Down-regulation of key genes involved in carbon metabolism in *Medicago truncatula* results in increased lipid accumulation in vegetative tissue

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ABSTRACT

This article has been accepted for publication and undergone full peer review but has not been through the copyediting, typesetting, pagination and proofreading process, which may lead to differences between this version and the Version of Record. Please cite this article as doi: 10.1002/csc2.20124.

Alfalfa (*Medicago sativa* L.), is the most widely grown perennial forage crop, which is a close relative of the model diploid legume *Medicago truncatula*. However, use of alfalfa lead to substantial greenhouse gas emissions and economic losses related to inefficiencies in rumen fermentation. The provision of supplemental lipids has been used as a strategy to mitigate these issues, but it is a costly approach. The ability to enhance lipid content within the vegetative tissues of alfalfa would therefore be very advantageous. As such, our aim was to assess and select gene candidates to increase total shoot lipid content in *M. truncatula* using a virus-induced gene silencing (VIGS) approach. We targeted gene homologs of the *SUGAR-DEPENDANT 1* (*SDP1*), *ADP-GLUCOSE-PYROPHOSPHORYLASE SMALL SUBUNIT 1* (*APS1*), *TRIGALACTOSYLDIACYLGLYCEROL 5* (*TGD5)* and *PEROXISOMAL ABC TRANSPORTER 1* (*PXA1)* in *M. truncatula* for silencing. Reduced target transcript levels were confirmed and changes of shoot lipid content and fatty acid composition were measured. Silencing of *SDP1, APS1* and *PXA1* each resulted in significant increases in shoot total lipid content. Significantly increased proportions of α-linolenic acid (18:3$\Delta^{9cis,12cis,15cis}$) were observed and stearic acid (18:0) levels significantly decreased in the total acyl lipids extracted from vegetative tissues of each of the *M. truncatula* silenced plants. In contrast, palmitic acid (16:0) levels were significantly decreased in only *SDP1* and *PXA1*-silenced plants. Genes of *PXA1* and *SDP1* would be ideal targets for mutation as a means of improving the quality of alfalfa for increasing feed efficiency and minimizing greenhouse gas emissions from livestock production in the future.

Key words: Virus-induced gene silencing, feed quality, forage legume, lipid metabolism, methane emissions

INTRODUCTION

Alfalfa (*Medicago sativa* L.), which is an obligatory outcrossing autotetraploid species, is one of the most extensively grown and studied forage crops in the world due to its protein content, palatability, adaptability, ability to fix nitrogen and yield components (Koivisto and Lane 2001; Radović et al. 2009). However, inefficiencies in rumen fermentation of plant materials such as alfalfa leads to the emission of substantial amounts of methane, which is a powerful greenhouse gas that has been estimated to have a 25 times greater impact on climate change than carbon dioxide (Solomon *et al*. 2007). Since there is considerable attention being paid to reducing methane emissions in numerous

countries in recent years (Jayasundara et al. 2016), which makes the improvement of forages for reduced methane production a priority for breeders and biotechnologists.

While lipid supplementation of feed appears to be a promising avenue in terms of reducing methane emissions (Beauchemin et al. 2008; Moate et al. 2011; Bayat et al. 2018) and intake without impacting livestock productivity or quality, this requires a substantial financial input by producers. Therefore, the use of forages with increased shoot (leaf and stem) lipid content would provide an attractive alternative. In forage species such as alfalfa, leaf tissues produce an abundance of polar lipids for the production of cell membranes, with a total lipid content of approximately 3% of dry matter (Barrett et al. 2015), and there appears to be little genetic variation in this trait. As such, little progress has been made using conventional breeding approaches to increase the lipid content of vegetative tissues in forages (Palladino et al. 2009; Glasser et al. 2013; Hegarty et al. 2013; Wijekoon et al. 2019b). Furthermore, while the alfalfa genome has been sequenced and assembled at the diploid level (www.alfalfatoolbox.org/doblast), such information is not available at the tetraploid level and functional genomics applications have thus been limited in this species (Biazzi et al. 2017).

Medicago truncatula has been previously used as a model species for functional genomics analyses in legumes (Grønlund et al. 2008), and is a close relative of *M. sativa*. Virus-induced gene silencing (VIGS) has become a powerful functional genomics tool in transient gene silencing studies and has been applied successfully in *M. truncatula* (Grønlund et al. 2008). While VIGS has also been successfully used in other forage legumes recently (Wijekoon et al. 2019a), the diploid nature of *M. truncatula* and availability of a genome sequence facilitates such research in this species. As such, in the current study, we endeavoured to harness progress that has been made in other plant species in terms of increasing the lipid content of vegetative tissues (reviewed by Chapman et al. 2013; Xu and Shanklin, 2016; Weselake, 2016; Vanhercke et al. 2019) by assessing the capacity of several candidate genes to enhance shoot lipid content when down-regulated using VIGS in *M. truncatula*. Putative homologs of *SUGAR-DEPENDANT 1* (*SDP1*), *ADP-GLUCOSE-PYROPHOSPHORYLASE SMALL SUBUNIT 1*(*APS1*), *TRIGALACTOSYLDIACYLGLYCEROL 5* (*TGD5)* and *PEROXISOMAL ABC TRANSPORTER 1* (*PXA1)*, which have all been found previously to increase leaf lipid content in *Arabidopsis thaliana* when down-regulated or mutated (Slocombe et al. 2009; Sanjaya et al. 2011; Kelly et al. 2013; Fan et al. 2015), were identified in *M. truncatula* and targeted for silencing. The results of this study provide a suite of candidate genes for targeting, using conventional or advanced molecular breeding

(e.g., genome editing) approaches, in order to enhance the quantity of shoot lipids in alfalfa and other agronomically important leguminous forages.

