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Benzoylation and sinapoylation of glucosinolate R-groups in Arabidopsis

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SUMMARY

Glucosinolates (GSLs) are nitrogen- and sulfur-containing metabolites that contribute to human health and plant defense. The biological activities of these molecules are largely dependent on modification of the GSL R-groups derived from their corresponding amino acid precursors. In Arabidopsis seeds, esterification of the R-group of hydroxylated GSLs (OH-GSLs) leads to the accumulation of benzoylated GSLs (BzGSLs) and sinapoylated GSLs (SnGSLs). BzGSLs were thought to be synthesized from OH-GSLs and benzoyl CoA by a BAHD acyltransferase, but no BAHD gene is strongly co-expressed with the two reference genes *BZO1* and *AOP3* that are required for BzGSL biosynthesis. In contrast, three genes encoding serine carboxypeptidase-like (SCPL) acyltransferases [SCPL5, SCPL17 and SCPL19 (SNG2)] do exhibit strong co-expression. Using a reverse genetic approach, we found that the GSL profile of the *scpl5* mutant was identical to that of wild-type, but both BzGSLs and SnGSLs were barely detectable in *scpl17* mutants and their amounts were decreased in the *sng2* mutant. In addition, both *scpl17* and *sng2* mutants accumulate the putative BzGSL precursors OH-GSLs and benzoylglucose. The results of further GSL analyses in other phenylpropanoid mutants and benzoate feeding experiments suggested that SCPL17 mediates the acyltransferase reaction directly, while the mutation in *sng2* causes a decrease in BzGSLs and SnGSLs via an unknown indirect mechanism. Finally, benzoate feeding experiments using *bzo1* mutants and BZO1 biochemical characterization indicated that the *in vivo* role of BZO1 is to synthesize the benzoate precursor cinnamoyl CoA rather than to generate benzoyl CoA from benzoate and CoA as previously predicted.

Keywords: glucosinolate, benzoate, serine carboxypeptidase-like acyltransferases, benzoylation, sinapoylation, Arabidopsis.

INTRODUCTION

Glucosinolates (GSLs) are nitrogen- and sulfur-containing secondary metabolites derived from amino acids that protect plants from pests and in some plants are involved in auxin biosynthesis (Agerbirk *et al.*, 2009; Bender and Celenza, 2009; Hopkins *et al.*, 2009). From a human perspective, GSLs are important as contributors of flavor to certain foods, as cancer prevention agents, and as biofumigants (Traka and Mithen, 2009). Because of the economic importance of GSLs, their biosynthetic pathways have been intensively studied using molecular genetics and systems biology approaches in Arabidopsis (Hirai, 2009; Albinsky *et al.*, 2010; Sønderby *et al.*, 2010).

In Arabidopsis, approximately 40 GSLs have been identified, most of which are derived from methionine (Met) and tryptophan (Trp) (Hogge *et al.*, 1988; Kliebenstein *et al.*, 2001a, 2007; Reichelt *et al.*, 2002). In some Met-derived GSLs, the R-group is hydroxylated by the 2-oxo acid-dependent dioxygenase AOP3 (Kliebenstein *et al.*, 2001b). In Arabidopsis Col-0, the two resulting hydroxylated GSLs (OH-GSLs), 3-hydroxypropyl GSL (3OHP) and 4-hydroxybutyl GSL (4OHB), are further benzoylated to give 3-benzoyloxypropyl GSL (3BZO) and 4-benzoyloxybutyl GSL (4BZO) or sinapoylated to give the corresponding 3-sinapoyloxypropyl GSL (3SIN) and 4-sinapoyloxybutyl GSL (4SIN) (Hogge *et al.*, 1988; Kliebenstein *et al.*, 2007). In addition, similar benzoylated GSLs (BzGSLs) with longer chain lengths, 5-benzoyloxypentyl GSL and 6-benzoyloxyhexyl GSL, have been detected, but the corresponding

OH-GSLs and sinapoylated GSLs (SnGSLs) have not (Hogge *et al.*, 1988). Further, two additional types of BzGSLs have been identified, i.e. BzGSLs in which benzoylation occurs on the glucose ring of GSL core structure (Reichelt *et al.*, 2002), and 2-benzoyloxybut-3-enyl GSL, the only Arabidopsis GSL in which hydroxylation and benzoylation occur after alkenylation (Kliebenstein *et al.*, 2001b). 3BZO and 4BZO accumulate abundantly in Col-0 seeds, but 2-benzoyloxybut-3-enyl GSL has not been detected in this ecotype and the amounts of other BzGSLs are small. Therefore, most studies that have been performed have focused on the changes of 3BZO or 4BZO, and hereafter the term BzGSLs refers these two BzGSLs only.

Previous isotope-labeling experiments together with genetic and biochemical studies in various plant species have identified two major biosynthetic routes to benzoate (BA), the β-oxidative and non-β-oxidative pathways, both of which begin with phenylalanine (Phe) (Ribnicky *et al.*, 1998; Abd El-Mawla and Beerhues, 2002; Orlova *et al.*, 2006). In the β-oxidative pathway, Phe is converted into cinnamate, which is in turn activated to its corresponding CoA thioester (Ribnicky *et al.*, 1998; Beuerle and Pichersky, 2002). Cinnamoyl CoA is then converted to benzoyl CoA by a series of side chain-shortening reactions shared with fatty acid degradation (Hertweck *et al.*, 2001). Finally, BA is released from benzoyl CoA by a thioesterase (Van Moerkercke *et al.*, 2009). In the non-β-oxidative pathway, benzaldehyde is synthesized first from cinnamate or cinnamoyl CoA and is then converted to BA by an aldehyde oxidase or benzaldehyde dehydrogenase (Schnitzler *et al.*, 1992; Abd El-Mawla and Beerhues, 2002; Boatright *et al.*, 2004). In Arabidopsis, BA biosynthesis has been studied mainly in the seeds because BzGSLs are abundant there and can be used as marker metabolites to measure BA biosynthesis activity. For example, BzGSLs decrease in the *aao4* mutant and are barely detectable in the *chy1* mutant. AAO4 displays aldehyde oxidase activity (Ibdah and Pichersky, 2009; Ibdah *et al.*, 2009), and Chy1 has been suggested to be involved in β-oxidation (Ibdah and Pichersky, 2009).

Another Arabidopsis gene, *BZO1*, has been identified by forward genetics screening using BzGSLs as reporter molecules for BA metabolism. Kliebenstein *et al.* (2007) showed that L*er bzo1* mutants lack 3BZO and have elevated levels of 3OHP in their seeds. Further, enzymatic assays with recombinant BZO1 revealed that the protein is capable of forming benzoyl CoA from BA and coenzyme A. Although these findings implicate benzoyl CoA as the acyl donor in benzoylated GSL synthesis, the identities of the acyltransferases involved in the benzoylation and sinapoylation reactions have not yet been determined. The benzoyltransferase may belong to the BAHD family (named on the basis of the first letter of the names of the first four enzymes recognized as belonging to this family: BEAT (benzylalcohol *O*-acetyltransferase), AHCT (anthocyanin *O*-hydroxycinnamoyltransferase), HCBT (*N*-hydroxycinnamoyl/benzoyltransferase) and DAT (deacetylvindoline 4-*O*-acetyltransferase); St-Pierre and De Luca, 2000), because these enzymes use acyl CoAs as donors, and benzoyl CoA was shown to be the acyl donor in synthesis of benzylbenzoate and taxol (D'Auria *et al.*, 2002; Walker *et al.*, 2002; Boatright *et al.*, 2004). An alternative to the BAHD acyltransferases are the serine carboxypeptidase-like (SCPL) acyltransferases, which use glucose esters such as sinapoylglucose (SG) as acyl donors to generate esters including sinapoylmalate, sinapoylcholine, 1,2-disinapoylglucose and sinapoylated anthocyanins (Lehfeldt *et al.*, 2000; Shirley *et al.*, 2001; Fraser *et al.*, 2007). Recently it was reported that SCPL1 from oat is required to transfer a benzoyl group to des-acyl avenacins, compounds that contribute to broad-spectrum disease resistance in this species (Mugford *et al.*, 2009).

