Characterization of SCP-2 from *Euphorbia lagascae* reveals that a single Leu/Met exchange enhances sterol transfer activity

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In *Euphorbia lagascae*, the storage triacylglycerol in the seed endosperm contains high amounts of the epoxidated fatty acid vernolic acid [(12S,13R)-epoxy-12-octadecenoic acid]. Vernolic acid has potential industrial applications in the production of paints, coatings and lubricants as an alternative to petroleum-derived oils [1]. Our research interest was initially to increase our knowledge about the enzymatic reactions involved in mobilization and oxidation of vernolic acid in order to improve our potential to develop valuable new crops [2]. The large size of *E. lagascae* seeds also makes them attractive for proteomic, biochemical and physiological studies of seed germination. We have previously applied proteomics to *E. lagascae* endosperm to identify novel components involved in endosperm degradation, nutrient recycling, lipid catabolism and β-oxidation [3].

In this report, we show that sterol carrier protein-2 (SCP-2) accumulates in the *E. lagascae* endosperm during seed germination. SCP-2 is a small intracellular basic protein domain implicated in peroxisomal β-oxidation. We extend our knowledge of plant SCP-2 by characterizing SCP-2 from *Euphorbia lagascae*. This protein consists of 122 amino acids including a PTS1 peroxisomal targeting signal. It has a molecular mass of 13.6 kDa and a pI of 9.5. It shares 67% identity and 84% similarity with SCP-2 from *Arabidopsis thaliana*. Proteomic analysis revealed that *E. lagascae* SCP-2 accumulates in the endosperm during seed germination. It showed *in vitro* transfer activity of BODIPY-phosphatidylcholine (BODIPY-PC). The transfer of BODIPY-PC was almost completely inhibited after addition of phosphatidylinositol, palmitic acid, stearoyl-CoA and vernolic acid, whereas sterols only had a very marginal inhibitory effect. We used protein modelling and site-directed mutagenesis to investigate why the BODIPY-PC transfer mediated by *E. lagascae* SCP-2 is not sensitive to sterols, whereas the transfer mediated by *A. thaliana* SCP-2 shows sterol sensitivity. Protein modelling suggested that the ligand-binding cavity of *A. thaliana* SCP-2 has four methionines (Met12, 14, 15 and 100), which are replaced by leucines (Leu11, 13, 14 and 99) in *E. lagascae* SCP-2. Changing Leu99 to Met99 was sufficient to convert *E. lagascae* SCP-2 into a sterol-sensitive BODIPY-PC-transfer protein, and correspondingly, changing Met100 to Leu100 abolished the sterol sensitivity of *A. thaliana* SCP-2.

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In this report, we show that sterol carrier protein-2 (SCP-2) accumulates in the *E. lagascae* endosperm during germination. SCP-2 is an intracellular, small, basic protein domain implicated in peroxisomal β-oxidation. We extend our knowledge of plant SCP-2 by characterizing SCP-2 from *Euphorbia lagascae*. This protein consists of 122 amino acids including a PTS1 peroxisomal targeting signal. It has a molecular mass of 13.6 kDa and a pI of 9.5. It shares 67% identity and 84% similarity with SCP-2 from *Arabidopsis thaliana*. Proteomic analysis revealed that *E. lagascae* SCP-2 accumulates in the endosperm during seed germination. It showed *in vitro* transfer activity of BODIPY-phosphatidylcholine (BODIPY-PC). The transfer of BODIPY-PC was almost completely inhibited after addition of phosphatidylinositol, palmitic acid, stearoyl-CoA and vernolic acid, whereas sterols only had a very marginal inhibitory effect. We used protein modelling and site-directed mutagenesis to investigate why the BODIPY-PC transfer mediated by *E. lagascae* SCP-2 is not sensitive to sterols, whereas the transfer mediated by *A. thaliana* SCP-2 shows sterol sensitivity. Protein modelling suggested that the ligand-binding cavity of *A. thaliana* SCP-2 has four methionines (Met12, 14, 15 and 100), which are replaced by leucines (Leu11, 13, 14 and 99) in *E. lagascae* SCP-2. Changing Leu99 to Met99 was sufficient to convert *E. lagascae* SCP-2 into a sterol-sensitive BODIPY-PC-transfer protein, and correspondingly, changing Met100 to Leu100 abolished the sterol sensitivity of *A. thaliana* SCP-2.
protein domain that in vitro enhances the transfer of lipids between membranes [4,5]. In mammals, SCP-2 is implicated in peroxisomal β-oxidation, which is the repeated cleavage of 3-oxoacyl-CoAs into acyl-CoAs and acetyl-CoAs. In oilseeds, this process provides metabolic energy and carbon skeletons to fuel germination and early postgerminative growth [6]. The exact function of the SCP-2 domain in β-oxidation is not clear, but it might facilitate the presentation and/or solubilization of the substrates and/or stabilization of the enzymes involved in catalysing the reaction cycles [7,8]. These hypotheses are mainly based on studies of the mammalian peroxisomal proteins, sterol carrier protein-X (SCP-X) and D-bifunctional protein (DBP), which both contain C-terminal SCP-2 domains. SCP-X consists of a 3-oxoacyl-CoA thiolase domain connected to a C-terminal SCP-2 domain [9], whereas DBP has domains for D-3 (equivalent to 3R)-hydroxacyl-CoA dehydrogenase, 2-enoyl-CoA hydratase and SCP-2 [10,11]. Thus, the enzymatic domain of SCP-X catalyses the last step of the peroxisomal β-oxidation pathway, and the enzymatic domains of DBP catalyse the second and third steps. Although genes for DBP and SCP-X have not been identified in plant genomes, we recently showed that plants encode and express SCP-2. PSCP (At5g42890) encodes the Arabidopsis thaliana SCP-2, which is a 13.6-kDa protein with pI of 9.2 predominantly localized in peroxisomes [12]. Curiously, A. thaliana SCP-2 and also all other plant SCP-2s that we have identified are single-domain polypeptides, whereas, as indicated above, SCP-2 domains in animals and many other eukaryotes are often present at the terminus of polypeptides, which carry multiple protein domains [13].