MATERIALS AND METHODS

PLANT MATERIAL

All experiments were carried out using the *M. truncatula* PI3077453 genotype supplied by Plant Gene Resources Canada, Saskatoon due to its high silencing efficiency (Wijekoon et al. 2019a). Seeds were sown individually in foam trays until roots were visible and then transplanted to soil in individual pots. Plants were grown in the greenhouse with a 16 hr/8 hr photoperiod and a light intensity of approximately150 $\mu E/m^2/s$.

BIOINFORMATICS

Coding and genomic sequences of putative *M. truncatula* homologs of Arabidopsis *SDP1* (AT5G097010), *APS1* (AT5G48300), *TGD5* (AT1G27695) and *PXA1* (AT4G39850) were identified through BLAST searches of the *M. truncatula* genome database (http://www.medicagogenome.org/). Local alignments were carried out using the Geneious 8.1.9 program (Biomatters Inc., Newark, NJ). Related legume sequences were obtained using a BLAST search against GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi), as well as the Alfalfa Gene Index and Expression Atlas Database (https://plantgrn.noble.org/AGED/index.jsp). Deduced amino acid sequences representing SDP1, APS1, TGD5 and PXA1 from several legumes were aligned and dendrograms (http://evolution.gs.washington.edu/phylip.html) were generated using the neighbor-joining algorithm with 1,000 bootstrap replications. Accession numbers for the deduced proteins from different legume species are presented in Table 1.

GENERATION OF VIRUS-INDUCED GENE SILENCING VECTORS

Total RNA was extracted from *M. truncatula* PI3077453 leaf tissue with the Spectrum Plant Total RNA kit (Sigma-Aldrich Canada, Oakville, ON) according to the manufacturer's instructions. The concentration of RNA was determined using a Nanodrop (Thermo Fisher Scientific, Whitby, ON) and RNA integrity was confirmed through agarose gel electrophoresis. First-strand cDNA was generated

using the Superscript IV First-Strand Synthesis System (Thermo Fisher Scientific) with an oligo-dT primer and approximately 3.5 µg total RNA as template. Quantitative real time (RT)-PCR was carried out using primers designed to amplify 316 bp (*MtSDP1*), 319 bp (*MtAPS1*), 251 bp (*MtTGD5*) and 316 bp (*MtPXA1*) fragments of each coding sequence (primer sequences are listed in Table 2) along with Platinum SuperFi Green Master Mix (Thermo Fisher Scientific). Thermocycling parameters were as follows: 98°C for 30 seconds (s), 35 cycles of 98°C for 10s, 57°C for 10s and 72°C for 30s followed by a final extension at 72°C for 5 min. The resulting amplicons were cloned into the pGEM-T Easy vector (Promega Corp., Madison, WI) and sequenced to confirm their identity. The sequence of each fragment was assessed for potential off-target effects using the BLAST program within the *M. truncatula* genome database.

The cloned fragments were then re-amplified using an identical set of primers with USER-defined sequences at the 5' terminus of each primer (GGCAATTU for the forward primers and GGTATTU for the reverse primers; Table 2), along with PfuTurbo Cx Hotstart DNA polymerase (Thermo Fisher Scientific) according to the manufacturer's recommendations. Thermocycling conditions comprised an initial denaturation at 95°C for 2 min, 30 cycles of 95°C for 30s, 57°C for 30s and 72°C for 1 min followed by a final extension at 72°C for 10 min. Bands were gel-purified using the QIAquick Gel Extraction kit (Qiagen Inc., Toronto, ON), and each purified fragment was inserted into SwaI-linearized pCAPE2 plasmid (Grønlund et al. 2008), which comprises a pea early browning virus (PEBV)-based VIGS vector, via USER cloning (New England Biolabs, Whitby, ON). The identity of each of the resulting VIGS vectors was confirmed through sequencing.

VIRUS-INDUCED GENE SILENCING

The plasmid constructs, along with empty pCAPE2 vector and pCAPE1 (Grønlund et al. 2008), were introduced into *Agrobacterium tumefaciens* (hereafter Agrobacterium) strain GV3101 using electroporation, respectively. For infiltration, overnight liquid cultures of Agrobacterium containing each construct were used to inoculate 500 mL of Luria Bertani medium containing 10 mM 2-(N-morpholino) ethanesulfonic acid (MES), 20 µM acetosyringone, and 50 µg mL^{-1} kanamycin and grown overnight at 28^{o}C with shaking (200 rpm). The Agrobacterium cultures were then pelleted at 3,000g for 15 min and resuspended in infiltration buffer (10 mM MES, 200 µM acetosyringone, 10 mM $MgCl_2$) to an OD600 of 2.5 and mixed with Agrobacterium cultures containing pCAPE1 in a 1:1 ratio

(v/v) according to Grønlund et al. (2008). After incubation of the mixture for one hour at room temperature, the abaxial surface of all leaves and apical meristems of two-three week-old seedlings were infiltrated using a 1-cc syringe. Ten to twenty plants were infiltrated with two replications each. Subsequent to infiltration, the plants were grown in a greenhouse with a 16 hour (hr)/8 hr photoperiod and a light intensity of approximately 150 $\mu E/m^2/s$. Five weeks following infiltration, plant shoots containing stems and leaves were harvested, immediately frozen in liquid nitrogen and stored at -80°C for subsequent analyses.

GENETIC ANALYSES OF PLANTS SUBJECTED TO VIRUS-INDUCED GENE SILENCING

Five weeks following infiltration, total RNA was extracted from the leaves of *M. truncatula* plants bearing each construct representing three biological samples from each plant using the Sigma Spectrum Plant Total RNA kit and reverse transcribed using the Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific). In every case, the leaves harvested for molecular analyses had developed subsequent to infiltration to avoid the possible presence of contaminating Agrobacterium. Complementary DNA was assessed for the presence of transcribed PEBV vector/coat protein sequence and the constitutive tubulin transcript (XM_013601854) using Platinum SuperFi Green PCR Master Mix and primers US2F, US2R, MTtubulinf and MTtubulinr, respectively, as described previously (Wijekoon et al. 2019a; Table 1). A thermocycling regime of 98^{o}C for 30s followed by 30 cycles of 98^{o}C for 15s, 55^{o}C for 30s, 72^{o}C for 30s and 72^{o}C for 5 min each was utilized. Plants that tested positive for the PEBV sequence were selected for quantification of transcript levels by qRT-PCR.

