Plant Biology ISSN 1435-8603

RESEARCH PAPER

Arabidopsis Chy1 null mutants are deficient in benzoic acid-containing glucosinolates in the seeds

M. Ibdah & E. Pichersky

Department of Molecular, Cellular and Developmental Biology, University of Michigan, Ann Arbor, MI, USA

Keywords:
Benzoic acid; benzoyloxyglucosinolate; Chy1; cinnamoyl-CoA; hydrolase; T-DNA mutation.

Correspondence
E. Pichersky, Department of Molecular, Cellular and Developmental Biology, University of Michigan, 830 North University Street, Ann Arbor, MI 48109, USA.
E-mail. lelx@umich.edu

Editor
B. Piechulla

Received: 1 July 2008; Accepted: 13 September 2008

doi:10.1111/j.1438-8677.2008.00160.x

ABSTRACT

The specific set of reactions that lead to the synthesis of benzoic acid in plants is still unclear, and even the subcellular compartment in which these reactions occur is unknown. Biosynthesis of both vegetative tissues and seeds of *Arabidopsis thaliana* contain a class of defense compounds termed glucosinolates, but only the seeds synthesize and store high levels of two glucosinolate compounds that contain a benzoic acid moiety. To identify genes involved in the synthesis of benzoic acid (directly or *via* benzaldehyde) in *Arabidopsis*, we analysed the levels of benzoylated glucosinolates in several lines that carry mutations in genes with homology to *Pseudomonas fluorescens* feruloyl-CoA hydratase, an enzyme that converts feruloyl-CoA to vanillin and acetyl-CoA, a reaction analogous to the conversion of cinnamoyl-CoA to benzaldehyde. We show here that mutations in the gene *At5g65940*, previously shown to encode a peroxisomal protein with β-hydroxyisobutyryl-CoA hydrolase activity and designated as *Chy1*, lead to a deficiency of benzoic acid-containing glucosinolates in the seeds. Furthermore, *Chy1* exhibits cinnamoyl-CoA hydrolase activity with a K_m of 2.9 μm. Our findings suggest that at least a part of benzoic acid biosynthesis occurs in the peroxisomes, although the specific pathway that leads to benzoic acid and the specific biochemical role of *Chy1* remain unclear.

INTRODUCTION

Benzoic acid (BA) and its derivatives serve as important structural elements in a large number of compounds in plants and have a range of functions. For example, both taxol and cocaine, produced by plants as defense compounds, contain a benzoyl group (Bjorklund & Leete 1992; Walker & Croteau 2000). Methylbenzoate and benzylbenzoate are common volatile esters found in the scent of many flowers, including snapdragon and petunia, and function to attract pollinators (Negre *et al.* 2003). Benzoyl moieties are not limited to specialized metabolites, as they are also present, for example, in some cytokinins, with *meta*-topolin being the most active compound among the aromatic compounds (Werbrouck *et al.* 1996).

Several hypothetical routes have been proposed for the biosynthesis of BA from a phenylpropanoid precursor in plants that, in principle, fall into two basic routes, β-oxidative and non-β-oxidative, and lead initially to either benzoyl-CoA or benzaldehyde, respectively (Fig. 1) (Beuerle & Pichersky 2002). It is possible that several of these pathways coexist in plants and might even be active simultaneously in the same tissue (Orlova *et al.* 2006). Experiments with stable isotope-labelled precursors in tobacco (*Nicotiana tabacum*) leaves (Ribnicky *et al.* 1998) suggest that benzoic acid is produced from phenylalanine-derived cinnamic acid *via* the β-oxidative pathway, mirroring the fatty acid β-oxidation pathway (Hertweck *et al.* 2001), and yielding benzoyl-CoA first, which is then hydrolysed by a thioesterase to free benzoic acid. In contrast, labelling experiments along with initial enzyme characterization in *Hypericum androsaemum* cell cultures (Abd El-Mawla & Beerhues 2002) support the existence of the non-oxidative conversion of cinnamic acid to benzaldehyde, with subsequent formation of benzoic acid, which can be further converted to benzoyl-CoA (Beuerle & Pichersky 2002). However, none

