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Activities of Arabidopsis sinapoylglucose:malate sinapoyltransferase shed light on functional diversification of serine carboxypeptidase-like acyltransferases

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ABSTRACT

Analysis of the catalytic properties of the serine carboxypeptidase-like (SCPL) 1-*O*-sinapoyl-β-glucose:L-malate sinapoyltransferase (SMT) from Arabidopsis showed that the enzyme exhibits besides its primary sinapoylation of L-malate, minor hydrolytic and disproportionation activities, producing free sinapic acid and 1,2-di-*O*-sinapoyl-β-glucose, respectively. The ability of the enzyme to liberate sinapic acid from the donor molecule 1-*O*-sinapoyl-β-glucose indicates the existence of a short-lived acylenzyme intermediate in the proposed random sequential bi–bi mechanism of catalysis. SMT-catalyzed formation of disinapoylglucose has been corroborated by docking studies with an established homology structure model that illustrates the possible binding of two 1-*O*-sinapoyl-β-glucose molecules in the active site and the intermolecular reaction of the two glucose esters. The *SMT* gene is embedded in a tandem cluster of five SCPL sinapoyltransferase genes, which encode enzymes with high amino acid sequence identities and partially overlapping substrate specificities. We assume that in recent duplications of genes encoding SCPL proteins, neofunctionalization of the duplicates to accept 1-*O*-sinapoyl-β-glucose as acyl donor was gained first, followed by subfunctionalization leading to different acyl acceptor specificities.

1. Introduction

Decisive steps in the evolution of genetic systems are gene duplication and subsequent neo- and subfunctionalization of the enzymes encoded by gene duplicates (Ohno, 1970; Roth et al., 2007). Thus, new enzymes emerge during speciation, often forming large gene families encoding enzymes with either new stringent functions or extended substrate specificities. Genetic dynamics is especially important in the evolution of plant secondary metabolism (Hartmann, 2007). The extraordinary diversity that characterizes plant chemistry is brought about by gene families coding for enzymes that modify various structure skeletons of phenylpropanoids, isoprenoids, alkaloids or polyketides, amounting to more than 200,000 compounds so far. Besides hydroxylation, methylation, glycosylation or prenylation, acylation particularly plays an important role to gain versatile properties of secondary metabolites. The latter includes reactions with acetate, malonate, malate or hydroxybenzoates and hydroxycinnamates.

In various plant species, transfer of acyl groups is driven by energy-rich β-acetal esters (1-*O*-β-glucose esters) instead of the more common coenzyme A thioesters (Steffens, 2000; Strack and Mock, 1993). An isobutyroyltransferase from wild tomato (Li and Steffens, 2000) and sinapoyltransferases of members of the Brassicaceae, i.e. 1-*O*-sinapoyl-β-glucose:L-malate sinapoyltransferase (SMT) and 1-*O*-sinapoyl-β-glucose:choline sinapoyltransferase (SCT) (Lehfeldt et al., 2000; Shirley et al., 2001; Fraser et al., 2007; Weier et al., 2008), use the β-acetal esters as acyl donors. These acyltransferases have sequence similarities to serine carboxypeptidases (SCPs), classifying them as serine carboxypeptidase-like (SCPL) proteins. The Arabidopsis genome encodes a large family of annotated SCPL proteins with a distinguished number of proteins that cluster with proven SCPL acyltransferases (Milkowski and Strack, 2004; Fraser et al., 2005).

The high overall sequence identity between the SCPL acyltransferases and their presumed origin from SCPs poses questions on molecular mechanisms that drive the functional shift from hydrolase to acyltransferase activity. First studies with artificial peptides established no concomitant peptidase activities for glucose ester-dependent acyltransferases (Li and Steffens, 2000; Shirley and Chapple, 2003).

The *SMT* gene in Arabidopsis is embedded in a tandemly arranged cluster of five SCPL acyltransferase genes, and it has been discussed that encoded enzymes of this cluster may exhibit overlapping substrate specificities (Fraser et al., 2007). Analysis of accumulating sinapate esters in transgenic Arabidopsis mutants indicated that SMT may also catalyze the formation of 1,2-di-*O*-sinapoyl-β-glucose (**3**), which is the defined activity of

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1-*O*-sinapoyl-β-glucose:1-*O*-sinapoyl-β-glucose sinapoyltransferase (SST) from *Raphanus sativus* (Dahlbender and Strack, 1986) and Arabidopsis (Fraser et al., 2007).