Quantitative real-time PCR was performed in triplicate on 4-6 selected plants using a QuantStudio 6 Flex real-time PCR system (Thermo Fisher Scientific). Reactions were performed in a final volume of 10 µL using Power UP SYBR PCR mix (Thermo Fisher Scientific), 0.5 µl of undiluted cDNA, and 5 µM of primers designed to anneal to a region of each target coding sequence that was distinct from the VIGS product. Primers MTEFf and MTEFr (Table 2) were used to amplify a 136 bp fragment of the constitutively expressed EF-1α transcript (XM_013595882), which was utilized as an internal control. Thermal parameters for amplification were as follows: 2 min at 50°C and 2 min at 95°C, followed by 40 cycles of 95°C for 15s and 60°C for 60s. Primer-pair specificity was validated for each qRT-PCR experiment through use of a dissociation curve, which demonstrated a single

amplicon for each of the targeted transcripts. Levels of gene expression were determined using the 2-ΔΔct method (Livak and Schmittgen, 2001) with expression data comprising mean values of biological replicates normalized to those of the EF-1α control.

ASSESSMENT OF TOTAL LIPID CONTENT USING NEAR-INFRARED SPECTROMETRY

Plants exhibiting substantial reductions in target transcript levels based on qRT-PCR analysis were tested for lipid content using near-infrared (NIR) spectrometry (Foss NIR systems 6500) as described previously (Wijekoon et al. 2019b). This approach is typically used for measuring forage quality parameters including protein, fat, dry matter and crude fibre, and has been used previously to assess foliar oil characteristics in a *Melaleuca cajuputi* breeding population (Schimleck et al. 2003). Powdered, oven-dried tissues were used directly for the analysis using a ring-type cell. Analyses were carried out on ten to twenty biological replicates per treatment with three technical replicates per sample.

LIPID EXTRACTION AND ANALYSIS USING GAS CHROMATOGRAPHY-MASS SPECTROMETRY

The fatty acid composition of total acyl lipids was analysed by gas chromatography (GC)-mass spectrometry (MS). Total lipids were extracted from mature *M. truncatula* shoots (containing leaves and stems) from three to five plants (three technical replicates of each) exhibiting down-regulation of each target gene, respectively, as well as empty vector controls, as described previously (Singer et al. 2016; Wijekoon et al. 2019a) with slight modifications. In brief, powdered, oven-dried tissues (100 mg) were transmethylated using 3N methanolic HCl at 80ºC for 2hrs. The resulting fatty acid methyl esters were then extracted twice with hexane, evaporated under a stream of nitrogen gas and re-dissolved in 500 µl iso-octane containing methyl heicosanoin (21:0 methyl ester, 0.1 mg/ml; Nu Check Prep Inc., Elysian, MN) as an internal standard. The extracted fatty acid methyl esters were analyzed using an Agilent 6890 Network GC system equipped with a DB 23 capillary column (30 m x 0.25 mm x 0.25 µm) with helium as the carrier gas (1.2 ml/min) and a 5975 inert XL Mass Selective Detector (Agilent Technologies Canada Inc., Mississauga, ON). A split/splitless inlet was used and the injection volume was 1 µl in the ten-to-one split mode. Peaks were identified using the software NIST MS Search 2.0 from the National Institute of Standards and Technology (NIST, Gaithersburg,

MD).The temperature program utilized was as follows: 100ºC, hold for 4 min, 10ºC/min to 180ºC, hold for 5 min, and 10ºC/min to 230ºC, hold for 5min.

STATISTICAL ANALYSIS

Experimental data was analyzed using PROC GLM in SAS program (SAS Institute, Cary, NC, USA). Analysis of variance (ANOVA) was carried out to observe if there were any significant ($P < 0.05$) changes on relative transcript levels, total lipid content and composition with each treatment. When the effect was significant, the least significant difference (LSD) ($P < 0.05$) test was used to separate treatment means.

RESULTS

IDENTIFICATION OF M. TRUNCATULA CARBON METABOLISM-RELATED HOMOLOGS

Putative homologs of four genes shown previously to increase leaf lipid accumulation in Arabidopsis when mutated/down-regulated (*SDP1*, *APS1*, *TGD5* and *PXA1*) were identified in *M. truncatula* through BLAST searches of the genome database. The coding sequence of *MtSDP1* (Medtr6g080170) exhibited 78.5% pairwise homology to its Arabidopsis homolog (At5g04040) at the nucleotide level and 70.6% identity at the amino acid level. Two other close relatives of this gene also exist in the *M. truncatula* genome (Medtr1g087300 and Medtr7g090470), which exhibit 90.4% and 77.9% pairwise identity with Medtr6g080170 at the nucleotide level, respectively. The coding sequence of *MtAPS1* (Medtr5g097010) exhibited 77.0% pairwise identity with its Arabidopsis homolog (At5g48300) at the nucleotide level and 86.2% identity at the amino acid level. A closely related homolog also exists in *M. truncatula* (Medtr3g082150), which exhibited 85.3% pairwise homology to Medtr5g097010 at the nucleotide level. A putative *MtTGD5* coding sequence was identified (Medtr3g102780) that exhibited 71.1% identity with its Arabidopsis homolog (At1g27695) at the nucleotide level and 65.9% identity at the amino acid level; however, its coding sequence length was more than double that of the Arabidopsis coding sequence (678 bp and 276 bp, respectively). The coding sequence of *MtPXA1* (Medtr3g087350) exhibited 73.4% pairwise identity with its Arabidopsis homolog (AT4G39850) at the nucleotide level, and 76.0% identity at the amino acid level. A dendrogram was constructed using deduced amino acid sequences of putative SDP1, APS1, TGD5 and PXA1 proteins from several

legume species as well a homologous protein from *Brassica napus* and *A. thaliana* using Arabidopsis homolog as an outgroup (Figure 1). Alfalfa and *M. truncatula* amino acid sequences grouped together and exhibited more than 80% homology for all four of the selected proteins. The proteins from *B. napus* did not fall into either of the legume clades and appeared to be more diverged in their amino acid sequences (Figure 1).

