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Biosynthesis of curcuminoids and gingerols in turmeric (*Curcuma longa*) and ginger (*Zingiber officinale*): Identification of curcuminoid synthase and hydroxycinnamoyl-CoA thioesterases

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Abstract

Members of the Zingiberaceae such as turmeric (*Curcuma longa* L.) and ginger (*Zingiber officinale* Rosc.) accumulate at high levels in their rhizomes important pharmacologically active metabolites that appear to be derived from the phenylpropanoid pathway. In ginger, these compounds are the gingerols; in turmeric these are the curcuminoids. Despite their importance, little is known about the biosynthesis of these compounds. This investigation describes the identification of enzymes in the biosynthetic pathway leading to the production of these bioactive natural products. Assays for enzymes in the phenylpropanoid pathway identified the corresponding enzyme activities in protein crude extracts from leaf, shoot and rhizome tissues from ginger and turmeric. These enzymes included phenylalanine ammonia lyase, polyketide synthases, *p*-coumaroyl shikimate transferase, *p*-coumaroyl quinate transferase, caffeic acid *O*-methyltransferase, and caffeoyl-CoA *O*-methyltransferase, which were evaluated because of their potential roles in controlling production of certain classes of gingerols and curcuminoids. All crude extracts possessed activity for all of these enzymes, with the exception of polyketide synthases. The results of polyketide synthase assays showed detectable curcuminoid synthase activity in the extracts from turmeric with the highest activity found in extracts from leaves. However, no gingerol synthase activity could be identified. This result was explained by the identification of thioesterase activities that cleaved phenylpropanoid pathway CoA esters, and which were found to be present at high levels in all tissues, especially in ginger tissues. These activities may shunt phenylpropanoid pathway intermediates away from the production of curcuminoids and gingerols, thereby potentially playing a regulatory role in the biosynthesis of these compounds.

Keywords: Polyketide synthase; Phenylpropanoid pathway; Rhizome; Acyltransferase; Phenylalanine ammonia lyase; O-methyltransferase; Medicinal plant

1. Introduction

Powdered turmeric (*Curcuma longa* L.) rhizome is widely used as a food additive, especially in curries, and has been very popular in traditional Asian medicine for the treatment of a number of conditions, including hepatic disorders and rheumatism (Miquel et al., 2002). In addition, anti-inflammatory, antiulcerogenic and antitumor activities, among others, have been described for turmeric (Claeson et al., 1994; Joe et al., 2004). The most important constituent of turmeric, curcumin (**1**), has been shown to possess many of these properties. Ginger (*Zingiber officinale* Rosc.), also a member of the Zingiberaceae and an important component of traditional Asian herbal medicine, is used for management of such symptoms as the common cold, digestive disorders, rheumatism, neuralgia, colic and motion-sickness, as well as being an important spice to flavor foods and beverages. [6]-Gingerol (**2**), the major gingerol in ginger rhizomes, has been found to possess many

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interesting pharmacological and physiological activities, such as anti-inflammatory, analgesic and cardotonic effects (Mascolo et al., 1989; Mustafa et al., 1993).

Because of their importance to human health and nutrition, some initial investigations into the biosynthesis of the curcuminoids and gingerols were performed over 25 years ago (Denniff and Whiting, 1976; Macleod and Whiting, 1979; Denniff et al., 1980). These initial radiotracer feeding studies suggested that these compounds are derived from intermediates in the phenylpropanoid pathway that are condensed with other molecules, derived in turn from the acetate and short and medium-chain fatty acid pathways. Based on these results, Schröder (1997) proposed that enzymes similar to polyketide synthases are most likely responsible for formation of the basic backbone structure of these compounds and would utilize coenzyme A derivatives of the intermediates that were suggested from the studies carried out during the 1970s. Based on these results, two biosynthetic pathways can readily be envisaged for the production of each of these two groups of compounds. The curcuminoids could be formed from condensation of two molecules of *p*-coumaroyl-CoA with one molecule of malonyl-CoA via the action of a polyketide synthase (or similar) enzyme, perhaps involving an additional diketide intermediate, as has been suggested recently by Bernd Schneider's group (Brand et al., 2006). The resulting bisdemethoxycurcumin (**3**) would then be transformed through demethoxycurcumin (**4**) into curcumin (**1**) via two sequential rounds of hydroxylation followed by *O*-methylation (see bottom pathway in Fig. 1). Alternatively, it is likely that the curcuminoid synthase enzyme may utilize the CoA esters of both *p*-coumaric acid (**5**) and ferulic acid (**6**) as substrates. In this case, the central pathway in Fig. 1 could be operative, and the hydroxylation and *O*-methylation reactions that lead to formation of the methoxyl functional groups in curcumin (**1**) would be the same reactions as those found in the general phenylpropanoid pathway. Likewise, the gingerols could be produced via related pathways, as outlined in Fig. 1, with the addition of reduction steps necessary to eliminate the double bond present in the *p*-coumaric acid-derived intermediate(s). This reduction could occur prior to condensation or after condensation of the phenylpropanoid derived moiety with the CoA ester of the corresponding short chain aliphatic alcohol. Enzymes in all of these proposed pathways would include one or more polyketide synthases, cytochrome p450 hydroxylases, and *S*-adenosyl-L-methionine-dependent *O*-methyltransferases. The present investigation

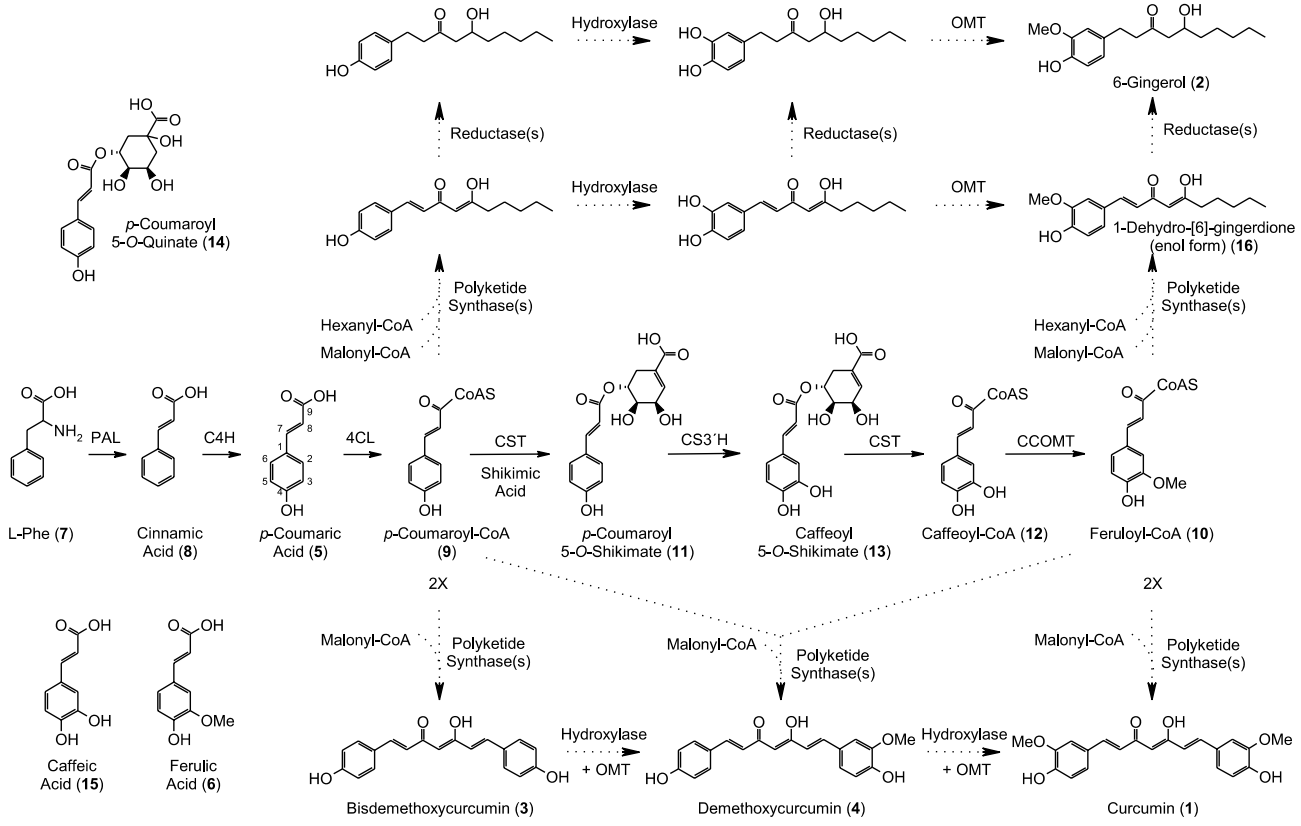

Fig. 1. Proposed biosynthetic pathway to curcuminoids and gingerols in turmeric and ginger. Enzymes are as follows: PAL = phenylalanine ammonia lyase; C4H = cinnamate 4-hydroxylase; 4CL = 4-coumarate:CoA ligase; CST = *p*-coumaroyl shikimate transferase; CS3′H = *p*-coumaroyl 5-*O*-shikimate 3′-hydroxylase; OMT = *O*-methyltransferase; CCOMT = caffeoyl-CoA *O*-methyltransferase. All conversions have been demonstrated in other species, except for those catalyzed by the polyketide synthases, the reductase, and the hydroxylases and OMTs that would convert bisdemethoxycurcumin (**3**) via demethoxycurcumin (**4**) to curcumin (**1**) (indicated by dashed arrows).

describes the identification of such enzyme activities in ginger and turmeric tissues, and provides the first direct evidence for enzymatic involvement of the phenylpropanoid pathway in the production of these compounds in these plants.