We report herein on the variable activities of the recombinant Arabidopsis SMT that produces not only sinapoyl-L-malate (**2**) but also free sinapic acid (**4**) and 1,2-di-*O*-sinapoyl-β-glucose (**3**). To substantiate the latter, we performed docking studies applying our previously established SMT structure model (Stehle et al., 2006). The resulting docking arrangements clearly demonstrate the binding of two 1-*O*-sinapoyl-β-glucose molecules (**1**) in the active site of the enzyme and illustrate the intermolecular reaction of the two glucose esters to form the disinapoylated product. In addition, based on hydrolytic activity of the SMT, we postulate the existence of a short-lived acylenzyme intermediate in a random sequential bi–bi mechanism of enzyme catalysis. Acylenzyme formation corresponds to that of SCPs; however, the SMT kinetics is in contrast to their double displacement (ping–pong) mechanism. We conclude that the Arabidopsis sinapoyltransferases, classified as SCPL proteins, originated from more recent gene duplications and are probably still at a stage of evolutionary diversification.

2. Results and discussion

2.1. SMT mediates SST activity and hydrolysis of sinapoylglucose

In SMT assays lacking the preferred acceptor L-malate, we observed by chromatographic and diode array detection analyses (HPLC–DAD) two minor products. One of these products, less polar than sinapoyl-L-malate (**2**), with absorption characteristics of sinapate esters (RT, 25.5 min; λ_{max}, 330 nm), was shown to be identical with 1,2-di-*O*-sinapoyl-β-glucose (**3**) by co-chromatography with standard compounds and LC–ESI-MS/MS analysis. The data are in agreement with those of the same compound isolated from *R. sativus* (Strack et al., 1984), *Brassica napus* (Baumert et al., 2005) and Arabidopsis (Fraser et al., 2007). The second minor reaction product was identified as free sinapic acid (**4**) (RT, 15.4 min; λ_{max}, 324 nm), indicating hydrolytic activity of SMT towards 1-*O*-sinapoyl-β-glucose (**1**).

The negative ion ESI mass spectrum of sinapic acid (**4**) showed a $[M-H]^-$ ion at *m/z* 223, prominent key ions at *m/z* 208 ($[M-H-Me]^-$), 193 ($[M-H-CH_2O]^-$), 179 ($[M-H-CO_2]^-$) and a base peak ion at *m/z* 164 ($[M-H-CO_2-Me]^-$). While 1-*O*-sinapoyl-β-glucose (**1**) ($[M-H]^-$ at *m/z* 385) displayed a significant loss of 162 mass units (hexose moiety) to form the ion at *m/z* 223 ([sinapate–H]$^-$) and the base peak ion at *m/z* 205, the ESI-CID mass spectrum of 1,2-di-*O*-sinapoyl-β-glucose (**3**) ($[M-H]^-$ at *m/z* 591) is characterized by key ions at *m/z* 367 ($[M-H-sinapate]^-$) and 223.

Fig. 1 illustrates the three activities of the recombinant SMT. Formation of 1,2-di-*O*-sinapoyl-β-glucose (**3**) is the prime activity of SST, first identified from *R. sativus* (Dahlbender and Strack, 1986) and recently also proposed for Arabidopsis (Fraser et al., 2007). The activity of SST was shown to be highest near pH 8.0, with residual activities of 25% at pH 6.0 (Dahlbender and Strack, 1986). When we measured SMT activity at pH 8.0, formation of sinapoyl-L-malate (**2**) decreased to about 1%, whereas that of