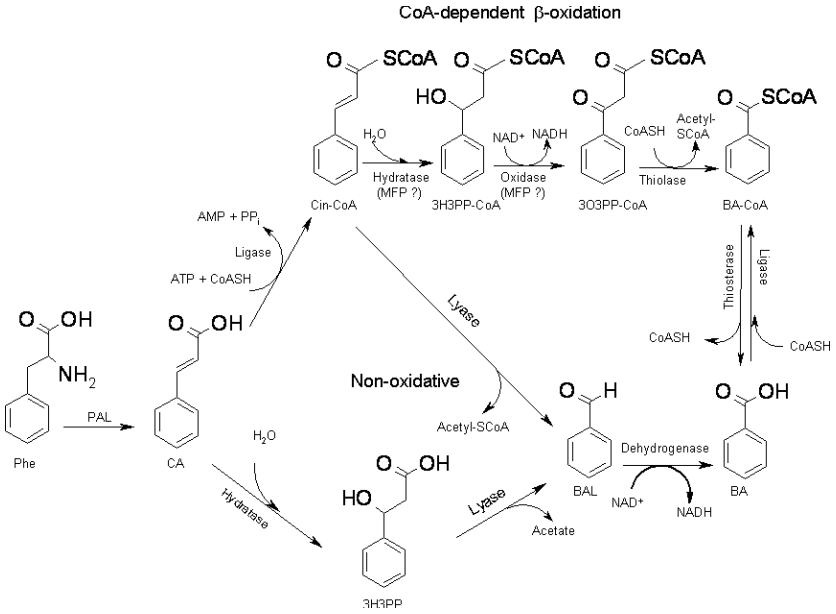

Fig. 1. Proposed biosynthetic pathway leading to benzoic acid in *Arabidopsis via* phenylalanine. The conversion of CA to BA could occur either by CoA-dependent β-oxidation or by non-oxidative routes. The non-oxidative pathway may use a free cinnamic acid or cinnamoyl-CoA. There is some evidence for the first pathway in plants, and an analogous reaction to the latter occurs in *Pseudomonas fluorescens* (see Wildermuth 2006).

of the genes or enzymes involved in any chain-shortening steps in BA biosynthesis has been identified or characterized in plants.

In the bacterium *Pseudomonas fluorescens*, a feruloyl-CoA hydratase/lyase was found that catalyses the metabolism of feruloyl-CoA to vanillin and acetyl-CoA *via* hydration and reverse aldol cleavage (Gasson *et al.* 1998; Mitra *et al.* 1999). This enzyme is active on a number of 4-hydroxycinnamoyl-CoA substrates, but not on cinnamoyl-CoA (Gasson *et al.* 1998; Mitra *et al.* 1999). This led to the postulation of a similar CoA-dependent non-oxidative pathway involved in BA biosynthesis in plants, and to a subsequent report of cinnamoyl-CoA hydratase/lyase activity in protein extracts of elicited *H. androsaemum* cells (Abd El-Mawla & Beerhues 2002). To date, this enzyme activity has not been further purified from *H. androsaemum* or any other plant species, so the identity of the protein possessing such activity is not known.

Arabidopsis and related species synthesize a class of compounds known as glucosinolates that serve in defense throughout the plant. These compounds all have the same basic β-thioglucose structure, but differ in the structure of the aliphatic side chain. Developing *Arabidopsis* seeds, but not other parts of the plant, synthesize large amounts of two types of glucosinolate whose side chain contains a benzoyl moiety (Graser *et al.* 2001). Because of the high amounts of benzoylated glucosinolates in *Arabidopsis* seeds, these seeds constitute an excellent system to examine the biosynthesis of benzoic acid. In this report, we demonstrate that *Arabidopsis* plants homozygous for null alleles in the gene *At5g65940 (Chy1)*, a distant homolog of *P. fluorescens* feruloyl-CoA hydratase/lyase that was previously shown to encode a peroxisomal protein that affects β-oxidization (Zolman *et al.* 2001), are deficient in benzoylated glucosinolates in the seeds.

MATERIAL AND METHODS

Plant materials

Arabidopsis thaliana (ecotype Columbia) plants were grown on soil at 23 °C under 16 h light/8 h dark for 4–6 weeks. SALK mutant lines were obtained from the ABRC. In addition, plants homozygous for two mutant *Chy1* alleles, *chy1-1* and *chy1-3*, were obtained from Dr Bonnie Bartel (Rice University, Houston, USA).