The physiological function of plant SCP-2 has not been determined, although its peroxisomal location and lipid-binding capabilities may suggest a role in peroxisomal β-oxidation. To extend our knowledge of the function and activity of plant SCP-2, we have compared the lipid-transfer activity of SCP-2 from E. lagascae and A. thaliana. There are similarities but also quite a few interesting differences. We showed previously that the A. thaliana SCP-2-stimulated transfer of BODIPY-phosphatidylcholine (BODIPY-PC) was unaffected by palmitic acid, indicating that this single-chain lipid is a poor transfer substrate for plant SCP-2 [12]. Therefore, we were surprised to discover that palmitic acid very efficiently inhibits BODIPY-PC transfer by E. lagascae SCP-2. Furthermore, in contrast with A. thaliana SCP-2, the E. lagascae SCP-2-mediated BODIPY-PC transfer was not affected by any of the sterols tested. To better understand the lipid–ligand binding mode of plant SCP-2 and to identify amino-acid substitutions that may explain the differences in ligand-transfer activity, we used protein modelling and site-directed mutagenesis. Replacement of Leu99 with Met99 was sufficient to convert E. lagascae SCP-2 into a sterol-sensitive BODIPY-PC-transfer protein, and, correspondingly, changing Met100 to Leu100 abolished the sterol sensitivity of the BODIPY-PC transfer mediated by A. thaliana SCP-2.

Results

E. lagascae SCP-2 accumulates in the endosperm during germination

We recently reported the initial characterization of the endosperm proteome of E. lagascae and its changes during seed germination [3]. In the previous 2D gel electrophoresis experiments, we used immobilized pH gradients of 3–10, and consequently did not obtain a perfect separation of proteins with high pI. In this set of experiments, our intention was to complement the previous report by focusing on the identification of small and basic proteins that accumulate in the E. lagascae endosperm during germination. The aim was to identify novel components involved in β-oxidation, endosperm degradation, or nutrient recycling. Protein extracts from E. lagascae endosperm, collected 2, 4 and 6 days after the seeds had been sown, were loaded on to dry polyacrylamide gel strips with immobilized pH gradients of 6–11. Electrophoresis in the second dimension was performed on 15% polyacrylamide gels. In the 2D gels, we identified spots which increased in size and density during germination. One such protein spot is indicated in Fig. 1. We cut this spot from the gels, digested the protein with trypsin, extracted the peptides, and finally sequenced the peptides using a mass spectrometer equipped with an electrospray ion source. The peptide sequence analysis revealed that this spot corresponded to E. lagascae SCP-2. The spot corresponding to SCP-2 is barely detectable in samples collected 2 days after sowing, whereas a distinct spot is seen in samples collected after 4 and 6 days (Fig. 1). Thus, there is an evident accumulation of SCP-2 in the endosperm during germination.

Cloning and sequence analysis of E. lagascae SCP-2

The peptide sequences, obtained from tandem MS analysis of the E. lagascae SCP-2 2D gel spot, were used to search the cDNA sequences in an expressed sequence tag library constructed from mRNA isolated from germinating E. lagascae seeds [14]. One of the
sequenced expressed sequence tag clones, BF12, was identified to encode the sequenced peptides derived from *E. lagascae* SCP-2. The clone BF12 has accession number BG507194 in GenBank. Complete sequence analysis of BF12 revealed that it lacked the 5'-end of the coding region. RACE-PCR was carried out on mRNA isolated from the endosperm of germinating seeds to obtain the full coding sequence of the *E. lagascae* SCP-2 cDNA.

*Euphorbia lagascae* SCP-2 cDNA sequence encodes a protein of 122 amino acids with a molecular mass of 13.6 kDa and pI of 9.5. It contains a PTS1 peroxisomal targeting signal at the C-terminus (SKL), suggesting that the protein is predominantly localized to peroxisomes. The amino-acid sequence of *E. lagascae* SCP-2 shares 67% identity and 84% similarity with *A. thaliana* SCP-2, 66% identity and 80% similarity with a putative SCP-2 from the monocotyledon *Oryza sativa*, and 58% identity and 75% similarity with a putative SCP-2 from the moss *Physcomitrella patens*. Thus, the amino-acid sequence of SCP-2 is well conserved among land plants. The amino-acid identity shared between SCP-2 from land plants and the green algae *Chlamydomonas reinhardtii* is less than 40%, which is about the same level of identity shared between land plant and mammalian SCP-2.

When confirmed and putative plant SCP-2 protein sequences from angiosperms, gymnosperms, ferns, mosses and green algae are aligned, it becomes evident that the C-terminal parts of the proteins are the most conserved regions (Fig. 2). Thus, from Gly86 in *E. lagascae* SCP-2, angiosperm SCP-2 domains share 100% similarity. As shown in Fig. 2, the start codons are well aligned in the plant SCP-2 sequences. Moreover, in *E. lagascae* SCP-2 cDNA, an in-frame stop codon was detected upstream of the start codon. These observations allowed us to conclude that *E. lagascae* SCP-2 is not encoded as a domain of a larger multifunctional protein.

**Expression pattern of SCP-2 in *E. lagascae***

Total RNA was isolated from various tissues, such as leaves, roots, stems, flowers and siliques of *E. lagascae* plants. We also isolated RNA from endosperm and hypocotyls of 4-day-old seedlings. The expression pattern of SCP-2 RNA was analysed by RT-PCR using gene-specific primers SCPElNE and SCPElCN. As a control for our RNA preparations and RT-PCR conditions, we also used primers ELEFF and ELEFR to analyse the expression of *E. lagascae* elongation factor 1-α (EF1-α), which is expected to show a stable expression pattern. SCPElNE and SCPElCN amplify a 384-bp fragment from *E. lagascae* SCP-2, and a 290-bp fragment is amplified from EF1-α, using ELEFF and ELEFR. Analysis of the PCR products revealed that a PCR product from EF1-α was obtained from all samples (Fig. 3). The *E. lagascae* SCP-2 primers SCPElNE and SCPElCN amplified a PCR product of the expected size from samples from hypocotyl, endosperm, flowers, siliques, leaves and stems. A particularly large accumulation of the SCP-2 amplification product relative to EF1-α was obtained from the endosperm sample, suggesting that SCP-2 mRNA is most abundant in the endosperm of germinating seeds. Moreover, our results indicate that expression is higher in hypocotyls, endosperm, flowers, siliques and capsules than leaves and stems. Thus, *E. lagascae* SCP-2 seems to be mainly expressed during germination, and also during flower and seed development. We did not detect any amplification product from the root sample, indicating that SCP-2 is expressed at very low levels in roots.
Lipid-transfer capability of *E. lagascae* SCP-2

Using a resonance energy transfer (RET) assay, we show that purified recombinant *E. lagascae* SCP-2 is capable of transferring fluorescently labelled phosphatidylcholine (BODIPY-PC) between bilayer vesicles (Fig. 4, CTRL trace). The matrix lipids of the bilayer vesicles need to be unfavourable substrates for the transfer protein to allow a good transfer signal. A mixture of bovine brain sphingomyelin with cholesterol was chosen as it gives a tight membrane matrix with fluid BODIPY-PC clusters. This tightly packed matrix membrane is as resistant to the SCP-2-mediated lipid transfer as possible, and therefore the transfer protein preferentially transfers the labelled BODIPY-PC. To examine the ability of *E. lagascae* SCP-2 to recognize lipids other than BODIPY-PC as potential substrates, we used a competition assay [12]. In this assay set-up, BODIPY-PC and the added unlabelled lipids compete as substrates for SCP-2. The unlabelled lipids were added as multilamellar aggregates. No additional increase in fluorescence intensity or light scattering was detected as a result of the addition of the unlabelled lipids. This indicates that they remain as a third distinct entity during the measurements. We analysed the inhibiting ability of a range of lipids (Table 1 and Fig. 4). The rate of transfer of BODIPY-PC after the addition of liposomes was almost completely inhibited by bovine liver phosphatidylinositol and palmitic acid, both lipids yielding a normalized decrease in BODIPY-PC transfer activity of 0.8. The decrease in transfer activity was 0.6 in the presence of stearoyl-CoA or vernolic acid and 0.3 in the presence of dimyristoylphosphatidic acid.

