

Characterization of a BAHD acyltransferase responsible for producing the green leaf volatile (Z)-3-hexen-1-yl acetate in *Arabidopsis thaliana*

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Summary

Green-leaf volatiles are commonly emitted from mechanically and herbivore-damaged plants. Derived from the lipoxygenase pathway, these compounds may serve as attractants to predators and parasitoids of herbivores, prevent the spread of bacteria and fungi, and induce several important plant defense pathways. In *Arabidopsis thaliana*, the major volatile released upon mechanical wounding of the leaves is the GLV ester, (Z)-3-hexen-1-yl acetate. We have characterized a member of the BAHD acyltransferase gene family At3g03480 which catalyzes the formation of (Z)-3-hexen-1-yl acetate from acetyl-CoA and (Z)-3-hexen-1-ol. The encoded acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase (CHAT) has the ability to accept several medium-chain-length aliphatic and benzyl-derived alcohols, but has highest catalytic efficiency with (Z)-3-hexen-1-ol. The highest expression of *CHAT* occurs in the leaves and stems. Upon mechanical damage, the (Z)-3-hexen-1-yl acetate emission peaked after 5 min and declined to pre-damage levels after 90 min. However, *CHAT* gene transcript levels increased much more slowly with the highest levels detected between 3 and 6 h after wounding. An increase in *CHAT* enzyme activity *in vitro* followed the transcript increase, with levels peaking between 10 and 12 h after wounding. Plants expressing either an RNAi cassette for the *CHAT* gene or plants harboring a T-DNA insertion in the *CHAT* coding region had greatly reduced (Z)-3-hexen-1-yl acetate emission, showing that the *CHAT* enzyme is responsible for the *in planta* production of this ester.

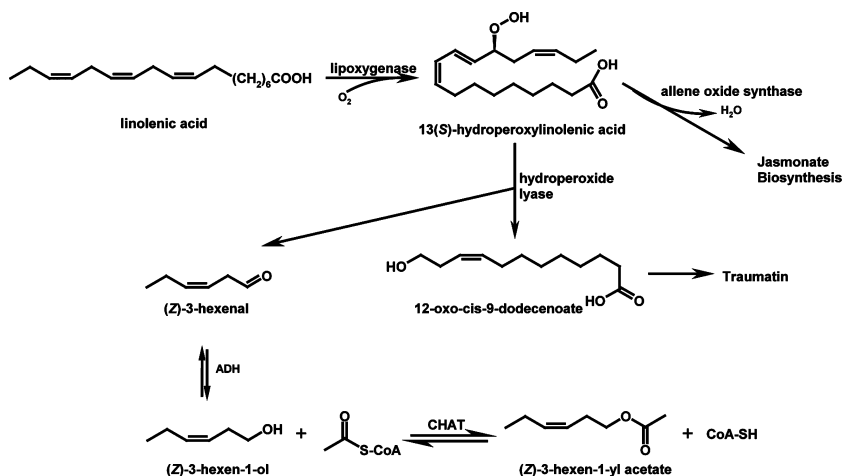
Key words: *Arabidopsis thaliana*, green leaf volatile, BAHD, acyltransferase, acetyl coenzyme A, (Z)-3-hexen-1-yl acetate, mechanical wounding.

Introduction

Plant volatiles are small-molecular-weight compounds with high vapor pressures at ambient temperatures (Gang, 2005). Although there are many different plant volatiles, most are synthesized via a few basic pathways and thus fall into several major groups, which include the terpenoids, benzenoids, phenylpropanoids and fatty acid derived compounds (Dudareva *et al.*, 2004). Once the basic skeletons are produced via these pathways, the diversity of volatiles is achieved via additional modification reactions such as acylation, methylation, oxidation/reduction and cyclic ring closure. Such modifications often result in increased volatility and changed olfactory properties (Pichersky *et al.*, 2006).

Recent work has identified several physiological and ecological roles for plant volatiles. Critical functions include the attraction of pollinators by floral scent and the attraction of enemies of feeding herbivores by volatiles emitted from injured as well as non-injured leaves, a phenomenon known as indirect defense (Arimura *et al.*, 2005). To date, more than 15 different species of plants have been documented to deploy volatile compounds for indirect defense when challenged by herbivore attack (Degenhardt *et al.*, 2003). Different plant species emit a specific blend of volatiles depending on the herbivore, although the predominant classes of compounds released include the mono- and sesquiterpenes, as well as green leaf volatiles (GLV), C₆ aldehydes, alcohols

Figure 1. The lipoxygenase pathway leading to the production of green leaf volatiles.



and esters derived from fatty acids through the action of lipoxygenases (Holopainen, 2004).

Green leaf volatiles are a class of compounds found throughout the plant kingdom. The most commonly documented GLVs are derived from linolenic and linoleic acids (Hatanaka, 1993; Matsui, 2006) (Figure 1). Their biosynthesis involves hydroperoxide formation catalyzed by lipoxygenase followed by cleavage at C₁₂, mediated by hydroperoxide lyase, resulting in the formation of a non-volatile C₁₂ acid and a volatile C₆ aldehyde. The latter is formed with a (*Z*) configuration, but isomerization to the (*E*) isomer commonly occurs. Although GLV release is frequently associated with vegetative tissue, wounded either mechanically or through the action of a herbivore, these compounds have also been found as volatile constituents in the flowers and fruits of many different plant species (Homatidou *et al.*, 1992; Knudsen *et al.*, 1993).

Green leaf volatiles function in a multitude of different roles. Several studies have shown that C₆ aldehydes act as anti-fungal and anti-bacterial compounds (Hamilton-Kemp *et al.*, 1998), and they have also been shown to reduce aphid feeding in tobacco plants (Hildebrand *et al.*, 1993). However, C₆ aldehydes may also be detrimental to the plant synthesizing them. It has been shown for example, that (*E*)-2-hexenal inhibits the germination and growth of soybean and *Arabidopsis thaliana*, as well as inhibiting pollen tube formation in apple (Bate and Rothstein, 1998; Gardner *et al.*, 1990; Hamilton-Kemp *et al.*, 1991).

Several recent studies have reported that GLVs may participate in plant-to-plant signaling. For example, tomato plants exposed to exogenously applied (*E*)-2-hexenal were shown to respond by increasing the production of terpenes involved in local and systemic responses (Farg and Pare, 2002). In addition, (*Z*)-3-hexen-1-ol treatment of maize plants was shown to induce several well known plant-defense-related genes but defense metabolite levels were unchanged (Farg *et al.*, 2005). In a similar experiment the exposure of

maize plants to GLVs was able to induce the production of blends of volatiles that were similar to those produced under herbivore attack (Ruther and Furstenau, 2005). Similarly, (*Z*)-3-hexenal, (*Z*)-3-hexen-1-ol and (*Z*)-3-hexen-1-yl acetate were found to induce defense responses in tomato (Engelberth *et al.*, 2004).

In the cruciferous plant *A. thaliana*, ecotype Columbia, exogenous application of (*E*)-2-hexenal as well as other GLVs similarly induces a subset of genes known to be involved in various plant-defense responses (Bate and Rothstein, 1998; Kishimoto *et al.*, 2005). The gene encoding hydroperoxide lyase (*HPL1*) is wound inducible, and the induction of C₆ aldehyde production following injury is extremely rapid, occurring as soon as 20 sec after mechanical damage to the leaves (Matsui *et al.*, 2000). More recently, it was discovered that the *HPL1* gene in the Columbia ecotype of *A. thaliana* contains a truncation in the first exon when compared with the gene from the ecotype Landsberg *erecta* (*Ler*). This truncation results in a drastic decrease in GLV production in Columbia, when compared with that of *Ler*, and is caused by a less active *HPL1* enzyme (Duan *et al.*, 2005).

We have previously identified a gene from *A. thaliana*, *At3g03480*, which encodes an enzyme similar to *Clarkia breweri* benzoyl CoA:benzyl alcohol benzoyl transferase (BEBT), an enzyme responsible for the production of benzylbenzoate (D'Auria *et al.*, 2002). These enzymes belong to a large family of plant acyltransferases known as the BAHD family, the name being derived from the first four enzymes to be identified and characterized (BEAT, AHCT, HCBT and DAT) (D'Auria, 2006; St Pierre and De Luca, 2000). In an effort to characterize *At3g03480*, we expressed it in *Escherichia coli* and were able to show that the enzyme it encodes did not have a high affinity for benzyl alcohol and benzoyl CoA, but rather that it was able to utilize acetyl CoA and the GLV alcohol (*Z*)-3-hexen-1-ol. Here we report on the biochemical characterization

Table 1 Green leaf volatiles (GLVs) emitted from intact *Ler* plants under various conditions

Treatment	(Z)-3-hexen-1-ol ^a	(Z)-3-hexen-1-yl acetate ^a
Control	0.23 ± 0.18 ^b	0.1 ± 0.2
Mechanically damaged	188 ± 72	342.4 ± 180
<i>Spodoptera exigua</i> damaged	22.6 ± 3.4	36.4 ± 12

^ang (gFW)⁻¹ collected over a 4-h period.

