**) to methylecgonone (**9**), which is reduced to methylecgonine (**10**) by the action of methylecgonone reductase. Cocaine (**2**) is formed by esterification of (**10**) with benzoyl coenzyme A through the enzyme cocaine synthase.



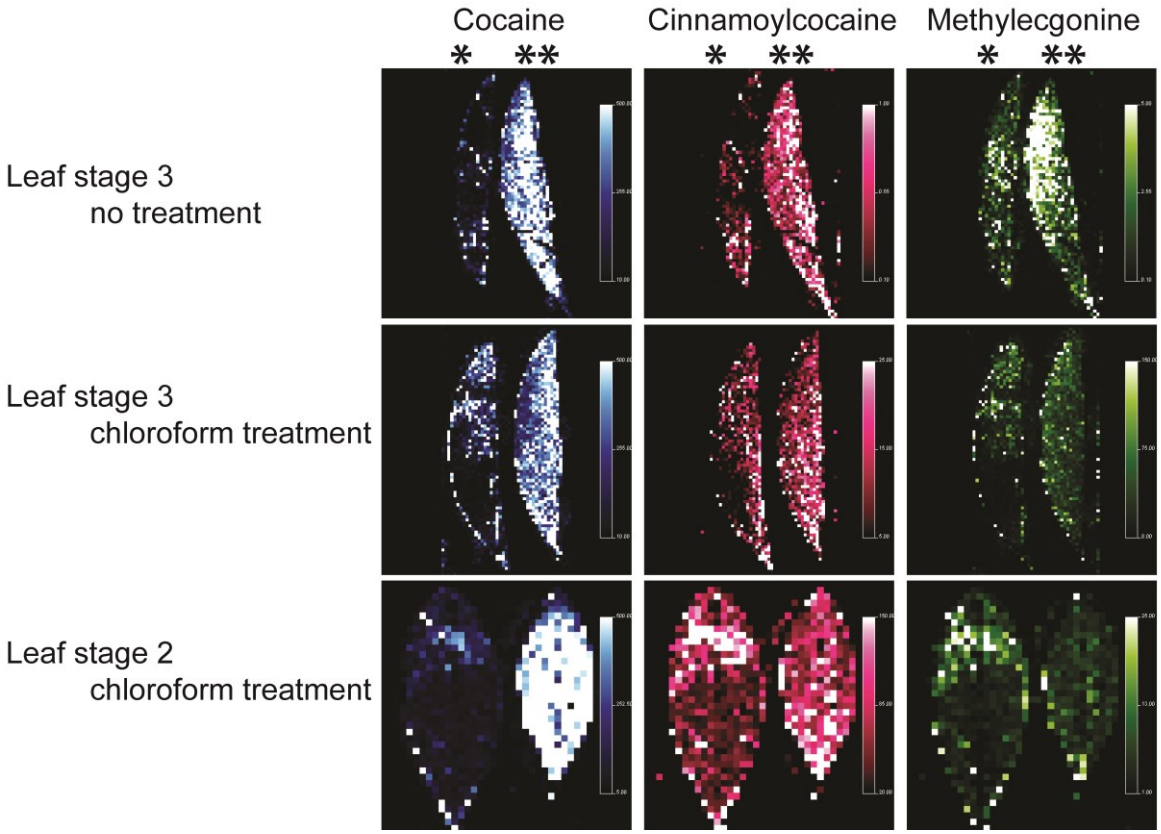
Supplemental Figure 2 | Immunoblot analysis to assess the potential cross-reactivity of the polyclonal anti-Cocaine Synthase antibody with other BAHD acyltransferase proteins from *E. coca*. Acyltransferases isolated from *E. coca* were heterologously expressed in *E. coli* carrying a 9x His-tag. Crude *E. coli* protein extracts were subject to SDS/PAGE and gels were blotted onto filters. The filters were first probed with anti-His antibodies (**a**) or anti-cocaine synthase antibodies (**b**), followed by incubation with secondary antibodies conjugated to horseradish peroxidase. Anti-His antibodies were used to prove that recombinant acyltransferases are present. Polyclonal antibodies recognize cocaine synthase and EcBAHD8. Bands were visualized using chemiluminescence.



Supplemental Figure 3 | Nucleotide alignment of EcBAHD7 (cocaine synthase) and the codon optimized version of EcBAHD7 (EcBAHD7opt)



Supplemental Figure 4 | MALDI-SRM/MS images with pixels of 1x1 mm. Cocaine trace is shown in blue at *m/z* 304 > *m/z* 182, cinnamoylcocaine trace is shown in pink at *m/z* 330 > *m/z* 182 and methylecgonine trace is shown in green at *m/z* 200 > *m/z* 182 in leaf stage 1, 2 and 3 leaves. The images are displayed after normalization with the SRM trace of cocaine-d_3. The vertical color scale represents the respective SRM signal intensity ratio of the analyte over the reference compound cocaine-d_3: white pixels give the highest signal; dark pixels give the lowest signal. Horizontal bars represent 10 mm scale for each image. *adaxial leaf surface; **abaxial leaf surface.



Supplemental Figure 5 | MALDI-SRM/MS images of non-treated and chloroform treated *E. coca* leaves after semi-automated spraying of the matrix. Cocaine trace is shown in blue at *m/z* 304 > *m/z* 182, cinnamoylcocaine trace is shown in pink at *m/z* 330 > *m/z* 182 and methylecgonine trace is shown in green at *m/z* 200 > *m/z* 182 in leaf stage 2 and 3 leaves. The images are displayed after normalization with the SRM trace of cocaine-d3. The vertical color scale represents the respective SRM signal intensity ratio of the analyte over the reference compound cocaine-d3: white pixels give the highest signal; dark pixels give the lowest signal. Images are acquired with pixels size of 1x1 mm. *adaxial leaf surface; **abaxial leaf surface.

Supplementary Tables

Supplemental Table 1 | Details of the abbreviated BAHD acyltransferases used for Fig. 3. Included in the table are which major acyl CoA unit is known, what the major class of products are formed and which species the particular acyltransferase was identified in.

Acyltransferase Name	NCBI genbank protein ID no.	Major Acyl CoA donor	Major product(s) formed	Species
DAT	AAC99311	Acetyl	Vindoline	*C. roseus*
MAT	AAO13736		Minovincinine	*C. roseus*
CbBEAT	AAC18062	Acetyl	Benzyl acetate	*C. breweri*
CbBEBT	AAN09796	Benzoyl	Benzyl benzoate	*C. breweri*
NtBEBT	AAN09798	Benzoyl	Benzyl benzoate	*N. tabacum*
CHAT	AAN09797	Acetyl	(*Z*)-3-hexen-1-yl acetate	*A. thaliana*
BPBT	AAU06226	Benzoyl	Benzyl benzoate/ Phenethyl benzoate	*Petunia x hybrida*
HCBT	CAB06430	Hydroxy-cinnamoyl/ Benzoyl	Dianthramides	*D. caryophyllus*
SAAT	AAG13130	Acetyl	Medium chain aliphatic and benzyl esters	*Fragaria x ananassa*
Vinorine synthase	CAD89104	Acetyl	Vinorine	*R. serpentina*
SalAT	AAK73661	Acetyl	Thebaine	*P. somniferum*
Ss5MaT2	AAR26385	Malonyl	Anthocyanins	*S. splendens*
Gt5AT	BAA74428	Hydroxy-cinnamoyl	Anthocyanins	*G. triflora*
Dm3MAT2	AAQ63616	Malonyl	Anthocyanins	*Chrysanthemum x morifolium*
Dm3MAT1	AAQ63615	Malonyl	Anthocyanins	*Chrysanthemum x morifolium*
Dv3MAT	AAO12206	Malonyl	Anthocyanins	*D. variabilis*
Vh3MAT1	AAS77402	Malonyl	Quercetin 3-*O*-6-*O*-malonylglucoside	*Verbena x hybrida*

Lp3MAT1	AAS77404	Malonyl	Quercetin 3-*O*-6-*O*-malonylglucoside	*L. purpureum*
Ss5MaT1	AAL50566	Malonyl	Anthocyanins	*S. splendens*
Sc3MaT	AAO38058	Malonyl	Anthocyanins	*P. cruenta*
Pf5MaT	AAL50565	Malonyl	Anthocyanins	*P. frutescens*
Pf3AT	BAA93475	Hydroxy-cinnamoyl	Anthocyanins	*P. frutescens*
ACT	AAO73071	Hydroxy-cinnamoyl	Hydroxy-cinnamoyl agamatine derivatives	*H. vulgare*
NtHCT	CAD47830	Hydroxy-cinnamoyl	Chlorogenic acid and derivatives	*N. tabacum*
AtHCT	NP_199704	Hydroxy-cinnamoyl	Chlorogenic acid and derivatives	*A. thaliana*
AsHHT1	BAC78633	Hydroxy-cinnamoyl	Avenanthramides	*A. sativa*
NtHQT	CAE46932	Hydroxy-cinnamoyl	Chlorogenic acid	*N. tabacum*
CmAAT(1-4)	CAA94432 AAL77060 AAW51125 AAW51126	Medium chain aliphatic	Medium chain and hydroxycinnamoyl acyl esters	*C. melo*
Pun1	AAV66311	unknown	Capsaicin pathway	*C. annum*
DBNTBT	AAM75818	Benzoyl	2′-deoxytaxol	*T. canadensis*
BAPT	AAL92459	*B*-phenylalanoyl	*N*-debenzoyl-2′-deoxytaxol	*T. cuspidata*
DBBT	Q9FPW3	Benzoyl	7,13-diacetylbaccatin III	*T. cuspidata*
TAT	AAF34254	Acetyl	taxa-4(20),11(12)-dien-5a-yl-acetate	*T. cuspidata*
DBAT	AAF27621	Acetyl	Baccatin III	*T. cuspidata*
Cer2	AAM64817	Acetyl?	C32 epicuticular waxes	*A. thaliana*
Glossy2	CAA61258	Acetyl?	C32 epicuticular waxes	*Z. mays*
AMAT	AAW22989	Anthaniloyl	Methyl anthranilate	*V. labrusca*

NtMAT1	BAD93691	Malonyl	Flavonoid and napthol glucosides	*N. tabacum*
MpAAT1	AAU14879	Short to medium chain aliphatic	Short to medium chain aliphatic volatile esters	*Malus x domestica*
HMT/HLT	BAD89275	Tigloyl	Quinolizidine alkaloids	*L. albus*
RhAAT1	AAW31948	Acetyl	Geranyl acetate and other volatile esters	*Rosa hybrid cultivar*
VAAT	AF193790_1	Short to medium chain aliphatic	A wide range of volatile esters in the fruit	*F. vesca*
BanAAT	AX025506	Short to medium chain aliphatic	A wide range of volatile esters in the fruit	*Musa acuminata* × *balbisiana*
At5MAT	NM_113880	Malonyl	Cyanidin anthocyanin	*A. thaliana*
AtSHT	NP_179497	Hydroxycinnamoyl	Acylated polyamines	*A. thaliana*
CsHCT	ACF37072	Hydroxycinnamoyl	Quinate esters	*Cynara cardunculus*
AtHHT1	NP_851111	Feruloyl	Acylated palmitate esters	*A. thaliana*
OsMAT1	NP_001046857	Malonyl	Acylated flavonoids	*O. sativa*
TpHCT2	ACI16631	Hydroxycinnamoyl	Malate esters	*T. pratense*
AtACT	NP_200924	Hydroxycinnamoyl	Acylated anthocyanin	*A. thaliana*
AtSDT	NP_179932	Sinapoyl	Spermidine amides	*A. thaliana*
AtSCT	AAP81804	Coumaroyl	Spermidine amides	*A. thaliana*
GmIF7MaT	NP_001237760	Malonyl	Isoflavone esters	*G. max*
CbRAS	CAK55166	Hydroxycinnamoyl	Rosmarinic acid	*C. blumei*
CcHQT	ABO77956	Hydroxycinnamoyl	Chlorogenic acid	*C. canephora*