Extraction and analysis of benzoylglucosinolates

Extracts were made using methods adapted from previously published protocols (Reichelt *et al.* 2002). Seeds (20 mg) were extracted in a 1-ml solution of 80% MeOH to which 30 μl of 10 mM sinigrin (Sigma-Aldricht, Allentown, PA, USA) were added as an internal standard (final concentration 300 μM). The extracts were centrifuged in a microcentrifuge at 2000 *g* for 10 min and the supernatant loaded onto a small (50 mg) column of DEAE Sephadex A25 (Sigma-Aldricht). Water was added to the DEAE Sephadex A25 to induce swelling. The column was rinsed successively with 1 ml 80% (aqueous) MeOH, 1 ml deionized water, and 1 ml 20 mM NaOAc, pH 5.5. After washing, 25 μl sulfatase (Sigma-Aldricht) solution, prepared according to Graser *et al.* (2001), were loaded on the column and the column was left standing overnight. The resulting desulfoglucosinolates were eluted from the column with 0.5 ml 20% (aqueous) MeOH.

Extraction and HPLC analysis of benzoic acid

The protocol for extraction of BA was modified from Chong *et al.* (2001). Plant material (10–20 mg seeds) was extracted with 2 ml 90% MeOH containing 10 μl 10 mM

cinnamic acid as an internal standard. After centrifugation in a microcentrifuge for 5 min at 14,000 rpm, the residue was extracted again with 2 ml 100% MeOH. The combined extracts were reduced to dryness under nitrogen. Samples were either analysed directly by HPLC for BA after resuspension in methanol, or dried extracts were resuspended in 1 ml water at 50 °C, saponified with 100 µl 1 M NaOH for 30 min at room temperature, neutralized with 30 µl 1 N HCL, centrifuged in a microcentrifuge for 5 min at 14,000 rpm, and then injected into the HPLC.

Cloning and expression of a cDNA of the *Arabidopsis At5g65940* (*Chy1*) gene

RNA from the siliques of *A. thaliana* Columbia ecotype Col-0 was isolated using methods adapted from previously published protocols (Maes & Messens 1992). Total RNA (5 µg) was reversed-transcribed using an oligo dT primer and MLV RT (Stratagene, La Jolla, CA, USA). The open-reading frame of the *Chy1* gene (*At5g65940*) was amplified by PCR using the primers 5′-ATGGCAGT-CGAGATGGCCTCTCA-3′ and 5′-CAGCTTTGCGATC-CCTAAAGCAGGCAAGTTATT C-3′, which eliminated the termination codon. The high-purity KOD-DNA polymerase (Novagen, Madison, WI, USA) was used and constructs were verified by DNA sequencing. The cDNA was ligated into the vector pEX5-CT/TOPO TA (Invitrogen, Carlsbad, CA, USA) to create a fusion of the open-reading frame with a His tag-coding extension at the C-terminus. The plasmid was transferred into *Escherichia coli* BL21 (DE3) cells. Protein expression was induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside at 18 °C for 14 h. Bacteria were lysed and soluble protein purified by Nickel chromatography and assayed for purity by SDS–PAGE as previously described (Koeduka *et al.* 2008).

Enzyme activity measurements

Enzyme assays were carried out in a buffer containing 50 mM Tris/HCl pH 7.0, with 20 µM substrate and 1–500 ng of total protein in a total volume of 100 µl. The assays were incubated at 30 °C for 30 min, after which reactions were terminated by the addition of 20 µl 3.5% TCA in 50% acetonitrile. The reaction products were analysed by reverse phase liquid chromatography on a Inertsil ODS-2 5 µm C18 column (150 × 4.6 mm i.d., 5 µm particle size) operated at 0.8 $ml \cdot min^{-1}$ and 30 °C, and eluted with a gradient (solvent A: 1.5% phosphoric acid in H_2O, solvent B: acetonitrile) of 0–10% B (2 min), 10–45% B (12 min), 45–50% B (16 min) 50–95% (17 min), 95% (1 min), 95–10% (25 min). Compound elution was monitored at 200–400 nm with a Waters 996 UV/visible photodiode array detector (Waters, Milford, MA, USA).

Characterization of Chy1 kinetic parameters

In all enzyme activity measurements, appropriate enzyme concentrations and incubation times were chosen so that the reaction velocity was linear during the incubation time, and at least three replicates were performed. pH optima were measured in 50 mM Tris-buffer ranging from pH 5.5 to 9.0.

