Variations on a theme: synthesis and modification of plant benzoic acids
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Plant benzoic acids (BAs) are critical regulators of a plant’s interaction with its environment. In addition, innumerable plant-derived pharmacological agents contain benzyol moieties. Despite the prevalence and import of plant BAs, their biosynthetic pathways are not well-defined. Mounting evidence suggests that BAs are synthesized both directly from shikimate/chorismate and from phenylalanine in plants; however, few genes in these pathways have been identified. Exciting progress has been made in elucidating genes that modify BAs via methylation, glucosylation, or activation with Coenzyme A. As these modifications alter the stability, solubility, and activity of the BAs, they impact the functional roles of these molecules. The combination of multiple BA biosynthetic routes with a variety of chemical modifications probably facilitates precise temporal and spatial control over active forms, as well as the channeling of intermediates to particular benzoate products.

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Current Opinion in Plant Biology 2006, 9:288–296

This review comes from a themed issue on Physiology and metabolism
Edited by Erin Pichersky and Krishna Niyogi

Available online 4th April 2006
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DOI 10.1016/j.pbi.2006.03.006

Introduction
Plant benzoic acids (BAs) and their derivatives are common and widespread mediators of plant responses to biotic and abiotic stress. For example, salicylic acid (SA, 2-hydroxybenzoic acid) is a key signaling molecule that mediates plant defense against a variety of pathogens in a number of species, including tobacco and Arabidopsis thaliana. Its accumulation is required for the establishment of local and systemic required resistance (SAR) responses [1]. SA is also synthesized in response to oxidative stressors, such as ozone, and in part responsible for induced plant protective responses [2]. The volatiles methyl benzoate (MeBA) and methyl salicylate (MeSA) are present in the floral scents of more than 100 species of 30 different families and function as pollinator attractants [3**]. In addition, MeSA is involved in tritrophic interactions; MeSA is emitted in response to herbivore damage (e.g. that caused by aphids or mites), attracting the predator of the herbivore [3**4]. As shown in Figure 1, BAs are also incorporated into numerous plant secondary metabolites that have established roles in plant-herbivore or plant-pathogen interactions, such as BA glucosinolate esters (e.g. in A. thaliana [5]), salicina (the major phenolic glycoside in willow [6]), xanthenes (e.g. in Hypericum androsaemum [7]), cocaine (in Erythroxylum coca [8]), and taxol (in Taxus cuspidate [9]). Many of these BA and benzyol compounds are also important pharmacological agents; for example, salicylate is the active ingredient in aspirin, taxol is a potent anti-cancer drug, and cocaine is a local analgesic.

Despite the importance and prevalence of plant benzoates, the biosynthesis of BAs is not well defined. It is likely that several BA biosynthetic pathways exist in a given species, providing fine control over spatial and temporal synthesis and channeling intermediates to particular benzoate products. A now classic example is the synthesis of the active C5-unit isopentenyl diphosphate (IPP), the building block of all isoprenoids. A cytosolic mevalonate pathway synthesizes IPP, which is used in the biosynthesis of sterols, sesquiterpenes, and triterpenoids. By contrast, the plastidic, non-mevalonate 1-deoxy-d-xylulose-5-phosphate pathway (similar to that used by bacteria) is involved in the biosynthesis of plastidic isoprenoids, including carotenoids, phytol (the side chain of chlorophylls), plasteadquinone-9, and isoprene [10]. Modifications of BA (and SA) that influence their volatility, membrane permeability, solubility, and activity are also crucial to their transport and function. Thus, understanding the biosynthesis and modification of BAs in plants is a crucial first step to understanding the regulation and function of these important molecules.

Biosynthesis of BAs in plants
Plant BAs have been reported to originate either from phenylalanine (Phe) or directly from a shikimate-derived product such as isochorismate, in which the carboxyl carbon of shikimate is retained in the BA (Figure 2). By contrast, the carboxyl carbon of BAs that are synthesized from Phe originates from the β-carbon of the Phe side chain. Predominant early studies supported BA synthesis from Phe, and thus most work has focused on Phe-derived pathways. As is readily apparent from Figure 2, few of the plant genes that encode the enzymes of these pathways have been cloned; thus, much work is
needed to validate, define, and refine the biosynthetic pathways for BAs.