EcBAHD1	JQ413184	unknown	unknown	*E. coca*
EcBAHD2	JQ413185	unknown	unknown	*E. coca*
EcBAHD3	JQ413186	unknown	unknown	*E. coca*
EcHQT	JQ413187	Hydroxycinnamoyl	Hydroxycinnamoyl quinate esters	*E. coca*
EcBAHD5	JQ413188	unknown	unknown	*E. coca*
EcBAHD6	JQ413189	unknown	unknown	*E. coca*
Cocaine synthase	KC140149	Benzoyl Cinnamoyl	Cocaine Cinnamoylcocaine	*E. coca*
EcBAHD8	KC140150	Benzoyl Cinnamoyl	Cocaine Cinnamoylcocaine	*E. coca*

Supplemental Table 2 | Primer sequences used for *cocaine synthase* and *EcBAHD8*

Primer name	Primer sequence (5‘ → 3‘)
EcCS_EC_Fwd	CTGGTTCCGCGTGGTTCCATGGAAATGTCTAAAAAGAAGCTAGAAATCA
EcCS _EC_Rev	CAAGAAAGCTGGGTCTGAATCACATGCGACAATATGCTG
EcCSopt_SC_Fwd	CTGGTTCCGCGTGGTTCCAAAAATGGAAATGTCTA
EcCSopt_SC_ Rev	CAAGAAAGCTGGGTCTTACATACGACAATAAGCA
EcCSopt_SC_C-Strep_Rev	CAAGAAAGCTGGGTCTTATTTTTCGAACTGCGGGTGGCTCCAAGCGCTCATACGACAATAAGCAGCCAGAACAG
EcCS_qPCR_Fwd	GGCAATTCAACTTACACTCTTCAACTG
EcCS_qPCR_Rev	AACAAACCTTCTCTTAACGAACTCTCC
EcBAHD8_EC_Fwd	CTGGTTCCGCGTGGTTCCATGGAAATGCCTAACTTTCTGGAAATG
EcBAHD8_EC_Rev	CAAGAAAGCTGGGTCTCACATGCGAGAATATGCTGCAAG
EcBAHD8_SC_Fwd	CTGGTTCCGCGTGGTTCCAAAAATGGAAATGCCTAACTTTCTGGAAATG
EcBAHD8_SC_C-Strep_Rev	CAAGAAAGCTGGGTCTTATTTTTCGAACTGCGGGTGGCTCCAAGCGCTCATGCGAGAATATGCTGCAAG
EcBAHD8_qPCR_Fwd	GCCGATTTCTTGAGCCACTTCTG
EcBAHD8_qPCR_Rev	GTTACTACTTTGCTGCTGCCTTCC

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3.4 Manuscript IV

Learning from nature: new approaches to the metabolic engineering of plant defense pathways

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Current Opinion in Biotechnology

Learning from nature: new approaches to the metabolic engineering of plant defense pathways

Jan Jirschitzka, Derek Joseph Mattern, Jonathan Gershenzon and John Charles D'Auria

Biotechnological manipulation of plant defense pathways can increase crop resistance to herbivores and pathogens while also increasing yields of medicinal, industrial, flavor and fragrance compounds. The most successful achievements in engineering defense pathways can be attributed to researchers striving to imitate natural plant regulatory mechanisms. For example, the introduction of transcription factors that control several genes in one pathway is often a valuable strategy to increase flux in that pathway. The use of multi-gene cassettes which mimic natural gene clusters can facilitate coordinated regulation of a pathway and speed transformation efforts. The targeting of defense pathway genes to organs and tissues in which the defensive products are typically made and stored can also increase yield as well as defensive potential.

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Introduction

Plants are some of nature's most prolific chemists, but this is a collective trait. Each species produces only a small fraction of the over 40 000 natural products known [1]. Breeding or engineering is required to increase the diversity of natural products in an individual plant species. The majority of plant natural products are believed to defend against herbivores and pathogens, and many of these are also important to humans as medicines, flavorings, fragrances, and industrial materials. Plant breeders have long attempted to alter the profiles of defense compounds and other natural products in plants. Classical breeding techniques have managed to increase the yield of some valuable products and decrease the amounts of various toxins. With the establishment of basic plant molecular biology methods, such as gene cloning and transformation, efforts began to augment classical breeding by trying to engineer metabolism. Engineering can modulate the levels of existing products, and can also introduce entirely new sets of metabolites by incorporating novel pathways. Over the past quarter century, plant metabolic engineering has had some notable successes [2], but it has proved much more difficult to modify chemical profiles than anticipated, especially compared to similar attempts at engineering microbes. Some of the most successful recent outcomes in plant engineering have come from efforts that closely emulate the way plants regulate their own metabolism. Here we review several newer approaches to engineering plant defense pathways that borrow heavily from the regulatory mechanisms developed by plants themselves over millions of years of evolution.

Exploiting the natural bounty of plant transcription factors

The ability to regulate an entire biosynthetic pathway at once has long been a goal in plant metabolic engineering. Plants accomplish this at the gene expression level with transcription factors that regulate the expression of multiple genes in a single pathway. Such transcription factors are known for defense pathways, and are drawn mainly from the bZIP, MYB, MYC, WRKY and ERF families [3]. For example, the ORCA3 transcription factor from *Catharanthus roseus* (Madagascar periwinkle) is a classic example of a defense pathway regulator. It controls a major portion of the terpenoid indole alkaloid pathway, and like various other defense transcription factors, its own expression is induced by jasmonic acid signaling [4] and by other transcription factors, including a basic helix-loop-helix protein known as CrMYC2 [5••].

Before starting to work with transcription factors, engineers of defense metabolism need to consider the complexity of these regulatory networks. In some instances, a single transcription factor can substantially modify the expression of genes encoding a major portion of a plant defense pathway making engineering efforts simple. Recent examples include the manipulation of proanthocyanidin formation in poplar [6], artemisinin biosynthesis in *Artemisia annua* [7] and nicotine synthesis in *Nicotiana tabacum* [8] (Table 1). However, there are also examples of transcription factors reported to control only a single pathway enzyme. These may be useful, but only when the targeted enzyme is responsible for a significant proportion of flux control in the pathway. For example,

Table 1

Transcription factors recently employed in the engineering of plant defense pathways

Family	Transcription factor	Plant species	Defense metabolites	GenBank	Ref.
bHLH	CrMYC2	*Catharanthus roseus*	Terpenoid indole alkaloids	AF283507	[5••]
	MYC2	*Arabidopsis thaliana*	Sesquiterpenes	At1g32640	[9]
	NbbHLH1	*Nicotiana benthamiana*	Nicotine	GQ859152	[11]
	NbbHLH2	*Nicotiana benthamiana*	Nicotine	GQ859153	[11]
MYB	MYB134	*Populus tremuloides*	Proanthocyanidins	ACR83705	[6]
	MYB28	*Arabidopsis thaliana*	Glucosinolates	Q9SPG2	[14•]
	MYB29	*Arabidopsis thaliana*	Glucosinolates	Q9FLR1	[14•]
	MYB76	*Arabidopsis thaliana*	Glucosinolates	Q9SPG5	[14•]
	DkMyb2	*Diospyros kaki*	Proanthocyanidins	BAI49719	[15]
	GmMYBZ2	*Glycine max*	Terpenoid indole alkaloids	DQ902861	[17]
	AtMYB12	*Arabidopsis thaliana*	Flavonoids	NM_130314	[19]
	ODORANT1	*Petunia hybrida*	Phenylpropanoids	AAV98200	[18]
WRKY	AaWRKY1	*Artemisia annua*	Artemisinin	FJ390842	[7]
	CrWRKY1	*Catharanthus roseus*	Terpenoid indole alkaloids	HQ646368	[10]
	SoWRKY1	*Spinacia oleracea*	Compounds responding to wounding and salicylic acid	N/A	[16]
ERF	ORC1	*Nicotiana tabacum*	Nicotine	CQ808982	[8]
	AaERF1	*Artemisia annua*	Artemisinin	JN162091	[12]
	AaERF2	*Artemisia annua*	Artemisinin	JN162092	[12]

Abbreviations: bHLH, basic helix-loop-helix; ERF, ethylene response factor.

transcription factors have been employed to overexpress single genes involved in sesquiterpene biosynthesis in *Arabidopsis thaliana* [9] and terpenoid indole alkaloid biosynthesis in *C. roseus* [10]. In the case of *C. roseus*, tryptophan decarboxylase (TDC) was targeted for upregulation by overexpression of CrWRKY1 (although the transcript levels of other pathway genes were also affected). This transformation resulted in the overproduction of the alkaloid serpentine. Curiously, overexpression of a repressive form of CrWRKY1 resulted in a dramatic increase in the amounts of two other terpenoid indole alkaloids, catharanthine and tabersonine, apparently owing to repression of other transcription factors. Although the mechanisms are not yet understood, both approaches may be valuable because serpentine, catharanthine and tabersonine are all pharmacologically important [10].

Multiple transcription factors that are closely related often interact to regulate a single defense pathway [11,12]. For example, the jasmonate-induced NbbHLB1 and NbbHLH2 transcription factors are both involved in nicotine biosynthesis in *Nicotiana benthamiana*. Overexpression of one or the other factor singly produced higher levels of nicotine, but expression of both together provided even more nicotine [11]. In the case of AaERF1 and AaERF2, which regulate artemisinin biosynthesis in *A. annua*, AaERF1 responds earlier to methyl jasmonate treatment than AaERF2 and so could be used to manipulate the timing of pathway activity [12]. Timing manipulation may be a crucial tool in the heterologous expression of the artemisinin pathway in plants since artemisinin and its semi-synthetic analogs are some of the best drugs currently available for the treatment of malaria.

For multiple transcription factors that control a single pathway, assigning specific roles is not a trivial exercise. The R2R3 MYB transcription factors that regulate aliphatic glucosinolate biosynthesis in *A. thaliana* do not control the pathway in a simple linear fashion where each binds to the promoter region of specific pathway genes [13]. Of the three aliphatic glucosinolate-controlling MYBs, MYB28 is the dominant regulator of the transcripts of the principal biosynthetic enzymes, MYB76 affects transcripts involved in secondary modifications of glucosinolates and influences spatial distribution within leaves, and MYB29 may represent an integration point for overall transcriptional control of the pathway [14•]. With further knowledge, these factors might eventually be useful in exercising specific control over glucosinolate amount and composition in different tissues.

