



Wax synthases from sunflower (*Helianthus annuus*) seeds

C. DeAndrés-Gil^{a, id}, M. Villoslada-Valbuena^{a, id}, M. Venegas-Calcrón^{a, id},
A.J. Moreno-Pérez^{a, b, id}, F. Beaudoin^c, S. Kurup^c, E. Martínez-Force^{a, id}, R. Garcés^a, J.J. Salas^{a, *}

^a Instituto de la Grasa, Ctra Utrera Km 1, Building 46, 41013, Sevilla, Spain

^b Universidad de Sevilla, Facultad de Biología, Av. Reina Mercedes s/n, 41013, Sevilla, Spain

^c Rothamsted Research Centre, Rothamsted, UK

ARTICLE INFO

Handling Editor: Ying HUANG Zhen-

Keywords:

Wax esters
Sunflower hull
Cover lipids
Yeast expression
Ester synthesis

ABSTRACT

Wax esters (WEs) are aliphatic esters of very-long-chain fatty acids and fatty alcohols that contribute to the lipid coatings of many plant organs. In the case of sunflower seeds, these compounds accumulate especially in the seed hull and are synthesized by the action of wax synthase (WS) enzymes through the esterification of acyl-CoA derivatives with very-long-chain fatty alcohols produced by fatty acyl reductases. Here, genes encoding 15 WSs were identified in the sunflower genome, of which 3 are expressed appreciably in the seed during the period of WE accumulation: *HaWS6*, *HaWS8*, and *HaWS11*. The localization and structure of these WSs were studied, and they were expressed in yeast in order to assay the microsomes prepared from them. The *HaWSs* all had very broad specificity profiles for alcohols and they were able to esterify short-chain alcohols (<10 C) with the acyl-CoA supplied. Indeed, simultaneous expression of *HaWS8* and *HaFAR5* resulted in the production in yeast of some WEs typically present in the sunflower hull.

1. Introduction

The surface of aerial plant tissues is covered by a hydrophobic layer known as the cuticle, which consists of a cutin polymer layer embedded in wax (the intracuticular wax layer), which is in turn covered by another external layer of wax, the epicuticular wax layer (Kunst and Samuels, 2003). The properties of cuticular waxes enable them to act as hydrophobic barriers that protect the plant organs against biotic and abiotic stresses, serving as a defence against pathogens, while also reducing non-stomatal water loss or participating in temperature regulation and solute transport (King et al., 2007; Li et al., 2008). Cuticular waxes are mainly composed of complex mixtures of very-long-chain fatty acids (those with 20 carbon atoms and more, VLCFA) and their derivatives, including free fatty acids, aldehydes, alkanes, primary and secondary fatty alcohols, ketones or wax esters (WEs: Kunst and Samuels, 2003). WEs are formed by CoA-activated fatty acids esterified to long-chain fatty alcohols, and their proportion in the cuticular wax layer varies depending on the specific plant or vegetal tissue. Furthermore, WEs are also found as storage lipids in jojoba (*Simmondsia chinensis*) seeds (Lardizabal et al., 2000), in animal hair coatings or feathers (Cheng and Russell, 2004), in beeswax and sperm-whale oil, and they are produced by some bacteria as a carbon store (Röttig and Steinbüchel,

2013).

Sunflower seeds are achenes consisting of a fibrous hull and an oil-rich kernel. The hull is rich in WEs (Garcés et al., 2023), which play a role in protecting the embryo from humidity and pest attack. WEs are extracted together with sunflower oil, causing a turbidity in the final product that is undesirable for the consumer. Therefore, sunflower oil requires additional refining through a process called winterization, which involves lowering the temperature to encourage the formation of crystals that can then be filtered out. This involves the loss of part of the oil and increases the cost of extraction (Dijkstra, 2015). However, sunflower WEs can be recovered during the oil refining process as a by-product, with applications in the cosmetics and food industry (Domergue and Miklaszewska, 2022).

The WEs in sunflower seed are long-chain WEs, mainly saturated species with an even number of carbon atoms in the range of WE40:0 to WE62:0, among which chain lengths from C44 to C48 were predominant (Garcés et al., 2023). WE synthases (WSs) catalyse the final step in wax biosynthesis, starting from the fatty alcohols derived from the reduction of VLCFAs by fatty acyl reductases (FARs; Rowland and Domergue, 2012; DeAndrés-Gil et al., 2024). In a second enzymatic reaction, an acyl chain from an activated fatty acid is transferred to a fatty alcohol, resulting in WEs as the final product (Lardizabal et al., 2000; Röttig and

* Corresponding author.

E-mail address: jjsalas@ig.csic.es (J.J. Salas).

<https://doi.org/10.1016/j.plaphy.2025.109692>

Received 1 August 2024; Received in revised form 20 February 2025; Accepted 22 February 2025

Available online 23 February 2025

0981-9428/© 2025 The Authors. Published by Elsevier Masson SAS. This is an open access article under the CC BY license (<http://creativecommons.org/licenses/by/4.0/>).

Steinbüchel, 2013).

WS have been identified in a wide range of organisms and they can be classified into three unrelated groups of acyltransferases (Röttig and Steinbüchel, 2013): bacterial-type bifunctional WS/acyl-CoA: diacylglycerol acyltransferase (DGAT), with additional triacylglycerol (TAG) synthesizing activity (Kalscheuer and Steinbüchel, 2003); jojoba-type WSs (Lardizabal et al., 2000) and mammalian WSs, such as those in mice and humans (Cheng and Russell, 2004). Interestingly, none of these three groups are related to one another based on their sequence data. The bacterial WS/DGAT type was first identified and characterized in the *Acinetobacter calcoaceticus* ADP1 strain (Kalscheuer and Steinbüchel, 2003), and it has a distinct origin to the jojoba-type or mammalian WSs. Orthologous genes have also been identified in some plants, such as *Arabidopsis thaliana* (Li et al., 2008) or *Petunia hybrida* (King et al., 2007), where they have been associated with the synthesis of the intracuticular WEs present in the leaves and stems. Jojoba-type WSs are necessary for the synthesis of WEs as storage lipids, whereas the mammalian WSs participate in the synthesis of esters in the sebaceous glands. WSs are usually associated with membranes or lipid inclusions, although their amphiphilic nature mean they can also be distributed in the cell cytoplasm (Röttig and Steinbüchel, 2013; Stöveken et al., 2005). This distribution is particularly relevant for enzymes belonging to the bifunctional WS/DGAT group, whereas eukaryotic DGATs and jojoba-type or mammalian WSs are exclusively membrane proteins (Lardizabal et al., 2000).

Here, the WS isoforms present in the sunflower genome were studied, among which was a group of 3 genes related to the WS/DGAT family that, based on their expression profiles, were considered to be the main candidates responsible for WE synthesis in sunflower seeds. Of these, the HaWS8 and HaWS11 isoforms were confirmed to be expressed in the sunflower seed hull, while HaWS6 was reported to be expressed strongly in seeds in previous massive sequencing results. The enzymes encoded by these genes were situated in the endoplasmic reticulum and WS activity was reproduced *in vitro* by expressing them in yeast. Finally, these enzymes were able to generate sunflower specific WEs when co-expressed with FAR enzymes in yeast. The possible impact of these genes on the synthesis of WE present in sunflower oil was discussed based on these results.

2. Experimental methods

2.1. Biological material and growth conditions

Sunflower (*Helianthus annuus* L.) plants from the CAS-6 line (Collection of Andalusian Sunflower, Instituto de la Grasa, CSIC, Seville, Spain) were cultivated in growth chambers on 25 °C/15 °C (day/night) cycles, with a 16 h photoperiod and a photon flux density of 250 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Sunflower seeds were harvested at different developmental stages (4, 7, 10, 12, 18, 25 days after anthesis, DAA) and while 4 to 10 DAA samples were used whole, samples from 12 to 25 DAA were divided into the kernel and hull. Vegetative tissues (leaf, stem, root and cotyledon) were collected 6 days after germination. All tissue samples were frozen immediately in liquid nitrogen and stored at -80°C .

The *Escherichia coli* DH5 α strain was used as the plasmid host for gene cloning, and the *Agrobacterium tumefaciens* strain GV3101 was used for agroinfiltration and transient expression in tobacco leaves. Both strains were grown at 37 °C in LB medium [pH 7] (1 % tryptone, 0.5 % yeast extract, 1 % NaCl) with the addition of the appropriate antibiotic.

The *Saccharomyces cerevisiae* strain H1246 (MAT α ADE2-1 can1-100 ura3-1 are1- Δ ::HIS3 are2- Δ ::LEU2, dga1- Δ ::KanMX4 lro1- Δ ::TRP1), a quadruple mutant yeast strain defective in neutral lipid accumulation (a kind gift of Fred Domergue, CNRS, Bordeaux, France), was used as a heterologous expression system to test WE synthase activity.

2.2. RT-qPCR studies of gene expression

For the quantitative gene expression analysis, mRNA was isolated from the samples collected and the corresponding cDNAs were synthesized as described previously (DeAndrés-Gil et al., 2024). Expression of the sunflower WSs identified was analysed in the cDNA samples by real time quantitative PCR (RT-qPCR) using specific primer pairs (Supplementary Table S1; all the primers were synthesized by Eurofins MWG Operon, Germany) and the Advanced universal SYBR Green supermix (Bio-Rad, USA) on a CFX96™ Real-Time PCR Detection System (Bio-Rad, USA). Efficiency curves were generated using sequential dilutions of the cDNAs. The relative expression of the genes was calculated by the Livak method (Livak and Schmittgen, 2001) and the sunflower actin gene (HaACT1, GenBank Accession FJ487620) was used as the internal reference to normalize the data, amplifying this with the specific HaActin-F4 and HaActin-R4 primers (Supplementary Table S1).

2.3. Gene cloning and sequence analysis

The WSD1 protein sequence from *A. thaliana* (At5g37300) was used as a query to identify genes encoding sunflower WS homologues in the Heliogene sunflower database (Heliogene Sunflower genome portal - <https://www.heliogene.org/>) and the putative sunflower WS genes were selected for cloning (named HaWS6, HaWS8 and HaWS11). The coding region of each gene was amplified by PCR from developing sunflower seed cDNA and directionally cloned into the yeast expression vector pYES2 (Thermo Fisher Scientific, United States) using specific primers (Supplementary Table S1). The nucleotide sequences were confirmed by sequencing (Eurofins MWG Operon, Germany).

Sunflower WS proteins were confirmed to be WSs through alignment with homologous sequences from other species using the MEGA X software tool, employing the default settings. These alignments were used to generate a phylogenetic tree based on the neighbour-joining algorithm, implementing a bootstrap analysis with 10000 replicates. The predictions of subcellular localization was performed using the prediction tools MultiLoc2, PredoTAR and DeepLoc 2.0. The hydrophobicity profiles and transmembrane tendency of the WS enzymes were determined using the ProtScale software and DeepTMHMM program.

