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Functional characterization and expression of GASCL1 and GASCL2, two anther-specific chalcone synthase like enzymes from *Gerbera hybrida*

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Highlights

- Two anther-specific chalcone synthases (ASCLs) from *Gerbera hybrida* were characterized
- GASCL1 and GASCL2 are tri- and tetraketidesynthases that utilize long chain acyl-CoA starters
- *GASCL1* and *GASCL2* have tapetum localized expression
- This study extends the well-characterized type III polyketide synthase family from *Gerbera hybrida* to two members that are very distinct from chalcone synthase

Abstract

The chalcone synthase superfamily consists of type III polyketidesynthases (PKSs), enzymes responsible for producing plant secondary metabolites with various biological and pharmacological activities. Anther-specific chalcone synthase-like enzymes (ASCLs) represent an ancient group of type III PKSs involved in the biosynthesis of sporopollenin, the main component of the exine layer of moss spores and mature pollen grains of seed plants. In the latter, ASCL proteins are localized in the tapetal cells of the anther where they participate in sporopollenin biosynthesis and exine formation within the locule. It is thought that the enzymes responsible for sporopollenin biosynthesis are highly conserved, and thus far, each angiosperm species with a genome sequenced has possessed two *ASCL* genes, which in *Arabidopsis thaliana* are *PKSA* and *PKSB*. The *Gerbera hybrida* (gerbera) PKS protein family consists of three chalcone synthases (GCHS1, GCHS3 and GCHS4) and three 2-pyrone synthases (G2PS1, G2PS2 and G2PS3). In previous

studies we have demonstrated the functions of chalcone synthases in flavonoid biosynthesis, and the involvement of 2-pyrone synthases in the biosynthesis of antimicrobial compounds found in gerbera. In this study we expanded the gerbera PKS-family by functionally characterizing two gerbera ASCL proteins. *In vitro* enzymatic studies using purified recombinant proteins showed that both GASCL1 and GASCL2 were able to use medium and long-chain acyl-CoA starters and perform two to three condensation reactions of malonyl-CoA to produce tri- and tetraketide 2-pyrones, usually referred to as alpha-pyrones in sporopollenin literature. Both *GASCL1* and *GASCL2* genes were expressed only in floral organs, with most expression observed in anthers. In the anthers, transcripts of both genes showed strict tapetum-specific localization.

Keywords: *Gerbera hybrida*; Asteraceae; type III polyketide synthase; ASCL; sporopollenin

1. Introduction

The surface of the pollen grain can be divided into three separate layers. The outer layer is called exine, a multilayered complex structure made up of a chemically resistant biopolymer called sporopollenin. The inner layer, intine, is mostly composed of cellulose. Pollen grains are also covered by a pollen coat, composed of lipids, proteins, pigments and aromatic compounds, which fill the cavities of exine. The exine gives protection against pathogen attack, dehydration, UV irradiation and plays a role in pollination by interacting with the female stigmatic cells. Based on present understanding, the components of the exine are synthesized by the surrounding sporophytic tapetal cells and transferred on the surface of developing microspores within the locule (Ariizumi and Toriyama, 2011; Edlund et al., 2004; Quilichini et al., 2015). Due to its high resistance to chemical and physical degradation, the exact structure of sporopollenin has been difficult to resolve, but phenolics and fatty acids have been suggested to be likely components (Ahlers et al., 1999; Guilford et al., 1988). The biosynthesis of sporopollenin has also remained unclear. Recent studies have identified the involvement of fatty acid biosynthesis-related genes in sporopollenin biosynthesis (Chen et al., 2011; de Azevedo Souza et al., 2009; Morant et al., 2007). Two independent research groups identified two enzymes similar to chalcone synthases involved in sporopollenin biosynthesis in *Arabidopsis*, and their physiological role in sporopollenin biosynthesis was shown through mutation studies. The enzymes were named PKSA and PKSB. Single mutations in the genes encoding these proteins led to abnormal exine patterning, and the double mutation to male sterility (Dobritsa et al., 2010; Kim et al., 2010; Mizuuchi et al., 2008).

The chalcone synthase superfamily consists of type III polyketidesynthases (PKSs), enzymes responsible for producing structurally different and biologically active plant secondary metabolites having roles in antimicrobial defense, UV photoprotection, flower pigmentation, and pollen fertility (Austin and Noel, 2003; Kim et al., 2010). Type III PKS enzymes are abundant in vascular plants but they have also been characterized from the moss *Physcomitrella patens* (Kim et al., 2013). Type III plant polyketide synthases are relatively small (40-45 kDa) homodimeric proteins that catalyze the sequential decarboxylative condensation reaction between malonyl-CoA and a variety of CoA-linked starter molecules. Starter molecules may vary from short to long chain aliphatic moieties, but also aromatic molecules such as *p*-coumaroyl-CoA are used. PKSs that are able to utilize methylmalonyl- or ethylmalonyl-CoA as an extender molecule in addition to malonyl-CoA have also been characterized (Jez et al., 2000; Schröder et al., 1998). The polyketide reaction is initiated by a starter molecule loading on to the active-site Cys, which is followed by

malonyl-CoA decarboxylation, polyketide chain elongation, and in many cases enzymatically catalyzed cyclization of the polyketide intermediate (Morita et al., 2007). The best characterized type III polyketide synthase is chalcone synthase (CHS), which catalyzes the first step in the biosynthesis of plant flavonoids. CHS is a tetraketide synthase that uses *p*-coumaroyl-CoA as a starter substrate in a condensation reaction with three keto units from malonyl-CoA to form naringenin chalcone (Ferrer et al., 1999).

Genes encoding enzymes with sequence similarity to CHS and shown to be expressed in the tapetum cells of the anther have been isolated from *Brassica napus* (Shen and Hsu, 1992), *Oryza sativa* (Hihara et al., 1996), *Nicotiana sylvestris* (Atanassov et al., 1998) and *Silene latifolia* (Barbacar et al., 1997). Atanassov et al. (1998) showed that the anther-specific CHS-like enzymes belong to a separate subfamily of plant type III PKSs. They relate to CHS in gene and amino acid sequence and catalytic mechanism, but have a different substrate preference and are often referred to as anther-specific CHS-like enzymes (ASCLs). Mizuuchi and coworkers (2008) characterized two Arabidopsis ASCLs, PKSA and PKSB, which accepted unusually long (up to C₂₀ chain length) fatty acyl CoAs as starter substrates and catalyzed the sequential condensation reaction of a starter-CoA and malonyl-CoA to produce alpha-pyrone *in vitro*. Nishikawa et al. (2005) found Arabidopsis mutants with reduced pollen-stigma adhesion (*less adhesive pollen, lap*) and discovered that the exine patterning was altered, resulting in structurally weakened pollen grains that easily collapsed in these lines. Dobritsa et al. (2010) mapped *LAP6* and *LAP5* to two CHS-like genes, *PKSA* and *PKSB*. At the same time, Kim et al. (2010) identified a role for *LAP6/PKSA* and *LAP5/PKSB* in sporopollenin biosynthesis, initially through coexpression with *ACOS5*, a key gene in the process. On the basis of these results and work done with fatty-acyl metabolism enzymes, a model for sporopollenin biosynthesis in Arabidopsis was established (Grienemberger et al., 2010; Quilichini et al., 2015). The model proposes that in a reaction sequence localized to the ER, ASCLs work together with cytochrome P450-enzymes (Morant et al., 2007), fatty acyl-CoA synthetase (ACOS) (de Azevedo Souza et al., 2009), and tetraketide alpha-pyrone reductases (Grienemberger et al., 2010) to produce polyhydroxylated tetraketide products incorporated directly or after modifications into the sporopollenin polymer. The polyhydroxylation of the polyketide has been suggested to provide multiple places for cross-linking the polyketide product and other potential sporopollenin components such as fatty acids and fatty alcohols by ester and ether bonds. This has been used to explain the cross-linked nature of sporopollenin and its resistance to chemical and physical degradation (Kim et al., 2010; Scott et al., 2004). It has also been suggested that sporopollenin biosynthetic enzymes could work as a metabolon (Lallemant et al., 2014).