Enzymatic synthesis and purification of aromatic coenzyme A esters

Cinnamoyl-CoA, p-coumaroyl-CoA, and 3-hydroxy-3-phenoylpropionoyl-CoA were prepared using tobacco 4Cl as described by Beuerle & Pichersky (2002).

RESULTS

A bioinformatic search for an *Arabidopsis* gene homologous to *Pseudomonas fluorescens* feruloyl-CoA hydratase/lyase

A bioinfomatic search for plant proteins with significant sequence similarity to *Pseudomonas fluorescens* feruloyl-CoA hydratase/lyase did not yield any protein with sequence identity >30%. However, in the *Arabidopsis* genome there are at least 13 genes encoding proteins with low (<30%) but statistically significant sequence similarity to *P. fluorescens* feruloyl-CoA hydratase/lyase (Fig. 2). These include the two peroxisomal multifunctional proteins encoded by genes *At4g29010* and *At3g06860,* each containing a hydratase domain known to be involved in the β-oxidation degradative pathway of fatty acids (Richmond & Bleecker 1999; Rylott *et al.* 2006), as well as the protein encoded by *At5g65940*, designated Chy1 and also shown to be localized to peroxisomes (Zolman *et al.* 2001). *chy1* mutants have been shown to be defective in the β-oxidization of fatty acids (Zolman *et al.* 2001; Lange *et al.* 2004). They are also defective in the conversion of indole-3-butyric acid (IBA) to indole-3-acetic acid (IAA), a reaction that requires the removal of two carbons from the butyric side chain and that has been postulated to occur through the β-oxidation pathway (Zolman *et al.* 2001). The only enzymatic activity so far demonstrated for *Chy1* is hydrolysis of β-hydroxyisobutyryl-CoA to β-hydroxyisobutyrate and CoA, a reaction that is part of the valine degradation pathway in animals (Shimomura *et al.* 1994; Zolman *et al.* 2001). The inhibitory affect of the *chy1* mutants on β-oxidation was hypothesized to occur *via* the accumulation of a toxic intermediate in the valine degradation pathway (Zolman *et al.* 2001). However, whether valine degradation in plants occurs in peroxisomes is still unknown (Lange *et al.* 2004; Lucas *et al.* 2007).

Seeds of mutants in the *Chy1* gene are deficient in benzoylated glucosinolates

We obtained T-DNA knockout lines (in *Arabidopsis* Col-0 background) of many of the 13 *Arabidopsis* homologs of *P. fluorescens* feruloyl-CoA hydratase/lyase and tested their seeds for the presence of benzoylated glucosinolates. Null mutations in the two multifunctional proteins of fatty acid

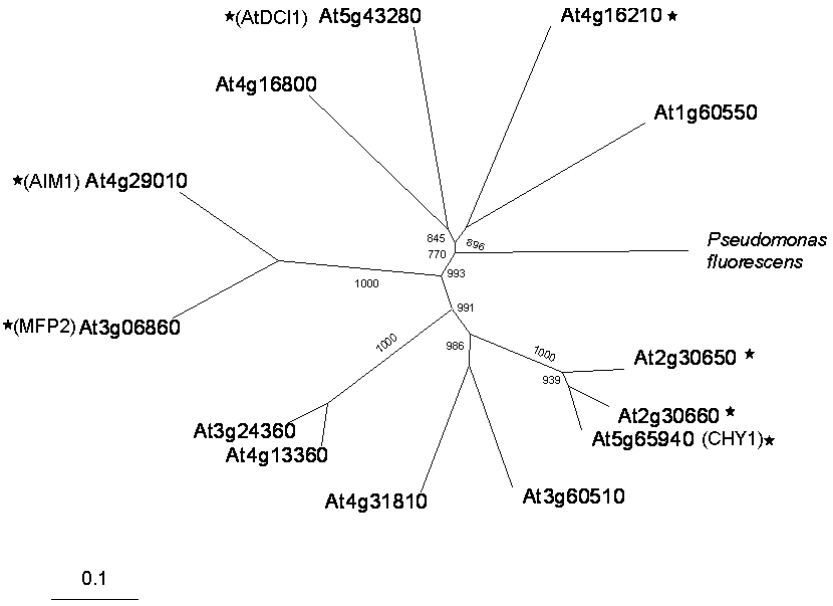

Fig. 2. A phylogenetic tree of *Pseudomonas fluorescens* feruloyl-CoA hydratase/lyase and 13 homologous *Arabidopsis* proteins. The unrooted tree was generated using CLUSTALW version 1.83 (EBI, http://www.ebi.ac.uk/clustalw/). The bootstrap method was performed for 1000 replicates. The asterisk next to the protein indicates that its C-terminus contains the peroxisomal targeting sequence AKL or SKL.

