Veratrole Biosynthesis in White Campion

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White campion (Silene latifolia) is a dioecious plant that emits 1,2-dimethoxybenzene (veratrole), a potent pollinator attractant to the nocturnal moth Hadena bicruris. Little is known about veratrole biosynthesis, although methylation of 2-methoxyphenol (guaiacol), another volatile emitted from white campion flowers, has been proposed. Here, we explore the biosynthetic route to veratrole. Feeding white campion flowers with [13C9]-phenylalanine increased guaiacol and veratrole emission, and a significant portion of these volatile molecules contained the stable isotope. When white campion flowers were treated with the phenylalanine ammonia lyase inhibitor 2-aminoindanol-2-phosphonic acid, guaiacol and veratrole levels were reduced by 50% and 63%, respectively. Feeding with benzoic acid (BA) or salicylic acid (SA) increased veratrole emission 2-fold, while [3H]BA and [3H]SA feeding indicated that the benzene ring of both guaiacol and veratrole is derived from BA via SA. We further report guaiacol O-methyltransferase (GOMT) activity in the flowers of white campion. The enzyme was purified to apparent homogeneity, and the peptide sequence matched that encoded by a recently identified complementary DNA (SIGOMT1) from a white campion flower expressed sequence tag database. Screening of a small population of North American white campion plants for floral volatile emission revealed that not all plants emitted veratrole or possessed GOMT activity, and SIGOMT1 expression was only observed in veratrole emitters. Collectively these data suggest that veratrole is derived by the methylation of guaiacol, which itself originates from phenylalanine via BA and SA, and therefore implies a novel branch point of the general phenylpropanoid pathway.

Many flowers emit volatile compounds that act as long-range, and sometimes short-range, pollinator attractants. Floral scents are often composed of a complex blend of odorants, which might include terpenoids, phenylpropanoids, fatty acid derivatives, and amino acid-containing compounds (Pichersky and Gershenzon, 2002; Dudareva et al., 2006; Knudsen et al., 2006). An enormous diversity exists in the composition of floral scents among species, and bouquets often vary even within a species. The evolution of scent might be aided by the ability of many pollinators, mostly insects, to discriminate and interpret complex floral mixtures by recognizing specific individual components in such mixtures (Ando et al., 2001; Lei and Vickers, 2008; Koeduka et al., 2009; Reinhard et al., 2010). While some insect pollinators are considered generalists and will visit many types of flowers with diverse bouquets, some are specialists and visit exclusively or predominantly just one plant species, which they recognize via its floral scent. The relationship between white campion (Silene latifolia) and the noctuid moth Hadena bicruris (lychnis moth) is a prime example of the latter.

White campion is a night-blooming dioecious plant that is native to Europe and emits approximately 50 different compounds from its male and female flowers during the night (Dötterl et al., 2005; Muhlemann et al., 2006; Waelti et al., 2008). H. bicruris is specifically attracted to the floral scent emitted by white campion at night, and pollination typically occurs during nectar ingestion (Brantjes, 1976; Labouche and Bernasconi, 2010). However, female moths also lay their eggs inside female flowers after foraging, and the larvae subsequently feed on the developing seeds (Bopp and Gottsberger, 2004). Approximately 25% of the seeds are consumed by the larvae, dramatically impacting the fitness of white campion in this nursery pollination system (Wolfe, 2002). Among the volatiles emitted by the male and female flowers of white campion is veratrole (1,2-dimethoxybenzene). Veratrole is one of only seven compounds emitted by white campion that elicit strong behavioral responses in H. bicruris (Dötterl et al., 2006). Following pollination, the emission of veratrole drastically decreases while the emission of other behaviorally active compounds remains unchanged (Muhlemann et al., 2006). The postpollination decrease in veratrole is believed to reduce visits by the moths to pollinated flowers, thus reducing further seed predation by H. bicruris (Muhlemann et al., 2006). Interestingly, veratrole is not a component of the floral bouquet in the related species Silene dioica, and rarely does H. bicruris oviposit into the flowers of this plant (Bopp and Gottsberger, 2004; Waelti et al., 2008).