Gerbera hybrida is a commercially important ornamental plant species that is a member of the sunflower family, Asteraceae. Gerbera has been used as a model plant for flower and inflorescence development (Broholm et al., 2008; Juntheikki-Palovaara et al., 2014; Tähtiharju et al., 2012; Zhao et al., 2016) and type III PKS research. We have previously shown that gerbera contains three chalcone synthases involved in the flavonoid pathway (Deng et al., 2014; Helariutta et al., 1995). Additionally it contains a triketide synthase G2PS1 that is responsible for the biosynthesis of triacetolactone (4-hydroxy-6-methyl-2-pyrone), a putative precursor for antimicrobial compounds, the aglycones of gerberin (5,6-dihydro-4-hydroxy-6-methyl-2-pyrone) and parasorboside (tetrahydro-4-hydroxy-6-methyl-2-pyrone) (Eckermann et al., 1998; Koskela et al., 2011). Recently we expanded the number of known gerbera PKS family proteins by showing

that the genome encodes two pentaketide synthases, G2PS2 and G2PS3, which are involved in the biosynthesis of 4-hydroxy-5-methylcoumarin, another antimicrobial compound in gerbera (Pietiäinen et al., 2016). In the present study, we further extend the gerbera PKS protein family by functionally characterizing two proteins, GASCL1 and GASCL2, orthologous to Arabidopsis PKSA and PKSB, as part of the growing number of ASCL proteins that form a family believed to be functionally and evolutionally conserved in land plants (Quilichini et al., 2015; Wang et al., 2013). Interestingly, while the first sequenced gymnosperm genomes (Nystedt et al. 2013; Neale et al., 2014) contain a single ASCL gene, all angiosperms with a genome sequenced possess these two genes for ASCL proteins. The proteins can be divided into two separate groups based on whether they possess either Ala or Val at position 220/214 (Arabidopsis numbering). All ASCLs can be identified as either Ala-clade or Val-clade proteins (Jepson et al., 2014). Gerbera ASCL-genes share 72-73 % sequence similarity with the Arabidopsis PKSA and PKSB genes and 77-79 % amino acid sequence identity with their encoded proteins. Phylogenetically, the genes are part of the ASCL-gene family, and GASCL1 belongs to the Ala-, and GASCL2 to the Val-subclade of ASCLs. The GASCL1 and GASCL2 genes are specifically expressed in the tapetal cells of the anther. *In vitro* enzyme assays with recombinant GASCL1 and GASCL2 proteins showed that they are able to use middle and long chain aliphatic acyl-CoA starters and make two to three condensation reactions with malonyl-CoA to produce tri- and tetraketide alpha-pyrone products.

2. Results and discussion

2.1 Analysis and isolation of gerbera ASCL-genes

RNA-sequencing of gerbera anther libraries indicated that anthers express two CHS-like genes that were not previously characterized. GASCL1 was obtained in full length; it contained a 1173 nucleotide long open reading frame (ORF) and showed 77 % sequence identity with Arabidopsis PKSA at the deduced amino acid level. GASCL2 lacked 59 nucleotides from the 5' of the gene. Using genome walking we were able to sequence the missing 5' end of GASCL2 and generate a 1167 nucleotide long ORF that shared 79 % sequence identity, at the encoded amino acid level, with the Arabidopsis PKSB protein. A phylogenetic tree based on known or putative ASCL nucleotide sequences (Fig. 1) showed that both genes are part of the ASCL gene family, separate from the plant non-ASCL type III PKSs. An amino acid alignment (Fig. S1) showed that GASCL1 has the amino acid Ala at the position 218, similar to PKSA (at position 220), and therefore belongs to the Ala-clade of ASCLs. GASCL2, on the other hand, belongs to the Val-clade proteins together with PKSB, as described by Jepson and coworkers (2014). A similar Ala-Val separation into two different sister-clades was also observed in the phylogenetic tree using DNA sequences.

2.2 *In vitro* enzyme activity of GASCL1 and GASCL2

Recombinant GASCL1 and GASCL2 proteins with N-terminal His-tags were produced in *E.coli* in a soluble form, purified using Ni-affinity chromatography and visualized in SDS-PAGE (Fig. 2). The enzymatic activity of recombinant GASCL1 and GASCL2 were tested using *in vitro* assays. When acetyl-CoA was used in the assays, only GASCL1 was able to make a product, the triketide triacetolactone (4-hydroxy-6-methyl-2-pyrone) (Table1, compound 1), based on retention time and the mass of the reference compound. GASCL2 did not show any activity with acetyl-CoA. This result is similar to that reported by Mizuuchi et al. (2008), who found that PKSA from Arabidopsis,

but not PKSB, was able to utilize short-chain CoA starters. Neither GASCL1 nor GASCL2 accepted *p*-coumaroyl-CoA or benzoyl-CoA as starters. Both enzymes accepted hexanoyl- or octanoyl-CoA as substrates, and single major peaks were observed in chromatograms. Other smaller peaks were observed in the mass spectra (Fig. S2), which appeared to be due to loss of CO₂ ([M-H-44]⁻) from the parent ion, indicating the presence of an alpha-pyrone ring. Based on the mass, elemental composition, fragmentation pattern and the enzyme activity of other known ASCLs (Jepson et al., 2014; Mizuuchi et al., 2008) the products were characterized as alpha-pyrone triketides (Table 1, compounds 2 and 3).

When stearoyl-CoA (C₁₈) was used in the assays, two peaks were observed in the chromatogram (Fig. 3A) with retention times of 8.39 min (m/z 391) and 8.94 min (m/z 349); these were not observed in the empty vector control (Fig. 3B). A similar loss of CO₂ ([M-H-44]⁻) from the parent ion was observed in the mass spectra of both products (Fig. 3C and 3D). Based on the mass, elemental composition, loss of CO₂ from the parent ion, and the results by Kim et al. (2010) who observed exactly the same m/z values in the products when C₁₈-CoA was used, the products were identified as triketide- (4) and tetraketide (5) alpha-pyrones. The triketide product (relative peak area 7159) was the main product observed in the MS-analysis compared to the tetraketide (relative peak area 52). Also, Jepson et al. (2014) observed that the ASCL from *Hypericum perforatum*, HpPKS1, was able to synthesize a tetraketide product when the carbon number was extended to C₁₄-CoA or C₁₈-CoA.

All known and characterized plant ASCL enzymes are able to synthesize tri- and tetraketide products from hydroxylated medium chain starters (e.g. 12-OH-C₁₈-CoA and C₁₈-CoA) and have a stronger preference for them over the analogous unsubstituted substrates. Therefore, ASCL enzymes are called hydroxyalkylpyrone synthases (Jepson et al., 2014; Quilichini et al., 2015). Unfortunately, these compounds were not available for this study. Grienemberger et al. (2010) characterized two tapetum-specific alpha-pyrone tetraketidereductases from Arabidopsis that reduce the carbonyl function of the tetraketide alpha-pyrone but do not act on the triketides synthesized by PKSA or PKSB. This suggests that the tetraketide is the physiological product, although the triketide product was the main compound produced in our experiments and also in other *in vitro* studies (Jepson et al., 2014; Kim et al., 2010). Our results suggest that a long chain starter would be the physiological substrate in gerbera, since both GASCL1 and GASCL2 produced the tetraketide from stearoyl-CoA only, but not from the shorter-chain CoAs.

2.3 Expression studies and *in situ* hybridization analysis

The expression profiles of *GASCL1* and *GASCL2* genes were tested in various gerbera organs, including a pooled anther sample from the early stages of anther development, as in Helariutta et al. (1993). The qPCR results showed that expression was low (in relation to *GGAPDH*) in all plant parts (Fig. 4). The highest expression was detected in flower organs, where it was strongest in anthers. Practically no expression was detected in the inflorescence stem (scape), leaves or roots. Kim et al. (2010) observed that *PKSB* was expressed exclusively in flowers, while *PKSA* had some expression in leaves, roots and stems. Little is known about the role of ASCLs outside non-anther tissues. Jepson et al. (2014) demonstrated that the expression of HpPKS1 from *Hypericum perforatum* was upregulated by abscisic acid (ABA) and downregulated by jasmonic acid (JA) and wounding in leaves. Based on these observations, they suggested that HpPKS1 and other Ala-clade ASCLs could be involved in the biosynthesis of minor cell wall components in non-anther tissues. Based on our observations, *GASCL1* and *GASCL2* are flower-specific, with highest expression

observed in anthers. Most likely they do not play any role in leaves since hardly any expression was observed there.

The *in situ* hybridization method was used to see if tapetum-specific expression could be observed as has been described in Arabidopsis (Kim et al., 2010). Gerbera anthers were collected for *in situ* studies and gene-specific antisense and sense probes were used. Both *GASCL1* and *GASCL2* antisense probes showed strong hybridization signal exclusively in the tapetum (Fig. 5). The sense probes did not give any hybridization signal.