Fig. 2. Multiple sequence alignment of plant SCP-2 from angiosperms (dicotyledons: *E. lagascae* and *A. thaliana*; monocotyledon: *O. sativa*), gymnosperm (*P. pinaster*), fern (*C. richardii*), moss (*P. patens*) and green algae (*C. reinhardtii*). Black boxes indicate that identical amino acids are present in at least 80% of the sequences, and shaded boxes indicate that amino acids with similar physicochemical properties are present in at least 80% of the sequences. The sequences included in the analysis have the following GenBank accession numbers: *E. lagascae*, AA142079; *A. thaliana*, NP_199103; *P. patens*, BJ200729.1; *C. richardii*, BE642073; *O. sativa*, AU030065.1; *P. pinaster*, BX249678.1; *C. reinhardtii*, BI729324.1.

**Fig. 3.** RT-PCR analysis of SCP-2 in *E. lagascae* tissues. Total RNA isolated from hypocotyls (HY), endosperm (EN), flower (FL), silique (SI), stem (ST), root (RO) and leaf (LE) was analysed for the expression of SCP-2 and EF1-α. The DNA size marker is shown to the left (MM), with numbers referring to sizes in bp of the corresponding bands.

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acid (DMPA). Ergosterol, sitosterol and cholesterol only showed a marginal competing effect, and steryl glucoside, trimyristin, monogalactosyldiacylglycerol, galactosylceramide and palmitoyl-sphingomyelin did not affect the transfer at all. The results are surprising bearing in mind that, with SCP-2 from *A. thaliana*, the transfer of BODIPY-PC was almost completely inhibited after the addition of ergosterol, whereas palmitic acid and stearoyl-CoA did not affect the transfer at all [12].

**Structural model of E. lagascae SCP-2 in Triton-bound conformation**

To study the basis for the ligand-binding preference of *E. lagascae* SCP-2, we constructed a structural model in the Triton-bound conformation (*E. lagascae* Tr-SCP-2) (Fig. 5A) based on the Triton X-100-bound structure of the SCP-2-like domain of human DBP [Protein Data Bank (PDB) code I1KT] [8]. The hydrophobic end of the Triton X-100 molecule is buried in

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**Fig. 4.** SCP-2 competition assay. BODIPY-PC transfer mediated by *E. lagascae* SCP-2 before (arrow A) and after (arrow B) addition of competing unlabelled lipids. The lipids were added as multilamellar aggregates that remain as a third entity in the assay. Phosphatidylinositol, palmitic acid, stearoyl-CoA and vernolic acid have a dramatic competing effect on BODIPY-PC transfer. DMPA has a weak effect, whereas the rest of the lipids analysed interfere marginally with *E. lagascae* SCP-2-mediated BODIPY-PC transfer. The control (CTRL) trace is *E. lagascae* SCP-2-mediated BODIPY-PC transfer without addition of any competitors. MGDG, monogalactosyldiacylglycerol; SPM, palmitoyl-sphingomyelin; GalCer, galactosyl ceramide; bIPI, bovine liver phosphatidylinositol.
Table 1. *E. lagascae* SCP-2 in vitro lipid-transfer activity. *E. lagascae* SCP-2-mediated (10 µg) lipid transfer was examined using a fluorescence competition assay. The values, given as decrease in BODIPY-PC transfer rate on introducing the lipid, are mean ± SD from at least four different analyses. blPI, bovine liver phosphatidylinositol.

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<tr>
<th>Lipid</th>
<th>Decrease in BODIPY-PC transfer rate</th>
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<tr>
<td>Palmitoyl-sphingomyelin</td>
<td>0.00 ± 0.00</td>
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<tr>
<td>Galactosyl ceramide</td>
<td>0.00 ± 0.00</td>
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<tr>
<td>Trimyristin</td>
<td>0.00 ± 0.00</td>
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<tr>
<td>Steryl glucoside (soybean)</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.11 ± 0.01</td>
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<tr>
<td>Cholesterol olate</td>
<td>0.12 ± 0.01</td>
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<tr>
<td>Sitosterol</td>
<td>0.13 ± 0.02</td>
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<tr>
<td>Ergosterol</td>
<td>0.15 ± 0.02</td>
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<tr>
<td>DMPA</td>
<td>0.27 ± 0.03</td>
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<tr>
<td>Vernolic acid</td>
<td>0.61 ± 0.03</td>
</tr>
<tr>
<td>Stearoyl-CoA</td>
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<tr>
<td>Palmitic acid</td>
<td>0.79 ± 0.01</td>
</tr>
<tr>
<td>blPI</td>
<td>0.83 ± 0.10</td>
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the inner cavity and the polar tail stretches out through the opening between helices D and E and β-strand V (Fig. 5C) [8]. According to the alignment used for modelling, the sequence identity is 36.7% between *E. lagascae* SCP-2 and the SCP-2-like domain of human DBP. Most of the residues that interact with the Triton X-100 molecule in the crystal structure of the SCP-2-like domain of human DBP are located on the C-terminal half of the protein sequence. The sequence identity between the C-terminal halves of *E. lagascae* SCP-2 and *A. thaliana* SCP-2 (starting from Asp77 and Asp78, respectively) is considerably higher (82.6%) than the identity between the N-terminal halves (58.4%), suggesting that the C-termini of *E. lagascae* SCP-2 and *A. thaliana* SCP-2 are important for ligand binding.

