In vitro inhibition of shikimate hydroxycinnamoyltransferase by acibenzolar acid, the first metabolite of the plant defence inducer acibenzolar-S-methyl

Jonathan Negrel, Agnès Klinguer, Marielle Adrian

▶ **To cite this version:**

Jonathan Negrel, Agnès Klinguer, Marielle Adrian. In vitro inhibition of shikimate hydroxycinnamoyltransferase by acibenzolar acid, the first metabolite of the plant defence inducer acibenzolar-S-methyl. Plant Physiology and Biochemistry, 2021, 163, pp.119-127. 10.1016/j.plaphy.2021.03.050 . hal-03309048

HAL Id: hal-03309048
https://hal.inrae.fr/hal-03309048

Submitted on 24 Apr 2023

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution - NonCommercial 4.0 International License

Version of Record: https://www.sciencedirect.com/science/article/pii/S0981942821001753
Manuscript_2f5d649ea7973037260cc022a5129f6d

In vitro inhibition of shikimate hydroxycinnamoyltransferase by acibenzolar acid, the first metabolite of the plant defence inducer acibenzolar-S-methyl.

Jonathan Negrel*, Agnès Klinguer, and Marielle Adrian

Agroécologie, AgroSup Dijon, CNRS, INRAE, Univ. Bourgogne Franche-Comté, F-21000 Dijon, France

*Corresponding author. Tel.: 33 (0)380693165; fax: 33 (0)380693753
Email addresses: jonathan.negrel@inrae.fr (J. Negrel); agnes.klinguer@inrae.fr (A. Klinguer); Marielle.Adrian@u-bourgogne.fr (M. Adrian)

Abstract:

Acibenzolar acid, the first metabolite formed *in planta* from the defence inducer acibenzolar-S-methyl (ASM), has been shown to be an inhibitor of the enzyme shikimate hydroxycinnamoyltransferase (HST), extracted from grapevine or tobacco cell suspension cultures. Using a purified recombinant *Arabidopsis thaliana* HST, the inhibition was found to be competitive, acibenzolar acid binding reversibly to the shikimate binding site of the HST:*p*-coumaroyl-CoA complex, with a *K*i value of 250 µM. The other hydroxycinnamoyltransferases tested in the course of this study, using either hydroxypalmitic acid, putrescine, tyramine, or quinic acid as acyl acceptors were not, or only slightly, inhibited by acibenzolar acid. To understand the specificity of the interaction of acibenzolar acid with HST, we analyzed the structure-activity relationship of a series of benzoic or acibenzolar acid analogues, tested either as *At*HST substrates or as inhibitors. This analysis confirmed previously published data on the substrate flexibility of HST and

© 2021 published by Elsevier. This manuscript is made available under the CC BY NC user license
https://creativecommons.org/licenses/by-nc/4.0/

demonstrated that both the carboxyl group and the thiadiazole moiety of acibenzolar acid are playing an important role in the interaction with the shikimate binding site. Acibenzolar acid, which cannot form an ester bond with *p*-coumaric acid, was however a less potent inhibitor than protocatechuic or 3-hydroxybenzoic acids, which are used as acyl acceptors by HST. Our results show that the interaction of acibenzolar acid with HST, which is probably directly linked to the substrate promiscuity of HST, is unlikely to play a direct role in the defence-inducing properties of ASM in plants.

Keywords: Shikimate hydroxycinnamoyltransferase, Enzyme promiscuity, Acibenzolar-S-methyl, acibenzolar acid, 3-mercaptobenzoic acid, *Arabidopsis thaliana, Vitis vinifera*.

1. Introduction

The enzyme hydroxycinnamoyl-CoA: shikimate *O*-hydroxycinnamoyl-transferase (HST, EC 2.3.1.133) catalyzes the synthesis of 5-*O*-*p*-coumaroylshikimate (Fig. 1, Ulbrich and Zenk, 1980), a key intermediate in the biosynthesis of the G and S units of lignin (Hoffmann et al., 2004, Zhao, 2016). In the course of a study of defence reactions in *Vitis vinifera* cell suspension cultures treated with elicitors, we observed that the activity of HST, which uses *p*-coumaroyl-CoA as substrate, increases concomitantly with the activity of stilbene synthase, which also uses *p*-coumaroyl-CoA as a substrate to synthesize resveratrol, the precursor of stilbene phytoalexins in grapevine (Krzyzaniak et al., 2018). This observation led us to look for HST inhibitors that could be supplied to the elicited cell suspensions to slow down the synthesis of lignin to measure the impact of this inhibition on the synthesis of stilbene phytoalexins. To our knowledge the potential competition between stilbene and lignin biosynthesis in grapevine has never been studied, contrary to the competition between stilbene and flavonoid synthesis (Fischert et al. 1997).

HST has originally been reported to be a highly specific enzyme (Ulbrich and Zenk, 1980) but is now paradoxically considered as a model of substrate-permissive acyltransferase (Levsh et al., 2016, Chiang et al., 2018). The substrate promiscuity of *Arabidopsis thaliana* HST has recently been exploited to identify non-native substrates such as protocatechuic acid (3,4-dihydroxybenzoic acid, 3,4-DHBA) which can be synthesized by transgenic plants to inhibit HST activity and, consequently, decrease lignin content *in planta* (Eudes et al., 2016). Since HST is a well-conserved enzyme among land plants and since its substrate promiscuity also appears to be well-conserved, we first tested on *Vitis vinifera* HST a series of hydroxybenzoic acid derivatives known to inhibit *At*HST (Eudes et al., 2016). In the course of this screening, we also tested several amino- or mercapto-benzoic acids and we observed that

surprisingly acibenzolar acid, the first metabolite of the plant defence inducer acibenzolar-S-methyl (ASM) (Fig. 2, FAO/WHO, 2016) is also a relatively potent inhibitor of *Vv*HST. Acibenzolar acid is known to be very rapidly formed from ASM *in planta* (Scarponi et al., 2001) and it is one of the main residues of ASM in food crops (FAO/WHO, 2016, Sun et al., 2021). It is generally considered that ASM is a propesticide (Jeschke, 2016), able to penetrate into plants before being converted into acibenzolar acid by methyl salicylate esterase (Tripathi et al., 2010). It is well established that acibenzolar acid is, by itself, a potent systemic acquired resistance (SAR) inducer able to trigger the expression of PR proteins (Ruess et al., 1996, Scarponi et al., 2001, Tripathi et al., 2010).

In this study we show that acibenzolar acid interacts with the shikimate binding site of HST. Our results show that this interaction is however unlikely to play a direct role in the plant defence inducing properties of ASM. New data on the catalytic and substrate promiscuity of *At*HST are also presented.

2. Material and methods

2.1 Plant material

Grapevine (*Vitis vinifera* cv. Gamay) cell suspensions were cultivated as previously described (Krzyzaniak et al., 2018). Tobacco cells (*Nicotiana tabacum* cv. Xanthi) were cultivated in the same conditions but in MS medium supplemented with 0,166 mg/L 2,4-D and 0,1 mg/L kinetin. Cells were filtrated 3 days after subculture, frozen in liquid nitrogen, and stored at −20 °C. Tomato (*Lycopersicum esculentum* cv. Marmande) roots were taken from 1-month-old plants grown in a controlled growth room as previously described (Negrel et al., 2016). Seedlings of *Medicago truncatula*, *Allium porum*, and *Sorghum bicolor* were grown in a controlled growth room at 23°C in vermiculite. The entire seedlings (shoots and roots) were harvested when the plants were 2-3 cm tall.

They were then frozen in liquid nitrogen and stored at -20°C before use. The different species were chosen because HST activity was readily detected in the corresponding enzymatic extracts.

2.2 Chemicals and biochemicals

Acibenzolar acid, acibenzolar-S-methyl, 3-dehydroshikimate, and the other molecules tested as potential HST substrates or inhibitors were all purchased from Sigma Aldrich (France). *p*-coumaroyl-CoA, caffeoyl-CoA, and feruloyl-CoA were prepared enzymatically from the corresponding acids, using recombinant tobacco 4-coumarate: CoA ligase (Beuerle and Pichersky, 2002). After purification on C18 SepPack columns and elution in water, the thioesters were quantified using the extinction coefficients described in Stöckigt and Zenk, 1975.