The value of transcription factors in plant pathway engineering is usually not limited to the original source species. In recent years several factors have been shown to be active in other species that are not necessarily of close taxonomic affinity to the source species [15–18]. For example, the transcription factor AtMYB12, which partially regulates the phenylpropanoid pathway in *A. thaliana*, also increases the concentration of phenylpropanoids and flavonoids in *N. tabacum* when heterologously expressed in that species. The resulting plants had increased resistance to larvae of *Spodoptera litura* and *Helicoverpa armigera* in comparison to wild-type tobacco [19].

A limitation on the use of transcription factors is their possible effects on multiple pathways that could lead to unwanted phenotypes. For example, the transcription factor PtMYB14 from *Pinus taeda* is involved in the regulation of terpene biosynthesis and flavonoid biosynthesis, but also affects vascular organization and development [20]. The fact that many defense pathway transcription factors are regulated by jasmonate signaling cascades raises the prospect of increasing defense production by engineering the jasmonate pathway or simply by direct application of jasmonates. However, continuous upregulation of jasmonate signaling could significantly inhibit growth by interfering with gibberellin signaling [21•]. In addition, because jasmonate and salicylate signaling are frequently antagonistic, upregulation of jasmonate may lead to a reduction in salicylate-triggered defenses, such as those involved in resistance to many pathogens [22].

Imitating multiple gene clusters with multi-gene cassettes

Metabolic engineers have sometimes tried to introduce an entire, multiple step defense pathway into a plant, even though the stable transformation of several genes, either sequentially or together at a single site in the genome, is a major challenge. However, in looking at natural plant genomes, there are increasing reports of several genes from a single plant defense pathway being found clustered together [23,24]. These genes are physically linked in the genome, in a similar way to a prokaryotic operon, but each is transcribed independently.

A recent spectacular example of such a multiple gene cluster involves the genes for producing noscapine, a phthalide isoquinoline alkaloid in the opium poppy (*Papaver somniferum*) [25•]. At least six of the eight steps needed to make noscapine from the intermediate scoulerine were encoded by genes found on one contiguous portion of DNA (Figure 1). Expression profiling showed a similar pattern of expression for all six genes in the cluster, suggesting a common mechanism of transcriptional control. The physical proximity of genes in a cluster is thought to facilitate their regulation in a coordinated manner via changes in chromatin organization or other nuclear factors [23]. Coordinated regulation of several pathway steps at once may be one of the driving forces behind the evolution of multiple gene clusters, and could also be a valuable goal in pathway engineering as well.

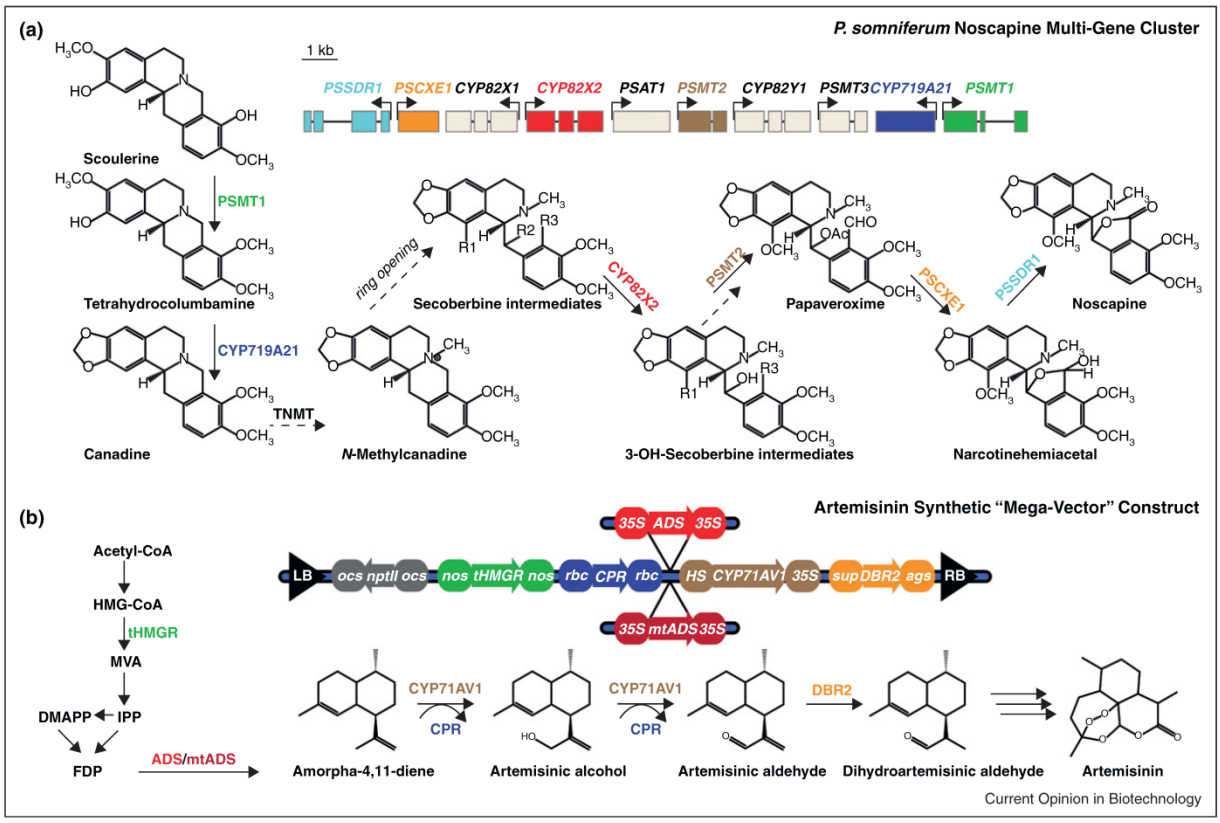
If plants could be engineered with artificial gene clusters via multi-gene cassettes, these might also allow coordinated regulation of the individual genes. Furthermore, transformation with such cassettes has additional benefits in that it avoids the problems of introducing a pathway by single gene transformation, which requires either lengthy crossing experiments of separately transformed lines or having to perform many sequential transformations. As the number of genes required to engineer a pathway is increased, the number of individual lines to analyze goes up exponentially. Further, finding suitable selectable markers for each gene becomes a major problem along with the likelihood of transgene silencing via positional affects [26]. Metabolic engineering efforts on multi-step pathways must also consider which promoter elements to use in order to obtain a high flux through the pathway while avoiding difficulties such as the accumulation of toxic intermediates. Such extensive promoter optimization was carried out when the mevalonate pathway for terpenoid biosynthesis was introduced in *Escherichia coli* [27], but efforts like this have yet to be performed in plants.

There are currently only three reported examples of the use of multi-gene cassettes in engineering plant defense pathways. The first involves the introduction of glucosinolates, normally found only in families of the Brassicales, into tobacco plants. In this case, the terminal three genes of the benzylglucosinolate pathway were synthesized in a polycistronic message under the control of a single CaMV 35S promoter [28,29]. The interspacing region was designed so that the ribosome would 'skip' during translation and produce single peptides. The other two examples concern the pathway for the production of the anti-malarial sesquiterpene artemisinin, the subject of considerable metabolic engineering experiments. In one approach, the transformation of subsets of multi-gene constructs for the artemisinin pathway genes was carried out in a sequential manner in tobacco. After selecting for lines with a suitable accumulation of the early intermediate amorphadiene, the authors performed subsequent transformations with genes encoding downstream enzyme steps but were not able to make it as far as artemisinic acid, one of the last intermediates [30]. Recently, a more successful approach was reported in which five genes were introduced into tobacco plants via a 'mega-vector' (Figure 1). In this case, each gene was under the control of a different constitutive promoter [31••]. In addition, a yeast gene encoding HMG-CoA reductase was added in order to increase the amounts of precursors to the artemisinin pathway. These modifications resulted in tobacco plants producing over 6.8 μg artemisinin/g dry weight. Further multi-gene vector technologies have been recently reviewed [32], and a new system has been introduced in which up to nine genes can be transformed at once into plants [33].

Emulating natural plant strategies for synthesizing and deploying defensive products

When contemplating the engineering of a plant defense pathway, it is appropriate to consider where in the plant the products are normally synthesized and stored. Certain defense metabolites are produced only in specific organs