2. Results and discussion

To investigate the potential role of specific phenylpropanoid pathway enzymes in the production of the curcuminoids and the gingerols, crude protein extracts obtained from young developing leaves, shoots and rhizomes from ginger and turmeric were assayed for these activities. These included activities for phenylalanine ammonia lyase (PAL); hydroxycinnamoyl-CoA transferases (HCTs), including *p*-coumaroyl shikimate transferase (CST), caffeoyl shikimate transferase (CaST), feruloyl shikimate transferase (FST) and *p*-coumaroyl quinate transferase (CQT); caffeic acid *O*-methyltransferase (COMT); caffeoyl-CoA *O*-methyltransferase (CCOMT); and polyketide synthases (PKS).

2.1. Phenylalanine ammonia lyase activity

Phenylalanine ammonia lyase (PAL) is the first enzyme of the phenylpropanoid pathway and as such is the entry point into the potential pathways leading to the formation of the curcuminoids and gingerols. This enzyme catalyzes the non-oxidative deamination of L-Phe (**7**) to afford *trans*-cinnamic acid (**8**) and ammonium ion (Nugroho et al., 2002; Costa et al., 2003). In this study, we subjected crude protein extracts from young developing turmeric and ginger tissues to assays for PAL activity using [U-^{14}C]L-Phe (**7**) as substrate and monitoring formation of [U-^{14}C]cinnamic acid (**8**) as product. As expected, all crude extracts from ginger and turmeric tissues possessed PAL activity (Fig. 2). The results of these assays demonstrated that PAL activity was generally higher in ginger tissues than in the comparable tissues from turmeric. In addition, the highest activity in ginger was found in crude extracts from developing leaves, which was significantly higher (about 3-fold higher) than PAL activity in developing shoots (*P*-value 1×10^{-11} in one-way ANOVA). There was no significant difference, however, in PAL activity between developing leaves and developing rhizomes of ginger. This high level of PAL activity in developing ginger leaves was surprising because ginger leaves are not known to possess high levels of flavonoids, lignins or other commonly found phenylpropanoid pathway derived compounds, yet the shoots and rhizomes would be expected to have significant PAL activity because of the production of lignin in the developing xylem. The high level of PAL activity in the developing leaves suggested that production of some group of phenylpropanoid pathway derived metabolites was enhanced in the leaves. In contrast, the highest activity in turmeric was in the developing rhizomes, which possessed PAL activity that was about 9-fold higher than in developing leaves or shoots (*P*-values of $<2.22 \times 10^{-14}$). Overall, the level of PAL activity in ginger and turmeric tissues was similar than that reported for young basil leaf tissue (Gang et al., 2001) although slightly less than that observed for isolated basil glandular trichomes, elicited alfalfa cells, elicited tobacco cells, or developing pine xylem (Gowri et al., 1991; Sharan et al., 1998; Gomez-Vasquez et al., 2004). Considering that these latter tissues are known to express enzymes in the phenylpropanoid pathway at very high levels, the PAL assay results from ginger and turmeric suggest that the phenylpropanoid pathway is very active in these tissues, especially in those tissues that accumulate the gingerols and curcuminoids.

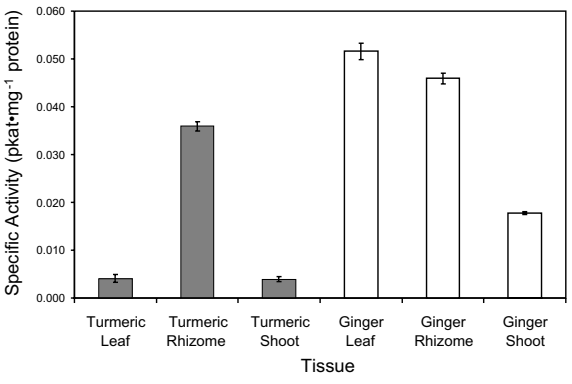

Fig. 2. Specific activities for PAL in ginger (white bars) and turmeric (gray bars) tissues. Bars indicate ±SE.

2.2. Hydroxycinnamoyl transferases

As suggested in Section 1, it is reasonable to hypothesize the route to curcumin (**1**) might go through bisdemethoxycurcumin (**3**), where only *p*-coumaroyl-CoA (**9**) would serve as a substrate for the "curcuminoid synthase" and feruloyl-CoA (**10**) would not. Methoxyl groups would be added by subsequent hydroxylase and *O*-methyltransferase activities that would work on the curcuminoid aromatic rings, and which would not be expected to be the enzymes involved with the central phenylpropanoid pathway. In this instance, the branchpoint enzyme leading to formation of feruloyl-CoA (**10**), known as *p*-coumaroyl-CoA:5-*O*-shikimate *p*-coumaroyl transferase (*p*-coumaroyl shimikate transferase, CST) (Gang et al., 2002), would not be involved in production of the curcuminoids. In fact, this enzyme (CST) might actually be suppressed, or at least it would not need to be highly active. We have previously shown that this enzyme activity is modulated in plants as a mechanism to control production of specific metabolites. Such a situation occurs in the glandular trichomes of certain sweet basil varieties. For example, basil varieties that produce large amounts of methylchavicol and very low levels of eugenol in their glandular trichomes have greatly reduced or no detectable CST activity (Gang et al., 2002). Methylchavicol does not possess a methoxyl group at the 3-position on the aromatic ring, whereas eugenol

does. In contrast, basil varieties that do produce large amounts eugenol or methyleugenol in their glandular trichomes have high levels of CST activity. We concluded that this enzyme activity is the one responsible for determining which compounds are produced. More recent work on this plant, where we have now identified evidence for an enzyme (called eugenol synthase) that can make both eugenol and chavicol, provide firm support for this conclusion (Koeduka et al., 2006; Vassão et al., 2006). Work in other plants, such as in tobacco (Hoffmann et al., 2004) has further supported the role of this enzyme in controlling production of methoxylated phenylpropanoids in plants. Thus, if this enzyme activity were found to be missing or very low in the tissues that produce the curcuminoids or the gingerols, then we could conclude that the pathway to curcumin (**1**) would likely proceed through bisdemethoxycurcumin (**3**), with all phenylpropanoid pathway derived moieties of the final curcuminoids being derived from *p*-coumaroyl-CoA (**9**) and not from feruloyl-CoA (**10**).

On the other hand, we also hypothesized that perhaps the enzymes involved in production of the core backbones of the curcuminoids and gingerols, the so-called curcuminoid and gingerol synthases, may utilize both *p*-coumaroyl-CoA (**9**) and feruloyl-CoA (**10**) as substrates. In this instance, the middle biosynthetic pathway in Fig. 1 would be responsible for formation of the curcuminoids, requiring the activity of CST or a comparable enzyme. Although it appears that *p*-coumaroyl-5-*O*-shikimate (**11**) is the intermediate in the general phenylpropanoid pathway (Gang et al., 2002) other hydroxycinnamoyl transferases (HCTs) have been identified in other plant species that utilize other acyl acceptors instead of or in addition to shikimate, such as CQT (Ulbrich and Zenk, 1979, 1980; Lofty et al., 1992; Hoffmann et al., 2004) or *p*-coumaroyl *p*-hydroxyphenyllactate transferase (CPLT) (Gang et al., 2002). Regardless of which substrate would actually serve as acyl acceptor, this pathway (middle pathway in Fig. 1) would require an active acyltransferase.

In an effort to distinguish between these two pathways (with or without involvement of the acyltransferase), we evaluated crude protein extracts from young leaves, shoots and rhizome from ginger and turmeric for hydroxycinnamoyl transferase activities. In these assays, we tested shikimic acid, 4-hydroxyphenyllactic acid, and quinic acid as potential acyl acceptors, and *p*-coumaroyl-CoA (**9**), caffeoyl-CoA (**12**) and feruloyl-CoA (**10**) as potential acyl donors. None of the crude protein extracts from either ginger or turmeric possessed activity that transferred any of the hydroxycinnamoyl groups to a hydroxyl functional group on 4-hydroxyphenyllactic acid (CPLT activity), which was not surprising since these plants do not produce rosmarinic acid, the downstream product of this activity (Petersen, 1993; Gang et al., 2002). In addition, feruloyl-CoA (**10**) did not serve as a substrate for any of these assays. This is consistent with known hydroxycinnamoyl transferase activities (Petersen, 1993; Gang et al., 2002).