TRANSIENT DOWN-REGULATION OF GENES INVOLVED IN CARBON METABOLISM

For each of the four target genes, qRT-PCR was utilized to amplify small regions of cDNA from *M. truncatula*, which were inserted into the pCAPE2-USER VIGS background vector (Grønlund et al. 2008). The *MtSDP1* VIGS fragment consisted of 292 nt near the 5' terminus of the coding sequence while the *MtAPS1* VIGS fragment consisted of 319 nt near the 3' terminus of the coding sequence. The *MtTGD5* VIGS fragment consisted of 251 nt near the 5' terminus of the coding sequence while the *MtPXA1* VIGS fragment consisted of 316 nt at the 3' terminus of the coding sequence. The *MtPXA1* and *MtTGD5* VIGS fragments did not exhibit significant nucleotide similarity to any other transcripts in the *M. truncatula* genome database. The *MtSDP1* fragment, however, exhibited 90.5% nucleotide identity with its closely related homolog Medtr1g087300, while the *MtAPS1* VIGS fragment exhibited 89.1% homology with its closely related homolog Medtr3g082150.

Agrobacterium-mediated infiltration of leaves and meristems was used to deliver *MtSDP1*, *MtAPS1* , *MtTGD5* or *MtPXA1* VIGS vectors, as well as an empty vector control, into *M. truncatula* PI3077453. Plants containing a 490 bp fragment of the pCAPE-USER viral vector sequence (Wijekoon et al. 2019a) were considered positive for successful infiltration. The success rate was about 80-90% (for example 8 to 9 plants containing pCAPE-USER viral vector out of 10 infiltrated plants). Plants containing the vector sequence were then selected for assessment of target transcript abundance of *MtSDP1*, *MtAPS1*, *MtTGD5* or *MtPXA1* and compared to the empty vector controls. Mean normalized transcript levels for each of the targeted genes were significantly ($P < 0.05$) reduced in plants infiltrated with Agrobacterium harboring experimental pCAPE2 constructs compared with the empty pCAPE2 control vector (Figure 2). The relative suppression of transcript levels in plants containing the vector sequence was in the range of about 50-75% overall (the amount of down-regulation typically seen in each plant), with *MtSDP1*-silencing showing the overall highest efficiency (Figure 2).

EFFECT OF GENE SILENCING ON TOTAL LIPID CONTENT OF VEGETATIVE TISSUES AND FATTY ACID COMPOSITION OF TOTAL ACYL LIPIDS

The average lipid content of vegetative tissue from empty vector control plants was approximately 3.4% DW based on NIR analyses. Significant increases in lipid content were observed in plants exhibiting silencing of *MtSDP1*, *MtAPS1* and *MtPXA1,* respectively, compared to empty vector controls, with average lipid contents over 4.5% DW in each case (Figure 3). The largest increase in lipid content was observed with silencing of *MtPXA1,* where lipid content increased by approximately 40% compared to empty vector control plants on a relative basis. None of the plants exhibiting high lipid content exhibited any morphological or developmental changes compared to empty vector or uninfiltrated plants. No significant alteration in total shoot lipid content was observed in *MtTGD5*-silenced plants.

Vegetative tissues from empty vector control plants and three to five *M. truncatula* plants showing reduced transcript levels of each targeted gene, respectively, were analyzed for fatty acid composition of total acyl lipids using GC-MS (Table 3). In each case, the major fatty acids observed in the total acyl lipid fraction were palmitic acid (16:0), palmitoleic acid (16:1Δ^{9cis}; hereafter 16:1), stearic acid (18:0), oleic acid (18:1Δ^{9cis}; hereafter 18:1), linoleic acid (18:2$\Delta^{9cis,11cis}$; hereafter 18:2), α-linolenic acid (18:3) and arachidic acid (20:0). In empty vector control plants, 18:3, 16:0 and 18:0 were the major molecular species representing approximately 50, 25 and 13% (w/w), respectively, of the total fatty acids. A reduction in the levels of *MtSDP1*, *MtAPS1*, *MtTGD5* or *MtPXA1* gene transcripts led to significant increases in 18:3 in each case, with the silencing of *MtSDP1* and *MtPXA1*, respectively, resulting in the most substantial relative increases of approximately 20% compared to empty vector control plants. The silencing of all four target genes, respectively, also resulted in significant reductions in 18:0 levels, suggesting that the increase in 18:3 was partially attributable to a decrease in 18:0 content. Levels of 18:2 also appeared to be diminished with the silencing of each gene target, but the observed average decreases were not significant. Significant decreases in 16:0 and 20:0 content were also observed in *MtSDP1* and *MtPXA1*-silenced plants, with significant reductions in 18:1 also evident in the case of *MtSDP1* silencing.

DISCUSSION

The majority of research aimed at reducing ruminant-derived methane emissions has centered on the use of various feed additives, such as the addition of moderate amounts of vegetable oil (reviewed by Singer et al. 2018). Indeed, it has been estimated that for every 1% absolute increase in oil within a ruminant's feed, methane emissions would be lowered by 3.5% - 5.6% (Beauchemin et al. 2008; Moate et al. 2011). This effect is due, at least in part, to the fact that the amount of methane produced by a ruminant is directly correlated to the amount of plant material eaten. Since lipids provide a higher caloric value than the other main energy sources in the vegetative tissues of forages (carbohydrates and protein), intake tends to decrease with lipid supplementation (Bayat et al. 2018). In line with this, supplementary feeding trials in sheep have shown that increasing dietary fat levels to 8% DW resulted in a 30% increase in feed conversion efficiency, and a reduction in intake of 16% (Cosgrove et al. 2004).