S-*p*-coumaroyl-3-mercaptobenzoic acid was synthesized from *p*-coumaroyl-*N*-hydroxysuccinimide (26,1 mg, 0,1 mmol) (Stöckigt and Zenk,1975) and 3-mercaptobenzoic acid (15,4 mg, 0,1 mmol) in 10 mL of a mixture of 0.1 M sodium bicarbonate and acetone (50/50, v/v). After 24 h at room temperature, acetone was evaporated under nitrogen, the mixture was acidified to pH 3 with acetic acid and the thioester was extracted twice with 5 mL ethyl acetate. After evaporation *in vacuo*, the residue was dissolved in a minimum volume of MeOH and purified by TLC using preparative Silica gel 60 F_{254} 2 mm glass plates. The main fluorescent product (Rf 0.6 after chromatography in $CHCl_3$-MeOH (85/15, v/v) was eluted with methanol, evaporated *in vacuo*, and dissolved in 1 mL MeOH. The identity of the thioester was checked by UPLC-Q-Tof-MS. Its mass spectrum was identical to the mass spectrum of the enzymatically formed product (Supplementary data, Fig.1). Both products displayed the same retention times and UV spectra, with a λ_{max} at 338 nm, corresponding to the λ_{max} of *p*-coumaroylthiophenyl esters (Stöckigt and Zenk, 1975).

2.3 Enzyme extraction

Frozen plant material (10 g fr. wt of cells, roots or seedlings) was manually ground in a pre-cooled mortar at 4 °C with sand, in 10 mL of 0.2 M Tris HCl buffer (pH 7.5) containing 2% ascorbic acid, 1 mM EDTA and 10 mM mercaptoethanol. Samples were centrifuged at 20,000×*g* for 15 min at 4 °C and the supernatant was made up to 80% saturation with solid ammonium sulphate. After 1 h stirring the precipitated proteins were collected by centrifugation (15 min at 12,000×*g*), dissolved in extraction medium (1 mL g^{-1} fr. wt), and dialyzed against 0.01 M Tris HCl (pH 7.5) containing 1 mM EDTA and 10 mM mercaptoethanol overnight at 4 °C. Extracts were then centrifuged at 20,000×g for 15 min, stored at 0°C in ice, and directly used as crude enzymatic extracts. In the case of tyramine hydroxycinnamoyltransferase (THT), a previously purified enzyme extracted from elicited tobacco cell suspensions (Negrel and Javelle 1997) and stored in our laboratory at -80°C was used.

2.4 Enzyme Activity Assays

HST activity was assayed spectrophotometrically using *p*-coumaroyl-CoA and shikimate as substrates (Ulbrich and Zenk, 1980). The enzyme extract (10 to 200 µL, corresponding to 0.15 µg pr. in the case of recombinant *At*HST and up to 0.6 mg pr. in the case of crude extracts) was mixed with 0.1 M potassium phosphate buffer pH 7 in a 1-mL microcell at 30 °C. *p*-coumaroyl-CoA (50 nmol, 10 µL) was added, the reference cell containing the same mixture. The reaction was initiated by the addition of shikimic acid (2 µmol, 10 µL). The decrease in absorbance at 350 nm was recorded for 10 min and the activity was calculated from the initial velocity of the reaction.

Quinate hydroxycinnamoyltransferase (HQT) activity was determined using the same assay, but using caffeoyl-CoA (50 µM) as acyl donor and quinic acid (4 mM) as acyl acceptor (Niggeweg et al., 2004).

Tyramine, 16-hydroxypalmitic acid, and putrescine hydroxycinnamoyl-transferase (THT, HHT, and PHT, respectively) activities were also monitored

spectrophotometrically, using saturating substrate concentrations, as previously described, (Negrel and Javelle, 1997, Lotfy et al., 1996, Negrel et al., 1992).

2.5 Recombinant AtHST expression and purification

The pGEX-KG plasmid containing the *At*HST gene coding region (Hoffmann et al., 2003) was transformed into the *E. coli* strain BL21(DE3)pLysS. A 10-mL preculture was grown overnight at 37 °C in LB medium containing 32 mg/L chloramphenicol and 35 mg/liter ampicillin and used to inoculate 125 mL of fresh medium. Bacteria were grown at 37 °C until OD_{600} reached 0.6 and then transferred to 20 °C overnight after the addition of 1 mM isopropyl-D-thiogalactopyranoside (IPTG) to induce protein expression. The *E. coli* cells were harvested by centrifugation, resuspended and washed with TB buffer (9.1 mM HEPES, 55 mM $MgCl_2$, 15 mM $CaCl_2$, 250 mM KCl, adjusted to pH 6.7), centrifuged and stored at – 80°C.

To purify HST, cells were resuspended in PBS lysis buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 140 mM NaCl, 2.7 mM KCl, pH 7.4), supplemented with 1 mg/mL lysozyme, 1 mM EDTA, and a Complete™ protease inhibitor mixture (Roche). After 30 min at room temperature to lyse the cells, DNase and 10 mM $MgCl_2$ were added and the mixture was left for an additional 30 min at 4°C. The lysis solution was then centrifuged at 45.000 g for one hour. After an additional one-hour centrifugation at 100.000 g the supernatant was passed through a 1 mL GSTrap FF column (GE Healthcare) fitted on an FPLC system (GE Healthcare) and washed with 10 bed volumes of 10 mM PBS buffer at pH 7.4. The GST-tagged protein was eluted using elution buffer (50 mM Tris-HCl, pH 8.0 supplemented with 10 mM reduced glutathione). The fractions containing HST activity were pooled, concentrated to 100 µL, and treated overnight with 2 µL biotinylated thrombin (Novagen, 1.64 unit/µL) in 2 mL 10 mM PBS buffer pH 7.3 at 25 °C. After removal of thrombin

with 50 µL avidin-Sepharose, glutathione was removed by gel permeation on a Superdex 75 column and residual GST removed by mixing the HST solution with 200 µL Glutathione-Sepharose 4B. The last steps of purification were assessed by electrophoresis on 12% SDS polyacrylamide gel (Supplementary data, Fig. 2). Recombinant HST (120 µg, 0.187 µkat/mg pr.) was stored for up to 14 days in ice at 0°C in Eppendorf LoBind tubes in 10 mM KPi buffer pH 7.3 containing 10 mM mercaptoethanol without measurable loss of activity. The specific activity was calculated after determination of protein concentration with the Bradford assay (Bradford, 1976).

2.6 Determination of Kinetic Parameters

*K*m and Vmax values were calculated from Lineweaver-Burk plots using the Enzyme mechanism software of a Beckman 640 spectrophotometer, equipped with a thermostated Auto 8 cell holder. For *K*m determinations, varying substrate concentrations were used depending on the substrate tested. *K*ms for acyl donors were determined using a 5 to 50 µM range with a fixed shikimate concentration (2 mM). *K*ms for acyl acceptors were determined using 0.1 to 2 mM range at a fixed *p*-coumaroyl-CoA concentration (50 µM), except for quinic acid and 2,5-DHBA (0,1 to 4 mM). Ki values were also determined using the Enzyme mechanism software from secondary plots (apparent *K*m *vs* inhibitor concentration), using inhibitor concentrations in the 50 to 500 µM range.

2.7 Quantification of VvHST inhibition by benzoic acid derivatives

100 µL of the crude enzymatic extract prepared from grapevine cell suspensions (0.375 mg pr., 28 pkat) were incubated with 870 µL 0.1 M KPi buffer at pH 7, 20 µL 2.5 mM *p*-coumaroyl-CoA, and 5 µL 0.1 M shikimate in the absence (control) or presence of the various benzoic acid derivatives (500 µM) listed in Fig. 3A. At the end of the incubation period (10 min at 30°C), the reaction was stopped with 50 µL acetic acid. After centrifugation 10 µL aliquots were

analyzed by HPLC, using a Beckman System Gold chromatography system equipped with a diode array detector Model 168, using a Phenomenex reverse-phase column (Kinetex 2.6 µm EVO C18, 4.6 x 100 mm) and an increasing gradient of acetonitrile in water containing 0.15 % phosphoric acid. The different products were eluted using a 30 min linear gradient from 5 to 40% acetonitrile with a flow rate of 1 mL/min. Quantification of *p*-coumaroylshikimate was performed from peak areas at 310 nm.