To identify the enzyme(s) required to acylate OH-GSLs, we targeted these two representative acyltransferase families that can potentially utilize activated forms of BA and sinapate as acyl donors. Here we report that the acyltransferase responsible for formation of the benzoylated and sinapoylated GSLs in Arabidopsis seeds belongs to the SCPL family, and probably uses benzoylglucose (BG) and SG as substrates. We further show that the enzyme encoded by *BZO1* is a cinnamoyl CoA ligase that catalyzes the formation of cinnamoyl CoA to provide the substrate for the pathway(s) by which this phenylpropanoid derivative is converted to BA.

RESULTS

Co-expression analysis reveals three SCPL genes as potential candidates in the synthesis of benzoylated/sinapoylated GSLs

It was previously shown using microarray analyses that many genes known to be involved in GSL biosynthesis are globally co-regulated by Myb transcription factors, and this information has been used to identify additional genes in this pathway (Gigolashvili *et al.*, 2009; Hirai, 2009). To discover genes encoding the enzymes responsible for the acylation of OH-GSLs, we searched for SCPL and BAHD family members co-expressed with *AOP3* and *BZO1* in the ATTED-II database (Obayashi *et al.*, 2007, 2009; Obayashi and Kinoshita, 2010). Three genes encoding SCPL acyltransferases (*SCPL5*, *SCPL17* and *SCPL19*), and no BAHD genes were identified among the top 100 hits.

We used the Botany Array Resource (BAR) expression browser (BAR, http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi) to examine the *in silico* expression patterns for the SCPL clade I genes for which expression data is available (Toufighi *et al.*, 2005) (Figure 1). This analysis revealed that *SCPL5*, *SCPL17* and *SCPL19* were highly expressed in seeds, distinct from the expression patterns of the other SCPL genes. Although SCPL19 is known to encode an enzyme with a

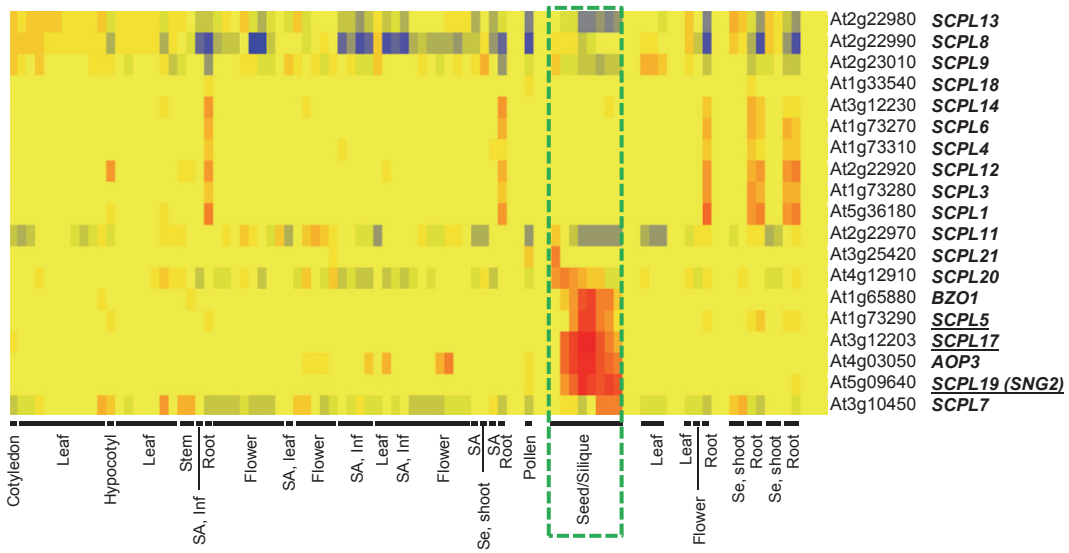

Figure 1. *In silico* analysis of candidate GSL benzoylation genes.
The heatmap shows the expression levels of clade I SCPL genes together with *BZO1* and *AOP3*. The AtGenExpress tissue series dataset was analyzed using the Botany Array Resource (BAR) expression browser. Each column represents tissues at different stages or under different conditions. Seed/silique tissues are highlighted by a green dotted box. SA, shoot apex; Inf, inflorescence; Se, seedling.

seed-specific function, sinapoylglucose:choline sinapoyltransferase (SCT) (Shirley *et al.*, 2001), the function of the other two members is unknown. It is important to note that all four Arabidopsis clade I SCPL proteins (SCPL8/SMT, SCPL9/SST, SCPL10/SAT and SCPL19/SCT) characterized to date are acyltransferases, as is a clade I SCPL protein from oat (Lehfeldt *et al.*, 2000; Li and Steffens, 2000; Fraser *et al.*, 2007; Mugford *et al.*, 2009).

Levels of both BzGSLs and SnGSLs are lower in the *scpl17* and *scpl19 (sng2)* mutants compared to wild-type

To determine whether any of these three SCPL genes are involved in the synthesis of BzGSLs and/or SnGSLs, we identified T-DNA insertion lines for each of them. One mutant was found for *SCPL5* (*scpl5-1,* SAIL_769_C05) and two for *SCPL17* (*scpl17-1*, SALK_075481; *scpl17-2*, SALK_126264 C). As described previously, a mutant in the *SCPL19* locus, designated *sinapoylglucose accumulator 2* (*sng2*), is defective in SCT, an enzyme required for the synthesis of sinapoylcholine in seeds, and accumulates SG instead of sinapoylcholine (Shirley *et al.*, 2001). We identified a new T-DNA insertion line for *SCPL19* (SALK_002255 C) and designated this mutant *sng2-2*. We also obtained two Col-0 *bzo1* mutant lines: *bzo1-4* (SALK_094196) and *bzo1-5* (SALK_143127). The three independent *bzo1* mutants previously reported were in the *Ler* ecotype (Kliebenstein *et al.*, 2007).

To determine whether any of these mutants display a GSL phenotype, desulfo-GSLs were isolated from dried seeds and analyzed by HPLC. The two *bzo1* mutants in the Col-0 ecotype were defective in BzGSL accumulation, consistent with previously reported results (Kliebenstein *et al.*, 2007). BzGSLs were barely detected in *bzo1-4,* which is a knockout mutant (Figure 2a,c). In *bzo1-5*, in which the T-DNA is integrated into the *BZO1* promoter, 3BZO and 4BZO accumulated to 33 and 19% of wild-type levels, respectively (Figure 2a,c, middle graph). Because the only benzoylated GSL accumulated by the L*er* ecotype is 3BZO, increased levels of 3OHP only were reported in the L*er* mutants *bzo1-1* and *bzo1-2* (Kliebenstein *et al.*, 2007). In contrast, the Col-0 ecotype synthesizes both 3BZO and 4BZO, enabling us to measure levels of 3OHP and 4OHB in our Col-0 *bzo* mutants. In both *bzo1-4* and *bzo1-5*, the levels of 3OHP remained unchanged but 4OHB levels were greater by 7.2 nmol mg tissue^{-1} (2.1-fold) and 4.9 nmol mg tissue^{-1} (1.8-fold), respectively, compared to the levels in the control (Figure 2c, upper graph). The overall increase in 4OHB in these two mutant lines was near equal to the decrease in 4BZO in each line (6.7 and 5.5 nmol mg tissue^{-1}, respectively; Figure 2c, middle graph).