Although such supplementation would be beneficial, its cost would be prohibitive to the vast majority of producers. Therefore, breeding forages with moderate (up to 5-6% DW) levels of lipids in their vegetative tissues would provide a promising alternative means of achieving reductions in methane emissions from livestock production. In the current study, we have examined the silencing of *SDP1*, *APS1*, *TGD5* or *PXA1* homologs in the model species *M. truncatula*, in an attempt to validate the capacity of several candidate gene targets to enhance the lipid content of vegetative tissues. Such candidate genes have the potential to be utilized for the downstream breeding of forage legumes such as alfalfa with increased shoot oil concentration. Each of the selected candidate genes function at a different level of carbon metabolism, with *SDP1* and *PXA1* being involved in the normal breakdown of TAG and fatty acids, respectively, *APS1* being required for starch biosynthesis and *TGD5* being involved in the transfer of lipids from the outer to inner plastid envelope. In line with this, their down-regulation/mutation has been found to lead to lipid accumulation in vegetative tissues as a result of decreased fatty acid/TAG breakdown (*PXA1*, *SDP1*), a re-distribution of cellular carbon from starch synthesis towards lipid synthesis (*APS1*), or elevations in fatty acid synthesis (TGD5; Slocombe et al. 2009; Sanjaya et al. 2011; Kelly et al. 2013; Fan et al. 2015).

Using a VIGS approach in *M. truncatula*, the down-regulation of *MtSDP1*, *MtAPS1* and *MtPXA1*, but not *MtTGD5*, were found to result in a significant relative increase in the lipid content of vegetative tissues compared to empty vector-infiltrated control plants (3 - 4% on a dry weight basis; Figure 3). While empty vector *M. truncatula* plants possessed mean shoot lipid content of 3.4%, those of *MtSPD1*, *MtAPS1* and *MtPXA1* plants were 4.69%, 4.67% and 4.81%, respectively.

Greater enhancements in vegetative lipid contents have been achieved previously, for example through the simultaneous modulation of several genes involved in the lipid biosynthesis and encapsulation in tobacco, which led to the accumulation of oilseed-like amounts (>30% DW) of TAG in vegetative tissues (Vanhercke et al. 2017). However, increases above approximately 8% DW would likely be detrimental in forage species due to possible associated effects on livestock performance and quality (reviewed by Singer et al. 2018).

The high homology of a target gene play a major role in efficiency of silencing genes by VIGS in legumes (Constantin et al., 2004; Grønlund et al., 2008). The target genes we used in this study are likely to exist as low copy genes in forage legumes assuming all the sequences silenced were homologs or paralogs of the gene target in the same species. Orthologous genes from far-related plant species were previously used in heterologous gene silencing (Senthil-Kumar et al. 2007; Wijekoon et al., 2019a). A highly homologous *PDS* gene fragment from field pea (Constantin et al., 2004) effectively silenced *PDS* genes in two perennial forage legumes (alfalfa and sainfoin) and an annual forage legume (fenugreek) by VIGS (Wijekoon et al., 2019a). Therefore, this approach can be extended and has a potentail to launch in the same forage legumes in order to modulate the lipid content in leaves using *MtSPD1*, *MtAPS1*, *MtPXA1* and *MtTGD5*.

In previous studies with Arabidopsis, enhanced lipid accumulation in the vegetative tissues of *SDP1*, *APS1*, and *PXA1* down-regulated/mutant lines was mainly associated with the accumulation of TAG, although this aspect was not explored in the current study. Vegetative tissues of higher plants typically contain very low levels of TAG, where it is thought to serve as transient reserve for excess fatty acids during membrane lipid turnover (Chapman et al. 2013). Indeed, in a previous survey of the leaves of 25 plants, TAG was detected in only 13 of the species assessed with levels up to 5 mg per g fresh weight (Lin and Oliver, 2008). In the case of forages, alfalfa has been found to have the lowest total fatty acid content of various forage grasses and legumes evaluated previously (Boufaïed et al. 2003a,b; Wijekoon et al. 2019b), which suggests that enhancing shoot lipid content would be particularly beneficial in this species.

In the current study, the silencing of all four target genes, respectively, resulted in significant alterations in the fatty acid composition of the total acyl lipids of vegetative tissues (Table 3), indicating that the down-regulation of each of these genes resulted in changes in lipid metabolism. For example, substantial increases in 18:3 content were observed in each case, which could translate

into improved feed quality. Previous studies have demonstrated that the 18:3 content of milk fat in dairy cows is directly influenced by the level of this polyunsaturated fatty acid in forage (Hebeisen et al. 1993; Penmetsa and Cook 2000). In addition, reducing levels of 16:0 and 18:0 resulted in increased levels of polyunsaturated fatty acids (such as 18:3) in *M. truncatula* leaves (Table 3) suggesting the same potential effect in forage crops. Beef rich in polyunsaturated fatty acids are associated with grazing or feeding forages containing lipids enriched in polyunsaturated fatty acids (Van Nevel and Demeyer 1996; LaBrune et al. 2008). However, few studies showed the proportions of 16:0 and 18:0 in milk and meat were unaffected by the diet in farm animals (reviewed in Wood et al., 2007; Schulz et al., 2018). Since both 18:2 and 18:3 are known to be important in protecting against cardiovascular diseases and hyperlipidemia (Henikoff and Comai 2003), the increased level of 18:2 and 18:3 could provide health benefits for consumers of livestock products. In addition, polyunsaturated fatty acids from feed can also undergo further modification in the rumen leading to the production of conjugated fatty acids, which are known to have numerous health benefits (Mir et al. 2004). Further to improving milk and meat for human consumption, increasing polyunsaturated fatty acid content in forages could also benefit livestock production in general since supplementing dairy cattle diets with polyunsaturated fatty acids has been shown to lead to improved reproductive performance (Moallem, 2018; Castro et al. 2019).

