The Plant Journal (1998) **14**(3), 297–304

Acetyl-CoA:benzylalcohol acetyltransferase – an enzyme involved in floral scent production in *Clarkia breweri*

Natalia Dudareva[†], John C. D'Auria, Kyoung Hee Nam, Robert A. Raguso[‡] and Eran Pichersky*
Department of Biology, University of Michigan, Ann Arbor, MI 48109–1048, USA

Summary

Volatile esters impart distinct characteristics to the floral scent of many plants, and are important in attracting insect pollinators. They are also important flavor compounds in fruits. The ester benzylacetate is a major constituent of the floral scent of *Clarkia breweri*, an annual plant native to California. The enzyme acetyl-CoA:benzylalcohol acetyltransferase (BEAT), which catalyzes the formation of benzylacetate, has been purified from *C. breweri* petals, and a cDNA encoding this enzyme has been isolated and characterized. The sequence of the 433-residue BEAT protein does not show high similarity to any previously characterized protein, but a 35-residue region from position 135–163 has significant similarity (42–56% identity) to several proteins known or suspected to use an acyl-CoA substrate. *E. coli* cells expressing *C. breweri BEAT* produced enzymatically active protein, and also synthesized benzylacetate and secreted it into the medium. Of the different parts of the *C. breweri* flower, petals contained the majority of *BEAT* transcripts, and no *BEAT* mRNA was detected in leaves. The levels of *BEAT* mRNA in the petals increased as the bud matured, and peaked at anthesis, paralleling changes in BEAT activity. However, three days after anthesis, mRNA levels began a steep decline, whereas BEAT activity remained high for the next two days, suggesting that the BEAT protein is relatively stable.

Introduction

Volatile esters are major constituents in many floral aromas (Knudsen *et al.*, 1993). In the moth-pollinated flowers of *Clarkia breweri*, an annual native to the coastal range of California, benzylacetate constitutes up to 40% of the total scent output. Two other esters, benzylbenzoate and methylsalicylate, contribute about 5% each (Raguso and Pichersky, 1995). Since benzenoid esters, and in particular benzylacetate, are emitted by a majority of moth-pollinated flowers, it was inferred that the moth pollinators find such benzenoid esters attractive (Knudsen and Tollsten, 1993). Raguso *et al.* (1996) recently tested this hypothesis by exposing captured individuals of *Hyles lineata*, the nocturnal hawkmoth that pollinates *C. breweri*, to various volatiles and measuring the electro-antennogram responses. They showed that responses following exposure to benzylacetate and methylsalicylate were among the strongest recorded.

Although some floral volatile esters, such as methylsalicylate and methyljasmonate, have important functions in some vegetative processes as well (Farmer and Ryan, 1990; Shulaev *et al.*, 1997), the biochemical synthesis of such esters has received little attention. We have recently developed enzymatic assays to test for the activities of the biosynthetic enzymes SAMT (salicylic acid methyltransferase) and acetyl CoA:benzylalcohol acetyltransferase (BEAT), the enzymes that catalyze the formation, respectively, of methylsalicylate (from SAM (S-adenosyl-l-methionine) and salicylate) and benzylacetate (from benzylalcohol and acetyl-CoA) (Dudareva *et al.*, 1998). We showed that the activities of these two enzymes in flowers of *C. breweri* are highest in petals and varied with the developmental stages of the flower.

Here, we report the purification of BEAT from petals of *C. breweri* flowers and the isolation of a cDNA clone encoding this enzyme. The sequence of the protein encoded by *BEAT* cDNA does not show extensive similarity to any other known protein sequences, but a short segment within it has significant similarity to short segments in other proteins known or hypothesized to use an acyl-CoA substrate. We have also examined *BEAT* mRNA levels in the different floral parts and throughout the development of the flower, and show that in general *BEAT* mRNA levels are positively correlated with BEAT enzymatic activity levels throughout bud development and anthesis.

Results

BEAT purification

Previous work has shown that in *C. breweri* flowers, BEAT is concentrated in the petals, with other floral parts containing lesser amounts (Dudareva *et al.*, 1998). We have also shown previously that some *C. breweri* plants emit more benzylacetate than others (Raguso and Pichersky, 1995). We therefore began our purification with petal material only,

Received 30 September 1997; revised 3 February 1998; accepted 4 February 1998.
*For correspondence (fax +1 734 647 0884; e-mail lelx@umich.edu).
[†]Present address: Horticulture Department, Purdue University, West Lafayette, IN 47907, USA.
[‡]Present address: Arizona Research Laboratories, Division of Neurobiology, Box 210077, University of Arizona, Tucson, AZ 85721–0077, USA.