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Despite the central role of veratrole in the white campion (*H. bicruris*) nursery pollination relationship and in other plant-pollinator interactions (Kumano-Nomura and Yamaoka, 2009), little is known about its biosynthesis. Based upon its structure, it is likely that veratrole is produced via methylation of guaiacol (2-methoxyphenol), which is also present in white campion flowers (Dötterl and Jürgens, 2005; Dötterl et al., 2005; Waelti et al., 2008). Indeed, we recently identified a complementary DNA (cDNA) in an EST database of white campion flowers that upon expression in *Escherichia coli* produced an enzyme that catalyzed this reaction in vitro and, therefore, was designated as guaiacol-\(\text{O}^+\)-methyltransferase (*SlGOMT1*; Gupta et al., 2012). However, direct evidence for GOMT activity in flowers of white campion was not presented in that work.

Guaiacol has been detected in several plants, most notably in tomato (*Solanum lycopersicum*), where it contributes to fruit flavor (Klee, 2010). In tomato, guaiacol is derived via the methylation of catechol (2-methoxycatechol), which is also present in white campion flowers (Dötterl and Jürgens, 2005; Dötterl et al., 2005; Waelti et al., 2008). Indeed, we recently identified a complementary DNA (cDNA) in an EST database of white campion flowers that upon expression in *Escherichia coli* produced an enzyme that catalyzed this reaction in vitro and, therefore, was designated as guaiacol-\(\text{O}^+\)-methyltransferase (*SIGOMT1*; Gupta et al., 2012). However, direct evidence for GOMT activity in flowers of white campion was not presented in that work.

Guaiacol has been detected in several plants, most notably in tomato (*Solanum lycopersicum*), where it contributes to fruit flavor (Klee, 2010). In tomato, guaiacol is derived via the methylation of catechol by catechol-\(\text{O}^+\)-methyltransferase (*CTOMT1*; Mageroy et al., 2012); however, the origin of catechol is still unknown. Although there is little overlap between the aroma compounds found in tomato fruit and white campion flowers, both produce guaiacol and methyl salicylate (MeSA), the latter of which is made from salicylic acid (SA; Ross et al., 1999; Tieman et al., 2010; Mageroy et al., 2012). Interestingly, tomato introgression lines with elevated fruit guaiacol levels, due in part to higher *CTOMT1* expression, also exhibit a modest decline in SA, suggesting a shared pathway between these two aroma compounds.

Here, we performed substrate, inhibitor, and stable isotope feeding experiments to elucidate the pathway to veratrole biosynthesis in white campion. We demonstrate that veratrole is likely derived from Phe via...
benzoic acid (BA) and SA. We also provide additional details on SIGOMT1, the methyltransferase involved in the final step of veratrole biosynthesis.

RESULTS

Evidence That Veratrole Is Derived from the Phenylpropanoid Pathway

Based upon the chemical structure of guaiacol and veratrole, it is reasonable to hypothesize that they are derived from the general phenylpropanoid pathway originating with L-Phe. To test this possibility, the stems of white campion flowers were placed in a solution containing 2-aminoindan-2-phosphonic acid (AIP). AIP is a competitive inhibitor of phenylalanine ammonia lyase (PAL), the enzyme that catalyzes the committed step in the phenylpropanoid pathway by converting Phe to trans-cinnamic acid (Cin). Following a 4-h treatment with the inhibitor, volatiles were collected by solid-phase microextraction (SPME) and analyzed by gas chromatography-mass spectrometry (GC-MS). AIP treatment caused a 50% decrease in the emission of both MeSA and guaiacol and a 60% decrease in veratrole (Fig. 1). There was no significant change in phenylacetaldehyde or in the sesquiterpene β-farnesene, two other volatiles in the floral bouquet. There was, however, a 4-fold increase in the emission of phenylethanol, which in plants is derived from Phe via phenylacetaldehyde (Kaminaga et al., 2006; Tieman et al., 2006), suggesting that the acetaldehyde reductase that converts 2-phenylacetaldehyde to 2-phenylethanol is not limiting and, therefore, that any excess of 2-phenylacetaldehyde that is not emitted (at the same rate as before) is quickly converted to 2-phenylethanol.