Our data indicate that *SDP1*, *APS1* and *PXA1* represent potentially useful targets for a loss-of-function approach in the conventional or molecular breeding of forage legumes to produce genotypes with enhanced lipid content in vegetative tissues. The increased lipid content of these legume forages could increase their energetic value as a feed for cattle and provide an environmental benefit through the reduced production of methane. Enhanced production of 18:3 in these forages could also potentially provide a feed for dairy and beef cattle which leads to milk and meat, respectively, with improved quality. Further research will be required to determine whether the increase in leaf lipid production through the down-regulation of these genes occurs at the expense of protein and or carbohydrate accumulation, or affects other agronomically important traits. In the future, it would also be interesting to explore possible synergistic effects on lipid biosynthesis by down-regulating two or more combinations of the four target genes.

ACKNOWLEDGEMENT

This work was funded in part by Alberta Beef Producers and the Agriculture and Agri-Food Canada Lethbridge Research and Development Centre (LeRDC). We acknowledge the help of Doug Friebel (Forage technician) and Udaya Subedi (MSc student) for their help with sampling and greenhouse trials at LeRDC. RJW is grateful for the support provided by the Canada Research Chairs program.

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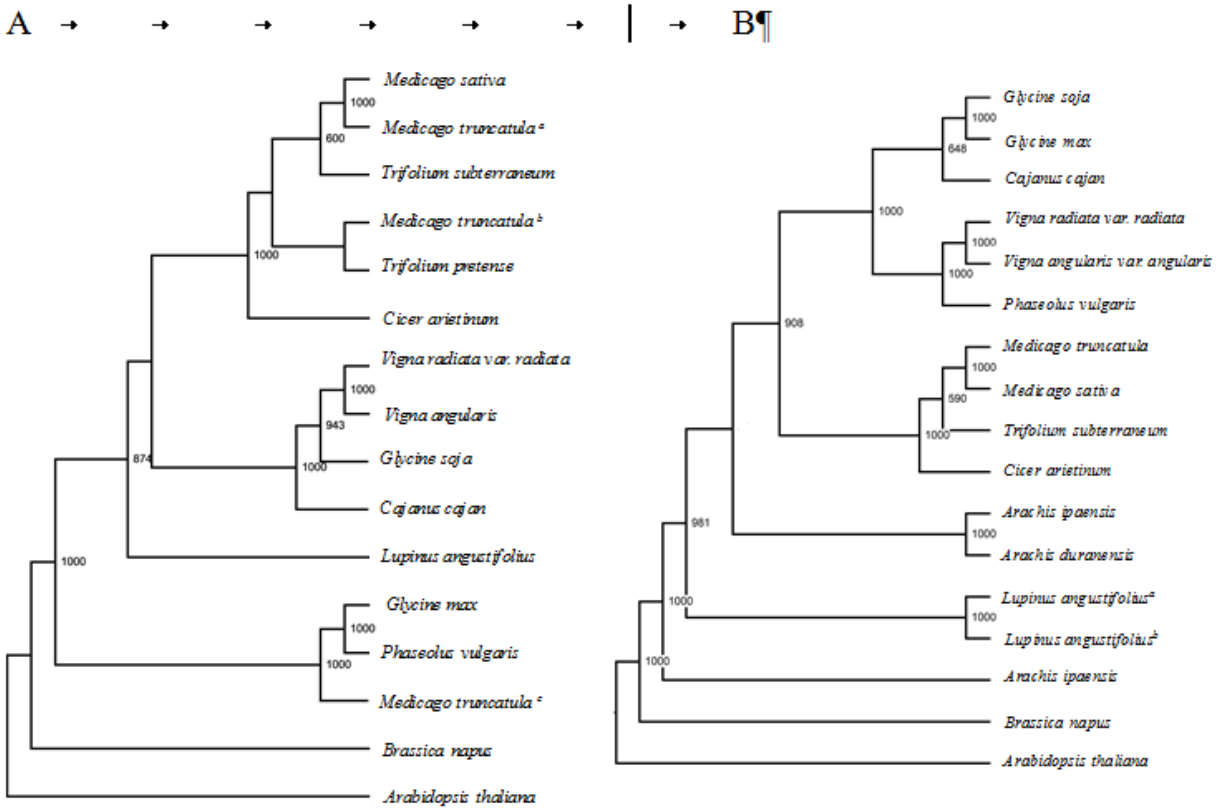
Figure 1. Phylogenetic analysis of predicted SDP1 (**A**), APS1 (**B**), TGD5 (**C**) and PXA1 (**D**) amino acid sequences from legume sequences rooted with their corresponding *Arabidopsis thaliana* homologs as an outgroup. The trees were constructed based on neighbor-joining calculations. The related sequences of *Medicago sativa* (alfalfa) and *M truncatula* (barrel medic) were grouped into the same clade in all four phylogenetic trees.