^bValues represent mean ± SD (*n* = 12).

and *in vivo* activity of Arabidopsis acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase (CHAT).

Results

The major volatile released from mechanically wounded Ler plants is (Z)-3-hexen-1-yl acetate

We performed dynamic headspace collection on non-wounded and mechanically wounded plants, and on plants damaged by the larvae (caterpillars) of the generalist herbivore *Spodoptera exigua*. Undamaged plants emitted only trace quantities of GLVs (Table 1). The major volatile released from mechanically wounded plants was the C₆ acetyl ester of (Z)-3-hexen-1-ol, of which 342.4 ± 180 ng were emitted per gram fresh weight (gFW)⁻¹ over the 4-h collection period (Table 1). Other GLVs detected included (Z)-3-hexen-1-ol [188 ± 72 ng (gFW)⁻¹], with only trace levels of (Z)-3-hexenal detected. Plants infested with *S. exigua* emitted quantities of (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate that were 10-fold less than the emissions from mechanically wounded plants.

To determine the timing of volatile release in more detail, we analyzed mechanically wounded *Ler* plants with a proton transfer reaction mass spectrometer (PTR-MS) (Figure 2). The GLV emitted first was the compound (Z)-3-hexenal, which reached maximum emission at approximately 30–45 s following damage. The peak emission of (Z)-3-hexen-1-ol occurred later, at approximately 2.5 min following mechanical damage, whereas the peak emission of (Z)-3-hexen-1-yl acetate occurred between 4.5 and 5.5 min after mechanical damage. The emission rate of the acetate ester returned to pre-damage levels after 90 min.

The enzymatic formation of (Z)-3-hexen-1-yl acetate also increases after mechanical wounding in the leaves of mechanically wounded Ler plants

To test for (Z)-3-hexen-1-yl acetate biosynthesis in the plant, we developed an assay (see Experimental procedures) to detect the activity of CHAT, the enzyme responsible for catalyzing the formation of the (Z)-3-hexen-1-yl acetate from (Z)-3-hexen-1-ol and acetyl-CoA. Leaves were damaged by

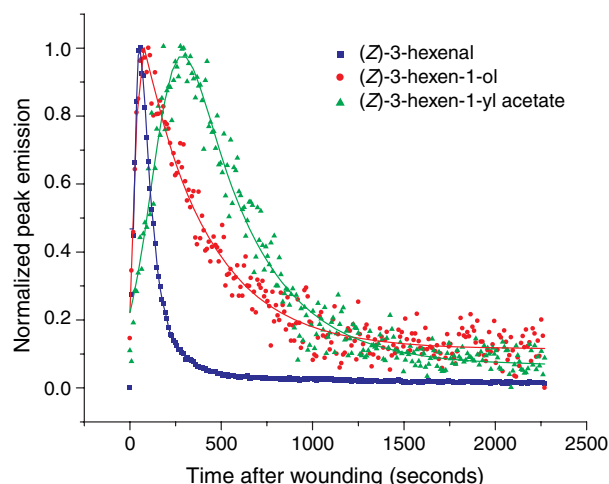


Figure 2. Proton-transfer reaction mass spectrometer (PTR-MS) analysis of green leaf volatile (GLV) emission following mechanical wounding in intact *Arabidopsis thaliana Ler* plants. Volatiles of mechanically wounded *Ler* plants were analyzed on-line using a PTR-MS (see Experimental procedures). Emission of the three major GLVs found in *A. thaliana*, (Z)-3-hexenal, (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate, were then normalized to peak emission. This graph represents the mean of four independent time courses consisting of five 4-week-old plants per trial. The solid lines are the result of non-linear curve-fitting analysis (using a Gaussian model).

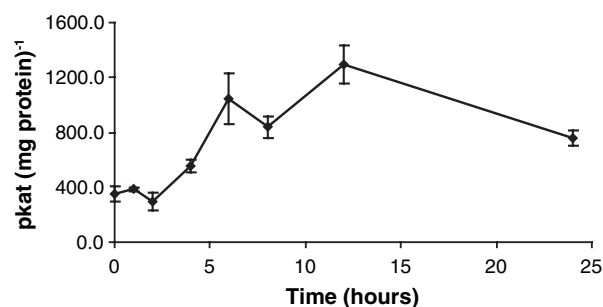


Figure 3. Acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase (CHAT) enzyme activity in mechanically wounded leaves. Leaf tissue was collected from mechanically wounded *Arabidopsis thaliana Ler* plants over a course of 24 h following damage. Each data point represents the mean of activity of at least three independent assays, with each assay consisting of a crude enzyme extract made from three different plants.

making two incisions to each side of the midvein and waiting for 1, 2, 4, 6, 8, 10, 12 and 24 h following treatment. Crude protein extracts of non-wounded leaf samples had a specific activity of 349 ± 53 pkat (mg protein)⁻¹. CHAT activity in the leaves following wounding increased over time, but this response lagged significantly behind (Z)-3-hexen-1-yl acetate release. A maximum specific activity of 1294 ± 138 pkat (mg protein)⁻¹ was reached 12 h after the initial wounding event. CHAT enzyme activity declined by 35% over the course of the next 12 h (Figure 3).

At3g03480 is responsible for (*Z*)-3-hexen-1-yl acetate production in mechanically wounded *Ler* plants

We had previously shown that *At3g03480* encoded a protein capable of the acetylation of (*Z*)-3-hexen-1-ol (D'Auria *et al.*, 2002). However, the members of the BAHD acyltransferase family sometimes have a broad substrate specificity (Beekwilder *et al.*, 2004; D'Auria *et al.*, 2002; El-Sharkawy *et al.*, 2005). To verify that *At3g03480* functions in (*Z*)-3-hexen-1-yl acetate formation *in vivo*, we down-regulated the expression of this gene using an RNAi construct consisting of the last 452 nucleotides of the open reading frame (see Experimental procedures). The absence of (*Z*)-3-hexen-1-yl acetate positively correlated with reduced *At3g03480* gene transcription compared with control plants, as measured by quantitative RT-PCR with gene-specific primers (Table 2; $r^2 = 0.479$; $P \leq 0.001$). RNAi transgenic plants exhibiting a severe reduction in *At3g03480* transcript levels emitted no (*Z*)-3-hexen-1-yl acetate, whereas several RNAi lines with *At3g03480* transcript levels that were either equal to or more

than those observed in the controls produced wild-type levels of (*Z*)-3-hexen-1-yl acetate.

To confirm our observations that a reduction in *At3g03480* activity results in a loss of (*Z*)-3-hexen-1-yl acetate production, we identified a T-DNA insertion line containing an insert within the coding region for *At3g03480*. The left border insertion site for SALK_025557 is at position 1024 in the coding region of the gene, as confirmed by sequencing. However, because the SALK lines were obtained from the Columbia ecotype (Alonso *et al.*, 2003), which is deficient in GLV biosynthesis (see above), this mutant was backcrossed with *Ler* plants for seven generations (see Experimental procedures) to assess the effect of the insertion on GLV emission. The BC7 plants containing the T-DNA insertion were allowed to self-fertilize and were analyzed by DNA gel blot (data not shown).

All plants analyzed had detectable levels of (*Z*)-3-hexen-1-ol (Figure 4a). However, those plants homozygous for the T-DNA insertion had no detectable levels of (*Z*)-3-hexen-1-yl acetate (Figure 4b). In contrast, plants containing at least one wild-type copy of *At3g03480* emitted (*Z*)-3-hexen-1-yl acetate at levels similar to either wild-type *Ler* or plants in the RNAi experiments with high *At3g03480* transcript levels. In addition, plants homozygous for the T-DNA insertion also had transcript levels that were less than 0.1% of the levels in plants containing at least one wild-type copy of the gene (data not shown), indicating that the aberrant transcript was not stable.