The small proportion of tapetal cells in the anther tissue explains why transcript levels in whole anthers are quite low. Tapetum is the innermost sporophytic layer of the anther and offers both lipidic and phenylpropanoid precursors for the developing microspores. Based on current knowledge, the components of the exine are synthesized in these surrounding sporophytic tapetal cells and transferred to the surface of developing microspores within the locule in Arabidopsis (Ariizumi and Toriyama, 2011; Quilichini et al., 2015; Wang et al., 2013). In the tapetal cells, the synthesized and reduced, esterified fatty acids yield CoAs. CoA esters are further processed by PKSs and reductases. The resulting polyhydroxylated tetraketide compounds are then exported from the tapetal cells by the ABCG26 transporter and/or LTPs to the developing microspores for polymerization and for further processing by unknown enzymes (Grienenberger et al., 2010; Quilichini et al., 2014; Quilichini et al., 2015). Our results on gerbera are in line with the idea that ASCLs catalyze biosynthetic reactions in the tapetum, where they take part in the metabolic pathway leading to sporopollenin biosynthesis.

4. Conclusions

The pollen cell wall's resistance to abiotic and biotic stresses has played a crucial role when plants invaded land (Blackmore et al., 2007). The enzymes responsible for the biosynthesis of sporopollenin are believed to be conserved in land plants. ASCLs form an ancient group of type III polyketide synthases that are involved in the biosynthesis of sporopollenin. Thus far there have been only four vascular plant species in which ASCLs have been functionally characterized (Jepson et al., 2014; Quilichini et al., 2015). In this study we examined two ASCLs from gerbera. Both *GASCL1* and *GASCL2* genes were expressed only in floral organs, with most expression observed in anthers. More specifically, they were both tapetum-specific. *In vitro* enzymatic studies showed that both *GASCL1* and *GASCL2* proteins were able to use medium and long chain acyl-CoAs with malonyl-CoA to perform two to threecondensation reactions to produce tri- and tetraketide alpha-pyrone. Further study is needed to explore the substrate preference of hydroxylated long chain CoAs as has been described in other species (Colpitts et al., 2011; Jepson et al., 2014; Wang et al., 2013).

In addition to the chalcone synthases from the flavonoid pathway and the 2-pyrone synthases involved in the biosynthesis of antimicrobial compounds, RNA-sequencing from anthers has shown that gerbera possess two ASCLs. *GASCL1* and *GASCL2*, described in this study, extend the gerbera type III PKS-family, which is now composed of eight members.

5. Experimental

5.1 Chemicals and plant material

Acetyl-CoA, benzoyl-CoA, hexanoyl-CoA, octanoyl-CoA, stearoyl-CoA, malonyl-CoA, 4-hydroxy-6-methyl-2-pyrone (triacetolactone) were purchased from Sigma-Aldrich (Steinheim, Germany) and *p*-coumaroyl-CoA from MicroCombiChem (Wiesbaden, Germany). The *Gerbera hybrida* (*G. jamesonii* Bolus ex Adlam × *G. viridifolia* Schultz-Bip) cultivar Terraregina was obtained from Terra Nigra B.V. (De Kwakel, The Netherlands). Plants were grown in greenhouse conditions as described previously (Deng et al., 2012).

5.2 Identification and phylogenetic analysis gerbera *ASCL* sequences

RNA sequencing from gerbera inflorescence stage eight anthers revealed two genes (*GASCL1* and *GASCL2*) which showed 72-73 % sequence similarity to Arabidopsis *PKSA* and *PKSB*. *GASCL1* was full length (1173 bp), but the ORF of *GASCL2* was lacking 59 nucleotides from the 5' end. A GenomeWalker Universal kit was used (Clontech, CA, USA) to obtain the missing 5' end. GenomeWalker DNA libraries were constructed according to the manufacturer's instructions. Libraries were used in the primary PCR with adaptor primer 1 (Table S1) and the gene specific primer in the 3' end. In the secondary PCR, the other adaptor primer 2 was used with the gene specific primer to produce fragments which were then cloned to the vector pJET1.2 blunt end cloning vector (Thermo Scientific). Multiple alignment (based on amino acids) of 26 identified or putative ASCLs, gerbera type III PKS family and a CHS from *Medicago sativa* was performed using the prank tool (ebi.ac.uk/goldman-srv/webprank), whereafter the aligned amino acid sequences were converted to the corresponding nucleotide sequences using an in-house program tool. The phylogenetic analysis using this nucleotide alignment was generated using RAxML (embnet.vital-it.ch/raxml-bb) with the GTR plus gamma model in a maximum likelihood bootstrap search. For the visualization of the phylogenetic tree, FigTree v1.4.2 was used (tree.bio.ed.ac.uk/software/figtree).

5.3 Protein expression in *E.coli*

GASCL1 was PCR amplified from cDNA made from stage eight gerbera anthers and for *GASCL2*, the full length cDNA in pJET1.2 vector was used as a template. PCR products were cloned into *NcoI* and *XhoI* sites of the expression vector pHIS8 (Ferrer et al., 1999), which creates 8 Histidines to the N-terminus of the expressed protein. Plasmids isolated from *E. coli* strain DH5α were verified by sequencing and then plasmids transformed to the strain Lemo21 (New England Biolabs). An overnight starter culture was used to initiate 50 ml cultures which were grown to OD₆₀₀ of 0.5 and induced by 0.5 mM IPTG. Cultures were grown at +16 °C for 22 hours. Pelleted cultures were lysed with B-PER Bacterial Protein Extraction Reagent (Thermo Scientific) supplemented with 20 mM imidazole, lysozyme (100 µg/ml), DNase I (5 U/ml) and a protease inhibitor (cOmplete mini EDTA free, Roche). Proteins were purified using His SpinTrap columns (GE Healthcare) according to the manufacturer's instructions. Finally PD MiniTrap G-25 columns (GE Healthcare) were used for buffer exchange.

5.4 Enzymatic assays

30 µg of purified protein was used in 100 µl of 0.1 M Tris-HCl assay buffer (pH 7.5) containing 2 mM DTT, 80 µM malonyl-CoA and 40 µM starter substrate. As a control, the empty pHIS8-vector was used. Activity with various starters was tested, including acetyl-CoA, benzoyl-CoA, hexanoyl-CoA, octanoyl-CoA, stearoyl-CoA and *p*-coumaroyl-CoA. Reaction was stopped after one hour with 20 µl of glacial acetic acid, followed by two times extraction with 300 µl ethyl acetate which was then evaporated to dryness.

5.5 UPLC-MS analysis

Reaction products were dissolved in 100 µl of methanol and the metabolites were separated on an Acquity UPLC® BEH C18 column (1.7 µm, 50 x 2.1 mm, Waters, Ireland) at 40°C. The mobile phase consisted of (A) water and (B) acetonitrile (Chromasolv® grade, Sigma-Aldrich, Steinheim, Germany) both containing 0.1% formic acid (Sigma-Aldrich, Steinheim, Germany). Waters Synapt G2 HDMS mass spectrometer (Waters, Milford MA, USA) was interfaced with the Waters Acquity UPLC® system (Waters, Milford MA, USA) via an ESI source. Mass range was 100 to 600. Samples were analyzed in negative ion mode, with capillary voltage at 3.0 kV. The source temperature was 120 °C and desolvation temperature was set to 360 °C. Linear gradient went from 95 % of A to 10 % in 10 min, then back to 95 % in 10.1 min and left to equilibrate for 2 min. The injection volume was 2 µl and flow-rate of the mobile phase was 0.6 ml/min.

5.5 Expression studies

RNA was extracted from various gerbera tissues using the pine tree method described by Chang et al. (1993). RNA was purified using NucleoSpin RNA Clean-up kit (Macherey-Nagel) and 500 ng of total RNA was used in cDNA synthesis using SuperScript III Reverse Transcriptase kit (Invitrogen). 180 µl of MQ water was added to dilute the cDNA for qRT-PCR which was done using LightCycler 480 Real-Time PCR detection unit (Roche). The PCR reaction contained 5 µl of diluted cDNA, 7.5 µl of LightCycler 480 SYBR Green I Master mix, 1 µl of MQ water and 0.5 µM forward and reverse primers. The PCR- program started with one initial cycle of 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 60 °C for 10 s and 72 °C for 10 s. Relative expression values were calculated by the method described by Pfaffl (2001) and normalized against the gerbera housekeeping gene *GAPDH* as described in Deng et al. (2014).