C → → → → → → → D··¶

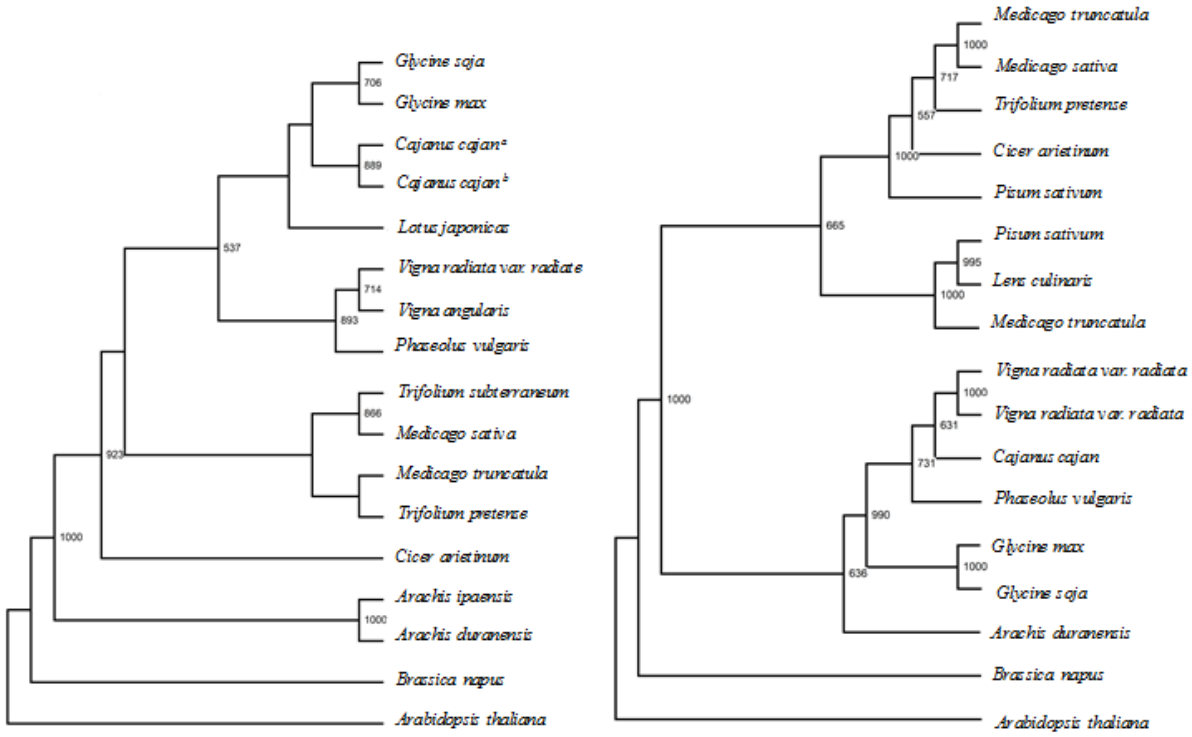


Figure 2. Quantitative real time RT-PCR using primers specific to *MtSDP1*, *MtAPS1*, *MtTGD5*, and *MtPXA1* in plants infiltrated with Agrobacterium harboring pCAPE1 and the indicated pCAPE2 vector constructs compared with empty pCAPE2 vector (EV) controls. Each bar represents the mean ± standard error of 3 technical replicates on each of 4--6 individual plants having pCAPE2 vector transcripts. The highest value of the empty vector in PXA1 was represented as 100%. Hatch marks at the y axis indicate 80 to 0 percentages, respectively. Asterisks indicate significant differences ($P < 0.05$) in the relative transcript levels of selected silenced plants compared with EV control plants.

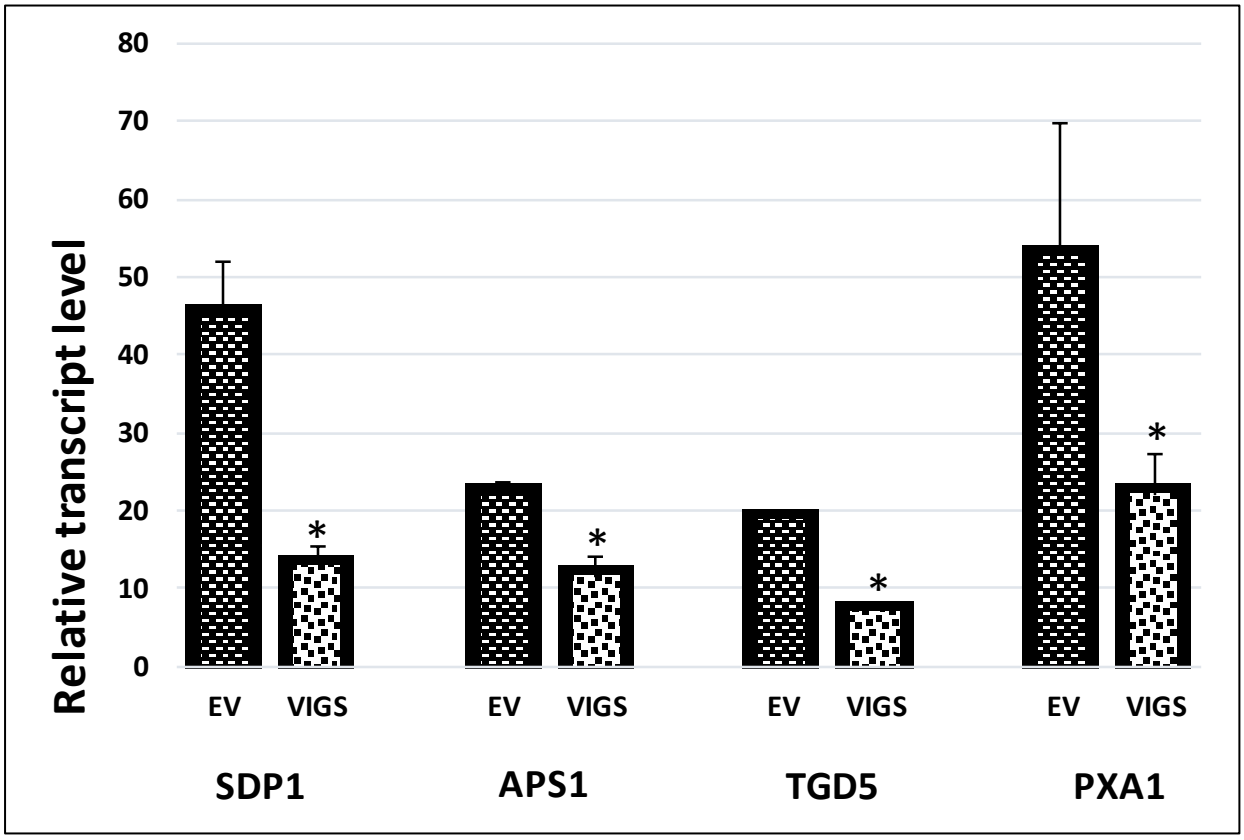


Figure 3. Shoot lipid accumulation in infiltrated *Medicago truncatula* plants. Total lipid content of vegetative tissue as % dry weight (DW) in *M. truncatula* plants exhibiting silencing of *SDP1*, *APS1*, TGD5 and *PXA1* genes, respectively, along with empty vector (EV)-infiltrated controls. Each bar represents the mean ± standard errors of 3 technical replicates on each of 4--6 selected individual plants based on lower gene transcript levels in figure 2. Asterisks indicate significant differences ($P < 0.05$) in the lipid content of the vegetative tissues of selected silenced plants compared with EV control plants.