The fold of the *E. lagascae* and *A. thaliana* Tr-SCP-2 models is a five-stranded (I–V) β-sheet covered on one side by five α-helices (A–E). The *E. lagascae* Tr-SCP-2 model (Fig. 5A) has an inner cavity that is lined by hydrophobic residues Ile10, Leu11, Leu13, Leu14, Phe17, Leu18, Val26, Phe36, Phe73, Leu75, Phe80, Leu83, Ala84, Pro90, Phe94, Leu99, Ile101, Leu105, Ala108, Phe111, Phe116, Pro117 and Pro119. Of these residues, Leu18, Val26, Leu75, Phe80, Pro90, Phe94 and Leu99 are conserved in the SCP-2-like domain of human DBP (supplementary Table S1). The hydrophobic cavity has two openings, of which the first one is formed by residues on helices A, C and E, and the second one is formed by residues on helices D and E and β-strand V (Fig. 5A). The polar residues Ser120 and Glu22 are located at the first opening of the cavity. At the second cavity opening, the *E. lagascae* Tr-SCP-2 model has Gln91, Gln109 and Thr112, corresponding to Gln90, Gln108 and Gln111 in the structure of the SCP-2-like domain of human DBP (supplementary Table S1).

The hydrophobic cavity of the *E. lagascae* Tr-SCP-2 model is extremely similar to the cavity of the *A. thaliana* Tr-SCP-2 model (Fig. 5B) [12]. More than half of the amino acids in the cavity are conserved, including the three polar amino acids at the second opening. Nevertheless, we could identify some interesting differences in the two proteins based on their structural models. The *A. thaliana* SCP-2 cavity has four methionines (Met12, 14, 15 and 100), which are replaced by leucines (Leu11, 13, 14 and 99) in *E. lagascae* SCP-2. Furthermore, the polar residue His18 in *A. thaliana* SCP-2 is replaced by Phe17 in *E. lagascae* SCP-2, and the polar residue Glu22 in *E. lagascae* SCP-2 is replaced by Ala23 in *A. thaliana* SCP-2. *E. lagascae* SCP-2 also has a phenylalanine at position 36, whereas this residue is an isoleucine (Ile37) in *A. thaliana* SCP-2. Vice versa, Phe76 in *A. thaliana* SCP-2 is replaced by Leu75 in *E. lagascae* SCP-2 (Fig. 5A,B; supplementary Table S1).

**Structural models of *E. lagascae* and *A. thaliana* SCP-2 with a bound palmitic acid**

Palmitic acid was shown to interfere with the BODIPY-PC transfer mediated by SCP-2 and would hence be a potential substrate of *E. lagascae* SCP-2 (Fig. 4), whereas BODIPY-PC transfer mediated by *A. thaliana* SCP-2 was not affected by the presence of palmitic acid [12]. This clearly indicates that interactions between the negative charge of palmitic acid and the positively charged SCP-2 s (at neutral pH) are not the sole inhibiting effect of BODIPY-PC transfer. To discover a structural reason for the difference in ligand preference, we constructed palmitic acid-bound models of *E. lagascae* and *A. thaliana* SCP-2 (pa-SCP-2) based on the mosquito SCP-2 structure (Fig. 5D) [15]. The initial assumption was that the binding mode of palmitic acid in *E. lagascae* SCP-2 might be similar to that in the mosquito SCP-2 structure, where the carboxylate moiety of the palmitic acid is bound by amino acids on a loop stretching upwards between helix A and β-strand I (Fig. 5D) [15]. The palmitic acid molecule in the mosquito SCP-2 structure is bound in the opposite direction compared with the Triton X-100 molecule in the SCP-2-like domain of human DBP (Fig. 5C) [8]. On the basis of the sequence alignments used for modelling, *A. thaliana* SCP-2 and *E. lagascae* SCP-2
SCP-2 share 19.2% and 21.4% sequence identity, respectively, with mosquito SCP-2. Apart from the palmitic acid-binding loop, the *E. lagascae* and *A. thaliana* pa-SCP-2 models are very similar to the Tr-SCP-2 models, and their inner cavities are lined by hydrophobic residues in the same way as in the Tr-SCP-2 models.

In the mosquito SCP-2 template structure, the negatively charged carboxylate group of the palmitic acid molecule is bound by the positively charged Arg24 (Fig. 5D) [15]. The corresponding residue in the *E. lagascae* pa-SCP-2 model is also a positively charged residue, Lys28, and the corresponding residue in the *A. thaliana* pa-SCP-2 model is the negatively charged Glu29. To examine whether Lys28 in *E. lagascae* SCP-2 is in fact involved in palmitic acid binding, we chose this residue for *in vitro* mutagenesis.

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**Fig. 5.** Structural models of (A) *E. lagascae* SCP-2 and (B) *A. thaliana* SCP-2 in Triton-bound conformation (stereo view). The amino acids lining the inner cavity are shown. Residues studied by *in vitro* mutagenesis are in orange. (C) The crystal structure of the SCP-2-like domain of human DBP [8], on which the models in (A) and (B) are based. (D) The crystal structure of yellow fever mosquito SCP-2 [15]. The amino acids (grey) and the two water molecules that participate in the binding of the palmitic acid carboxylate are shown. The side chain of Arg24 (yellow) makes a direct bond to the carboxylate group. According to the palmitic acid-bound model of *E. lagascae* SCP-2, based on the mosquito SCP-2 structure, the residue corresponding to Arg24 (yellow) is Lys28 in *E. lagascae* SCP-2. Mosquito SCP-2 has four methionines (orange) positioned in a row in the inner cavity. Mosquito SCP-2 Met90 corresponds to Met100 in *A. thaliana* SCP-2. The figures were prepared using MOLSCRIPT 2.1.2 [51], RASTER3D 2.7b [52], and GIMP 2.2 (http://www.gimp.org).

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SCP-2 from *Euphorbia lagascae*  
Docking of ergosterol into the models of A. thaliana SCP-2 and E. lagascae SCP-2 in Triton-bound conformation

Ergosterol was one of the preferred substrates of A. thaliana SCP-2 [12], whereas E. lagascae SCP-2 only showed a slight transfer of ergosterol (Fig. 4). To discover a structural explanation for this substrate selectivity, we docked ergosterol into the E. lagascae and A. thaliana Tr-SCP-2 models (Fig. 5A,B) using the program GOLD 2.2 [17,18]. This found two alternative binding modes for ergosterol with the A. thaliana Tr-SCP-2. The docking result with the higher fitness positioned ergosterol with the hydrocarbon chain pointing towards the cavity opening located between helices D and E and β-strand V (supplementary Fig. S1A). The hydroxyl group of ergosterol was positioned close to His18 from helix A. The other docking result, which had a lower fitness, positioned ergosterol in the opposite way (supplementary Fig. S1B). The hydrocarbon chain of ergosterol pointed towards the opening located between helices A, C and E, and the hydroxy group was positioned in the centre of the cavity near Met100. The same two binding modes for ergosterol with the E. lagascae Tr-SCP-2 model were found with GOLD, but with a lower fitness than with the A. thaliana Tr-SCP-2 model. On the basis of the docking results, A. thaliana SCP-2 residues Met14, Met15, His18, Met100 and the corresponding E. lagascae SCP-2 residues were chosen for in vivo mutagenesis.