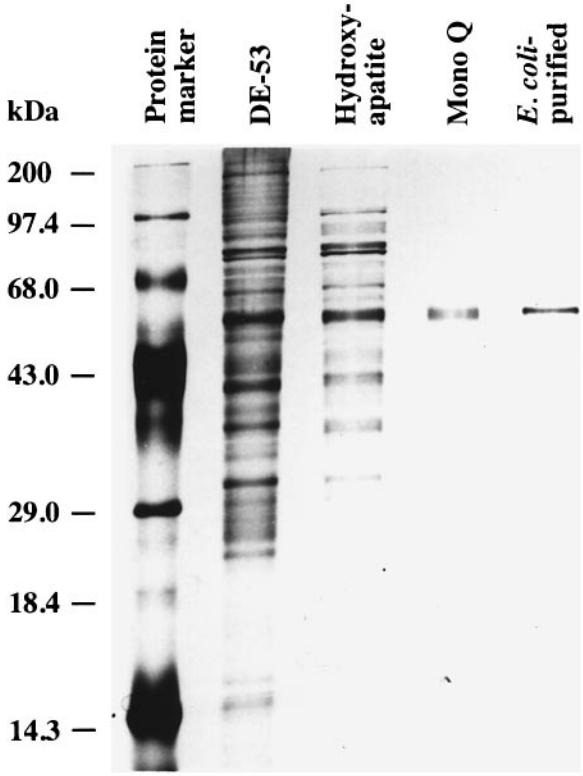

Figure 1. Purification of BEAT from petal tissue of *C. breweri* and from *E. coli* cells expressing plant BEAT.
Samples of peak fraction from successive purification steps were run on 13% SDS–PAGE. Protein bands were visualized by silver staining. The lane labeled 'Mono-Q' was loaded with approximately 1 μg protein from the fraction containing peak activity eluting from the Mono-Q column. The lane labeled '*E. coli*-purified' was loaded with 1 μg protein purified from inclusion bodies by lysis, centrifugation and washing (Betts *et al.*, 1994).

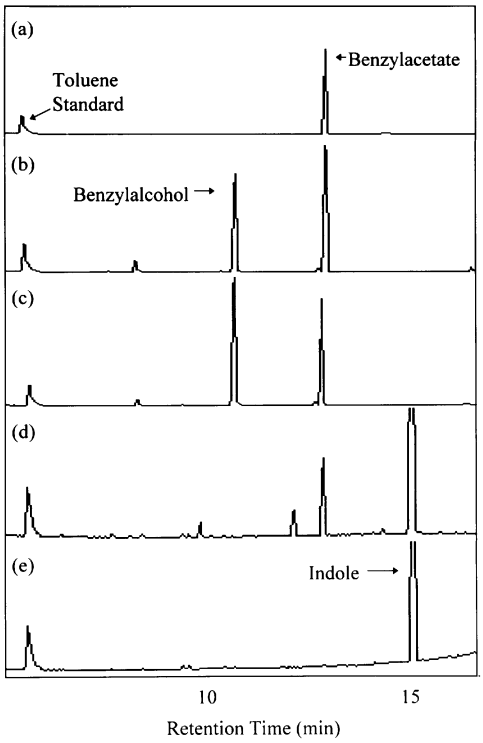

Figure 2. GC analysis of BEAT activity *in vitro* and *in vivo*.
(a) Benzylacetate standard.
(b) Analysis of product formed *in vitro* in a reaction catalyzed by plant-purified enzyme.
(c) Analysis of product formed *in vitro* in a reaction catalyzed by the plant enzyme purified from *E. coli* cells expressing *BEAT* cDNA.
(d) Analysis of spent media in which *E. coli* cells expressing *BEAT* cDNA grew.
(e) Analysis of spent media in which *E. coli* cells which did not possess *BEAT* cDNA grew.
Toluene was added to all samples for quantification purpose. Peaks were analyzed by MS for identification.

using a *C. breweri* line that has high BEAT activity (317 fkat mg^{-1} petal FW). Starting with 90 ml of crude petal extract, the enzyme was successively purified through a DE53 column, hydroxyapatite column, and an FPLC Mono-Q column (Figure 1). The yield was 55–65% (by activity) on each column, with a final cumulative yield of 20%.

After Mono-Q chromatography, we obtained approximately 100 μg of a protein with apparent molecular mass of 58 kDa and little or no additional proteins (Figure 1). This protein was active in catalyzing the formation of benzylacetate from benzylalcohol and acetyl CoA (Figure 2b), with a specific activity of 2.4 nkat mg^{-1} protein (nkat = nanomoles product per second). We also tested the enzyme with several naturally occurring and artificial substrates (Table 1). It was not active with the benzylalcohol derivatives 2-hydroxybenzylalcohol, 3-hydroxybenzylalcohol and 4-hydroxybenzylalcohol. In addition, it had no activity with linalool, an acyclic monoterpene alcohol which is another major component of the *C. breweri* scent. However, it had some activity with cinnamylalcohol, 2-phenylethanol, and 2-naphthalene-ethanol.

Protein sequence determination and isolation and characterization of a BEAT *cDNA*

N-terminal sequencing of this protein was unsuccessful due to a blocked N-terminus. We therefore prepared several peptides by CNBr cleavage of 10 μg of purified BEAT and determined a partial sequence from three of them (Figure 3). The peptide sequences were used to construct oligonucleotides for PCR amplification of a 200-nucleotide fragment of the *BEAT* coding sequence (see Experimental procedures). This fragment was in turn used to screen a *C. breweri* floral cDNA library (Dudareva *et al.*, 1996). Several cDNA clones, all containing the same open reading frame of 433 codons (starting with a methionine codon), were isolated. The sequence of the longest cDNA clone, together with the sequence of the encoded protein, is shown in Figure 3. The encoded protein contains all the peptide sequences determined experimentally (underlined sequences). The *BEAT* cDNA clone contains a total of 1564 nucleotides, with 115 nucleotides at the 5′ non-coding

Table 1. Relative activity of *C. breweri* petal BEAT with benzylalcohol and related substrates