2.8 Analysis of the different products formed by AtHST by HPLC-DAD

To determine which substrate was conjugated to *p*-coumaric acid, 2 µmoles of each potential acyl acceptor were incubated for 1 h at 30°C in the presence of 0.15 µg *At*HST in 1 mL KPi buffer pH 7 containing 50 nmoles *p*-coumaroyl-CoA. The reaction was stopped with 50 µL concentrated acetic acid. The relative activity of the different substrates was measured by comparing the consumption of *p*-coumaroyl-CoA in the same conditions after 15, 30, or 45 min.

After centrifugation, 10 µL aliquots of the incubation mixtures were analyzed by HPLC-DAD, as described in §*2.7*. The different *p*-coumaric acid conjugates formed by *At*HST were detected after comparison with control chromatograms obtained either after incubation of *At*HST and *p*-coumaroyl-CoA, without acyl acceptor, or after incubation of the boiled enzyme with *p*-coumaroyl-CoA and the acyl acceptor. Reaction products were characterized by their retention time, and their UV absorption spectra: *p*-coumaroyl-quinic acid (Rt: 9.90 min, λmax 308 nm), 5-*O*-*p*-coumaroyl-3-dehydroshikimic acid (12.65 min, 313 nm), 5-*O*-*p*-coumaroylshikimic acid (12.95 min, 312 nm), *O*-*p*-coumaroyl-3-hydroxybenzoic acid (14.48 min, 305 nm), *p*-coumaroyl-CoA 14.95 min, 333nm), *N*-*p*-coumaroyl-3-aminobenzoic acid (16.67 min, 303 nm), 3-*O*-*p*-coumaroyl-3,4-DHBA (18.61 min, 318 nm), 3-*O*-*p*-coumaroyl-2,5-DHBA (20.75 min, 310 nm), 3-*O*-*p*-coumaroyl-2,3-DHBA (20.91 min, 316 nm), *S*-*p*-

coumaroyl-3-mercaptobenzoic acid (21.39 min, 338 nm). Quantification of *p*-coumaroyl-CoA was performed from peak areas at 333 nm.

2.9 UPLC/Q-ToF-MS

To check the *in vitro* formation of *p*-coumaroyl-3-dehydroshikimic acid and *p*-coumaroyl-3-mercaptobenzoic acid by *At*HST, samples previously analyzed by HPLC-DAD were diluted 10-fold with acetonitrile/water (1/9, v/v) and reanalyzed by UPLC. Analyses were carried out in (-) ESI mode using a UPLC (Dionex Ultimate 3000, Thermo Fischer Scientific, Waltham, MA USA) coupled to a MaXis plus MQ ESI-QqTOF mass spectrometer (Bruker, Bremen, Germany), using an Acquity BEH C18 1.7 µm, 100 x 2.1 mm column (Waters, Guyancourt, France), as previously described (Romanet et al. 2020).

3. Results and discussion

3.1 Acibenzolar acid inhibits VvHST activity in crude enzymatic extracts

Both *At*HST and switchgrass HST have previously been shown to be efficiently inhibited *in vitro* by several non-canonical substrates such as 3,4-DHBA or 3-hydroxybenzoic acid (Eudes et al., 2016). We first attempted to test these inhibitors using crude enzymatic extracts prepared from *Vitis vinifera* cell suspensions, using the cell line in which we previously detected a strong HST activation upon elicitation (Krzyzaniak et al., 2018). The inhibition of *p*-coumaroylshikimate synthesis in these extracts was first monitored by HPLC. Several substituted benzoic acid derivatives were tested as inhibitors in this experiment which confirmed the inhibiting activities of 3-hydroxybenzoic acid, 3,4-DHBA, 2,3-DHBA, and 3-aminobenzoic acid, benzoic acid itself being inactive (Fig.3A). 3-mercaptobenzoic acid was also a very potent inhibitor,

while acibenzolar acid surprisingly turned out to be a relatively effective inhibitor, its activity being comparable with that of 3-aminobenzoic acid in the assay conditions used (Fig.3A). The inhibition of *Vv*HST by acibenzolar acid was then confirmed using a spectrophotometric assay to monitor HST activity (Fig.3B). *Vv*HST displayed Michaelis-Menten kinetics with a *K*m value of 279 µM for shikimate and the apparent *K*i value for acibenzolar acid, directly estimated from the Lineweaver Burk plots, was *ca.* 216 µM (Fig.3B). Precise determination of the mode of action of acibenzolar acid was however difficult, *p*-coumaroyl-CoA being partly hydrolyzed in the enzymatic extracts prepared from grapevine cell suspensions, as previously reported with other crude protein extracts (Negrel and Javelle, 2010).

The photometric assay was also used to determine whether acibenzolar acid could inhibit HST in protein extracts prepared from different plant species. Table 1 shows that the inhibition was not limited to *Vv*HST, comparable inhibitions being observed in crude enzymatic extracts prepared from different plant species and different plant organs (Table1). Benzoic acid, used as a control at the same concentration, always exhibited very low activity whatever the protein extract tested (Table 1). Interestingly the level of inhibition of HST in the presence of acibenzolar acid was different according to the plant species, ranging from 24 to 48%, in the assay conditions used. Higher inhibition was for example detected with the tobacco HST than with the tomato enzyme, although the amino acid sequences of the two transferases are almost identical (94% identity). Careful examination of the kinetic properties of the two enzymes revealed however that, unexpectedly, the affinity for shikimate of the tomato enzyme, extracted from roots (*K*m=0.65 mM), was lower than that of the tobacco transferase, extracted from cell suspensions (*K*m=0.4 mM). A comparison of the kinetic properties of the different transferases would be necessary to analyse the possible relationship between the sequences of the HSTs and the inhibition levels presented in Table 1.

3.2 Acibenzolar acid specifically inhibits shikimate hydroxycinnamoyl-transferase in crude tobacco enzymatic extracts.

We then attempted to determine whether this inhibition was specific for HST by testing acibenzolar acid on several tobacco hydroxycinnamoyltransferases. We chose tobacco instead of grapevine as an enzyme source because numerous well-characterized hydroxycinnamoytransferases have been described in tobacco. HST belongs to the BAHD family of acyl-CoA-dependent transferases, which includes many plant transferases that use hydroxycinnamoyl-CoA thioesters as donors for the transfer reaction (D'Auria 2006). We tested 3 tobacco transferases belonging to the same family, *i.e.* putrescine hydroxycinnamoyltransferase (PHT), 16-hydroxypalmitic acid hydroxycinnamoyltransferase (HHT), and quinate hydroxycinnamoyltransferase (HQT). In tobacco HST and HQT catalyze very similar reactions but are known to be distinct enzymes with distinct substrate preferences for shikimate and quinate (Niggeweg et al., 2004). Acibenzolar acid was almost completely inactive on PHT and HHT (Table 2), both transferases exhibiting a very high affinity for their substrates (Negrel et al., 1992, Lotfy et al., 1996). Weak inhibition of HQT was detected in the presence of saturating quinate concentrations (Table 2) but the comparison of the apparent *K*i values for HQT (479 µM) and HST (140 µM) confirmed that acibenzolar acid was a much stronger inhibitor of HST than HQT. Acibenzolar acid was also inactive on THT (Table 2), a dimeric enzyme belonging to another family of hydroxycinnamoyltransferases (Petersen, 2016), and displaying a high affinity for tyramine (Negrel and Javelle, 1997). To better understand the mechanism and the specificity of the interaction of acibenzolar acid with HST, we therefore attempted to check the preliminary results obtained with *Vv*HST and *Nt*HST using a purified enzyme. Since the alignment of the *At*HST amino acid sequence

with *Nt*HST and *Vv*HST confirmed that *At*HST is very similar to the other two transferases (79% identity in both cases), we used a plasmid containing the *Arabidopsis* gene that was already available in our laboratory to produce the corresponding recombinant enzyme.

3.3 Acibenzolar acid is a competitive inhibitor of recombinant AtHST

This study was performed using an *At*HST expressed in *E. coli* in frame with glutathione S-transferase, and purified after thrombin cleavage of the fusion protein (Supplementary data, Fig. 2). Since the vector used (pGEX-KG) introduces a small glycine-rich peptide immediately after the thrombin cleavage site to improve the thrombin cleavage efficiency (Hakes and Dixon, 1992), we carefully checked the properties of the recombinant protein before using it in inhibition experiments (Table 3). The purified protein displayed the characteristic properties of HSTs, *i.e.* a marked specificity for *p*-coumaroyl-CoA and shikimate (only canonical substrates were tested at this stage), a strong affinity for *p*-coumaroyl-CoA (*K*m= 4 µM), and a high *K*m for shikimate (213 µM). Interestingly 3-dehydroshikimate was conjugated much more efficiently to *p*-coumaric acid than quinic acid, indirectly confirming both the preference of the enzyme for shikimate and the regiospecificity of the enzyme (Table 3 and Supplementary data, Fig. 3).