For *in situ* hybridization, anthers were collected from outermost disc flowers at stage six of inflorescence development (Helariutta et al., 1993). Samples were fixed overnight, dehydrated in an ethanol gradient series and embedded to paraffin as described earlier (Elomaa et al., 2003). Anthers were then transversely cut to 10 µm sections and spread on precharged slides. For sense and antisense *GASCL1* and *GASCL2* probe synthesis, 154 and 215 bp fragments with no sequence similarity to each other were PCR-amplified from the cloned pHIS8-constructs. A T7 polymerase binding site was added to the forward primer for sense amplification or to the reverse primer for antisense amplification. For the probe synthesis, DIG RNA labelling kit (Roche) was used. Hybridization protocol was carried out as described earlier (Karlgrén et al., 2009) except that hybridizations were done at 42 °C.

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7. Figure legends, tables and references

Fig 1. Phylogenetic tree of gerbera *GASL1* and *GASL2* genes together with 26 known *ASCLs*. As plant non-*ASCL* type III PKSs, gerbera chalcone synthases (*GCHS1-3*), gerbera 2-pyrone synthases (*G2PS1-3*) and a *CHS* from *Medicago sativa* were included. Ala and Val subclades are marked to the tree. List of plant species and accession numbers can be found in Table S2.

Fig 2. SDS-page of soluble proteins produced in *E. coli*. Lanes 1, 4 and 7 show the protein standard. Lanes 2 and 3, the total soluble fractions of *E. coli* expressing *GASL1* and *GASL2*, respectively. Lanes 5 and 6, the purified *GASL1* and *GASL2* proteins.

Fig. 3. UPLC-MS analysis of GASCL1 enzyme activity when assayed with malonyl-CoA and stearoyl-CoA. Base peak intensity (BPI) chromatogram of enzyme activity with GASCL1 (A) and vector control (B). Triketide (m/z 349, Rt 8.94 min) and tetraketide (m/z 391, Rt 8.39 min) products were observed in the enzyme assays when either GASCL1 or GASCL2 was used but not in the empty vector control. The peak with retention time of 9.0 min in the vector control was a different compound (m/z 383). (C and D) Mass spectra of the triketide (C) and tetraketide (D) products. Loss of CO₂ was observed in both products indicating presence of an alpha-pyrone ring. With these substrates, the enzyme activities were identical with GASCL1 and GASCL2.

Fig. 4. Expression pattern of *GASCL1* (blue) and *GASCL2* (orange) in different gerbera tissues. Expression in anthers was measured from pooled samples collected at stages six, seven and eight of gerbera inflorescence development as described in Helariutta et al. (1993). Expression of the genes were normalized against the *GAPDH* gene from gerbera. Error bars represent standard error of the mean, number of replicates was 3.

Fig. 5. *GASCL1* and *GASCL2* mRNA were localized to the tapetal cells of the anthers using gene specific antisense and sense probes. The control (sense) probe did not give any hybridization signal. Scale bars, 50 µm.

Table 1. *In vitro* products in the GASCL1 and GASCL2 enzyme assays. Both enzymes had identical activity except that GASCL2 did not produce compound 1 (triacetolactone).

Starter CoA	Proposed structure of the compound	Elemental composition	Retention time	m/z [M-H] ⁻	m/z [M-CO ₂ -H] ⁻ (rel. int. to major ion) 15 eV
Acetyl - CoA	1	C ₆ H ₅ O ₃	0.38	125.0234	81.0365 (-)
Hexanoyl-CoA	2	C ₁₀ H ₁₃ O ₃	2.09	181.0878	137.0977 (100)
Octanoyl-CoA	3	C ₁₂ H ₁₇ O ₃	3.65	209.1185	165.1289 (100)
Stearoyl-CoA	4	C ₂₂ H ₃₇ O ₃	8.94	349.2751	305.2848 (22)
Stearoyl-CoA	5	C ₂₄ H ₃₉ O ₄	8.39	391.2855	347.2982 (12)

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Appendix A. Supplementary data

Table S1. Primers used in the study.

Table S2. Plant species used in construction of the phylogenetic tree and amino acid alignment.

Figure S1. Figure S1. Amino acid alignments of 26 identified or putative ASCLs, gerbera type III PKS family and a CHS from *Medicago Sativa* generated with prank (www.ebi.ac.uk/goldman-srv/webprank). Grouping to Ala- or Val clades of ASCL proteins is based on the position marked with an asterisk. For generating the phylogenetic tree in Figure 1, the alignment was converted to nucleotides.

Fig. S2. Figure S2. Mass spectra of triketide products produced by GASCL1 and GASCL2. Loss of CO₂ was observed in the mass spectra when either hexanoyl-CoA (A), octanoyl-CoA (B) or acetyl-CoA (C and D) was used in the enzyme assays and remained ions [M-CO₂-H]⁻ with m/z 165 (A), 137 (B) and 81 (D – MSMS data) were observed.

Supporting information

Table S1. Primers used in the study.

Name	Sequence (5'-3')	Application
GASCL1_qPCR_F	GGCAACTCTTCAGCAACTAGG	qPCR
GASCL1_qPCR_R	GGATGTGGCCATGAAAGAGA	qPCR
GASCL2_qPCR_F	CACTAATTGCGATGATCCTGAAC	qPCR
GASCL2_qPCR_R	CTCACAAGCCTGCATCAAAA	qPCR
qPCR GGAPDH_F	CCAGGAACCCAGAGGAGATACC	qPCR
qPCR GGAPDH_R	GGAGCGGATATGATGACCTTCTTG	qPCR
GASCL1_F_SET7	CATAATACGACTCACTATAGGGGGCAACTCTTCAGCAACTAGG	<i>in situ</i>
GASCL1_R_AST7	CATAATACGACTCACTATAGGGTCTCTTTCATGGCCACATCC	<i>in situ</i>
GASCL2_F_SET7	CATAATACGACTCACTATAGGGGCACTAATTGCGATGATCCTGAAC	<i>in situ</i>
GASCL2_R_AST7	CATAATACGACTCACTATAGGGTTTTGATGCAGGCTTGTGAG	<i>in situ</i>
GASCL1_pHIS8_F	CGTGGTTCCTCGAGTTAGTTGAGGCTACGAAGAAGAATGC	pHIS8 cloning
GASCL1_pHIS8_R	TCGAATTCCTCGAGTTAGTTGAGGCTACGAAGAAGAATGC	pHIS8 cloning
GASCL2_pHIS8_F	CGTGGTTCCTCGAGTTAGTTGAGGCTACGAAGAAGAATGC	pHIS8 cloning
GASCL2_pHIS8_R	TCGAATTCCTCGAGTTAGTTGAGGCTACGAAGAAGAATGC	pHIS8 cloning
GASCL2-B1	ATGGTTCATGAAGAGATGACAACTAAGACC	pJET1.2 cloning
GASCL2-end	TTACACTACTAAGTTCTTTGTAAGAATGCCTTCAAATGTAACC	pJET1.2 cloning
AP1	GTAATACGACTCACTATAGGGC	GENOMEWALKER
AP2	ACTATAGGGCACGCGTGGT	GENOMEWALKER

Table S2. Plant species used in construction of the phylogenetic tree and amino acid alignment.