In contrast, all of the crude protein extracts from ginger and turmeric showed CST and CQT activities. However, the ratio of these activities varied by tissue (see Fig. 3), demonstrating that at least two distinct enzymes exist in these tissues that are responsible for these activities (Gang et al., 2002). This is not surprising, because ginger produces chlorogenic acid (Rababah et al., 2004) (downstream product of CQT activity) as well as products of the general phenylpropanoid pathway (such as lignins downstream of CST activity). Also, previous investigations in other plants (Ulbrich and Zenk, 1979, 1980) have shown presence of two separate enzymes.

CST activity in ginger shoots was significantly higher (*P*-values $<1.7\times10^{-5}$) than in ginger leaves (2-fold) or in ginger rhizome (1.5-fold), which would be expected for an enzyme involved in processes such as lignification. In turmeric tissues, the activity for CST in shoots was about 1.6-fold higher compared with turmeric leaves and rhizome (*P*-values 2.8×10^{-6}). For both plants, CST was shown to be active with *p*-coumaroyl-CoA (**9**) and caffeoyl-CoA (**12**) thioesters as acyl donors. The activity of CST with caffeoyl-CoA (**12**) was 8- to 11.6-fold lower than the activity with *p*-coumaroyl-CoA (**9**) in turmeric (*P*-value 8.27×10^{-3}) and 5.5- to 11.5-fold lower in extracts from ginger tissues (*P*-value 1.24×10^{-2}). These results indicate that CST activities have a preference for *p*-coumaroyl-CoA (**9**) as acyl donor in all extracts from ginger and turmeric, and that multiple enzymes are responsible for these activities. This could be explained by either the presence of multiple isoforms of CST or by the activity of an additional HCT (such as CQT) with a broader substrate preference. We also measured CQT activity in these tissues, and the highest level of activity was found in shoots from ginger and turmeric. This activity was 2.2- to 4.3–fold lower than CST activity (*P*-value 4.53×10^{-2}) in extracts from turmeric tissues, whereas in ginger tissues the ratio varied from 1.3 to 3.4 depending on the tissue.

These results suggest that the general phenylpropanoid pathway is fully operational in all of the tissues examined, which was not really a surprise. However, these results also demonstrated that differences in substrate utilization and ultimately of product formation from the phenylpropanoid pathway intermediates are likely to occur. Our approach of using crude protein extracts from complex tissues such as leaves, shoots and rhizomes, however, was unable to fully distinguish the role of these different acyltransferases in production of specific compounds. Future work involving isolation and characterization of genes for acyltransferases in these plants will help to resolve this question. In addition, these results do not eliminate the bottom pathway in Fig. 1 from consideration.

2.3. *O*-Methyltransferases (COMT and CCOMT)

Just because a tissue possesses good activity for the acyltransferase that leads to production of 3-methoxylated compounds of the phenylpropanoid pathway does not

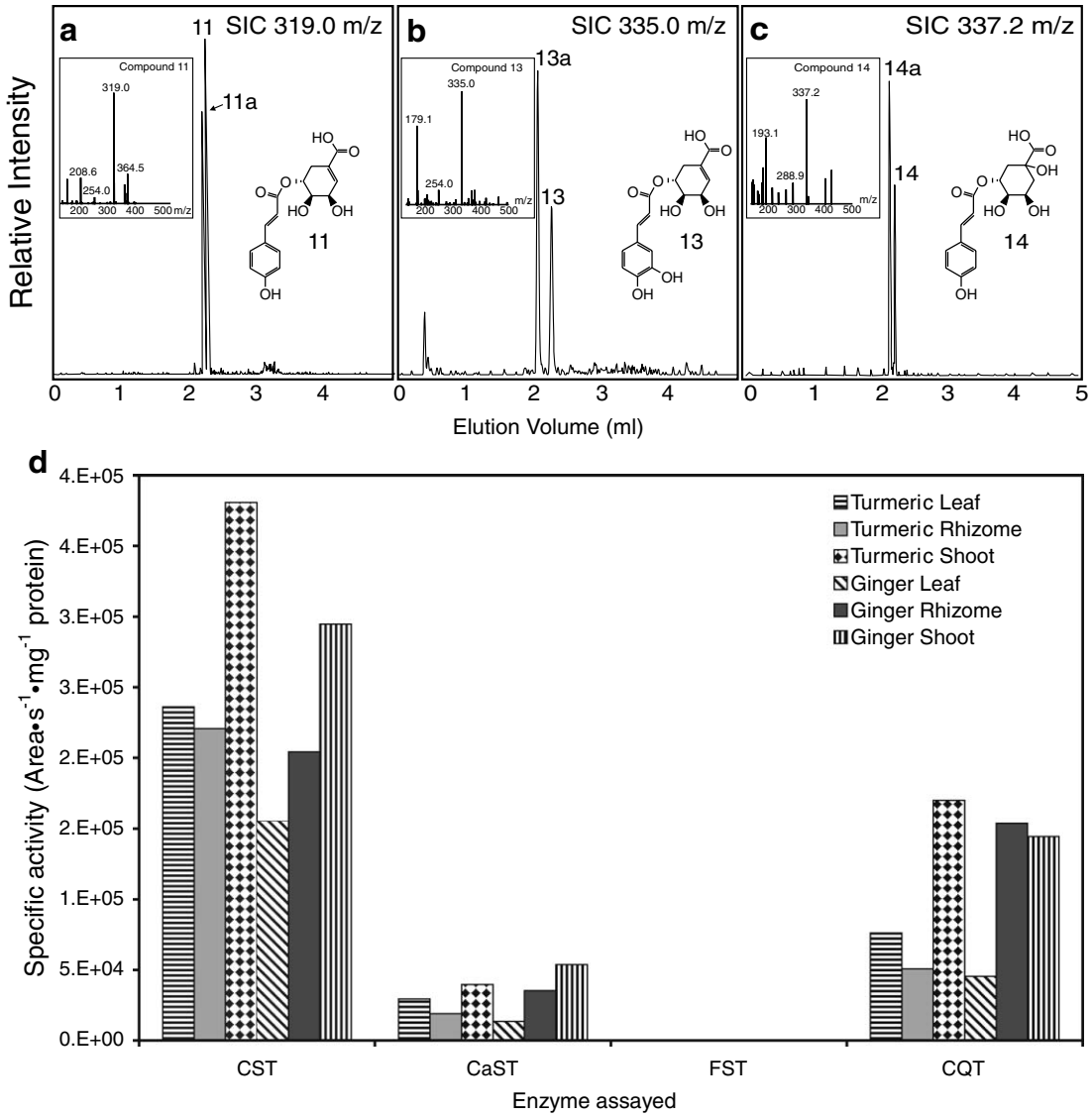

Fig. 3. Hydroxycinnamoyl transferases are very active in ginger and turmeric tissues. (a)–(c) LC–MS verification of reaction products. (a), assay with *p*-coumaroyl-CoA (**9**) and shikimic acid as substrates. Elution trace is the selected ion chromatogram at 319.0 mass-to-charge ratio (*m*/*z*). (b), assay with caffeoyl-CoA (**12**) and shikimic acid as substrates. Elution trace is the selected ion chromatogram at 335.0 *m*/*z*. (c), assay with *p*-coumaroyl-CoA (**9**) and quinic acid as substrates. Elution trace is the selected ion chromatogram at 337.2 *m*/*z*. Insets, electrospray ionization negative mode mass spectra for selected peaks. Peaks were identified as follows: 11, *p*-coumaroyl-5-*O*-shikimate; 11a, *p*-coumaroyl-4-*O*-shikimate; 13, caffeoyl-5-*O*-shikimate; 13a, caffeoyl-4-*O*-shikimate; 14, *p*-coumaroyl-5-*O*-quinate; 14a, *p*-coumaroyl-4-*O*-quinate. (d), specific HCT activities in ginger and turmeric tissues. CST, CaST, FST, and CQT are HCT activities, respectively, with *p*-coumaroyl-CoA/shikimate, caffeoyl-CoA/shikimate, feruloyl-CoA/shikimate, and *p*-coumaroyl-CoA/quinate as substrate pairs. Note that the relative HCT activities vary between different tissues (compare CST to CaST or CST to CQT).

mean that it will also possess the corresponding general phenylpropanoid pathway *O*-methyltransferase activities. Thus, the question was still open about whether the 3-methoxyl groups on the aromatic rings of the curcuminoids and the gingerols were produced before or after the condensation reaction(s) that leads to production of the molecular backbones of these compounds. Therefore, we sought to investigate the potential roles of specific *O*-methyltransferases (OMTs) in the pathway to the curcuminoids and the gingerols. *O*-Methyltransferase enzymes catalyze the transfer of a methyl group from *S*-adenosyl-L-methionine (SAM) to the hydroxyl group of an acceptor molecule, forming a methyl ether derivative. In the present investigation, we carried out assays in an attempt to identify the activities of the two core OMTs of the phenylpropanoid pathway: CCOMT and COMT, which methylates 5-hydroxyconiferyl aldehyde, 5-hydroxyferulic acid, and caffeic acid (**15**) (Gang, 2005). We did not at this time evaluate the corresponding potential OMT activities that would work on intermediates between bisdemethoxycurcumin (**3**), demethoxycurcumin (**4**) and curcumin (**1**), if the bottom pathway were to be operational, because such compounds are not available.