Feeding white campion flowers with a 5 mM solution of Phe for 4 h in the dark caused a 1.6-fold increase

![Figure 3. Stable isotope incorporation into white campion volatiles. Detached flowers were fed water (control) or solution containing 2 mM BA-d5 or 2 mM SA-d6 for 4 h in the dark, and volatiles were then analyzed by GC-MS. The mass spectra (total ion current [TIC]) of veratrole, guaiacol, MeSA, and phenylethanol obtained from plants treated with water (control), BA-d5, or SA-d6 are shown. Note the 4-mass-unit shift of the parent ions of veratrole (138), guaiacol (124), and MeSA (152) upon feeding with BA-d5 or SA-d6. Mass spectra are representative of a minimum of six feeding experiments. m/z, Mass-to-charge ratio.](image-url)
in veratrole emission (Fig. 2), and when \(^{13}\text{C}_9\)L-Phe uniformly labeled with the stable isotope \(^{13}\text{C}\) was used at a concentration of 2 mM, approximately 8% of the veratrole molecules (and approximately 7% of the guaiacol molecules) had a 6-D increase in their molecular mass, indicating that their benzene ring was derived from the labeled Phe (Supplemental Fig. S1).

Since Phe appeared to be a precursor of veratrole, we tested whether certain downstream intermediates in phenylpropanoid metabolism as well as other potential substrates could serve as precursors for veratrole synthesis when fed to white campion flowers. These precursor candidates (for a complete list, see Supplemental Table S1) were fed at a concentration of 5 mM for 4 h in the dark, and then volatiles were collected. Only those compounds that resulted in a significant increase in veratrole emission are presented in Figure 2. Feeding with Cin increased veratrole emission by 1.7-fold. Feeding with guaiacol, the proposed precursor of veratrole, caused a 30-fold increase, while feeding with catechol, the precursor to guaiacol (Mageroy et al., 2012), increased veratrole emission by 6-fold. Surprisingly, both BA and SA feeding increased veratrole emission by approximately 2-fold.

Stable Isotope Feeding Suggests That Veratrole Is Derived from BA via SA

To validate that BA and SA are bona fide precursors of veratrole in planta and are not simply inducing veratrole synthesis when fed to white campion flowers, the feeding experiments were repeated with deuterium-labeled \(^{2}\text{H}_5\)benzoic acid (BA-d5) and \(^{2}\text{H}_6\)salicylic acid (SA-d6), and the incorporation of deuterium into the main volatiles emitted was analyzed by GC-MS. When BA-d5 at a concentration of 2 mM was fed to detached white campion flowers for 4 h in the dark, we observed that 15% of the veratrole molecules emitted had a 4-D shift in the parent ion mass. A shift of 15% of the emitted molecules was also observed for both guaiacol and MeSA (Fig. 3). No apparent mass shift was observed in phenylethanol (Fig. 3) or in phenylacetaldehyde or \(\beta\)-farnesene, the other main volatiles in the bouquet. When SA-d6 was fed to detached flowers, a 4-D shift was again observed in 50%, 60%, and 80% of the parent ions of emitted veratrole, guaiacol, and MeSA, respectively (Fig. 3). It should be noted that only four deuterium atoms remain on the SA-d6 label in solution, since the carboxyl group on SA (pKa of approximately 3) completely ionizes at physiological pH, while the deuterium atom on the ring-bound hydroxyl group readily undergoes H/D back exchange. The 4-D shift in the mass of guaiacol and veratrole (as well as MeSA) with either BA-d5 or SA-d6 feeding suggests that both BA and SA are converted into these volatiles, with the retention of only the deuterium atoms directly bound to the benzene ring carbons that are not functionalized. Since a higher percentage of guaiacol and veratrole (as well as MeSA) showed the 4-D shift when SA-d6 was fed compared with BA-d5 feeding, it also suggests that SA is downstream from BA in the pathway to these volatiles.