β-oxidation are either lethal or sterile (Richmond & Bleecker 1999; Rylott *et al.* 2006) so it was not possible to test seeds from such mutants. Homozygous lines for a T-DNA insertion in genes *At4g16800*, *At4g16210*, and *At1g60550* also could not be obtained. Seeds of homozygous lines with T-DNA insertions in genes *At5g43280*, *At2G30650*, *At3g06860*, *At2g30660*, and *At4g16210* had normal levels of the two benzoylated glucosinolates found in *Arabidopsis* Col-0 wild-type seeds, 3-benzoyloxypropylglucosinolate (3BZO) and 4-benzoyloxybutylglucosinolate (4BZO) (see for example T-DNA insertion mutant of *At2g30650*, Fig. 3A and B). In contrast, seeds of plants from a homozygous line with a T-DNA insertion (SALK_025417) in gene *At5g65940* (*Chy1)* had no detectable levels of these two benzoylated glucosinolates. Since several EMS-induced point mutations in *Chy1* in the *Arabidopsis* Col-0 background have been reported (Zolman *et al.* 2001), we obtained two of those, *chy1-1* and *chy1-3*, and tested their seeds for the presence of 3BZO and 4BZO. These lines also had no detectable levels of either compound (see Fig. 3 for *chy1-1*).

Seeds of mutants in the *Chy1* gene contain no benzoic acid

To determine if mutants in the *Chy1* gene do not accumulate 3BZO and 4BZO because of a defect in their synthesis rather than in the process of linking them to the glucosinolate moiety, we measured the total levels of BA in these seeds. Methanolic extraction of seed material detected no free BA in any seed line, including the wild type (data not shown). When methanolic extracts were subjected to base hydrolysis, BA was detected in extracts from wild-type plants and from the line homozygous for a T-DNA insertion in gene *At2g30650* but not from seeds of the *chy1* mutants SALK_02541 and *chy1-1* (Fig. 4A and B).

Enzymatic activity of the Chy1 protein

The three amino acids at the C-terminus of the 378-residue Chy1 protein are AKL, a known peroxisomal targeting signal (Nakai & Horton 1999; Emanuelsson *et al.* 2000; Reumann 2004), and previous reports have demonstrated that Chy1 is present in peroxisomes (Zolman *et al.* 2001). Since Chy1 mutants are defective in BA biosynthesis as well as in peroxisomal β-oxidation (Zolman *et al.* 2001), we tested Chy1 for activity with the substrate cinnamoyl-CoA, which may be an intermediate in the biosynthesis of BA through β-oxidation (Fig. 1). Because Chy1 is homologous to the *P. fluorescens* feruloyl-CoA hydratase/lyase, we also checked to see if Chy1 is capable of producing benzaldehyde directly from cinnamoyl-CoA or 3-hydroxy-3-phenoylpropionoyl-CoA in a reaction analogous to that catalysed by *P. fluorescens* feruloyl-CoA hydratase/lyase (Fig. 1).

We used His-tagging and affinity chromatography to purify the *E. coli*-expressed *Arabidopsis* Chy1 protein (Fig. 5). Incubation of cinnamoyl-CoA with purified Chy1 led to the production of free cinnamic acid, and incubation of 3-hydroxy-3-phenoylpropionoyl-CoA with Chy1 led to the production of free 3-hydroxy-3-phenoylpropionate. In addition, incubation of *p*-coumaroyl-CoA with Chy1 led to the production of free *p*-coumaric acid. In contrast, Chy1 had no hydrolase activity with benzoyl-CoA, consistent with the previously published report (Zolman *et al.* 2001).

The K_m of Chy1 with cinnamoyl-CoA was 2.9 ± 1.0 μM, with a V_{max} of 12.6 ± 1.9 $\mu M \cdot s^{-1} \cdot mg^{-1}$ (Fig. 6). The enzyme had a broad pH optimum of 7.0 (Fig. 7), and the cations Mg^{2+}, Fe^{2+}, Cu^{2+}, Zn^{2+}, and Mn^{2+} at concentrations 1 mM had no effect on the hydrolase activity of Chy1, while Cu^{2+} at this concentration caused a >90% inhibition of Chy1 enzyme activity.