Mutant lipid-transfer activity

To test the importance of specific amino acids for lipid-transfer activity, we constructed genes encoding variants of A. thaliana and E. lagascae SCP-2. In particular, we replaced some of the Met residues in the hydrophobic cavity of A. thaliana SCP-2 with Leu, and vice versa in E. lagascae SCP-2. Furthermore, we converted His18 of A. thaliana SCP-2 into Phe18, and Lys28 of E. lagascae SCP-2 into Glu28. The BODIPY-PC-transfer activity for the different mutants differed only slightly from each other and from the wild-type E. lagascae or A. thaliana SCP-2. Replacing Leu99 with Met is sufficient to convert E. lagascae SCP-2 into a protein that is sensitive to sterols, as the rate of BODIPY-PC transfer was clearly diminished after the addition of ergosterol. The normalized decrease in BODIPY-PC-transfer activity after ergosterol addition was 0.15 for wild-type E. lagascae SCP-2 and 0.81 for the Leu99Met mutant (Table 2). In comparison with the wild-type, BODIPY-PC-transfer activity in the presence of ergosterol did not change for the other E. lagascae mutants, Lys28Glu and Leu13Met/Leu14Met. Changing Met100 to Leu abolished the sterol sensitivity of A. thaliana SCP-2 BODIPY-PC transfer. The normalized decrease in activity in the presence of ergosterol was 0.91 for wild-type A. thaliana and 0.11 for the A. thaliana SCP-2 Met100Leu mutant (Table 2). For the A. thaliana SCP-2 triple mutant, Met14Leu/Met15Leu/His18Phe, the decrease in BODIPY-PC-transfer activity after the addition of ergosterol was large (0.78) and not significantly different from that of wild-type A. thaliana SCP-2. None of the mutations in E. lagascae SCP-2 or A. thaliana SCP-2 caused any changes in BODIPY-PC-transfer activity in the presence of palmitic acid (Table 2).

Table 2. Lipid-transfer activity of E. lagascae and A. thaliana SCP-2 mutants. Normalized decrease in BODIPY-PC transfer rate mediated by different A. thaliana and E. lagascae SCP-2 mutants (10 µg) upon introducing ergosterol or palmitic acid to the sample. The values are mean ± SD from at least four different analyses.

<table>
<thead>
<tr>
<th>Plant</th>
<th>Type</th>
<th>Ergosterol</th>
<th>Palmitic Acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. lagascae</td>
<td>Wild-type</td>
<td>0.15 ± 0.02</td>
<td>0.79 ± 0.01</td>
</tr>
<tr>
<td>E. lagascae</td>
<td>Lys28Glu</td>
<td>0.14 ± 0.02</td>
<td>0.78 ± 0.02</td>
</tr>
<tr>
<td>E. lagascae</td>
<td>Leu99Met</td>
<td>0.81 ± 0.10</td>
<td>0.76 ± 0.06</td>
</tr>
<tr>
<td>E. lagascae</td>
<td>Leu13Met/Leu14Met</td>
<td>0.11 ± 0.02</td>
<td>0.77 ± 0.02</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>Wild-type</td>
<td>0.91 ± 0.08</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>Met100Leu</td>
<td>0.11 ± 0.02</td>
<td>0.00 ± 0.00</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>Met14Leu/Met15Leu/His18Phe</td>
<td>0.78 ± 0.09</td>
<td>0.09 ± 0.01</td>
</tr>
</tbody>
</table>

Discussion

The sequence identity between E. lagascae SCP-2 and A. thaliana SCP-2 is high, and the inner cavities of the two proteins are accordingly extremely similar, which would suggest that the proteins have similar ligands. Therefore, we were surprised to discover that palmitic acid very efficiently inhibits BODIPY-PC transfer mediated by E. lagascae SCP-2, whereas we showed previously that the A. thaliana SCP-2-stimulated transfer of BODIPY-PC was unaffected by palmitic acid.
We first thought that the binding mode of palmitic acid to *E. lagascae* SCP-2 might be the same as in the mosquito SCP-2 structure (Fig. 5D) [15], where Arg24 binds the carboxylate group of palmitic acid. The structural model of palmitic acid-bound *E. lagascae* SCP-2 suggests that Lys28 is important in palmitic acid binding. To test this hypothesis, Lys28 in *E. lagascae* SCP-2 was mutated to Glu, which is the corresponding residue in *A. thaliana* SCP-2. This Lys28Glu mutant showed no decrease in BODIPY-PC-transfer activity compared with the wild-type when palmitic acid was present. Therefore, we now conclude that Lys28 is not crucial for palmitic acid binding to *E. lagascae* SCP-2.

How does *E. lagascae* SCP-2 then actually bind palmitic acid? One possibility is that another residue from the loop between helix A and β-strand I is responsible for the binding. Suitable candidates for binding would be Lys25 and Gln27, corresponding to Glu26 and Thr28 in *A. thaliana* SCP-2. Another possibility is that *E. lagascae* SCP-2 binds palmitic acid in a similar way to the binding of Triton X-100 to the SCP-2-like domain of human DBP [8], i.e. with the charged moiety in the completely opposite direction. In this binding mode, the polar residues (Gln91, Gin109 and Thr112) on helix E in *E. lagascae* SCP-2 (Fig. 5A) could provide a suitable surrounding for the carboxylate group of palmitic acid. These residues are, however, completely conserved in *A. thaliana* SCP-2 (Fig. 5B) and therefore cannot contribute to the different binding preferences of *A. thaliana* and *E. lagascae* SCP-2. A possible explanation is that the hydrophobic residues, Leu75, Leu83 and Leu99 in *E. lagascae* SCP-2 (Fig. 5A), corresponding to Phe76, Val84 and Met100 in *A. thaliana* SCP-2 (Fig. 5B), make the shape and size of the binding cavities in *E. lagascae* and *A. thaliana* SCP-2 somewhat different and, thus, affect ligand specificity.