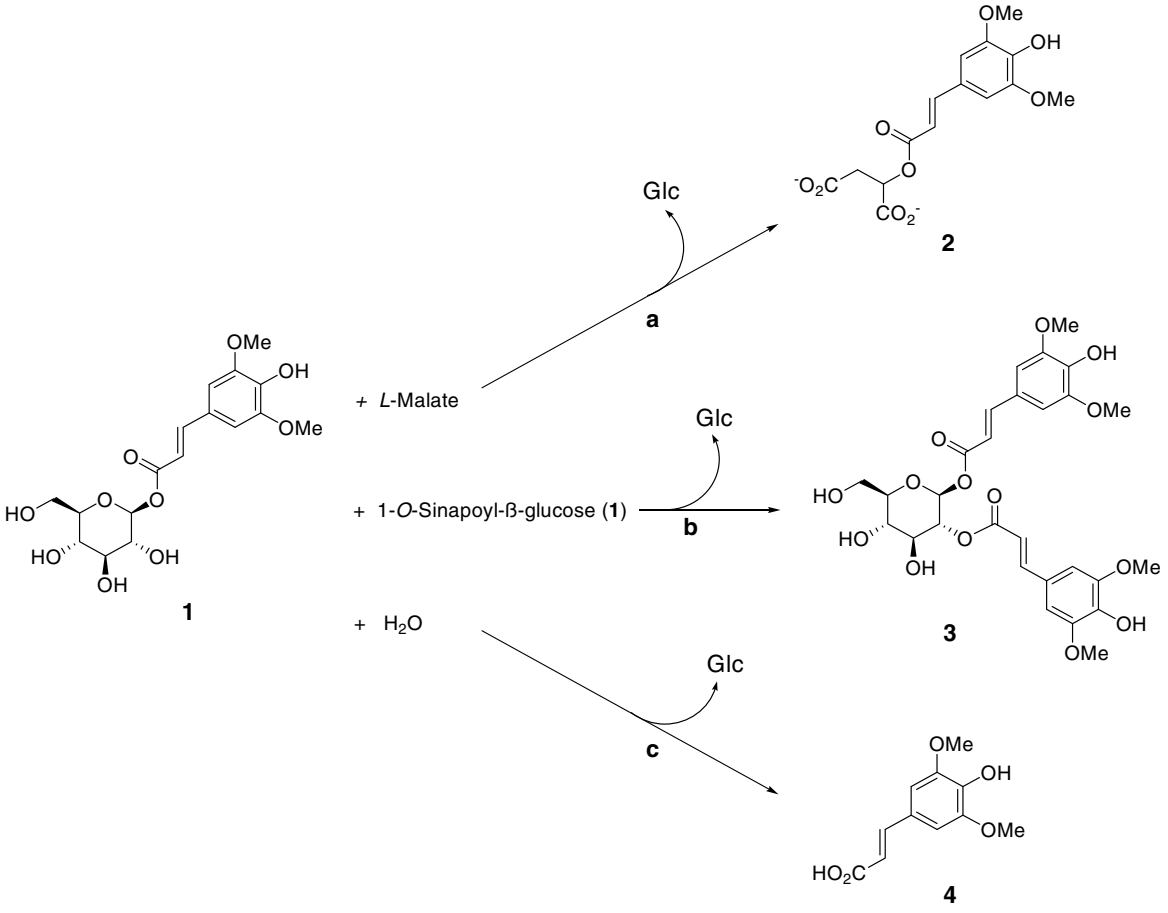

Fig. 1. Scheme of the SMT-catalyzed reactions. (a) Primary activity of SMT in the formation of sinapoyl-L-malate (**2**) with 1-*O*-sinapoyl-β-glucose (**1**) and L-malate under standard conditions (pH 6.0); (b) formation of 1,2-di-*O*-sinapoyl-β-glucose (**3**) in the absence or weak binding of L-malate at pH 8.0; (c) minor hydrolytic SMT activity under standard conditions to liberate sinapic acid (**4**), strongly increasing in the absence of L-malate and at pH 8.0 (see Table 1).

1,2-di-*O*-sinapoyl-β-glucose (**3**) increased, along with free sinapic acid (**4**). Table 1 summarizes the different SMT activities at pHs 6.0 and 8.0 with and without L-malate, using the recombinant protein produced in *Saccharomyces cerevisiae* cultures.

2.2. The SMT structure model supports SST activity

The unexpected SMT-catalyzed formation of 1,2-di-*O*-sinapoyl-β-glucose (**3**) prompted us to examine this reaction using our previously developed Arabidopsis SMT structure model (Stehle et al., 2006). Although we recently established a high yield expression system for heterologous production of active SMT (Stehle et al., 2008), we were not able yet to produce this protein in amounts that would facilitate crystallographic approaches for protein structure elucidation. However, further studies on expression optimization possibly pave the way to experimentally access structure–function relationships of the Arabidopsis SMT.

Table 1
SMT activities (pkat mg^{-1} protein; mean ± s.d.) at pHs 6.0 and 8.0 with and without L-malate, catalyzing the formation of sinapoyl-L-malate (**2**), sinapic acid (**4**) and 1,2-di-*O*-sinapoyl-β-glucose (**3**)

Substrates		Products		
Sinapoylglucose (**1**)	Malate	Sinapoylmalate (**2**)	Sinapic acid (**4**)	Disinapoylglucose (**3**)
pH 6.0				
+	+	23,069 ± 129	189 ± 37	–
+	–	–	2268 ± 18	27 ± 4
pH 8.0				
+	+	185 ± 14	1934 ± 5	173 ± 4
+	–	–	1903 ± 40	186 ± 1

Presence and absence of compounds is marked by "+" and "–", respectively.
Minor amounts of non-enzymatically liberated sinapic acid (**4**) in control assays containing heat-denatured enzyme was taken into consideration.