Table 2 Characterization of transgenic plants harboring a *CHAT* RNAi construct

Plant line	(<i>Z</i>)-3-hexen-1-ol emission ^a	(<i>Z</i>)-3-hexen-1-yl acetate emission	Percentage <i>CHAT</i> transcript ^b
CHAT <i>Ler</i> RNAi #1	1.1	0.01	8
CHAT <i>Ler</i> RNAi #2	2.1	0.00	10
CHAT <i>Ler</i> RNAi #3	1.2	0.05	9
CHAT <i>Ler</i> RNAi #4	1.5	0.79	122
CHAT <i>Ler</i> RNAi #5	2.1	0.02	55
CHAT <i>Ler</i> RNAi #6	2.1	0.08	70
CHAT <i>Ler</i> RNAi #7	1.7	0.03	58
CHAT <i>Ler</i> RNAi #8	2.4	0.03	39
CHAT <i>Ler</i> RNAi #9	1.5	0.00	25
CHAT <i>Ler</i> RNAi #10	1.6	0.00	49
CHAT <i>Ler</i> RNAi #11	1.3	0.32	94
CHAT <i>Ler</i> RNAi #12	1.6	0.05	6
CHAT <i>Ler</i> RNAi #13	1.5	0.00	26
CHAT <i>Ler</i> RNAi #14	1.2	0.00	37
CHAT <i>Ler</i> RNAi #15	1.6	0.03	58
CHAT <i>Ler</i> RNAi #16	1.9	0.13	102
CHAT <i>Ler</i> RNAi #17	1.8	0.05	63
CHAT <i>Ler</i> RNAi #18	1.4	0.02	46
CHAT <i>Ler</i> RNAi #19	1.2	0.10	89
<i>Ler</i> non-transformed	1.3	0.49	100
<i>Ler</i> empty vector	1.5	0.41	100

^aQuantification of GLV emission was performed using the zNose GC on excised mechanically damaged leaves (see Experimental procedures). Emission is expressed as ng (mg FW)⁻¹ per injection, where each injection consisted of volatiles collected during a 15-s sampling time.

^b*CHAT* (*At3g03480*) transcript was measured from each individual plant line in triplicate and the average gene expression was compared with levels of transcript in the control leaves (calibrator) using the gene *APT1* (*At1g27450*) as a normalizer.

The At3g03480 (CHAT) protein shows a preference for (Z)-3-hexen-1-ol as a substrate

For *E. coli* expression, the coding region of *At3g03480* was cloned into a vector that introduced an N-terminal 6× *His*-tag. After purification using *His*-tag affinity chromatography, the resulting CHAT protein was compared with a partially purified CHAT protein from transgenic *A. thaliana* in a *Ler* background containing a construct for the constitutive over-expression of the *At3g03480* cDNA under the control of the 35S Cauliflower mosaic virus (CaMV) promoter (see Experimental procedures). Gel exclusion chromatography performed on both the native plant protein and *His*-tagged protein showed that CHAT behaves as a monomer with a size of 49 and 51 kDa, respectively. The predicted sizes of the native protein and the *His*-tagged protein are 50.4 and 52.45 kD, respectively.

The *His*-tagged and native CHAT proteins have a Michaelis-Menton constant (K_m) value for acetyl-CoA of 38 ± 5 and $12 \pm 4 \mu\text{M}$, respectively, and a K_m value of 251 ± 33 and $126 \pm 16 \mu\text{M}$ for (*Z*)-3-hexen-1-ol, respectively. The use of two other CoA thioesters, malonyl CoA and benzoyl CoA, yielded no enzyme activity above that of the non-enzyme controls. We also compared the relative activity of the *E. coli*-expressed CHAT with various other alcohols (Figure 5). CHAT

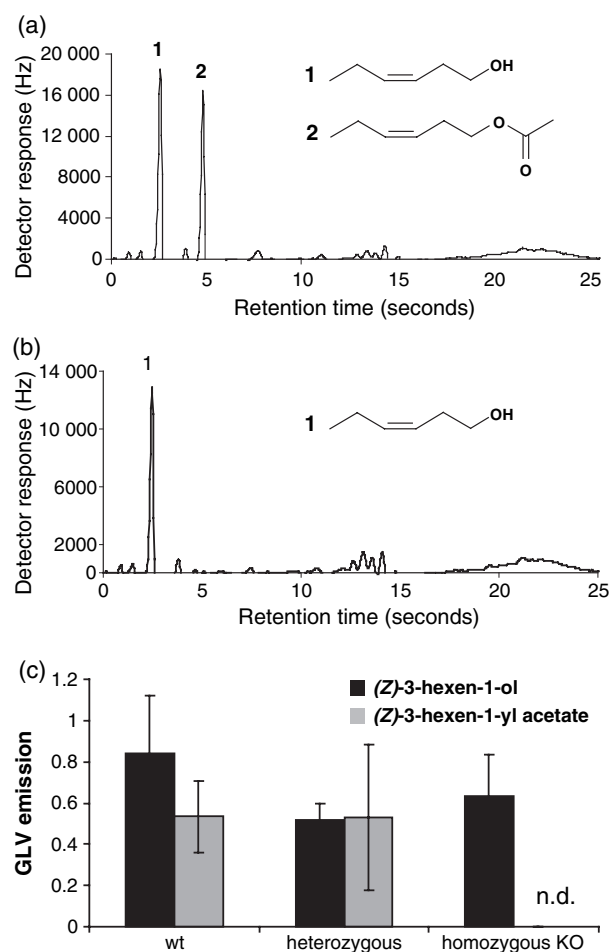


Figure 4. Analysis of 7th generation segregants from a backcross of SALK_025557 to *Arabidopsis thaliana* Ler plants. Excised wounded leaves of individual plants were analyzed for green leaf volatile (GLV) production using the zNose, a portable, rapid-separation GC with a surface acoustic wave detector.

(a) The zNose chromatogram for excised leaves of *Ler* plants homozygous for the wild-type (wt) *CHAT* gene.

(b) The zNose chromatogram for excised wounded leaves of selfed BC7 plants homozygous for the T-DNA insertion in the *CHAT* open reading frame.

(c) GLVs were quantified for all plants ($n = 80$) and data are represented as averages for those plants segregating as homozygous wt, heterozygous and homozygous knockout based on the results from DNA gel blot analysis. Solid bars represent (*Z*)-3-hexen-1-ol levels, whereas gray bars represent (*Z*)-3-hexen-1-yl acetate levels from mechanically damaged excised leaves. Emission is expressed as ng (mg FW)^{-1} per injection, where each injection consisted of volatiles collected during a 15-s sampling time (n.d. = not detected).

prefers primary, medium-chain-length, aliphatic alcohols, and had a very low activity with alcohols of more than 10 carbon atoms in length. Curiously, CHAT was 20% more active with 1-octanol than with (*Z*)-3-hexen-1-ol. However, detailed kinetic analysis showed that although the K_m value of CHAT for 1-octanol, $174 \pm 27 \mu\text{M}$, is similar to that of (*Z*)-3-hexen-1-ol, the catalytic efficiency (k_{cat}) for 1-octanol was two orders of magnitude lower than that for (*Z*)-3-hexen-1-ol. The k_{cat}/K_m values for the two alcohols (Table 3) thus indicate that

CHAT prefers (*Z*)-3-hexen-1-ol over 1-octanol by a factor of 625. Moreover, 1-octanyl acetate has not yet been reported from any ecotype of *A. thaliana*. These data support the proposition that the most likely *in vivo* acyl acceptor for CHAT is (*Z*)-3-hexen-1-ol.

The optimal pH range for both CHAT proteins was between 7.1 and 7.3, with only 89 and 45% activity remaining at a pH of 6.3 and 9.0, respectively. The enzyme was 100% stable for 30 min at 30°C, and 87% stable for 30 min at 37°C. However, incubation of the protein for 30 min at 50°C resulted in a total loss of enzyme activity. In addition to temperature stability, the effects of monovalent and divalent cations on enzyme activity were also tested. None of the cations tested proved to have a stimulatory effect. However, the divalent cations Mg^{2+} , Ca^{2+} , Co^{2+} , Zn^{2+} and Cu^{2+} had a strong inhibitory effect at a final concentration of 5 mM, leading to a 60–100% reduction in enzyme activity.

Organ-specific expression and differences in transcript levels of CHAT and HPL1 following mechanical wounding

CHAT and *HPL1* transcripts were measured with RNA isolated from intact organs of 6-week-old *Ler* plants to identify which tissue had the highest levels of gene transcripts. *CHAT* gene transcripts were highest in leaves compared with stems, flowers, siliques and roots. Both siliques and roots had transcript levels that are approximately two orders of magnitude lower than levels present in leaves (Figure 6a). *HPL1* transcripts were approximately 10-fold higher in the flowers compared with the leaves. This is in contrast to the fivefold lower levels of *CHAT* transcripts in the flowers compared with leaves.