Another intriguing discovery was that ergosterol, which efficiently inhibited BODIPY-PC transfer by *A. thaliana* SCP-2, has only a marginal effect on *E. lagascae* SCP-2-mediated BODIPY-PC transfer. The docking of ergosterol in *A. thaliana* SCP-2 gave two solutions (supplementary Fig. S1A,B) which are quite similar to the respective binding modes of Triton X-100 in the SCP-2-like domain of human DBP (Fig. 5C) [8] and palmitic acid in mosquito SCP-2 (Fig. 5D) [15]. A comparison of the hydrophobic cavities in *A. thaliana* and *E. lagascae* SCP-2 revealed that *A. thaliana* SCP-2 has four methionines, which are replaced by leucines in *E. lagascae* SCP-2 (Figs 2 and 5A,B). On the basis of the docking analysis, we suggest that the methionines in *A. thaliana* SCP-2 are involved in the binding of ergosterol (supplementary Fig. S1A). The competition assay showed that the *A. thaliana* SCP-2 Met100Leu mutant had lost its ergosterol sensitivity, and the *E. lagascae* SCP-2 Leu99Met mutant had acquired ergosterol sensitivity. Hence, Met100 is crucial for ergosterol binding in *A. thaliana* SCP-2, and, even more surprising, introducing this methionine to the *E. lagascae* SCP-2 cavity is enough to provide sterol-binding properties. However, the other methionines located on helix A in *A. thaliana* SCP-2 are apparently of little or no importance for ergosterol binding, as, in the presence of ergosterol, the *A. thaliana* SCP-2 Met14Leu/Met15Leu/His18Phe and *E. lagascae* SCP-2 Leu13Met/Leu14Met mutants had similar BODIPY-PC-transfer activities to their corresponding wild-type proteins. The role of His18 in ligand binding to *A. thaliana* SCP-2 was also examined using the Met14Leu/Met15Leu/His18Phe mutant. His18 seems to have no effect on binding of ergosterol, as wild-type *A. thaliana* SCP-2 and the *A. thaliana* Met14Leu/Met15Leu/His18Phe mutant showed similar decreases in BODIPY-PC-transfer activity after addition of ergosterol.

The X-ray crystallographic structures of SCP-2 domains also show methionines in their hydrophobic cavity, and it is, thus, tempting to speculate that these residues are important for sterol binding. Mosquito SCP-2 has five methionines, four of which are positioned close to each other in a row (Fig. 5D) [15]. One of these methionines (Met90) corresponds to Met100 in *A. thaliana* SCP-2, and there are experimental results showing that yellow fever mosquito SCP-2 has high affinity for cholesterol [19]. Mammalian SCP-2 has also been shown to transfer cholesterol [20,21], and rabbit SCP-2 has two methionines in its hydrophobic cavity [16]. Furthermore, there are published structures of protein–sterol complexes in which methionines participate in binding of the sterol, e.g. the crystal structures of the fungal protein β-cryptogein in complex with ergosterol and cholesterol show that three methionines positioned close to each other in the binding cavity interact with the two methyl groups of the sterol molecules (PDB code 1BXM) [22] (PDB code 1LRI) [23]. In the crystal structure of human 17β-hydroxysteroid dehydrogenase in complex with estradiol, two methionines are in contact with the sterol (PDB code 1FDS) [24]. The O3-hydroxy group of estradiol is bound by a histidine, but one of the two methionines is close to the hydroxy group. Further studies are needed to elucidate how *A. thaliana* SCP-2 binds to ergosterol and to determine the exact role of Met100 in the binding process.
Being basic at neutral pH, SCP-2 is likely to interact with negatively charged interfaces. Thus, we cannot rule out that negatively charged lipids will inhibit to some extent the SCP-2-mediated transfer of its substrates and that SCP-2 might be sensitive to negatively charged membranes analogous to the glycolipid transfer protein [25]. However, it is important to note that whereas some negatively charged lipids, such as bovine liver phosphatidylinositol, efficiently inhibited the BODIPY-PC transfer mediated by \textit{E. lagascae} SCP-2, other negatively charged lipids, such as DMPA, only had a rather marginal inhibitory effect. Furthermore, \textit{A. thaliana} SCP-2 is also positively charged at neutral pH and seems able to transfer its substrate from negatively charged surfaces [12].

The expression pattern, the lipid binding and transfer capability, and the peroxisomal targeting signal allow us to suggest that \textit{E. lagascae} SCP-2 is involved in the peroxisomal oxidation of lipids. It was of particular interest that vernolic acid interfered with BODIPY-PC transfer, as the storage triacylglycerols of \textit{E. lagascae} largely consist of this fatty acid. These results from our indirect assay suggest that vernolic acid could be a favoured transfer substrate for \textit{E. lagascae} SCP-2 and supports our hypothesis that \textit{E. lagascae} SCP-2 is involved in the catabolism of storage triacylglycerols in the endosperm. Our data from studies of \textit{A. thaliana} suggest that \textit{A. thaliana} SCP-2 is also involved in this process (B. S. Zheng and J. Edqvist, unpublished results). According to the current model of β-oxidation in \textit{A. thaliana} [6], the ABC transporter CTS (also referred to as PED3 or PXA1) delivers fatty acids into the peroxisomes [26–28]. In the peroxisome, the acyl-CoA synthetases activate the fatty acids to acyl-CoA esters [29]. Finally, the β-oxidation enzymes [acyl-CoA oxidases ACX1–6, the multifunctional proteins MFP2 and AIM1, and the 3-oxoacyl-CoA thiolases (PED1, KAT1 and Pkt2)] catalyse the repeated cleavage of the acyl-CoA esters to yield acetyl-CoA [30–34].

How does SCP-2 fit into this scheme of peroxisomal β-oxidation in plants? In \textit{A. thaliana}, SCP-2 is tightly coexpressed with MFP2 (Zheng and J. Edqvist, unpublished results) indicating a partnership between these two proteins. We suggest that SCP-2 interacts with the multifunctional protein to form a cavity for the hydrophobic tails of some β-oxidation substrates, as has been suggested for the SCP-2-like domain of mammalian DBP [8]. The extended hydrophobic cavity will increase the accessibility and solubility for at least some of the β-oxidation substrates and consequently improve the catalytic rate of the β-oxidation process. Thus, the role of SCP-2 would mainly be to facilitate β-oxidation of some substrates. Plant SCP-2 may also interact with other β-oxidation enzymes such as the 3-oxoacyl-CoA thiolases and acyl-CoA oxidases. Alternatively or additionally, SCP-2 may improve the catalytic rate of the β-oxidation process by recruiting substrates to the active sites of the β-oxidation enzymes.

It is possible that the distinctly different lipid-transfer properties of \textit{E. lagascae} and \textit{A. thaliana} SCP-2 reflect the lipid composition of the respective plant species. The differences may also indicate that \textit{E. lagascae} and \textit{A. thaliana} SCP-2 have adopted slightly different or overlapping physiological functions. We speculate that the sterol-binding property of \textit{A. thaliana} SCP-2 may indicate an involvement of this protein in nonvesicular trafficking of sterols as well as its suggested role in peroxisomal β-oxidation. On the other hand, the lack of sterol-transfer activity shown for \textit{E. lagascae} SCP-2 would suggest that it has a more specialized function in fatty acid β-oxidation. Our finding that changing one leucine residue to a methionine increased the affinity of SCP-2 for sterols will hopefully open the way for other experiments directed towards learning more about the biological function of SCP-2. For instance, it will be of interest to express SCP-2 proteins with altered ligand-binding properties in plant or animal models in which phenotypes for deletion and overexpression of SCP-2 proteins have already been assessed.