We then used this recombinant enzyme to study the mechanism of HST inhibition by acibenzolar acid (Fig. 4). It is known that *Sorghum* HST catalyzes the transfer of *p*-coumaric acid from *p*-coumaroyl-CoA to shikimic acid in an ordered mechanism that initially involves the binding of *p*-coumaroyl-CoA to the enzyme (Walker et al., 2013). This mechanism has later been confirmed with other HSTs (Levsh et al., 2016, Eudes et al., 2016). The inhibition pattern of *At*HST observed in the presence of acibenzolar acid again confirmed this result. The inhibition was completely reversed in the presence of high concentrations of shikimate (Fig. 4A) whereas uncompetitive inhibition, with a decrease in both

*K*m and Vmax, was observed when increasing concentrations of *p*-coumaroyl-CoA were added in the presence of a fixed concentration of shikimate (Fig. 4B). These results demonstrate that acibenzolar acid does not inhibit the initial binding of *p*-coumaroyl-CoA to the transferase but instead competes with shikimate for the same binding site on the HST:*p*-coumaroyl-CoA complex. The *K*i for acibenzolar acid, calculated from the replots of the apparent *K*m *vs* inhibitor concentration (Fig. 4C), was *ca*. 250 µM, a concentration of the same order of magnitude as the *K*m for shikimate.

3.4 The interaction of acibenzolar acid with HST is probably a consequence of the substrate promiscuity of the transferase

To better understand how acibenzolar acid, the structure of which is not at first sight directly related to the structure of shikimate, can interact with the shikimate binding site of HST, we analyzed the structure-activity relationship of a series of benzoic or acibenzolar acid analogues, tested either as *At*HST substrates or as inhibitors (Table 4). Several families of potential substrates or inhibitors were tested: mono- or dihydroxy-benzoic acids, amino- or mercapto-benzoic acids and acibenzolar acid analogues (Table 4 and Supplementary data, Fig. 4). Each of the potential acyl acceptors was first incubated with *At*HST and *p*-coumaroyl-CoA to check by HPLC-DAD analysis that a *p*-coumaric acid conjugate was actually formed. Acibenzolar acid and its analogues (**10** to **13**), which lack an alcohol group and cannot form an ester bond, or any other covalent bond with *p*-coumaric acid, were not used as substrates. *p*-Coumaric acid conjugates were detected after incubation with 3-hydroxybenzoic acid, 2,5-DHBA, 2,3-DHBA, 3,4-DHBA, 3-aminobenzoic acid, and 3-mercaptobenzoic acid (Table 4). The formation of a *p*-coumaric acid adduct from these substrates by *At*HST has previously been reported in *in vivo* activity assays and the identity of the different conjugates checked by HPLC-MS (Eudes et al., 2016), except in the case of 3-mercaptobenzoic acid. In this case, the identity of the product

formed *in vitro* was confirmed by UPLC-Q-Tof-MS (Supplementary data, Fig.1) and by comparison of the properties of the enzymatically formed thioester with those of the chemically synthesized standard, obtained by reacting 3-mercaptobenzoic acid with *p*-coumaroyl-*N*-hydroxysuccinimide ester (see Material and methods section). It was already known that HST can catalyze the formation of amide bonds (Moglia et al., 2010, Sander and Petersen, 2011, Eudes et al., 2016) but, to our knowledge, the formation of a thioester has never been reported. The activity detected by HPLC-DAD in the presence of the different substrates was also detectable with the photometric assay at 350 nm, except in the case of 3-mercaptobenzoic acid, the UV spectrum of the thioester formed from 3-mercaptobenzoic acid being almost identical to the UV spectrum of *p*-coumaroyl-CoA in the 300-400 nm region. The relative activity of the different substrates was therefore compared by measuring the consumption of *p*-coumaroyl-CoA by HPLC (Table 4). The optical assay, which allows a precise measurement of initial velocities at different concentrations of a given substrate, was only used to measure *K*m and *K*i values. No attempt was made to determine *K*m and *K*i values in the case of 3-mercaptobenzoic acid. Practically, calculation of some *K*m values was not feasible, either because the activity was too weak (3,4-DHBA) or because saturating substrate concentration could not be reached, even at 4 mM (3-hydroxy- and 3-amino-benzoic acids).

Table 4 shows that *At*HST only catalyzed the *p*-coumaroyl transfer to the OH, NH_2, or SH groups in the *meta*-position of benzoic acid. *Ortho*- and *para*-hydroxybenzoic acids were not used as substrates. No coumaric acid adduct formation could be either detected when anthranilic acid (2-aminobenzoic acid) or thiosalicylic acid (2-mercaptobenzoic acid) were tested as potential substrates (data not shown). Hence, non-native substrates used by *At*HST exhibit a group in *meta*-position that plays the role of the shikimate 5-hydroxyl group in the ester bond formation (Fig.1). Our results thus confirmed previously published data on the substrate promiscuity of HSTs (Sander and Petersen, 2011, Eudes

2016, Chiang et al., 2018). They also confirmed that *At*HST exhibits a higher affinity for 2,3-DHBA than for shikimate (Table 4), as previously reported for *Coleus blumei* HST (Sander and Petersen, 2011). The ability of the shikimate binding site of HST to accommodate benzoic acid derivatives substituted in both *ortho-* and *meta-* positions may partly explain why it can interact with acibenzolar acid.

We then attempted to compare the *K*i of acibenzolar acid (Fig.4) with the *K*i of the different benzoic acid derivatives used as substrates by HST (Table 4). The inhibition observed in the presence of these derivatives was competitive, the inhibition being completely reversed in the presence of a high concentration of shikimate, except in the case of 3-mercaptobenzoic acid which displayed a more complex mode of action and was not further studied. *K*i values for 2,5-DHBA and 2,3-DHBA, which are good alternate substrates of HST are equal to the respective *K*m values. Other *K*i values were calculated from secondary plots (*K*'m *vs* [I]), after measuring the inhibition of HST at different inhibitor concentrations, taking advantage of the fact that the formation of a *p*-coumaric adduct from these inhibitors was very slow in comparison with the formation of *p*-coumaroylshikimate, which could thus be directly monitored with the photometric assay.

Comparison of the different *K*i values (Table 4) shows that the most potent inhibitor of *At*HST is 3,4-DHBA, followed by 3-hydroxybenzoic acid, 2,3-DHBA, 3-aminobenzoic acid, and acibenzolar acid. The *K*i for ASM could not be determined because of its very low solubility in water (7.7 mg L^{-1} at 20°C, *i.e.* 36.6 µM). We tested it on *At*HST at 50 µM at 30°C, without detecting any increase in the apparent *K*m for shikimate. Acibenzolar acid was therefore a more potent inhibitor than ASM or benzoic acid, demonstrating that both the free carboxyl group and the thiadiazole moiety of acibenzolar acid are playing an important role in the interaction with the shikimate binding site (Table 4). Moreover, 4-methyl-1,2,3-thiadiazole-5-carboxylic acid (**12**, Supplementary

data, Fig.4), the active moiety of tiadinil, a SAR inducer in tobacco (Yasuda et al., 2006), was also a less potent inhibitor than acibenzolar acid (Table 4), demonstrating the importance of the benzoic acid moiety of the molecule in the interaction with the enzyme.

The structural basis of substrate recognition of *At*HST has previously been determined (Levsh et al., 2016). It is well established that the interaction between the guanidine group of Arg356 and the carboxylic group of shikimate is playing a key role in shikimate recognition and to orient the shikimate 5-hydroxyl toward the catalytic center. This hydroxyl group is deprotonated to create a nucleophile that attacks the carbonyl carbon of *p*-coumaroyl-CoA, resulting in the release of CoA and the formation of the ester. (Levsh et al., 2016). On this basis, and in view of our results, it seems possible that the carboxylic group of acibenzolar acid could also interact with the guanidine group of Arg356 in *At*HST. Since 3-aminobenzoic acid is used as a substrate by *At*HST, it is tempting to speculate that the electronic doublet of the nitrogen in *meta*-position of acibenzolar acid may interact with the carbonyl carbon of *p*-coumaroyl-CoA, leading to the formation of an unproductive enzyme-substrate complex.