We performed docking studies with our homology structure model to simulate the possible binding of a second molecule of 1-*O*-sinapoyl-β-glucose (**1**). These analyses suggest that there are no marked alterations in the protein structure compared to the structure model with bound L-malate (Fig. 2A). The docking arrangement with the highest fitness-value of the second 1-*O*-sinapoyl-β-glucose (**1**), located close to the first molecule, is shown in Fig. 2B. The binding of the acyl donor is not affected and that of the acceptor molecule is achieved by the formation of only two hydrogen bonds, instead of five bonds with L-malate to Lys268, His272, Asp278 and Arg322. The first bond with the acceptor 1-*O*-sinapoyl-β-glucose (**1**) is formed between Gly75 and the C3 hydroxyl group of the glucose moiety and the second between the ε-nitrogen of Arg322 and the C3 methoxyl group of the sinapoyl moiety. This weak binding of the acyl acceptor compared to the strong fixation of L-malate may explain why measurable *in vitro* disproportionation reactions of the SMT are detectable only in the absence of L-malate.

When the acylenzyme is formed, the second 1-*O*-sinapoyl-β-glucose molecule (**1**) slightly changes the docking arrangement in the active site due to more available space (Fig. 2B). Then the acceptor molecule is stabilized by π–π-interactions between the hydrophobic benzyl ring and the imidazol ring of His272. Further stabilization of the sinapoyl moiety is achieved by hydrogen bonds between the hydroxyl and methoxyl groups and the amino acid residues Thr412 and Glu414, respectively. Arg322 forms hydrogen bonds to the carbonyl oxygen, while the glucose moiety is still fixed at Gly75. This arrangement enables the catalytic His411 to abstract a proton from the C2 hydroxyl group. As a result, the activated acceptor 1-*O*-sinapoyl-β-glucose (**1**) is in an optimal po-

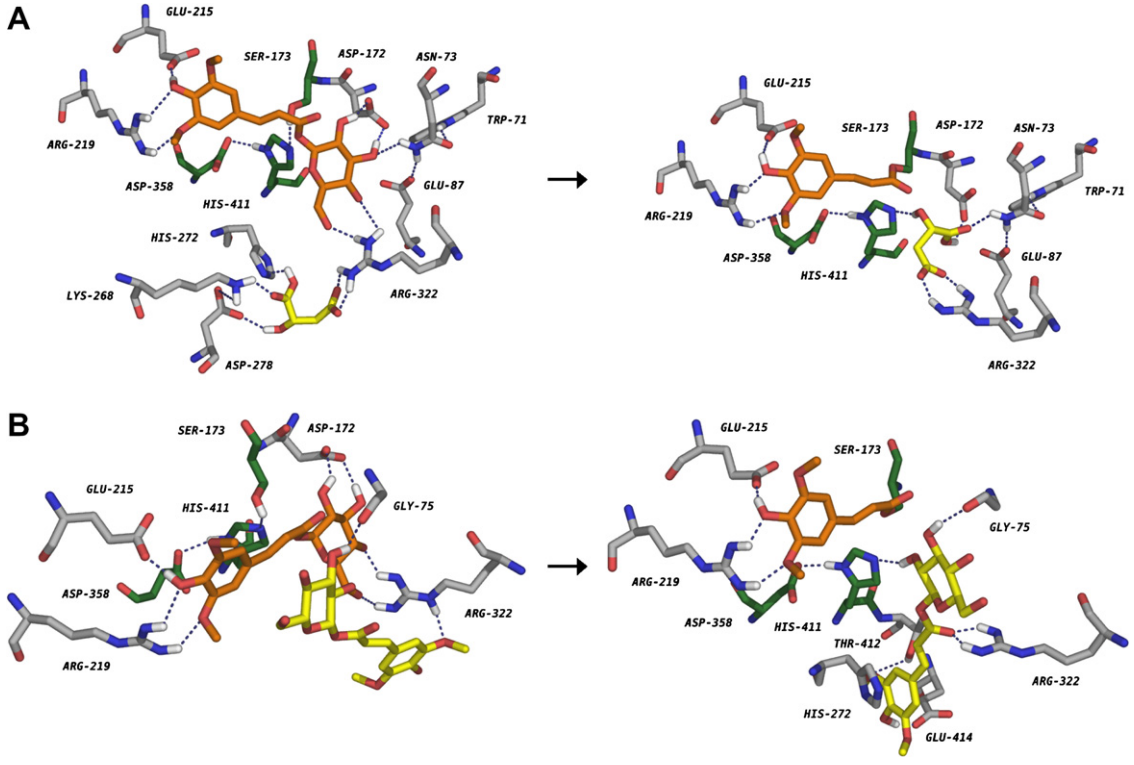

Fig. 2. Homology-based structure model of the active site of the SMT with the docked acyl donor (orange) and acyl acceptors (yellow). The residues of the catalytic triad Ser173, His411 and Asp358 are highlighted in green. Dotted lines indicate hydrogen bonds. (A) SMT with the two docked substrates 1-*O*-sinapoyl-β-glucose (**1**) and L-malate (left) and the corresponding acylenzyme complex (right) (after Stehle et al., 2006); (B) SMT with the two docked 1-*O*-sinapoyl-β-glucose molecules (**1**) (left) and the corresponding acylenzyme complex (right). For better visualization of hydrogen bonds, a different view is shown for the disproportionation reaction.

sition to attack the acylenzyme complex to form 1,2-di-*O*-sinapoyl-β-glucose (**3**).