Substrate name	Substrate formula	Plant-purified enzyme[a]	*E. coli*-expressed enzyme[a]
Benzylalcohol	$C_6H_5CH_2OH$	100%	100%
Cinnamylalcohol	$C_6H_5CHCHCH_2OH$	88%	97%
2-Naphthaleneethanol	$C_{10}H_7CH_2CH_2OH$	33%	57%
2-Phenylethanol	C_6H_5CH (OH)CH3	9%	28%
2-Hydroxybenzylalcohol	2-OH (C_6H_5)CH_2OH	<5%	<5%
3-Hydroxybenzylalcohol	3-OH (C_6H_5)CH_2OH	<5%	<5%
4-Hydroxybenzylalcohol	4-OH (C_6H_5)CH_2OH	<5%	<5%
3,7-dimethyl-3-hydroxy-1,6-octadiene (linalool)	$(CH_3)_2CCHCH_2CH_2C$ (CH_3) (OH)$CHCH_2$	<5%	<5%

[a]Values are averages of 2–4 independent measurements. All substrates were tested at 1 mM concentration. The activity level of BEAT with benzylalcohol was set arbitrarily at 100%.

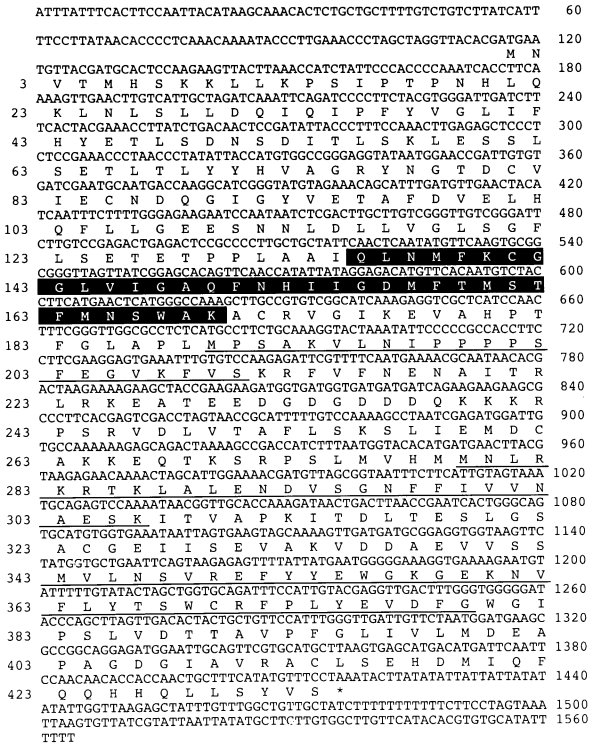

Figure 3. Nucleotide sequence of *C. breweri BEAT* cDNA.
The predicted protein sequence is shown below the nucleotide sequence. Numbers on the right refer to the nucleotide sequence; numbers on the left refer to the protein sequence. Peptide sequences obtained experimentally are underlined. The conserved motif found in other acyl-CoA-binding proteins is shown in black boxes with white letters. The EMBL accession number of this sequence is AF043464.

region and 150 nucleotides at the 3′ non-coding region. That the methionine codon at position 116–118 is the initiating codon is indicated by the presence of nonsense codons in-frame upstream of it. Primer extension experiments (data not shown) also indicate that this cDNA clone is missing only 10–13 nucleotides from its 5′ end.

The complete sequence of the BEAT protein does not share extensive sequence similarity to any sequences of enzymatically characterized proteins currently in the databanks. However, a small region of 35 residues (amino acid positions 135–163, shown in white text on a black background in Figure 3) has a significant similarity to several proteins known or hypothesized to use an acyl-CoA substrate. For example, this region is 56% identical to the corresponding region in the CER2 protein of *Arabidopsis* (Negruk *et al.*, 1996) and 45% identical to its homolog Glossy2 from maize (Tacke *et al.*, 1995), both believed to be involved in the elongation step of C_{30} to C_{32} in the biosynthesis of cuticular wax and which therefore might interact with acetyl-CoA. (Recently, however, CER2 has been reported to be localized to the nucleus (Xia *et al.*, 1997), although this observation, if confirmed, does not preclude CER2 from being involved in an acyl transfer reaction.) Region 135–163 in BEAT is also 42% identical to the corresponding region in hydroxycinnamoyl/benzoyl-CoA:anthranilate hydroxycinnamoyl/benzoyltransferase (HCBT) from carnation (Yang *et al.*, 1997), an acyl transferase that uses benzoyl-CoA or hydroxycinnamoyl-CoA as substrates and transfers them to anthranilate, a compound that also contains a benzyl ring. In addition, this region shows 40–50% identity to corresponding regions in several unidentified *Arabidopsis* genes whose sequences have recently been determined through the *Arabidopsis* genome project (e.g. accession number AC000103), and to an unidentified cDNA clone, MEL2, obtained from a cDNA library of ripening melon fruit (Aggelis *et al.*, 1997).

The C-terminus of BEAT shares a very limited sequence similarity with the corresponding region in HCBT, centered around the pentapeptide DFGWG found in both proteins (residues 377–381 in BEAT, 392–396 in HCBT). Also, a search of the EST databank revealed that the N-terminal 50 residues of *C. breweri* BEAT protein share approximately 45% identity with the corresponding region of one unidentified *Arabidopsis* cDNA clone, represented by several ESTs (e.g. accession number R83945), but for which only a limited sequence has been determined so far.

The protein encoded by *BEAT* has a predicted molecular mass of 48.2 kDa, considerably smaller than the 58 kDa

apparent molecular mass calculated from its migration in SDS–PAGE gels. Such discrepancies are not uncommon (e.g. Oh-oka *et al.*, 1986), and may be caused in this case, at least in part, by an unusual concentration of charged residues in positions 222–247. In this region of 26 amino acids, nine residues are negatively charged and eight residues are positively charged, with eight of the negatively charged amino acids clustered in the center of this short segment. Since the BEAT protein produced in the *E. coli* expression system (see below) has the same apparent molecular mass on SDS–PAGE as BEAT purified from petals, it is unlikely that post-translational modifications are responsible for the increase in apparent molecular mass.