As OH-GSLs are a common biosynthetic precursor to both BzGSLs and SnGSLs, we speculated that the increased levels of OH-GSLs in *bzo1* mutant lines may result in an increase in the levels of SnGSLs. To test this hypothesis, we compared UV chromatograms of the previous GSL analyses at 330 nm, a wavelength diagnostic for SnGSLs (Figure 2b). These analyses revealed that *bzo1* mutants do have elevated levels of SnGSLs (Figure 2b,c, lower graph). In addition to the increase in the two SnGSLs, 3SIN and 4SIN, that are found in wild-type plants, we found additional unidentified putatively sinapoylated GSLs in *bzo1-4* (Figure 2b, arrows).

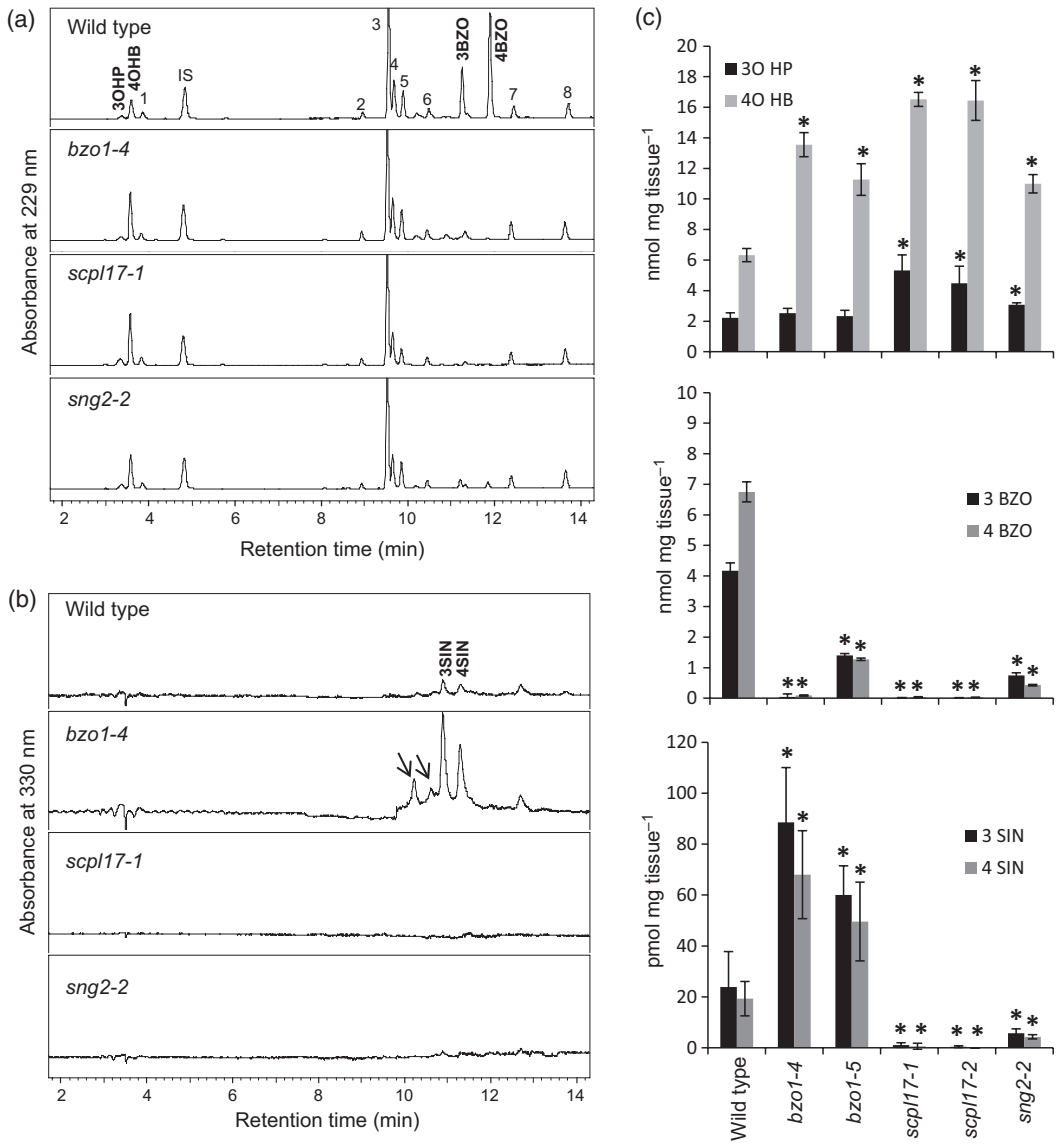

Figure 2. GSL analyses in wild-type, *bzo1*, *scpl17* and *sng2* seeds.
(a, b) Total GSL profiles at 229 nm (a) and SnGSL profiles at 330 nm (b) were extracted from the same diode array detector dataset, and the *y* axis of each chromatogram is scaled identically. Arrows indicate unidentified putative sinapoylated GSLs. The existence of the sinapoyl group was confirmed by LC-MS, but the structures of the molecules were not identified.
(c) The levels of OH-GSLs, BzGSLs and SnGSLs were quantified. Plants were grown under the same conditions, and four independent seed stocks harvested from different plants were analyzed for each genotype. $*P < 0.05$ versus wild-type (Student's *t* test). Values are means ± SD. IS, internal standard sinigrin; 3OHP, 3-hydroxypropyl; 4OHB, 4-hydroxybutyl; 3BZO, 3-benzoyloxypropyl; 4BZO, 4-benzoyloxybutyl; 3SIN, 3-sinapoyloxypropyl; 4BZO, 4-sinapoyloxybutyl. Peak 1, 4-methylsulfinylbutyl GSL; peak 2, 7-methylsulfinylheptyl GSL; peak 3, 4-methylthiobutyl GSL; peak 4, 8-methylsulfinyloctyl GSL; peak 5, indol-3-ylmethyl GSL; peak 6, 5-methylthiopropyl GSL; peak 7, 7-methylthioheptyl GSL; peak 8, 8-methylthiooctyl GSL.

We next evaluated whether any of the *scpl* mutants displayed related GSL phenotypes. There were no significant differences in the GSL profile of the *scpl5* mutant compared to wild-type; however, in the two independent *scpl17* mutants, BzGSLs and SnGSLs were near the limits of detection (Figure 2a,c). Surprisingly, in the *scpl19* mutant *sng2-2*, significant decreases in levels of BzGSLs and SnGSLs were observed. As observed in the *bzo1* mutants, a general increase in the levels of OH-GSLs was also seen in the *scpl17* and *sng2* mutants (Figure 2a,c, upper graph). The increase was higher in the *scpl17* mutants, which showed a more significant reduction of BzGSLs and SnGSLs compared to *sng2-2*. The increase in *scpl17* was also higher than in *bzo1* mutants, which, rather than lower levels of both BzGSLs and SnGSLs, contained lower levels of BzGSLs but elevated levels of SnGSLs. Except for OH-GSLs, BzGSLs and SnGSLs, there was no significant reproducible change in other GSLs in the *scpl17* and *sng2* mutants (Table S1).

SCPL17 and *SNG2* expression complemented the mutant phenotypes

To unambiguously determine whether the metabolite phenotype of the *scpl17* and *sng2* mutant lines was due to the identified mutations, we transformed these lines with functional copies of the respective genes. BzGSL and SnGSL synthesis was restored, and the amounts of OH-GSLs were reduced to the levels found in wild-type in all plants containing the transgenes (Figures S1 and S2).