2.4. Heterologous expression in yeast and microsomal membrane preparation

The constructs generated in pYES2 and the empty vector were transformed into the mutant *Saccharomyces cerevisiae* strain H1246. Transgenic yeast cells were inoculated into SC-Ura liquid medium in the presence of 2% (w/v) raffinose and grown overnight at 30 °C on a rotating platform (250 rpm). The OD600 was measured, and a volume of the culture required to inoculate 100 mL of liquid medium at an OD600 of 0.2 was removed and centrifuged. These yeast cultures were then resuspended in 100 mL of SC-Ura medium supplemented with 1% (w/v) raffinose and 2% (w/v) galactose to induce expression over 48 h. The cells were then collected by centrifugation and washed twice with distilled water before preparing microsomes according to the method described previously (Miklaszewska et al., 2013). The protein concentration of the microsomal fraction was measured by the BCA method (Thermo Scientific kit), according to the manufacturer's instructions.

2.5. Transient expression in *Nicotiana benthamiana* and confocal microscopy

For transient expression in a tobacco plant, HaWS sequences without their termination codons were amplified from cDNA (using the primers listed in Supplementary Table S1), as was the cytosolic marker RanBP1 protein from *Nicotiana benthamiana*. The corresponding fragments were cloned into the pENTR/D-TOPO vector (Invitrogen), and subsequently

transferred into the pK7WGF2 and pK7RWG2 vectors of the GATEWAY recombinational cloning system using the attL x attR (LR) recombination sites, resulting in a transcriptional fusion between the WS-EGFP and RanBP1-ERFP genes. Constructs were transferred into the *A. tumefaciens* GV3101 strain and individual clone cultures were co-infiltrated with the 35S:p19 viral suppressor construct. Transformed *Nicotiana benthamiana* leaves were then incubated for 3 days under normal growth conditions and visualised on a Zeiss LSM780 confocal microscope. The fluorescence signal was monitored at 525/550 nm after excitation at a wavelength of 488 nm for the GFP and RFP constructs, and at 600/622 nm after excitation at 561 nm for mCherry. Confocal images were obtained on a Zeiss LSM 780 confocal microscope with a 514 nm excitation laser and collecting the emissions between 597 and 650 nm.

2.6. Three-dimensional modelling of the HaWS structures

Homology modelling studies of the putative HaWS6, HaWS8 and HaWS11 protein structures were performed using the AlphaFold server (<https://alphafold.ebi.ac.uk/>). Molecular docking was carried out with SwissDock (<http://www.swissdock.ch/>) using stearyl-CoA and octadecyl alcohol as substrates and employing the default parameters. The resulting structures were visualised and the residues mapped using the UCSF Chimera package.

2.7. WS in vitro enzyme assays

WS activity was assayed for 45 min at 30 °C using 1–2 µg of microsomal proteins in a reaction mixture consisting of 10 mM MgCl₂, 5 mM ATP, 1 mM dithiothreitol (DTT), 1 mM CoASH, 1 mg/mL bovine serum albumin (BSA), 1.27 nM [1-¹⁴C]18:0-CoA substrate (140000 dpm) and 10 mM fatty alcohols (ethanol, butanol, hexanol, octanol, nonanol or decanol) in 100 µL of 50 mM potassium phosphate buffer (pH 7.2). The reactions were stopped by adding 1 mL of 1 M acetic acid in 2-propanol, and the lipids were extracted by adding 1.5 mL hexane and 1.5 mL of 6.7% Na₂SO₄. Aliquots of 0.1 mL of the organic phase were used to measure the total label incorporated by counting in a liquid scintillation counter (LS6500, Beckman Coulter, United States). The remainder was recovered, dried under nitrogen, resuspended into 100 µL hexane and separated by thin layer chromatography (TLC) using hexane/diethyl ether (90:10, v/v) as the solvent. Radiolabelled products were visualised and quantified on the plates by electronic autoradiography in a RITA star detector (Elysia Raytest). The band corresponding to WEs was found by comparison of the R_f with that from the corresponding standard.

2.8. WE extraction and purification

To study the wax accumulation in sunflower seed coatings throughout development, coatings were obtained from 7, 10, 12, 18, 25 and 50 DAA seed samples. About 0.4 g of the uncrushed samples were immersed in 2.5 mL of 3:2 (v/v) hexane/isopropanol, briefly shaking the samples and then incubating them at 80 °C for 30 min. The solvent was then transferred to a clean tube, and evaporated under nitrogen at 40 °C. The resulting residue was dissolved in hexane and analysed by gas chromatography coupled to mass spectrometry (GC/MS) as depicted below.

In the yeast expression studies, yeast cultures were grown and induced as indicated previously for the microsome preparations. In this case the yeast pellet was dried at 40 °C under nitrogen until a constant weight was reached and it was then subjected to lipid extraction as indicated previously (Mapelli-Brahm et al., 2020). The total lipids extracted were evaporated under nitrogen and dissolved in a 2 mL volume of chloroform, and the neutral lipids were separated in a solid phase silica gel cartridge of 0.5 g. The cartridge was equilibrated in chloroform, the lipid extract was loaded and the neutral lipids were eluted with 10 mL of chloroform. The solvent was then removed under nitrogen and the residue was dissolved in 2 mL hexane/ethyl ether 98:2.

The WEs were separated from the TAGs and other neutral lipids in another cartridge, this time equilibrated in hexane/ethyl ether (98:2). Once the neutral lipids were loaded, the WEs were eluted free of TAGs by adding 7 mL of the same solvent to perform GC/MS analysis.

2.9. WE GC/MS analysis

The WEs were separated and quantified by GC/MS as indicated previously (Garcés et al., 2023), using an Agilent Technologies (USA) 8890 GC/MS System equipped with a Quadrex 007-65HT medium polarity capillary column (30 m length, 0.25 mm internal span and 0.10 µm phase thickness) and an Agilent 5977B MS detector. The injector temperature was set to 340 °C and hydrogen was used as carrier gas at a flow rate of 1.6 mL/min, with injection performed in split mode at a ratio of 1:40. The oven was programmed at an initial temperature of 230 °C, which was maintained for 5 min and then increased to 330 °C at a flow rate of 2 °C/min. The MS detector was initially set to scan mode, covering a mass/molecular weight range of 145–905 without integrating peaks below 0.2 %. In SIM (Selected Ion Monitoring) mode, the detector was adjusted to the specific molecular ion using a precise window for each compound of interest (±0.5 Da), based on their retention times. Quantification was performed applying external calibration using C38:0 WE as the standard.

2.10. Data analysis

In all cases, the experiments were performed with 3–6 biological replicates, where average values were obtained from 2 to 4 technical replicates. Data were generally represented as their means ± standard deviation. Statistical significance group analysis was performed using a one-way ANOVA with Tukey's post hoc analysis at a significance level of $p = 0.05$. All analyses were conducted using IBM SPSS Statistics software.

3. Results and discussion

3.1. Gene structure analysis

Taking the amino acid sequence of an *Arabidopsis thaliana* WS (AtWSD1; At5g37300) as reference, the sunflower line XRQ genome (available in the Heliogene database, 2018 version - <https://www.heliogene.org/>) was searched for homologous sequences using the tblastn tool. A total of 15 sequences were identified as genes possibly encoding WSs, designated as HaWS1 to HaWS15, and distributed on chromosomes 4, 6, 10, 12, 12, 16 and 17 (Supplementary Fig. S1A, and see Supplementary Table S2 for the Heliogene codes of the genes identified, as well as the accession codes to their mRNA sequences deposited in the NCBI Genbank database - <http://www.ncbi.nlm.nih.gov/>). Some of the HaWS genes are associated with more than one Heliogene code as they are located in a region of the genome assembled from two contigs, or from assembly segments used during the sequencing of the sunflower genome. The percentage identity of all the HaWS amino acid sequences identified ranged from 32 to 40 % when compared to AtWSD1, except HaWS13 that was only 25.5 % identical (Supplementary Fig. S2).

The structure of the sunflower WS genes was analysed based on their genomic sequences and corresponding mRNAs. This analysis included examining the sequences of the introns, exons and 3'/5' untranslated regions of the genes identified, as well as their size (Supplementary Fig. S1B). Both the *Arabidopsis* AtWSD1 gene and the HaWS genes, with the exception of HaWS6, HaWS7, HaWS8, HaWS10, HaWS11 and HaWS13, are made up of 7 exons, although with structural and length differences. By contrast, the HaWS6, HaWS7, HaWS8, HaWS10 and HaWS11 genes have 6 exons, and the intronic sequences in HaWS7 were longer than those in the rest of the genes. Finally, the HaWS13 gene has 5 exons. These variations in genomic structure indicate phylogenetic differences between the sunflower HaWS genes. The differences in the

position of the exons, as well as in the length of the introns in the sunflower genes relative to their Arabidopsis counterparts could be attributed to the large number of transposable elements present in the sunflower genome. These elements are generally responsible for the modifications in gene structure and contribute to the increase in genome size (Kawakami et al., 2010).

3.2. Phylogenetic analysis

A phylogenetic tree was built based on the amino acid sequences of the sunflower *HaWSs* identified and homologous proteins from other plant species in order to evaluate the evolutionary relationships of the *WSs* (Fig. 1; Supplementary Table S3). Protein sequences from two bacterial *WS* (*MaWS/DGAT* and *MaWS2* from *Marinobacter aquaeolei*) were used as an outgroup to root the tree and the *WSs* segregated into three groups with different evolutionary origins: jojoba-type *WS*, first identified in this plant (Lardizabal et al., 2000); vertebrate-type *WS*, referred to as *AWAT* (Acyl-CoA wax-alcohol acyltransferases); and *WS*

related to bacterial bifunctional enzymes *WS/DGAT*, first identified in the *Acinetobacter baylyi* ADP1 strain (Kalscheuer and Steinbüchel, 2003; Stöveken et al., 2005). In plants like *Petunia hybrida* (King et al., 2007), *Arabidopsis thaliana* (Li et al., 2008), *Helianthus annuus* (Shalini and Martin, 2020; Zhang et al., 2022) or *Elaeis guineensis* (Rosli et al., 2018), genes have been characterized that fall into the same family as bacterial *WSs*, although there is less than 25% identity between their protein sequences. Despite the similarity in their structure, these proteins do not appear to have a common evolutionary origin. Thus, the structural and functional conservation may be a case of convergent sequence evolution in evolutionary unrelated species due to environmental selective pressure (Stern, 2013).