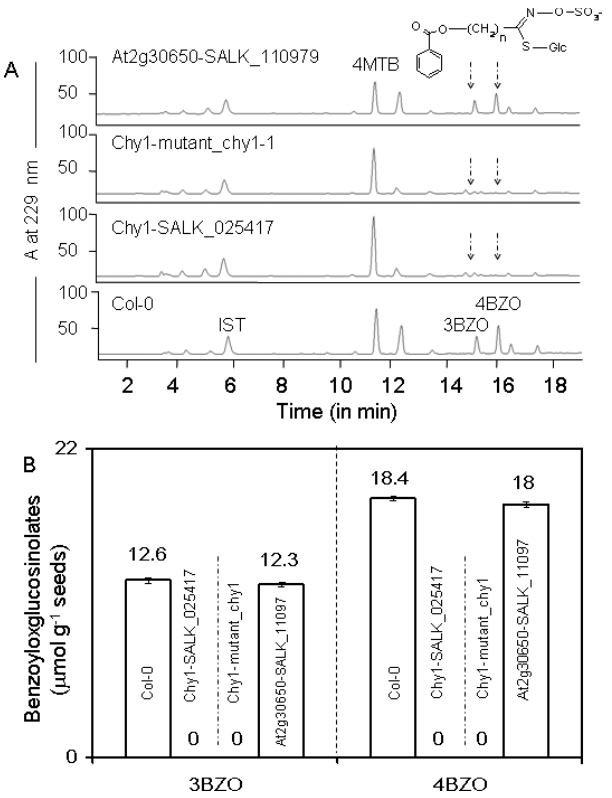

Fig. 3. Mutants in the *Chy1* gene show no accumulation of benzoyloxyglucosinolates in seeds. A: Analysis of benzoyloxyglucosinolates in seeds of different *Arabidopsis* lines by HPLC. Relative abundance is shown in arbitrary units, based on absorbance at 229–280 nm. Inset shows the structures of 3- and 4-benzoyloxyglucosinolate. 3-Benzoyloxypropylglucosinolate (3BZO) and 4-benzoyloxybutylglucosinolate (4BZO) were identified by absorption profile and retention time in comparison to standards. The peak containing the main non-benzoylated glucosinolate compound in these seeds, 4-methylthiobutylglucosinolate (4MTB), is also labeled. IST, internal standard (sinigrin). B: Comparison of the levels of 3BZO and 4BZO found in wild-type and *Chy1* mutant lines. Each bar represents three independent measurements.

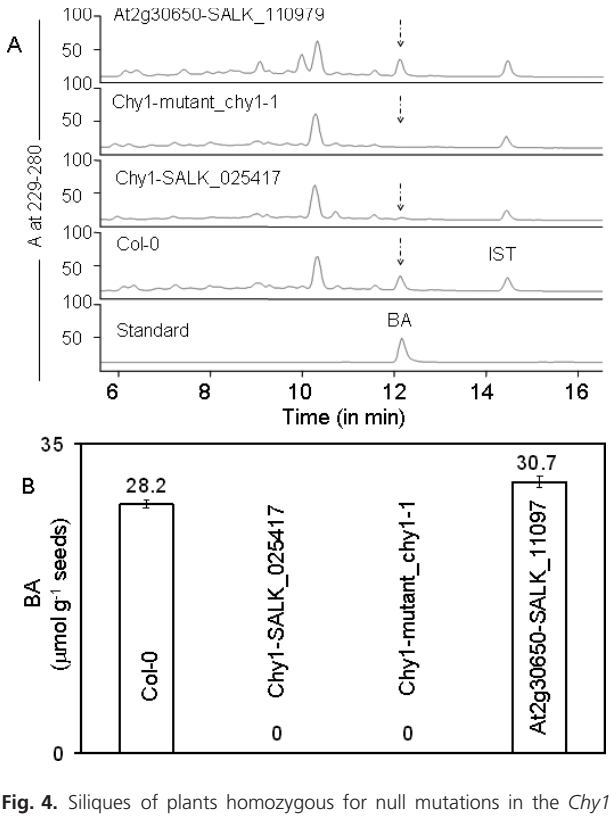

Fig. 4. Siliques of plants homozygous for null mutations in the *Chy1* gene show altered BA accumulation in the seeds. A: Identification of BA in base-hydrolysed *Arabidopsis* seed extracts by HPLC. Relative abundance is shown in arbitrary units, based on absorbance at 229–280 nm. Benzoic acid was identified by its absorption profile and retention time in comparison to standards. IST, internal standard (CA). B: Comparisons of BA levels found in wild-type and *Chy1* mutants lines. Each bar represents three independent measurements.