The synthesis of BA from Cin via the \(\beta\)-oxidation pathway in the peroxisomes has recently been demonstrated (Van Moerkercke et al., 2009, Klempien et al., 2012, Lee et al., 2012, Qualley et al., 2012). The simultaneous inhibition of PAL, which should decrease the internal pool of BA, together with exogenously supplied BA-d5 would be predicted to cause increases in the proportions of veratrole, guaiacol, and MeSA molecules with a 4-D mass increase. A similar prediction could be made for feeding SA-d6 in combination with AIP. While approximately 15% of the emitted veratrole, guaiacol, and MeSA molecules showed the 4-D mass shift following feeding with BA-d5 alone,
over 60% of these volatiles showed such a shift when feeding was performed in the presence of AIP (Fig. 4A). Similarly, if SA is derived from BA, as some reports have indicated (Klämbt, 1962; León et al., 1993; Yalpani et al., 1993; Meuwly et al., 1995; Silverman et al., 1995; Coquoz et al., 1998; Ribnicky et al., 1998), an increase in the percentage of labeled volatile molecules would be expected in similar experiments involving SA-d6 feeding in the presence versus absence of AIP. Indeed, while SA-d6 feeding alone resulted in 50%, 60%, and 80% of guaiacol, veratrole, and MeSA molecules showing the 4-D mass shift, respectively, in the presence of AIP, the label was incorporated into more than 90% of these volatile compounds (Fig. 4A).

To test whether veratrole is synthesized from Phe with SA as an intermediate, an isotope-trapping experiment was performed in which [13C9]L-Phe was fed alone or together with unlabeled SA. Feeding with [13C9]L-Phe at a concentration of 10 mM labeled approximately 20% of the emitted veratrole. However, less than 4% of the veratrole was labeled when [13C9]L-Phe was coadministered with an equimolar amount of SA (Fig. 4B). Moreover, the amount of phenylethanol that was labeled by feeding [13C9]L-Phe increased from 13% to 28% when SA was coadministered (Fig. 4B), likely due to the buildup of the trapped label.

Guaiacol O-Methyltransferase Activity in White Campion Flowers

Substrate and stable isotope feeding experiments suggest that guaiacol is the immediate precursor to veratrole in white campion flowers. Consistent with this observation, we recently identified a cDNA from a white campion EST library that encodes a protein that can convert guaiacol to veratrole in vitro (Fig. 5A) and, therefore, was designated as guaiacol O-methyltransferase (SIGOMT1; Gupta et al., 2012). To examine GOMT activity in white campion flowers, we tested desalted crude protein extracts from white campion flowers with guaiacol and the universal methyl donor S-adenosyl Met. Following incubation at room temperature, volatile compounds were collected by SPME and analyzed by GC-MS. Veratrole was readily detected after a 15-min incubation with as little as 5 μg of crude protein extract (Fig. 5B). When the assay was conducted in the presence of 14C-labeled S-adenosyl Met, a single product was resolved by thin-layer chromatography that comigrated with an authentic veratrole standard (Fig. 5C). The mass spectrometry spectra of the reaction product also matched that of an authentic veratrole standard (Supplemental Fig. S2), and veratrole could not be detected in either reaction when guaiacol was omitted from the assay or when the crude protein extract was denatured beforehand.

To probe the tissue specificity of veratrole biosynthesis, various parts of male and female plants were tested for veratrole emission and GOMT activity. The vegetative tissues of white campion male and female
plants are morphologically similar. However, strict genetic control of sex expression leads to anatomically distinct flowers (Fig. 5D, top panels). GOMT activity was detected in all parts of male and female flowers, but by far the highest levels were found in petal tissue (Fig. 5D, bottom panels). In comparison with petal tissue, GOMT activity in leaves, roots, and stems was several hundred-fold lower. Consistent with the distribution of GOMT activity and as reported previously (Gupta et al., 2012), veratrole emission was only detected from flowers of either sex.