We carried out assays using [methyl-^{14}C]SAM and either caffeoyl-CoA (**9**) or caffeic acid (**15**) as substrates, respectively, as described in Section 3. For detection of

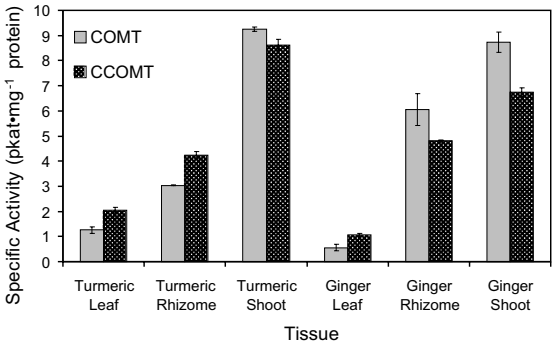

Fig. 4. CCOMT and COMT are highly active in ginger and turmeric tissues.

CCOMT activity, a base-catalyzed hydrolysis step was required prior to extraction with organic solvent and scintillation counting (Gang et al., 2001). Using this assay system, all crude protein extracts from ginger and turmeric appeared to possess both COMT and CCOMT activities. The results of these assays showed that the apparent specific activities for these enzymes were significantly higher in extracts from shoots when compared to leaves and rhizomes for both ginger and turmeric (see Fig. 4), consistent with a role for these enzymes in xylem development. For ginger, COMT activity in shoots was about 14-fold higher than in leaves and about 1.4-fold higher than in rhizomes (P-values $< 9.9 \times 10^{-7}$). For turmeric, COMT activity in shoots was 7.4-fold higher than in leaves and 3.0-fold higher than in rhizomes (P-values $< 1.5 \times 10^{-12}$). Thus, a similar pattern of activity was found for COMT, when different tissues were compared, as was found for the acyltransferase activities described above.

For the CCOMT activity assays, we performed controls where the product was not base-hydrolyzed prior to analysis. These controls displayed a high level of background activity that was observed for all crude extracts from ginger. These results suggest that the caffeoyl-CoA (**12**) was partially hydrolyzed to caffeic acid (**15**) under the CCOMT assay conditions and was then methylated by COMT (these were crude protein extracts, not purified enzymes) to yield ferulic acid (**6**), which was detected in the radiochemical-based assay. However, no apparent activity was found in the controls containing all reaction components except for the crude protein extracts (no-protein controls). This suggested that the hydrolysis of caffeoyl-CoA was catalyzed by the crude enzyme extracts. Such hydrolysis was not observed by us in previous investigations with other plants using the exact same assay system. For example, crude protein extracts from sweet basil glandular trichomes did not possess this CoA-ester hydrolyzing activity (Gang et al., 2001). The presence of a thioesterase activity capable of hydrolyzing hydroxycinnamoyl-CoA esters has been observed in other plants, however, such as in tobacco (Hoffmann et al., 2003). Such hydroxycinnamoyl-CoA thioesterase activities in ginger and turmeric are discussed in more detail below. Because of the presence of these thioesterase activities, however, the results obtained from the CCOMT assays do not clearly show the activity of CCOMT (because of potential contribution of COMT to the observed activity). Nevertheless, because significant differences were observed for CCOMT vs. COMT activity comparisons for ginger and turmeric tissues (see Fig. 4), it was possible to conclude that CCOMT activity was present at significant levels in these tissues. But, no conclusions regarding differences in CCOMT activity between tissues or between plants could be made. Even so, the results of these assays clearly indicate that the central phenylpropanoid pathway outlined in Fig. 1 is operable in these tissues at high levels and could contribute to production of the methoxylated groups observed on the aromatic rings of the curcuminoids and the gingerols.

2.4. Curcuminoid and gingerol synthase activities

As mentioned in Section 1, our potential biosynthetic pathway to the gingerols and curcuminoids (see Fig. 1) proposes that the enzymes that catalyze the formation of curcuminoid and gingerol backbone structures are or are related to polyketide synthases. The corresponding enzymes would be known as curcuminoid synthase and gingerol synthase, respectively. One major question regarding these enzymes is that of substrate specificity. As outlined in Fig. 1 and discussed above, two reasonable pathways to curcumin (**1**) can be envisaged. In one of these pathways, curcumin (**1**) is formed with bisdemethoxycurcumin (**3**) and demethoxycurcumin (**4**) acting as intermediates, where the curcuminoid synthase (CURS) would act (primarily) on *p*-coumaroyl-CoA (**9**). In the second pathway, the curcuminoids are each formed directly from phenylpropanoid pathway intermediates, where the curcuminoid synthase would have a broader substrate specificity, or where separate enzymes would be involved in production of each curcuminoid. One way to eliminate the latter pathway from consideration, would have been to find that the general phenylpropanoid pathway was not completely operable in the tissues that produce the curcuminoids (or gingerols). As described above, that was not the case. Therefore, purification of the curcuminoid synthase and gingerol synthase enzymes or cloning of the genes encoding these enzymes will be required before the actual pathway(s) can be verified.

Before such work can begin, verification that curcuminoid synthase and gingerol synthase are indeed polyketide synthases must be obtained. With this in mind, and based on reported assays for other plant polyketide synthases (Jez et al., 2000, 2001; Austin and Noel, 2003; Austin et al., 2004; Noel et al., 2005), we developed assays to monitor the formation of compounds such as curcumin (**1**), demethoxycurcumin (**4**) and bisdemethoxycurcumin (**3**) (with *p*-coumaroyl-CoA (**9**), feruloyl-CoA (**10**) and malonyl-CoA as substrates) and the formation of the [6]-gingerol (**2**) precursors, e.g., 1-dehydro-[6]-gingerdione (**14**) (using the same substrates plus the addition of hexanyl-

CoA). Formation of [6]-gingerol (**2**) from phenylpropanoid intermediates would require the action of additional reductases (to reduce the double bond and the ketone on the potential intermediate), and we did not seek to identify such reductases at this time.

Two general types of assays were performed using desalted crude extracts from leaf, shoot and rhizome tissues from different lines of ginger and turmeric. In the first case, radiolabeled precursor molecules ([2-^{14}C]malonyl-CoA) with unlabeled *p*-coumaroyl-CoA (**9**) and feruloyl-CoA (**10**, and unlabeled hexanyl-CoA as appropriate) were used. These assays were not able to detect curcumininoid or gingerol synthase activity, however, because the controls without substrates added (only [2-^{14}C]malonyl-CoA and protein solution added) showed very high background activity. These results suggest that the crude extracts from ginger and turmeric possess other enzyme activities that use malonyl-CoA as substrate to form other types of compounds (such as fatty acids, malonated flavonoid glycosides, etc.).

The second type of assay was carried out using unlabeled precursors. The results of these assays showed detectable curcuminoid synthase activity in the extracts from turmeric, with the highest activity found in extracts from leaves (Fig. 5), using malonyl-CoA with *p*-coumaroyl-CoA (**9**) and feruloyl-CoA (**10**) as combined substrates. Assays containing only *p*-coumaroyl-CoA (**9**) plus malonyl-CoA or only feruloyl-CoA (**10**) plus malonyl-CoA did not show any activity, which was a puzzling result. Likewise, no activity was detected (i.e., no formation of curcuminoids was observed) either in the controls without protein added or in controls without substrates added. No extracts from any tissue from ginger showed detectable

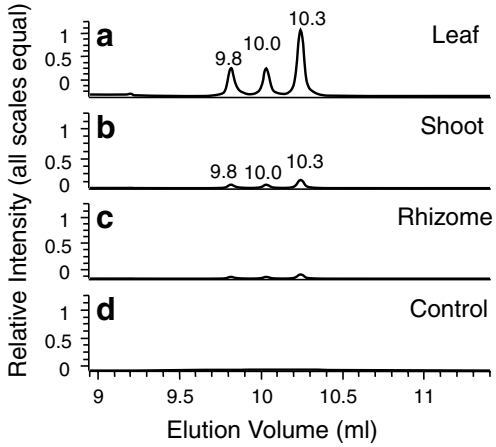

Fig. 5. Curcuminoid synthase activity is detectable in turmeric tissues. Assays with crude protein extracts from leaf (a), shoot (b), rhizome (c), and control (d, no protein) were performed using both *p*-coumaroyl-CoA and feruloyl-CoA as combined substrates. The peaks at 9.8, 10, and 10.3 ml are bisdemethoxycurcumin (**3**), demethoxycurcumin (**4**), and curcumin (**1**), respectively, monitored by HPLC at 425 nm. LC–MS analysis of these peaks verified their identities. These samples were analyzed using a long elution gradient (60 min gradient) to ensure baseline separation of the compounds in the UV detector.