Expression of BEAT *cDNA in* E. coli

To further verify that the cDNA we isolated encodes BEAT, we cloned it into the pET-T7(11a) expression vector, transformed *E. coli* cells with the recombinant plasmid, and induced the expression of this foreign gene. *E. coli* expressing *C. breweri BEAT* contained large amounts of a protein with apparent molecular mass on SDS–PAGE of 58 kDa, the same as the plant-purified BEAT, both in insoluble inclusion bodies (Figure 1) and in soluble form (data not shown). This protein had a very similar enzymatic activity to that of plant BEAT (Figure 2c and Table 1). *E. coli* cells harboring a pET-T7(11a) plasmid without the *BEAT* coding region did not have any BEAT activity, nor did bacteria lacking this plasmid entirely (data not shown). Furthermore, the spent medium in which *E. coli* cells expressing *BEAT* grew contained significant amounts of benzylacetate (2 μg ml^{-1}) (Figure 2d), whereas bacterial cells not containing the recombinant plasmid did not (Figure 2e). Both types of cultures produced copious amounts of indole. It should be noted that the cells were grown in regular LB medium (containing the antibiotic ampicillin) without the addition of any benzylalcohol.

Southern blot analysis

We performed Southern blot analysis with *C. breweri* genomic DNA digested with five different restriction enzymes, using the *Eco*RI fragment of *BEAT* cDNA from nucleotide 1–1150 as a probe. A single band was observed with *Bam*HI, *Eco*RV and *Xba*I digestions (Figure 4), suggesting that there is a single *BEAT* gene locus in the *C. breweri* genome. Several bands were observed when the DNA was digested with *Eco*RI and *Hin*dIII. The coding region of *BEAT* has one *Hin*dIII site spanning the region covered by the probe, and additional *Eco*RI and *Hin*dIII sites may occur in introns.

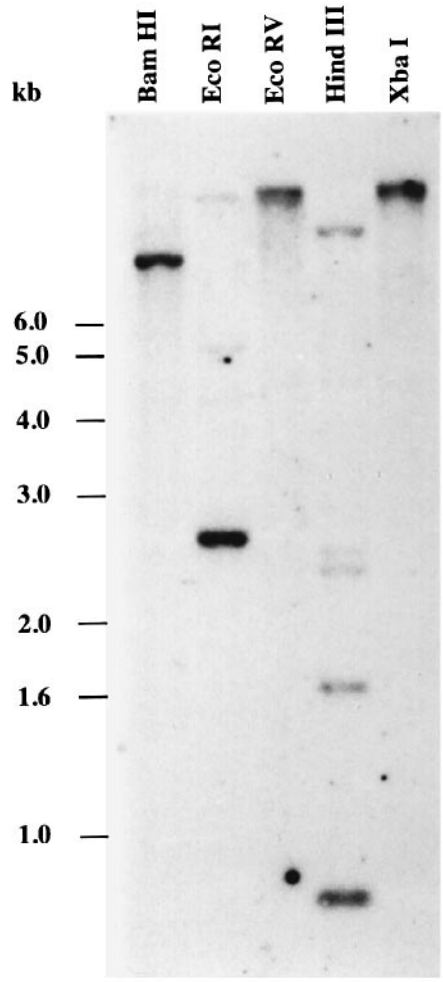

Figure 4. Southern blot analysis.
A blot of *C. breweri* genomic DNA digested with five restriction enzymes and probed with a probe derived from nucleotides 1–1150 of *BEAT* cDNA.

Tissue-specific expression of BEAT

The levels of *BEAT* mRNA in different parts of the *C. breweri* flower on the day of anthesis, when peak benzylacetate emission occurs (Dudareva *et al.*, 1998), were examined. Isolation of total RNA from leaves, style and stamens resulted in similar yields of approximately 0.2 μg mg^{-1} FW, with the yield from the stigma being 0.4 μg mg^{-1} FW, and the yields from the petals and sepals 0.1 μg mg^{-1} FW. Samples from different floral parts, each containing 7 μg of total RNA, were analyzed by Northern blots (Figure 5). The amount of 18S rRNA in each sample was monitored by hybridization with an 18S rDNA probe (Dudareva *et al.*, 1996). Both when normalized to equal 18S rRNA levels (an approximation for amount per cell) or when compared per mg fresh weight of tissue, highest levels of *BEAT* message were observed in petals, followed by style, sepals and stamens. However, stylar tissue appears to have a substantial level of *BEAT* mRNA, close to that of the petals, when RNA samples from equal amounts of fresh weight of

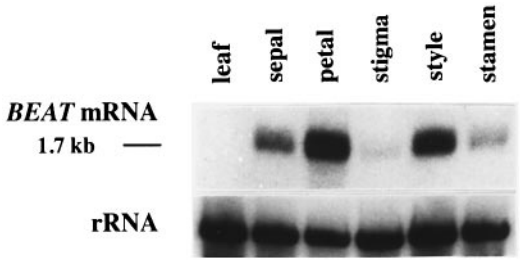

Figure 5. Expression of *BEAT* in flower parts.
Northern blot hybridization of RNA from different tissues. Each lane contained 7 μg total RNA. The blot was rehybridized with an 18S rDNA probe to standardize samples.

tissue are compared. Very low levels of *BEAT* mRNA were observed in the stigma, and none in leaves. These mRNA levels broadly parallel the BEAT activity profile previously obtained, in which highest enzyme activity is found in petals and none in the leaves (Dudareva *et al.*, 1998). However, stylar tissue is an exception, since its *BEAT* mRNA levels seem unproportionally high relative to its levels of BEAT enzymatic activity. At present, we have no explanation for this discrepancy.