Both *CHAT* and *HPL1* transcripts were induced after mechanical wounding in 4-week-old plants. The highest transcript levels of both genes occurred 3 h after the initial wounding event (Figure 6b), and transcript levels gradually declined thereafter, returning to pre-wounding levels between 8–10 h after wounding. No significant changes were observed in non-wounded tissue tested at identical times (data not shown).

To explore the correlation between *CHAT* transcript levels and (*Z*)-3-hexen-1-yl acetate emission, the transformants expressing *At3g03480* under the control of a CaMV 35S promoter were compared with wild-type *Ler* for (*Z*)-3-hexen-1-yl acetate production before and after mechanical wounding. Three independent transgenic lines with similar specific activities [approx. $500\text{--}1000 \text{ nkat (mg protein)}^{-1}$] were used. Both non-wounded and mechanically damaged 35S::*CHAT* plants showed a significant increase in (*Z*)-3-hexen-1-yl acetate production when compared with the non-transformed plants under identical conditions (Figure 7). Plants transformed with the same vectors containing no *CHAT* cDNA showed similar patterns to those of non-transformed *Ler* plants (data not shown).


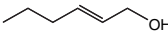
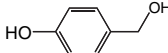

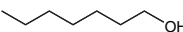
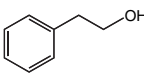

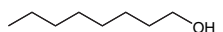
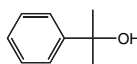


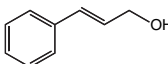
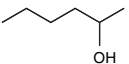
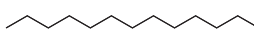
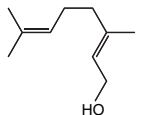
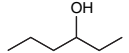
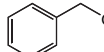

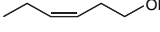
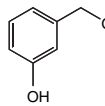
Alcohol substrate	%(Z)-3-hexen-1-ol	Alcohol substrate	%(Z)-3-hexen-1-ol	Alcohol substrate	%(Z)-3-hexen-1-ol
methanol	 0	(E)-2-hexen-1-ol	 34	4-OH benzyl alcohol	 0
ethanol	 0	1-heptanol	 90	2-phenyl ethanol	 14
1-butanol	 1	1-octanol	 123	2-phenyl 2-propanol	 0
1-hexanol	 44	1-decanol	 6	cinnamyl alcohol	 2
2-hexanol	 0	1-dodecanol	 0	geraniol	 36
3-hexanol	 0	benzyl alcohol	 5	linalool	 0
(Z)-3-hexen-1-ol	 100	3-OH benzyl alcohol	 0		

Figure 5. Relative activity of *Escherichia coli* expressed and purified acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase (CHAT) with a variety of alcohol substrates. All assays were performed with acetyl-CoA and one of the given alcohols. Activities are shown as a percentage of that with (Z)-3-hexen-1-ol. The specific activity of CHAT with acetyl-CoA and (Z)-3-hexen-1-ol as substrates was 32.7 nkat (mg protein)⁻¹.

Table 3 Kinetic parameters of CHAT

	K_m (μM) ^c	k_{cat} (s ⁻¹)	k_{cat}/K_m (nM ⁻¹ s ⁻¹)
CHAT 6xHis ^a			
(Z)-3-Hexen-1-ol	251 ± 33.4	11.3	45
1-Octanol	173.6 ± 27	0.125	0.072
Acetyl-CoA	38.5 ± 4.92	23	597
w/(Z)-3-Hexen-1-ol			
CHAT (native partially purified) ^b			
(Z)-3-Hexen-1-ol	126 ± 16	ND	ND
Acetyl-CoA	12 ± 4	ND	ND
w/(Z)-3-Hexen-1-ol			

^aPurified from *E. coli* over-expressing the At3g03480 product fused to a 6x HisTag.

^bPartially-purified from *A. thaliana* over-expressing At3g03480 cDNA behind a cauliflower mosaic virus (CaMV) 35S promoter.

^cValues represent the mean ± SD ($n = 12$).

Discussion

CHAT is a member of the BAHD acyltransferase family

CHAT belongs to a large group of plant acyltransferases commonly referred to as the BAHD family (St Pierre and De Luca, 2000). The BAHD acyltransferases are recognized by

several common motifs, including the HXXXD motif, important in the active site for general base catalysis, and the structural motif DFGWG near the C terminus (Ma *et al.*, 2004, 2005). Characterized acyltransferases of this family transfer a range of different acyl substrates from their CoA thioesters to various plant secondary compounds, which contain hydroxyl groups and nitrogen atoms to produce esters and amides, respectively. The *A. thaliana* Columbia genome contains 64 recognized members of the BAHD family (D'Auria, 2006). In addition to CHAT, the function of only one other Arabidopsis BAHD enzyme has been determined. Gene At5g48930 encodes quinate/shikimate ρ -OH coumaroyl/cinnamoyl CoA transferase, an enzyme involved in the production of lignin precursors (Hoffmann *et al.*, 2005). Several other BAHD members in *A. thaliana* have been implicated in the production of other acylated compounds, such as acyl-modified anthocyanins (Tohge *et al.*, 2005), but have not yet been shown to have a specific function in the plant.

CHAT (encoded by At3g03480) is responsible for (Z)-3-hexen-1-yl acetate production in Arabidopsis

Two independent lines of evidence demonstrate that gene At3g03480 encodes an acetyl-CoA:(Z)-3-hexen-1-ol acetyl-

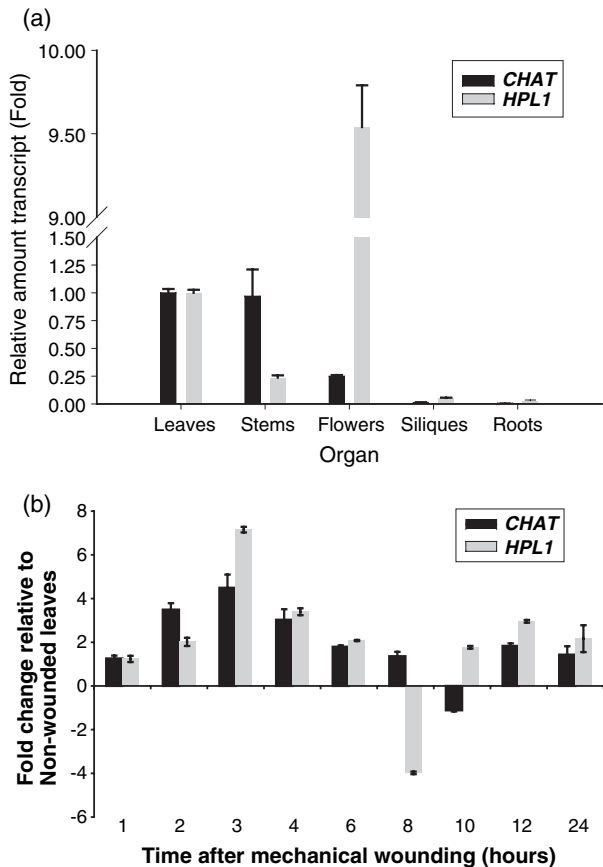


Figure 6. *CHAT* and *HPL1* transcript levels in various organs of *Arabidopsis thaliana* *Ler* plants after mechanical wounding. Comparative quantification of *CHAT* and *HPL1* transcripts in (a) non-wounded tissue representing leaves, stems, flowers, siliques and roots of 6-week-old *A. thaliana* *Ler* plants. (b) *CHAT* and *HPL1* transcript profiling following mechanical wounding consisting of two incisions made to each side of the midvein. Tissue was collected 1, 2, 3, 4, 6, 8, 10, 12 and 24 h after the initial wounding took place on 4-week-old plants. Black boxes represent the *CHAT* transcript whereas gray boxes represent *HPL1* transcripts. Each histogram represents the data obtained from three biological replicates each consisting of the RNA isolated from three individual plants. These samples were then studied in triplicate. The levels of transcript in all tissues were compared with those in the leaves (calibrator) using the gene *APT1* (*At1g27450*) as a normalizer.

transferase *in vivo*. Reduction of gene transcript levels by both RNA interference technology and by T-DNA insertion led to corresponding reductions in the levels of (Z)-3-hexen-1-yl acetate emission following mechanical damage to the leaves. The gene fragment used in the RNAi construct contained the last 452 nucleotides from gene *At3g03480*. This region was chosen because it does not include any stretch of 21 nucleotides or longer found in any other gene in the genome, and thus eliminates the possibility that other BAHD genes might have been silenced as well. As mechanically damaged leaves of some RNAi-suppressed and T-DNA inactivated *At3g03480* lines were completely devoid of (Z)-3-hexen-1-yl acetate emission, we conclude that *At3g03480* is the only gene encoding CHAT activity in these organs.