Figure 1

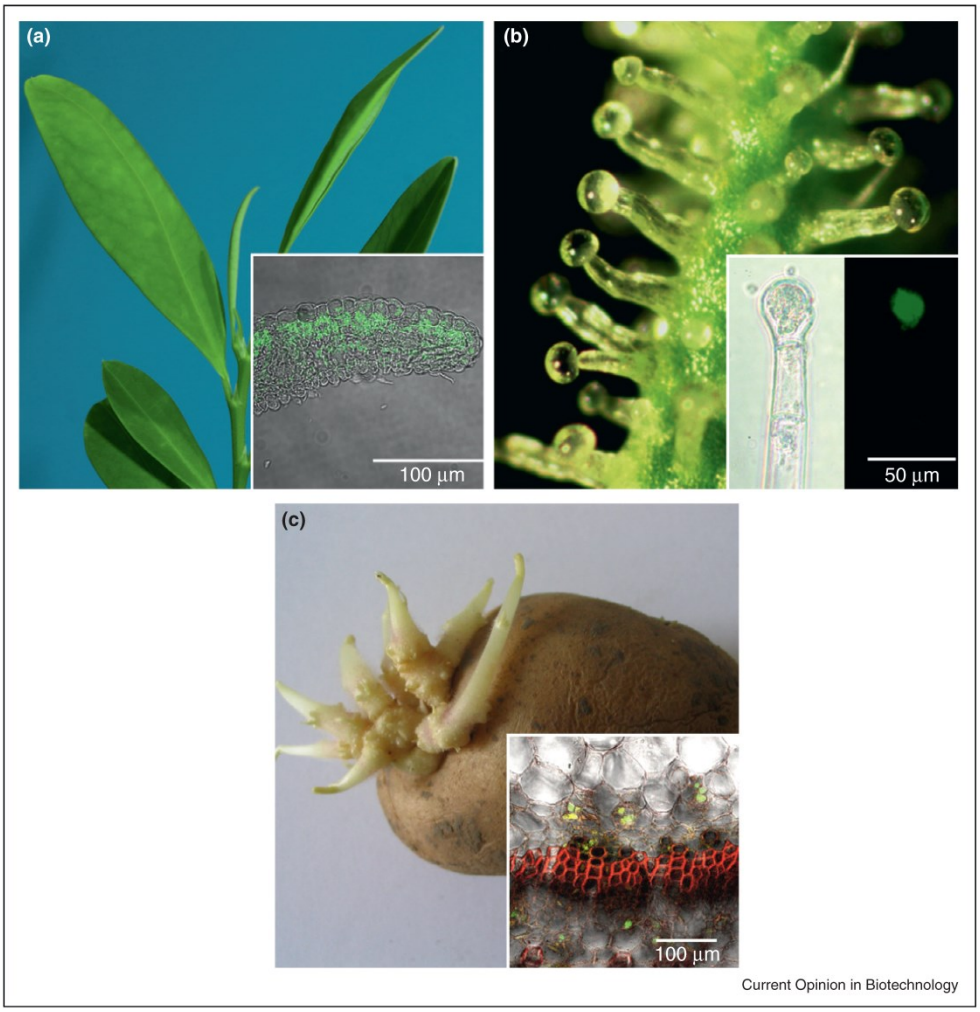


A natural plant multiple gene cluster compared with a synthetic multi-gene cassette used in engineering. **(a)** The naturally occurring noscapine gene cluster of *Papaver somniferum* encoding the biosynthetic pathway to this medicinal alkaloid with a scheme of the pathway below. **(b)** A synthetic construct used for engineering the formation of the anti-malarial sesquiterpene artemisinin in *Nicotiana tabacum* with the biosynthetic pathway below. In both illustrations, arrows represent the genomic orientation. Genes and their corresponding enzymes are depicted in the same color. The figure in panel (a) was modified from [25•] and reprinted with permission of the American Association for the Advancement of Science. The figure in panel (b) was modified and reprinted by permission from Macmillan Publishers Ltd: Nature Biotechnology [31••], copyright (2011).
Abbreviations: 35S, cauliflower mosaic virus (CaMV) 35S; ADS, amorphadiene synthase; mtADS, mitochondria targeted ADS; ags, agrocinopine synthase; CPR, cytochrome P450 reductase; CYP, cytochrome P450 family; DBR2, artemisinic aldehyde reductase; HS, hps18.1 promoter; nos, nopaline synthase; ocs, octopine synthase; PSAT1, *P. somniferum* acetyltransferase; PSCXE1, *P. somniferum* carboxylesterase; PSMT, *P. somniferum* *O*-methyltransferase; PSSDR1, *P. somniferum* short-chain dehydrogenase/reductase; rbc, rubisco; sup, superpromoter; tHMGR, truncated 3-hydroxy-3-methylglutarylcoenzyme A reductase; TNMT, tetrahydroprotoberberine cis-*N*-methyl transferase.

and tissues. For example, the pyrrolidine and tropane alkaloids of the Solanaceae (including nicotine, atropine and scopolamine) are made in the roots and then transported to the other parts of the plant [34]. This may be a consequence of the fact that their polyamine precursors are most abundant in the roots. Interestingly, the tropane alkaloids of the Erythroxylaceae (such as cocaine) are synthesized exclusively in the leaves [35]. In trying to engineer altered quantity or composition of these compounds, it seems best to target the tissues in which they are usually made (Figure 2).

After synthesis, plant defense compounds are often stored in specialized structures, including glandular trichomes (e.g., terpenoids and phenylpropanoids in mint) [36], resin ducts (e.g., terpenoid resins in spruce) [37], secretory cavities (e.g., gossypol in cotton), laticifers (e.g., alkaloids in poppies) [38], or sulfur-rich phloem cells (e.g., glucosinolates in cabbage) [39]. Specialized storage structures may serve to minimize the further metabolism of plant defenses by preventing access to downstream modification enzymes, limit their toxic effects on a plant's own tissues [40], and enhance their defensive role by deploying them on the outer surfaces of plants or releasing them in high concentrations at wound sites. Hence targeting engineering efforts to storage structures may improve yield, avoid autotoxicity and increase crop protection.

Figure 2



Plant defense pathways are localized in specific organs and tissues. **(a)** Shoot apex of a coca plant (*Erythroxylum coca*). Cocaine and other tropane alkaloids are synthesized in the leaves. Inset shows the immunolocalization of an enzyme of the cocaine biosynthetic pathway, methylecgonone reductase (green), to the palisade and spongy mesophyll tissues of a young leaf. **(b)** The cannabinoids of marijuana (*Cannabis sativa*) are synthesized and stored in glandular trichomes found on the female flower. Inset shows the localization (green) of an enzyme of the cannabinoid biosynthetic pathway, tetrahydrocannabinolic acid synthase, using a green fluorescent protein-labeled probe to the head of a glandular trichome. **(c)** Calystegine alkaloids are synthesized in the green sprouts of potato (*Solanum tuberosum*) tubers. Inset shows the immunolocalization (green) of an enzyme of calystegine biosynthesis, tropinone reductase II, in sprout tissue.
The glandular trichome image is courtesy of Dr. Jonathan E. Page. The sprouting potato image is courtesy of Prof. Birgit Dräger. The localization image shown in the inlay in panel (b) was taken from Sirikantaramas S, Taura F, Tanaka Y, Ishikawa Y, Morimoto S, Shoyama Y: **Tetrahydrocannabinolic acid synthase, the enzyme controlling marijuana psychoactivity, is secreted into the storage cavity of the glandular trichomes**. *Plant Cell Physiol* 2005, **46**:1578–1582, by permission of Oxford University Press. The immunolocalization image shown in the inlay in panel (c) is taken from Figure 6 of Kaiser H, Richter U, Keiner R, Brabant A, Hause B, Dräger B: **Immunolocalisation of two tropinone reductases in potato (*Solanum tuberosum L.*) root, stolon, and tuber sprouts**. *Planta* 2006, **225**;134 (Copyright © Springer-Verlag 2006) and is reproduced with kind permission from Springer Science and Business Media.

Most plant engineering approaches to date have used a constitutive promoter to drive expression of transgenes in all cells of a plant, regardless of organ, tissue or developmental stage. The CaMV 35S promoter from the cauliflower mosaic virus has been very popular owing to its activity in many different species [28,41–44]. In one successful case of the use of the CaMV 35S promoter, the antifungal protein KP4 was introduced into maize plants, rendering them resistant to the corn smut fungus, *Ustilago maydis* [41]. Additionally, overexpression of a lipid transport protein (NtLTP1) in tobacco plants increased secretion of trichome exudates. When given

Table 2

A selection of location-specific, plant promoter sequences from the recent literature

Organ or tissue	Name	Source	Ref.
Root	*pMe1*	*Manihot esculenta* (cassava)	[47]
	pDJ3S	*Dioscorea japonica* (yam)	[47]
	SRD1	*Ipomoea batatas* (sweet potato)	[53]
	AtMDK4-20	*Arabidopsis thaliana*	[46]
Syncytia (multi-nucleate feeding structures of root cyst nematodes)	*Pdf2.1*	*Arabidopsis thaliana*	[54]
Stem	Pro_{DIR16}	*Saccharum hybrid* (sugarcane)	[55]
	Pro_{OMT}	*Saccharum hybrid* (sugarcane)	[55]
	FAR6	*Arabidopsis thaliana*	[56]
Green tissue	P_{DX1}	*Oryza sativa* (rice)	[57]
Xylem	*DX*	*Populus maximowiczii x nigra* (poplar)	[58]
Pistil	*S12-RNase*	*Pyrus pyrifolia* (Chinese pear)	[59]
Floral tissue	*SlPS*	*Solanum lycopersicum* (tomato)	[60]
	SlPS	*Capsicum annuum* (pepper)	[60]
	SlPS	*Solanum tuberosum* (potato)	[60]
Glandular trichome	*NsCBTS-2a*	*Nicotiana sylvestris* (tobacco)	[61]
	cyp71av1	*Artemisia annua* (sweet wormwood)	[62]
Fruit	*Des*	*Elaeis guineensis* (oil palm)	[48]
	CuLea5	*Citrus unshiu* (satsuma mandarin)	[63]
	CsExp	*Cucumis sativus* (cucumber)	[64]
Endosperm	*PzsS3a*	*Zea mays* (maize)	[65]
	MS	*Cyamopsis tetragonoloba* (guar)	[66]
Pollen	*TaPSG719*	*Triticum aestivum* (wheat)	[67]
	OsGEX2	*Oryza sativa* (rice)	[68]

Abbreviations: *AtMDK4-20*, *Arabidopsis thaliana* MDK4-20 gene (At5g54370); *CsExp*, *Cucumis sativus* expansin; *CuLea5*, *Citrus unshiu* group 5 late embryogenesis abundant protein; *cyp71av1*, cytochrome p450 gene *cyp71av1*; *Des*, promoter of stearoyl-ACP desaturase gene; *DX*, developing xylem gene; *FAR6*, fatty acyl-CoA reductase 6 gene; *MS*, mannan synthase; *NsCBTS-2a*, *Nicotiana sylvestris* cembratrien-ol synthase 2a gene; *OsGEX2*, *Oryza sativa* gamete expressed 2 gene (Os09g25650); *Pdf2.1*, plant defensin 2.1 gene; *pDJ3S*, promoter of *Dioscorea japonica* storage protein dioscorin 3 small subunit; *PDX1*, promoter of green tissue-specific expression gene *DX1* (LOC_Os12g33120); *pMe1*, promoter of *Manihot esculenta* unknown gene; *ProDIR16*, promoter of dirigent (*SHDIR16*) gene; *ProOMT*, promoter of *O*-methyl transferase (*SHOMT*) gene; *PzsS3a*, promoter of starch synthase gene *zsS3a*; *S12-RNase*, S-RNase 12 gene; *SlPS*, promoter of prosystemin gene; *SRD1*, sweet potato MADS-box protein gene; *TaPSG719*, *Triticum aestivum* pollen exine synthesis gene PSG719.

a choice test between RNAi lines or those overexpressing NtLTP1 with the aphid *Aphis gossypii*, the knockdown lines were preferred [42]. Unfortunately, untargeted gene expression can lead to spurious side products that can drain plant resources and cause undesirable phenotypes. For example, overexpressing a geraniol synthase gene in maize under a constitutive ubiquitin promoter for producing the antifungal monoterpene, geranic acid, led to unwanted glycosylated metabolites [45]. More targeted expression may be desirable in many cases [46–49]. For example, rice (*Oryza sativa*) plants transformed with a construct overexpressing the pathogenesis protein OsNPR1 are more resistant to fungal infection by *Xanthomonas oryzae pv. oryzae* (Xoo) when using the native *OsNPR1* promoter as opposed to a similar construct using the constitutive ubiquitin promoter [50].

Tissue/cell-specific expression of plant genes to enhance defense requires a diverse assortment of promoter sequences. Fortunately, these are being reported at an increasing rate. Table 2 provides a list of recently discovered location-specific promoters which show potential in engineering plant defense pathways. For example, root-specific promoters have recently been utilized to provide targeted gene expression of a nematode-repellent peptide [46] and two protease inhibitors [51], both of which significantly increased plant resistance. Directing a defensive compound to storage structures was successfully accomplished by the glandular trichome-specific expression of two *N. tabacum* genes involved in the production of the labdane diterpene, (*Z*)-abienol, an important flavor and aroma precursor in tobacco. In this instance, the genes were overexpressed in *Nicotiana sylvestris* using their native, *N. tabacum* glandular trichome-specific promoters and the desired compound accumulated in the glandular trichomes of *N. sylvestris* [52•].