Name of the gene/protein	Species	Amino acid accession no.	Nucleotide accession no.
PpASCL	<i>Physcomitrella patens</i>	XP_001781520	XM_001781468
SmPKS2	<i>Selaginella moellendorffii</i>	XP_002985571	XM_002985525
PrCHSL	<i>Pinus radiata</i>	AAB80804	U90341
Os10g0484800 (YY2)	<i>Oryza sativa</i>	NP_001064891	NM_001071426
Os07g0411300	<i>Oryza sativa</i>	NP_001059449	NM_001065984
HpPKS1	<i>Hypericum perforatum</i>	JQ073294	JQ073294
PtCHSL4	<i>Populus trichocarpa</i>	XP_002302511	XM_002302475
PtCHSL5 XP_002326830	<i>Populus trichocarpa</i>	XP_002326830	XM_002326794
PtCHSL6 XP_002305482	<i>Populus trichocarpa</i>	XP_002305482	XM_002305446
RcCHSL	<i>Ricinus communis</i>	XP_002529257	XM_002529211
NsCHSLK	<i>Nicotiana glauca</i>	CAA74847	Y14507
AtPKSA	<i>Arabidopsis thaliana</i>	At1g02050	NM_100085
AtPKSB	<i>Arabidopsis thaliana</i>	At4g34850	NM_119651
AtCHSL	<i>Arabidopsis thaliana</i>	At4g00040	NM_116221
AhCHSL1	<i>Arabidopsis halleri</i>	AAZ81872	DQ062307
AhCHSL2	<i>Arabidopsis halleri</i>	AAZ23686	DQ062345
ZmCHSL	<i>Zea mays</i>	NP_001149508	NM_001156036
HvCHSL	<i>Hordeum vulgare</i>	AAV49989	AH014393
TaCHSL1	<i>Triticum aestivum</i>	EU408770	EU408770
TaCHSL	<i>Triticum aestivum</i>	CAJ15412	CT009735
AtaCHSL	<i>Aegilops tauschii</i>	CAJ13966	CT009625
Sb01g018950	<i>Sorghum bicolor</i>	XP_002467058	XM_002467013
Sb02g009850	<i>Sorghum bicolor</i>	XP_002461886	XM_002461841
SiCHSL	<i>Silene latifolia</i>	BAE80096	AB182106
VvCHSL1	<i>Vitis vinifera</i>	CAO47307	XM_002276617
VvCHSL2	<i>Vitis vinifera</i>	CAO70579	XM_002276606
GhASCL1	<i>Gerbera hybrida</i>		KX550276
GhASCL2	<i>Gerbera hybrida</i>		KX550277
Gh2PS1	<i>Gerbera hybrida</i>	P48391	Z38097
Gh2PS2	<i>Gerbera hybrida</i>	AMQ09636	KR107540
Gh2PS3	<i>Gerbera hybrida</i>	CAA62683	KR107541
GhCHS1	<i>Gerbera hybrida</i>	P48390	Z38096
GhCHS3	<i>Gerbera hybrida</i>	P48392	Z38098
GhCHS4	<i>Gerbera hybrida</i>	CAP20328	AM906210
MsCHS	<i>Medicago sativa</i>	P30074	L02902

MsCHS	-----
GhCHS1	-----
GhCHS3	-----
GhCHS4	-----
Gh2PS1	M-----G-----
Gh2PS3	M-----GS-----
Gh2PS2	M-----GS-----
PtCHSL4	-MS-----E---S---D---S-----N---GAS-----
PtCHSL5	-MS-----K---T---I---G-----N---GAS-----
HpPKS1	-MS-----N---L---E---T-----N---GSS-----
VvCHSL2	-MS-----Q---M---E---R-----N---GAP-----
NsCHSLK	-----
GhASCL1	-MS-----K---A---N---G-----N---G-----
AtPKSA	-MS-----N---S---R---M-----N---GVE-----
AhCHSL1	-MS-----N---S---R---M-----N---GVE-----
AtCHSL	MLV-----S---A---R-----VE-----
Os10g0484800_YY2_	-MA-----D---L---GFGDARSG-----N---GSR-----
TaCHSL1	-MS-----G---L---G---G-----L---GSI -GGGG
Sb01g018950	-MT-----G-----G---A-----N---GRS-----
PrCHSL	-MS-----A---S---N---GT-----N---GVV-----
Os07g0411300	MVS-----T---N-AGGI A---SK-----Q---ASS-----
HvCHSL	MVSARDVD-----T---N---K---QQ-----QQQATCL-----
TaCHSL	MVSARGVD-----TTTAAN---K---QQ-----Q---ATC-----
AtaCHSL	MVSARGVD-----TTTAAN---K---QQ-----Q---ATC-----
ZmCHSL	MVS-----SSMD-----T---T---S---DK-----R---ASS-----
Sb02g009850	MVS-----SAMD-----T---TA---S---SK-----H---ASS-----
PtCHSL6	MGS-----E---Q---I---GQ-----G---GLT-----
RcCHSL	MGS-----E-----A---GLS-----
VvCHSL1	MGS-----G---T---V---EQ-----V---GSL-----
AtPKSB	MGS-----I---D---A---AV-----L---GSE-----
AhCHSL2	MGS-----I---D---A---EV-----L---GSE-----
SI CHSL	MGF-----E---N---I---KL-----N---GMG-----
GhASCL2	MVH-----E---E-----MT-----
SmPKS2	-MS-----N---GSS-----
PpASCL	MAS-----RRVEAA---F---D---GQAVELGATI PAAN---GNG-----

MsCHS	-----M-----VSVSEI RKA--QRAEGP--ATI LAI GTANPANCVEQSTYPDIFYKI
GhCHS1	-----MASS---VDMKAI RDA--QRAEGP--ATI LAI GTATPANCVYQADYPDYYFRI
GhCHS3	-----MATSPAVI DVETI RKA--QRAEGP--ATI LAI GTATPANCVYQADYPDYYFRV
GhCHS4	-----M-----VNI EEFKRA--QRAEGP--ATI MAI GTATPSNCVFQDTPDYDYFRV
Gh2PS1	---S-YSS-----DDVEVI REA--GRAOGL--ATI LAI GTATPPNCVQAADYADYYFRV
Gh2PS3	---S-YGT-----VDVEAI REA--QRAOGL--ATI LAI GTATPPNCFI QADYPDYYFRV
Gh2PS2	---S-YPA-----I DVEAI RES--ORGQGL--ATI LAI GTAVPPYCFYQADYPDYYFRV
PtCHSL4	---K-HCT-----TPS--RRAPTLGKATLLAI GKAFPSQLI PQECLVEGYI RD
PtCHSL5	---K-HYA-----TLT--RRSPTPGKATI LATGKAFPSQLVPOECLVEGYMRD
HpPKS1	---R-N-----GRAPTPGKATVLAMGKAFPKQHVPQECLVEKFI SD
VvCHSL2	---K-HDV-----ALA--RRLPMPGKATVLAMGKAFPSQLI PQECLVEGYFRD
NsCHSLK	-----MGKAFPAQLVPQDCLVEGYI RD
GhASCL1	---N-GNS-----SAT--RRAPTPGKATVLAI GKAFPGQLVPQDCLVEGFFRD
AtPKSA	---K-LSS-----KST--RRVANAGKATLLALGKAFPSQVVPQENLVEGFRLD
AhCHSL1	---K-LSS-----I ST--RRVANPRKATLLALGKAFPSQVVPQENLVEGFRLD
AtCHSL	---K-----Q--KRVAYQGKATVLALGKALPSNVVSOENLVVEEYLR
Os10g0484800_YY2_	---S-QCS-----R-----GKAMLLALGKGLPEQVLPQEKVVETYLQD
TaCHSL1	NGI S-HGS-----G-----GKAKLLALGKGLPEQVLPQEKLVETYLQD
Sb01g018950	---Q-QSS-----R-----GKAMLLALGKGFPDQVLPQEKVVESYLQD
PrCHSL	---A-VKS-----RRQ--HR---PGKTTAMAFGRAFPDQLVMOEFLVDGYFRN
Os07g0411300	---M-----APNPGKATI LALGHAFPOQLVMQDYVVDGFMNRN
HvCHSL	---L-----APNPGKATI LALGHAFPOQLVMQDYVVEGFMNRN
TaCHSL	---L-----APNPGKATI LALGHAFPOQLVMQDYVVEGFMNRN
AtaCHSL	---L-----APNPGKATI LALGHAFPOQLVMQDYVVEGFMNRN
ZmCHSL	---ML-----APNPGKATI LALGHAFPOQLVMQDYVVDGFMKN
Sb02g009850	---ML-----APNPGKATI LALGHAFPOQLVMQDYVVDGFMKN
PtCHSL6	---S-----KASPGKATI LALGKAFPHQLVMOEFLVDGYFKN
RcCHSL	---K-----MANPGKATI LALGKAFPHQLVMOEFLVDGYFKN
VvCHSL1	---K-----KANPGKATI LALGKAFPHQLVMOEFLVDGYFRN
AtPKSB	---K-----KSNPGKATI LALGKAFPHQLVMOEFLVDGYFKT
AhCHSL2	---K-----KSNPGKATI LALGKAFPHQLVMOEFLVDGYFKT
SI CHSL	---K-----KPTPGKATVLSLGKGFPHTLVMOEFLVDGYFRN
GhASCL2	---T-----KTDI GKANI LALGKAFPHQLVMDLLVDGYFRD
SmPKS2	---S-GVK-----RKN--LRAARPGKATLLAFGKALPDTVVKQEYLVVDGYFRD
PpASCL	---T-HQS-----I KVPGRHQVTPGKTTI MAI GRAVPANTTFNDGLADHYI QE