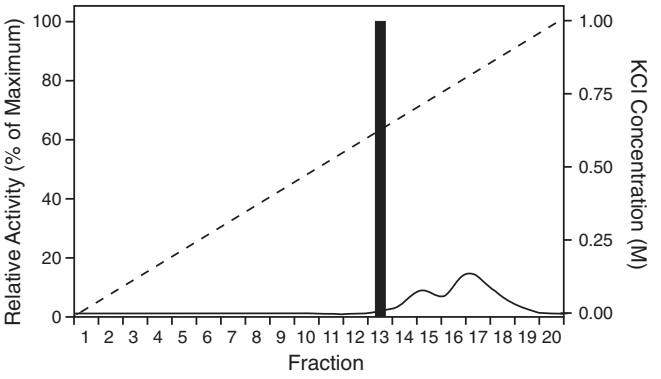

Fig. 6. Partial purification of curcuminoid synthase from turmeric leaves. Solid line: protein elution profile from strong anion exchange column, monitored at 280 nm. Dashed line: KCl concentration gradient used for elution of the protein. Solid bars show corresponding relative specific activity in each fraction. The results indicate maximum relative activity in fraction 13.

polyketide synthase activity, of any type. Even chalcone synthase activity was not detectable in the ginger extracts (this activity was also not detected for turmeric using this assay system).

Once we identified curcuminoid synthase activity in crude protein extracts from turmeric leaf, we began to develop methods to purify the corresponding protein(s). Separation of crude protein extract over a strong anion-exchange (Hi-trap Q) protein column led to the production of several semi-purified fractions, which were evaluated for enzyme activity. The results of curcuminoid synthase activity assays with these fractions indicated that only one fraction (#13, Fig. 6) contained this activity. Fig. 7 shows LC–MS analysis of curcuminoid synthase activity assay products, using fraction 13 from the protein fractionation and *p*-coumaroyl-CoA (**9**) and feruloyl-CoA (**10**) as substrates (plus malonyl-CoA). Although the activity was low, and only small amounts of products were formed, the three curcuminoids were detected as products of these assays, as supported by coelution with authentic standards, and by matching UV and MS spectra. Interestingly, all three activities (those forming curcumin (**1**), demethoxycurcumin (**4**), and bisdemethoxycurcumin (**3**)) coeluted. This suggests that there is either a single enzyme responsible for formation of the curcuminoids, or that if multiple enzymes do exist, that their properties are similar. Future work will seek to further characterize these enzymes.

2.5. Hydroxycinnamoyl-CoA thioesterases

As mentioned above (Section 2.3), we found what appeared to be hydroxycinnamoyl-CoA thioesterase (HTE) activities in all tissues in ginger and turmeric. In order to further characterize these putative hydroxycinnamoyl-CoA thioesterase activities in crude extracts from ginger and turmeric tissues, we developed assays that could detect the hydrolysis of *p*-coumaroyl-CoA (**9**), feruloyl-CoA (**10**), and caffeoyl-CoA (**12**) to the corresponding free

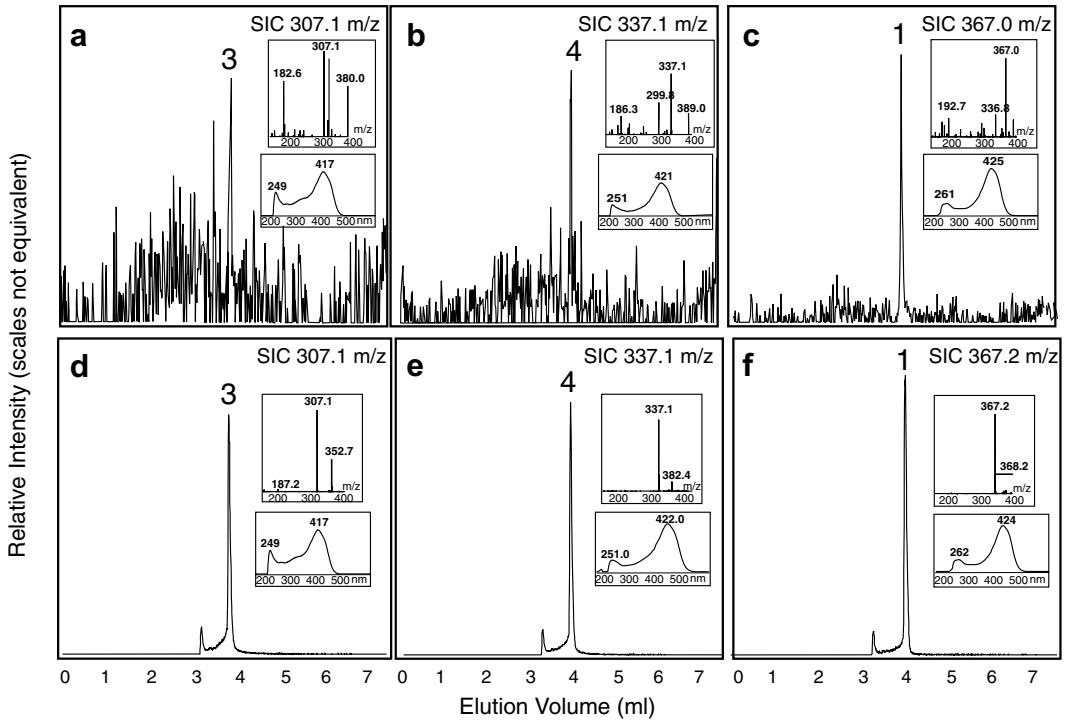

Fig. 7. LC–MS analysis of curcuminoid synthase assays using fraction 13 from turmeric leaf protein fractionation experiments (Fig. 6). (a)–(c), selected ion chromatograms (SICs) of eluting product peaks; (d)–(f), SICs of standard compounds. Insets: electrospray ionization negative mode mass spectra (upper) and UV chromatograms for selected peaks (lower). Peaks were identified as follows: bisdemethoxycurcumin (**3**), demethoxycurcumin (**4**), curcumin (**1**). These samples were analyzed using a short elution gradient (25 min gradient) to allow for faster sample analysis and because the products were analyzed by MS as well as UV.

acids and coenzyme A. Both compounds were observed to result from such assays. The results of these assays are shown in Fig. 8, showing how the free hydroxycinnamic acid products were analyzed. All the crude extracts from ginger and turmeric showed HTE activity with *p*-coumaroyl-CoA (**9**) and feruloyl-CoA (**10**). Interestingly, crude extracts from ginger showed significant HTE activity toward caffeoyl-CoA (**12**), whereas this latter activity was reduced in turmeric extracts (data not shown). Other enzyme activities have been reported from plants that are able to degrade hydroxycinnamoyl-CoA esters. For example, coenzyme A phosphohydrolases were first reported over 2 decades ago from barley, oat, wheat, maize, pea and tobacco (Negrel and Smith, 1984). However, these latter enzymes cleave the CoA moiety itself, leading to formation of hydroxycinnamoyl-4′-phosphopantetheines and AMP. Subsequent thioesterase activities then are able to cleave the resulting hydroxycinnamoyl-4′-phosphopantetheines to form the free acids. Because we believe that one of the products of our assays was in fact free coenzyme A, we are not convinced that such an activity was in fact present in our plant extracts. However, it could not be completely ruled out. Nevertheless, we did observe formation of the free hydroxycinnamic acids, indicating that thioesterase activity associated with degradation of the hydroxycinnamoyl-CoA esters was in fact present in these plant extracts. The nature of any intermediates in this conversion is yet to be determined.

Thus, it is possible that these HTE activities (perhaps in conjunction with CoA phosphohydrolase activities) may compete for the substrates (especially *p*-coumaroyl-CoA (**9**) and feruloyl-CoA (**10**)) of the potential curcuminoid or gingerol synthases. This finding is very significant, because such enzymatic activities may shunt phenylpropanoid pathway intermediates away from the production of curcuminoids and gingerols in certain cell types in these tissues, thereby potentially playing a regulatory role in the biosynthesis of these compounds. This hypothesis is further supported by the substrate specificity observed for these activities: where *p*-coumaroyl-CoA (**9**) and feruloyl-CoA (**10**), but not caffeoyl-CoA (**12**), were good substrates for the HTE activities in turmeric. At a more practical level, these hydroxycinnamoyl-CoA thioesterase activities appear to have confounded attempts to purify the enzyme responsible for curcuminoid synthase activity in turmeric and may have prevented detection of the enzyme activity responsible for formation of gingerols in ginger.