Conclusions

Plants are widely known as metabolic engineers in their own right. They employ a diverse hierarchy of controls to regulate their biosynthesis of defense metabolites at multiple levels of organization. In addition to direct

transcriptional and post-transcriptional controls of biosynthetic genes and enzymes, the abundance of gene transcripts is controlled by a variety of upstream signals and regulatory factors. Further, the operation of the pathway is usually restricted to particular organs and tissues and certain stages of development. All of these regulatory aspects have presumably been shaped by natural selection to optimize plant protection in coordination with growth and reproduction. Human attempts to engineer plant defense pathways can only benefit from greater knowledge of these natural regulatory mechanisms. Here we have shown that regulatory features, such as transcription factors, multiple gene clusters and tissue-specific synthesis and storage can be successfully exploited in engineering efforts. Further information about these and other controls, including upstream signaling pathways, subcellular protein targeting, multi-enzyme complexes, substrate supply and transporters, can only improve the success of defense pathway engineering in plants.

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4 Discussion

Tropane alkaloids represent an important group of secondary metabolites, ecologically important as defense compounds and valued by mankind for their pharmacological properties. Albeit at the same time there is a flip side to their benefits. Although tropine and scopolamine (also known as hyoscine) are classified by the world health organization (WHO) as essential drugs needed for a basic healthcare system, the structurally similar cocaine causes worldwide socioeconomic problems (Streatfeild, 2001; WHO, 2011). Even if atropine and scopolamine are essential in medicine, they are poisonous to livestock (Scientific Opinion, 2008). Considering the impact of tropane alkaloids to mankind, it is striking that there are many gaps in our knowledge of their biosynthesis and we have only information on biosynthesis in the Solanaceae (Roberts et al., 2010). The discoveries of methylecgonone reductase and cocaine synthase are therefore important findings for elucidating the pathways of tropane alkaloid biosynthesis. In addition, these two newly discovered enzymes provide insight into the evolution of tropane alkaloids. The independent recruitment of an aldo-keto reductase and a short-chain dehydrogenase / reductase in two distinct plant families to perform a similar biosynthetic step in tropane alkaloid production is evidence of the multiple origins of the pathway. Thus more enzymes in tropane alkaloid biosynthesis and even the entire pathway may differ among the separate lineages producing these compounds.

4.1 Tropane alkaloids are restricted to certain angiosperm plant families

The scattered distribution of tropane alkaloids in different families of the angiosperms makes their evolutionary origin a matter of debate. One hypothesis is that the ability to make tropane alkaloids was achieved in an early angiosperm possibly as a result of horizontal gene transfer HGT and parts of the pathway have been subsequently lost throughout evolutionary history (Wink, 2008a). Another hypothesis is that the tropane alkaloid biosynthesis pathway evolved more than once

in the angiosperms (Docimo et al., 2012). This debate will only be resolved by the further identification of genes involved in their synthesis (Pichersky and Lewinsohn, 2011). Ideally, all tropane alkaloid producing plant families need to be investigated. There are in total ten of these families within seven plant orders (see **Appendix**). The most ancient tropane alkaloid containing family in this context is the Proteaceae, which shared a common ancestor with the other TA producing families approximately 125 million years ago (Magallon and Castillo, 2009). Besides phytochemical reports about the presence of tropanes in the Proteaceae, there is nothing known about their biosynthetic enzymes (Bick et al., 1981; Butler et al., 2000). If the tropane alkaloid biosynthesis pathway was an early acquisition during evolution, then the enzymes involved found in Solanaceae and / or Erythroxylaceae should be similar to the enzymes involved in tropane alkaloid biosynthesis in the Proteaceae. If the tropane alkaloid biosynthesis pathway (or parts of it) evolved independently more than once, then the responsible enzymes in the Proteaceae could be very different from tropane alkaloid biosynthesis enzymes found in Solanaceae and Erythroxylaceae.

One step of tropane alkaloid biosynthesis pathway has been investigated in more than one plant family. The reduction of tropinone / methylecgonone has been biochemically characterized in three plant families, the Brassicaceae, Erythroxylaceae, and Solanaceae (Dräger, 2006; Brock et al., 2008). The last common ancestor shared between these three families lived approximately 115 million years ago (Magallon and Castillo, 2009). The Brassicaceae and Erythroxylaceae shared their last common ancestor approximately 108 million years ago (Magallon and Castillo, 2009). The tropinone reductases in the Solanaceae and Brassicaceae are members of the short-chain dehydrogenase-reductase (SDR) protein family, while the methylecgonone reductase of the Erythroxylaceae is a member of the aldo-keto reductase (AKR) family. The utilization of SDR enzymes for the reduction of tropinone in the Solanaceae and the Brassicaceae is an example of divergent evolution. The ability to reduce tropinone appears to have arisen from an oxidoreductase of the SDR enzyme group in both

families, but the final outcome is different. The brassicaceous tropinone reductase reduces tropinone equally to the stereosiomers, tropine and pseudotropine, and therefore is considered a prototype tropinone reductase (Brock et al., 2008). However, in the Solanaceae, two types of tropinone reductases reducing tropinone to either tropine or pseudotropine have been identified (Dräger, 2006). Meanwhile, in the Erythroxylaceae the utilization of an AKR enzyme to specifically reduce methylecgonone to methylecgonine is a case of convergent evolution when compared to the SDR family tropinone reductase enzymes of the Solanaceae and the Brassicaceae. Thus the biological function of reducing the tropane alkaloid with a keto function at the C-3 position of the tropane ring evolved independently in Brassicaceae/Solanaceae and Erythroxylaceae. Tropinone reductases and methylecgonone reductase can both reduce tropinone, but only methylecgonone reductase is able to reduce methylecgonone (2-carbomethoxy-3-tropinone) (Couladis et al., 1991; Hashimoto et al., 1992). To date, the metabolite methylecgonone is only found in the plant family Erythroxylaceae, whereas the metabolite tropinone is found in most tropane alkaloid producing plant families like Solanaceae and Brassicaceae. Thus it can be speculated that the SDR enzyme utilization seems to be the more ancient and that the recruitment of an AKR in tropane alkaloid biosynthesis more recent in evolutionary terms. Even if both enzyme families use different reductase sequences, there are indications for a common evolutionary origin for SDR and AKR enzymes. Both enzyme superfamilies have different structural motifs, but contain alternating arrangements of α-helix and β-sheets suggesting they may have arisen from a now extinct common structural ancestor (Jez et al., 1997).

The evolutionary origin of cocaine synthase (CS) from *Erythroxylum coca* is more difficult to ascertain. Cocaine synthase is the first BAHD identified to be involved in tropane alkaloid biosynthesis. Even though coenzyme A-dependent acyltransferase activities were reported in Solanaceae and suggested for Erythroxylaceae, no sequence for the responsible enzyme has yet been described (Leete et al., 1988; Robins et al., 1991; Rabot et al., 1995). It is very likely that the

enzyme responsible for the esterification at the C-3 position of the tropane ring in the other tropane alkaloid producing plant families is also an enzyme of the BAHD superfamily. However, without further information evolutionary speculations on the origin of the esterification step in tropane alkaloid formation in different plant families is difficult.

4.2 Impact of methylecgonone reductase and cocaine synthase

In order to understand a biosynthetic pathway which is shared among different families of plants, it is important to not focus too much on any single family, especially if generalities about the pathway are to be made (Facchini and De Luca, 2008). In some families, the substrates utilized might be different or there might be different additional end products. In case of tropane alkaloids, the previously accepted model for the biosynthetic pathway was derived exclusively from solanaceous plants. Focus on this pathway may hide some of the alternate routes available. Indeed the early steps in tropane alkaloid biosynthesis in the Erythroxylaceae have already been suggested to be different from those in the Solanaceae (Docimo et al., 2012). The first evidence in support of this theory is methylecgonone reductase from *E. coca*.

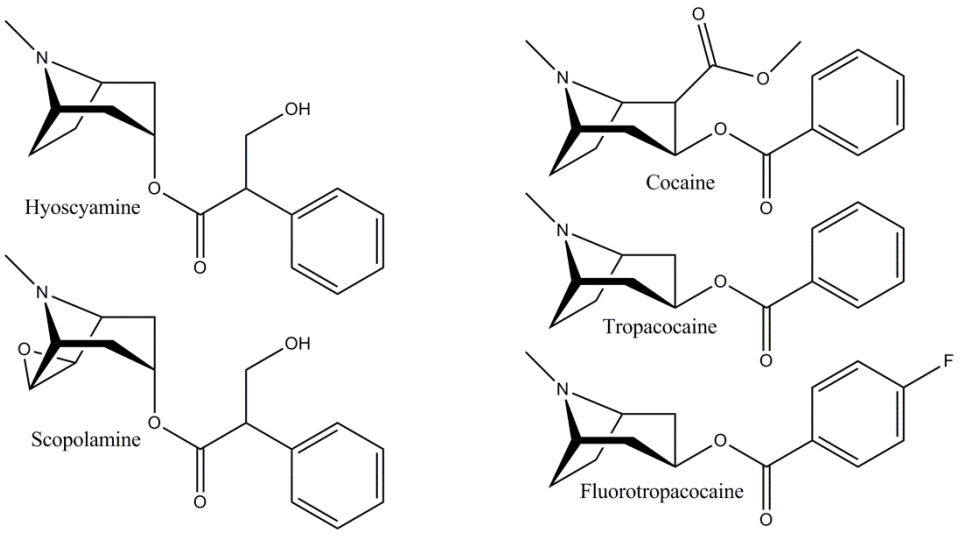

Figure 5 | Selected structures of C-3α (left) and C-3β (right) esterified tropane alkaloids

Besides broadening our evolutionary understanding, methylecgonone reductase and cocaine synthase extended the amount and diversity of genes available for the engineered production of tropane alkaloids. The limited amount of structural genes available has restricted those interested in metabolic engineering. Studies that have attempted engineering of the tropane alkaloid pathway have endeavored to increase the production of atropine or scopolamine in hairy root cultures (Zhang et al., 2004; Liu et al., 2010; Kang et al., 2011; Yang et al., 2011; Kai et al., 2012a; Kai et al., 2012b). The structural genes used in these studies were 6β-hydroxy hyoscyamine epoxidase (H6H), tropinone reductases (TR), putrescine *N*-methyltransferase (PMT) and ornithine decarboxylase (ODC). Overexpression under strong constitutive promoters has been the method of choice to achieve higher alkaloid content. Methylecgonone reductase (MecgoR) may be utilized in a similar fashion as tropinone reductase II, since it is capable of reducing methylecgonone, tropinone, nortropinone, and 6-hydroxytropinone in a stereospecific fashion forming the β-alcohol at the C-3 position of the tropane ring. It has therefore a slightly different substrate spectrum then tropinone reductase II. Cocaine synthase is the first acyltransferase available as potential target in metabolic engineering tropane alkaloids. It esterifies very specifically C-3β-tropane alcohols like pseudotropine or methylecgonine, and is unable to utilize C-3*α*-tropane alcohols. The CoA substrates accepted are not limited to benzoyl-CoA and include cinnamoyl-, hexanoyl- and *p*-coumaroyl-CoA. Therefore, both recently discovered enzymes from coca could be used together to produce C-3β-esterified tropane alkaloids.