Benzoylcholine and benzoylglucose accumulate in *scpl17* mutants

The reduction in both BzGSLs and SnGSLs in mutants of *SCPL17* and *SCPL19* (*SNG2*) suggested that the enzymes encoded by these two genes are directly or indirectly involved in the synthesis of BzGSLs and SnGSLs from OH-GSLs. To further elucidate the biochemical pathways leading to BzGSLs and SnGSLs, LC-MS/MS analyses were performed in precursor ion scanning mode to identify possible benzoylated or sinapoylated pathway intermediates that may accumulate in these mutants. This analysis identified one benzoylated compound that hyper-accumulated in the *scpl17* mutant line compared to the *sng2 scpl17* double mutant and wild-type plants (Figure 3). This putative intermediate was identified as benzoylcholine (BC) based on an MS spectrum displaying a major peak at *m*/*z* 208, corresponding to intact BC.

To test whether SCT, the enzyme encoded by the *SNG2* gene, can produce BC in addition to its known product sinapoylcholine, *SNG2* was transiently expressed in *Nicotiana benthamiana*. Desalted protein extract was prepared from leaves expressing *SNG2*. Incubation of this crude protein extract with BG and choline resulted in generation of BC (Figure 4). BC was not synthesized when BG or choline were omitted from the reaction, or when BG and choline were incubated with protein extract prepared from leaves infiltrated with infiltration solution or agrobacteria transformed with the empty vector (Figure 4).

We further quantified the amounts of BC and its precursor BG in *scpl17* and *sng2* mutant seeds using LC-MS, and found that levels of BC, and, to a lesser extent, BG increased in the *scpl17* mutants (Figure 5). It is likely that the increase of BG was not observed in the previous LC-MS/MS analyses because of the low levels of BG found in *scpl17* mutant seeds and the different sensitivity limits of the two methods. Parallel analyses indicated that BG levels are also elevated in *sng2-2* seeds (Figure 5a), but there was no difference in BC levels (Figure 5b).

SCPL17 is required for benzoylation of GSLs

To further examine the metabolism of benzoylated compounds, we administered 1 mM BA to developing siliques of relevant genotypes. In these experiments, BG accumulated to elevated levels in all genotypes (Figure 6a). BC levels also increased in all mutants except *sng2*, strongly suggesting that SCT is required for BC synthesis *in vivo*. We also analyzed the change in GSLs after feeding BA. In wild-type and *sng2* siliques, BA treatment generally caused a modest decrease in OH-GSLs, with no change in 4OHB in *sng2* and a corresponding increase in the levels of BzGSLs, although only a few of these changes were statistically significant (Figure 6b,c). In contrast, in *scpl17*, BzGSL levels remained very low even though BG levels were elevated (Figure 6), indicating that SCPL17 may play a direct role in benzoylation of GSLs.

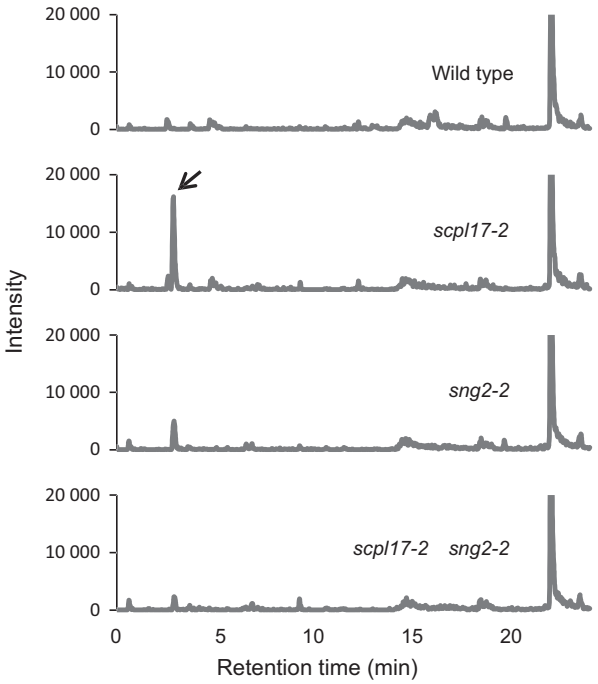

Figure 3. LC-MS/MS analyses in wild-type, *scpl17*, *sng2* and *scpl17 sng2* seeds.
Benzoylated compounds were identified by scanning daughter ions of *m*/*z* 105. In the *scpl17* mutant, a benzoylated compound accumulated (arrow) and was identified as benzoylcholine.

Levels of BzGSLs and SnGSLs in some phenylpropanoid mutants are lower than in wild-type

To explore whether other perturbations in phenylpropanoid metabolism affect GSL synthesis, we analyzed GSLs in the *bright trichomes 1* (*brt1-1*), *reduced epidermal fluorescence 1* (*ref1-4*) and *ferulic acid hydroxylase 1* (*fah1-2*) mutants (Meyer *et al.*, 1996; Nair *et al.*, 2004; Sinlapadech *et al.*, 2007). FAH1 and REF1 catalyze late steps in the synthesis of sinapate, and BRT1 converts sinapate and UDP-glucose into SG in the penultimate step of sinapoylmalate biosynthesis. The levels of both SnGSLs and BzGSLs were lower in all of

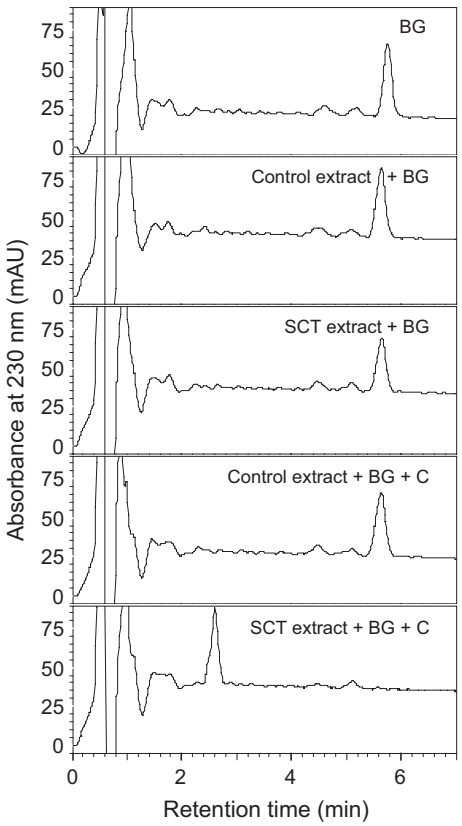

Figure 4. SCT enzyme assay.
Tobacco leaves were infiltrated with agrobacteria containing an *SNG2* overexpression vector. Total protein was isolated after 4 days incubation, desalted, and incubated with benzoylglucose (BG) or choline (C).

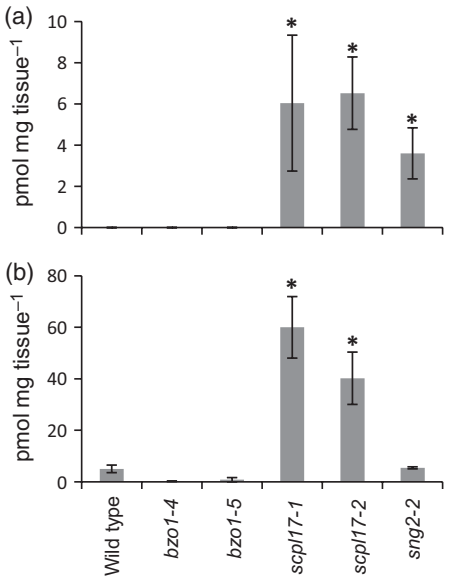

Figure 5. Levels of benzoylglucose (a) and benzoylcholine (b) in wild-type, *bzo1*, *scpl17* and *sng2* seeds.
From 5 mg dried seeds, 50% MeOH extracts were isolated and analyzed using LC-MS under ESI-positive mode. *$P < 0.05$ versus wild-type (Student's *t* test).

these mutants but the levels of OH-GSLs were elevated (Figure 7). These data are consistent with a role for SG in SnGSL synthesis, and suggest there may be changes in phenylpropanoid metabolism in the mutants that have more distant effects on BA or BzGSL synthesis.