**Purification of GOMT from White Campion Flowers**

The protein responsible for the conversion of guaiacol to veratrole in GOMT assays with crude protein extracts from white campion flower petal tissue was purified to apparent homogeneity. Compared with the crude protein extract, the specific activity was enriched by 42-fold with a recovery of 1.1%. Each step of the purification procedure is outlined in Table I. The GOMT activity eluted as a single peak on four different column matrices, which included anion exchange on DEAE cellulose, mixed-mode separation on hydroxypatite, affinity separation on adenine agarose, and hydrophobic interaction on butyl Sepharose (Fig. 6A). The protein fractions from the various purification steps were analyzed by SDS-PAGE and revealed the progressive enrichment of an approximately 40-kD protein (Fig. 6B). This protein was noticeably absent in fractions that were not retained by adenine agarose affinity (Fig. 6B), and GOMT activity was undetectable in this particular protein fraction. The molecular subunit mass of the purified protein was also similar in size to previously reported orthodiphenol O-methyltransferases (Pellegrini et al., 1993). The purified protein was subjected to in-gel tryptic digestion, and the amino acid sequence of the resulting peptides was determined. All peptide sequences that were obtained matched the protein encoded by SIGOMT1 (Gupta et al., 2012). Based on the nucleotide sequence of SIGOMT1, the full-length cDNA was expressed recombinantly in E. coli with a C-terminal His_6 tag. Recombinant SIGOMT1 was purified by Ni^{2+} affinity chromatography and subsequently tested with 15 potential substrates under standard GOMT assay conditions (Supplemental Fig. S3, A–C). Only guaiacol proved to be an efficient substrate and exhibited Michaelian kinetics with kinetic constants similar to those reported previously (Gupta et al., 2012).

**Veratrole Emission Is Associated with SIGOMT1 Expression**

The North American populations of white campion exhibit a significantly higher variation in floral scent compared with their European counterparts, from which they originated (Dötterl et al., 2005). Most notably, veratrole is absent in the floral scent of many individuals from North America, while it is a characteristic odorant in European populations (Dötterl and Jürgens, 2005; Dötterl et al., 2005; Waelti et al., 2008). Therefore, we sampled a small number of individuals (n = 27) of white campion plants collected at a single location in North America (Delph et al., 2010) for volatile emission. Indeed, several male (n = 5) and female (n = 6) plants were identified that did not emit veratrole (Fig. 7A). Among these individuals, we tested three males and three females for floral GOMT activity in crude protein extracts and found that this activity was only associated with those plants that emit veratrole (Fig. 7B). Furthermore, when SIGOMT1 expression was probed by reverse transcription (RT)-PCR using gene-specific primers, we could only detect transcripts in veratrole emitters (Fig. 7C), suggesting that SIGOMT1 expression is required for veratrole emission in this North American population.

**Veratrole Emission during the Day/Night Cycle**

The emission of floral volatiles in white campion, as in many other nocturnally pollinated plants, is confined to the night (Dötterl and Jürgens, 2005; Dötterl et al., 2005). However, the precise time at which veratrole is emitted has not been reported. Therefore, we performed a detailed time-course analysis of veratrole emission during a typical 12-h-light/12-h-dark photoperiod. In addition, given the relationship between SIGOMT1 and veratrole emission, we also assayed GOMT activity in male and female flowers of white campion during this time period. With our North American accession, we

<table>
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<th>Purification Step</th>
<th>Total Protein (mg)</th>
<th>Total Activity (pkat)</th>
<th>Specific Activity (pkat/mg)</th>
<th>Purification Fold</th>
<th>Yield (%)</th>
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<td>3.6</td>
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<td>309</td>
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did not detect significant differences in veratrole emission between male and female flowers (Supplemental Table S2). Veratrole was not detected during the day, and emission commenced upon the onset of darkness at 6 PM, peaked during the hours of 10 PM and 2 AM, and concluded at 5 AM (Fig. 8). On the other hand, GOMT