2.3. SMT employs a short-lived acylenzyme complex

It was previously shown that the SMT reaction follows a random sequential bi–bi mechanism (Stehle et al., 2008). Both substrates have to be present in the active site before the reaction is initiated. However, in the SMT-mediated SST reaction the displacement of the weakly bound second 1-*O*-sinapoyl-β-glucose molecule (**1**) by water is facilitated. This leads to hydrolysis resulting in an increased liberation of sinapic acid (**4**) (Table 1) that points to a pivotal question concerning our model of the SMT kinetic mechanism. So far, based on modeling studies and kinetic analysis of sinapoyl-L-malate (**2**) formation (Stehle et al., 2006, 2008), we were not able to distinguish clearly between two conceivable modes for the SMT kinetic mechanism – including an acylenzyme complex (sinapoylseryl-SMT), characteristic for the serine carboxypeptidases (Douglas et al., 1976), or the catalytic serine acting as a base, described for a C–C hydrolase (Fleming et al., 2000). In standard enzyme assays (pH 6.0), containing L-malate as second substrate, only minute amounts of liberated sinapic acid (**4**) are detectable. Thus, water is probably not recognized as acyl acceptor and unable to initiate the reaction. Hence it follows that if the SMT activity would depend on a general base mechanism, hydrolysis should not proceed, given the sequential reaction mechanism of the enzyme, demanding the presence of both substrates in the active site that is not accomplished when water displaces the acyl acceptor. However, the observation of the hydrolytic reaction of SMT most likely indicates the existence of a short-lived acylenzyme intermediate that can be attacked by a water molecule.

We achieved probably weak binding of L-malate at pH 8.0, the pH optimum determined for SST activity from *R. sativus* (Dahlbender and Strack, 1986), and observed the formation of 1,2-di-*O*-sinapoyl-β-glucose (**3**) and sinapic acid (**4**) in appreciable amounts, along with sinapoyl-L-malate (**2**). At this pH value L-malate is mostly deprotonated and therefore His272 in the structure with the two docked substrates and Asp172 in the acylenzyme complex are no longer able to stabilize the acceptor by hydrogen bonds (Fig. 2A). This assumption was supported by an enzymatic activity of 185 pkat mg^{-1} protein for sinapoyl-L-malate (**2**) formation, indicating a strong decrease to a residual activity value below 1% compared to the activity at pH 6.0, whereas the hydrolytic activity increased about 12-fold (Table 1). Additionally, the SMT catalyzed the disproportionation reaction of two 1-*O*-sinapoyl-β-glucose molecules (**1**) resulting in an activity of 170 pkat mg^{-1} protein.

In enzyme assays lacking L-malate, hydrolase activity was remained at the same level at both pH values, whereas the formation of 1,2-di-*O*-sinapoyl-β-glucose (**3**) at pH 8.0 increased about sevenfold, comparable to the activity of sinapoyl-L-malate (**2**) formation at pH 8.0. These results show that SMT specificity towards L-malate is favoured by more acidic pH values (optimum at pH 6.0), which is