2.6. Conclusions

The results of this investigation lead to several significant findings regarding the biosynthesis of curcuminoids and gingerols (and of related compounds) in turmeric and ginger. First, because all tested enzymes of the central phenylpropanoid pathway (PAL, CST/CQT, CCOMT, COMT) are very active in the tissues that are involved in

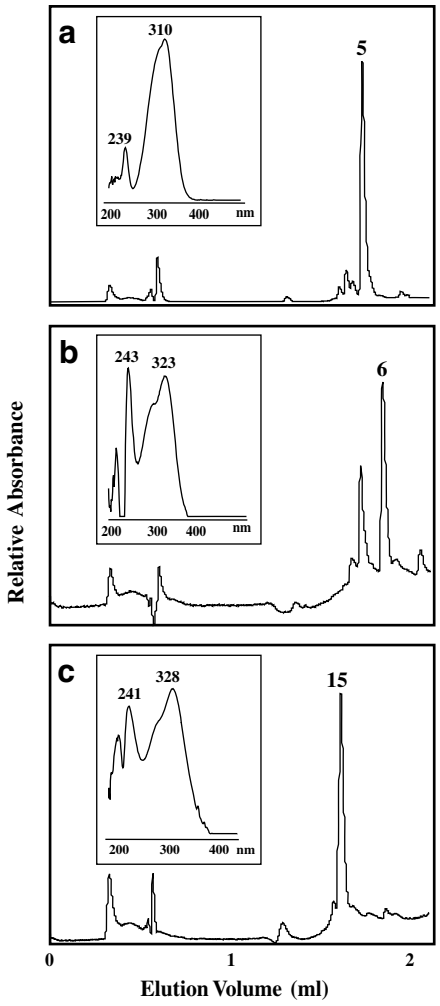

Fig. 8. Thioesterase activity is high in ginger and turmeric tissues. HPLC analyses of thioesterase assays using crude protein extracts from ginger leaf are shown in (a)–(c). Similar results were observed for extracts from other ginger tissues and from turmeric tissues. (a), assay with *p*-coumaroyl-CoA (**9**) as substrate. Elution trace is the selected UV absorbance chromatogram at 310 nm. (b), assay with feruloyl-CoA (**10**) as substrate. Elution trace is the selected UV absorbance chromatogram at 323 nm. (c), assay with caffeoyl-CoA (**12**) as substrate. Elution trace is the selected UV absorbance chromatogram at 328 nm. Insets are UV spectra of labeled peaks and match those of authentic standards. Peaks were identified as follows: **5**, *p*-coumaric acid; **6**, ferulic acid; **15**, caffeic acid.

the production of these compounds, we were not able to distinguish between the two proposed biosynthetic routes to these compounds illustrated in Fig. 1, i.e., we could not determine for certain whether the methoxyl functional groups on the aromatic rings are introduced prior to or after condensation of the precursors into the gingerol or curcuminoid backbone structures. Both pathways are still open possibilities. Second, we identified an enzymatic activity (curcuminoid synthase) capable of forming the curcuminoids in turmeric. This is the first report of an enzyme activity capable of producing these compounds. This activity required malonyl-CoA and phenylpropanoid pathway-derived hydroxycinnamoyl-CoA esters as substrates, suggesting that the corresponding protein is a polyketide synthase or an enzyme that is closely related. This activity could be the result of a single enzyme or of multiple enzymes in sequence. The recent identification of a type III polyketide synthase from *Wachendorfia thyrsiflora* that is able to produce a diketide (Brand et al., 2006), which could serve as intermediate to the curcuminoids, suggests that the latter may be the case. This will certainly be an interesting question to answer. The most puzzling result regarding the curcuminoid synthase activity was the requirement for both *p*-coumaroyl-CoA (**9**) and feruloyl-CoA (**10**) to be present in the assay in order for any product to be observed. Assays with only one of these two compounds, but containing all other assay components, did not yield detectable products. However, because of the low activity that we observed, this result may be due to assay conditions that are far from optimal. Because the gingerols and curcuminoids are produced in ginger and turmeric slowly throughout the growing season (Baranowski, 1986), the low activity that we measured is not unreasonable. Finally, identification of the thioesterase activities that hydrolyze very efficiently the hydroxycinnamoyl-CoA precursors to the curcuminoids and the gingerols leads to the conclusion that an alternative approach to isolation and characterization of curcuminoid synthase and gingerol synthase must be undertaken. Thus, a genomics-based approach may be the most efficient means to identify the genes that are responsible for direct formation of the curcuminoids and gingerols in these important medicinal plants.

3. Experimental

3.1. Plant material

Zingiber officinale Rosc. (ginger) and *C. longa* L. (turmeric) rhizomes were grown as previously described (Jiang et al., 2005a,b).

3.2. Reagents

All the solvent and reagents were molecular biology grade or reagent grade and were obtained from Sigma (St. Louis), Aldrich (Milwaukee, WI), or Fisher Scientific (Hampton, NH). [Methyl-^{14}C]*S*-adenosyl-L-methionine (60 mCi/mmol), [U-^{14}C]L-Phe (469 mCi/mmol), and [2-^{14}C]Malonyl CoA (52 mCi/mmol) were purchased from Amersham Biosciences (now GE Healthcare, Waukesha, WI).

3.3. Synthesis of CoA esters

Preparative enzymatic synthesis of hydroxycinnamoyl-CoA esters was carried out according to the method described by Beuerle and Pichersky (2002). Briefly, tobacco (*Nicotiana tabacum*) 4-coumarate:coenzyme A ligase (4CL) was expressed in *Escherichia coli* and purified using a Ni-

chelating column. Purified 4CL was used to synthesize *p*-coumaroyl-CoA (**9**), feruloyl-CoA (**10**) and caffeoyl-CoA (**12**). To synthesize the CoA esters, 3.3 mg hydroxycinnamic acid, 2 mg CoA, and 6.9 mg ATP were dissolved in a total volume of 10 ml of 50 mM Tris–HCl pH 7.5 buffer containing 2.5 mM $MgCl_2$. The reaction was started by the addition of 0.25 mg purified 4CL. After 5 h at room temperature, 6.9 mg ATP, 2 mg CoA, and 0.25 mg enzyme were again added and the reaction continued. After an additional 12 h, 0.4 g of ammonium acetate was added. The resulting hydroxycinnamoyl-CoA esters were purified using solid-phase extraction cartridges (1000 mg Chromabond C_{18} ec, Macherey-Nagel) preconditioned with consecutive washes of MeOH, ddH_2O, and 4% ammonium acetate solution (5 column vol. each). After the crude enzyme reaction was loaded on the preconditioned cartridge, the column was rinsed with 4% ammonium acetate solution until the flow through showed the absence of free CoA (determined by spectrophotometry). The hydroxycinnamoyl-CoA esters were recovered by elution with distilled H_2O. Fractions containing the hydroxycinnamoyl-CoA esters were identified by their UV spectrum and lyophilized overnight. Lyophilized hydroxycinnamoyl-CoA esters were stored for several months at −80 °C without noticeable degradation.

3.4. Instrumentation

LC–MS analysis was performed using a LCQ Advantage mass spectrometer (ThermoElectron) attached to Surveyor HPLC system containing system controller, quaternary MS pump, built-in solvent degassing unit, auto injection system, built-in column oven, and PDA detector with a 5 cm flow cell. HPLC separation was performed on a Supelco Discovery HS C_{18} column (15 cm × 2.1 mm i.d., 3.0-μm film thickness) protected by a C_{18} guard column. Electrospray ionization was achieved in negative ion mode by setting the source voltage to 5.25 kV and setting the capillary voltage to 31 V. The capillary temperature was set at 275 °C. All other parameters were based on autotuning with curcumin (**1**) as standard compound (Jiang et al., 2006a,b).

Radiochemical incorporation rates were determined by scintillation counting in a Beckman LS1800 liquid scintillation counter. The raw data (counts per minutes) were converted to picomoles of product produced per minutes, based on the specific activity of the substrate.

3.5. Enzyme extraction

Soluble protein extracts were made from fresh tissue (rhizome, leaves and shoots). The tissue was weighed and then ground under N_2(l) in a mortar. Ice-cold protein extraction buffer was added (1:3, w/v), consisting of 50 mM Bis–Tris [2-[bis(hydroxyethyl)amino]-2-(hydroxymethyl)-1-propane-1,3-diol]HCl, pH 8.0, 1 mM phenylmethanesulfonyl fluoride, 10 mM dithioerythritol, and 5 mM EDTA disodium salt, with 2% (w/v) polyvinylpolypyrrolidone added to the extraction mixture. After incubation on ice for 30 min, the crude protein extracts were obtained by centrifuging the ground mixture at 14,000 rpm for 30 min at 4 °C and transferring the clarified supernatant to a new tube. Protein extracts were used immediately or stored at −80 °C (with 10% glycerol added) until needed. The determination of protein concentration was carried out according to Bradford (1976) using IgG as standard.

3.6. Enzyme assays and products analysis

Extensive optimization was performed for each of the assays described below, where different buffer, salt, pH, temperature, incubation time, protein concentration, and protein extraction conditions were evaluated. For all assays, the best conditions that produced the highest activity are described.

3.6.1. Radiolabeled phenylalanine ammonia lyase (PAL) assays

PAL activity was determined using a method previously reported (Gang et al., 2001). This assay measures the conversion [U-^{14}C]L-Phe (**7**) into [U-^{14}C]cinnamic acid (**8**). In a 1.5-ml microfuge tube in a final assay volume of 50 μl were added: 0.1 M sodium borate, pH 8.8, [U-^{14}C]L-Phe (1 μl, 469 mCi/mmol, Amersham Biosciences), and 20 μl of protein extract diluted to 0.77 μg/μl and 0.84 μg/μl for ginger and turmeric tissues, respectively. Assays were initiated by addition of protein to the other reaction components and allowed to incubate at 37 °C for 5 h. Controls included assays containing all reaction components without protein added. To stop the reaction, 5 μl of 6 N HCl was added with mixing, followed by EtOAc (100 μl) to quantitatively extract the cinnamic acid (**8**) product. The microcentrifuge tubes were vortexed 30 s, followed by a 3-min centrifugation at 14,000 rpm in a microcentrifuge, and then 40 μl of the organic phase (on top and clear in color) were transferred a scintillation vial, followed by 3 ml of nonaqueous scintillation fluid (Bio-Safe II NA, Research products International, Mount Prospect, IL).