Of the 3 *WS* groups mentioned, the sunflower *WSs* identified belong to the group of *WS* related to bacterial *WS/DGAT* enzymes (*WSD*). Phylogenetic analysis grouped these sunflower enzymes into 3 different clusters (Fig. 1). The first cluster includes *HaWS1*-5, 9, 12, 14 and 15, related to the proteins from *Vitis vinifera* (*VvWSD1*), *Ricinus communis* (*RcWSD11*) or *Petunia hybrida* (*PhWS1*). This first cluster is the closest to

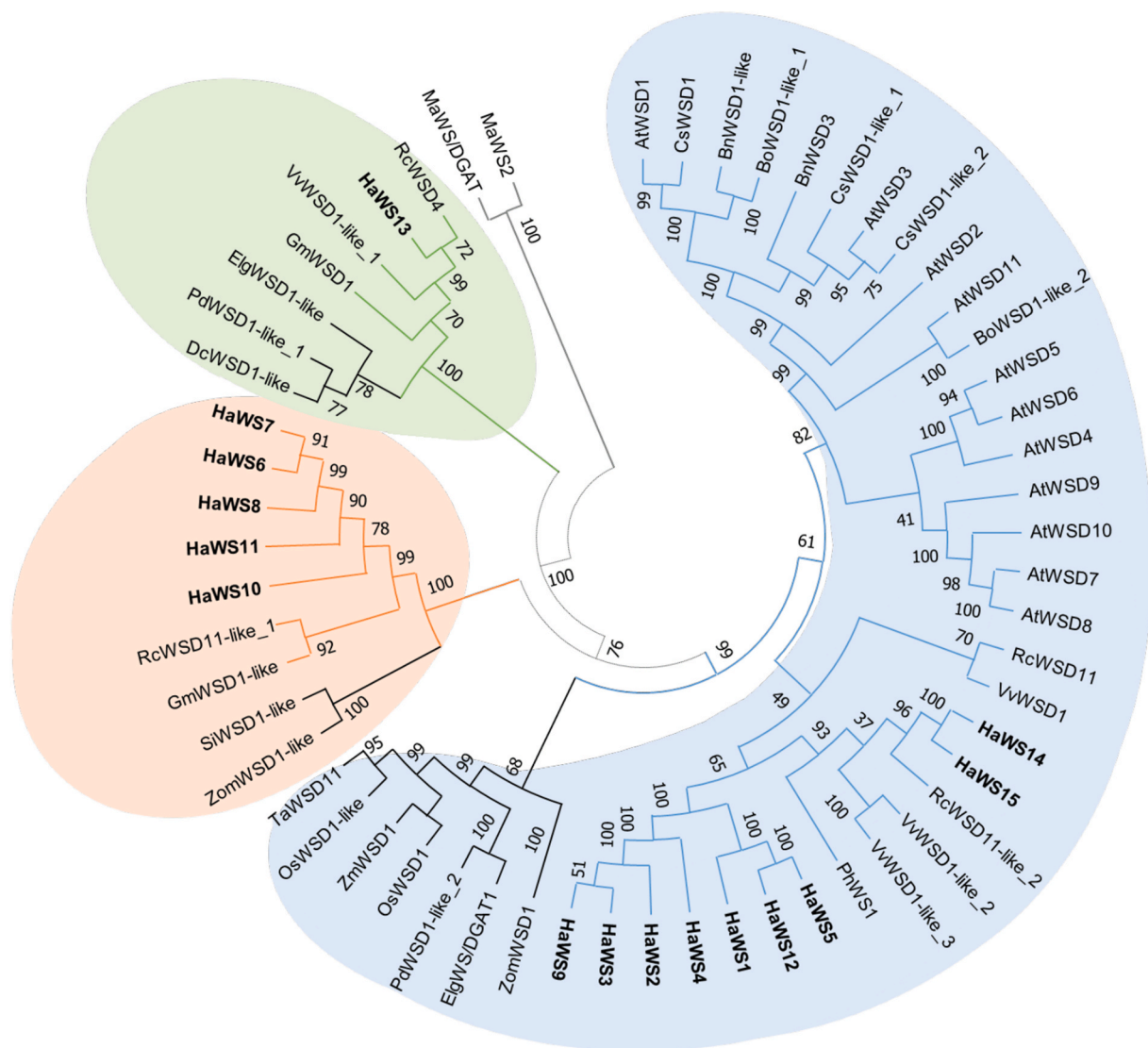


Fig. 1. Phylogenetic tree of plant wax synthases, including: At, *Arabidopsis thaliana*; Bn, *Brassica napus*; Bo, *Brassica oleracea*; Cs, *Camelina sativa*; Dc, *Dioscorea cayenensis*; Elg, *Elaeis guineensis*; Gm, *Glycine max*; Ha, *Helianthus annuus*; Ma, *Marinobacter aquaeolei*; Os, *Oryza sativa*; Pd, *Phoenix dactylifera*; Ph, *Petunia hybrida*; Rc, *Ricinus communis*; Si, *Spirodela intermedia*; Ta, *Triticum aestivum*; Vv, *Vitis vinifera*; Zm, *Zea mays*; and Zom, *Zostera marina*. Two *Marinobacter aquaeolei* (Ma) sequences were used as an outgroup to root the tree (in grey). The clades marked in blue, orange and green represent the 3 groups into which the sunflower *HaWS* are divided, as highlighted in bold. Within each group, the sub-trees marked in black represent the monocots. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

the WSD forms in *Arabidopsis*, grouped together with enzymes from other plants belonging to the Brassicaceae family, such as the WSDs from *Brassica napus*, *Brassica oleracea* and *Camelina sativa*. The second cluster contains the *HaWS*6, 7, 8, 10 and 11 enzymes, grouped with other isoforms from *Ricinus communis* or *Glycine max*. The third cluster is the most evolutionarily close to bacterial WSs and it contains the *HaWS*13 enzyme related to the *Glycine max* and *RcWSD4* isoforms from castor bean. All sunflower *HaWS* have homologous forms in monocots, grouped into a sub-clade within each cluster.

3.3. Expression of WS/DGAT-type *HaWS*s

From previous data, bacterial type or WS/DGAT WSs are related to the synthesis of surface lipids in plants (Li et al., 2008). To establish which WS isoforms might be implicated in WE synthesis, their expression in sunflower hulls was studied during the period that WEs accumulate. The accumulation of WEs in this organ was determined by applying the method described previously (Garcés et al., 2023: Fig. 2A).

WES began to accumulate in the first stages of seed formation when they are deposited in the seed hull, and they accumulate linearly for up to 24 DAA, a period in which the expression of the *HaWS* isoforms in the seed hull was assessed. Previous expression and massive sequencing data in public sunflower databases was first retrieved, such as that published by NCBI and Heliagene based on EST sequencing (Supplementary Fig. S3). This expression data was normalized using different methods, making direct comparison difficult but offering a general idea of the relative expression of the *HaWS* genes in seeds. Furthermore, data from the sunflower XRQ line does not specify the developmental stage of the seeds used for RNA extraction. Both massive sequencing experiments indicated that *HaWS*6, *HaWS*8 and *HaWS*11 were the genes expressed predominantly in sunflower seeds, with weaker expression of other genes like *HaWS*2, *HaWS*3, *HaWS*13 and *HaWS*14, which is in good agreement with previous results (Shalini and Martin, 2020). The expression of different *HaWS* genes was also assessed more recently in developing seeds (from 10 to 38 DAA), with only weak expression detected (Zhang et al., 2022). The expression of all the *HaWS* candidate

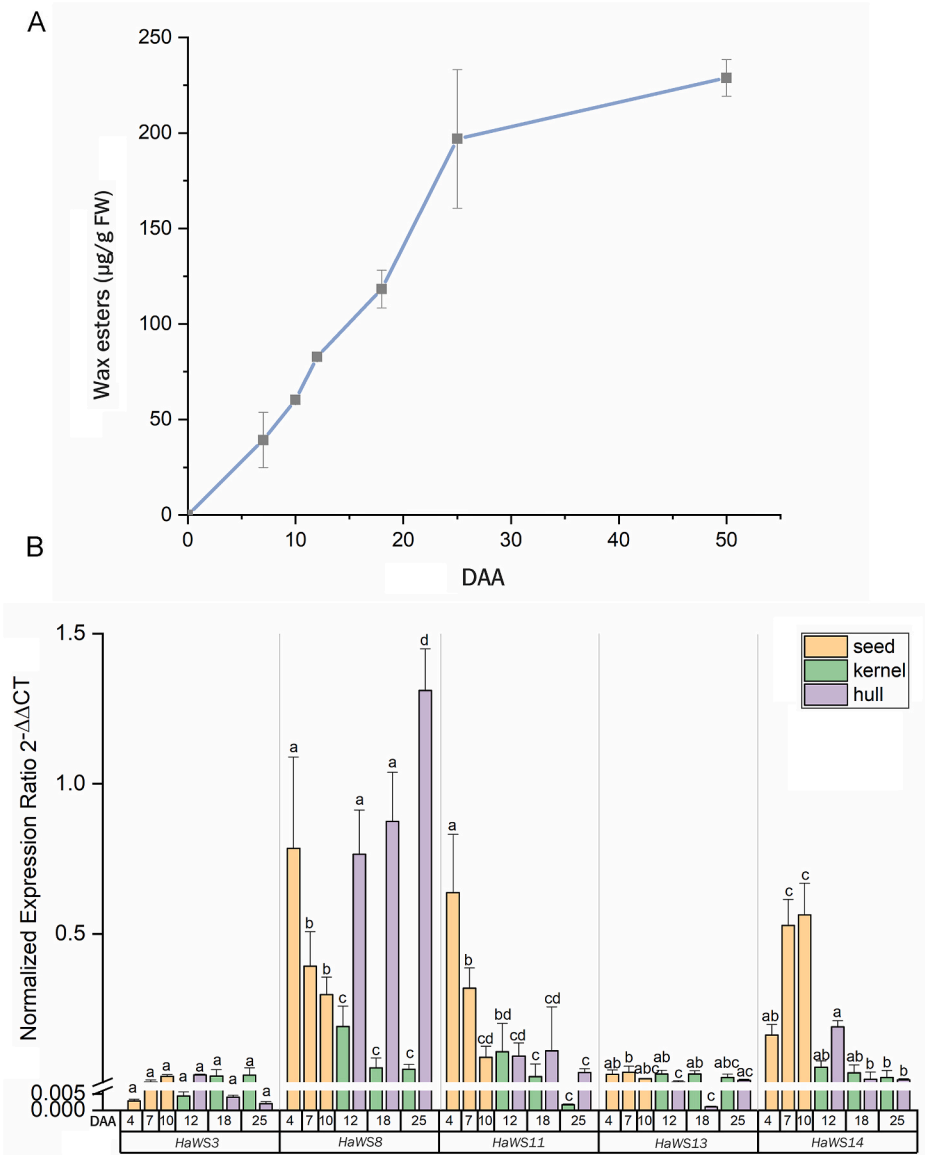


Fig. 2. (A) Wax accumulation curve (µg/g fresh weight) in sunflower seeds throughout development (7, 10, 12, 18, 25 and 50 DAA). (B) Expression of WS genes in *Helianthus annuus* seeds at different developmental stages. The expression values were normalized to the sunflower actin gene (*HaACT1*) and the data represent the mean values (±SD) from 3 independent biological samples: “a, b, c, d” represent the statistical significance according to one-way ANOVA with Tukey’s *post hoc* analysis at $p = 0.05$ significance. DAA, days after anthesis.

genes was investigated in developing seeds and vegetative tissues by RT-qPCR. As such, cDNA from seeds at different developmental stages and from different tissues (root, stem, leaf and cotyledon) were studied. Indeed, whole seeds 4, 7 and 10 DAA were assessed, as well as seeds separated into kernel and hull tissues once these tissues had differentiated and synthesized most of their surface waxes (at 12, 18 and 25 DAA). This analysis was carried out on the *HaWS3*, *HaWS8*, *HaWS11*, *HaWS13* and *HaWS14* genes, as primer pairs designed to amplify the *HaWS2* and *HaWS6* genes were not sufficiently efficient (qPCR *HaWS2_F*/qPCR-*HaWS2_R* and qPCR *HaWS6_F*/qPCR *HaWS6_R*: see [Supplementary Table S1](#) for the sequences of the primer pairs used to amplify each gene).