BZO1 is a cinnamoyl CoA ligase

BZO1 was suggested to function as a benzoyl CoA ligase *in vivo* based on its similarity to CoA-activating enzymes, its activity with BA *in vitro*, and the observation that *bzo1* mutants lack BzGSLs (Kliebenstein *et al.*, 2007). However, the observation that SCPL17, which is likely to use a glucose ester as an acyl donor rather than a CoA thioester, is required to synthesize BzGSLs, as well as our recent observation that a petunia protein with high similarity to BZO1 acts as cinnamoyl CoA ligase (Klempien *et al.*, 2012), prompted us to reconsider the activity of BZO1 as a cinnamoyl CoA ligase.

To test whether BZO1 functions as a cinnamoyl CoA ligase, we first measured the levels of BA, BG and BC in *bzo1* mutants. Although BA and BG were near or below detectable limits in wild-type and *bzo1* seeds (Figure 5a), BC was readily detected in wild-type but reduced to only 10–15% of wild-type levels in *bzo1-4* and *bzo1-5* (Figure 5b). In the BA feeding experiments described above, the levels of BzGSLs in *bzo1-5* siliques increased and the amounts of OH-GSLs decreased to values similar to those seen in wild-type (Figure 6b,c). These results suggest that the decrease of BzGSLs in *bzo1-5* is due to the lack of BA, not the lack of benzoyl CoA ligase activity. Similar results were obtained when BA was provided to siliques of the *bzo1-4* null mutant, which contains lower residual levels of 3BZO and 4BZO (Figure S3).

We next examined BZO1 enzymatic activity with five phenolic substrates. BZO1 was expressed in *Escherichia coli*, and *in vitro* enzyme assays were performed using purified His-tagged protein. BZO1 showed highest and lowest activity toward cinnamate and ferulate respectively (cinnamate > coumarate > BA > caffeate > ferulate) (Figure 8). The relative activity of BZO1 with BA was only 27% of that with cinnamate. More detailed kinetic analysis revealed that BZO1 displayed slightly higher affinity and significantly greater turnover for cinnamate compared to BA (Table 1).

DISCUSSION

SCPL17 is required for GSL acylation but the effects of *sng2* on GSL biosynthesis may be indirect

Side-chain modifications contribute to the structural and functional diversity of GSLs. For example, benzoylation and sinapoylation reactions generate BzGSLs (approximately 15% of total GSLs) and SnGSLs (approximately 0.06% of

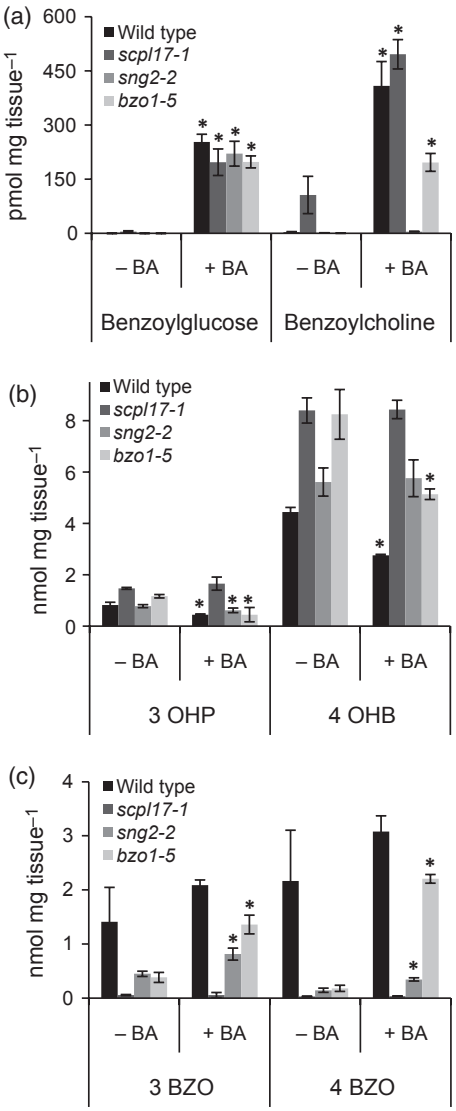

Figure 6. Benzoate feeding in wild-type, *scpl17*, *sng2* and *bzo1* siliques.
Three maturing siliques were detached from the main inflorescence of a 6-week-old Arabidopsis plant. At this stage, siliques at the bottom of each inflorescence were just beginning to turn yellow. The fourth to sixth siliques from the bottom were used for each feeding assay. After 2 days of treatment with 1 mM BA, siliques were pooled, and benzoylglucose and benzoylcholine (a), OH-GSLs (b) and BzGSLs (c) were quantified. –BA, siliques treated with water (control); +BA, siliques treated with 1 mM benzoate. Three independent plants were analyzed for each genotype. $*P < 0.05$ versus control (Student's *t* test). Values are means ± SD ($n = 3$).

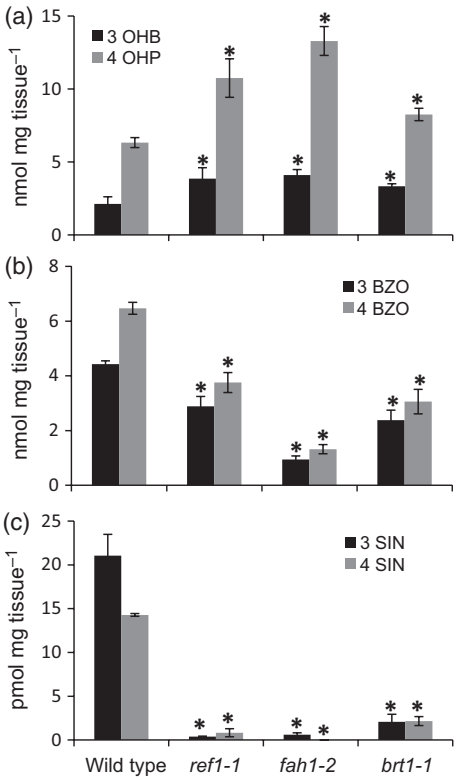

Figure 7. GSL analyses in the phenylpropanoid mutants.
Plants were grown under the same conditions, and three independent seed stocks harvested from different plants were analyzed for each genotype. $*P < 0.05$ versus wild-type (Student's *t* test). Values are means ± SD.

total GSLs) from OH-GSLs (calculated based on quantified amounts of GSLs displayed in Figure 2a,b). Investigations of the biosynthetic pathway of these GSLs have focused on BzGSLs, not only because BzGSLs are relatively abundant, but also because BzGSLs are major BA-derived compounds that may be used to dissect BA biosynthesis. Feeding experiments with isotope-labeled precursors in Arabidopsis siliques showed that the benzoyl group and aliphatic portion of BzGSLs are derived from Phe and Met, respectively (Graser *et al.*, 2001). Simultaneous labeling of 4-methylthiobutyl GSL (4MTB), 4-methylsulfinylbutyl GSL, 4OHB and 4BZO after feeding with [1,2-^{14}C]desulfo-4MTB suggested that 4MTB is converted to 4BZO via 4-methylsulfinylbutyl GSL and 4OHB. This hypothetical pathway has been further elucidated by identifying the flavin-dependent mono-oxygenases that convert methylthio-GSLs into methylsulfinyl GSLs (Hansen *et al.*, 2007; Li *et al.*, 2008), and AOP3, which converts methylsulfinyl GSLs to OH-GSLs (Kliebenstein *et al.*, 2001b). Despite these advances, the acyltransferase(s) that convert OH-GSLs to BzGSLs and SnGSLs has not been identified.