3.6.2. Acyltransferase assays

The acyltransferase assays were performed according the method described by Gang et al. (2002) with some modifications. The reactions consisted of 50 mM potassium phosphate buffer (pH 7), 0.5 mM ascorbate (Na^+ salt), 2.5 mM ATP, 1 mM CoA, 0.5 mM hydroxycinnamic acid (from 25 mM stock in ethanol), and 0.02 mg ml^{-1} purified recombinant 4CL. After incubation at room temperature for 4 h, the synthesis mixture was used directly as substrate/buffer for acyltransferase assays. These 50 μl assays were initiated by addition to 35 μl of hydroxycinnamoyl-CoA substrate/buffer mixture of 5 μl of 20 mM acyl acceptor (shikimic acid, quinic acid or 4-hydroxyphenyllactic acid), and 10 μl of protein solution (7.7 μg of protein for

ginger assays and 8.4 μg for turmeric). Controls included assays containing all reaction components without protein added, and assays without substrates added (only protein solution and buffer). The assays were incubated at 37 °C for 1 h and quenched by addition of 4 μl of 88% formic acid. After centrifugation to remove precipitated protein, 35 μl of remaining assay mixtures were transferred to vials for LC–MS analysis. The samples were analyzed by LC–MS using a gradient of 5 mM ammonium acetate–0.1% formic acid in H_2O (A) and CH_3CN (B) with a flow rate of 0.250 ml min^{-1}. The linear gradient consisted of 5% B in A for 2 min, followed by 5–100% B in 10 min, and 100% B for 2 min, followed by return to 5% B and re-equilibration for 10 min prior to injection of the next sample. Total run time was 25 min. The column temperature set to 40 °C. In-line UV spectra were obtained using an attached ThermoElectron photodiode array detector. Electrospray ionization was achieved in negative ion mode as described under Instrumentation above. Products were identified by HPLC elution volume, UV spectrum, and mass spectrum. Quantification of assay products was performed by calculating peak area of the molecular ion (M−H) peak.

3.6.3. Radiolabeled caffeic acid O-methyltranferase and caffeoyl-CoA O-methyltranferase assays

The assays for COMT and CCOMT activities were performed as previously described (Gang et al., 2001). The assays for COMT activity were prepared by adding to a 1.5 ml microcentrifuge tube: 5 μl of enzyme (3–5 μg of protein), 10 μl of assay buffer (250 mM Tris–HCl [pH 7.5]), 10 mM DTE, 1 μl of 50 mM caffeic acid (**15**) in EtOH, 1 μl [methyl-^{14}C]*S*-adenosyl-L-methionine (60 mCi/mmol in 10 mM ethanol: sulfuric acid), and H_2O (33 μl) to bring the assay to volume to 50 μl. Assays were incubated at 30 °C for 30 min in a heating block, after which 4 μl of 6 N HCl was added to stop the reaction. CCOMT activity assays were carried out in the same manner, with substitution of caffeoyl-CoA (**12**) for caffeic acid (**15**) as substrate, and, prior to acidification (with 16 μl of 6 N HCl) and extraction with EtOAc, the hydroxycinnamoyl-CoA esters were hydrolyzed by base treatment (10 μl of 10 N NaOH, heating at 70 °C for 10 min). Controls included assays containing all reaction components without protein added and an additional control for CCOMT activity assays where the assays were not base hydrolyzed prior to acidification. The radioactively labeled methylated products were extracted by addition of EtOAc (100 μl). Radiochemical incorporation rates were determined by scintillation counting of 50 μl of the EtOAc phase.

3.6.4. Evaluation of curcuminoid and gingerol synthase activities

Curcuminoid synthase and gingerol synthase activities were assayed in 50 mM Bis–Tris propane buffer pH 8, containing 50 μM malonyl-CoA, and three different combinations of the other substrates: (1) 100 μM *p*-coumaroyl-CoA (**9**); (2) 100 μM of feruloyl-CoA (**10**); or (3) 50 μM *p*-coumaroyl-CoA (**9**) and 50 μM of feruloyl-CoA (**10**). For assays performed with extracts from ginger tissues, we also added 50 μM hexanyl-CoA in all of the assays. Reactions were initiated by the addition of 1–90 μg crude or partially purified protein in a final assay volume of 200 μl. Controls included: assays containing all reaction components without protein added; assays without substrates added (only protein solution and buffer); and assays containing protein solution, malonyl-CoA and buffer (i.e., the hydroxycinnamoyl-CoA esters were excluded). The incubations were carried out at 28 °C for 8 h and were quenched by addition of 5 μl of 6 N HCl. In the case of the radiolabeled products, these were extracted by adding EtOAc (100 μl), vortexing for 30 s, and centrifuging at 14,000 rpm for 3 min. Radiochemical incorporation rates are determined by scintillation counting of 40 μl of the EtOAc phase. The assays performed using unlabeled substrates were subjected to two sequential extractions with EtOAc (0.2 ml) that were combined for each sample. The combined extracts were evaporated to dryness and re-dissolved in MeOH (25 μl). The resuspended extracts were analyzed by LC–MS as described above for acyltransferase assays, but which used either: a 5–100% B gradient that lasted 60 min instead of 10 min, for a total of 75 min per run (for assays with crude protein extracts from the plants); or a 5–100% B gradient that lasted 25 min instead of 10 min, for a total of 40 min per run (for assays with partially purified protein samples). Eluting compounds were identified by comparison of HPLC elution volume, UV spectra and mass spectra to those of known standard compounds.

3.6.5. Hydroxycinnamoyl-CoA thioesterase assays

Hydroxycinnamoyl-CoA thioesterase activity was measured in 50 mM Bis–Tris propane buffer pH 8, containing 100 μM of either *p*-coumaroyl-CoA (**9**), feruloyl-CoA (**10**) or caffeoyl-CoA (**12**) as substrate. Reactions were initiated by the addition of 1–90 μg crude or partially purified protein in a final assay volume of 200 μl. Controls included assays containing all reaction components without protein added and assays without substrates added (only protein solution and buffer). The incubations were carried out at 28 °C for up to 8 h and were quenched by addition of 4 μl of 88% formic acid. After centrifugation to remove precipitated protein, 100 μl of the remaining assay mixtures were transferred to vials for HPLC analysis. The samples were separated by HPLC using the same method as described above. Products were identified by UV absorbance spectrum and retention time match to authentic standards.

3.7. Partial purification of curcuminoid synthase enzyme from turmeric leaves

Crude protein extracts from turmeric leaves (up to 1 L extracts) were subjected to ammonium sulfate fractionation. At the end of this procedure, we obtained three frac-

tions, representing the 20%, 70% and 95% saturation fractions. Because the volume and protein concentration of fractions 1 (20% saturation) and 3 (95% saturation) were very low, after desalting using a PD-10 column, fraction 2 was used for further protein purification. This fraction was applied to a Hi-trap Q anion exchange column (1-ml bed volume) pre-equilibrated in buffer A (20 mM Bis–Tris [pH 8.0]). After washing to remove unbound proteins, proteins were eluted using a linear gradient in 30 min from 0 to 1 M KCl in buffer A. Flow rate: 1 ml min^{-1}. Fractions (1 ml) were desalted into buffer A and concentrated to 0.3 ml prior to assays for polyketide synthase activity.

3.8. Statistical analysis

All enzyme activity assays were performed in duplicate (technical replicates) using four biological replicates per tissue. The results are expressed a means ± SEM. Single factor ANOVA was performed (Microsoft Excel XP) in order to determine the statistical significance of intergroup comparisons. $P < 0.05$ was considered to be statistically significant, at the 95% confidence level.

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References

Austin, M.B., Noel, A.J.P., 2003. The chalcone synthase superfamily of type III polyketide synthases. Nat. Prod. Rep. 20, 79–110.

Austin, M.B., Izumikawa, M., Bowman, M.E., Udwary, D.W., Ferrer, J.L., Moore, B.S., Noel, J.P., 2004. Crystal structure of a bacterial type III polyketide synthase and enzymatic control of reactive polyketide intermediates. J. Biol. Chem. 279, 45162–45174.

Baranowski, J.D., 1986. Changes in solids, oleoresin, and (6)-gingerol content of ginger during growth in Hawaii. Hortscience 21, 145–146.

Beuerle, T., Pichersky, E., 2002. Enzymatic synthesis and purification of aromatic coenzyme A esters. Anal. Biochem. 302, 305–312.

Bradford, M.M., 1976. Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein–dye binding. Anal. Biochem. 72, 248–254.

Brand, S., Hölscher, D., Schierhorn, A., Svatos, A., Schröder, J., Schneider, B., 2006. A type III polyketide synthase from *Wachendorfia thyrsiflora* and its role in diarylheptanoid and phenylphenalenone biosynthesis. Planta 224, 413–428. doi:10.1007/s00425-006-0228-x.