MsCHS	TNSEHKTELKEKFORMCDKSMI KRRYMYLTEEI LKENPNVCEYM---APSLDARQDMVVV
GhCHS1	TKSEHMVDLKEKFKRMCDKSMI RKRYMHI TEEYLKQNPNCAYM---APSLDVRQDLVVV
GhCHS3	TESEHMVDLKEKFORMCDKSMI RKRYMHI TEEFLKENPSMCKFM---APSLDARQDLVVV
GhCHS4	TKSEHKTELKEKFKRMCDKSMI KRRYMYLTEEI LEEKPNVCAYM---APSLNERODI VVV
Gh2PS1	TKSEHMVDLKEKFKRI CEKTAI KKRYLALTEDYLOENPTMCEFM---APSLNARODLVVT
Gh2PS3	TKSEHMVELKEKFKRI CDKTAI KKRYLALTEEYLKQNPMSMCEFM---APSLNARODLI VP
Gh2PS2	TKSEHMVDLKEKFKRVCEKTAI KKRYLVLTEDYLEKNPNMGEFM---APSFNARODLVVP
PtCHSL4	TKCDDAS-I KEKLERLCKTTTVKTRYTVMSREI LDKYP---ELATEGTPTI QORLEI ANP
PtCHSL5	TKCDDAS-I KEKLERLCKTTTVKTRYTVMSKEI LEKYP---ELATEGSPTI QORLEI ANP

TKCTDAA-I KEKLERLCETTTVKTRYTVMCKEVLDPKY---ELAVEGSPTI RQRFEI ANP
TKCQDLA-I KEKLERLCKTTTVKTRYTVMCKEIL EKY---ELATEGSPTI KQKLEI ANP
TNCQDLA-I KEKLERLCKTTTVKTRYTVMCKEILDPKY---ELATEGTPTI KORLEI ANP
TNCMDVA-MKEKLERLCKTTTVKTRYTVMCKEILDPKY---ELATEGSPTI TORLDI ANP
TKCDDAF-I KEKLEHLCKTTTVKTRYTVLTREI LAKY---ELTTEGSPTI KORLEI ANP
TKCDDAF-I KEKLEHLCKTTTVKTRYTVLSREI LDKY---ELTTEGSPTI KORLEI ANE
IKCDNLS-I KDKLOHLCKSTTVKTRYTVMSRETLHKY---ELATEGSPTI KORLEI AND
TI CDDPA-TRAKLERLCKTTTVRTRYTVMSKELLDEHP---ELRTEGTPTLTPRLDI CNA
TSCDDPA-TRAKLERLCKTTTVRTRYTVMSKELLDEYP---ELKTEGSPTLTPRLDI CNA
SSCDDPA-TRAKLERLCKTTTVRTRYTVMSKELLDKHP---ELKMEGTPTLTPRLDI CNA
TNCQDPV-LROKLERLCKTTTVKTRYVVMSEI LAQHP---ELAVEGATVQRLEI SNV
TNCDDPE-LKEKLTRLCKTTTVKTRYVVMSEI LKSY---ELAQEGOPTMKORLDI SNK
TNCNDPE-LKEKLTRLCKTTTVKTRYVVMSEI LKSY---ELAQEGLPTMKORLDI SNK
TNCNDPE-LKEKLARLCKTTTVKTRYVVMSEI LKSY---ELAQEGLPTMKORLDI SNK
TNCNDPE-LKEKLARLCKTTTVKTRYVVMSEI LKSY---ELAQEGLPTMKORLDI SNK
TNCDDPE-LKEKLTRLCKTTTVRTRYVVMSEI LKNYP---ELAQEGLPTMNORLDI SNA
TNCDDPE-LKEKLTRLCKTTTVKTRYVVMSEI LKNYP---ELAQEGLPTMKORLDI SNA
TNCDDPE-LKOKLTRLCKTTTVKTRYVVMSEI LKNYP---ELAI EGI PTI KORLDI CND
TNCDDPE-LKOKLTRLCKTTTVKTRYVVMSEI LEKYP---ELAVEGLPTLKORLDI CNE
TNCDDPD-LKEKLARLCKTTTVKTRYVVMSEI LRKY---ELVI EGQPTVKORLDI CNK
TKCDDPE-LKOKLTRLCKTTTVKTRYVVMSEI LKKYP---ELAI EGGSTVORLDI CND
TKCDDPE-LKOKLTRLCKTTTVKTRYVVMSEI LKKYP---ELAI EGGSTVORLDI CND
TNCDDPE-LKOKLTRLCKTTTVKTRYVVMSEI LKKCP---ELAMAGOATVKORLDI CND
TNCDDPE-LKRRKI RLCKTTTVKTRYVVMSEI LKKYP---ELALEGLPTVKORLDI CNK
TKVENAA-LKEKLQRLCKTTTVKTRYVVMSEI LENHP---EFYTPGTPSI RQRLEI AGE
FNLQDPV-LQAKLRRLCETTTVKTRYLVVNI LDEHP---EELVDGAATVSRQAI TGA

EVPRKLGEAAVKAI KEWGQPKSKI THLI VCTTSGVDMPGADYQLTKLLGLRPVVKRYMMY
 EVPKLGKEAAMKAI KEWGHPSKSI THLI FCTTSGVDMPGADYQLTKLLGLRPSVKRFMMY
 EVPKLGKEAATKAI KEWGQPKSKI THLVFCTTSGVDMPGADYQLTKLLGLRPSVKRLMMY
 EVPKLGKEAATRAI KEWGQPKSKI THLVFCTTSGVDMPGADYQLTKLLGLRPSVKRLMMY
 GVPMLGKEAAVKAI DEWGLPKSKI THLI FCTTAGVDMPGADYQLVKLLGLSPSVKRYMLY
 GVPMLGKEAAVNAI QEWGHPKSKI THLI FCTTAGVDMPGADYQLI KLLGLSPSI SRYMMY
 EVPKLGKEAAI KAI DEWGHPSKSI THLI FCTTAGVDMPGADYQLTKLLGLSPSVKRYMMY
 AVVEMAKSMACI NEWGGSVEDI THI VVYSSSEVRLPGGDLYLASQLGLRNDVGRVMLY
 AVVEMALKASI ACI NEWGGSVKDI THVVVYSSSEI RLPGGDLYLASQLGLRNDVGRVMLY
 AVVEMAI EASHACI KEWGRPI SEI THVVVYSSSELRLPGGDLYLASELGLRNDVGRVMLY
 AVLQAMEASLACI EEWGRPVGI THI VVYSSSEI RLPGGDLYLASQLGLRSDVGRIMLY
 AVVEMAKQASQACI KEWGRSAEEI THI VVYSSSEI RLPGGDLYLATELGLRNDI GRVMLY
 AVTEMAKDSLACI KOWGRPAGDI THVVVYSSSEI RLPGGDLYLASELGLRNDVNRVMLY
 AVVEMALEASLGC I KEWGRPVEDI THI VVYSSSEI RLPGGDLYLAKLGLRNDVNRVMLY
 AVVEMALEASLGC I KEWGRPVEDI THI VVYSSSEI RLPGGDLYLAKLGLRNDVNRVMLY
 AVVQMAEASLVC I KEWGRAVEDI THLVVYSSSEFRLPGGDLYLSAQLGLSNEVORVMLY
 AVL ELGATAARAALGEWGRPAADI THLVYI SSELRLPGGDFLATRLGLHPNTVRTSLI
 AVL ELGAAAAARAALGEWGRPAADI THLVYI SSELRLPGGDFLATRLGLSPNTVRTSLI
 AVI DLGAAAAARAALDWGRPAADI THLI YI SSELRLPGGDVHLAARLGLSPNTVRTSLI
 AVTDMADVACDRDLKEWGRPVSEI THLVVYSSSEI RLPGGDLYLASRLGLRSDVSRVMLY
 AVTQIMATEASLACVRSWGGLSEI THLVVYSSSEARFPGGDHLARALGLSPDVRRVMLA
 AVTQIMATEASLACVEAWGGDLSAI THLVVYSSSEARFPGGDHLARALGLSPDVRRVMLA
 AVTQIMATEASLACVKAWGGDLSAI THLVVYSSSEARFPGGDHLARALGLSPDVRRVMLA
 AVTQIMATEASLACVKAWGGDLSAI THLVVYSSSEARFPGGDHLARALGLSPDVRRVMLA
 AVTQIMATEASLSCVRSWGGLSSI THLVVYSSSEARFPGGDHLARALGLSPDVRRVMLA
 AVTQIMATEASLSCVRSWGGLSSI THLVVYSSSEARFPGGDHLARALGLSPDVRRVMLA
 AVTQIMAI GASRACI KKWGRSVSDI THMVVYSSSEARLPGGDLYLAGGLGLSPETQRVMLY
 AVTKMAI EASOKI KNWGRPI SDI THLVVYSSSEARLPGGDHLARLGLSPETQRTVLY
 AVTQIMAI DASKACI KKWGRSVSEI THLVVYSSSEARLPGGDLYLAKGLGLSPETHRVOLY
 AVTEMAVEASRACI KNWGRSI SDI THVVVYSSSEARLPGGDLYLAKGLGLSPDTHRVLLY
 AVTEMAVEASRACI KNWGRSI SEI THLVVYSSSEARLPGGDLYLAKGLGLSPDTHRVLLY
 AVTEMAI DASKACI SDWGRPI SDI THLVVYSSSEARLPGGDLYLAKGLGLSPETNRVMLY
 AVTQIMAI EASQACI KNWARP I SDI THLVVYSSSEARLPGGDLYLAKGLGLSPNTNRVMLY
 AVTKMGVAAATQALAEWGRPI SDI THLVVYSSSEVRFPGGDLYLSKHLGLSSDI SRVMLY
 AVTLQGHAEATAAI KEWGRPASEI THLVVYSSSEI RLPGGDLYLAQLI GLRSDVNRVMLY