The *HaWS8*, *HaWS11* and *HaWS14* genes were each expressed in seeds, with *HaWS8* expressed most strongly overall ([Fig. 2B](#)). *HaWS8* expression decreased progressively over the early stages of seed development (4–10 DAA), and this trend persisted throughout seed kernel development. However, this gene was strongly expressed in the seed hull, with transcripts increasing progressively from 12 to 25 DAA, representing the *HaWS* gene with the highest level of expression in this tissue. *HaWS11* followed a similar expression pattern to *HaWS8* in the kernel, so it was expressed at high levels at earlier stages of development. In hulls, *HaWS11* was expressed weakly, although its transcripts were detected throughout the study. *HaWS14* was expressed significantly at the initial stages of seed development but this expression dropped to barely detectable levels after 12 DAA. *HaWS3* and *HaWS13* were expressed very weakly in seeds relative to the other genes studied. The only gene expressed significantly in developing seed hulls was *HaWS8*, suggesting it is probably involved in wax synthesis in sunflower seeds. Furthermore, based on earlier EST sequencing data, the contribution of *HaWS6* to this process is also probable ([Supplementary Fig. S3](#)) and hence, it was studied further. Of the genes expressed weakly in seeds, *HaWS11* was also characterized as it was expressed in the seed hull, and there were significant EST counts in the NCBI and HeliGene databases.

Regarding vegetative tissues, no specific expression patterns were evident among the different genes studied but generally, they were more strongly expressed in stems and less so in roots relative to the other tissues ([Fig. 3](#)). This pattern was consistent with the expression of the homologous *AtWSD1* gene in *Arabidopsis*, which is expressed predominantly in flowers, stems and leaves, and only weakly in roots ([Li et al., 2008](#); [Patwari et al., 2019](#)). Moreover, it is consistent with the role of

this gene in wax production in the cuticle of the aerial tissues of this plant, especially in stems where the wax content is much higher than in the *Arabidopsis* leaves ([Suh et al., 2005](#)). Suberin is the predominant surface lipid in the plant roots, far exceeding waxes ([Schreiber et al., 1999](#)), which explains the weak expression of the *WS* genes in this organ. In sunflower, the gene most strongly expressed in the stems and leaves was *HaWS14*, which was particularly prominent in stems. In addition, *HaWS3* and *HaWS8* were also expressed more intensely in stems as opposed to the other tissues analysed. *HaWS13* was expressed similarly in stems, leaves and cotyledons, whereas *HaWS11* was expressed very weakly in all the tissues analysed.

3.4. Structure

The residues that are candidates to be involved in the catalytic activity of *HaWS* were determined using information from two crystallized and characterized *Marinobacter aquaeolei* *WS* enzymes: *MaWS/DGAT* ([Petronikolou and Nair, 2019](#); accession code A1TX06) and *MaWS2* ([Villa and Cabezas, 2014](#); accession code A1U572). Aligning these protein sequences with the *HaWS6*, *HaWS8* and *HaWS11* sequences, and those of other homologous proteins from *Arabidopsis thaliana* (*AtWSD1*) and *Petunia hybrida* (*PhWS1*: [Supplementary Fig. S2](#)), allowed the identification of homologous residues and key motifs associated with enzyme activity. The presence of these highly conserved residues and motifs among the proteins from different species highlights their importance in the catalytic mechanism of these enzymes throughout their evolutionary history. The identity between the protein sequences of the three sunflower *WS* enzymes was 63.5%, and they had around 31% identity with *AtWSD1*, and between 32 and 34% identity with the *PhWS1* protein sequence. Moreover, *HaWS6*, *HaWS8* and *HaWS11* share around 20% identity with the *Marinobacter aquaeolei* enzymes (see [Table 1](#) for detailed information regarding the number of amino acids, molecular weight and isoelectric point of the *HaWS6*, *HaWS8* and *HaWS11* protein sequences).

Following a search of the protein databases (InterPro, [Paysan-Lafosse et al., 2023](#); PROSITE), it was concluded that the *HaWS6*, *HaWS8* and *HaWS11* amino acid sequences contained two conserved domains characteristic of the acyltransferase family: a *WS*/acyltransferase domain at the N-terminal end (Pfam PF03007), which includes the catalytic motif described below; and a *WS*/acyltransferase domain at the C-terminal end (Pfam PF06974). All the *WSDs* analysed had a highly conserved HHXXXDG motif that has been implicated in the direct catalysis of the acyl chain transfer reaction ([Supplementary Fig. S2](#)). This motif has also been found in other acyltransferases, as well as in condensation domains of non-ribosomal peptide synthetases, chloramphenicol acyltransferases or polyketide-associated proteins (PAPs) of *Mycobacteria* ([Buglino et al., 2004](#)). The second histidine residue of this domain acts as the general base, promoting deprotonation of the alcohol hydroxyl group to generate the reactive alkoxide and hence, catalyse the transfer of the acyl group ([Petronikolou and Nair, 2019](#)). In addition to the residues forming part of the catalytic motif, there are other conserved residues forming characteristic motifs of *WSDs*. The PLW residues (Pro-Leu-Trp) in the N-terminal domain of these proteins form motif I, conserved in all the *WSDs* analysed ([Supplementary Fig. S2](#), marked in blue). The location of this motif suggests it plays a structural role in the correct folding of the catalytic centre. The conserved ND residues (Asn-Asp) form motif II and they could establish interactions

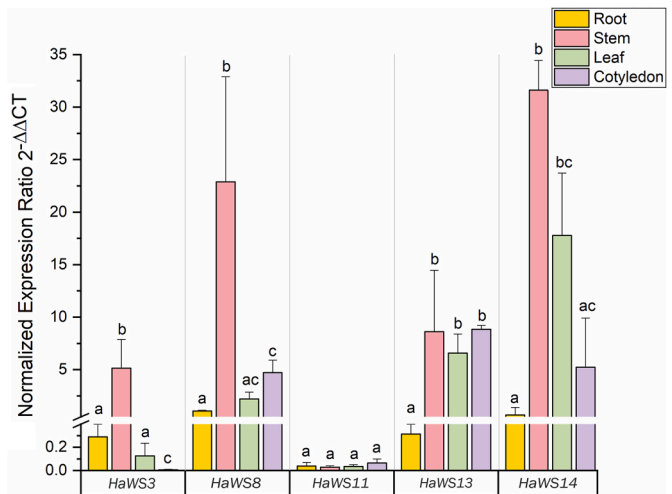


Fig. 3. Expression of the wax synthases in vegetative tissues of *Helianthus annuus*, normalising the values to the sunflower actin gene (*HaACT1*). The data represent the mean values ($\pm$ SD) from 3 independent biological samples: “a, b, c” represent the statistical significance according to one-way ANOVA with Tukey’s *post hoc* analysis at $p = 0.05$ significance. DAA, days after anthesis.

Table 1
Characteristics of the wax synthase proteins cloned from sunflower.

	<i>HaWS6</i>	<i>HaWS8</i>	<i>HaWS11</i>
GenBank Accession No.	XP_022018443.1	XP_022036387.1	KAF5786970.1
Amino acids	456	455	474
Molecular weight (kDa)	50.79	50.68	52.63
pI	8.65	8.62	8.55

with phosphate groups of the acyl-CoA substrates. In addition, the conserved arginine residue (marked with a purple arrow in [Supplementary Fig. S2](#)) could interact through an ionic bond with the aspartic acid residue of the catalytic motif. This interaction plays a critical role in maintaining the correct distance between protein domains and an adequate conformation, as well as aiding in the positioning of the catalytic loop for the reaction ([Walker et al., 2013](#)).

The AlphaFold structure prediction server (<https://alphafold.ebi.ac.uk/>) was used to understand the three-dimensional structure of *HaWS6*,

HaWS8 and *HaWS11*. As there was only moderate identity between the sunflower WSs (63.5%), some differences were apparent in the models generated ([Supplementary Fig. S4](#)). The 3 *HaWS*s studied were predicted to adopt a monomeric structure that is divided into the two domains indicated above, the N-terminal WS/acyltransferase domain (between residues 60–247 in *HaWS8* and 66–256 in *HaWS11*) and the C-terminal WS/acyltransferase domain (residues 304–449 in *HaWS8* and 312–457 in *HaWS11*; [Supplementary Fig. S4](#)). In *HaWS8* and *HaWS6*, a total of 11 α helices and 13 β strands were identified, while 14 α helices and 14 β

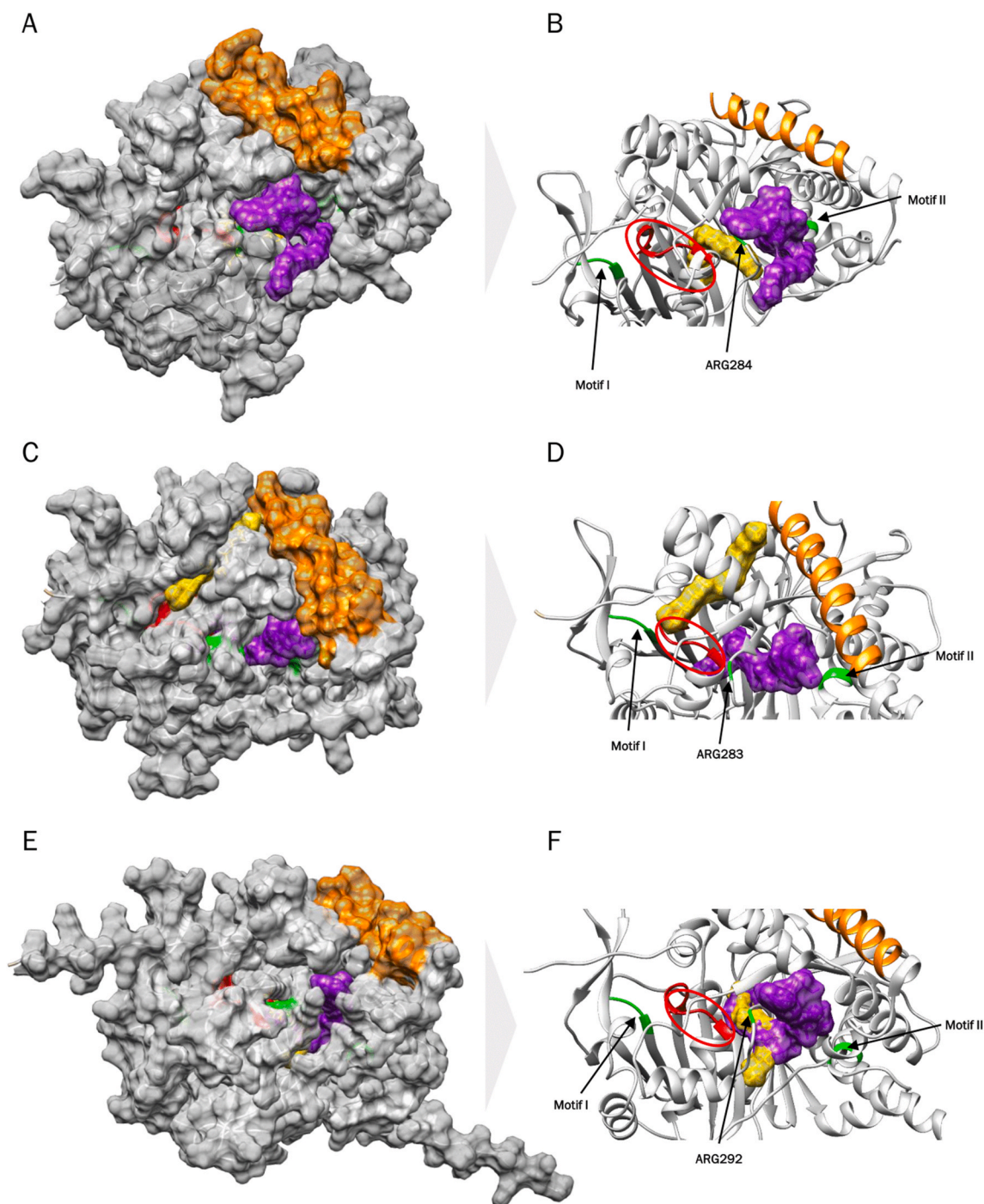


Fig. 4. Proposed tertiary structure models for *HaWS6*, *HaWS8* and *HaWS11*. Representation of the *HaWS6* (A), *HaWS8* (C) and *HaWS11* (E) molecular surface in which the entrance of the cavity for the substrates can be seen. Ribbon diagrams reflecting the interaction of *HaWS6* (B), *HaWS8* (D) and *HaWS11* (E) with model substrates. The C16:0-CoA (purple) and C18:0-OH (yellow) substrates are represented in 3D. Residues with structural and substrate-interacting functions are marked in green, while the protein's catalytic motif is circled in red. The residues marked in orange are hydrophobic residues that could be part of a transmembrane region. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