Claeson, P., Tuchinda, P., Reutrakul, V., 1994. Naturally occurring 1,7-diarylheptanoids. J. Indian Chem. Soc. 71, 509–521.

Costa, M.A., Collins, R.E., Anterola, A.M., Cochrane, F.C., Davin, L.B., Lewis, N.G., 2003. An in silico assessment of gene function and organization of the phenylpropanoid pathway metabolic networks in *Arabidopsis thaliana* and limitations thereof. Phytochemistry 64, 1097–1112.

Denniff, P., Whiting, D.A., 1976. Biosynthesis of [6]-gingerol, pungent principle of *Zingiber officinale*. J. Chem. Soc. Chem. Commun., 711–712.

Denniff, P., Macleod, I., Whiting, D.A., 1980. Studies in the biosynthesis of [6]-gingerol, pungent principle of ginger (*Zingiber officinale*). J. Chem. Soc., Perkin Trans. 1, 2637–2644.

Gang, D.R., 2005. Evolution of flavors and scents. Annu. Rev. Plant Biol. 56, 301–325.

Gang, D.R., Wang, J., Dudareva, N., Nam, K.H., Simon, J.E., Lewinsohn, E., Pichersky, E., 2001. An investigation of the storage and biosynthesis of phenylpropenes in sweet basil. Plant Physiol. 125, 539–555.

Gang, D.R., Beuerle, T., Ullmann, P., Werck-Reichhart, D., Pichersky, E., 2002. Differential production of *meta* hydroxylated phenylpropanoids in sweet basil (*Ocimum basilicum* L.) peltate glandular trichomes and leaves is controlled by the activities of specific acyltransferases and hydroxylases. Plant Physiol. 130, 1536–1544.

Gomez-Vasquez, R., Day, R., Buschmann, H., Randles, S., Beeching, J.R., Cooper, R.M., 2004. Phenylpropanoids, phenylalanine ammonia lyase and peroxidases in elicitor-challenged cassava (*Manihot esculenta*) suspension cells and leaves. Ann. Bot. 94, 87–97.

Gowri, G., Paiva, N.L., Dixon, R.A., 1991. Stress responses in alfalfa (*Medicago sativa* L.). 12. Sequence-analysis of phenylalanine ammonia-lyase (PAL) cDNA clones and appearance of PAL transcripts in elicitor-treated cell-cultures and developing plants. Plant Mol. Biol. 17, 415–429.

Hoffmann, L., Maury, S., Martz, F., Geoffroy, P., Legrand, M., 2003. Purification, cloning, and properties of an acyltransferase controlling shikimate and quinate ester intermediates in phenylpropanoid metabolism. J. Biol. Chem. 278, 95–103.

Hoffmann, L., Besseau, S., Geoffroy, P., Ritzenthaler, C., Meyer, D., Lapierre, C., Pollet, B., Legrand, M., 2004. Silencing of hydroxycinnamoyl-coenzyme A shikimate/quinate hydroxycinnamoyl transferase affects phenylpropanoid biosynthesis. Plant Cell 16, 1446–1465.

Jez, J.M., Austin, M.B., Ferrer, J.L., Bowman, M.E., Schroder, J., Noel, J.P., 2000. Structural control of polyketide formation in plant-specific polyketide synthases. Chem. Biol. 7, 919–930.

Jez, J.M., Ferrer, J.L., Bowman, M.E., Austin, M.B., Schroder, J., Dixon, R.A., Noel, J.P., 2001. Structure and mechanism of chalcone synthase-like polyketide synthases. J. Ind. Microbiol. Biotechnol. 27, 393–398.

Jiang, H., Solyom, A., Timmermann, B.N., Gang, D.R., 2005a. Characterization of gingerol-related compounds in ginger rhizome (*Zingiber officinale* Rosc.) by high-performance liquid chromatography/electrospray ionization mass spectrometry. Rapid Commun. Mass Spectrom. 19, 2957–2964.

Jiang, H., Xie, Z., Koo, H., McLaughlin, S.P., Timmermann, B.N., Gang, D.R., 2005b. Metabolic profiling, phylogenetic analysis and anti-inflammatory investigation of Zingiber species: tools for authentication of ginger (*Zingiber officinale* Rosc.). Phytochemistry 67, 232–244. doi:10.1016/j.phytochem.2005.1008.1001.

Jiang, H.L., Somogyi, A., Jacobsen, N.E., Timmermann, B.N., Gang, D.R., 2006a. Analysis of curcuminoids by positive and negative electrospray ionization and tandem mass spectrometry. Rapid Commun. Mass Spectrom. 20, 1001–1012.

Jiang, H.L., Timmermann, B.N., Gang, D.R., 2006b. Use of liquid chromatography–electrospray ionization tandem mass spectrometry to identify diarylheptanoids in turmeric (*Curcuma longa* L.) rhizome. J. Chromatogr. A 1111, 21–31.

Joe, B., Vijaykumar, M., Lokesh, B.R., 2004. Biological properties of curcumin-cellular and molecular mechanisms of action. Crit. Rev. Food Sci. Nutri. 44, 97–111.

Koeduka, T., Fridman, E., Gang, D.R., Vassão, D.G., Jackson, B.L., Kish, C.M., Orlova, I., Spassova, S.M., Lewis, N.G., Noel, J.P., Baiga, T.J., Dudareva, N., Pichersky, E., 2006. Eugenol and isoeugenol, characteristic aromatic constituents of spices, are biosynthesized via

reduction of coniferyl alcohol esters. Proc. Natl. Acad. Sci. USA 103, 10128–10133.

Lofty, S., Fleuriet, A., Machiex, J.-J., 1992. Partial purification and characterization of hydroxycinnamoyl CoA:transferases from apple and date fruits. Phytochemistry 31, 767–772.

Macleod, I., Whiting, D.A., 1979. Stages in the biosynthesis of [6]-gingerol in *Zingiber officinale*. J. Chem. Soc., Chem. Commun., 1152–1153.

Mascolo, N., Jain, R., Jain, S.C., Capasso, F., 1989. Ethnopharmacological investigation of ginger (*Zingiber officinale*). J. Ethnopharmacol. 27, 129–140.

Miquel, J., Bernd, A., Sempere, J.M., Diaz-Alperi, J., Ramirez, A., 2002. The curcuma antioxidants: pharmacological effects and prospects for future clinical use. A review. Arch. Gerontol. Geriat. 34, 37–46.

Mustafa, T., Srivastava, K.C., Jensen, K.B., 1993. Drug development report. 9. Pharmacology of ginger, *Zingiber officinale*. J. Drug Dev. 6, 25–39.

Negrel, J., Smith, T.A., 1984. The phosphohydrolysis of hydroxycinnamoyl-coenzyme A thioesters in plant extracts. Phytochemistry 23, 31–34.

Noel, J.P., Austin, M.B., Bomati, E.K., 2005. Structure–function relationships in plant phenylpropanoid biosynthesis. Curr. Opin. Plant Biol. 8, 249–253.

Nugroho, L.H., Verberne, M.C., Verpoorte, R., 2002. Activities of enzymes involved in the phenylpropanoid pathway in constitutively salicylic acid-producing tobacco plants. Plant Physiol. Biochem. 40, 755–760.

Petersen, M., 1993. Purification of rosmarinic acid synthase from cell-cultures of *Coleus blumei* Benth. Planta 191, 18–22.

Rababah, T.M., Hettiarachchy, N.S., Horax, R., 2004. Total phenolics and antioxidant activities of fenugreek, green tea, black tea, grape seed, ginger, rosemary, gotu kola, and ginkgo extracts, vitamin E, and *tert*-butylhydroquinone. J. Agric. Food Chem. 52, 5183–5186.

Schröder, J., 1997. A family of plant-specific polyketide synthases: facts and predictions. Trends Plant Sci. 2, 373–378.

Sharan, M., Taguchi, G., Gonda, K., Jouke, T., Shimosaka, M., Hayashida, N., Okazaki, M., 1998. Effects of methyl jasmonate and elicitor on the activation of phenylalanine ammonia-lyase and the accumulation of scopoletin and scopolin in tobacco cell cultures. Plant Sci. 132, 13–19.

Ulbrich, B., Zenk, M.H., 1979. Partial purification and properties of *p*-hydroxycinnamoyl-CoA:quinate hydroxycinnamoyl transferase from higher plants. Phytochemistry 18, 929–933.

Ulbrich, B., Zenk, M.H., 1980. Partial purification and properties of *p*-hydroxycinnamoyl-CoA:shikimate-*p*-hydroxycinnamoyl transferase from higher plants. Phytochemistry 19, 1625–1629.

Vassão, D.G., Gang, D.R., Kueduka, T., Jackson, B., Pichersky, E., Davin, L.B., Lewis, N.G., 2006. Chavicol formation in sweet basil (*Ocimum basilicum*): cleavage of an esterified C9 hydroxyl group with NAD(P)H-dependent reduction. Org. Biomol. Chem. 4, 2733–2744. doi:10.1039/b605407b.