### Experimental procedures

**Plants, bacteria and chemicals**

\textit{Euphorbia lagascae} Spreng. was germinated and grown as previously described [3]. Tissues were stored at −80°C, for shorter periods of time, until used. The \textit{E. lagascae} seeds were a gift from S. Stynne, Department of Crop Science, SLU, Alnarp, Sweden. Cloning was performed in \textit{Escherichia coli} DH5α, and \textit{E. coli} BL21 cells were used for over-expression of \textit{E. lagascae} and \textit{A. thaliana} SCP-2. The fluorescent probes BODIPY FL C12 PC and Dil-C18 were from Invitrogen (Carlsbad, CA, USA). Egg sphingomyelin and bovine liver phosphatidylinositol were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Galactosylcereamide, trimyristin, cholesterol oleate, steryl glycoside (soybean), ergosterol, β-sitosterol, DMPA, palmitic acid, stearyl-CoA and vernolic acid [(+)-(12S,13R)-epoxy-cis-9-octadecenoic acid methyl ester] were from Larodan AB (Malmö, Sweden). Cholesterol was from Sigma (St Louis, MO, USA). Palmitoyl-sphingomyelin was purified from egg sphingomyelin using RP-HPLC (Supelco, Bellefonte,
Proteomics and bioinformatics

Total protein extracts were prepared from the endosperm of 2-, 4- and 6-day-old seedlings as described in [3]. The protein solutions were loaded on to dry polyacrylamide gel strips with immobilized pH gradients of 6–11 (GE Healthcare, Chalfont St Giles, UK). First and second dimension gel electrophoresis, silver staining, and analysis of gels were carried out as previously described [3]. Peptide analysis by MS was performed by a slightly modified version [3,35] of a method described by Wilm et al. [36]. To acquire peptide sequence data, a quadrupole time-of-flight mass spectrometry instrument (Micromass Q-Tof; Waters Corp., Milford, MA, USA) with a nanospray ion source was used. The capillary voltage was set to 800–900 V and the cone voltage to 40 V. Argon was used as collision gas, and the kinetic energy was set to between 20 and 40 eV. Peptide sequence data were analysed using the BIOLYNX program of the MassLynx NT software package (version 3.4; Waters Corp.). Peptide sequences obtained by MassLynx were subjected to BLAST using BLASTP [37] at the National Center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov/BLAST/). Multiple sequence alignment was created using CLUSTALX [38], and the resulting similarities were then visualized by subjecting the alignment to Boxshade (http://www.ch.embnet.org/software/BOX_form.html).

RACE and RT-PCR

Euphorbia lagascae total RNA was isolated from various tissues using a guanidine hydrochloride method previously described by Logemann et al. [39], or with the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). RACE-PCR, to obtain the full-length cDNA sequence of E. lagascae SCP-2, was performed with the FirstChoice RLM-RACE kit (Ambion, Austin, TX, USA). Outer (STOP, 5′-GAC AAATTCGATCCAAATCCA-3′) and inner (STPI, 5′-CAG TATGCCTACAGCCTCGG) E. lagascae SCP-2 specific primers were designed based on the sequence of cDNA clone BF12 [14]. For RT-PCR, 2 μg total RNA was used for cDNA synthesis together with 200 ng poly(dT) primer in a polymerization reaction catalysed by Superscript III for cDNA synthesis together with 200 ng poly(dT) primer. For RT-PCR, 2 primers were designed based on the sequence of cDNA TATGCCTCACAGCTTCG (E. lagascae).

Structural modelling of E. lagascae SCP-2

A structural model of E. lagascae SCP-2 in the Triton-bound conformation was constructed based on the crystal structure of the SCP-2-like domain of human DBP (PDB code 1HKT) [8], which has bound a Triton X-100 molecule (Fig. 5C). This model is referred to as E. lagascae Tr-SCP-2. Structural models of E. lagascae SCP-2 and A. thaliana SCP-2 with a bound palmitic acid were also constructed based on the crystal structure of yellow fever mosquito SCP-2 (PDB code 1PZ4) [15], which has a palmitic acid molecule bound to its inner cavity (Fig. 5D). These models are referred to as E. lagascae pa-SCP-2 and A. thaliana pa-SCP-2, respectively. The sequences of E. lagascae SCP-2, A. thaliana SCP-2, rabbit SCP-2, mosquito SCP-2 and the SCP-2-like domain of human DBP were submitted to the PredictProtein server (available at http://cubic.bioc.columbia.edu/predictprotein/), which predicts the secondary structure of a protein sequence [40]. The known SCP-2 crystal structures were superimposed using the program VERTAA in the Bodil modelling environment [41]. The E. lagascae and A. thaliana SCP-2 sequences were aligned with the template sequences using the program MALIGN within Bodil [42,43]. The sequence alignments used for modelling were refined in the same way as described in Edqvist et al. [12] in order to construct models comparable to the previously made models of A. thaliana SCP-2 in Triton-bound conformation (A. thaliana Tr-SCP-2). The models were constructed using the program MODELLER [44], and the representative model was selected as previously described [12]. The cavities within the models and the known structures were identified by using the program SURFNET [45] and visualized and examined with Bodil [41].

The docking program GOLD 2.2 [17,18] was used to dock ergosterol into the E. lagascae and A. thaliana Tr-SCP-2 models. Hydrogens were added to the models before the GOLD runs using the program REDUCE [46]. The ergosterol structure was downloaded from the HIC-Up site (Hetero-compound Information Centre–Uppsala, http://xray.hmc.uu.se/hicup/) [47]. Ten independent genetic algorithm runs were made in GOLD for the ligand, using the default docking parameters. The binding site in the models was restricted within a 15 Å radius of the side-chain hydrogen (HZ) of Phe112 and Phe111 in the A. thaliana and E. lagascae Tr-SCP-2 models, respectively. The docking was stopped if the three best scoring solutions were within 1.5 Å rmsd of each other. The docking results were visualized and examined in Bodil [41].
Expression and purification of plant SCP-2 in E. coli

The coding region was amplified from E. lagascae cDNA by the use of primers SCPEINE (5'-ACTGGGAACTCAAGTCTTCTGAG-3') and SCPEICN (5'-TCATGCAGCCGCTCAAGTCTGAG-3'). The PCR fragment obtained was digested with restriction enzymes EcoRI and NotI, and subcloned into the EcoRI–NotI site of the vector pGEX-5X-2 (GE Healthcare) to obtain a gene fusion between gluthatione S-transferase (GST) and SCP-2 insert. The obtained plasmid pGEX-SCPEl was confirmed by DNA sequencing. Plasmid pGEX-SCPEl was transformed into E. coli BL21 cells. The bacteria were cultured at 27 °C. When A600 reached 1.1, the expression of GST-ElSCP-2 was induced by the addition of 0.6 mM isopropyl β-d-thiogalactoside. Expression of A. thaliana SCP-2 in E. coli was performed as described previously [12]. Purification of the fusion proteins (supplementary Fig. S2) and cleavage with Factor Xa were carried out according to the handbook for the GST Gene Fusion System (GE Healthcare) as described in [12].