**Biosynthesis of BAs directly from shikimate/chorismate**

Bacteria provided the first evidence for BA biosynthesis directly from shikimate/chorismate. For example, SA and 2,3-dihydroxybenzoic acid (DHBA) are synthesized from chorismate via isochorismate, and are precursors for siderophores such as pyochelin in *Pseudomonas aeruginosa* [11] and enterobactin in *Escherichia coli* [12]. In plants, evidence for the shikimate/chorismate direct pathway of BA synthesis includes two $^{13}$C NMR studies in which the $^{13}$C-labeling of the carboxyl carbon is consistent with synthesis directly from shikimate/chorismate for gallic acid (3,4,5-trihydroxybenzoic acid) in the tree *Rhus typhina* [13] and 3-hydroxybenzoate (3HBA), which is incorporated into amarogentin in *Swertia chirata* ([14*]; Figure 1). In *A. thaliana*, the bulk of the SA that is produced in response to pathogens is synthesized from isochorismate and not from Phe, as confirmed by the lack of induced SA accumulation in isochorismate synthase (ics1) mutants [15]. This study provided the first genetic evidence for a shikimate/chorismate direct pathway for the synthesis of BAs in plants. Additional studies supporting, but not providing direct evidence for, this pathway include; first, the correlation of DHBA accumulation with ICS activity in elicited *Catharanthus roseus* cell cultures [16,17], and second, the incorporation of radiolabeled 3HBA (but not of Phe, trans-cinnamic acid [t-CA], or BA) into the benzyol moiety of xanthone coupled with lack of induction of
Biosynthesis of BAs in plants by the direct shikimate/chorismate pathway and via phenylalanine. The carboxyl carbon of shikimate is labeled \(^{13}C\) as is the \(\beta\)-carbon of phenylalanine (\(*\)). The plant enzymes involved in BA biosynthesis for which genes have been cloned are indicated, as are the chorismate-utilizing enzymes anthranilate synthase (AS) and aminodeoxychorismate synthase (ADCs). Pathways from trans-cinnamic acid alone are shown for simplicity; similar pathways from precursors in which either hydroxyl or methoxyl functionalities decorate the benzene ring (e.g. \(p\)-coumaric acid) have been reported. In addition, possible glucosylated precursors are not shown. C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase; CM, chorismate mutase; ICS, isochorismate synthase; PAL, phenylalanine ammonia lyase.

Biosynthesis of BAs from phenylalanine

BA biosynthesis from Phe has been shown to proceed from \(\alpha\)-CA, which is produced from Phe via PAL. The conversion of \(\alpha\)-CA to BA (or benzoyl-CoA) requires the cleavage of two carbons from the \(C_3\) side-chain of the precursor. As shown in Figure 2, three possible routes have been reported for this production of BA from \(\alpha\)-CA: CoA-dependent \(\beta\)-oxidation (Figure 2a), a CoA-independent non-oxidative route (Figure 2b), and a CoA-dependent, non-oxidative pathway (Figure 2c). To distinguish between these pathways, researchers have used the identification of the intermediate benzoaldehyde as confirmation of the non-oxidative route and a requirement for CoA and the enzyme 4-coumarate:CoA ligase (4CL) in support of the CoA-dependent \(\beta\)-oxidation pathway. Support for the more recently proposed third route (Figure 2c) includes a requirement for CoA and 4CL coupled with detection of the benzoaldehyde intermediate. There is an additional possibility that BA (or BA-CoA) could be produced from Phe via phenylpyruvate in a manner similar to that used by bacteria to catabolize Phe [19]. As limited conclusive evidence exists for the reported plant pathways from Phe to BA, alternative possibilities such as this should be explored.