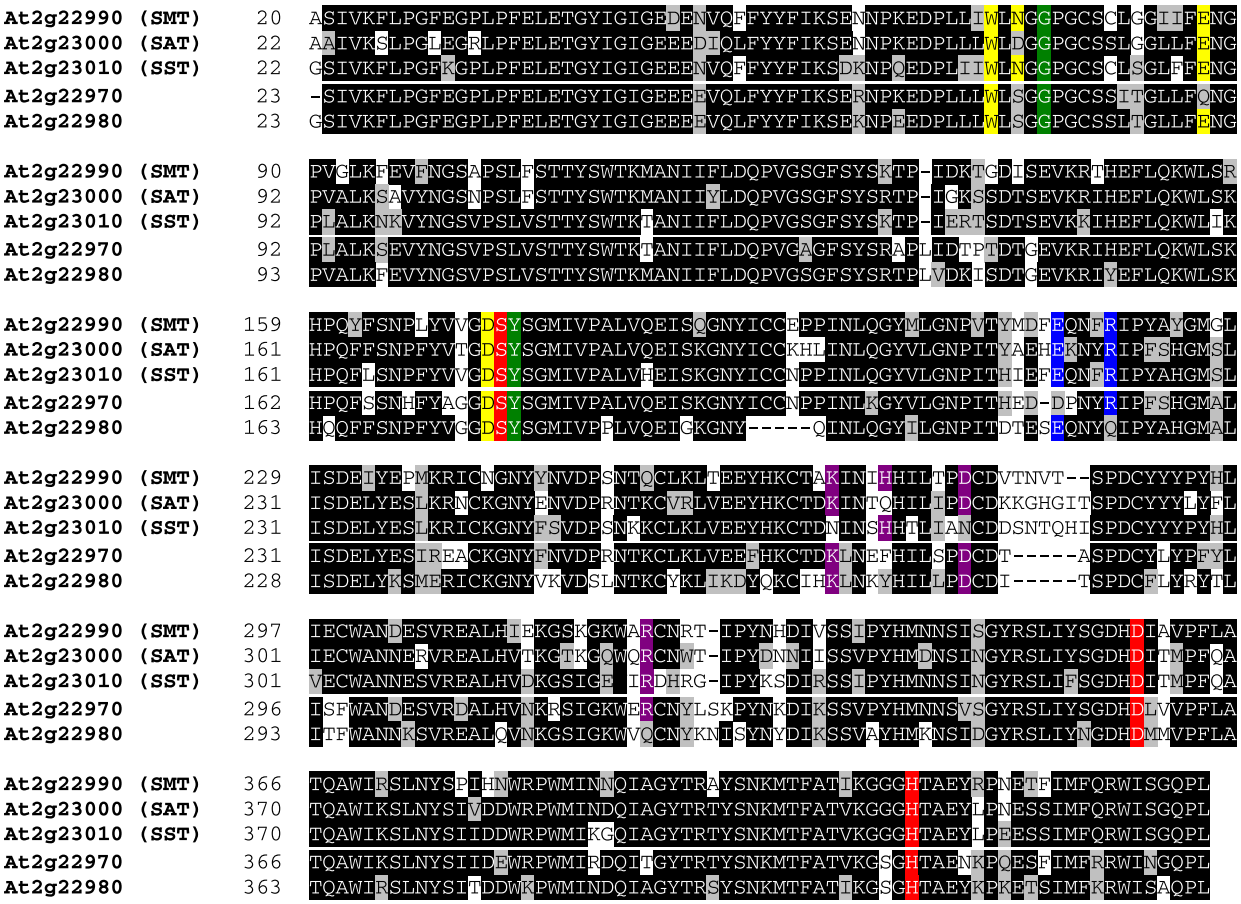

Fig. 3. Alignment of amino acid sequences of the Arabidopsis SCPL acyltransferases, encoded by the SCPL gene cluster on chromosome 2. The cDNA-encoding amino acid sequence of SMT was aligned with the sequences of the identified SAT and SST (Fraser et al., 2007) and the two putative acyltransferases encoded by At2g22970 and At2g22980 by CLUSTAL W using the BLOSUM 62 matrix (Henikoff and Henikoff, 1992, 1993). The N-terminal leader sequences have been removed. Fully conserved residues are shaded in black and those in grey indicate conservation of at least 50%. Residues shaded in different colours are part of the reaction mechanism and substrate recognition that was found out for the SMT (Stehle et al., 2006); the catalytic triad in red, the oxyanion hole in green, recognition of the sinapoyl moiety of the donor molecule in blue, the hydrogen bond network in yellow and the primary L-malate recognition in the SMT in magenta.

consistent with SMT localization to the vacuoles of Arabidopsis leaves (Hause et al., 2002). Nevertheless, SMT does not exhibit strict substrate specificity *in planta*, which was shown in analysis of an *sng1-6* transgenic line, harbouring the *SMT* gene (Fraser et al., 2007), that still accumulated 1,2-di-*O*-sinapoyl-β-glucose (**3**).

2.4. SCPL sinapoyltransferases are still on evolutionary diversification

The *SMT* gene is one of five SCPL acyltransferase genes on Arabidopsis chromosome 2. Of these genes, three have been considered as potentially mediating SST activity *in planta* (Fraser et al., 2007). While a major SST activity has been postulated for the protein encoded by At2g23010, minor SST activities have also been ascribed to the At2g22980 gene product and to SMT (At2g22990). For the latter, *in vitro* activity has been proven in a recent biochemical approach (Stehle et al., 2008).