In vitro mutagenesis of A. thaliana and E. lagascae SCP-2

The following oligonucleotides were used for in vitro mutagenesis: ELM13M14 (5'-AAGTCCAAAATATTATGGA TATGATGGCTCATTCTTGAG-3'); ELE28 (5'-ATG AAATCCGATGCAATCCTGGACCTGCTG-3'); ELM99 (5'-ATGAGGGGTGCGCTGAAGATCAAGG-3'); ATL14-Li5F18 (5'-AAATCCGATGCAATCTGGACCTGCTG AAGGAATTTCTCTCCACCGACGCC-3'). For construction of A. thaliana SCP-2 variants Met100Leu and Met14Leu/Met15Leu/His18Phe and E. lagascae SCP-2 variants Leu13Met/Leu14Met, Lys28Glu and Leu99Met, we used the megaprimer method [48]. The first round of PCR was performed with the mutagenic primer in combination with the M13 reverse primer. In the second round, the PCR product of the first round was used as primer in combination with the M13 forward primer. Plasmids pGEX-SCPAI [12] and pGEX-SCPEl served as template for both rounds of PCR. In a third round of PCR, the PCR products from round two were, in the case of A. thaliana SCP-2, amplified with primers SCPAtCN and SCPAtNE, or, in the case of E. lagascae SCP-2, amplified with primers SCPEINE and SCPEICN. PCRs were performed with Phusion DNA polymerase (Finnzymes, Espoo, Finland). The obtained PCR fragments carrying the SCP-2 gene variants were digested with restriction enzymes EcoRI and NotI, and subcloned into the EcoRI–NotI site of the vector pGEX-5X-2 (GE Healthcare) to obtain gene fusions between GST and the SCP-2 genes. The sequences of the SCP-2 inserts were confirmed by DNA sequencing. Plasmids carrying SCP-2 variants were transformed into E. coli BL21 cells.

RET assay for monitoring SCP-2-mediated lipid intermembrane transfer

The RET method used for measuring the transfer of lipids between bilayer vesicles, which is based on a method published previously [49], has been described briefly [12]. BODIPY-PC (1%) was used as the energy donor and DiI-C18 (3%) was the energy acceptor. The labelled lipids were mixed with bovine brain sphingomyelin and cholesterol (6:1), dried under nitrogen and then dissolved in sodium phosphate buffer containing 10 mM sodium dihydrogen phosphate, 1 mM dithiothreitol and 1 mM EDTA. The small unilamellar vesicles (both donors and acceptors) were prepared by sonication for 10 min on ice with a Branson (Danbury, CT, USA) 250 sonifier, and then centrifuged for 15 min at 15 000 g with a Sorvall Biofuge Pico centrifuge (Thermo Electron Corporation, Waltham, MA, USA) to remove titanium probe particles, multilamellar vesicles and undispersed lipids (negligible amount). The acceptor vesicles were used in 10-fold excess over donor vesicles. The transfer was started by introducing 10 µg protein to the assay.

Competition assay for monitoring SCP-2-mediated lipid intermembrane transfer

To analyse the ability of plant SCP-2 to transfer unlabelled lipids, a competition assay [12,50] was used. Although indirect, this assay enables us to address if unlabelled lipids interfere with the transfer of BODIPY-PC in an experimental set-up that does not require the large amount of material that is often the case in conventional binding assays. The RET assay with BODIPY-PC, which has been shown to be a substrate, was started by addition of SCP-2. Two minutes after the injection of the protein, when the BODIPY-PC transfer was still ongoing, we introduced an unlabelled lipid (sonicated small unilamellar aggregates) to the sample. If the added lipid was a substrate for SCP-2, it would result in a decrease in the rate of transfer of SCP-2 because of the competition from the presence of a new distinct transferable lipid pool. When the added lipid was not a substrate for SCP-2, no deviation in the slope of the transfer rate would occur. All the unlabelled lipids were sonicated (Finnsonic Bath Sonifier; Finnsonic, Lahti, Finland) for 6 min at 25 °C. The amount of the added lipid was 400 nmol. No additional increase in the fluorescence intensity or light scattering was detected as a result of the addition of the multilamellar aggregates. This would indicate that no fusion or aggregation of the assay component occurred during the time frame of the measurements. The values are derived from using a control assay,
where the BODIPY-PC transfer is allowed to continue without disturbance. The fluorescence intensity increase at 3 min for the BODIPY-PC transfer without a competing lipid is set to 0. A straight line, indicating no BODIPY-PC transfer, i.e. complete inhibition, is set to 1. The fraction of decrease in the fluorescence intensity compared with the control at 3 min after injection of the competitive lipid is given as the values in Tables 1 and 2.

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References


The following Supplementary material is available online:

**Fig. S1.** Docking of ergosterol. The GOLD [17,18] docking analysis gave two solutions for the binding of ergosterol in the structural mode of ergosterol in the structural model of *A. thaliana* SCP-2. The binding mode in (A) has higher fitness than the binding mode in (B). The polar amino acids at the opening between helices D and E and β-strand V are shown as well as the amino acids studied by in vitro mutagenesis.

**Fig. S2.** SDS/PAGE of purified recombinant *E. lagascae* and *A. thaliana* SCP-2 proteins. 1.5 μg of purified proteins were applied to each lane and analyzed on 16% polyacrylamide gels using Coomassie Blue staining. Lane S1, *E. lagascae* SCP2 Lys28Glu; lane S2, *E. lagascae* SCP2 Leu99Met; lane S3, *E. lagascae* SCP2 Leu13Met/Leu14Met; lane S4, *A. thaliana* SCP2 Met100Leu; lane S5, *A. thaliana* SCP2 Met14Leu/Met15Leu/His18Phe; lane S6, *E. lagascae* SCP2 wild-type; lane S7, *A. thaliana* SCP2 wild-type; lane M, PageRuler™ Prestained Protein Ladder. The numbers on the side of the marker lane correspond to the size in kDa of the molecular markers. Samples were prepared as described in Experimental procedures.

**Table S1.** Cavity amino acids in the Triton-bound conformation of SCP-2. The amino acids lining the cavities in the *E. lagascae* and *A. thaliana* Tr-SCP-2 models and in the structure of the SCP-2-like domain of human DBP were identified using the program surfnet [45]. Amino acids conserved in at least two of the SCP-2s are shown in bold. Residues in parentheses are not part of the cavity according to the surfnet calculations.

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