BEAT expression in petals during flower development

Since petals constitute slightly over 50% the total mass of the *C. breweri* flower, excluding the unscented hypanthium (Pichersky *et al.*, 1994), and BEAT enzyme activity per fresh weight is highest in petals, it is not surprising that 90% of the total BEAT activity is found in petals (Dudareva *et al.*, 1998). *BEAT* mRNA levels per mg fresh weight are also clearly highest in the petals (Figure 5), and, because of the total mass of the petals (which is 6.5-fold greater than that of the style, the organ with the next highest levels of *BEAT* mRNA), the bulk of the *BEAT* transcripts in the flower must also be found in the petals. We therefore examined the steady-state levels of *BEAT* mRNA in petals during the lifespan of the flower. Since *C. breweri* flowers usually last only 4–6 days, even when not pollinated, we analyzed samples (3 μg each) of total RNA extracted from petals of maturing buds and from petals of open flowers 1–4 days after anthesis (Figure 6a). RNA yields per mg fresh weight of petals of all ages were very similar, and the amount of 18S rRNA in each sample was monitored by hybridization with an 18S rDNA probe. A plot of the variation in relative amount of *BEAT* mRNA during the lifespan of the petal, normalized to equal amount of 18S rRNA, is given in Figure 6(b).

BEAT mRNA was first detected in petal cells just before the flower opened, and its level increased until it peaked on the day of anthesis. After the second day post-anthesis, mRNA levels declined sharply. In contrast, we have previously shown (Dudareva *et al.*, 1998) that BEAT specific activity continued to increase after day 2 and only began

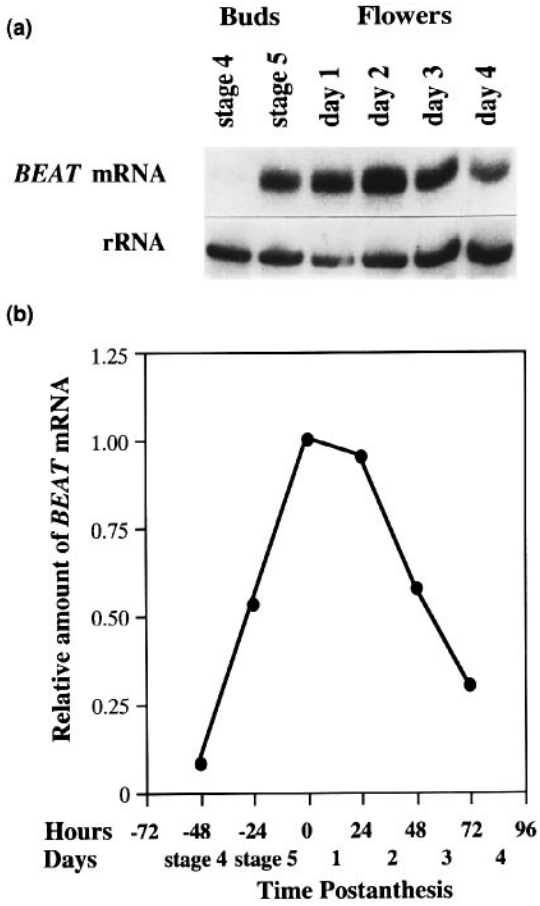

Figure 6. Expression of *BEAT* in petals throughout the lifespan of the flower.
(a) Northern blot hybridization with RNA from petals at different stages of development. Total RNA (3 μg) was obtained from petals of different stages of development.
(b) Plot of the variation in levels of petal *BEAT* mRNA. Values were obtained by scanning blots with a phospho-imager. Each point is the average of two experiments (including the one shown in (a)) and values were corrected by normalizing for 18S RNA levels.

to decrease on day 5 after anthesis. This observation suggests that the BEAT protein is relatively stable in petal tissue.

Discussion

Characterization of acetyl CoA:benzylalcohol acetyltransferase

Although volatile esters endow the floral scent of many flowers with their distinct character and are important in attracting insect pollinators, their biosynthesis has not been much investigated. Benzylacetate in particular is one of the most commonly found esters in moth-pollinated flowers (Knudsen and Tollsten, 1993), and is often found in the aromas of other flowers as well (Knudsen *et al.*, 1993; Van Dort *et al.*, 1993; Watanabe *et al.*, 1993). In addition, benzylacetate and other benzylalcohol esters are

important flavorants in ripe fruits and as ingredients in perfumes (Croteau, 1977; Croteau and Karp, 1991; Watanabe *et al.*, 1993).

In the present study, we have purified the enzyme BEAT, which catalyzes the formation of the ester benzylacetate, a major constituent of the floral scent of *C. breweri* (Figure 1). An interesting observation is that, at least *in vitro*, BEAT could transfer the acetyl group, with varying levels of efficiency, to several alcohol substrates which contain a benzyl ring or even a naphthyl ring system (the list of substrates tested so far is of course limited). Among enzymes of secondary metabolism, it is not uncommon for an enzyme to have activity with several substrates (Wang and Pichersky, 1998). A very similar situation occurs with the acyltransferase HCBT, which has been shown to catalyze the formation *in vivo* of *N*-benzoylanthranilate from benzoyl-CoA and anthranilate (Yang *et al.*, 1997). *In vitro*, however, HCBT has even higher catalytic activities with cinnamoyl-CoA or 4-coumaroyl-CoA, the transfer of which to anthranilate results in products that are not found *in vivo* (Yang *et al.*, 1997).