The CoA-dependent \(\beta\)-oxidation pathway mirrors fatty acid \(\beta\)-oxidation [20; Figure 2a). In this pathway, \(\alpha\)-CA is activated by formation of the cinnamoyl-CoA ester, which is then hydrated to form 3-hydroxy-3-phenylpropionyl-CoA. This hydroxyl group is oxidized to a ketone, and the \(\beta\)-keto thioester is cleaved by a reverse Claisen condensation to form benzoyl-CoA. 4CL catalyzes the activation of \(\alpha\)-CA but the subsequent enzymes have not been identified. In support of this pathway, radiolabeled Phe
was not incorporated into benzaldehyde in tobacco mosaic virus (TMV)-inoculated tobacco (Nicotiana tabacum) leaf tissue [21], cucumber (Cucumis sativus) seedlings, or smoke-treated coyote tobacco (Nicotiana attenuata) plants [22], whereas radiolabeled 3-hydroxy-3-phenylpropanoic acid was detected as an intermediate [22]. Similarly, in Lithospermum erythrorhizon cell free extracts, p-hydroxybenzoic acid (4HBA) was synthesized in a CoA-dependent manner requiring 4CL activity, and p-hydroxybenzaldehyde was not detected as an intermediate [23].

The CoA-independent, non-β-oxidative pathway (or non-oxidative pathway [Figure 2b]) involves the hydration of the free acid, side-chain degradation via a reverse aldol condensation, and NAD(P)-dependent oxidation of the intermediate aldehyde. Supporting evidence comes from studies examining 4HBA biosynthesis from p-coumarate in elicitor-treated carrot (Daucus carota) cell cultures and extracts [24], potato tuber cell free extracts [25], and L. erythrorhizon cell extracts [26]; CoA was required and p-hydroxybenzaldehyde was detected. However, there has been no subsequent work to provide detailed confirmation of these findings. By contrast, a later in vitro study with L. erythrorhizon supports the β-oxidation pathway from p-coumaryl-CoA [23] as the major 4HBA biosynthetic pathway; this difference in results is largely attributable to modifications of in vitro assay conditions. (In vitro assay conditions such as pH, redox status, and substrate availability might differ from those in planta; thus care must be taken in interpreting all in vitro studies.)

In Pseudomonas fluorescens, a feruloyl-CoA hydratase/lyase was isolated that catalyzes the metabolism of feruloyl-CoA to vanillin and acetyl-CoA via hydration and reverse aldol cleavage [27,28]. This enzyme was active on a number of 4-hydroxycinnamoyl-CoA substrates, but not on cinnamoyl-CoA [27]. This led to the postulation of a similar CoA-dependent, non-oxidative pathway involved in BA biosynthesis in plants (Figure 2c), and to the subsequent discovery of a cinnamoyl-CoA hydratase/lyase activity in protein extracts of elicited H. androsaemum cells [7]. To date, this enzyme has not been further purified or characterized.

Modification of BAs
Modifications of small molecules not only alter the critical properties (i.e. volatility, stability, and activity) and thus the functional roles of these molecules, but also allow for precise temporal and spatial control over active forms. In plants, these modifications can include methylation, glycosylation, amino acid conjugation, and activation with CoA (Figure 3). Over the past few years, exciting progress has been made in identifying the first genes and enzymes that are involved in modifying the small molecule BA in plants. Some of these enzymes can also use SA or other structurally related molecules as a substrate with varied affinity or catalytic efficiency.

Methylation
Benzoic carboxyl methyltransferases catalyze the transfer of the methyl group of S-adenosyl-l-methionine (SAM) to the free carboxyl of BAs, forming volatile methyl esters such as MeBA and MeSA. These methyl esters are important constituents of many plant floral scents and the first benzoid carboxyl methyltransferases were identified and isolated from floral tissue [3**]. More recent work has also identified benzoid carboxyl methyltransferases that have a role in defense [29,30]. For example, a reverse genomics approach was used to identify a putative Arabidopsis SA carboxyl methyltransferase (AtBSMT, At3g11480) that has a role in defense from among the 24 predicted Arabidopsis SABATH family members. Expression of this gene correlated with induced MeSA emission in response to a variety of treatments, including moth herbivory and treatment with alamethicin, a voltage-gated ion-channel-forming antibiotic from the fungus Trichoderma viride [29]. This induction of MeSA emission in Arabidopsis is similar to that observed in soybean, in which emitted MeSA has been shown to attract predators of the aphid herbivore [4]. In addition, highly localized induction of AtBSMT around the site of feeding by piercing-sucking thrips suggests that AtBSMT might play additional roles in defense [29]. Similar to other SABATH family members, AtBSMT is able to utilize SA, BA, and structurally similar molecules, including antranilic acid and 3HBA [29]. Further experiments employing knockout and overexpresses Arabidopsis lines are therefore needed to confirm and dissect the in planta function(s) of AtBSMT.