Certain C-3β-esterified tropane alkaloids like tropacocaine, the ester of benzoyl-CoA and pseudotropine, have medicinal properties. The cocaine analogue, tropacocaine (see **Fig. 5**), has the same local anesthetic properties as cocaine but is far less toxic (Novák et al., 1984). It is found in species belonging to the Erythroxylaceae and the Solanaceae. Tropacocaine has also been suggested to be useful as replacement medication for cocaine addicts (Snow, 2004). To produce tropacocaine, solanaceous root cultures can be employed overexpressing methylecgonone reductase and cocaine

synthase in a similar manner to the existing engineered root cultures producing atropine and scopolamine (Kai et al., 2012b). The production of tropacocaine could also be further utilized for the semi synthetic production of the currently most potent cocaine replacement medications (Rush and Stoops, 2012). Unfortunately, engineering of tropacocaine may pose further regulatory problems since it can serve as a precursor for the production of illicit designer drugs such as fluorotropacocaine (Biliński et al., 2012; Kavanagh et al., 2012).

Currently, the tropane alkaloids with the most medicinal value are those containing C-3α-esters (Dewick, 2009; Kai et al., 2012b). The worldwide production of scopolamine is based on leaf extraction of cultivated Australian *Duboisia species* which contain up to 3% dry weight of alkaloids (Ohlendorf, 1996; Dewick, 2009). Metabolic engineering is beginning to be employed in order to supply the ever increasing demand for this important anticholinergic drug (Dewick, 2009; Kai et al., 2011; Kai et al., 2012b). In order to achieve these goals, esterification of tropine (a C-3α-tropane alcohol) is needed. Cocaine synthase cannot perform this reaction in its current state so structure-function analysis is needed to reveal which residues result in its specificity for esterifying C-3β-tropane alcohols only. This information could then be used to alter its specificity to esterify C-3α-tropane alcohols like tropine. In addition, the discovery of cocaine synthase can help identify the enzymes in the Solanaceae responsible for the esterification of tropine forming littorine, the precursor of hyoscyamine.

4.3 Ecological aspects of tropane alkaloids

Despite the medicinal value of tropane alkaloids, plants clearly produce these secondary metabolites for reasons of their own. Tropane alkaloids are important defense compounds (Wink, 1998a, b). For example, natural concentrations of cocaine were shown to be an effective toxin against the tobacco hornworm (*Manduca sexta*) when sprayed on tomato leaves (Nathanson et al., 1993). Similar effects were reported

for scopolamine. The mortality of the worm *Tubifex tubifex* and the insect *Spodoptera frugiperda* is increased by scopolamine (Wink, 1998b; Alves et al., 2007). In addition to the toxic effects of esterified tropane alkaloids, scopolamine was shown to be a feeding deterrent for honey bees (*Apis mellifera*) (Wink, 1998a). Furthermore, forced tropane alkaloid uptake changes the behavior of honey bees (Barron et al., 2009). After cocaine treatment, the likelihood of honey bee conducting a dance for its nest mates to indicate the location of sucrose or pollen was increased in a dose-dependent manner. It is suggested that cocaine binds to dopamine reuptake transporters in insects similar to the mammalian mode of action (Barron et al., 2009). But some insects have specialized on host plants containing tropane alkaloids. They evade the toxic effects of tropane alkaloids and often use them for their own purposes. Examples are the winter cherry bug (*Acanthocoris sordidus*) and the aposematic butterfly (*Placidula euryanassa*), which use scopolamine from their host plants, *Duboisia leichhardtii* and *Brugmansia suaveolens*, respectively, for their own defense (Freitas et al., 1996; Kitamura et al., 2004). Another specialist, the coca moth (*Eloria noyesi*), feeds exclusively on coca plants (*Erythroxylum coca*). Cocaine is sequestered in larval stages and retained in adult moths (Blum et al., 1981). Besides *Eloria noyesi*, there are other lymantrids known to feed exclusively on Erythroxylaceae. *Eloria subapicalis* feeds only on few *Erythroxylum species* like *E. deciduum*, one of the not cultivated wild Erythroxylaceae known to contain cocaine (Oliveira et al., 2010; Diniz et al., 2011). These examples demonstrate the role of tropane alkaloids as defense compounds. On the molecular level their mode of action is through binding to amine transporters (Singh, 2000). However, the esterification at the C-3 position is important for their physiological function (Schmeller et al., 1995). Non-C-3-esterified tropane alkaloids have little or no affinity to amine transporters compared to esterified tropane alkaloids (Williams et al., 1977; Ritz et al., 1990). Therefore, non-esterified tropane alkaloids have only little or no effect on the nervous system (Williams et al., 1977).

Non-esterified tropane alkaloids have different molecular effects. Calystegines, which are polyhydroxy, non-esterified tropane alkaloids, are accumulated by several

lepidopteran species in order to make them indigestible to potential predators (Nash and Watson, 1995). This is possible through the polyhydroxylation of the tropane ring. Calystegines mimic sugars and can act as glucosidase inhibitors (Biastoff and Dräger, 2007). In total, there are five groups of polyhydroxy alkaloids and the non-esterified polyhydroxy tropane alkaloids are just one of them (Asano et al., 2000). The latex of *Morus alba* (Moraceae) contains up to 2.5% sugar-mimicking alkaloids. Non-specialized caterpillars feeding latex-containing diets of *Morus alba* showed high mortality (Konno et al., 2006). Only specialized silkworm caterpillars are able to feed on sugar-mimicking, alkaloid rich diets (Konno et al., 2006). Latex-free diets did not show any toxic effects to non-specialized caterpillars. Besides their toxicity to insects, sugar-mimicking alkaloids defend the plant against nematodes. The sugar-mimicking alkaloid 2R, 5R-dihydroxymethyl-3R, 4R-dihydroxypyrrolidine (DMDP), found in legumes such as *Lonchocarpus costaricensis* or *Derris elliptica* (both Fabaceae), affects nematodes in various ways (Welter et al., 1976; Birch et al., 1993). Several nematode species were affected in cyst hatch, mobility or root galling. These ecological effects are assumed to be similar for all sugar-mimicking alkaloids, but detailed studies using calystegines need to be performed (Biastoff and Dräger, 2007).

Even though tropane alkaloids are potent plant defense compounds, their use in plant protection as potential insecticides has not been strongly considered since this would make a crop plant producing medicinal compounds or food toxic to humans as well. However, non-esterified tropane alkaloids are less toxic to the human nervous system (Williams et al., 1977). They exhibit potential anti-nematode and insecticidal properties, and these properties could be used for a targeted engineering approach to bring non-esterified tropane alkaloids into crop plants (Biastoff and Dräger, 2007). For example, a root targeted expression of tropane alkaloids in crop plants would protect the plant from below ground enemies, and theoretically above ground crop plant parts would be unchanged. But in order to engineer plants using tropane alkaloids, the tropane alkaloid biosynthesis pathway must be better understood.

4.4 Future prospects of tropane alkaloid enzyme discovery

Many steps in the tropane alkaloid biosynthesis pathway remain unknown. For example, the second ring formation leading to the tropane skeleton remains elusive. In addition, reports of C-3α-tropane alcohols and esters in other *Erythroxylum* species and *E. coca* varieties have yet to been the target of biochemical or molecular studies (Oliveira et al., 2010). Since methylecgonone reductase from *E. coca* cannot produce the C-3α-alcohol of methylecgonone or tropinone, there should be a reductase enzyme producing C-3α-tropane alcohols as well as an acyltransferase able to produce C-3α-tropane esters in the Erythroxylaceae. In the Solanaceae there are two distinct tropinone reductases (TRI and TRII) producing either tropine or pseudotropine, respectively. Assuming a similar scenario in Erythroxylaceae, there should be a methylecgonone reductase (MecgoRII) responsible for the formation of the C-3α-tropane alcohol of methylecgonone (which would be then called pseudomethylecgonine, accordingly) and tropine (Dräger, 2006). Furthermore, it is possible that the acyltransferase using tropine and benzoyl-CoA (producing 3α-benzoyloxytropane, the stereoisomer of tropacocaine) would as well be a member of the BAHD superfamily and be very similar to cocaine synthase. In order to approach these questions, I was able to obtain wild relatives of coca from Kenya (*Erythroxylum emarginatum* and *E. fischeri*) and Mauritius (*E. hypericifolium*, *E. laurifolium*, *E. macrocarpum* and *E. sideroxyloides*). These Old World *Erythroxylum species* are not cultivated like *E. coca*, nor do they produce cocaine. The cocaine producing ability seems to be linked only to New World *Erythroxylum species* (Schulz, 1907; Oliveira et al., 2010). Transcriptome sequencing should allow the discovery of the putative MecgoRII and cocaine synthase-like enzyme sequences. Research on this subject is currently in progress.

5 Summary

Tropane alkaloids are pharmacologically valuable plant secondary metabolites found in ten angiosperm plant families. Despite their medicinal value and socioeconomic impact on mankind, their biosynthesis and ecological function remains to be understood. The most biochemically investigated angiosperm family producing tropane alkaloids is the Solanaceae (order Solanales), predominantly known for atropine and scopolamine from belladonna (*Atropa belladonna*), datura (*Datura stramonium*), henbane (*Hyoscyamus niger*) and mandrake (*Mandragora officinalis*). The Erythroxylaceae (order Malpighiales), another important tropane alkaloid producing angiosperm family, is predominantly known for cocaine from the coca plant (*Erythroxylum coca*).

In this thesis, I review the current state of tropane alkaloid biosynthesis in plants, their occurrence in the angiosperms and their ecological functions. Besides the Solanaceae, biochemical investigations on tropane alkaloid production in other plant families have been neglected. Therefore the last two steps of tropane alkaloid biosynthesis in *E. coca* were investigated. Interestingly, the penultimate step in cocaine biosynthesis in Erythroxylaceae is performed by a different family of oxidoreductase enzymes than reported from the Solanaceae. Short chain reductases / dehydrogenases (SDR) reduce tropinone in Solanaceae and an aldo-keto reductase (AKR) reduces 2-carbomethoxy-3-tropinone in the Erythroxylaceae. The utilization of both SDR and AKR enzymes in tropane alkaloid biosynthesis in angiosperms is an example of convergent evolution. In addition, the enzyme responsible for the last step of cocaine biosynthesis, the esterification of 2-carbomethoxy-3β-tropine and benzoyl-CoA, in *E. coca* was characterized. The enzyme belongs to the BAHD acyltransferase enzyme superfamily. These enzymes are reported to be involved in other alkaloid biosynthetic pathways, but were shown for the first time to be involved in tropane alkaloid biosynthesis.

The importance of biochemical investigations of plant secondary metabolite pathways is also reviewed in this thesis in the context of metabolic engineering. In

order to manipulate or engineer plants defense metabolite pathways, the *in planta* mechanisms controlling the pathway need to be understood. Furthermore, concepts of secondary metabolite pathway organization like multi-gene cassettes could be used as template for engineering approaches. Finally, the two enzymes described in this thesis are discussed in the context of plant metabolic engineering.