DISCUSSION

The biosynthesis of BA in plants has remained unclear despite repeated attempts at elucidation (Wildermuth 2006). While labeling experiments have given conflicting results (summarized in Wildermuth 2006), no clear enzymatic reactions responsible for the shortening of the propyl side chain have been conclusively demonstrated. In addition to the lack of biochemical data, mutants directly defective in the synthesis of BA have not yet been reported, suggesting that perhaps the BA biosynthetic pathway (or pathways) is either essential to plant viability or that it contains redundancies.

Benzoic acid biosynthesis in developing *Arabidopsis* seeds for the production of benzoylated glucosinolates appears to constitute a system that is amenable to genetic analysis. A mutant that lacks seed benzoylated glucosinolates has recently been found in *Arabidopsis*, and further analysis identified the mutated gene as *BZL1* (*At1g65880*) (Kliebenstein *et al.* 2007). Biochemical analysis suggested that *BZL1* is capable of catalysing the formation of BA-CoA using BA, CoA, and ATP (Kliebenstein *et al.* 2007). BA-CoA is believed to be the substrate of a benzoyltranferase that links the benzoyl moiety to the glucosinolate moiety (Graser *et al.* 2001; Kliebenstein *et al.* 2007). Interestingly, *BZL1* has a peroxisomal targeting sequence at its C-terminus (Kliebenstein *et al.* 2007), although the synthesis of benzoylated glucosinolates is believed to be in the cytosol, not the peroxisomes (Chen & Andreasson 2001).

Here, we show that mutations that inactivate the Chy1 protein, previously demonstrated to be in peroxisomes, also lead to a deficiency in benzoylated glucosinolates in developing *Arabidopsis* seed. Extracts of seeds of *chy1* mutants also contain no BA after hydrolysis, suggesting that the BA detected in similarly treated extracts of wild-type seeds is derived from hydrolysis of benzoylated glucosinolates. The absence of BA in *chy1* mutant seeds

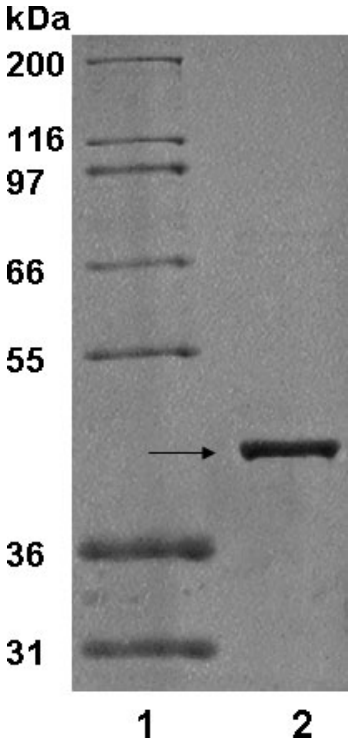

Fig. 5. SDS–PAGE analysis of purified recombinant CHY1. Lanes 1: Molecular mass markers; 2: soluble extract from recombinant CHY1 after Ni-NTA matrix chromatography. The purified CHY1 protein is marked with an arrow.

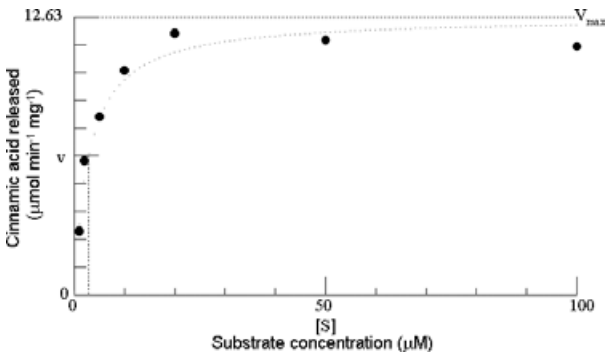

Fig. 6. Cinnamoyl-CoA hydrolase activity of recombinant CHY1 protein. The graph shows the saturation kinetics of CHY1 at pH 7.0 using cinnamoyl-CoA as substrate.

suggests that its biosynthesis occurs, at least partly, in the peroxisomes, although some reactions in this pathway may occur elsewhere.