strands were detected in *HaWS11*. The N-terminal domain of this enzyme consists of 7 β strands surrounded by 7 α helices, while the C-terminal domain contains 5 β strands and 3 α helices, which cover the outer part of the domain. Both domains are connected by a β strand and an α helix. In the case of *HaWS11* (Supplementary Fig. S4), the N-terminal domain is formed by 8 β strands and 8 α helices, and it is connected by 2 α helices and 1 β strand to the C-terminal domain that is composed of 5 β strands and 4 α helices. The functional motifs and residues involved in *HaWS* activity were represented (Fig. 4), and the substrate docking models of *HaWS6*, *HaWS8* and *HaWS11* were predicted with the SwissDock server, along with the palmitoyl-CoA (C16:0-CoA) and octadecanol (C18:0-OH) substrates that are both necessary for the enzymatic reaction. The HHXXXXG residues that make up the catalytic motif in the N-terminal domain of the proteins are His131-Gly137 in *HaWS6*, His130-Gly136 in *HaWS8* and His142-Gly148 in *HaWS11* (Supplementary Fig. S4, highlighted in red). Motif I is involved in the structural organization of the active site and it corresponds to the residues Pro113-Trp115 in *HaWS6*, Pro112-Trp114 in *HaWS8* and Pro124-Trp126 in *HaWS11*. The residues involved in the interaction with the acyl-CoA substrates form motif II, corresponding to the amino acids Asn246-Asp247 in *HaWS6*, Asn245-Asp246 in *HaWS8* and Asn254-Asp255 in *HaWS11* (Fig. 4). The arginine residue involved in maintaining the correct conformation between domains corresponds to residues Arg284, Arg283 and Arg292 in *HaWS6*, *HaWS8* and *HaWS11*, respectively (Fig. 4). Moreover, the possible transmembrane regions of both proteins are indicated, with more consistent predictions for *HaWS8* than for the other two proteins. In both cases, this possible transmembrane region corresponds to an α helix located at the end of the N-terminal domain (Fig. 4). The docking models of the three sunflower proteins revealed the formation of a cavity in the three-dimensional structure that acts as an entrance for the substrates and that allows them to interact with the catalytic motif. Both substrates (C16:0-CoA and C18:0-OH) are placed with the aliphatic chains facing towards the interior of the cavity and its terminal carboxyl or hydroxyl ends facing outward. In addition, the residues that make up motif II and that are implicated in binding to the acyl-CoA molecule are close to this area in all 3 docking models (Fig. 4).

3.5. Subcellular location

To determine the location of these enzymes, the presence of signal peptides was first evaluated and the subcellular localization of *HaWS6*, *HaWS8* and *HaWS11* proteins was assessed using different prediction platforms (TargetP 2.0; DeepLoc 2.0). No signal peptides were detected in any of the sunflower proteins analysed, although the DeepLoc 2.0 prediction suggested a probable location in the endoplasmic reticulum (ER), similar to the homologous AtWSD1 protein in Arabidopsis (Supplementary Table S4). Moreover, hydrophobicity profiles generated with the KD scale highlighted regions with strong hydrophobicity that might be involved in membrane anchorage (Supplementary Fig. S5), consistent with the predicted transmembrane (TM) regions: approximately residues 180–200 for *HaWS8* and 190–210 for *HaWS11* (Supplementary Fig. S4). According to the predictions, part of the protein sequence before the N-terminal TM region would be located in cytoplasm, harbouring the catalytic motif and motif I. By contrast, the region after the TM domain would reside in the ER lumen, such that the conserved motif II and arginine residue would be situated therein.

The data provided by the prediction platforms were complemented by the *in vivo* subcellular localization studies of *HaWS8* and *HaWS11*. The coding sequences of these genes without the stop codons were amplified and the constructs were fused to the green fluorescent protein (GFP) to generate *HaWS*-GFP using the GATEWAY cloning system. In addition, the NbRanBP1-RFP construct was generated as a cytosolic marker and the CD3-959-mCherry construct was used as an ER marker. Both the sunflower WS constructs and the markers were transiently expressed in *Nicotiana benthamiana* leaves by agroinfiltration, and the

signals were monitored by confocal microscopy. The fluorescence analysis of these two *HaWS* revealed that both co-localized with the ER marker (CD3-959-mCherry; Fig. 5A) but not with the cytosolic RanBP1-RFP marker (Fig. 5B). These results were consistent with the *in silico* predictions and with the localization of another sunflower WS that corresponds to the *HaWS2* gene previously characterized in Arabidopsis protoplast cells (Zhang et al., 2022). The *in vivo* localization of *HaWS6* was not studied, although the structural similarities and bioinformatics predictions indicate that a reticular location is very likely.

There is evidence that most enzymes involved in the synthesis of substrates necessary to produce surface lipids in plants are located in the ER, such as the VLCFAs or fatty alcohols. This is the case for fatty acid elongases (FAE complexes) or FARs (Wang et al., 2016). In sunflower, the ketoacyl-CoA synthase enzymes that constitute the FAE elongation system are associated with the ER membrane (González-Mellado et al., 2019), whereas the localization of the FARs did not offer conclusive results, suggesting a cytosolic location with some interaction with the ER (DeAndrés-Gil et al., 2024). Since most surface lipid components are generated in the ER, the entire wax biosynthetic pathway would be expected to be associated with this compartment. However, not all the enzymes belonging to this family of plant WSs have been defined as ER enzymes. In the oil palm (*Elaeis guineensis*), some of these enzymes were predicted to be soluble enzymes (Rosli et al., 2018), while the subcellular localization of some bacterial forms was ambiguous. For example, the AtfA enzyme from *Acinetobacter baylyi* is a WS primarily associated with the cytoplasmic membrane and lipid inclusions, although it was also partially soluble in the cytoplasm (Stöveken et al., 2005).

3.6. In vitro characterization

The activity of the *HaWS*s cloned from sunflower was evaluated *in vitro*, transforming the quadruple yeast mutant strain H1246 with the genes of interest cloned into the pYES2vector. This mutant does not accumulate neutral lipids due to the absence of the DGA1 and LRO1 genes involved in TAG synthesis, and of ARE1 and ARE2 that participate in sterol ester formation. This mutant strain was chosen to avoid any interference from the yeast's endogenous machinery when analysing WS activity. Considering the possible TM regions in sunflower WSs and the high probability of their ER localization, the microsomal fraction of yeast expressing these proteins was analysed *in vitro*. Indeed, other WSs like those from *jojoba*, *Marinobacter hydrocarbonoclasticus* or *Petunia hybrida* have already been characterized *in vitro* using microsomes (King et al., 2007; Miklaszewska et al., 2018).

The enzymes *HaWS6*, *HaWS8*, and *HaWS11* were characterized in terms of substrate specificity by measuring ester formation from various alcohols and [1-¹⁴C]18:0-CoA-labeled acyl-CoA as the acyl donor. The physiological substrates of these enzymes, based on the composition of WEs in the sunflower hull, were very-long-chain saturated alcohols. However, the use of these substrates was hindered by the difficulty in solubilizing them in the assay medium. In this regard, additional assays were conducted in a micellar phase, emulsifying the VLC alcohols in mixtures of DMSO, Triton X-100, and BSA. Reproducible activity measurements were also not obtained in this case, likely because the solvents and surfactants altered the aggregation state of these membrane-bound proteins. Nonetheless, assays were performed with both saturated and unsaturated short-chain alcohols to gather as much information as possible about them, bearing in mind that these are likely not their physiological substrates. The microsomal preparations from yeast expressing the three sunflower enzymes exhibit WS activity (Fig. 6), indicative of a membrane location of these enzymes. The microsomes from yeast expressing *HaWS8* had significantly higher activity than those from yeast expressing the other WSs, with a maximum activity of 454 pmol/min/mg prot relative to the maximum activities of 50 and 25 pmol/min/mg prot for *HaWS6* and *HaWS11*, respectively (Fig. 6B). The specificity profiles of all 3 enzymes retained some similarities, such as activity with all alcohols tested that have more than 6 carbons.