We have also isolated and characterized a cDNA encoding BEAT (Figure 3). The protein encoded by *BEAT* cDNA has 433 amino acids, and its sequence does not show high similarity to any previously characterized protein. It does have a small region of 35 residues (residues 135–163, see Figure 3) with 40–55% identity to a corresponding region in several proteins known or hypothesized to catalyze the transfer of an acyl group from an acyl-CoA substrate. Besides HCBT from carnation, and CER2 from *Arabidopsis* and its homolog GLOSSY2 from maize, the list of proteins with this 35-residue sequence motif includes an unidentified protein, MEL2, from ripening melon fruit, and several *Arabidopsis* proteins whose sequence was determined through the *Arabidopsis* genome project. This 35-residue motif is likely to be involved in the interaction between the protein and its acyl-CoA substrate and/or in the transfer reaction. The additional, limited sequence similarity between BEAT and HCBT at the C-terminus might be due to the fact that both BEAT and HCBT bind substrates with a benzyl ring.

It is interesting that all these proteins have a length in the range of 420–460 amino acids, and that the 35-residue motif occurs at approximately the same position along the polypeptide chain in all of them. These proteins might all belong to a class of acyl transferases, whose functions are diverse. For example, it is known that esters are among the aroma constituents of ripening melon (Yabumoto *et al.*, 1977), and MEL2 may thus be involved in catalyzing the formation of one of these esters. Since acylation is a common reaction, the several *Arabidopsis* proteins with the 35-residue motif whose sequences have already been deposited in the databanks may represent only a small fraction of the total number of acyl transferases present in plant genomes.

Expression of BEAT *cDNA in* E. coli

E. coli cells growing in a standard LB medium and expressing *BEAT* not only produced enzymatically active protein, but also synthesized benzylacetate and secreted it into the medium. We are not aware of any reports concerning the synthesis of benzylalcohol in *E. coli*. However, our results clearly indicate that benzylalcohol is synthesized by this bacterium, perhaps only as a pathway intermediate which the over-expressed BEAT enzyme is capable of scavenging into benzylacetate synthesis. The medium in which the bacteria expressing *BEAT* grew also contained, in addition to benzylacetate, a few other unidentified volatiles in lesser amounts (e.g. the minor peak at 12 min, Figure 2d) which we could not definitely identify but which MS analysis indicated contained an acetyl group. It appears that the over-expression of *BEAT* in the bacterial cells caused acetylation of other compounds, perhaps structurally similar to benzylalcohol. This is not surprising in view of the less-than-strict substrate specificity of the enzyme (Table 1).

Tissue- and developmental stage-specific BEAT *expression*

Due to the several-fold variation in levels of rRNA per cell and in yield of total RNA per mg fresh weight in preparations from different tissues, it is difficult to make rigorous quantitative comparisons among mRNA levels from different tissues. However, our results clearly demonstrate that *BEAT* is not expressed at detectable levels in leaves, and that among floral organs, the bulk of the *BEAT* mRNA transcripts are found in the petals. When *BEAT* mRNA steady-state levels from petals at different stages are normalized to equal 18S RNA levels and then compared with each other (same-tissue normalization is more likely to be valid), it is also clear that BEAT mRNA levels in petal cells peak 1–2 days post-anthesis. This result is similar to those previously described for other genes encoding scent biosynthetic enzymes in the *C. breweri* flower, whose mRNA levels also peak at or around anthesis (Dudareva *et al.*, 1996; Wang *et al.*, 1997), suggesting a common regulatory mechanism.

Experimental procedures

Plant material and growth conditions

Details of the construction of true-breeding *C. breweri* stocks and growing conditions are as described in Raguso and Pichersky (1995).

BEAT enzyme assays and product analysis

Details of the extraction buffers and assay conditions were as described in Dudareva *et al.* (1998). Throughout the purification procedure, enzyme activity was assayed in a total volume of 50 μl, and the radioactive product was quantified by hexane extraction and scintillation counting as previously described (Dudareva *et al.*, 1998). Product verification was performed by radio-TLC and by GC–MS. For the GC–MS analysis, the enzymatic reaction was scaled up to 1 ml final volume of solution containing 50 mM Tris–HCl pH 7.5, 3 mM 2-mercaptoethanol, 0.5 mM acetyl-CoA (non-radioactive), 1 mM benzylalcohol, and 30 μl of purified BEAT from either *C. breweri* petals or the *E. coli* expression system (≈ 0.5–1.2 μg). The reaction was carried out for 1 h, and product was extracted with 1 ml hexane, concentrated, and injected into the GC–MS system.

Enzyme purification

BEAT was purified in a series of chromatographic steps involving a DEAE anion exchange column (DE53, Whatman), an hydroxyapatite column (Bio-Rad), and another anion exchange column, Mono-Q, on Pharmacia's FPLC system. Running conditions were identical to the ones described in Pichersky *et al.* (1995). Enzyme eluted from DE53 in the 0.2–0.3 M KCl range, from the hydroxyapatite column in the 0.13–0.18 M sodium phosphate range, and from the Mono-Q column in the 0.14–0.17 M KCl range.