The BAHD and the SCPL acyltransferases are good candidate families to which the acyltransferase(s) in question may belong. On one hand, it has been suggested that BAHD acyltansferase(s) may synthesize BzGSLs and SnGSLs based upon biochemical precedents and the known involvement of BZO1 in BzGSL synthesis (Kliebenstein *et al.*, 2007; Ibdah and Pichersky, 2009; Ibdah *et al.*, 2009; Sønderby *et al.*, 2010). On the other hand, there are a number of examples in which sinapoylated metabolites are synthesized in Arabidopsis from the activated sinapate donor SG

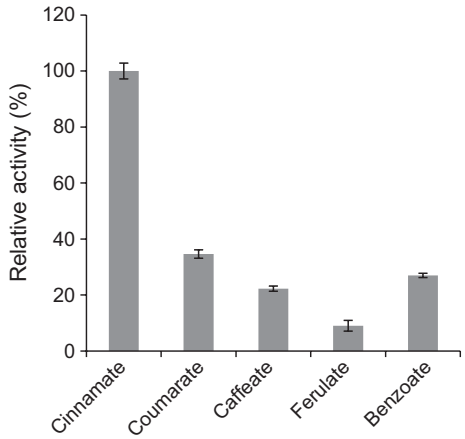

Figure 8. Substrate specificity of BZO1.
Relative activities of BZO1 were measured for hydroxycinnamates (0.8 mM) and CoA (2 mM). Activity with cinnamate was 10 533 pkat mg^{-1} protein and was set as 100%. Values are means ± SD (n = 3).

through the action of an SCPL acyltransferase (Lehfeldt *et al.*, 2000; Shirley *et al.*, 2001; Fraser *et al.*, 2007). Co-expression analyses suggested that one or more of the several SCPL genes expressed in developing seeds may mediate these reactions (Figure 1). Previous reports have shown that *AOP3* and *BZO1* are abundantly expressed in seeds compared to siliques, whereas other GSL biosynthesis genes working at earlier steps display the opposite pattern (Nour-Eldin and Halkier, 2009). This indicates that precursor GSLs are synthesized in maternal tissues and that side-chain modifications leading to BzGSL biosynthesis occur in seeds. Specifically, these reactions may occur in embryos, where seed GSLs are accumulated (Kliebenstein *et al.*, 2007). Co-expression of SCPL genes with the two reference genes suggest that these genes may function with *AOP3* and *BZO1* in seed embryos.

Our SCPL-mediated acylation hypothesis was supported by the observation that mutations in two SCPL genes, *SNG2*/*SCPL19* and *SCPL17*, led to decreases in both BzGSLs and SnGSLs (Figure 2). While approximately 10% residual BzGSLs and SnGSLs remain in *sng2*, these GSLs were near the limits of detection in *scpl17*. In addition to these BzGSLs and SnGSLs, similar changes were observed for a pair of putative benzoylated and sinapoylated GSLs that eluted after the two known major BzGSLs and SnGSLs. The *m*/*z* values for these unknown compounds are 524 and 626 under ESI-negative mode, which match the masses of benzoylated and sinapoylated 5-methylthiobutyl GSL (5MTB), respectively. Their amounts decreased in *scpl17* and *sng2* mutants, while the benzoylated form decreased and the sinapoylated form increased in *bzo1*. Benzoylated 4-methylthiopropyl GSL, in which the benzoyl unit is linked to the thioglucose moiety, has been identified in Arabidopsis (Reichelt *et al.*, 2002), but acylated forms of 5MTB have not. Even though their identities need to be confirmed, this finding suggests that SCPL enzyme(s) may have broad substrate specificity for benzoylating GSLs.

The fact that mutations in both *SCPL17* and *SNG2* strongly reduce BzGSL and SnGSL accumulation suggests two possible models for involvement of the corresponding proteins. According to a sequential model, SCT synthesizes sinapoylated and benzoylated intermediates, possibly choline esters, which are then used to acylate OH-GSLs by SCPL17. Consistent with this model, SCT, which is known to synthesize sinapoylcholine (Shirley *et al.*, 2001), is required for BC accumulation *in vivo* (Figures 3 and 6), and can also generate BC when assayed *in vitro* (Figure 4). Further, BC accumulates in *scpl17* mutants, suggesting that it may be a substrate for the enzyme that *SCPL17* encodes. On the other hand, choline esters are expected to be poor benzoyl and sinapoyl donors in comparison to 1-*O*-glucose esters, which are known to have a high free energy of hydrolysis (Mock and Strack, 1993).

In the second and preferred indirect model, SCPL17 catalyzes both benzoylation and sinapoylation of OH-GSLs using BG and SG as donors, but the impact of *sng2* mutations is indirect (Figure 9). This model is consistent with the stronger GSL phenotype of *scpl17* versus *sng2* mutants, and the observation that BzGSL accumulation is enhanced when *sng2* siliques, but not *scpl17* siliques, are provided with exogenous BA (Figure 6c). In this model, BC accumulation in *scpl17* mutants occurs only because a pool of BG accumulates when BzGSL synthesis is blocked, and this pool becomes available to SCT for trans-esterification. Unfortunately, attempts to directly test the ability of SCPL17 heterologously expressed in *Nicotiana benthamiana* to acylate OH-GSLs using BG (and BC) *in vitro* were unsuccessful even though control experiments measuring SCT

Table 1 Kinetic parameters for BZO1

Substrate	K_m (μM)	V_{max} (pKat mg^{-1})	k_{cat} (sec^{-1})	k_{cat}/K_m (mM^{-1} sec^{-1})
t-cinnamate	138 ± 17	2325 ± 187	0.153 ± 0.012	1.12 ± 0.05
Benzoate	169 ± 7	710 ± 111	0.047 ± 0.007	0.28 ± 0.04
CoA	229 ± 1	1910 ± 3	0.126 ± 0.000	0.55 ± 0.00

Values are means ± SD (n = 3).