QOQCFAGGTVLRLAKDLAENN-KGARVLVVCSEVTAVTFRGPSTHLDLSLVGOALFGDGA
QOQCFAGGTVLRLAKDLAENN-KGARVLVVCSEI TAVTFRGPNDTHLDLSLVGOALFGDGA
QOQCFAGGTVLRLAKDLAENN-KGARVLVVCSEI TAVTFRGPNEGHLDSLVGOALFGDGA
QOQCFAGGTVLRLAKDLAENN-KGARVLVVCSEI TAVTFRGPDETHLDLSLVGOALFGDGA
QOQCAAGGTVLRLAKDLAENN-KGSRVLI VCSEI TAI LFHGPNNHLDLSLVGOALFGDGA
QOQCSAGGMVLRLAKDLAENN-KGSRVLVVCCEI TAI I FRGPNNHLDLSLVGOALFGDGA
QOQCAAGGMVLRLAKDLAENN-KGSRVLVI CSEI TTI I FRGPDEQI DLSLVGOALFGDGA
FLGCYGGVTGLRVAKDI AENN-PGSRVLLTTSETTI LGFRPPSKARPYDLVGAALFGDGA
FLGCYGGVTGLRVAKDI AENN-PGSR I LTTSETTI LGFRPPNKARPYDLVGAALFGDGA
FLGCYGGVTGLRVAKDI AENN-PGSRVLLTTSETTI LAFRPPSNARPYDLVGAALFGDGA
FLGCYGGVTGLRVAKDI AENN-PGSRVLLTTSETTI LGFRPPNKERPVDLVGAALFGDGA
FLGCYGGVTGLRVAKDI AENN-PGSRVLLTTSETTI LGFRPPNKARPYDLVGAALFGDGA
FLGCYGGVTGLRI AKDI AENN-PGSRVLLTTSETTI LGFRPPNKSRPYDLVGAALFGDGA
FLGCYGGVTGLRVAKDI AENN-PGSRVLLTTSETTI LGFRPPNKARPYDLVGAALFGDGA
FLGCYGGVTGLRVAKDI AENN-PGSRVLLTTSETTI LGFRPPNKARPYDLVGAALFGDGA
FLGCYGGVTGLRVAKDI AENN-PGSRVLLTTSETTI LGFRPPNKARPYDLVGAALFGDGA
FLGCYGGVTGLRVAKDI AENN-PGSRVLLTTSETTVLGFRRPPNKARPYNLVGAALFGDGA
FLGCSSGGAALRTAKDI AENN-PGSRVLVAAETTVLGFRRPPSPDRPYDLVGAALFGDGA
FLGCSSGGAALRTAKDI AENN-PGSRVLI AAETTVLGFRRPPSPDRPYDLVGAALFGDGA