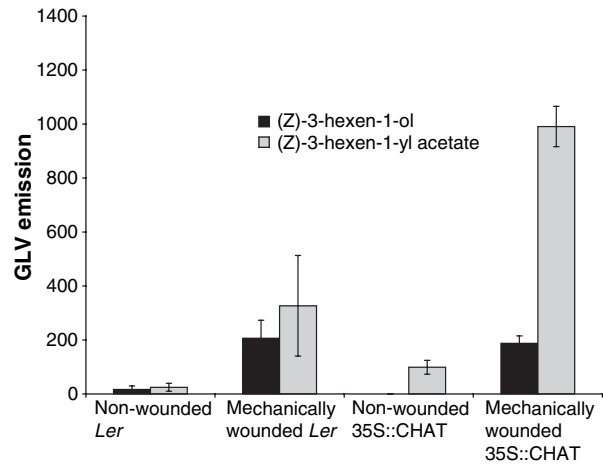


Figure 7. (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate emissions from non-transgenic and transgenic *Arabidopsis thaliana* *Ler* plants expressing *CHAT* under a 35S cauliflower mosaic virus promoter. Six plants each of 35S::CHAT *Ler* containing high acetyl CoA:(Z)-3-hexen-1-ol acetyltransferase (*CHAT*)-specific activity and wild-type *Ler* were either mechanically wounded or left as non-wounded controls. Dynamic headspace sampling using activated charcoal filters was performed for 4 h and the volatiles collected were measured by GC-MS. Emission is presented as ng (gFW)⁻¹ collected over a 4-h period.

Leaves wounded by mechanical treatment emit more GLVs than herbivore-damaged leaves

The production of wound-induced GLVs is well documented for a number of different plant species. For example, cotton plants attacked by the herbivore *S. exigua* emit (Z)-3-hexen-1-yl acetate as well as a host of other isoprenoid-derived compounds and indole (Rose *et al.*, 1998). Interestingly, continuous mechanical damage can sometimes induce greater quantities of (Z)-3-hexen-1-yl acetate in lima bean plants than that observed with continuous herbivory (Mithofer *et al.*, 2005). Following this trend, our results indicate that in mechanically damaged *Arabidopsis* leaves (two lateral incisions on each side of the midvein), (Z)-3-hexen-1-yl acetate is emitted at levels 10-fold higher than after damage by the generalist herbivore *S. exigua* (where approx. 25% of the leaf area is removed). In a similar fashion, mechanical wounding of *Arabidopsis* plants, but not herbivory wounding by the specialist herbivore *Pieris rapae*, resulted in (Z)-3-hexen-1-yl acetate emission at levels similar to those found in our study (Van Poecke *et al.*, 2001). It is known that insect damage and mechanical wounding induce a different subset of genes in *A. thaliana* (Reymond *et al.*, 2000, 2004). In the plant *Medicago truncatula*, herbivore feeding and caterpillar saliva can decrease gene transcript levels of genes encoding enzymes for terpenoid biosynthesis (Bede *et al.*, 2006). However, the mechanism underlying this phenomenon and its biological consequences are not known. Further studies will need to be performed to test possible explanations such as post-translational modification of lipoxygenase pathway enzymes or

the possibility that inhibitory agents are present in the saliva of herbivore species. For example, the enzyme glucose oxidase found in the salivary glands of *Helicoverpa zea* is sufficient to reduce the accumulation of nicotine in *Nicotiana tabacum* (Musser *et al.*, 2005).

Our study also showed that the production of (*Z*)-3-hexen-1-yl acetate after leaf injury is very rapid (Figure 2). Contrary to results reported by Duan *et al.* (2005), we did not detect the (*E*)-isomers of the GLVs in *Ler* plants. Other cruciferous plants have also been shown to make solely the (*Z*)-isomers of GLVs in whole-leaf samples, with the (*E*)-isomers present only in macerated samples (Tollsten and Bergstrom, 1988). In addition, unlike previous studies that used leaf homogenates (Bate *et al.*, 1998a,b; Duan *et al.*, 2005), we detected (*Z*)-3-hexenal only in low levels, irrespective of the headspace collection technique used.

The quantity of (Z)-3-hexen-1-yl acetate emitted after leaf damage is limited by different factors

The emission of (*Z*)-3-hexen-1-yl acetate from mechanically wounded *Ler* plants in this study peaked between 4.5 and 5.5 min after wounding (Figure 2). However, levels of CHAT transcripts and enzymatic activity showed no response in this time frame, but instead increased much more slowly peaking after 3 h (transcript) and 6–12 h (enzyme activity) after damage was inflicted (Figures 3 and 6). The time delay between peak transcript levels and maximal enzyme activity is often seen for genes encoding enzymes of secondary metabolism. For example, the maximal enzyme activity of benzoyl-CoA:benzyl alcohol/phenylethanol benzoyltransferase in petunia is delayed by several days from peak transcript levels in the flowers (Boatright *et al.*, 2004). Therefore, the (*Z*)-3-hexen-1-yl acetate emitted from the leaves in the first 5–10 min after damage must be synthesized by the CHAT protein already present in the leaf prior to injury. At this time, synthesis of (*Z*)-3-hexen-1-yl acetate is likely to be limited by the quantity of substrate [(*Z*)-3-hexen-1-ol] available. As the level of (*Z*)-3-hexen-1-ol increases the levels of CHAT become limiting, as indicated by the observation that Arabidopsis plants overexpressing CHAT under the control of the 35S promoter emit much higher levels of (*Z*)-3-hexen-1-yl acetate following mechanical damage than do wild-type plants (Figure 7). The observation that detached leaves emit significant levels of (*Z*)-3-hexen-1-ol also suggests that CHAT limits the formation of the corresponding acetate ester (Table 2 and Figure 4b). As heavier damage to excised leaves from surgical clamp damage would be expected to produce more (*Z*)-3-hexen-1-ol (compared with the razor-blade treatment) than can be converted into the corresponding acetate within the short period of sampling time.

A recent study on the exogenous application of GLVs on maize plants has shown that (*Z*)-3-hexen-1-ol, and to a lesser

extent (*Z*)-3-hexenal, are converted *in vivo* into the corresponding acetate ester, suggesting that maize also has CHAT activity in healthy, non-wounded plants (Yan and Wang, 2006). In addition, the application of a series of other medium-chain-length alcohols with different degrees of saturation show that the maize CHAT enzyme has similar biochemical properties as that from *A. thaliana*. For example, the maize CHAT shows a preference for primary alcohols and exhibits a reduction in the turnover of the (*E*)-2-hexen-1-ol isomer when compared with the turnover of the (*Z*)-3-hexen-1-ol isomer. The study by Yan and Wang (2007) also reaffirms the potential signaling roles of GLVs to induce defense-related metabolites in maize plants.

Plants overexpressing CHAT protein emit more (*Z*)-3-hexen-1-yl acetate even without injury than uninjured, untransformed controls (Figure 7). These plants may have a more active lipoxygenase pathway because of a GLV-induced feedback loop, similar to the 'priming' response observed in (*Z*)-3-hexen-1-ol-treated maize plants (Pare *et al.*, 2005). At 15–20 min after wounding, emission of (*Z*)-3-hexen-1-yl acetate starts to decline (Figure 2) because of a lack of sufficient (*Z*)-3-hexen-1-ol, the substrate for CHAT from the lipoxygenase pathway. However, over the next 12 h, CHAT transcript and activity increased three- to fourfold. A similar pattern occurs for *HPL1* (Figure 6b, and Bate *et al.*, 1998b) and for lipoxygenase (*Lox1* and *Lox2*) transcripts (Bell and Mullet, 1993; Melan *et al.*, 1993). This may prepare the plant to respond with greater (*Z*)-3-hexen-1-yl acetate emission to a second round of wounding.

(*Z*)-3-Hexen-1-ol is most likely synthesized from the respective aldehyde by the action of an alcohol dehydrogenase (ADH) (Figure 1). It has been reported that a mutation in the *ADH1* gene in *A. thaliana* results in a 51% reduction in (*Z*)-3-hexen-1-ol levels after mechanical damage (Bate *et al.*, 1998a). In addition, the gene encoding ADH1 (At1g77120) has also been shown to be inducible following mechanical damage, with similar results to those found with CHAT and *HPL1* here, and so may also influence the supply of substrate for CHAT (Delessert *et al.*, 2004). Many plant species are known to store GLV alcohols in the form of glucosides. For example, (*Z*)-3-hexenyl β -D-glucopyranoside has been found as a bound aroma precursor in both tea (*Camellia sinensis*), and Melón de Olor (*Sicana odorifera*) (Kobayashi *et al.*, 1994; Parada *et al.*, 2000). We have looked for the presence of glycosidically bound lipoxygenase-derived alcohols in *A. thaliana*, but were unable to detect them (data not shown). Thus the glycosylation of (*Z*)-3-hexen-1-ol seems unlikely to affect the supply of substrate for CHAT.