Figure 6. Purification of GOMT activity from white campion flowers. A, Elution profiles of GOMT activity from DE53 cellulose, hydroxyapatite, and butyl Sepharose. At each step of the purification, a single peak of GOMT activity was resolved. B, SDS-PAGE of fractions obtained during the successive steps of GOMT purification. Fractions are the crude extract (approximately 10 mg; lane 1), DEAE cellulose (approximately 10 mg; lane 2), hydroxyapatite (approximately 10 mg; lane 3), proteins not retained by adenosine agarose that did not exhibit GOMT activity (approximately 10 mg; lane 4), adenosine agarose (approximately 3 mg; lane 5), and butyl Sepharose (approximately 1 mg; lane 6). Molecular mass markers are indicated on the left in kD, and proteins were visualized by Coomassie Brilliant Blue staining.

Figure 7. Veratrole emission depends on the expression of SIGOMT1. A, Representative gas chromatography traces of the volatile compounds emitted from flowers that produce veratrole (top panel) and those that do not (bottom panel). The missing veratrole peak in the bottom panel is indicated with an arrow. The other peaks are indicated by the following numbers: 1, phenylacetaldehyde; 2, MeSA; 3, β-farnesene; 4, guaiacol; 5, phenylethanol. TIC, Total ion current. B, Total GOMT activity in crude flower tissue extracts from veratrole emitters (+) and nonemitters (−). Data are mean values ± SE obtained from three independent extractions of pooled tissue from the corresponding groups. C, Gene expression of SIGOMT1 in veratrole emitters and nonemitters. A group of six plants (three male and three female) that emit veratrole (+) and another group of six plants (three male and three female) that do not (−) were identified. The expression of SIGOMT1 was probed in these plants by RT-PCR (38 PCR cycles) using pooled tissue from the corresponding groups. As a positive control, RT-PCR using actin-specific primers was performed on the same set of samples.
activity stayed at relatively constant levels during the day and night (Fig. 8).

DISCUSSION

Veratrole and MeSA Are Derived from Phe with BA and SA as Intermediates in White Campion Flowers

While long suspected (Klämbt, 1962; Yalpani et al., 1993; Meuwly et al., 1995; Silverman et al., 1995; Coquoz et al., 1998; Ribnicky et al., 1998), it has recently been convincingly demonstrated in petunia (Petunia hybrida) and Arabidopsis (Arabidopsis thaliana) that BA is made by β-oxidation of Cin (Van Moerkercke et al., 2009; Colquhoun et al., 2012; Klempien et al., 2012, Lee et al., 2012; Qualley et al., 2012). The synthesis of SA is not as clearly resolved. In tobacco (Nicotiana tabacum), a 2-hydroxylase that converts BA to SA was reported (Yalpani et al., 1993; León et al., 1995). However, genetic evidence suggests that in Arabidopsis, SA is made from isochorismate, a side product of the shikimate pathway (Wildermuth et al., 2001), although a recent report also showed a decrease in SA production in Arabidopsis PAL mutants (Huang et al., 2010).

Since our initial labeling experiments with [13C9]-Phe resulted in the incorporation of the label into MeSA, guaiacol, and veratrole (Supplemental Fig. S1), we followed with labeling experiments using BA-d5 and SA-d6. Since feeding with BA-d5 showed incorporation into the benzene ring of MeSA, as well as an increase in the percentage of MeSA molecules with the deuterium label from 15% to 60% upon inhibition of PAL with AIP treatment, our data indicate that the pathway Phe → BA → SA predominates in white campion flowers.