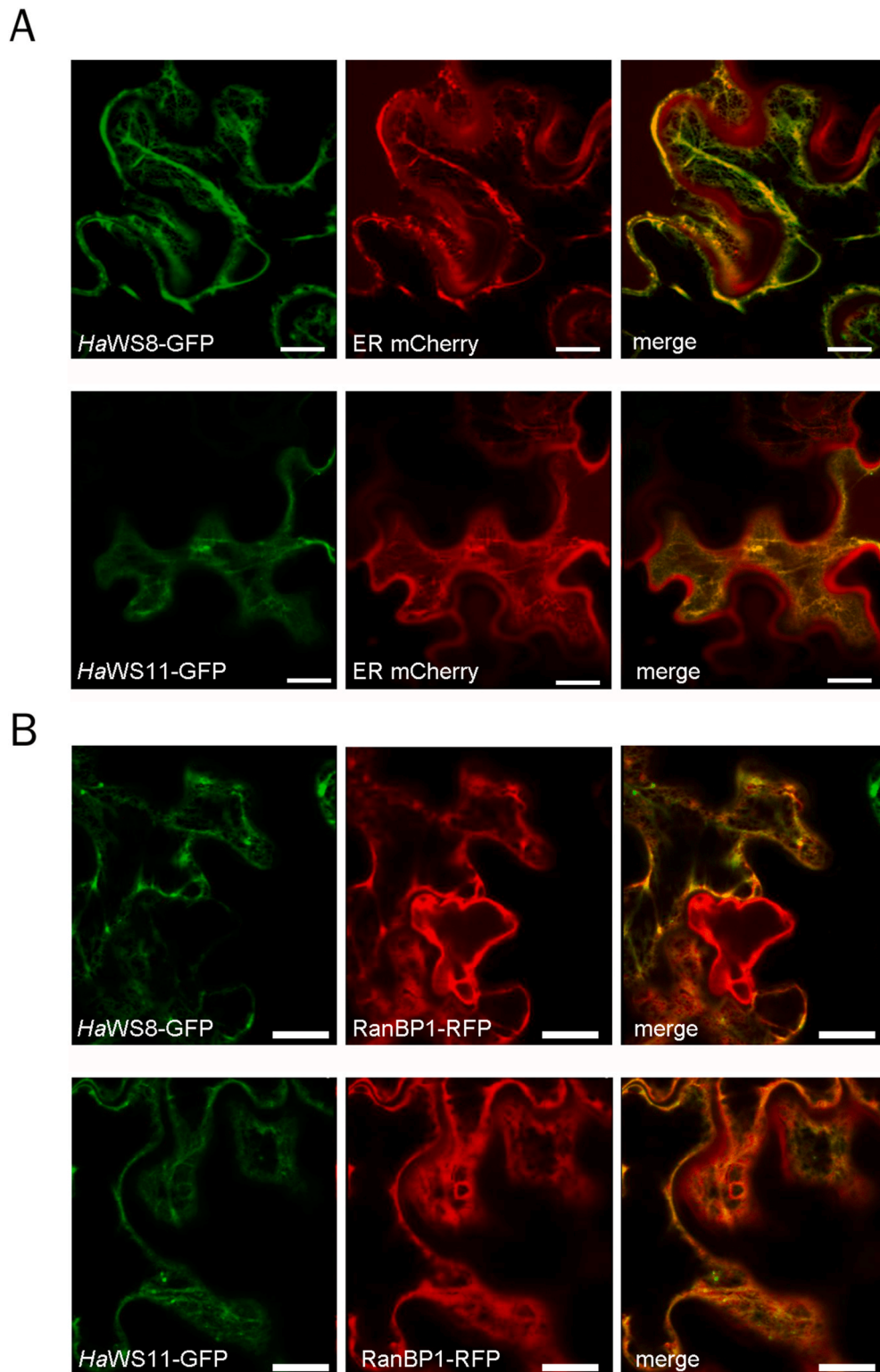


Fig. 5. Subcellular localization of *HaWS8* and *HaWS11*. (A) Confocal images of tobacco leaves expressing *HaWS8*-GFP and *WS11*-GFP constructs together with the ER marker CD3-959-mCherry. (B) Confocal images of *HaWS8*-GFP and *WS11*-GFP constructs together with the cytosolic marker NbRanBP1-RFP. Scale bar = 20 μ m.

Unexpectedly, *HaWS8* and *HaWS6* displayed highest activities with unsaturated 3Z-hexenol, while for *HaWS6* this was the case with 2E-hexenol. *HaWS6* was not active on short alcohols like ethanol and butanol, whereas *HaWS11* had stronger relative activity with these substrates (Fig. 6A). The waxes identified in sunflower seeds mainly contain saturated species with very-long-chain alcohols (Garcés et al., 2023) and

thus, the activity profile identified here would not justify this composition. The activity exhibited by sunflower WS towards short saturated and unsaturated substrates could be due to the experimental conditions employed *in vitro* deviating from the physiological ones. Thus, short and unsaturated substrates tend to be more soluble in aqueous solutions, making them more available to the enzymes than longer substrates.

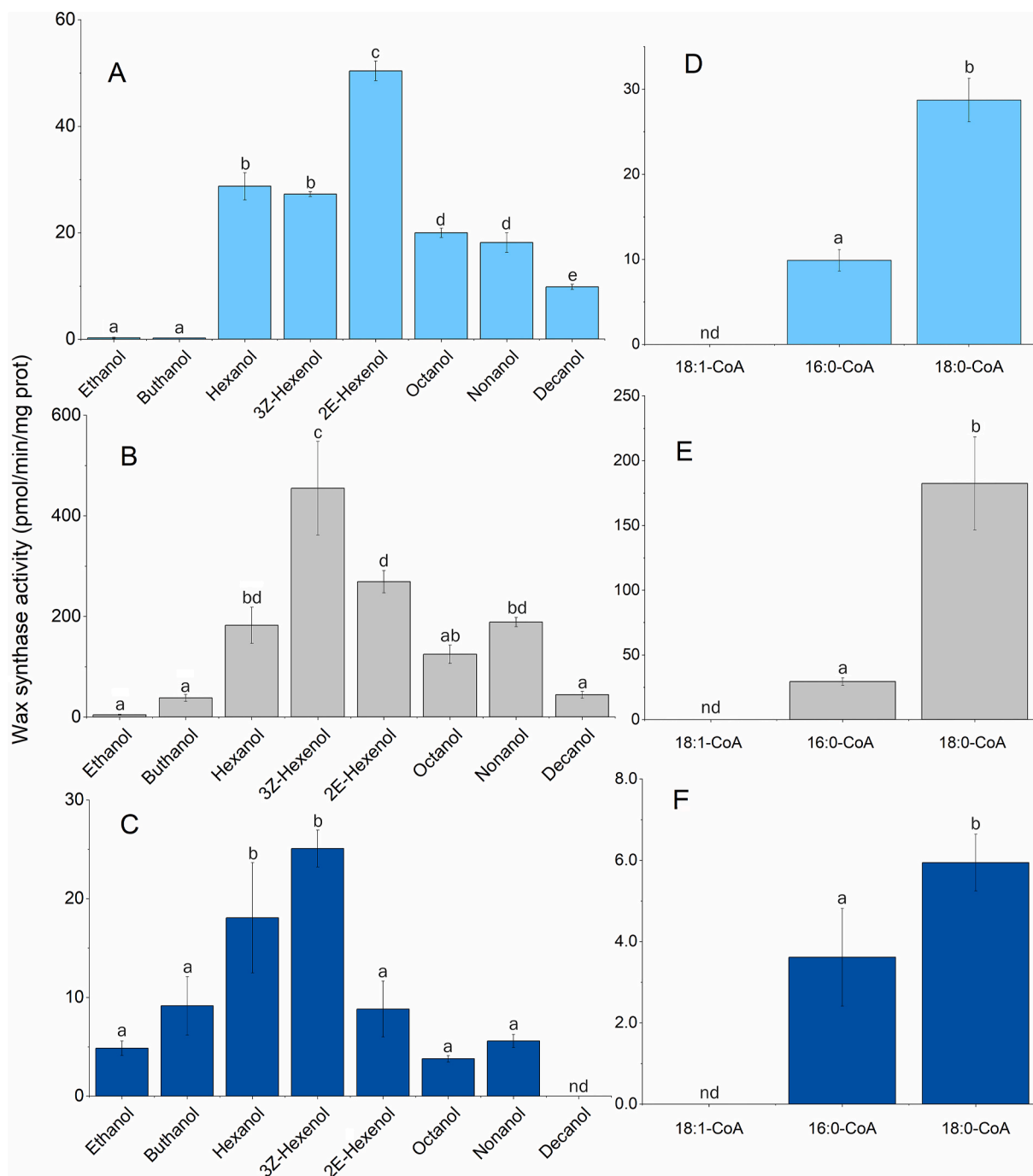


Fig. 6. *HaWS6* (A) *HaWS8* (B) and *HaWS11* (C) enzyme activity on different fatty alcohol substrates, using $[1-^{14}\text{C}]18:0\text{-CoA}$ as an acyl donor. The enzyme activity of the same enzymes using hexanol and different radioactively labeled acyl-CoA substrates ($[1-^{14}\text{C}]18:1\text{-CoA}$, $[1-^{14}\text{C}]16:0\text{-CoA}$ and $[1-^{14}\text{C}]18:0\text{-CoA}$) is represented in the right panels (*HaWS6*, D; *HaWS8*, E; and *HaWS11*, F). The data is represented as the mean ($\pm$ SD) of three independent replicates and the letters “a” and “b” represent statistical clustering at the $p = 0.05$ significance.

Furthermore, the enzyme’s environment in these assays is potentially very different to that in the cells of the developing seed, where endogenous proteins could be involved in facilitating the enzyme’s interaction with long-chain alcohols.

The specificity of the 3 cloned *HaWSs* towards different acyl-CoA substrates was also studied, using hexanol as the acyl acceptor. Radio-labelled acyl-CoA substrates with different chain lengths and degrees of saturation were assessed: $[1-^{14}\text{C}]16:0\text{-CoA}$, $[1-^{14}\text{C}]18:0\text{-CoA}$, and $[1-^{14}\text{C}]18:1\text{-CoA}$ (Fig. 6B). The three enzymes displayed a similar profile, with lower activity towards the $[1-^{14}\text{C}]16:0\text{-CoA}$ substrate than to the radioactive stearyl-CoA, suggesting a preference for longer chain

acyl-CoA substrates. No activity was detected for the unsaturated $[1-^{14}\text{C}]18:1\text{-CoA}$ substrate, indicating that the *HaWSs* selectively exclude unsaturated acyl-CoA substrates (Fig. 6D and E). This would be consistent with the composition of waxes in sunflower seeds discussed previously, which contain mainly saturated species, in particular saturated fatty alcohols and saturated 20 or 22 carbon fatty acid species (Broughton et al., 2018; Garcés et al., 2023).

The data suggest that sunflower *WSs* have a relatively broad range of specificity towards fatty alcohols and that their activity is determined by the availability of substrates in the enzyme’s environment. This broad specificity profile has been described previously for other *WSs*. The *in*

vitro characterization of five WSs from different organisms (*Acinetobacter baylyi*, *Marinobacter hydrocarbonoclasticus*, or *Mus musculus*) expressed in yeast cells showed that these enzymes were active on short and medium-chain alcohols (C2-C18) when combined with [$1-^{14}\text{C}$]16:0-CoA as the acyl donor. These enzymes had a stronger preference for long-chain substrates, with distinct specificities among the different enzymes studied (Shi et al., 2012). The activity of other bacterial WSs purified from *Escherichia coli* crude extracts also reflected preferences for a broad range of substrates, including short and medium-chain alcohols from ethanol to hexadecanol in conjunction with long-chain acyl-CoA substrates (C14 and C16; Barney et al., 2012; Kalscheuer et al., 2004). As for PhWS1, enzymatic assays also indicated a wide range of substrate specificities with a preference for saturated acyl-CoA substrates. This result is consistent with the docking models (Fig. 4), in which acyl-CoA fits into a deeper hydrophobic pocket while alcohols remaining at the enzyme's surface, without needing to fit tightly into a defined cavity, but rather in a more external and open region of the enzyme, which would explain the broader specificity profile for alcohols.