Protein sequence determination

Sequence analysis was performed on peptides produced from cyanogen bromide cleavage of purified BEAT as previously described (Dudareva *et al.*, 1996).

cDNA isolation and characterization

For PCR-amplification of fragments of *BEAT* cDNA, we synthesized several pairs of degenerate primers based on the peptide sequences. PCR experiments were performed as previously described using the *C. breweri* flower cDNA library as the target (Dudareva *et al.*, 1996). PCR experiments using the 20-mer oligonucleotide 5′-GG (GATC)AA (TC)TT (TC)TT (TC)AT (CTA)GT (GATC)GT-3′, designed based on the peptide sequence GNFFIVV (sense orientation), and the 23-mer oligonucleotide 5′-TT (TC)TT (TC)TC (GATC)CC (CT)TT (GATC)CCCCA (CT)TC-3′, based on the peptide sequence EWGKGEKN (antisense orientation), gave a product of 200 nucleotides. This fragment was eluted from a 2% agarose gel, labeled, and used to screen a *C. breweri* floral cDNA library. Several clones were isolated and characterized by restriction enzyme digests and by sequencing. All proved to encode the same protein. The sequence of the longest clone was completely determined on both strands.

RNA isolation and RNA gel-blot analysis

Total RNA from floral tissues and petals at different stages of development was isolated and analyzed as described in Dudareva *et al.* (1996) and Wang *et al.* (1997). A 1.15 kb *Eco*RI fragment containing the 5′ end of *BEAT* cDNA was used as a probe in Northern blot analysis. Total RNA levels were initially measured by spectrophotometry. For determination of tissue specificity, 7 μg of total RNA was loaded in each lane, and for time-course assays, 3 μg of total RNA was loaded. Hybridization signals were counted in a phospho-imager, and *BEAT* mRNA transcript levels were normalized to 18S RNA levels for graph presentation (Dudareva *et al.*, 1996).

DNA gel blot analysis

Genomic DNA (10 μg) was digested with different restriction enzymes under standard conditions, separated on 0.7% agarose gel and transferred to nitrocellulose membrane (BioTrace^R NT). Hybridization was carried out in 6× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate), 5× Denhardt's solution (0.1% BSA, 0.1% Ficoll 400 and 0.1% PVP 360), 0.1% SDS and 1 mM EDTA at 65°C. Blots were washed at 65°C twice with 5× SSC and twice with 2× SSC and autoradiographed with an intensifying screen on Kodak XAR film at –80°C.

Expression of BEAT *in* E. coli

The coding region of *BEAT* was amplified with the forward 25-mer oligonucleotide 5′-CCATATGAATGTTACGATGCACTCC-3′, and the backward 34-mer oligonucleotide 5′-TGGATCCTTAGGAAACG-TATGAAAGCAGTTGGTG-3′. The forward oligonucleotide introduced an *Nde*I site at the initiating methionine ATG codon. The backward nucleotide introduced a *Bam*HI site downstream of the stop codon, and also eliminated the single original *Nde*I site in *BEAT* cDNA which occurs 6–11 nucleotides upstream of the stop codon. The elimination of this *Nde*I site was accomplished by changing the original T at the third position of the codon for tyrosine 431 to C, thus maintaining a tyrosine codon. The PCR-amplified 1.3 kb fragment was cloned into the *Nde*I–*Bam*HI site of the expression vector pET-T7(11a), the resulting plasmid was transferred into *E. coli* BL21 cells, and the expression of *BEAT* cDNA was induced by the addition of 0.4 mM IPTG at A_{600} of 0.5 with 3 h incubation at 37°C (Wang *et al.*, 1997). Cell cultures (25 ml) were harvested by centrifugation, resuspended in 100 mM NaCl, 50 mM Tris–HCl pH 8.0, 1 mM EDTA and 1 mM PMSF, and sonicated on ice with a microtip probe for four time intervals of 30 sec each. After spinning this lysate at 12 000 ***g*** for 5 min, soluble and insoluble fractions were assayed for enzyme activity and analyzed by SDS–PAGE. The *E. coli*-expressed BEAT protein was further purified by DE53 anion exchange and Mono-Q chromatography.

Extraction of benzylacetate from E. coli *cells and GC–MS analysis*

BL21 cells expressing *BEAT* and those without the pET-T7(11a)–*BEAT* plasmid were grown under conditions described above. After harvesting the cells for protein purification, the spent medium (25 ml) was extracted with 5 ml of hexane, and the hexane phase removed, placed in a glass tube, and reduced to 0.2 ml by passing N_2 at the opening of the tube. The samples (3 μl of the hexane concentrate) were analyzed by GC–MS.

Acknowledgements

This research was funded by grant IBN-9417582 from the National Science Foundation to E.P. R.A.R. was supported in part by an NIH/Genetics Training Grant fellowship.

References

Aggelis, A., John, I., Karvouni, Z. and Grierson, G. (1997) Characterization of two cDNA clones for mRNAs expressed during ripening of melon (*Cucumis melo* L.) fruits. *Plant Mol. Biol.* **33**, 313–322.

Betts, S.D., Hachigan, T.M., Pichersky, E. and Yocum, C.F. (1994) Reconstitution of the spinach oxygen-evolving complex with recombinant *Arabidopsis* manganese-stabilizing protein. *Plant Mol. Biol.* **26**, 117–130.

Croteau, R. (1977) Biosynthesis of benzaldehyde, benzyl alcohol and benzyl benzoate from benzoic acid in cranberry (*Vaccinium macrocarpon*). *J. Food Biochem.* **1**, 317–326.