CHAT is predicted to be a cytosolic enzyme and contains no obvious signal peptides. On the other hand, the enzymes upstream of CHAT, namely lipoxygenase and hydroperoxide lyase, are predicted to be found in the chloroplast (Bate *et al.*, 1998b; Feussner and Wasternack, 2002). Thus, the substrate for CHAT needs to cross into the cytosol, although

a disruption of the cell, as occurs during wounding, may alleviate this requirement.

The role of (Z)-3-hexen-1-yl acetate in plant defense

Many research groups have shown that aldehydes and alcohols derived from the lipoxygenase pathway have strong anti-fungal and anti-bacterial properties. For example, the growth of fungal hyphae from *Aspergillus flavus* can be inhibited by C₆ aldehydes and alcohols found in infected maize and cotton plants (Zeringue and McCormick, 1989; Zeringue *et al.*, 1996). Other fungal species show similar growth inhibition (Vaughn *et al.*, 1993). In addition, GLVs also show inhibitory effects on the growth of phytopathogenic bacteria including different pathovars of *Pseudomonas syringae* (Croft *et al.*, 1993; Deng *et al.*, 1993; Prost *et al.*, 2005). The alcohols are generally less effective at inhibiting growth than the aldehydes (Andersen *et al.*, 1994). However, (Z)-3-hexen-1-yl acetate was shown to be an effective inhibitor of the white mold *Sclerotium rolfsii* (Cardoza *et al.*, 2002). Public databases for gene expression in *A. thaliana* show that the gene encoding CHAT is up-regulated after *P. syringae* treatment (<http://affymetrix.arabidopsis.info/narrays/experimentbrowse.pl>).

If (Z)-3-hexen-1-yl acetate has a role as a defense against microorganisms, CHAT induction following injury may better prepare the plant to resist infection. The increase in CHAT levels observed here would mean that subsequent injury should result in increased levels of (Z)-3-hexen-1-yl acetate formation. A similar delayed timecourse of induction has been found for terpene-producing enzymes in the gymnosperm *Abies grandis*, where induction peaks several days after mechanical damage (Funk *et al.*, 1994; Steele *et al.*, 1998). We are currently studying the potential priming function of (Z)-3-hexen-1-yl acetate *in planta* and how it applies to both herbivores and pathogens.

In addition to being toxic to pathogenic organisms, the C₆ aldehydes produced after either damage or infection may also be harmful to the plant. (E)-2-hexenal induces DNA damage and the formation of 1,N²-(1,3-propano)-2'-deoxyguanosine adducts in mammalian cells (Golzer *et al.*, 1996). If this and other aldehydes have a similar effect in plants, another function of CHAT may be to aid the conversion of certain lipoxygenase-derived aldehydes to less toxic derivatives as suggested by Farag *et al.* (2005). The availability of *A. thaliana* lines with altered CHAT levels now provides opportunities to test these roles in future experiments.

Experimental procedures

Plant materials and treatments

Arabidopsis thaliana (L.) Heynh (ecotype *Ler*) plants were grown on soil in a climate-controlled growth chamber similar to that reported

by Chen *et al.* (2003) Either glufosinate-ammonium- or kanamycin-resistant seedlings were transferred to soil and later the respective transgenes were confirmed by PCR. All treatments were pre-formed with 4–6-week-old rosette stage (non-bolting) plants of *A. thaliana* (ecotype *Ler*). For the mechanical wounding treatment, two lateral incisions to each side of the midvein of the leaves were made with a sterile razor blade. Eggs of *S. exigua* (Lepidoptera: Noctuidae) were obtained from Aventis (Frankfurt, Germany) and were reared on an artificial wheat-germ diet (Heliothis mix; Stonefly Industries, Bryan, TX, USA) for about 10–15 days at 22°C under an illumination of 750 μmol m⁻² s⁻¹. For the herbivory treatments, two-third instar larvae were placed on the rosette portion of each plant and were allowed to feed for the duration of the headspace collections.

The T-DNA insertion line SALK_025557 was obtained from the Arabidopsis Biological Resource Center (ABRC) and analyzed for resistance to kanamycin as described above (Alonso *et al.*, 2003). In addition, a PCR-based assay was designed to detect the presence of the T-DNA insertion by utilizing a primer from the T-DNA left border, LBB1, 5'-GCGTGGACCGCTTGCTGCAACT-3', in conjunction with an internal primer specific for At3g03480, 5'-CCACCCGTATGGAACCGACA-3'. In addition, a second primer specific for a region downstream of the T-DNA insertion was also used to test for zygosity, 5'-TCATCCTTTAGACACATTTAGCACTCC-3', by performing a PCR reaction with both gene-specific primers. Plants homozygous for the T-DNA insert and segregating for kanamycin resistance were then backcrossed for seven generations to *Ler* plants. In every generation, 30 progeny were screened for the presence of the T-DNA insert and a minimum of five positive plants, based on the PCR assay, were used as the pollen donors for backcrossing. Plants positive for a T-DNA insert after the seventh generation of backcrossing were allowed to self-fertilize and were subsequently used for analysis.

Reagents

All solvents and reagents were either molecular biology or reagent grade and were obtained from Fluka (Munich, Germany), Sigma-Aldrich (Munich, Germany), or Roth (Karlsruhe, Germany), unless otherwise noted.

Radiolabeled [1-¹⁴C]acetyl-CoA (57.5 mCi mmol⁻¹) was purchased from Amersham Biosciences (Uppsala, Sweden). [7-¹⁴C]Benzoyl-CoA was enzymatically synthesized from [7-¹⁴C]benzoic acid (57 mCi mmol⁻¹; Movarek Biochemicals, Brea, CA, USA) as previously described (Beuerle and Pichersky, 2002). Radiolabeled [2-¹⁴C]malonyl-CoA (53 mCi mmol⁻¹) was purchased from Movarek.

Enzyme extraction and assay

Crude protein extracts were prepared and assayed as previously reported (D'Auria *et al.*, 2002). For each time point, three replicates consisting of leaves from three different plants were combined, and assays were repeated at least three times. Assay samples were prepared by adding the following to a 0.6-ml microcentrifuge tube: 5.0 μl of crude extract, 10.0 μl of assay buffer (250 mM Bis-Tris propane, pH 7.7), 1.0 μl of 100 mM alcohol substrate dissolved in dimethyl sulfoxide, 1.0 μl of [1-¹⁴C]acetyl-CoA, and 33 μl of water to bring the assay volume to 50 μl.

The assays were carried out at room temperature (25°C) for 30 min and the radio-labeled acetate was counted as previously described (D'Auria *et al.*, 2002). The raw data (disintegrations per minute) were converted to picokatals based on the specific activity of the radiolabeled substrate and the known efficiency of the

scintillation counter used. Assays in which no alcohol substrate was added were performed to test background thioesterase activity, which was found to be less than 5 pkat mg protein⁻¹. In addition, boiled enzyme extracts were substituted for intact enzyme to test the non-enzymatic breakdown of the CoA thioester via a reaction with reducing agents in the enzyme extract. The identities of the products were verified by GC-MS as described previously using authentic standards (Dudareva *et al.*, 1998).

Volatile collection and analysis

Volatile collections used for GC-MS analysis from rosette plants were performed in a climate-controlled growth chamber [22°C, 55% relative humidity, and 150 µmol m⁻² s⁻¹ photosynthetically active radiation (PAR)] in 1-l flat-flanged glass vessels (Schott, Mainz Germany). The dynamic headspace collection was performed by supplying compressed air (2 l min⁻¹) that was purified by passage over a charcoal filter linked to a ZeroAir generator (Parker Hannifin Corp., Haverhill, MA, USA). The air was then pumped out of the glass vessel at a flow rate of 1 l min⁻¹ and passed over a filter containing 1.5 mg of activated charcoal (CLSA-Filter; Le Ruisseau de Mont-brun, Daumazan sur Arize, France). The remaining air escaped through the opening around the base of the chamber providing a positive pressure barrier against the entrance of ambient air. Headspace was collected for 4 h and the filters were then eluted with 100 µl CH₂Cl₂ containing 100 ng nonyl acetate as an internal standard. The volatile headspace collections were analyzed on a Hewlett-Packard 6890 gas chromatograph coupled to a Hewlett-Packard 5973 quadrupole mass-selective detector. Separation was performed on a (5%-phenyl)-methylpolysiloxane (DB5) column (J&W Scientific, Folsom, CA, USA) of 30 m × 0.25 mm inner diameter × 0.25-µm thickness. Helium was the carrier gas (flow rate 2 ml min⁻¹), a splitless injection (2 µl) was used and a temperature gradient of 5°C min⁻¹ from 40°C (3-min hold) to 240°C was applied. Parameters for electron impact ionization in the quadrupole mass detector were as follows: repeller, 30 V; emission, 34.6 µA; electron energy, 70 eV; quadrupole temperature, 150°C; source temperature, 230°C; transfer line temperature, 250°C. The mass spectrometer was run in the scan mode with an m/z ration of 40–350.