3.5 The inhibition of HST by acibenzolar acid is unlikely to play a direct role in the defence-inducing properties of ASM

ASM has been introduced as a plant defence activator 25 years ago (Ruess et al., 1996) and it is well established that it works by activating the SAR signal transduction pathway, usually induced by pathogens, by a mechanism independent of salicylic acid accumulation (Lawton et al., 1996). This implies that ASM, or one of its metabolites, acts either directly as a salicylic acid analogue, or at a step downstream of salicylic acid accumulation (Friedrich et al., 1996). Although it has been suggested that ASM could directly bind to NPR1 (Wu et al., 2012), a key regulator of SAR (Cao et al., 1997), and although

the role of the NPR1 gene in ASM perception is well established (Canet et al., 2010), the exact mechanism of action of ASM has remained uncertain, partly because the role of NPR1 as a salicylic acid receptor has been questioned until recently (Fu et al., 2012) and because of the occurrence of numerous salicylic acid-binding proteins in plants (Pokotylo et al., 2019). Moreover, ASM has been shown to inhibit several plant enzymes, including catalase, ascorbate peroxidase (Wendehenne et al., 1998), and mitochondrial oxidase (Van der Merwe and Dubery, 2006), suggesting that its mode of action could be relatively complex.

In this context, it was logical to address the question of the role that HST inhibition by acibenzolar acid could play in the mode of action of ASM. It is well established that acibenzolar acid is the first metabolite formed *in planta* from ASM. It is thus likely that HST can actually interact with acibenzolar acid in ASM-treated plants. But ASM is known to act at very low concentration (Lawton et al., 1996) and it is not obvious that acibenzolar acid could reach a sufficiently high concentration in ASM-treated plants to significantly inhibit the activity of HST, especially since it can be further metabolized by hydroxylation or conjugation (FAO/WHO, 2016). It is in any case highly unlikely that the inhibition of HST by acibenzolar acid could play a role in PR gene expression. It is well established that 3-hydroxybenzoic acid, which is a much more potent HST inhibitor than acibenzolar acid (Table 4), is unable to induce the synthesis of PR1 in tobacco (Abad et al., 1988). Moreover, 3-hydroxybenzoic acid has been found to be inactive in priming assays with salicylic acid analogues in both *N. tabacum* and *A. thaliana* (Kohler et al., 2002, Beckers et al., 2009). Further experiments are however needed to fully support this conclusion. It would, for example, be interesting to investigate whether ASM-induced defence responses are impaired in plants with a strongly reduced lignin level resulting from down-regulation of HST. These experiments may however be difficult to interpret since the synthesis of PR proteins is known to be often constitutively expressed

in these plants, in which the reduced lignin level is usually associated with a severe growth reduction (Gallego-Giraldo, 2020).

Surprisingly the time course of release of acibenzolar acid in ASM-treated plants has rarely been studied (Scarponi et al., 2001) and it is thus difficult to predict, simply from the comparison of *K*i and *K*m values, whether HST could be inhibited *in planta* without measuring the concentrations of shikimate and acibenzolar acid. It is noteworthy however that shikimate is known to be only an intermediate of the shikimic acid pathway and that its concentration in plants is usually very low (Shaner et al. 2005). Because of its relatively low affinity for shikimate, HST has been proposed to implement a metabolic flux control in the phenylpropanoid pathway to regulate the consumption of *p*-coumaroyl-CoA in response to the availability of cytosolic shikimate (Schoch et al., 2006). According to this model, the synthesis of *p*-coumaroylshikimate-derived phenolics, especially the G and S units of lignin, would be decreased when shikimate is present in limiting amounts in the cytosol to give the priority to the synthesis of aromatic amino acids and to the synthesis of compounds directly derived from *p*-coumaroyl-CoA, such as flavonoids, stilbenes, or the H units of lignin (Schoch et al., 2006, Adams et al., 2019). The inhibition of HST by acibenzolar acid could thus theoretically play a role in the reallocation of phenylpropanoid precursors in metabolic pathways involved in defence at the expense of growth. This mechanism could for example promote the accumulation of chlorogenic acid which has been described in ASM-treated cell suspensions (Mhlongo et al., 2016, Ncube et al., 2016) or the formation of stilbenes and anthocyanins in ASM-treated grapevine cells (Iriti et al., 2004).

4. Conclusion

Although acibenzolar acid is not a close analogue of shikimic acid, our results show that it is a relatively potent inhibitor of HST *in vitro*. This inhibition is probably a consequence of the substrate promiscuity of HST, since

the shikimate binding site of the transferase can accommodate structurally diverse benzoic acid derivatives. Further work is necessary to monitor the time course of release of acibenzolar acid in ASM-treated plants to compare the concentration reached by acibenzolar acid with the concentration of shikimate in order to determine whether HST inhibition could also occur *in planta*. Although this inhibition is unlikely to play a direct role in the defence-inducing properties of ASM, it could theoretically have an impact on *p*-coumaroyl-CoA metabolism in ASM-treated plants.

Acknowledgements

We thank Laurent Hoffmann (LRSV Toulouse, France) and Pascaline Ullmann (IBMP, Strasbourg, France) for generously supplying the plasmid containing the *At*HCT gene. We also thank Régis Gougeon and the DiVVA platform (Institut Universitaire de la Vigne et du Vin) for performing the UPLC/Q-ToF-MS analyses.

References

Abad, P., Maret, L., Cardin, L., Poupet, A., Ponchet, M., 1988. The effect of benzoic acid derivatives on *Nicotiana tabacum* growth in relation to PR-b1 production. Antiviral Res. 9, 315-327.

Adams, Z.P., Ehlting, J., Edwards, R., 2019. The regulatory role of shikimate in plant phenylalanine metabolism. J. Theor. Biol. 462, 158-170.

Beckers, G.J.M., Jaskiewicz, M., Liu, Y., Underwood, W.R., He, S.Y., Zhang, S., Conrath, U., 2009. Mitogen-activated protein kinases 3 and 6 are required for full priming of stress responses in *Arabidopsis thaliana*. Plant Cell, 2, 944-953.

Beuerle, T., Pichersky, E., 2002. Enzymatic synthesis and purification of aromatic coenzyme A esters. Anal. Biochem. 302, 305-312.

Bradford, M., 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248-254.

Canet, J.V., Dobon, A., Roig, A., Tornero, P., 2010. Structure-function analysis of *npr1* alleles in *Arabidopsis* reveals a role for its paralogs in the perception of salicylic acid. Plant Cell Environ. 33, 1911-1922.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., Dong, X., 1997. The *Arabidopsis* NPR1 gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell 88, 57-63.

Chiang, Y.C., Levsh, O., Lam, C.K., Weng, J.K., Wang, Y., 2018. Structural and dynamic basis of substrate permissiveness in hydroxycinnamoyltransferase (HCT). PLoS Comput. Biol. 14 (10) : e1006511. doi.org/ 10.1371/journal.pcbi.1006511

D'Auria, J.C., 2006. Acyltransferases in plants: a good time to be BAHD. Curr. Opin. Plant Biol. 9, 331–340.

Eudes, A., Pereira, J. H., Yogiswara, S., Wang, G., Benites, V.T., Baidoo, E.E.K., Lee, T.S., Adams, P.D., Keasling, J. D., Loque, D., 2016. Exploiting the Substrate Promiscuity of Hydroxycinnamoyl-CoA: Shikimate Hydroxycinnamoyl Transferase to Reduce Lignin. Plant Cell Physiol. 57, 568–579.

FAO/WHO (Food and Agriculture Organization of the United Nations/World Health Organization). 2016. *Pesticide residues in food 2016—Special session of the joint FAO/WHO meeting on pesticide residues*. Rome, Italy: Food and Agriculture Organization of the United Nations/World Health Organization. Retrieved from http://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/JMPR/Evaluation2016/ACIBENZOLAR-S-METHYL.pdf

Fischert, R., Budde, I., Hain, R., 1997. Stilbene synthase gene expression causes changes in flower colour and male sterility in tobacco. Plant J., 11, 489-498.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Gut Rella, M., Meier, B., Dincher, S., Staub, T., Uknes, S., Métraux, J.-P., Kessmann, H., Ryals, J., 1996. A benzothiadiazole derivative induces systemic acquired resistance in tobacco. Plant J., 10, 61–70.