In tobacco, a potential methyl salicylate esterase (NtSABP2) catalyzing the reverse reaction (i.e. the release of free SA from MeSA) has been identified by structural and biochemical studies [31**]. SABP2-silenced tobacco plants failed to develop SAR after inoculation with TMV [32]. As SAR development is dependent on SA, this suggests a possible role for MeSA in defense-related SA-dependent signaling and/or transport [31**,32]. Verification of this role requires measurement of MeSA levels in the SABP2-silenced tobacco plants, particularly because neither the methylesterase activity of NtSABP2 on structurally similar substrates (such as MeBA, methyl antranilate, and methyl 3HBA) nor possible hydroxynitrile lyase activity was reported. (SABP2 shares significant amino acid (45%) and structural similarity with hydroxynitrile lyases [31**].)

Glucosylation
UDP-glucosyltransferases (UGTs) have been identified that catalyze the transfer of glucose to the carboxyl group of BA, forming a benzoyl glucose ester (BAE) in tobacco [33] and Arabidopsis [34] in vitro. These enzymes can also
Glucosylation structurally related molecules such as SA, forming the salicyloyl glucose ester (SAE) or 2-O-β-D-glucoside (SAG) [33,34]. As there are numerous UGTs in plants (e.g. 107 in A. thaliana), evidence of in vitro biochemical activity and the correlation of induction with the accumulation of the glucose ester or glucoside has been used to support specific functional roles. To date, no single UGT knockouts deficient in the accumulation of BA, SAE, or SAG has been identified.

Glucosylation alters the hydrophilicity, stability, subcellular localization and bioactivity of the acceptor molecule [35]. For example, the bulk of pathogen-induced SA accumulates as SAG in the plant vacuole [36]. Because free SA is phytotoxic, the accumulation of the glucoside allows the storage of SA in a stable, non-toxic, inactive form that can be hydrolyzed to active free SA [37]. Glucosylation might play a role in the homeostasis and usage of BAs through the controlled interconversion of the acids and glucose esters/glucosides. Furthermore, glucosylated compounds might also serve as intermediates in the biosynthesis of BAs. For example, the cinnamoyl-glucose ester has been proposed as an intermediate in the formation of BA (and BA) in tobacco [38], and glucosylated o-coumarate has been proposed as a precursor of SAE, SAG, and the salicyloyl-diglucose ester in a number of plants, including willow [6]. The discovery of tobacco TOGT1, a UGT that shows high activity with t-CA and o-coumaric acid, supports this possibility [39]. TOGT1 is induced during the tobacco response to TMV, in parallel with the accumulation of BA, BAE, SA, and SA–glucose conjugates [38,39]. As the bulk of BA (and SA) is present as a glucose conjugate [38], identifying and characterizing the genes and proteins that are responsible for the formation, hydrolysis, and transport of these glucose esters and glucosides is essential.
Amino acid conjugation

Conjugation of a plant hormone to amino acids can activate the hormone (e.g. jasmonic acid [40**]) or inactivate it (e.g. auxin [indole-3-acetic acid [IAA]] [41**, 42**]), therefore playing a crucial role in phytohormone regulation and action. The enzymes that catalyze the formation of these amide-linked phytohormone conjugates are GH3-like proteins belonging to the firefly luciferase superfamily [43]. In addition, in Arabidopsis, a number of IAA–amino acid amidohydrolases that catalyze the reverse reaction have been identified through genetic screens [42**]. Though no BA-specific amino acid synthetases or amidohydrolases have yet been identified, it is possible that amino acid conjugates play a role in the temporal and spatial control of free benzoates. Of the 19 putative phytohormone amino acid synthetases in Arabidopsis [43, 44], there are currently two candidate SA–amino acid synthetases: GH3.5 and PBS3. Recombinant GH3.5 (M4122.70, At4g27260) exhibited adenylase activity with SA (and IAA) [43]. PBS3 expression is highly correlated with expression of the pathogen-induced SA biosynthetic gene ICS1 (Spearman rank coefficient = 0.83 using AtGen Pathogen Treatment subset of NASCAArray Database [45**]), and pbs3 mutants are more susceptible to Pseudomonas syringae ([46]; R Innes, M Wildermuth, unpublished).