However, this observation by itself does not distinguish between the possibilities that BA is synthesized *via* β-oxidation or retro-aldol cleavage. Since Chy1 is homologous both to the hydrates domain of the multi-functional protein (MFP) of β-oxidation, as well as to *P. fluorescens*

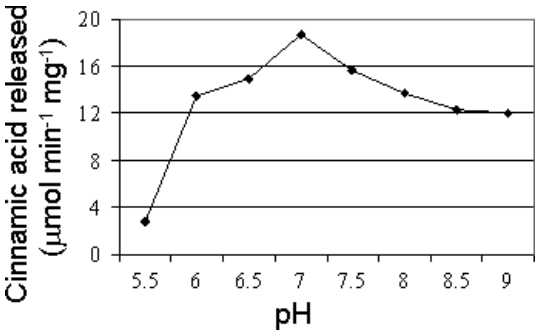

Fig. 7. Determination of the pH optimum of Chy1 hydrolase activity with cinnamoyl-CoA as substrate.

feruloyl-CoA hydratase/lyase, we considered two competing hypotheses: that Chy1 binds cinnamoyl-CoA and hydrates it to 3-hydroxy-3-phenoylpropionoyl-CoA, which is then further metabolized through the β-oxidation pathway, or that it uses cinnamoyl-CoA to produce benzaldehyde, perhaps through a 3-hydroxy-3-phenoylpropionoyl-CoA intermediate (Fig. 1). Since both hypotheses postulate that cinnamoyl-CoA is the substrate of Chy1, we incubated Chy1 with cinnamoyl-CoA, but the only product of this reaction observed was free cinnamic acid. Incubation of Chy1 with 3-hydroxy-3-phenoylpropionoyl-CoA or with p-coumaroyl-CoA also gave the respective free acids.

These results suggest that Chy1 acts *in vitro* primarily as a hydrolase. Zolman *et al.* (2001) previously reported that Chy1 could specifically hydrolyse β-hydroxyisobutyryl-CoA but no other acyl-CoAs tested, including BA-CoA and several other straight-chain and branched-chained acyl-CoAs. Cinnamoyl-CoA, however, was not tested in that study. The K_m for β-hydroxyisobutyryl-CoA was 3.7 μM, similar to the K_m we measured for Chy1 with cinnamoyl-CoA (2.9 μM). Since Chy1 mutants are defective in β-oxidation of fatty acids as well as the conversion of the IAA precursor IBA to IAA, Zolman *et al.* (2001) hypothesized that the mutation in Chy1 leads to accumulation in the peroxisomes of methylacrylyl-CoA, a precursor of β-hydroxyisobutyryl-CoA in the valine degradation pathway, to toxic levels and that the β-oxidation pathway is consequently inhibited. Lange *et al.* (2004) presented labeling data that suggest that valine degradation indeed occurs in the peroxisomes, but recent results presented by Lucas *et al.* (2007) concluded that valine degradation in plants is more likely to occur in the mitochondria and not in the peroxisomes. While our results do not resolve this question, we show here that cinnamoyl-CoA is as good a substrate of Chy1 as β-hydroxyisobutyryl-CoA. We note, however, that hydrolysis of cinnamoyl-CoA is not part of either of the two alternative pathways to BA depicted in Fig. 1. One possibility is that the activity of the Chy1 protein on cinnamoyl-CoA changes when associated with other proteins. Alternatively, Chy1 exerts its influence on BA synthesis indirectly. Thus, while our data strongly suggest that BA is synthesized at least partly in

the peroxisomes, and further indicate that Chy1 has a strong affinity for cinnamoyl-CoA, the exact role that Chy1 plays in BA biosynthesis remains to be determined.

ACKNOWLEDGEMENT

The authors thank Dr Bonnie Bartel for the gift of *chy1-1* and *chy1-3* mutants and for valuable discussion. We also thank Drs. Jonathan Gershenzon and Michael Reichelt for providing glucosinolate standards as well as advice on the isolation and analysis of glucosinolates.

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