3.7. *In vivo* activity characterization

As mentioned earlier, the sunflower WSs identified in this study belong to the family of bacterial WSs of which AtfA from *Acinetobacter baylyi* was the first characterized enzyme (Kalscheuer and Steinbüchel, 2003; Stöveken et al., 2005), a bifunctional enzyme with dual WS and DGAT activity. Hence, the pYES2:HaWS8 and pYES2:HaWS11 constructs were used to evaluate any potential DGAT activity in the sunflower WSs, introducing them into the H1246 strain deficient in neutral lipid synthesis. *Saccharomyces cerevisiae* is an appropriate model to study plant DGAT activity as this strain is capable of synthesizing diacylglycerols (DAGs) formed by long-chain saturated fatty acids, similar to those found in vegetable oils (King et al., 2007). The potential dual WS/DGAT activity of the sunflower enzymes was studied using different positive and negative controls. As positive controls, the *dgat1A* gene from sunflower (GenBank EU543809.1, previously cloned by our group) was cloned into the pYES2 plasmid and expressed in the H1246 strain, and the wild-type W303-1A strain expressing the empty pYES2 plasmid were used. The *dgat1A* gene encodes a DGAT involved in TAG synthesis in sunflower seeds. As a negative control, the H1246 strain expressing the empty pYES2 plasmid was used (see Supplementary Table S5 for all the constructs and strains used). The proteins were expressed heterologously, and the impact of HaWS8 and HaWS11 on neutral lipid formation in the H1246 strain was analysed. After protein induction, and the extraction and separation of neutral lipids, the TAGs, DAGs and free fatty acids (FFAs) in the samples were analysed by HPLC. The expression of sunflower HaWSs did not restore TAG synthesis in the mutant yeast strain as TAG formation was only observed in the positive controls (Supplementary Fig. S6). There was a significant accumulation of FFAs and DAGs in both the negative control and in the strains expressing HaWS6, HaWS8 and HaWS11, confirming that the H1246 strain synthesizes the necessary precursors for TAG synthesis. Hence, the enzymes studied apparently lack DGAT activity *in vivo*. This was consistent with the activity of the previously characterized HaWS2 (Zhang et al., 2022) and there are other examples of WSs lacking this activity, although they are related to the bifunctional WSD family, such as the plant PhWS1 (King et al., 2007), EgDGAT/WS.1 (Yuan et al., 2020), or the bacterial WS proteins like MaWSD5 from *Marinobacter aquaeolei* (Vollheyde et al., 2020) or MhWS2 from *Marinobacter hydrocarbonoclasticus* (Miklaszewska et al., 2018). Of the WS enzymes from plants belonging to this family, only AtWSD1 from *Arabidopsis thaliana* has dual WS and DGAT activity, although DGAT activity was only detected *in vitro* (Li et al., 2008).

To reconstruct the complete metabolic pathway for sunflower wax synthesis, a HaFAR and HaWS were co-expressed heterologously in the *Saccharomyces cerevisiae* eukaryotic expression system. The HaFAR5 and HaWS8 genes were selected for this co-expression based on their activity

demonstrated *in vivo* and *in vitro*. HaFAR5 produced the largest amount of fatty alcohols when heterologously expressed in the wild-type yeast strain (DeAndrés-Gil et al., 2024), whereas HaWS8 had the strongest activity *in vitro* relative to HaWS6 and HaWS11 when yeast microsomal fractions were studied. Heterologous expression was carried out by cloning the HaFAR5 and HaWS8 coding fragments into the pYES3 and pYES2 expression vectors, respectively, both driven by the inducible GAL1 promoter. The wild-type yeast strain W303-1A was used, with a genotype suitable to use the two auxotrophies necessary for the selection of the pYES2 (-Ura) and pYES3 (-Trp) vectors. As a control, the W303-1A strain expressing both empty vectors was used to verify if the yeast produced waxes endogenously. Moreover, a second control was used in which only HaFAR5 was expressed, enabling the specific contribution of HaFAR5 to wax synthesis in yeast to be assessed. Once the proteins of interest were expressed, the waxes produced were analysed and compared with the control lines.

Heterologous expression of HaFAR5 (DeAndrés-Gil et al., 2024) and HaWS8 in the W303-1A strain led to the production of wax species ranging from 32 to 46 carbons in length (Fig. 7), significantly different to the empty control in all cases. HaFAR5 expression increased the WE content, although not significantly compared to the empty plasmid (Fig. 7). This may be due to the presence of non-specific acyltransferases

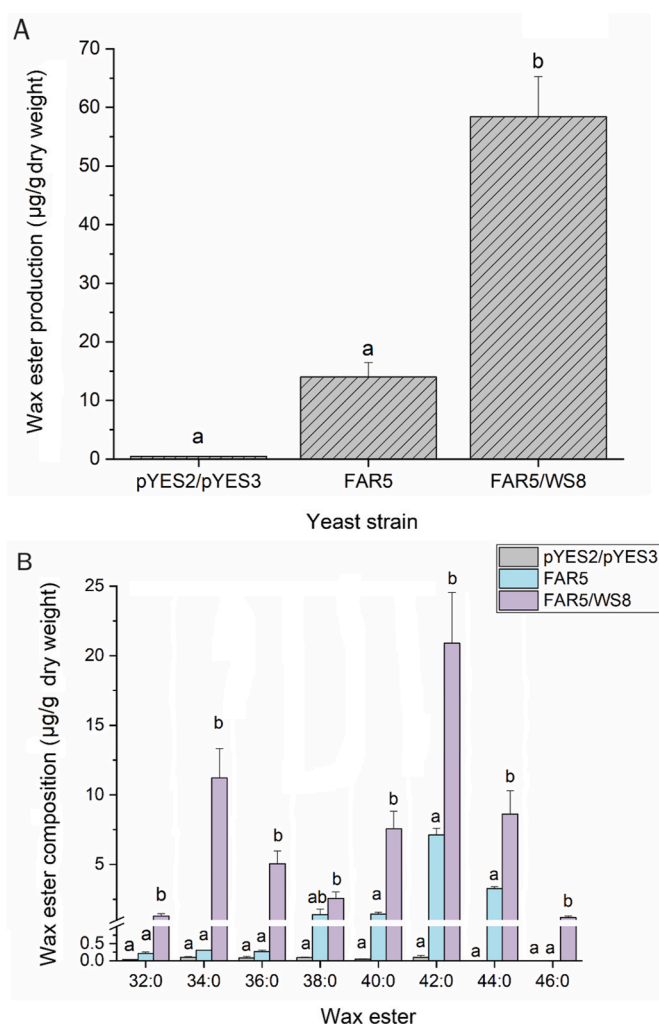


Fig. 7. Analysis of the waxes produced in the wild-type W303-1A yeast strain expressing HaFAR5 and HaWS8 under the control of the GAL1 promoter. Empty pYES2 and pYES3 vectors were used as a negative control. A second control expressing HaFAR5 was also assessed. (A) Quantification of the waxes produced in µg/g dry weight. (B) Changes in the wax species produced (µg/g dry weight). The letters "a" and "b" represent statistical clustering at $p = 0.05$ significance.

that can catalyse esterification between the acyl chains of acyl-CoA molecules and alcohols. Enzymes like ATF1 and ATF2 have been identified in *Saccharomyces cerevisiae*, alcohol acetyltransferases capable of synthesizing acetate esters from acetyl-CoA and different short-chain alcohols (Nancolas et al., 2017). *Saccharomyces cerevisiae* also possesses acyl-CoA:ethanol acyltransferases, such as the EHT1 and EEB1 enzymes that contribute to the synthesis of ethyl esters from short- or medium-chain acyl-CoA molecules and ethanol (Saerens et al., 2006). Since *Saccharomyces cerevisiae* is unable to synthesize long-chain fatty alcohols endogenously as substrates (Shi et al., 2012), heterologous expression with the empty control did not lead to the identification of waxes longer than C32 in length. However, by heterologously expressing the sunflower *HaFAR5* reductase, this enzyme can catalyse the production of the long-chain alcohol substrates necessary for the alcohol acyltransferases of the W303-1A strain, and waxes could be formed if its broad substrate specificity is considered. Nevertheless, co-expressing *HaWS8* along with *HaFAR5* quadrupled wax production relative to that produced by *HaFAR5* alone (Fig. 7A). Regarding the composition, significant differences were also observed in the production of all the wax species identified, except for C38:0 (Fig. 7B). The results obtained are consistent with the predominant wax species in sunflower seeds, saturated 42:0, 44:0 and 46:0 species (Garcés et al., 2023), suggesting that the co-expression of *HaFAR5* and *HaWS8* is capable of reconstituting at least partially the wax synthesis pathway in sunflower seeds. This could indicate that the waxes present in sunflower seeds are largely synthesized by the *HaFAR5* and *HaWS8* enzymes, without excluding the involvement of other enzymes like *HaFAR3* and *HaWS6*.

4. Conclusions

In the present work we have investigated which WSs were involved in the synthesis of the waxes present in the hull of sunflower seeds. Once the different WS sequences in the sunflower genome were identified, the *HaWS6*, *HaWS8* and *HaWS11* were seen to be expressed in seeds, and very strong *HaWS8* expression in the developing sunflower hulls was confirmed. These enzymes were cloned and their structure modelled to understand which features might affect their activity. Fluorescent protein labelling studies also indicated a probable ER localization and when characterised functionally in yeast, their activity was evident in the microsomal fraction *in vitro*. The substrate specificity of the three enzymes was very similar with a very broad profile for all the alcohols tested, including unsaturated and saturated ones. In the case of acyl-CoA, the enzymes were only active with saturated substrates. The enzymes did not display DGAT activity and in the case of *HaWS8*, it could produce WEs in yeast similar to those of the sunflower seed when expressed with the *FAR5* of that species, reinforcing the initial hypothesis that they are the enzymes responsible for the biosynthesis of sunflower seed WEs.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

This work was funded by Spanish Ministerio de Ciencia e Innovación through the grants AGL2017 83449R and PID2020–113134RB-I00/MCIN/AEI/10.13039/501100011033, and the CSIC Project I-LINK 2021 Ref. LINKB20078.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.plaphy.2025.109692>.

Data availability

Data will be made available on request.