Activation with CoA

The incorporation of a benzoyl moiety into secondary metabolites occurs by the transfer of an activated BA intermediate, such as benzoyl-CoA, via an acyltransferase. Though benzoate:CoA ligases have been isolated and characterized from benzoate-degrading microorganisms [47–50], the characterization of benzoate:CoA ligases from plants is more recent. A 3HBA:CoA ligase was isolated and characterized from the plant Centaurea erythraeum [51], and the purification and characterization of a BA:CoA ligase (BZL) from Clarkia breweri flowers followed [52]. In addition to showing activity with BA, BZL also exhibited significant activity with other structurally related compounds, including anthranilic acid (50% relative activity compared with BA) and 3HBA (25% relative activity) [52]. Though benzoyl-CoA could be formed by alternative pathways (Figure 2), the presence of a benzoyl-CoA ligase could allow for the refined spatial and temporal control of benzoyl-CoA (and BA) availability. This could facilitate the specific channeling of benzoyl-CoA to the appropriate acyltransferase for addition of the benzoyl moiety to a given secondary metabolite. In fact, an acyltransferase that is capable of synthesizing benzyl benzoate (a volatile ester) from benzoyl-CoA and benzyl alcohol has been identified in C. breweri flowers [53], though we do not yet know whether this enzyme and C. breweri BZL are co-expressed and co-localized.

An acyl-CoA thioesterase could catalyze the reverse reaction to release BA from benzoyl-CoA. As CoA esters are not membrane permeable, conversion of benzoyl-CoA to free BA would facilitate the inter- and intracellular transport of BAs. Alternatively, benzoyl-CoA could be transported across membranes by an ABC [54], or other, transporter. Acyl-CoA thioesterases are an extremely diverse set of enzymes that comprise at least five distinct families present in a variety of subcellular locations, including the cytosol, endoplasmic reticulum, mitochondrion, and peroxisome. In plants, acyl-CoA thioesterase activity is quite prevalent [55**, 56, 57], but the first acyl-CoA thioesterase (A. thaliana ACH2) was cloned only recently [55**]. Elucidating the function of plant acyl-CoA thioesterases is an exciting area of research, and it will be interesting to determine whether a BA-specific acyl-CoA thioesterase plays a role in modulating benzoate biosynthesis, modification, and/or addition to benzoyl-containing secondary metabolites.

Conclusions

Many readers might be surprised that we do not yet know precisely how BA and other BAs are synthesized in plants. The observed complexity is probably due to the simplicity of these molecules and their evolving chemical and functional elaboration. Evidence is building to support the idea that BAs could be synthesized both directly from shikimate/chorismate and from Phe in most plants. There is a pressing need, however, to determine in what tissues and under which conditions these distinct pathways are utilized. In addition, it is likely that these pathways intersect and impact each other, complicating analysis and interpretation. Multiple routes for the synthesis of BAs from each of these two major pathways are possible, perhaps facilitating fine control and channeling of intermediates to particular benzoxia products. Finally, the modification of BAs might not only control activity and function but also be essential to their synthesis.

To date, as seen in Figures 2 and 3, few of the genes that are involved in BA biosynthesis (and modification) in plants have been identified. However, the use of genetic/genomic model plants such as A. thaliana has and can facilitate the identification of these genes and provide the framework for careful and integrated dissection of the complexity associated with BA synthesis, modification, and function [15, 29]. In particular, SA and benzoyl glucosinolate biosynthesis can be readily examined in Arabidopsis, and it appears that the shikimate/chorismate direct and Phe-derived pathways are operational in Arabidopsis [5, 15, 58**, 59**]. Because the shikimate and phenylpropanoid pathways are closely associated, it is possible that genetically altering or inhibiting the activity of one pathway could impact another. These impacts might be assessed readily in Arabidopsis by global expression profiling. Comparative genomic and biochemical approaches could then be used to extend (and diversify) findings to other plants. In parallel, detailed biochemical studies to elucidate benzenoid
metabolism in systems that have elaborated and analyzable benzoate products (e.g. floral scent from petunia petals [60**]) are both complementary and invaluable.