Our data also show that SA is an intermediate in the pathway to veratrole biosynthesis in white campion. The 4-D shift observed in both guaiacol and veratrole after feeding with SA-d6 indicates that the hydroxyl group in the ortho-position of SA-d6 is retained and the carboxyl group is substituted with a hydroxyl group prior to the incorporation of SA-d6 into guaiacol and veratrole. These results are also consistent with the increase in labeled guaiacol and veratrole when SA-d6 is fed together with AIP and with the decrease in labeled guaiacol and veratrole when [13C9]-Phe is fed...
together with unlabeled SA (Fig. 4). Taken together, these results indicate that BA is first hydroxylated at the ortho-position to SA and then converted to catechol (1,2-dihydroxybenzene), which is next methylated to guaiacol. SIGOMT1 then converts guaiacol to veratrole (Fig. 9). It is most likely that SA is converted directly to catechol by oxidative decarboxylation, because a simple decarboxylation will produce phenol first, and phenol does not appear to be a precursor for veratrole (Supplemental Table S1). While the enzymatic conversion of SA to catechol has not yet been documented in plants, it has been reported that yeast and microbes oxidatively decarboxylate SA to catechol as part of the modified 3-oxoadipate and meta-cleavage pathways (You et al., 1990; Eppink et al., 1997; Bosch et al., 1999; Holesova et al., 2011).

SA is a well-studied hormone in plants (for review, see Dempsey et al., 2011). However, not only has its mode of degradation been uncertain, but how plants degrade this hormone has also not been resolved. Our evidence suggests that, similar to bacteria and fungi, plants have enzymes that convert SA to catechol. It will be of interest to determine if the plant enzymes are homologous to the microbial ones or whether plants evolved a different type of enzyme(s). Regardless of the origin of the SA-degrading enzyme in plants, it appears that in white campion, such an enzyme has been recruited for the synthesis of veratrole, a key pollinator attractant in this species. A similar evolutionary adaptation may have occurred in tomato, whose fruit accumulates SA, catechol, and the flavor molecule guaiacol (Mageroy et al., 2012). The synthesis of SA and other hormones in terminal organs of the plant (flowers, fruits) and the conversion of such hormones to end products that serve an ecological role appear to be common phenomena, with the scent volatiles methyl jasmonate and jasmonate, derived from the hormone jasmonic acid, being another example (Knudsen et al., 2006).

The Synthesis of Veratrole Is Catalyzed by SIGOMT1

Our results show that both male and female flowers of white campion possess GOMT activity, which was purified to apparent homogeneity through a four-step chromatographic procedure. We identified this enzyme as SIGOMT1, a recently characterized O-methyltransferase that can convert guaiacol to veratrole in vitro (Gupta et al., 2012). SIGOMT1 was found exclusively in petal tissue, which is the site of veratrole emission, and specifically methylates guaiacol with kinetic properties that are well within the range of previously reported plant orthodiphenol O-methyltransferases (Gang et al., 2002; Lavid et al., 2002; Mageroy et al., 2012). Since GOMT activity eluted as a single peak on four different chromatographic matrices and because plants that fail to emit veratrole do not express SIGOMT1 nor have GOMT activity, we conclude that SIGOMT1 is the enzyme responsible for the final step in veratrole biosynthesis. Despite the fact that SIGOMT1 expression critically impacts veratrole emission, in vitro GOMT enzyme activity changes little during the day and night, suggesting that SIGOMT1 could be allosterically regulated in vivo or that veratrole emission is controlled at the level of substrate availability.

The Evolution of Veratrole Emission

Since the introduction of white campion to North America approximately 200 years ago (McNeill, 1977), a noticeable change has occurred in the floral scent composition of this population compared with its European counterpart (Dötterl and Jürgens, 2005; Dötterl et al., 2005). The majority of variation in odor between the bouquets of the flowers from the two continents is attributed to veratrole emission, which is frequently absent in the scent bouquet of North American white campion (Dötterl and Jürgens, 2005; Dötterl et al., 2005). Although veratrole is a potent attractant to H. bicruris (Dötterl et al., 2006), this specialist pollinator did not accompany white campion to North America (Wolfe, 2002). In this population, the flower visitors are typically generalist insect pollinators (Young, 2002); thus, selection pressure on veratrole synthesis is presumably absent, contrary to European white campion, whose reproductive success apparently depends on the emission of this floral volatile (Dötterl et al., 2006).