6 Zusammenfassung

Tropanalkaloide sind pharmakologisch wertvolle, pflanzliche Sekundärstoffe, die in zehn Pflanzenfamilien der Bedecktsamigen Pflanzen (Angiospermae) vorkommen. Trotz der medizinischen und sozioökonomischen Bedeutung für die Menschheit, sind Biosynthese und Ökologie der Tropanalkaloide immer noch unbekannt. Die biochemisch am meisten untersuchte bedecktsamige Pflanzenfamilie, die Tropanalkaloide produziert, ist die Familie der Solanaceen (Ordnung Solanales). Zu den bekannten Solanaceen, die vorwiegend Atropin und Scopolamin enthalten, zählen die schwarze Tollkirsche (*Atropa belladonna*), der gemeine Stechapfel (*Datura stramonium*), das schwarze Bilsenkraut (*Hyoscyamus niger*) und die gemeine Alraune (*Mandragora officinalis*). Eine weitere wichtige bedecktsamige und tropanalkaloidproduzierende Pflanzenfamilie, ist die Familie der Erythroxylaceen (Ordnung Malpighiales). Sie ist vorwiegend durch das Kokain von der Kokapflanze (*Erythroxylum coca*) bekannt.

In meiner Dissertation fasse ich den derzeitigen Wissenstand zu Biosynthese, Vorkommen in den Angiospermae und Ökologie der Tropanalkaloide zusammen. Abgesehen von den Solanaceen wurde die biochemische Erforschung der Tropanalkaloidbiosynthese in den anderen Pflanzenfamilien vernachlässigt. Deshalb untersuchten wir die zwei letzten Schritte der Tropanalkaloidbiosynthese in *E. coca*. Interessanterweise wird der vorletzte Schritt der Kokainbiosynthese in Erythroxylaceen von einer anderen Enzymfamilie der Oxidoreduktasen durchgeführt, als das bei den Solanaceen der Fall ist. Die Enzymfamilie der kurzkettigen Reduktasen/Dehydrogenasen (SDR) reduziert Tropinon in den Solanaceen und die Enzymfamilie der Aldo-Keto Reduktasen (AKR) reduziert 2-Carbomethoxy-3-Tropinon in den Erythroxylaceen. Der Einsatz von zwei Enzymfamilien (SDR und AKR) in der Tropanalkaloidbiosynthese, ist ein Beispiel für konvergente Evolution. Weiterhin wurde von uns das letzte Enzym der Kokainbiosynthese, das 2-Carbomethoxy-3β-Tropin und Benzoyl-CoA verestert, aus *E. coca* charakterisiert. Dieses Enzym gehört zur Enzymsuperfamilie der BAHD Acyltransferasen.

Bekanntermaßen sind BAHD Acyltransferasen an der Biosynthese anderer Alkaloide beteiligt, jedoch ist dies die erste Beschreibung der Beteiligung an der Tropanalkaloidbiosynthese.

Weiterhin wird in dieser Arbeit die Bedeutung der biochemischen Untersuchung von Biosynthesewegen sekundärer Pflanzenstoffe in Zusammenhang mit Metabolic Engineering gebracht. Um die Biosynthesewege pflanzlicher Abwehrstoffe in Pflanzen zu manipulieren oder zu konstruieren, müssen zunächst die zugrundeliegenden Kontrollmechanismen verstanden werden. Außerdem kann die Organisation der Biosynthesewege pflanzlicher Sekundärstoffe (wie Multigen Kassetten) als Vorbild für Metabolic Engineering Ansätze dienen. Abschließend werden in dieser Arbeit die zwei charakterisierten Enzyme in Zusammenhang mit Metabolic Engineering von Pflanzen gestellt.

7 References

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9 Eigenständigkeitserklärung

Entsprechend der zurzeit gültigen Promotionsordnung der Biologisch-Pharmazeutischen Fakultät der Friedrich-Schiller-Universität Jena erkläre ich, daß ich die vorliegende Arbeit selbständig und nur unter Verwendung der angegebenen Hilfsmittel und Literatur angefertigt habe. Personen, die an der experimentellen Durchführung, Auswertung des Datenmaterials oder bei der Verfassung der Manuskripte beteiligt waren, sind am Beginn der Arbeit ("Overview of the manuscripts") und jedes Manuskriptes angegeben. Die Hilfe eines bezahlten oder unbezahlten Promotionsberaters wurde nicht in Anspruch genommen. Die vorgelegte Arbeit wurde weder an der Friedrich-Schiller-Universität Jena, noch an einer anderen Hochschule als Dissertation eingereicht.

Jena, den 20. Januar 2014

Jan Jirschitzka

10 Appendix

Table 1: List of angiosperm plant families reported to contain tropane alkaloids.

Family	Genus	Species	Reference
Brassicaceae	*Aethionema*	*A. grandiflora*	(1)
	Brassica	*B. campestris*	(1)
		B. oleracea var. gongylodes	(1)
		B. oleracea var. silvestris	(1)
		B. oleracea var. gemmifera	(1)
		B. nigra	(1)
	Calepina	*C. irregulans*	(1)
	Camelina	*C. alyssum*	(1)
		C. sativa	(1)
	Capsella	*C. bursa-pastoris*	(1)
	Cochlearia	*C. anglica*	(1)
		C. arctica (also C. groenlandica)	(2)
		C. danica	(1)
		C. glastifolia	(1)
		C. megalosperma	(1)
		C. officinalis	(3)
		C. polonica	(1)
		C. pyrenaica	(1)
	Cheiranthus	*C. cheiri (also Erysimum cheiri)*	(1)
	Crambe	*C. cordifolia*	(1)
		C. kotschyana	(1)
		C. maritime	(1)
		C. orientalis	(1)
	Diplotaxis	*D. tenuifolia*	(1)
	Iberis	*I. amara*	(1)
	Hersperis	*H. matronalis*	(1)
	Lepidium	*L. sativum*	(1)
	Moricandia	*M. arvensis*	(1)
Convolvulaceae	*Aniseieae*	*A. martinicensis*	(4)
	Argyreia	*A. capitata*	(5)
		A. hookeri	(5)
		A. mollis	(5)
		A. nervosa	(4)
	Astripomoea	*A. malvacea*	(4)
	Bonamia	*B. brevifolia*	(4)
		B. semidigyna var semidigyna	(5)
		B. spectabilis	(5)
		B. trichantha	(4)
	Calystegia	*C. japonica*	(6)
		C. macrostegia ssp. cyclostegia	(4)
		C. sepium	(7)
		C. silvatica	(4)
		C. soldanella	(6)
	Convolvulus	*C. althaeoides*	(4)
		C. arvensis	(7)
		C. canariensis	(4)
		C. cantabrica	(4)
		C. caput-medusae	(5)
		C. chilensis	(4)

	C. cneorum	(5)
	C. pseudocantabricus	(8)
	C. sabatius ssp. mauritanicus	(9)
	C. siculus	(9)
	C. subhirsutus	(10)
	C. demissus	(4)
	C. dorycnium	(4)
	C. elongates	(4)
	C. erinaceus	(4)
	C. farinosus	(4)
	C. floridus	(4)
	C. glandulosus	(4)
	C. graminetinus	(4)
	C. hamadae	(4)
	C. hermanniae	(4)
	C. humilis	(4)
	C. kilimandschari	(4)
	C. krauseanus	(4)
	C. lanatus	(11)
	C. lineatus	(4)
	C. lopezsocasii	(4)
	C. sagittatus	(4)
	C. scoparius	(4)
	C. subauriculatus	(4)
	C. tricolor ssp. tricolor	(4)
Cuscuteae	*C. australis*	(4)
Dichondra	*D. micrantha*	(4)
	D. repens	(5)
	D. sericea	(4)
Erycibe	*E. elliptilimba*	(12)
	E. hainanensis	(4)
	E. malaccensis	(5)
	E. micrantha	(4)
	E. obtusifolia	(4)
	E. parvifolia	(5)
	E. rheedii	(4)
	E. schmidtii	(4)
Evolvulus	*E. alsinoides var. decumbens*	(4)
	E. argyreus	(5)
	E. glomeratus	(4)
	E. nummularius	(4)
	E. sericeus var. holoceriseus	(13)
Falkia	*F. repens*	(5)
Hewittia	*H. sublobata*	(4)
Ipomoea	*I. abrupta*	(4)
	I. alba	(5)
	I. anisomeres	(4)
	I. aquatic	(5)
	I. arborescens	(4)
	I. argillicola	(4)
	I. asarifolia	(4)
	I. batatas	(5)
	I. batatoides	(4)
	I. cairica	(4)
	I. calobra	(14)
	I. carnea	(15)

	I. chiriquiensis	(4)
	I. cholulensis	(4)
	I. cristulata	(4)
	I. eremnobrocha	(5)
	I. eriocarpa	(4)
	I. hederacea	(4)
	I. hederifolia	(5)
	I. indica var. acuminate	(4)
	I. involucrate	(4)
	I. lobata	(4)
	I. lonchophylla	(4)
	I. mauritiana	(4)
	I. muelleri	(4)
	I. nil	(4)
	I. obscura	(6)
	I. pes-caprae	(6)
	I. pes-tigridis	(4)
	I. plebeian	(4)
	I. polpha	(14)
	I. pubescens	(4)
	I. purpurea	(4)
	I. quamoclit	(4)
	I. ramosissima	(4)
	I. regnellii	(4)
	I. reticulate	(4)
	I. rubens	(4)
	I. sepiaria	(4)
	I. setifera	(5)
	I. shirambensis	(4)
	I. sloteri	(4)
	I. squamosal	(4)
	I. tenuirostris	(4)
	I. tiliacea	(4)
	I. trichosperma	(4)
	I. tricolor	(4)
	I. trifida	(4)
	I. triloba	(4)
	I. turbinate	(4)
	I. velutina	(4)
	I. violacea	(4)
	I. wightii	(4)
Iseia	*I. luxurians*	(5)
Jacquemontieae	*J. pentantha*	(4)
	J. tamnifolia	(4)
Lepistemon	*L. urceolatum*	(4)
Maripa	*M. nicaraguensis*	(4)
	M. panamensis	(5)
Merremia	*M. aegyptia*	(4)
	M. aurea	(4)
	M. cissoids	(5)
	M. dissecta	(4)
	M. emarginata	(4)
	M. gemella ssp. gemella	(4)
	M. guerichii	(4)
	M. hederacea	(4)
	M. kentrocaulos	(4)