Acknowledgements
I thank UC Berkeley and the US National Science Foundation (NSF) MCB-0420267 for financial support, Marcus Strawn for assistance with figures, and collaborator Roger Innes for allowing the inclusion of information prior to publication.

References and recommended reading
Papers of particular interest, published within the annual period of review, have been highlighted as:

* of special interest
** of outstanding interest

12. Gehring AM, Bradley KA, Walsh CT, Enterobactin biosynthesis in Escherichia coli: isochorismate lyase (EntB) is a bifunctional enzyme that is phosphopantetheinylated by EntD and then acylated by EntE using ATP and 2,3-dihydroxybenzoate. Biochemistry 1997, 36:8495-8503.
See annotation for [13].
18. Abd El-Mawla AM, Schmidt W, Beerhues L: Cinnamic acid is a precursor of benzoic acids in cell cultures of Hypericum androsaemum, but not in cell cultures of Centaurea erythraea. RAaN. Planta 2001, 212:298-293.
22. Jarvis AP, Scaife O, Oldham NJ: 3-Hydroxy-3-phenylpropanoic acid is an intermediate in the biosynthesis of benzoic acid and salicylic acid but benzoaldehyde is not. Planta 2000, 212:119-126.

Crystal structure and biochemical studies reveal that SABP2 is likely to function as a MeSA esterase. This work highlights the importance of obtaining crystal structures for plant enzymes; structure supports a role for SABP2 as a MeSA esterase and not as a lipase.

Kumar D, Klessig DF: High-affinity salicylic acid-binding protein 2 is required for plant innate immunity and has salicylic acid-stimulated lipase activity. Proc Natl Acad Sci USA 2003, 100:16101-16106.


Staswick PE, Tiryaki I: The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in Arabidopsis. Plant Cell 2004, 16:2117-2127.

The first report of plant hormone activation by conjugation to an amino acid.


The authors identify putative IAA-amino acid synthetases in Arabidopsis and describe an approach for dissecting the complexity (including partial redundancy) of these enzymes’ function. Note that GH3.5 mutants exhibit weak auxin phenotypes; thus, a potential role for GH3.5 as an SA–aminio acid synthetase remains a possibility.


An excellent review on the well-studied phytohormone auxin. The authors provide a framework for thinking about the synthesis, modification, activity, and function of small molecules in plants.


A great resource for those employing in silico reverse genetic approaches in Arabidopsis. It is possible to examine gene expression patterns and determine correlated genes in response to diverse treatments (including abiotic and biotic stressors), in diverse tissues, etc.


The most detailed purification and biochemical characterization of a plant BA:CoA ligase to date. No protein sequence — or cDNA.


The authors describe the first cloning and characterization of a plant acyl-CoA thioesterase. The biochemical activity and expression patterns of this enzyme suggest that it is not involved in fatty-acid oxidation.


Use of the PAL inhibitor AIP impacted the growth and reproduction of an avirulent isolate of Peronospora parasitica, but not of a virulent isolate. Total SA accumulation was reduced by about two-thirds in the avirulent interaction when AIP was employed. AIP treatment abrogated induced PAL expression; thus, the specificity of its impact needs to be examined.

By coupling the use of a mutant in SA biosynthesis (via ICS) with a PAL inhibitor, the authors found that local resistance to Botrytis appears to be mediated by SA that is derived from t-CA.


A very nice integrated approach (involving modeling, isotope-labeling, and metabolite analysis) for analyzing the complexity of benzenoid metabolism in petunia petal tissue. The authors predicted a benzyl benzoate intermediate, isolated a cDNA encoding a protein that is capable of synthesizing benzyl benzoate, and characterized this enzyme biochemically.