MATERIALS AND METHODS

Chemicals and Reagents

Sodium benzoate-d5 (99.3% atom deuterium) and 2-hydroxybenzoic acid-d6 (98.8% atom deuterium) were from CDN Isotopes. L-Phe-U-13C9 (97%–98%) was from Cambridge Isotope Laboratories. AIP was a kind gift from Dr. Till Beuerle (Technical University Braunschweig). S-[Methyl-14C]adenosyl-L-Met (47 mCi mmol⁻¹) was from Perkin-Elmer. Adenosine agaro agar affinity gel (5′-AMP-agaro, C8 attachment) was from Sigma, hydroxyapatite bio-gel HT was from Bio-Rad, and DE53 DEAE cellulose was from Whatman. All other chemicals were from Sigma.

Plant Material and Growth Conditions

Seed for white campion (Silene latifolia) was a kind gift from Dr. Lynda Delph and originated from a naturally occurring population near Blacksburg, Virginia. Plants were grown in potting soil (Fafard) supplemented with Osmocote (Scotts) and maintained in a growth chamber under a 12-h photoperiod (approximately 300 µmol m⁻² s⁻¹; mixed cool-white and incandescent bulbs). Temperature was maintained at 22°C during the light period and 18°C during darkness.

Feeding White Campion Flowers and Volatile Analysis

Flowers from either male or female white campion plants were excised at the base of the calyx and placed in a 10-ml feeding solution right before the onset of darkness. Flowers in the feeding solution were transferred to a sealed vacuum desiccator for 4 h in the dark. Following the feeding period, a SPME fiber was inserted through the top of the desiccator, and volatiles were collected for 15 min in the dark. Under these conditions, the amount of total volatiles collected using SPME fibers from the head space of detached flowers correlated linearly with time from 2 to 30 min. Substrate feeding was performed at a concentration of 5 mM, and stable isotope feeding was at a concentration of 2 mM. The
optimal stable isotope feeding concentration was determined by measuring the incorporation of BA-d5 into veratrole over a concentration range of 0.1 to 30 μM BA-d5. The deuterium label incorporated into veratrole was linear up to 20 μM BA-d5. Volatiles were analyzed on a GC17-A coupled to a QP-5000 GC-MS system (Shimadzu) equipped with an EC-WAX column (Grace Davison; 30-m length, 0.25-mm film thickness) using the following temperature program: 44°C for 3.5 min, ramp of 5°C min−1 up to 200°C, ramp of 70°C min−1 up to 275°C, and hold for 1 min. The injector temperature was 220°C operating in the splitless mode, and the interface temperature was 280°C. The identification of volatiles emitted by white campion flowers was based on comparisons of the retention time and mass spectrometry spectra of authentic standards. For the quantification of veratrole emission, standard concentration curves using authentic 1,2-dimethoxyphenol (veratrole), guaiacol, and MeSA were constructed. Other floral volatiles were first normalized to β-farnesene, a volatile terpene whose emission is not affected by the various treatments, and then expressed as a percentage of untreated control plants.

**Protein Extraction and GOMT Enzyme Assays**

Plant material was flash frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle. Protein was extracted in 100 mM Tris-HCl, pH 7.5, containing 5 mM MgCl2, 10 mM β-mercaptoethanol, 10% glycerol [v/v], and 3% insoluble polyvinylpoly-pyrrolidone [w/v]. Soluble crude extracts were clarified by centrifugation at 4°C and desalted into 50 mM potassium phosphate, pH 6.5. Active fractions were pooled, desalted into buffer B, and then expressed as a percentage of untreated control plants.

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Supplemental Data

The following materials are available in the online version of this article.

**Supplemental Figure S1.** Stable isotope feeding of white campion flowers with [13C6]Phe.

**Supplemental Figure S2.** Mass spectral analysis of the reaction product obtained in GOMT assays with crude protein extracts from white campion flowers.

**Supplemental Figure S3.** Enzymatic characterization of SIGOMT1.

**Supplemental Table S1.** List of substrates used in white campion feeding experiments.

**Supplemental Table S2.** Veratrole emission from male and female flowers.

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