The identities of (Z)-3-hexen-1-yl acetate, (Z)-3-hexen-1-ol and (Z)-3-hexenal were determined by a comparison of retention times and mass spectra with those of authentic standards and with mass spectra in the National Institute of Standards and Technology (NIST) and Wiley (West Sussex, England) libraries. For quantification, response factors were computed using a seven-point standard curve made for each compound of interest using concentrations ranging from 897 pg µl⁻¹ to 89 ng µl⁻¹. The efficiency of the dynamic headspace collection system was tested by dissolving several different known concentrations of the three major *A. thaliana* GLVs in methanol. This solution (200 µl) was then placed in a 3-ml glass vial. Dynamic headspace collections were performed on duplicate samples under identical conditions as described for plant volatile collections. At the end of the experiment, the vials and filters were each extracted with 100 µl dichloromethane and analyzed alongside an unused portion of the same standard by GC-MS. After taking into account dilution factors, the efficiency of collection was determined by dividing the total quantity of compound collected on the charcoal filter by the total quantity of compound placed in the chamber subtracted from what was left over in the vial after the experiment. These factors were then taken into account and used as normalizers for the quantification of all GLVs collected from the dynamic system. For example, the efficiency of collection for (Z)-3-hexen-1-ol and

(Z)-3-hexen-1-yl acetate were found to be 60 and 50%, respectively, in the range normally emitted by plants in this study.

Headspace analysis of wild-type plants, those expressing an *At3g03480* RNAi hairpin construct and T-DNA knockout plants was accomplished using a zNose model 4200, a portable, rapidly-separating GC using an internal pump for vapor samples and a quartz, surface acoustic wave detector (SAW) (Electronic Sensor Technology, Newbury Park, CA, USA) in a similar fashion to that previously described by Kunert *et al.* (2002). For the analysis of *At3g03480* RNAi and T-DNA knockout plants, one leaf was excised from each plant, weighed, squeezed four times with a surgical clamp, and then placed in a 2-ml teflon sealed glass vial. After 15 min, the leaf headspace was sampled by inserting a small syringe needle through the cap and inserting the needle-like entrance port of the zNose into the vial. Air was sampled from the vial for 15 s and adsorbed onto a stainless steel GC column (length 1 m; DB 5; film thickness 0.25 µm; inner diameter 0.25 mm). Helium was the carrier gas at a flow rate of 3 ml min⁻¹ with the column temperature starting at 40°C and increasing to 180°C at a rate of 5°C sec⁻¹, while keeping the SAW at a constant 40°C for the duration of the run. The quantification of compounds was achieved by making a seven-point standard curve using standards for (Z)-3-hexen-1-yl acetate, (Z)-3-hexen-1-ol and (Z)-3-hexenal. Standards were introduced into the zNose using a heated glass desorber tube connected to a model 3100 vapor calibrator (Electronic Sensor Technology,) set at 210°C. The zNose method used in the analysis of *A. thaliana* GLVs is suitable for the separation of the three most common GLV compounds found in the *Ler* ecotype. The detector response for all three compounds was found to be linear over a range of 1–1000 ng, which is within the physiological ranges observed in our headspace collections.

Details of the PTR-MS instrument have been described in detail previously (de Gouw *et al.*, 2003; Lindinger *et al.*, 1998). The PTR-MS was operated at an E/N of 90 Td. The transmission of masses was calibrated and included in the calculated count rate per second measured for each mass, given here in arbitrary units (ncps; normalized counts per second). The detection limit of the PTR-MS was given by the background signal measured from contaminant-free air and the count rate per second, and was lower than 1 ncps.

The GLVs (Z)-3-hexenal, (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate fragment in proton-transfer reactions using H₃O⁺ under the settings described giving the following pattern of mass fragments. Because only singly charged ions play a role in PTR-MS, ions are referred to by their mass rather than by their mass-to-charge ratio. (Z)-3-Hexenal: m99 (31%, parent ion [RH⁺]), m81 (51%) and m57 (18%); (Z)-3-hexen-1-ol: m101 (1%, RH⁺), m83 (76%), m81 (1%), m67 (1%) and m55 (22%); (Z)-3-hexen-1-yl acetate: m143 (2%, RH⁺), m83 (73%), m61 (4%), m57 (1%) and m55 (18%). As a result of an overlap of fragment masses at m83 and m55, the abundance of (Z)-3-hexenal, (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate were corrected by the respective factor contributing to the sum of all fragments. This method for correction of the fragmentation pattern of (Z)-3-hexen-1-ol and (Z)-3-hexen-1-yl acetate was validated in a test experiment where four mixtures of a known ratio of (Z)-3-hexen-1-ol:(Z)-3-hexen-1-yl acetate were measured with the PTR-MS. The measured ratio was calculated by the correction factor as described above. In all ratios tested, the deviation from the expected outcome was less than 5%.

Quadruplicate experiments were carried out using five plants of the *Ler* ecotype of *A. thaliana* per experiment. For simultaneous measurements of PTR-MS on-line and zNose GC, a 2 l glass desiccator supplied with volatile organic compound

(VOC)-free, ozone-scrubbed air at a flow rate of 4 l min⁻¹ was used. The desiccator had one outlet line connected to the PTR-MS and one outlet for sampling with the z-Nose. The plants were set in the desiccator, wounded and immediately measured. The time resolution of the PTR-MS data was 9.2 s and data was integrated according to the time of air exchange of the desiccator to 27 s.

Isolation and cloning of At3g03480 (CHAT) cDNA and expression in E. coli and A. thaliana (ecotype Ler)

The cDNA for *At3g03480* (accession AF500201) was isolated as previously described (D'Auria *et al.*, 2002). The coding region of *At3g03480* was subcloned into the pET-T7 (28a) vector (Novagen, Madison, WI, USA) by introducing an *NdeI* site on the 5' end using the forward primer 5'-ACATATGGACCATCAAGTGTCTCTGCCAC-3' and a *BamHI* site on the 3' end after the stop codon with the backward primer 5'-TGGATCCTCATCCTTTAGACACATTTAGACTCC-3', which introduced a 6× *His* N-terminal tag. Expression and harvesting of the recombinant protein in BL21(DE3) *E. coli* cells was accomplished as previously described (D'Auria *et al.*, 2002).

The open reading frame of *CHAT* was subcloned into the binary vector pJML5 (courtesy of Jianming Li, University of Michigan, Ann Arbor, MI, USA), a derivative of the binary vector pCGN1547 (McBride and Summerfelt, 1990). This vector contains a CaMV 35S promoter, followed by a pBluescriptSK multicloning site (Stratagene, La Jolla CA, USA), and is flanked by the RbcSE9 terminator (accession no. M21375). The *At3g03480* cDNA was subcloned into the vector by insertion between the *XbaI* and *BamHI* restriction sites. The 5' end forward primer used was 5'-CTCTAGAATGGACCATCAAGTGTCTCTGCCAC-3', as well as the *BamHI* reverse primer mentioned above. Sequencing of all subcloned products in this study revealed that no PCR errors were obtained. The resulting plasmid, CHAT-pJML5, was subsequently transformed into *Agrobacterium tumefaciens* strain ASE101, which was used to transform *A. thaliana* ecotype *Ler* using the floral vacuum infiltration method (Bechtold *et al.*, 1993). Several independent lines were maintained as single-insertion homozygous lines based on their segregation of kanamycin resistance, increased enzyme activity and RT-PCR for the presence of the corresponding transgene.

Purification of recombinant proteins

All work with harvesting and purification of recombinant proteins was performed either on ice or in a refrigerated chamber operating at 8°C. Partial purification of CHAT from *A. thaliana* plants harboring the CHAT-pJML5 transgene construct was achieved by the use of DE53 anion exchange chromatography, followed by MonoQ, and finally by gel sizing on Superdex 75 (Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described (D'Auria *et al.*, 2002). Fractions were tested by radioactive enzyme assay and those fractions containing peak activity were pooled and used for subsequent purification steps.