		M. peltata	(4)
		M. pterygocaulos	(4)
		M. quinata	(4)
		M. quinquefolia	(5)
		M. tuberosa	(4)
		M. umbellate	(5)
		M. vitifolia	(4)
	Odonellia	*O. hirtiflora*	(4)
	Operculina	*O. aequisepala*	(4)
		O. riedeliana	(4)
	Polymeria	*P. ambigua*	(4)
		P. calycina	(4)
		P. longifolia	(4)
		P. pusilla	(4)
	Porana	*P. volubilis*	(5)
	Quamoclit	*Q. angulate*	(6)
	Stictocardia	*S. campanulata*	(5)
		S. laxiflora	(4)
		S. tiliaefolia	(4)
		S. mojangensis	(4)
	Turbina	*T. abutiloides*	(5)
		T. corymbosa	(4)
Elaeocarpaceae	*Peripentadenia*	*P. mearsii*	(16),(17)
Erythroxylaceae	*Erythroxylum*	*E. alaternifolium*	(18)
		E. amazonicum	(19)
		E. andrei	(19)
		E. argentinum	(18)
		E. austral	(18)
		E. caatingae	(20)
		E. campestre	(18)
		E. citrifolium	(18)
		E. coca	(18)
		E. confusum	(19)
		E. cumanense	(18)
		E. cuneatum	(18)
		E. cuneifolium	(19)
		E. deciduum	(18)
		E. dekindtii	(18)
		E. densum	(19)
		E. ecarinatum	(18)
		E. ellipticum	(18)
		E. emarginatum	(18)
		E. fimbriatum	(21)
		E. foetidum	(19)
		E. glaucum	(21)
		E. glazioui	(19)
		E. gonocladum	(19)
		E. gracilipes	(21)
		E. havanense	(18)
		E. hypericifolium	(18)
		E. hypoleucum	(19)
		E. aff. impressum	(19)
		E. incrassatum	(21)
		E. laetevirens	(22)
		E. lealcostae	(19)
		E. ligustrinum	(19)

		E. lucidum	(18)
		E. macrocarpum	(18)
		E. macrocnemium	(21)
		E. macrophyllum	(19)
		E. magnoliifolium	(19)
		E. mamacoca	(23)
		E. martii	(19)
		E. mattos-silvae	(19)
		E. mexicanum	(19)
		E. microphyllum	(18)
		E. mikanii	(19)
		E. monogynum	(18)
		E. moonii	(18)
		E. mucronatum	(19)
		E. myrsinites	(19)
		E. novogranatense	(18)
		E. ochranthum	(19)
		E. orinocense	(19)
		E. ovalifolium	(19)
		E. panamense	(18)
		E. passerinum	(19)
		E. pelleterianum	(18)
		E. pervillei	(18)
		E. pictum	(19)
		E. pulchrum	(18)
		E. pungens	(24)
		E. recurrens	(21)
		E. rotundifolium	(18)
		E. rufum	(18)
		E. shatona	(19)
		E. sideroxyloides	(18)
		E. steyermarkii	(21)
		E. suberosum	(19)
		E. ulei	(23)
		E. vacciniifolium	(18)
		E. zambesiacum	(18)
		E. zeylanicum	(18)
Moraceae	*Morus*	*M. alba*	(25)
		M. bombycis	(26)
Olacaceae	*Heisteria*	*H. olivae*	(27)
Phyllanthaceae	*Phyllanthus*	*P. discoides*	(28)
Proteaceae	*Agastachus*	*A. odorata*	(29)
	Bellendena	*B. montana*	(29)
	Darlingia	*D. darlingiana*	(29)
		D. ferruginea	(29)
	Knightia	*K. deplanchei*	(29)
		K. strobilina	(29)
	Triunia	*T. erythrocarpa*	(30)
Rhizophoraceae	*Bruguiera*	*B. cylindrical*	(31)
		B. exaristata	(32)
		B. sexangula	(33)
	Crossostylis	*C. biflora*	(34)
		C. multiflora	(34)
		C. sebertii	(34)
	Pellacalyx	*P. axillaris*	(35)

Solanaceae	*Anthocercis*	*A. angustifolia*	(36)
		A. anisantha	(36)
		A. fasciculate	(36)
		A. genistoides	(36)
		A. gracilis	(36)
		A. ilicifolia	(36)
		A. intricate	(36)
		A. littorea	(36)
		A. uiscosa	(36)
		A. viscosa	(36)
	Anthotroche	*A. myoporoides*	(36)
		A. pannosa	(36)
		A. walcottii	(36)
	Atropa	*A. acuminate*	(36)
		A. baetica	(36)
		A. belladonna	(36)
		A. belladonna var. lutea	(36)
		A. caucasica	(36)
		A. komarovii	(36)
		A. pallidiflora	(36)
	Brugmansia	*B. arborea*	(37)
		B. aurea	(38)
		B. candida	(36)
		B. chlorantha	(36)
		B. cornigera	(36)
		B. sanguinea	(36)
		B. suaveolens	(36)
	Brunfelsia	*B. nitida*	(39)
	Capsicum	*C. annuum var. angulosum*	(40)
		C. frutescens	(40)
	Crenidium	*C. spinescens*	(36)
	Cyphanthera	*C. albicans*	(36)
		C. anthocercidea	(36)
		C. microphylla	(36)
		C. mycosotidea	(36)
		C. odgersii	(36)
		C. scabrella	(36)
		C. tasmanica	(36)
	Cyphomandra	*C. betacea*	(36)
	Datura	*D. discolor*	(36)
		D. candida x candida	(36)
		D. ceratocaula	(36)
		D. discolor x stramonium var. godronii	(36)
		D. ferox	(36)
		D. ferox x stramonium	(36)
		D. innoxia	(36)
		D. innoxia x leichardtii	(36)
		D. leichardtii	(36)
		D. metel	(36)
		D. quercifolia	(36)
		D. stramonium	(36)
		D. stramonium var. godronii	(36)
		D. stramonium var. inermis	(36)
		D. stramonium var. stramonium	(36)
		D. stramonium var. tatula	(36)
		D. tatula	(41)

	D. wrightii	(36)
Duboisia	*D. hopwoodii*	(36)
	D. leichardtii	(36)
	D. myoporoides	(36)
	D. leichardtii x myoporoides	(36)
Granunosolen	*G. dixonii*	(36)
Hyoscyamus	*H. albus*	(36)
	H. arachnoideus	(42)
	H. aureus	(36)
	H. boveanus	(43)
	H. bohemicus	(36)
	H. canariensis	(36)
	H. desertorum	(36)
	H. kurdicus	(44)
	H. muticus	(36)
	H. niger	(36)
	H. niger var. pallidus	(36)
	H. orientalis	(36)
	H. pusillus	(36)
	H. reticulatus	(36)
	H. senecionis	(36)
	H. turcomanicus	(36)
Latua	*L. pubiflora*	(36)
	L. venenosa	(36)
Lycium	*L. chinense*	(45)
Lycopersicum	*L. esculentum*	(40)
Mandragora	*M. caulescens*	(36)
	M. chinghaiensis	(36)
	M. officinarum var. autumnalis	(36)
	M. officinarum var. vernalis	(36)
Nicandra	*N. physaloides*	(36)
Nierembergia	*N. hippomanica*	(46)
Physalis	*P. alkekangi*	(36)
	P. alkekengi var. francheti	(47)
	P. divaricate	(48)
	P. exocarpa	(40)
	P. ixocarpa	(48)
	P. peruviana	(36)
	P. pubescens	(48)
Physochlaina	*P. alaica*	(36)
	P. dubia	(36)
	P. infundubularis	(36)
	P. orientalis	(36)
	P. physaloides	(36)
	P. praealta	(36)
Przewalskia	*P. shebbearei*	(36)
	P. tangutica	(36)
Salpichroa	*S. organifolia*	(36)
Schizanthus	*S. alpestris*	(36)
	S. grahamii	(36)
	S. hookerii	(36)
	S. littoralis	(36)
	S. pinnatus	(36)
	S. porrigens	(49)
	S. tricolor	(50)

Scopolia	*S. acutangula*	(36)
	S. anomala	(36)
	S. carniolica	(36)
	S. himalaiensis	(36)
	S. japonica	(36)
	S. lurida	(36)
	S. parviflora	(36)
	S. sinensis	(36)
	S. tangutica	(36)
	S. sinensis x tangutica	(36)
Solandra	*S. grandiflora*	(36)
	S. longiflora	(36)
	S. guttata	(36)
	S. hartwegii	(36)
	S. hirsute	(36)
	S. macrocantha	(36)
Solanum	*S. dimidiatum*	(51)
	S. dulcamara	(51)
	S. kwebense	(51)
	S. melongena	(51)
	S. scabrum	(40)
	S. sodomaeum	(39)
	S. tuberosum	(51)
Symonanthus	*S. aromaticus*	(36)
Withania	*W. frutescens*	(39)
	W. somnifera	(36)

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11 Curriculum Vitae

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Education

2013	Visiting researcher: Department of Chemistry & Biochemistry, Texas Tech University, Lubbock, USA
2010-present	PhD student: MPI for Chemical Ecology, Department of Biochemistry, Jena, Germany
2008	Internship: Department of Medical Biochemistry and Biophysics Umeå University, Umeå, Sweden
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Publications

Jirschitzka, J., F. Dolke and J. C. D'Auria (2013). "**Increasing the pace of new discoveries in tropane alkaloid biosynthesis**." *New Light on Alkaloid Biosynthesis and Future Prospects*. G.-G. h. Nathalie, Academic Press. 68: 39-72.

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Jirschitzka, J., G. W. Schmidt, M. Reichelt, B. Schneider, J. Gershenzon and J. C. D'Auria (2012). "**Plant tropane alkaloid biosynthesis evolved independently in the Solanaceae and Erythroxylaceae.**" *Proceedings of the National Academy of Sciences of the United States of America* 109(26): 10304-10309.

Oral presentations

Jirschitzka J. **Identification of the penultimate enzymes in the biosynthesis of cocaine and other tropane alkaloids in *Erythroxylum coca* and other *Erythroxylum* species.** IMPRS Evaluation Symposium 2013, MPI for Chemical Ecology, IMPRS, Jena, Germany, Apr 2013

Poster presentations

Jirschitzka J. **Current status of cocaine research in *Erythroxylum coca*.** SAB Meeting 2012, MPI for Chemical Ecology, Jena, Germany, Oct 2012

Jirschitzka J., Gershenzon J., D'Auria J. **Cocaine biosynthesis in *Erythroxylum coca*: Insight on the evolution of tropane alkaloid formation in angiosperms and its diversity.** 3rd Banff Conference on Plant Metabolism, Alberta, Canada, Jul 2012

Jirschitzka J., Gershenzon J., D'Auria J. **Methylecgonone reductase in *Erythroxylum coca* and other species.** 11th IMPRS Symposium, MPI for Chemical Ecology, Dornburg, Germany, Feb 2012

Jirschitzka J., Gershenzon J., D'Auria J. **Methylecgonone reductase: The penultimate step in cocaine biosynthesis.** ICE Symposium, MPI for Chemical Ecology, Jena, Germany, Sep 2011

Jirschitzka J., Gershenzon J., D'Auria J. **Methylecgonone reductase: The second to last step in cocaine production.** Gordon Research Conference - Plant Metabolic Engineering, Waterville Valley, NH, USA, Jul 2011

Jirschitzka J., Gershenzon J., D'Auria J. **Methylecgonone reductase: The second to last step in cocaine production.** Trends in Natural Products Research: A Young Scientists' Meeting, Phytochemical Society of Europe (PSE), Kolymvari, Crete, Greece, Jun 2011

Jirschitzka J., Schmidt G., D'Auria J. **Biochemical characterization of the two terminal steps of cocaine biosynthesis in *Erythroxylum coca*.** SAB Meeting 2010, MPI for Chemical Ecology, Jena, Germany, Oct 2010

Awards

Poster award, 3rd Banff Conference on Plant Metabolism, Alberta, Canada, Jul 2012