In a homology-based SMT structure model, amino acid residues of the active site could be identified. By site-directed mutagensis these were proven to play an important role in enzyme catalysis (Stehle et al., 2006). Particularly, the residues for recognition of 1-*O*-sinapoyl-β-glucose (**1**) as well as those forming the hydrogen bond network and the oxyanion hole are conserved in the SMT-related enzymes SST and SAT (1-*O*-sinapoyl-β-glucose:anthocyanin acyltransferase) (Fig. 3). Most interestingly, these enzymes display at a conserved position the functionally important residue Asn73 (SMT), involved in binding and approximation of both 1-*O*-sinapoyl-β-glucose (**1**) and L-malate. Among the functionally important amino acid residues, only the SMT-Asn73 homolog, part of the hydrogen bond network, has changed to Asp in the SAT enzyme.

In contrast, the residues that potentially play a role for L-malate recognition of SMT (Stehle et al., 2006) are neither completely conserved in the two related acyltransferases, SAT and SST, nor in the two putative acyltransferases, encoded by At2g22970 and At2g22980 (Fraser et al., 2007). At2g22980 was assumed to encode an enzyme with minor SST activity, but the function of At2g22970 protein is unknown. The amino acid sequence derived from the At2g22970 ORF carried the conserved residues Lys268 (SMT), Asp278 (SMT) and Arg322 (SMT) involved in L-malate recognition by SMT. Moreover, the At2g22970 sequence contains the conserved residues Trp71 (SMT) and Asp172 (SMT) that form together with Asn73 (SMT) the hydrogen bond network in SMT. In At2g22970, however, the Asn73 (SMT) homolog has changed to a Ser residue.

In a current study on molecular evolution of the SCPL acyltransferase genes in Arabidopsis, we cloned the At2g22970 cDNA and were able to prove the formation of sinapoyl-L-malate (**2**) from 1-*O*-sinapoyl-β-glucose (**1**) and L-malate in activity assays containing the recombinant At2g22970 protein, when we specifically replaced some amino acids with those implicated in the SMT-like reaction (not documented). The mutant variants were generated based on the alignment of this protein with the SMT amino acid sequence (Fig. 3) and a model structure using SMT as template protein (Stehle et al., 2006). These results clearly classify this protein as another SCPL sinapoyltransferase, but a targeted metabolomics approach on Arabidopsis mutants and detailed biochemical work is needed to understand the nature of this enzyme.

The multiple alignments in Fig. 3 suggests that the most diverse sequence positions are located near the residues involved in acyl acceptor recognition, indicating that this region may be a target for evolution-driven structural alterations without changing the chemical identity of the functional residues. Interestingly, these amino acid residues are also highly conserved in the related SCT – except of those for acyl acceptor recognition (Stehle et al., 2006). This is remarkable, since the SCT clusters in the SCPL acyltransferase family (Milkowski and Strack, 2004; Fraser et al., 2005) but a post-translational endoproteolysis during enzyme maturation distinguished this protein from the other group members. Nevertheless, the present results establish the molecular basis required for gaining further insights into acyl donor and acceptor specificities for SCPL acyltransferases.

3. Concluding remarks

So far the ability to catalyze hydrolysis is only proven for the SMT, but it is most likely, that other SCPL acyltransferases retained functionally redundant hydrolytic activities. Interestingly, enzymes with bifunctional activities, transacylation and hydrolysis, have also been described from other systems. Kowalczyk et al. (2003) showed that indole-3-acetyl transferase from *Zea mays* catalyzes both synthesis and hydrolysis of indole-3-acetylinositol. In *S. cerevisiae* two acyl-coenzyme A:ethanol *O*-acyltransferases, Eht1 and Eeb1, with an α/β-hydrolase fold and a catalytic triad consisting of the amino acid residues Ser–Asp–His exhibit acyltransferase as well as esterase activities (Saerens et al., 2006). Therefore, the change from a hydrolytic α/β-hydrolase enzyme to an acyltransferase seems to be a common phenomenon.

The different enzymatic activities of SMT suggest that evolution of this enzyme has not yet reached strict substrate specificity and loss of hydrolytic properties. We assume that *SMT* and the other four SCPL acyltransferase genes on Arabidopsis chromosome 2 originated from more recent gene duplications and might be still at a stage of evolutionary diversification. Because of the high sequence similarities of all five proteins (Fig. 3), encoded by tandemly arranged genes, and the fact that the enzymes all use 1-*O*-sinapoyl-β-glucose (**1**) and exhibit partially overlapping acyl acceptor specificities, it is likely that neofunctionalization of the duplicates towards acyl donor specificity was gained first, followed by subfunctionalization resulting in specificities for different acyl acceptors.