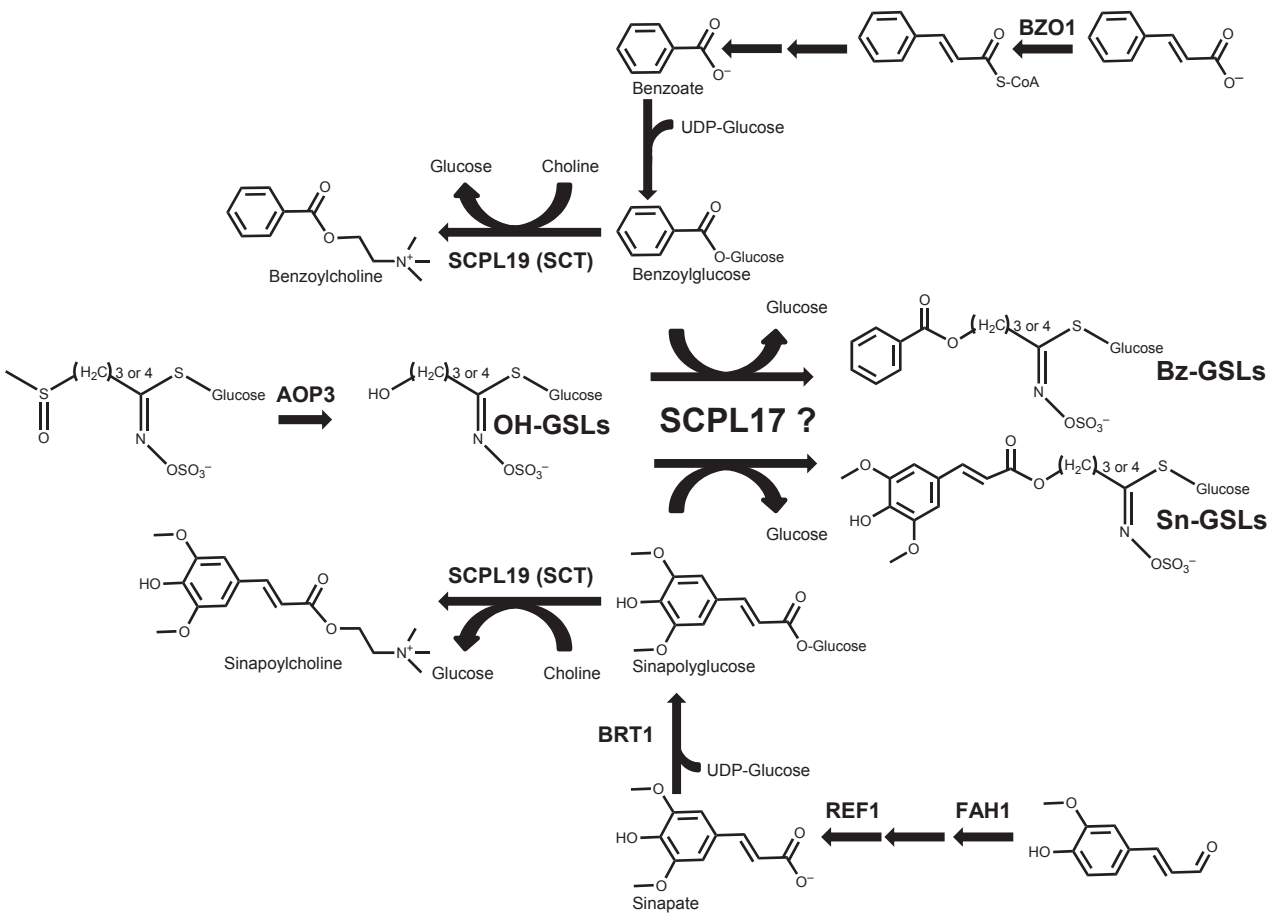

Figure 9. Preferred model explaining BzGSL and SnGSL biosyntheses.
SCPL17 may mediate final acyltransferase reactions by using glucose esters as acyl donors. BZO1 works as a cinnamoyl CoA ligase and provides benzoate for BzGSL biosynthesis.

and sinapoylglucose:malate sinapoyltransferase (SMT) (Lehfeldt *et al.*, 2000) activities using previously established assay conditions (Lehfeldt *et al.*, 2000; Shirley *et al.*, 2001; Shirley and Chapple, 2003) were not problematic. For these transient expression studies in tobacco, we used the same CsMV:*SCPL17* vector that we used to complement *scpl17* mutants, and thus would expect successful protein transient expression in tobacco as well. Although it is unclear why these experiments were unsuccessful, previous attempts to heterologously express SCPL proteins have been challenging (Lehfeldt *et al.*, 2000; Shirley *et al.*, 2001; Shirley and Chapple, 2003), perhaps due to the large number of disulfide cross-links that need to be made correctly in order for this class of proteins to function. Alternatively, these data may indicate that the substrate for the enzyme is not the mature glucosinolate, or that the enzyme requires specific assay conditions, essential co-factors or partner proteins for activity that we have not yet identified.

Although our second model appears more likely at this time, it is unclear how the postulated indirect effect of *sng2* mutations on BzGSL and SnGSL accumulation is mediated. Mutations in *SNG2* are known to dramatically alter soluble phenylpropanoid pools, and it is possible that the accumulation of SG has a direct impact on SCPL17-mediated catalysis. Alternatively, these alterations in pool sizes may act through the same mechanism that leads to alterations in BzGSL levels in *brt1*, *ref1* and *fah1* (Figure 7). Finally, previous studies have demonstrated interactions between the glucosinolate and phenylpropanoid biosynthetic pathways (Hemm *et al.*, 2003), and it is possible that the same metabolic interplay extends to the level of the OH-GSL acyltransferase encoded by *SCPL17*.

BZO1 functions as a cinnamoyl CoA ligase

The observation that BA feeding restores BzGSL accumulation in *bzo1* indicates that the benzoyl CoA ligase activity of BZO1 is not required for BzGSL synthesis and that BZO1 must have another catalytic function elsewhere in the pathway. Our results showing that SCPL17 is the acyltransferase involved in transferring the benzoyl moiety from BG

to OH-GSLs, and the kinetic characterization of BZO1 *in vitro* (Table 1) showing that the enzyme prefers cinnamate to BA, demonstrate that both of these conclusions are correct. These findings place BZO1 at the beginning of the BA synthetic pathway where it functions in the peroxisome as a cinnamoyl CoA ligase as recently reported in petunia (Klempien *et al.*, 2012). These findings shed more light on the BA biosynthetic pathway in Arabidopsis. The near absence of BA-derived compounds such as BC, BG and BzGSLs in *bzo1* and *chy1* mutants (Ibdah and Pichersky, 2009) suggests that BA biosynthesis in Arabidopsis seeds occurs exclusively via cinnamoyl CoA, and that the non-β-oxidative pathway mediated by direct conversion of cinnamate into benzaldehyde is of negligible importance, at least in developing seeds. Although either BA or benzaldehyde is the likely end product of cinnamoyl CoA metabolism, synthesis of BzGSLs probably occurs in the vacuole where SCPL proteins are thought to be localized (Fraser *et al.*, 2005).

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

Arabidopsis thaliana Columbia-0 was used as the wild-type for all experiments. T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center stock center. Plants were grown in Redi-earth Plug & Seedling Mix (Sun Gro Horticulture, www.sungro.com) supplemented with Scotts Osmocote Plus controlled release fertilizer (Hummert International, www.hummert.com) at 22°C under a 16 h light/8 h dark photoperiod.

Vector construction for complementation

Columbia wild-type genomic DNA was used as a template to amplify genomic DNA fragments for cloning. Genomic fragments containing ORFs were amplified using primers CC2628 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGGGGAAAGAGTGCTACTACT-3′) and CC2714 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTATCTAGTGTGCTATTTGGCGA-3′) for *SCPL17*, and CC2237 (5′-GGGGACAAGTTTGTACAAAAAAGCAGGCTCCATGAGAAACCTTAGCTTCATCG-3′) and CC2238 (5′-GGGGACCACTTTGTACAAGAAAGCTGGGTAAACTGTAACAACAACAAAGAGAGAA-3′) for *SNG2*. In addition, the upstream promoter region (849 bp from ATG) and the ORF of *SCPL17* were amplified using CC2713 (5′-GGGGACAACTTTTGTATACAAAGTTGTTCTCTTTGATCAAAAGTTTTTGA-3′) and CC2714. All these fragments were recombined with pCC1155 (Bonawitz *et al.*, 2012), a donor vector modified from pDONR221 (Invitrogen, www.invitrogen.com). The resulting entry vectors were recombined with two types of binary destination vectors: pCC1136 (Weng *et al.*, 2011) to clone the open reading frame under the control of its endogenous promoter and pCC1607 in which gene expression is driven by the cassava vein mosaic virus (CsMV) promoter. pCC1607 was constructed from pCC1136 by inserting the cassava vein mosaic virus (CsMV) promoter in front of the Gateway cassette using *Hind* III and *Xho* I sites. Finally, the recombined constructs pCC1702 (CsMV:*SCPL17*), pCC1703 (*SCPL17*:*SCPL17*) and pCC1705 (CsMV:*SNG2*) were introduced into *Agrobacterium tumefaciens* C58 pGV3850, and transformed into the mutants by the floral-dip method (Clough and Bent, 1998).

LC-MS/MS and LC-MS analyses for soluble seed metabolites

For LC-MS/MS and LC-MS analyses, soluble metabolites were extracted from 5 mg seeds. Seeds were ground after soaking in 50% MeOH (20 μl mg^{-1}) at 65°C for 10 min, and then further incubated at 65°C for 20 min. After centrifuging (11000 g, 25°C, 10 min), the supernatant was removed and extracted with an equal volume of hexane, and 10 μl of the MeOH phase was injected for each analysis.