Croteau, R. and Karp, F. (1991) Origin of natural odorants. In *Perfumes – Art, Science and Technology* (Muller, P.M. & Lamparsky, D., eds). New York: Elsevier Applied Science, pp. 101–126.

Dudareva, N., Cseke, L., Blanc, V.M. and Pichersky, E. (1996) Evolution of floral scent in *Clarkia*: novel patterns of *S*-linalool synthase gene expression in the *C. breweri* flower. *Plant Cell*, **8**, 1137–1148.

Dudareva, N., Raguso, R.A., Wang, J., Ross, J.R. and Pichersky, E. (1998) Floral scent production in *Clarkia breweri.* III. Biosynthesis and emission of benzenoid esters. *Plant Physiol.* **116,** 599–604.

Farmer, E.E. and Ryan, C.A. (1990) Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. *Proc. Natl. Acad. Sci. USA*, **87**, 7713–7716.

Knudsen, J.T. and Tollsten, L. (1993) Trends in floral scent chemistry in pollination syndromes: floral scent composition in moth-pollinated taxa. *Bot. J. Linn. Soc.* **113**, 263–284.

Knudsen, J.T., Tollsten, L. and Bergstrom, G. (1993) Floral scents – a check-list of volatile compounds isolated by head-space techniques. *Phytochemistry*, **33**, 253–280.

Negruk, V., Yang, P., Subramanian, M., McNevin, J.P. and Lemieux, B. (1996) Molecular cloning and characterization of the CER2 gene of *Arabidopsis thaliana*. *Plant J.* **9**, 137–145.

Oh-oka, H., Wada, K., Kuwabara, T. and Murata, N. (1986) Complete amino acid sequence of 33 kDa protein isolated from spinach photosystem II particles. *FEBS Lett.* **197**, 63–66.

Pichersky, E., Raguso, R.A., Lewinsohn, E. and Croteau, R. (1994) Floral scent production in *Clarkia* (Onagraceae). I. Localization and developmental modulation of monoterpene emission and linalool synthase activity. *Plant. Physiol.* **106**, 1533–1540.

Pichersky, E., Lewinsohn, E. and Croteau, R. (1995) Purification and characterization of *S*-linalool synthase, and enzyme involved in the production of floral scent in *Clarkia breweri*. *Arch. Biochem. Biophys.* **316**, 803–807.

Raguso, R.A. and Pichersky, E. (1995) Floral volatiles from *Clarkia breweri* and *C. concinna* (Onagraceae): recent evolution of floral scent and moth pollination. *Plant. Sys. Evol.* **194**, 55–67.

Raguso, R.A., Light, D.M. and Pichersky, E. (1996) Electro-antennogram responses of *Hyles lineata* (Sphingidae: Lepidoptera) to volatile compounds from *Clarkia breweri* (Onagraceae) and other moth-pollinated flowers. *J. Chem. Ecol.* **22**, 1735–1766.

Shulaev, V., Silverman, P. and Raskin, I. (1997) Airborne signaling by methyl salicylate in plant pathogen resistance. *Nature*, **385**, 718–721.

Tacke, E., Korfhage, C., Michel, D., Maddaloni, M., Motto, M., Lanzini, S., Salamini, F. and Doring, H.-P. (1995) Transposon tagging of the maize *Glossy2* locus with the transposable element *En/Spm*. *Plant J.* **8**, 907–917.

Van Dort, H., Jaegers, P., Ter Heide, R. and Van der Weerdt, A. (1993) *Narcissus trevithian* and *N. geranium*: analysis and synthesis of compounds. *J. Agric. Food Chem.* **41**, 2063–2075.

Wang, J. and Pichersky, E. (1998) Characterization of *S*-adenosyl-L-methionine:(iso)eugenol *O*-methyltransferase involved in floral scent production in *Clarkia breweri*. *Arch. Biochem. Biophys.* **349**, 153–160.

Wang, J., Dudareva, N., Bhakta, S., Raguso, R.A. and Pichersky, E. (1997) Floral scent production in *Clarkia breweri* (Onagraceae). II. Localization and developmental modulation of the novel enzyme SAM:(iso)eugenol *O*-methyltransferase and phenylpropanoid emission. *Plant Physiol.* **114,** 213–221.

Watanabe, N., Watanabe, S., Nakajima, R., Moon, J.H., Shimokihira, K., Inagaki, J., Etoh, H., Asai, T., Sakata, K. and Ina, K. (1993) Formation of flower fragrance compounds from their precursors by enzymic action during flower opening. *Biosci. Biotech. Biochem.* **57**, 1101–1106.

Xia, Y., Nikolau, B.J. and Schnable, P.S. (1997) Developmental and hormonal regulation of the *Arabidopsis* CER2 gene that codes for a nuclear-localized protein required for the normal accumulation of cuticular waxes. *Plant Physiol.* **115**, 925–937.

Yabumoto, K., Jennings, W.G. and Yamaguchi, M. (1977) Volatile constituents of cantaloupe, *Cucumis melo* and their biogenesis. *J. Food Sci.* **42**, 32–37.

Yang, Q., Reinhard, K., Schiltz, E. and Matern, U. (1997) Characterization and heterologous expression of hydroxycinnamoyl/ benzoyl-CoA:anthranilate *N*-hydroxycinnamoyl/ benzoyltransferase from elicited cell cultures of carnation, *Dianthus caryophyllus* L. *Plant Mol. Biol.* **35**, 777–789.

The EMBL accession number of this sequence is AF043464.