PtCHSL6	I EGFCHKLI GV-----A-G--LTDKD-----YNKMFVAVHP
RcCHSL	I EGFQKLMGV-----A-G--LTDKD-----YNKLFVAVHP
VvCHSL1	I EAFCDKLI RN-----V-G--FSDED-----YNKI FVAVHP
AtPKSB	VENFCKKLI GK-----A-G--LAHKN-----YNQMFVAVHP
AhCHSL2	VENFCKKLI GK-----A-G--LAHKN-----YNQMFVAVHP
SI CHSL	I EAFCDKLMSS-----V-G--LTSKD-----YNDMFVAVHP
GhASCL2	VKGFCDKLLGH-----I -G--PNEMG-----YNDLFVAVHP
SmPKS2	VEGFI QGLLKRSGRVEA-G--GV--D-----YDRLFVAI HP
PpASCL	I QGFCDP I LKR-----A-G--GL--K-----YNDI FVAVHP
MsCHS	GGPAI LDQVEQKLALKPEKMNATREVLSEYGNMSSACVLFI LDEMRRKKST-Q---NGL--
GhCHS1	GGPAI LDQVELKGLKKEEKL RATHVLSEYGNMSSACVLFI I DEMRRKSS-E---NGA--
GhCHS3	GGPAI LDQVEFKLGLREEKL RASRHLSEYGNMSSACVLFI LDEMRRKSI -K---DGK--
GhCHS4	GGPAI LDQVEEKLGLEPDKLRATHVLSEYGNMSSACVLFI LNMERHSSA-T---DGL--
Gh2PS1	GGRAI LDQVERKLNKEDKLRASRHLSEYGNLI SACVLFI I DEVRKRSM-A---EGK--
Gh2PS3	GGRAI LDQVELKLDLKEEKL RASRHLSEYGNLTSACVLFI I DEMRRKSV-E---EGK--
Gh2PS2	GGRAI LDEVELKLNKKEEKL RASRHLSEYGNLTSACVLFI I DEMRRKSM-E---DGK--
PtCHSL4	GGPAI LNRLESKLKLNEEKLECSRRALMDYGNVSSNTI VYVLEYMRDE-L-K---RG--
PtCHSL5	GGPAI LNRLESNKLNTEKLECSRRALI NYGNVSSNTI VYVLEYMKEE-L-K---RE--
HpPKS1	GGPEI LNRVESRLGLNADKLECSRRALMDFGNVSSNTVLYVMEYLREE-L-K---RK--
VvCHSL2	GGPAI LNRLESTLKLRESEKLECSRRALMDYGNVSSNTI FYVMEYMREA-L-K---RK--
NsCHSLK	GGPAI LNRLENTLKLQSEKLDCSRRLMDYGNVSSNTI FYVMEYMREE-L-K---NK--
GhASCL1	GGPAI LNRLETTLKLRGDKLDCSRRLMDFGNVSSNTI FYVMEYMRDE-L-M---SK--
AtPKSA	GGPAI LNRLETKLKLEKLESSRRALVDYGNVSSNTI LYVMEYMRDE-L-K---KK--
AhCHSL1	GGPAI LNRLETKLKLEKLESSRRALVDYGNVSSNTI LYVMEYMREE-L-K---KK--
AtCHSL	GGPAI LSGLETKLKLEKLECSRRALMDYGNVSSNTI FYI MDKVRDE-L-E---KK--
Os10g0484800_YY2_	GGPAI LNRLEVCLLEPEKLLKI SRKALMNYGNVSSNTVYVLEYLRDE-L-K---K--
TaCHSL1	GGPAI LNRLEVCLLEPEDKLKI SRKALMNYGNVSSNTVYVLEYLRDE-L-K---K--
Sb01g018950	GGPAI LNRLEFCLELOPEKLLKI SRKALKNYGNVSSNTI FYVLEYLRDE-L-K---K--
PrCHSL	GGPAI LNRLEKKLSLGEPEKLYYSRQALADYGNASSNTI VYVLDAMR-Q-L-K-----
Os07g0411300	GGPAI LTKMEGRLGLDGGKLRASRSALRDFGNASSNTI VYVLENMVEETR-Q---RR--
HvCHSL	GGPAI LTKMEGRLGLDGGKLRASRSALRDFGNASSNTI VYVLENMVEESRQ---RM--
TaCHSL	GGPAI LTKMEGRLGLDGGKLRASRSALRDFGNASSNTI VYVLENMVEESR-Q---R--
AtaCHSL	GGPAI LTKMEGRLGLDGGKLRASRSALRDFGNASSNTI VYVLENMVEESR-Q---R--
ZmCHSL	GGPAI LTKMEGRLGLGADKLRASRCALRDFGNASSNTI VYVLENMVEDTR-R---RR-L
Sb02g009850	GGPAI LTKMEGRLGLGADKLRASRCALRDFGNASSNTI VYVLENMVEETR-R---RK-L
PtCHSL6	GGPAI LNRMEKRLDLLPDKLNASRRALMDYGNASSNTI VYVLEYMI EESR-K---MK--
RcCHSL	GGPAI LNRMEKRLDLFPEKLNASRRALMDYGNASSNTI VYVLEYMI EESL-K---MK--
VvCHSL1	GGPAI LNRMEKRLDLLPEKLNASRRALADYGNASSNTI VYVLEYMLEESS-K---TKR--
AtPKSB	GGPAI LNRI EKRLNLSPEKLSPSRRALMDYGNASSNSI VYVLEYMLEESK-KVRN-MN--
AhCHSL2	GGPAI LNRMEKRLNLSPEKLSPSRRALMDYGNASSNSI VYVLEYMLEESK-KVRN-MN--
SI CHSL	GGPAI LNRLEKRLDLPDKLSASRRALTDYGNASSNTI VYVMEYMI EEGL-K---RK--
GhASCL2	GGPAI LNRLEKKFDLWPEKLSASRRALADYGNASSNTI VYVLEYLI EESL-K---MK--
SmPKS2	GGPAI LNACEKKLQLAGDKLKCSROVLSDYGNI NGSTI I YVLDYMRQV-----
PpASCL	GGPAI LNAVQKQLDLAPEKLQATAROVLRDYGNI SSSTCI YVLDYMRHQSL-K---LK--
MsCHS	--KTTG-----E-----GLEWGVLFGFGPGLTI ETVVLRSV---
GhCHS1	--GTTG-----E-----GLEWGVLFGFGPGLTVETVVLHVS---
GhCHS3	--TTTG-----E-----GLEWGVLFGFGPGLTVETVVLHSL---
GhCHS4	--KTTG-----E-----GLEWGVLFGFGPGLTVETVVLHVS---
Gh2PS1	--STTG-----E-----GLDCGVLFGFGPGMTVETVVLRSV---
Gh2PS3	--RTTG-----E-----GLDLGVLFGFGPGLTVETVVLRLG---
Gh2PS2	--KTTG-----E-----GLDWGVLFGFGPGMTVETVVLHSL---
PtCHSL4	---GG-----E-----WGLALAFGPFI TFEGI LLRSL---
PtCHSL5	---GG-----E-----EWGLALAFGPFI TFEGI LLRSL---
HpPKS1	---GS-----E-----EWGLALAFGPFI TFEGVLLRSL---
VvCHSL2	---GG-----E-----EWGLALAFGPFI TFEGI LI RSLYNI---
NsCHSLK	--KNGG-----E-----EWGLALAFGPFI TFEGI LLRSL---
GhASCL1	--EN-G-----E-----EWGLALAFGPFI TFEGI LLRSL---
AtPKSA	--GDAA-----Q-----EWGLGLAFGPFI TFEGLLI RSL---
AhCHSL1	--GDAA-----Q-----EWGLGLAFGPFI TFEGLLI RSL---
AtCHSL	--GTEG-----E-----EWGLGLAFGPFI TFEGLMRNL---
Os10g0484800_YY2_	--GMI R-----E-----EWGLI LAFGPFI TFEGLMVRGI ---
TaCHSL1	--GAI R-----E-----EWGLI LAFGPFI TFEGLMVRGI ---
Sb01g018950	--GAI S-----E-----EWGLI LAFGPFI TFEGLLVRGV---
PrCHSL	---GG-----E-----KQSPWGLI LAFGPFI TFEGI LARSL---
Os07g0411300	--EEAA-----E-----EEDCEWGLI LAFGPFI TFEGI LARNL---
HvCHSL	--TEAPVLENMVEESRRORTTEPEMEP-----ECEWGLI LAFGPFI TFEGI LARNL---
TaCHSL	--AEAP-----EPEPEPEGA-DOECEWGLI LAFGPFI TFEGI LARNL---
AtaCHSL	--AEAP-----EPEPEPEG-EEECEWGLI LAFGPFI TFEGI LARNL---
ZmCHSL	LAADDG-----GEDCEWGLI LAFGPFI TFEGI LARNL---
Sb02g009850	LAAPAG-----D-----GEDCEWGLI LAFGPFI TFEGI LARNL---
PtCHSL6	--AG-----AANCWGLI LAFGPFI TFEGI LARNL---
RcCHSL	--EE-----NVNNEWGLI LAFGPFI TFEGI LARNL---
VvCHSL1	--QD-----QGDGEWGLI LAFGPFI TFEGI LARNL---
AtPKSB	--EE-----E--NEWGLI LAFGPGVTTFEGI I ARNL---
AhCHSL2	--EE-----E--NEWGLI LAFGPGVTTFEGI I ARNL---
SI CHSL	--N-G-----D-----KNDNDWGLI LAFGPGL-----
GhASCL2	--SE-G-----K-----KGGPELGLI LAFGPGVTTFEGI LTKNL---
SmPKS2	--NAAA-----D-----KPDPEWGLI LAFGPGVTMEGMVARNL---
PpASCL	--EAND-----N-----VNTEPEWGLLLAFGPGVTI EGALLRNL---

MsCHS	-----A-----I-----
GhCHS1	-----PTTVTVAV-----
GhCHS3	-----PATI SVAT-----Q-----N
GhCHS4	-----P-----I-----
Gh2PS1	-----R-----V-----TAAVANGN
Gh2PS3	-----P-----V-----ATPVANGN
Gh2PS2	-----P-----T-----TTLVANGI
PtCHSL4	-----
PtCHSL5	-----
HpPKS1	-----CL-----
VvCHSL2	VGKRYWDNI TL I QKTQLNFKLT-----
NsCHSLK	-----
GhASCL1	-----N-----
AtPKSA	-----TSS-----
AhCHSL1	-----
AtCHSL	-----
Os10g0484800_YY2_	-----N-----
TaCHSL1	-----N-----
Sb01g018950	-----K-----KTFTS-----
PrCHSL	-----V-----
Os07g0411300	-----Q-----ARARARD-----
HvCHSL	-----Q-----ARI AA-N-----
TaCHSL	-----Q-----ARLGA-N-----
AtaCHSL	-----Q-----ARLGA-N-----
ZmCHSL	-----Q-----ATARA-SAQL-----
Sb02g009850	-----Q-----ATARA-SAQL-----
PtCHSL6	-----T-----I-----
RcCHSL	-----T-----V-----
VvCHSL1	-----T-----V-----
AtPKSB	-----D-----V-----
AhCHSL2	-----D-----V-----
SI CHSL	-----
GhASCL2	-----V-----V-----
SmpKS2	-----V-----A-----
PpASCL	-----C-----

Figure S1. Amino acid alignments of 26 identified or putative ASCLs, gerbera type III PKS family and a CHS from *Medicago Sativa* generated with prank (www.ebi.ac.uk/goldman-srv/webprank). Grouping to Ala- or Val clades of ASCL proteins is based on the position marked with an asterix. For generating the phylogenetic tree in Figure 1, the alignment was converted to nucleotides.

Figure S2. Mass spectra of triketide products produced by GASCL1 and GASCL2. Loss of CO_2 was observed in the mass spectra when either hexanoyl-CoA (A), octanoyl-CoA (B) or acetyl-CoA (C and D) was used in the enzyme assays and remained ions $[\text{M}-\text{CO}_2-\text{H}]^-$ with m/z 165 (A), 137 (B) and 81 (D – MSMS data) were observed.