The identification of compounds that contain a benzoylated and sinapoylated moiety was accomplished by LC-MS/MS. Separations were performed on an Agilent (www.home.agilent.com) 1200 system using a Shimadzu (www.shimadzu.com) Shim-pack XR-ODS column (2.2 μm particle size, 75 × 3.0 mm internal diameter) with a flow rate of 0.6 ml min^{-1}. The mobile phase consisted of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile). The starting eluent was 90% A/10% B. The proportion of B was increased linearly to 25% over 14 min, then to 70% over 8 min, then to 95% over 3 min, and held at 95% for 3 min. The eluent was adjusted to initial conditions over 1 min and held for 4 min to re-equilibrate the column. Following separation, the column effluent was passed through a photodiode array detector and then introduced into a Agilent 6460 triple-quadrupole MS utilizing positive mode JetStream ESI (capillary voltage 3.3 kV, nebulizer gas pressure 450 kPa; gas temperature 350°C; drying gas flow rate 11 L min^{-1}; sheath gas temperature 250°C; sheath flow rate 7 L min^{-1}). The tandem mass spectrometer was set to run in precursor ion mode. MS1 scanned between 40 to 800 *m*/*z*. MS3 toggled between *m*/*z* 207.1 for sinapoylated compounds and *m*/*z* 105.1 for benzoylated compounds. A collision energy of 24 eV was used. A response was only recorded if loss of sinapate or BA was observed. MS1 then recorded the mass of the parent compound. Spectroscopic and mass data were collected and analyzed using AGILENT MASSHUNTER B.03 software (Agilent, www.chem.agilent.com).

Quantification of BG and BC was accomplished by LC-MS. Separations were performed on an Agilent HPLC 1100 system using a Shimadzu Shim-pack XR-ODS column (2.2 μm particle size, 75 × 3.0 mm internal diameter) with a flow rate of 0.6 ml min^{-1}. The mobile phase consisted of eluent A (0.1% formic acid in water) and eluent B (0.1% formic acid in acetonitrile). The starting eluent was 90% A/10% B. The proportion of B was increased linearly to 13.5% over 2 min, then to 50% over 3 min, then to 90% over 0.1 min, and held at 90% for 1.9 min. The eluent was adjusted to initial conditions over 0.1 min and held for 2.9 min to re-equilibrate the column. The column effluent was introduced into a photodiode array detector, and then introduced into an Agilent MSD-TOF MS under ESI-positiive mode (capillary voltage 3.5 kV; nebulizer gas pressure 480 kPa; gas temperature 350°C; drying gas flow rate 11 L min^{-1}; fragmentor voltage 125 V; skimmer 65 V; octopole radio frequency voltage 250 V). The acquisition mass range was 70–1500 *m*/*z*. Spectroscopic and mass data were collected and analyzed using AGILENT MASSHUNTER B.03 software. BG and BC quantification was accomplished using multi-level calibration curves constructed using authentic standards.

Glucosinolate analyses

For GSL isolation, a simple 1.5 ml microcentrifuge tube method was established based on a previously published method (Glover *et al.*, 1988). A sample of 5 mg seeds was incubated at 65°C in 200 μl 50% MeOH after adding 10 μl of 5 mM sinigrin and 10 μl of 0.3 M lead acetate. After 10 min, the wet seeds were ground and further incubated at 65°C for 20 min. Each sample was centrifuged (11000 g, 15

min, 25°C), and 150 μl of the supernatant was taken and mixed with 100 μl QAE Sephadex (Sigma, www.sigmaaldrich.com) equilibrated with water in a new microcentrifuge tube. After mixing and brief centrifugation, the supernatant was removed after brief centrifugation, and the resin was washed twice with 1 ml 50% MeOH and twice with water. To each sample was added 60 μl of an aqueous aryl sulfatase solution, which contains 3 μl sulfatase stock solution prepared from sulfatase powder (20 000 U g^{-1}, Sigma) as described by Graser *et al.* (2000). Then, samples were incubated for 6 h at 37°C. After incubation, 100 μl water was added to each sample and 70 μl was removed for analysis.

Isolated desulfo-GSLs (30 μl) were separated using HPLC (Shimadzu) using a Varian Microsorb C18 reversed-phase column (Fisher Scientific, www.fishersci.com) (5 μm particle size, 150 × 4.6 mm internal diameter) using a flow rate of 1 ml min^{-1}: 2 min at 5% acetonitrile, 2 min gradient from 5 to 8% acetonitrile, 4 min gradient from 8 to 32% acetonitrile, 9 min gradient from 32 to 59% acetonitrile, 1 min gradient from 59 to 95% acetonitrile, 4 min at 95% acetonitrile, 1 min gradient from 95 to 5% acetonitrile, and 4 min at 5% acetonitrile. Each desulfo-GSL was identified based on comparison of retention times with published results and LC-MS analyses. For quantification of each desulfo-GSL, the peak area at 229 nm relative to the internal standard (desulfo-sinigrin) was calculated, and the absolute amount was obtained using appropriate response factors (Haughn *et al.*,1991; Brown *et al.*, 2003). To quantify sinapoylated desulfo-GSLs, the peak area was integrated at 330 nm and sinapic acid was used as a standard.

Enzyme assays

Agrobacterium tumefaciens C58 pGV3850 transformed with constructs pCC1607 (empty vector) or pCC1705 (CsMV:*SCT*) was infiltrated into leaves of *Nicotiana benthamiana*. After 4 days, leaf tissue was harvested, ground in liquid nitrogen and suspended in 2.6 ml of extraction buffer (50 mM MOPS, pH 7.0), and incubated at 4°C for 15 min with gentle shaking. After centrifugation, protein extracts were filtered through Miracloth (Calbiochem, www.calbiochem.com) and desalted using Amersham Biosciences PD-10 desalting columns (GE Healthcare, www.gelifesciences.com).

For the SCT enzyme assay, 16 μl of protein extract was mixed with 2 μl of 1 mM SG or 2 μl of 1 mM BG and 2 μl of 1 M choline. After incubation for 2 h at 30°C, the reaction was terminated and proteins were precipitated with 90 μl of cold 100% MeOH. SG was purified from *sng1* mutant leaves (Lehfeldt *et al.*, 2000), and BG was synthesized from BA and UDP-glucose using UGT74F2 expressed in *E. coli* (Lim *et al.*, 2002) or from Arabidopsis leaves fed with BA.

For the BZO1 assay, the coding region of *BZO1* (At1g65880) was amplified using the sense oligonucleotide CCATATGATGGA TGATTTGGCATTATG and the antisense oligonucleotide GCTCGA GAAGCCGCGAAATAAAATGTC, and sub-cloned into the *Nde*I–*Xho*I site of expression vector pET-32a (Novagen, http://www.emdmillipore.com/life-science-research/novagen/) in-frame and upstream of the His_6 tag. Induction, harvesting and protein purification by affinity chromatography on Ni-NTA agarose (Qiagen, www.qiagen.com) were performed as described previously (Klempien *et al.*, 2012). The affinity-purified BZO1 proteins were used for kinetic analyses. Enzyme assays and assessment of kinetic properties were performed as described previously (Klempien *et al.*, 2012).

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Complementation analyses of *scpl17* mutant seeds.

Figure S2. Complementation analyses of *sng2-2* mutant seeds.

Figure S3. Benzoate feeding in wild-type and *bzo1-4* siliques.

Table S1. Abundances of glucosinolates in seeds of wild-type, *bzo1*, *scpl17* and *sng2* mutants of Arabidopsis.

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