Fu, Z.Q., Yan, S., Saleh, A., Wang, W., Ruble, J., Oka, N., Mohan, R., Spoel, S.H., Tada, Y., Zheng, N., Dong, X., 2012. NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature 486, 228-232.

Gallego-Giraldo, L., Liu, C., Pose-Albacete, S., Pattathil, S., Peralta, A.G., Young, J., Westpheling, J., Hahn, M.G., Rao, X., Knox, J.P., De Meester, B., Boerjan, W., Dixon, R.A., 2020. Arabidopsis Dehiscence Zone Polygalacturonase 1 (ADPG1) releases latent defense signals in stems with reduced lignin content. PNAS, 117, 3281–3290.

Hakes, D.J., Dixon, J.E., 1992. New vectors for high level expression of recombinant proteins in bacteria. Anal. Biochem. 202, 293-298.

Hoffmann, L., Maury, S., Martz, F., Geoffroy, P., Legrand, M., 2003. Purification, cloning, and properties of an acyltransferase controlling shikimate

and quinate ester intermediates in phenylpropanoid metabolism. J. Biol. Chem. 278, 95–103.

Hoffmann, L., Besseau, S., Geoffroy, P., Ritzenthaler, C., Meyer, D., Lapierre, C., Pollet, B., Legrand, M., 2004. Silencing of hydroxycinnamoyl-Coenzyme A shikimate/quinate hydroxycinnamoyltransferase affects phenylpropanoid biosynthesis. Plant Cell 16, 1446–1465.

Iriti, M., Rossoni, M., Borgo, M., Faoro, F., 2004 Benzothiadiazole enhances resveratrol and anthocyanin biosynthesis in grapevine, meanwhile improving resistance to *Botrytis cinerea*. J. Agric. Food Chem. 52, 4406-4413.

Jeschke, P., 2016. Propesticides and their use as agrochemicals. Pest Manag. Sci. 72, 210-225.

Kohler, H., Schwindlng, S., Conrath, U., 2002. Benzothiadiazole-induced priming for potentiated responses to pathogen infection, wounding, and infiltration of water into leaves requires the NPR1/NIM1 gene in *Arabidopsis* Plant Physiol. 128, 1046-56.

Krzyzaniak, Y., Negrel, J., Lemaitre-Guillier, C., Clément, G., Mouille, G., Klinguer, A., Trouvelot, S., Héloir, M.C., Adrian, M., 2018. Combined enzymatic and metabolic analysis of grapevine cell responses to elicitors. Plant Physiol. Biochem. 123, 141–148.

Lawton, K.A., Friedrich, L., Hunt, M., Weymann, K., Delaney, T., Kessmann, H., Staub, T., Ryals, J., 1996. Benzothiadiazole induces disease resistance in *Arabidopsis* by activation of the systemic acquired resistance signal transduction pathway. Plant J. 10, 71-82.

Levsh, O., Chiang, Y.C., Tung, C.F., Noel, J.P., Wang, Y., Weng, J.K., 2016. Dynamic conformational states dictate selectivity toward the native substrate in a substrate-permissive acyltransferase. Biochemistry, 55, 6314–6326.

Lotfy, S., Javelle, F., Negrel, J., 1996. Purification and characterization of hydroxycinnamoyl-Coenzyme A: ω-hydroxypalmitic acid *O*-hydroxycinnamoyltransferase from tobacco (*Nicotiana tabacum* L.) cell suspension cultures. Planta, 199: 475-480.

Mhlongo, M.I., Steenkamp, P.A., Piater, L.A., Madala, N.E., Dubery, IA., 2016. Profiling of altered metabolomic states in *Nicotiana tabacum* cells induced by priming agents. Front. plant sci. 7, 1527. doi.org/10.3389/fpls.2016.01527

Moglia, A., Comino, C., Lanteri, S., de Ros, R., de Waard, P., van Beek, T.A., Goitre, L., Retta, S.F., Beekwilder, J., 2010. Production of novel antioxidative phenolic amides through heterologous expression of the plant's chlorogenic acid biosynthesis genes in yeast. Metab. Eng. 12, 223-232.

Ncube, E.N., Steenkamp, P.A., Madala, N.E., Dubery, I.A., 2016. Stimulatory effects of acibenzolar-S-methyl on chlorogenic acids biosynthesis in *Centella asiatica* cells. Front. Plant Sci. 7, 1469. doi.org/10.3389/fpls.2016.01469

Negrel, J., Paynot, M., Javelle, F., 1992. Purification and properties of putrescine hydroxycinnamoyltransferase from tobacco (*Nicotiana tabacum*) cell suspensions. Plant Physiol. 98, 1264-1269.

Negrel, J., Javelle, F., 1997. Purification, characterization and partial amino acid sequencing of hydroxycinnamoyl-CoA: tyramine N-hydroxycinnamoyl-

transferase from tobacco cell-suspension cultures. Eur. J. Biochem. 247, 1127-1135.

Negrel, J., Javelle, F. 2010. The biosynthesis of acetovanillone in tobacco cell-suspension cultures. Phytochemistry 71, 751-759.

Negrel, J., Javelle, F., Morandi, D., Lucchi, G., 2016. Characterization and purification of a bacterial chlorogenic acid esterase detected during the extraction of chlorogenic from arbuscular mycorrhizal tomato roots. Plant Physiol. Biochem. 109, 308-318.

Niggeweg, R., Michael, A.J., Martin, C., 2004. Engineering plants with increased levels of the antioxidant chlorogenic acid. Nat. Biotechnol. 22, 746-754.

Petersen, M., 2016. Hydroxycinnamoyltransferases in plant metabolism Phytochem. Rev. 15: 699. doi.org/10.1007/s11101-015-9417-1

Pokotylo, I., Kravets, V., Ruelland, E., 2019. Salicylic acid binding proteins (SABPs): The hidden forefront of salicylic acid signaling. Int. J. Mol. Sci.,20, 4377. doi:10.3390/ijms20184377

Romanet, R., Bahut, F., Nikolantonaki, M., Gougeon, R.D., 2020. Molecular characterization of white wines antioxidant metabolome by Ultra High Performance Liquid Chromatography High-Resolution Mass Spectrometry Antioxidants, *9*, 115; doi:10.3390/antiox9020115

Ruess, W., Mueller, K., Knauf-Beiter, G., Staub, T., 1996. Plant activator CGA 245704: an innovative approach for disease control in cereals and tobacco. In Brighton crop protection conference – Pests and diseases (pp53-60)

Sander, M., Petersen, M., 2011. Distinct substrate specificities and unusual substrate flexibilities of two hydroxycinnamoyltransferases, rosmarinic acid synthase and hydroxycinnamoyl-CoA: shikimate hydroxycinnamoyltransferase, from *Coleus blumei* Benth. Planta 233, 1157-1171.

Scarponi, L., Buonaurio, R., Martinetti, L. 2001. Persistence and translocation of a benzothiadiazole derivative in tomato plants in relation to systemic acquired resistance against *Pseudomonas syringae* pv tomato. Pest Manag. Sci. 57, 262-268.

Schoch, G.A., Morant, M., Abdulrazzak, N., Asnaghi, C., Goepfert, S., Petersen, M., Ullmann, P., Werck-Reichhart, D., 2006. The *meta*-hydroxylation step in the phenylpropanoid pathway: a new level of complexity in the pathway and its regulation. Environ. Chem. Lett. 4:127–136.

Shaner, D.L., Nadler-Hassar, T., Henry, W.B., Koger, C.H., 2005. A rapid *in vivo* shikimate accumulation assay with excised leaf discs. Weed Science, 53, 769-774.

Stöckigt, J., Zenk, M.H., 1975. Chemical syntheses and properties of hydroxycinnamoyl Coenzyme A derivatives. Z. Naturforsch. Sect. C Biosci. 30, 352-358.

Sun, H., Luo, F., Zhang, X., Zhou, L., Lou, Z., Chen, Z., 2021. Residue analysis and dietary exposure risk assessment of acibenzolar-S-methyl and its metabolite acibenzolar acid in potato, garlic, cabbage, grape and tomato. Ecotox. Environ. Safety 207, 111178.