References

- Broughton, R., Ruiz-Lopez, N., Hassall, K.L., Martínez-Force, E., Garcés, R., Salas, J.J., Beaudoin, F., 2018. New insights in the composition of wax and sterol esters in common and mutant sunflower oils revealed by ESI-MS/MS. *Food Chem.* 269, 70–79. <https://doi.org/10.1016/j.foodchem.2018.06.135>.
- Barney, B.M., Wahlen, B.D., Garner, E.L., Wei, J., Seefeldt, L.C., 2012. Differences in substrate specificities of five bacterial wax ester synthases. *Appl. Environ. Microbiol.* 78 (16), 5734–5745. <https://doi.org/10.1128/AEM.00534-12>.
- Buglino, J., Onwueme, K.C., Ferreras, J.A., Quadri, L.E.N., Lima, C.D., 2004. Crystal structure of PapA5, a phthiocerol dimycocerosyl transferase from *Mycobacterium tuberculosis*. *J. Biol. Chem.* 279 (29), 30634–30642. <https://doi.org/10.1074/jbc.M404011200>.
- Cheng, J.B., Russell, D.W., 2004. Mammalian wax biosynthesis. II. Expression cloning of wax synthase cDNAs encoding a member of the acyltransferase gene family. *J. Biol. Chem.* 279 (36), 37798–37807. <https://doi.org/10.1074/jbc.M406226200>.
- DeAndrés-Gil, C., Moreno-Pérez, A.J., Villoslada-Valbuena, M., Halsey, K., Martínez-Force, E., Garcés, R., Kurup, S., Beaudoin, F., Salas, J.J., Venegas-Calderón, M., 2024. Characterisation of fatty acyl reductases of sunflower (*Helianthus annuus* L.) seed. *Plant Sci.* 341, 111992. <https://doi.org/10.1016/j.plantsci.2024.111992>.
- Dijkstra, A.J., 2015. Oil refining. In: Martínez-Force, E., Salas, J.J., Dunford, N.T. (Eds.), *Sunflower: Chemistry, Production, Processing, and Utilization*. AOCS Press, pp. 227–258. <https://doi.org/10.1016/B978-1-893997-94-3.50015-5>.
- Domergue, F., Miklaszewska, M., 2022. The production of wax esters in transgenic plants: towards a sustainable source of bio-lubricants. *J. Exp. Bot.* 73 (9), 2817–2834. <https://doi.org/10.1093/jxb/era046>.
- Garcés, R., de Andrés-Gil, C., Venegas-Calderón, M., Martínez-Force, E., Moreno-Pérez, A.J., Salas, J.J., 2023. Characterization of sunflower seed and oil wax ester composition by GC/MS, a final evaluation. *Lwt* 173, 114365. <https://doi.org/10.1016/j.lwt.2022.114365>.
- González-Mellado, D., Salas, J.J., Venegas-Calderón, M., Moreno-Pérez, A.J., Garcés, R., Martínez-Force, E., 2019. Functional characterization and structural modelling of *Helianthus annuus* (sunflower) ketoacyl-CoA synthases and their role in seed oil composition. *Planta* 249 (6), 1823–1836. <https://doi.org/10.1007/s00425-019-03126-1>.
- Kalscheuer, R., Steinbüchel, A., 2003. A novel bifunctional wax ester synthase/acyl-CoA: Diacylglycerol acyltransferase mediates wax ester and triacylglycerol biosynthesis in *Acinetobacter calcoaceticus* ADP1. *J. Biol. Chem.* 278 (10), 8075–8082. <https://doi.org/10.1074/jbc.M210533200>.
- Kalscheuer, R., Luftmann, H., Steinbüchel, A., 2004. Synthesis of novel lipids in *Saccharomyces cerevisiae* by heterologous expression of an unspecific bacterial acyltransferase. *Appl. Environ. Microbiol.* 70 (12), 7119–7125. <https://doi.org/10.1128/AEM.70.12.7119-7125.2004>.
- King, A., Jixiang, J.N., Hilliard, J., Jaworski, J.G., 2007. Cuticular wax biosynthesis in *petunia* petals: cloning and characterization of an alcohol-acyltransferase that synthesizes wax-esters. *Planta* 381–394. <https://doi.org/10.1007/s00425-007-0489-z>.
- Kunst, L., Samuels, A.L., 2003. Biosynthesis and secretion of plant cuticular wax. *Prog. Lipid Res.* 42 (1), 51–80. [https://doi.org/10.1016/S0163-7827\(02\)00045-0](https://doi.org/10.1016/S0163-7827(02)00045-0), 2003.
- Kawakami, T., Strakosh, S.C., Zhen, Y., Ungerer, M.C., 2010. Different scales of Ty1/copia-like retrotransposon proliferation in the genomes of three diploid hybrid sunflower species. *Heredity* 104 (4), 341–350. <https://doi.org/10.1038/hdy.2009.182>.
- Lardizabal, K.D., Metz, J.G., Sakamoto, T., Hutton, W.C., Pollard, M.R., Lassner, M.W., 2000. Purification of a jojoba embryo wax synthase, cloning of its cDNA, and production of high levels of wax in seeds of transgenic *Arabidopsis*. *Plant Physiol.* 122 (3), 645–655. <https://doi.org/10.1104/pp.122.3.645>.
- Li, F., Wu, X., Lam, P., Bird, D., Zheng, H., Samuels, L., Jetter, R., Kunst, L., 2008. Identification of the wax ester synthase/acyl-coenzyme A:diacylglycerol acyltransferase WSD1 required for stem wax ester biosynthesis in *Arabidopsis*. *Plant Physiol.* 148 (1), 97–107. <https://doi.org/10.1104/pp.108.123471>.
- Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2[−]ΔΔCT method. *Methods* 25 (4), 402–408.
- Mapelli-Brahm, A., Sánchez, R., Pan, X., Moreno-Pérez, A.J., Garcés, R., Martínez-Force, E., Weselake, R.J., Salas, J.J., Venegas-Calderón, M., 2020. Functional characterization of lysophosphatidylcholine: acyl-CoA acyltransferase genes from sunflower (*Helianthus annuus* L.). *Front. Plant Sci.* 11, 529568.
- Miklaszewska, M., Kawiński, A., Banaś, A., 2013. Detailed characterization of the substrate specificity of mouse wax synthase. *Acta Biochim. Pol.* 60 (2), 209–215. <https://doi.org/10.18388/abp.2013.1973>.
- Miklaszewska, M., Dittrich-Domergue, F., Banaś, A., Domergue, F., 2018. Wax synthase MhWS2 from *Marinobacter hydrocarbonoclasticus*: substrate specificity and biotechnological potential for wax ester production. *Appl. Microbiol. Biotechnol.* 102 (9), 4063–4074. <https://doi.org/10.1007/s00253-018-8878-8>.
- Nancolas, B., Bull, I.D., Stenner, R., Dufour, V., Cumow, P., 2017. *Saccharomyces cerevisiae* Atf1p is an alcohol acetyltransferase and a thioesterase *in vitro*. *Yeast* 34 (6), 239–251. <https://doi.org/10.1002/yea.3229>.
- Patwari, P., Salewski, V., Gutbrod, K., Kreszies, T., Dresen-Scholz, B., Peisker, H., Steiner, U., Meyer, A.J., Schreiber, L., Dörmann, P., 2019. Surface wax esters

- contribute to drought tolerance in *Arabidopsis*. *Plant J.* 98 (4), 727–744. <https://doi.org/10.1111/tpj.14269>.
- Paysan-Lafosse, T., Blum, M., Chuguransky, S., Grego, T., Beatriz, L., Salazar, G.A., Bileschi, M.L., Bork, P., Bridge, A., Colwell, L., Gough, J., Haft, D.H., Letuni, I., Marchler-bauer, A., Mi, H., Natale, D.A., Orengo, C.A., Pandurangan, A.P., Rivoire, C., et al., 2023. InterPro in 2022. *Nucleic Acids Res.* 51 (D1), D418–D427. <https://doi.org/10.1093/nar/gkac993>.
- Petronikolou, N., Nair, S.K., 2019. Structural and biochemical studies of a biocatalyst for the enzymatic production of wax esters. *ACS catalysis* 8 (7), 6334–6344. <https://doi.org/10.1021/acscatal.8b00787>.
- Röttig, A., Steinbüchel, A., 2013. Acyltransferases in bacteria. *Microbiol. Mol. Biol. Rev.* 77 (2), 277–321. <https://doi.org/10.1128/mmb.00010-13>.
- Rowland, O., Domergue, F., 2012. Plant fatty acyl reductases: enzymes generating fatty alcohols for protective layers with potential for industrial applications. *Plant Sci.* 193–194, 28–38. <https://doi.org/10.1016/j.plantsci.2012.05.002>.
- Rosli, R., Chan, P.L., Chan, K.L., Amiruddin, N., Low, E.T.L., Singh, R., Harwood, J.L., Murphy, D.J., 2018. In silico characterization and expression profiling of the diacylglycerol acyltransferase gene family (DGAT1, DGAT2, DGAT3 and WS/DGAT) from oil palm, *Elaeis guineensis*. *Plant Sci.* 275, 84–96. <https://doi.org/10.1016/j.plantsci.2018.07.011>.
- Saerens, S.M.G., Verstrepen, K.J., Van Laere, S.D.M., Voet, A.R.D., Van Dijck, P., Delvaux, F.R., Thevelein, J.M., 2006. The *Saccharomyces cerevisiae* EHT1 and EEB1 genes encode novel enzymes with medium-chain fatty acid ethyl ester synthesis and hydrolysis capacity. *J. Biol. Chem.* 281 (7), 4446–4456. <https://doi.org/10.1074/jbc.M512028200>.
- Schreiber, L., Hartmann, K., Skrabs, M., Zeier, J., 1999. Apoplastic barriers in roots: chemical composition of endodermal and hypodermal cell walls. *J. Exp. Bot.* 50 (337), 1267–1280.
- Shalini, T., Martin, A., 2020. Identification, isolation, and heterologous expression of sunflower wax synthase for the synthesis of tailored wax esters. *J. Food Biochem.* 44 (10), e13433. <https://doi.org/10.1111/jfbc.13433>.
- Shi, S., Octavio Valle-Rodríguez, J., Khoomrung, S., Siewers, V., Nielsen, J., 2012. Functional expression and characterization of five wax ester synthases in *Saccharomyces cerevisiae* and their utility for biodiesel production. *Biotechnol. Biofuels* 5 (1), 1–10. <https://doi.org/10.1186/preaccept-1932279820621895>.
- Stern, D.L., 2013. The genetic causes of convergent evolution. *Nat. Rev. Genet.* 14 (11), 751–764. <https://doi.org/10.1038/nrg3483>.
- Stöveken, T., Kalscheuer, R., Malkus, U., Reichelt, R., Steinbüchel, A., 2005. The wax ester synthase/acyl coenzyme A:diacylglycerol acyltransferase from *Acinetobacter* sp. strain ADP1: characterization of a novel type of acyltransferase. *J. Bacteriol.* 187 (4), 1369–1376. <https://doi.org/10.1128/JB.187.4.1369-1376.2005>.
- Suh, M.C., Samuels, A.L., Jetter, R., Kunst, L., Pollard, M., Ohlrogge, J., Beisson, F., 2005. Cuticular lipid composition, surface structure, and gene expression in *Arabidopsis* stem epidermis. *Plant Physiol.* 139 (4), 1649–1665. <https://doi.org/10.1104/pp.105.070805.1>.
- Villa, J.A., Cabezas, M., 2014. Use of limited proteolysis and mutagenesis to identify folding domains and sequence motifs critical for wax ester synthase/acyl coenzyme A:diacylglycerol acyltransferase activity. *Appl. Environ. Microbiol.* 80 (3), 1132–1141. <https://doi.org/10.1128/AEM.03433-13>.
- Vollheyde, K., Yu, D., Hornung, E., Herrfurth, C., Feussner, I., 2020. The fifth WS/DGAT enzyme of the bacterium *Marinobacter aquaeolei* VT8. *Lipids* 55 (5), 479–494. <https://doi.org/10.1002/lipd.12250>.
- 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 (2), 640–651. <https://doi.org/10.1104/pp.113.217836>.
- Wang, M., Wang, Y., Wu, H., Xu, J., Li, T., Hegebarth, D., Jetter, R., Chen, L., Wang, Z., 2016. Three TaFAR genes function in the biosynthesis of primary alcohols and the response to abiotic stresses in *Triticum aestivum*. *Sci. Rep.* 6 (1), 1–14. <https://doi.org/10.1038/srep25008>.
- Yuan, Y., Arondel, V., Domergue, F., 2020. Characterization and heterologous expression of three DGATs from oil palm (*Elaeis guineensis*) mesocarp in *Saccharomyces cerevisiae*. *Biochimie* 169, 18–28. <https://doi.org/10.1016/j.biochi.2019.09.010>.
- Zhang, C., Yang, J., Meng, W., Zeng, L., Sun, L., 2022. Genome-wide analysis of the WSD family in sunflower and functional identification of HaWSD9 involvement in wax ester biosynthesis and osmotic stress. *Front. Plant Sci.* 13, 975853. <https://doi.org/10.3389/fpls.2022.975853>.