CHAT protein containing a 6× *His* tag was harvested in *His*-Tag lysis buffer (50 mM Bis-Tris, pH 7.0, 500 mM NaCl, 10 mM imidazole and 10% w/v glycerol). BL21(DE3) cells containing heterologously expressed CHAT protein were sonicated twice with a Sonopuls HD2070 sonicator (Bandelin, Berlin, Germany) with the microprobe attachment at 60% full power for a 5-min 20% cycle. Cell debris was removed by centrifugation at 20 000 g. The supernatant was used for FPLC chromatography using a 1-ml

*His*Trap Ni Sepharose column (Amersham Biosciences). The elution of protein was performed in a three-step gradient consisting of 100, 250 and 500 mM imidazole. Fractions containing peak CHAT activity were pooled and concentrated using an Amicon 8200 concentrator with a 63.55-mm polyethersulfone filter with a 10-kDa molecular weight cut-off (Millipore, Bedford, MA, USA). The enzyme was then desalted into assay buffer (50 mM Bis-Tris, pH 7.0, 5 mM DTT and 10% w/v glycerol) using an Econo-Pac 10DG desalting column (Bio-Rad, Hercules, CA, USA). All purification steps were analyzed by SDS-PAGE gel electrophoresis followed by either Coomassie Brilliant Blue or silver staining of the gel. CHAT protein concentration was determined as previously described (Bradford, 1976).

Biochemical characterization

Determinations of biochemical properties for the purified CHAT enzyme including pH range, temperature stability, relative alcohol activity, effectors and kinetics were performed as previously described (D'Auria *et al.*, 2002).

Construction of the At3g03480 RNAi vector

In order to clone the *At3g03480* RNAi construct, the binary vector pFGC5941 for dsRNA production was obtained from the Arabidopsis Biological Resource Center (Columbus, OH, USA; stock no. CD3-447). A 452-bp segment from the 3' end of the *CHAT* cDNA (position 914–1365) was amplified by PCR using the forward primer, 5'-AATCTAGAGCGCGCCAGAAACCACCGCTAGAGCC-3', and backward primer, 5'-AAGGATCCATTTAAATTCATCCTTTAGACACATTTAGACTC-3'. The resulting fragment was cloned and placed into the TOPO-4 vector (Invitrogen, Carlsbad, CA, USA) for maintenance and sequencing. The vector containing the RNAi fragment was digested with *AscI* and *SwaI* and ligated into pFGC5941 to generate the plasmid CHAT-447.5. The vector was also digested with *BamHI* and *XbaI*, and ligated into CHAT-447.5 to generate the plasmid CHAT-447. The resulting product has a copy of the 452-bp fragment inserted between the *AscI* and *SwaI* sites, and an inverted repeat of the fragment inserted between the *BamHI* and *XbaI* sites. A spacer consisting of the chalcone synthase intron from *Petunia hybrida* lies between the two inserted sequences. CHAT-447 was transformed into *A. tumefaciens* strain GV3850, and was then used for the transformation of *A. thaliana* (ecotype *Ler*) via floral vacuum infiltration (Bechtold *et al.*, 1993). Several independent lines were maintained as single insertion homozygous stocks based on segregation with glufosinate ammonium, and the presence of the transgene.

DNA extraction and DNA gel blot analysis

Genomic DNA extraction and hybridization conditions for DNA gel blot analysis were performed as previously described (Dudareva *et al.*, 1998). Isolated DNA from individual plants was digested with the restriction enzyme *DraI* (New England Biolabs, Frankfurt am Main, Germany), run on a 1% agarose gel and transferred to HybondXL membrane (Amersham Biosciences). Transfer of the DNA to the membrane under alkaline conditions was carried out according to the manufacturer's protocol. The probe used in the hybridization was the 475-bp *XbaI/BamHI* fragment obtained from the CHAT-447 vector. This fragment was labeled using the Redi-Prime II labeling system (Amersham Biosciences) according to the manufacturer's protocol.

RNA extraction and determination of CHAT, HPL1 and APT1 gene expression by quantitative RT-PCR

Plant RNA was either extracted as previously described (D'Auria *et al.*, 2002) or via an LiCl precipitation method. Regardless of the isolation method, all RNA was subjected to an on-column DNase treatment (Qiagen, Hilden, Germany) to remove traces of genomic DNA. An aliquot of the RNA was measured by a spectrophotometer in the range of 230–300 nm. Based on the spectrophotometer reading, a dilution was made to 100 ng ml⁻¹ and the RNA was further analyzed on an Agilent 2100 Bioanalyzer RNA 6000 Nano Lab kit (Agilent Technologies, Palo Alto, CA, USA). The RNA concentration for all subsequent steps was based on the Bioanalyzer estimate.

Standardization of cDNA qRT-PCR templates was carried out according to a standardized protocol (Phillips M. and D'Auria J., manuscript in preparation). For reverse transcription, Superscript III (Invitrogen) was used according to the manufacturer's instructions. RNA was removed after the reaction by a 30-min digestion with 5 µl Rnase A (10 µg ml⁻¹) at 37°C. The resulting first-strand cDNA was purified on a Qiagen QIAquick PCR column by the addition of 10× volume of PB buffer. The purified cDNA was analyzed with the Bioanalyzer on a RNA 6000 Pico labchip (Agilent Technologies) without any dilution. Using smear analysis software (Agilent Technologies), the cDNA fragments in the range of approximately 500 bp–6 kb were used to estimate the effective cDNA concentration. All cDNA samples were diluted to 100 pg µl⁻¹ with nuclease-free water and used as templates in subsequent quantitative real time PCR experiments.

All quantitative RT-PCR experiments were performed on a Stratagene Mx3000P qRT-PCR machine using SYBR® green I with 6-carboxyl-X-rhodamine (ROX) as an internal standard according to the manufacturer's protocol. Each 20-µl reaction contained 100 pg purified cDNA as a template with the exception of non-RT controls, in which the cDNA was substituted with 100 pg total RNA. The thermal program was run as follows: 96°C for 10 min followed by 40 cycles of 30 s at 96°C, 30 s at 60°C and 30 s at 72°C. Fluorescence was read following both the primer annealing and elongation phases. All runs were followed by a melting curve analysis in which the temperature range was 55–95°C with a 1°C change per second. In all cases, the primers were optimized for efficiency by performing a series of five, fourfold dilutions on the purified cDNA template of known concentration. In this way, the linearity of the cycle times (C_t) values was also tested over three orders of magnitude against the log of template concentrations. R² values and primer efficiencies were calculated using the MxPro software (Stratagene, Amsterdam, The Netherlands). For each primer pair tested, three independent biological replicates were tested with similar template concentrations. In addition, three technical replicates were performed for each biological replicate.

The primers used in this study were all designed by BEACON DESIGNER version 4.0 software (Premier Biosoft, Palo Alto, CA, USA). All primer pairs incorporated an intron-spanning region so that a larger gene product would be found in the presence of genomic DNA. Among the criteria for primer design was the rejection of any potential primers displaying either high secondary structure or cross homology with all known similar sequences. The primers designed for *At3g03480* are 5'-AGCTTCTCTTGGATGTGGAAGGC-3' for the forward primer, and 5'-GAAGCGGAGTGCGAAGATAAATC-3' for the backward primer. The subsequent cDNA amplicon is 107 bp in length. The primers for *HPL1 At4g15440* are 5'-GGCGTTCGTGTTGGAGTTTATC-3' for the forward primer, and 5'-GGATTCGATTGTTCCCCAGAA-3' for the backward primer. The subsequent cDNA amplicon is 190 bp in length. The primers designed for *APT1 At1g27450* are 5'-GTTGCAGGTGTTGAAGCTA-

GAGGT-3' for the forward primer, and 5'-TGGCACCA-ATAGCCAACGCAATAG-3' for the backward primer. The subsequent cDNA amplicon is 64 bp in length. All primers were HPLC purified and have efficiencies between 90–100%. In addition, the products of each primer combination were cloned and sequenced a minimum of three independent times to confirm the sequence of the amplicon.

Comparative quantification experiments included a minimum of two biological replicates with three technical replicates for each sample unless otherwise stated. The $\Delta\Delta C_t$ method of comparative quantification employing efficiency correction was used to judge the relative quantification of the starting template for all genes of interest (Pfaffl, 2001). Data were not used further when the non-template (H₂O) controls had an amplified product within five C_t values of the highest C_t for the true biological samples.

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