Tripathi, D., Jiang, Y.L., Kumar, D., 2010. SABP2, a methyl salicylate esterase is required for the systemic acquired resistance induced by acibenzolar-*S*-methyl in plants. FEBS Letters 584, 3458-3463.

Ulbrich, B., Zenk, M. H., 1980. Partial purification and properties of p-hydroxycinnamoyl-CoA: shikimate-p-hydroxycinnamoyl transferase from higher plants. Phytochem. 19, 1625-1629.

Van der Merwe, J.A., Dubery, I.A., 2006. Benzothiadiazole inhibits mitochondrial NADH:ubiquinone oxidoreductase in tobacco. J. Plant Physiol. 163, 877-882.

Walker, A.M., Hayes, R.P., Youn, B., Vermerris, W., Sattler, S.E., Kang, C., 2013. Elucidation of the structure and reaction mechanism of sorghum hydroxycinnamoyltransferase and its structural relationship to other Coenzyme A-dependent transferases and synthases. Plant Physiol. 162, 640–651.

Wendehenne, D., Durner, J., Chen, Z., Klessig, D., 1998 Benzothiadiazole, an inducer of plant defences, inhibits catalase and ascorbate peroxidase. Phytochem. 47, 651-657.

Wu, Y, Zhang, D., Yan Chu, J., Boyle, P., Wang, Y., Brindle I.D., De Luca, V., Després, C., 2012. The *Arabidopsis* NPR1 protein is a receptor for the plant defense hormone salicylic acid. Cell Rep. 1, 639-647.

Yasuda, M., Kusajima, M., Nakajima, M., Akutsu, K., Kudo, T., Yoshida, S., Nakashita, H., 2006. Thiadiazole carboxylic acid moiety of tiadinil, SV-03, induces systemic acquired resistance in tobacco without salicylic acid accumulation. J. Pest. Sci., 3, 329-334.

Zhao, Q., 2016. Lignification: Flexibility, biosynthesis and regulation. Trends Plant Sci. 21, 713-721

Supplementary data, Fig. 1 HPLC chromatograms showing the *in vitro* formation of *S*-*p*-coumaroyl-3-mercatobenzoic acid by *At*HST

Supplementary data, Fig. 2: Coomassie blue-stained SDS-polyacrylamide gel showing the last steps of recombinant *At*HST purification.

Supplementary data, Fig. 3: HPLC chromatograms showing the *in vitro* formation of 5-*O*-*p*-coumaroyl-3-dehydroshikimic acid by *At*HST

Supplementary data, Fig. 4: Structures of the different benzoic or acibenzolar acid derivatives tested as substrates or inhibitors in the HST assay.

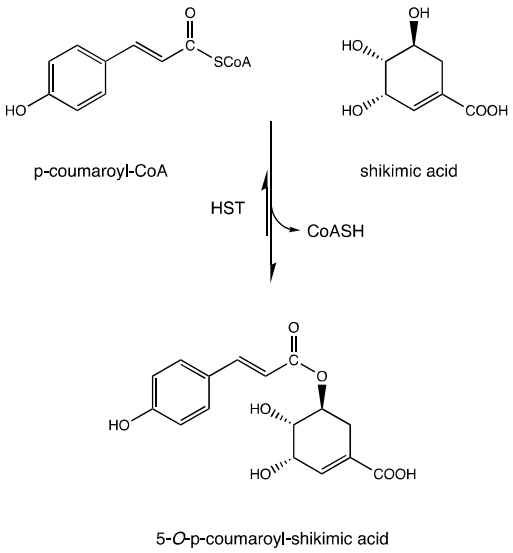
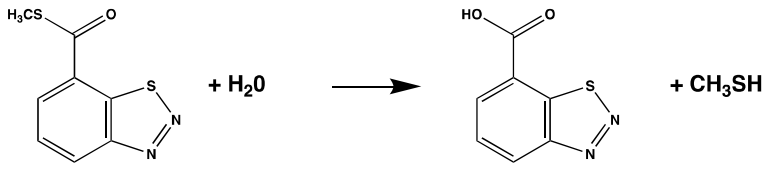


Fig. 1. *p*-coumaroylshikimate synthesis catalyzed by HST



Acibenzolar-S-Methyl **Acibenzolar acid**

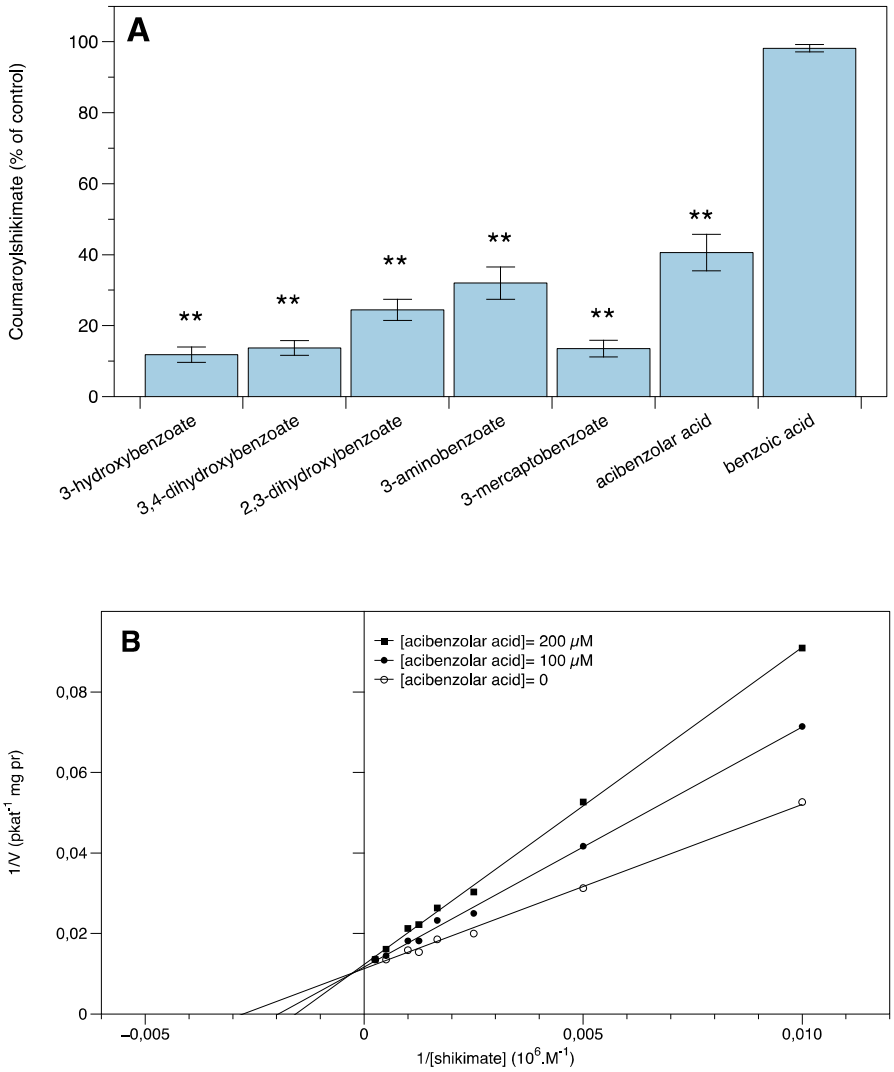


Fig. 3. *In vitro* inhibition of *Vitis vinifera* HST activity.

A: The synthesis of *p*-coumaroylshikimate in crude enzyme extracts prepared from cell suspensions was measured in the absence (control) or presence of different benzoic acid derivatives. Samples were analyzed by HPLC after incubation with p-coumaroyl-CoA (50 µM), shikimate (500 µM) and various benzoic acid derivatives (500 µM). The figure shows the mean ± SE of 3 repetitions. Asterisks indicate significant differences from the control using the Student's t-test (**P < 0.005)

B: Representative Lineweaver-Burk plot of HST activity showing the variations of 1/V at constant and saturating concentrations of p-coumaroyl-CoA (50 µM) with varying concentrations of shikimate, in the absence or presence of acibenzolar acid (100 or 200 µM).