4. Experimental

4.1. Enzyme assays

The SMT protein used in this study has been produced in *S. cerevisiae* cultures, transformed with the Arabidopsis SMT cDNA that has been optimized for high expression levels (Stehle et al., 2008). The enzyme assays with the recombinant protein contained 1 mM 1-*O*-sinapoyl-β-glucose (**1**) in the presence or absence of 10 mM L-malate in a total volume of 100 μl 100 mM MES buffer (pH 6.0) or 100 mM Tris–HCl (pH 8.0), both containing 5% (v/v) DMSO. After incubation at 30 °C at varying time intervals, the reaction was terminated by adding TFA to a final concentration of 10% (v/v). Product formation was analyzed by HPLC. After centrifugation, assay aliquots were injected onto a Nucleosil C18 column (5 μm; 250 × 4 mm i.d.; Macherey-Nagel, Düren, Germany). Separation was achieved using a 40-min linear gradient at a flow rate of 1 ml min^{-1} from 10% to 40% MeCN in 1.5% aq. H_3PO_4. Compounds were photometrically detected (maxplot between 210 and 500 nm) by a Waters 2996 photodiode array detector. Co-chromatography was achieved by adding standard compounds (Baumert et al., 2005) to the terminated enzyme assay before HPLC analysis. The quantitative data, calculated as equivalents of 1-*O*-sinapoyl-β-glucose (**1**), represent mean values (±s.d.) from three independent measurements.

4.2. LC–ESI-MS/MS

The negative ion ESI mass spectra of sinapic acid (**4**), 1-*O*-sinapoyl-β-glucose (**1**) and 1,2-di-*O*-sinapoyl-β-glucose (**3**) were obtained from a Finnigan MAT TSQ Quantum Ultra AM system equipped with a hot ESI source (HESI), electrospray voltage 3.0 kV, sheath gas: nitrogen; vaporizer temperature: 50 °C;

capillary temperature: 250 °C. The MS system is coupled with a Surveyor Plus micro-HPLC (Thermo Electron), equipped with a Ultrasep ES RP18E column (5 μm, 1 × 100 mm, SepServ). HPLC separation was achieved by using a 15-min linear gradient at a flow rate of 50 μl min^{-1} from 10% to 95% MeCN in 0.2 % aq. HOAc, with the latter held at 95% MeCN for another 15 min. The collision-induced dissociation (CID) mass spectra of sinapic acid (**4**) and 1-*O*-sinapoyl-β-glucose (**1**) were recorded during the HPLC run with a collision energy of 15 eV for the $[M-H]^-$-ions at *m/z* 223 (**4**) and 385 (**1**) by using a skimmer voltage of 25 eV, respectively. For 1,2-di-*O*-sinapoyl-β-glucose (**3**) a collision energy of 20 eV was used (collision gas: argon; collision pressure: 1.5 mTorr).

Sinapic acid (**4**): RT_{HPLC} = 11.7 min, ESI-CID mass spectrum (*m/z*, rel. int. (%)): 223 ($[M-H]^-$, 41), 208 (97), 193 (39), 179 (12), 164 (100), 149 (77), 121 (15).

1-O-Sinapoyl-β-glucose (**1**): RT_{HPLC} = 4.6 min, ESI-CID mass spectrum (*m/z*, rel. int. (%)): 385 ($[M-H]^-$, 18), 265 (3), 247 (10), 223 (24), 205 (100), 190 (22).

1,2-Di-O-sinapoyl-β-glucose (**3**): RT_{HPLC} = 15.2, ESI-CID mass spectrum (*m/z*, rel. int. (%)): 591 ($[M-H]^-$, 14), 367 ($[M-H-sinapate]^-$, 84), 223 ($[sinapate-H]^-$, 100), 205 (6).

4.3. Sequence analysis

The multiple sequence alignment was generated with the program CLUSTAL W (Thompson et al., 1994) using the BLOSUM 62 matrix (Henikoff and Henikoff, 1992, 1993).

4.4. Docking studies

Based on the recently published SMT homology model (pdb = 2drf; Stehle et al., 2006), docking studies were preformed to predict putative binding of a second 1-*O*-sinapoyl-β-glucose molecule (**1**). For this purpose, L-malate at the active site has been replaced by 1-*O*-sinapoyl-β-glucose (**1**). Subsequently, two different docking investigations using the automatic docking function of the program GOLD (GGOLD© Genetic Optimized Ligand Docking, Cambridge Crystallographic Data Center, 1998, Cambridge, UK) were carried out. The first one was done with 1-*O*-sinapoyl-β-glucose (**1**) as acyl donor bound to the enzyme as in the original model. The second docking run was performed with the acylenzyme intermediate, where the donor sinapoyl moiety is covalently linked to the catalytic active Ser173. In each case, 30 different docking arrangements were produced.

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