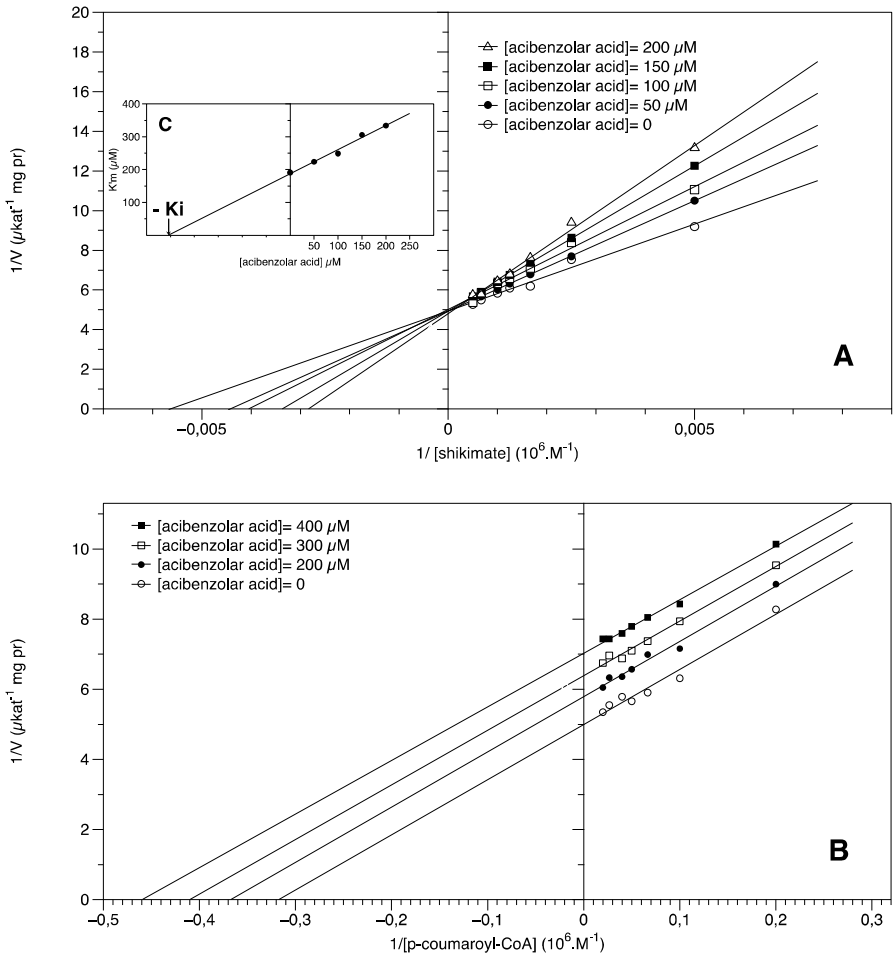


Fig. 4. Inhibition of *At*HST by acibenzolar acid.

Representative Lineweaver-Burk plots of *At*HST activity showing the variations of 1/V at constant and saturating concentrations of one substrate (**A**: [p-coumaroyl-CoA] = 50 µM, competitive inhibition; **B**: [shikimate] = 2 mM, uncompetitive inhibition) with varying concentrations of the second substrate, in the absence or presence of various concentrations of acibenzolar acid. A replot (**C**) of the apparent *K*m *vs* inhibitor concentration is shown.

Table 1: Inhibition of HST extracted from various plant species by acibenzolar acid.

Plant species	**% of control***	
	Inhibitor tested	
	Acibenzolar acid	Benzoic acid
Allium porrum (seedlings)	74 ±6	95 ±3
Medicago truncatula (seedlings)	52 ±3	93 ±3
Nicotiana tabacum (cell suspensions)	53 ±2	99 ±1
Solanum lycopersicum (roots)	66 ±9	97 ±2
Sorghum bicolor (seedlings)	61 ±5	99 ±1
Vitis vinifera (cell suspensions)	76 ±5	94 ±2

* mean of 3 replicates ± SE. HST activity was measured photometrically at 350 nm in crude enzymatic extracts using fixed substrate concentrations (50 µM p-coumaroyl-CoA and 2 mM shikimate), in the absence (control) or presence of 500 µM acibenzolar acid. The inhibition was compared to the inhibition measured in the presence of benzoic acid, at the same concentration (500 µM).

Table 2: Inhibition of different tobacco hydroxycinnamoyltransferases by acibenzolar acid.

Transferase[a]	Acyl donor[b] (50 µM)	Acyl acceptor[b]	Wavelength (nm)	% inhibition[c]
THT	feruloyl-CoA	tyramine (1 mM)	356	1 ±0.5
BAHD acyltransferases				
HST	*p*-coumaroyl-CoA	shikimate (2 mM)	350	30 ±5
PHT	caffeoyl-CoA	putrescine (1 mM)	400	1 ±0.4
HHT	feruloyl-CoA	16-hydroxypalmitic acid (0.1 mM)	360	2 ±1
HQT	caffeoyl-CoA	quinate (4 mM)	350	5 ±2

(a) The same crude tobacco cell suspension extract was used to compare the inhibition of the 4 BADH acyltransferases, whereas a purified enzyme, extracted from elicited cells, and available in our laboratory, was used to measure THT inhibition.

(b) Acibenzolar acid was tested on the 5 transferases using saturating substrate concentrations, for both acyl acceptors and donors.

(c) Mean of 3 replicates ±SE. The activity of each transferase was measured photometrically at the indicated wavelength in the absence or presence of acibenzolar acid (500 µM).

Table 3: Kinetic properties of the recombinant *At*HST used in this study.

Substrate (a)	Vmax (nkat/mg pr.)	*K*m (µM)	Vmax/*K*m (kat/g.mol)
Shikimate	187	213	0.878
3-Dehydroshikimate (b)	25	366	0.068
Quinate	59	4328	0.013
p-coumaroyl-CoA	187	4.04	46.28
caffeoyl-CoA	34.9	12.5	2.79
feruloyl-CoA	2.9	14	0.20

(a) Kinetic parameters for the acyl acceptors were determined in the presence of 50 µM p-coumaroyl-CoA. Parameters for the acyl donors were determined using shikimate (2 mM) as acyl acceptor.

(b) The formation of a p-coumaric acid ester from 3-dehydroshikimic acid was checked by HPLC and UPLC-Q-ToF-MS (Supplementary Fig. 3)

Table 4: Activity of various benzoic or acibenzolar acid derivatives tested as substrates or inhibitors of *At*HST.

Substrate or inhibitor tested (a)		**Activity as substrate (b)**			**or inhibitor (c)**
		Relative activity (%)	*K*m (µM)	Vmax/*K*m kat g^{-1} M^{-1}	*K*i (µM)
	shikimate	100	213	0.878	-
	Monohydroxybenzoic acids				
1	3-hydroxybenzoic acid	2	-	-	75
2	2-hydroxybenzoic acid	nd	-	-	
3	4-hydroxybenzoic acid	nd	-	-	
	Dihydroxybenzoic acids (DHBA)				
4	2,5-DHBA	8	440	0.034	440
5	2,3-DHBA	4	92	0.080	92
6	3,4-DHBA	0.4	-	-	31
7	3,5-DHBA	nd	-	-	
	Amino- or mercapto-benzoic acids				
8	3-aminobenzoic acid	2	-	-	194
9	3-mercaptobenzoic acid	5	-	-	
	Acibenzolar acid analogues				
10	acibenzolar acid	nd	-	-	254
11	acibenzolar-S-methyl	nd	-	-	(d)
12	4-methyl-1,2,3-thiadiazole-5-carboxylic acid	nd	-	-	458
13	benzoic acid	nd	-	-	(d)

(a) Numbers in the first column refer to the structures in Supplementary Fig. 4.

(b) The formation a p-coumaric adduct was checked by HPLC after incubating *At*HST for 1 h with 50 µM p-coumaroyl-CoA and 2 mM acyl acceptor (nd: no product detected). 100% corresponds to 187 nkat/mg pr. Relative activities (mean of 2 replicates) were determined from the HPLC data by comparing the consumptions of *p*-coumaroyl-CoA in the presence of the different substrates. *K*m values were determined from Lineweaver-Burk plots using the photometric assay at 350 nm, when saturating substrate concentration was reached at 4 mM (–: no feasible measurement).

(c) *K*i values for 2,5-DHBA and 2,3-DHBA are equal to *K*m values. Other *K*i values were calculated from secondary plots (*K*'m *vs* [I]), after measuring the inhibition of HST at different inhibitor concentrations, from 50 to 500 µM.

(d) The apparent *K*m for shikimate did not increase in the presence of 50 µM ASM nor in the presence of 500 µM benzoic acid.