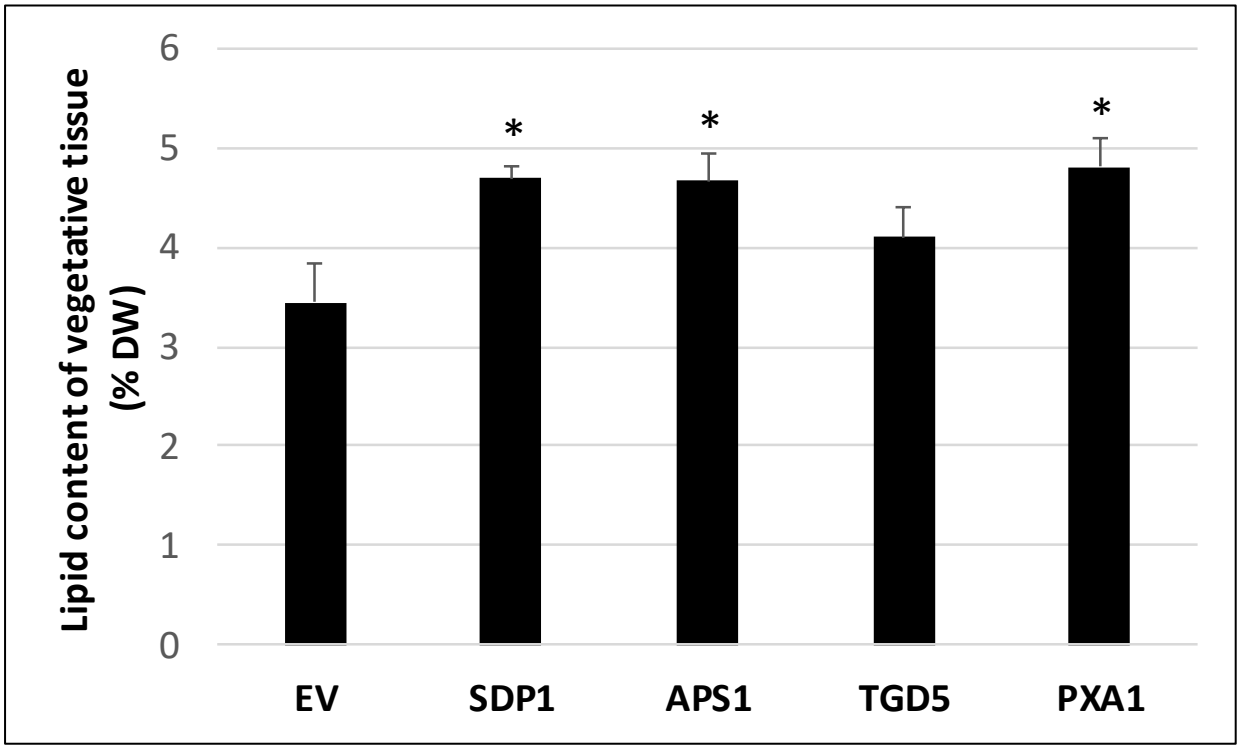


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Table 1. Amino acid sequences used in this study for bioinformatics analyses

Protein	Plant name	Accession number
SDP1	*Medicago truncatula* [a]	Medtr6g080170
	Medicago truncatula [b]	Medtr1g087300
	Medicago truncatula [c]	Medtr7g090470
	Arabidopsis thaliana	XP_020877580
	Cicer arietinum	XP_004514583
	Cajanus cajan	XP_020223686
	Trifolium pretense	PNY12629
	Trifolium subterraneum	GAU29594
	Lupinus angustifolius	XP_019434991
	Glycine max	XP_003521151
	Vigna radiata var. radiate	XP_014514806
	Glycine soja	KHN10036
	Vigna angularis	XP_017415378
	Trifolium subterraneum	GAU29594
	Phaseolus vulgaris	XP_007162133
	Medicago sativa	contig_95605
APS1	*Medicago truncatula* [a]	Medtr5g097010
	Medicago truncatula [b]	Medtr3g082150
	Arabidopsis thaliana	AT5g48300
	Cicer arietinum	XP_004491558
	Trifolium pretense	PNY11360
	Pisum sativum	CAA65540
	Glycine max	XP_014622539
	Cajanas cajan	XP_020208966
	Glycine soja	KHN23805
	Pisum sativum	CAA65539
	Vigna radiata var. radiata	XP_014505054
	Lens culinaris	ACX48912
	Vigna angularis	XP_017430739
	Phaseolus vulgaris	XP_007142479
	Arachis duranensis	XP_015972779
	Medicago sativa	contig_12997
	Brassica napus	XP_013644286
TGD5	*Medicago truncatula*	XM_024779213
	Arabidopsis thaliana	At1g27695
	Trifolium subterraneum	GAU36504
	Trifolium pretense	PNY08556
	Glycine soja	KHN04454
	Glycine max	XP_006578046
	Cicer arietinum	XP_004501534
	Lotus japonicas	AFK35920

	Vigna angularis	KOM42239
	Cajanas cajan [a]	XP_020213413
	Cajanas cajan [b]	XP_020211954
	Vigna radiata var. radiata	XP_014502439
	Arachis duranensis	XP_015935150
	Phaseolus vulgaris	XP_007136739
	Medicago sativa	contig 5113
	Medicago truncatula	XM_003617877
	Brassica napus	XP_013685326
PXA1	*Medicago truncatula*	XM_003601918
	Arabidopsis thaliana	AT4G39850
	Cicer arietinum	XP_012571862
	Cajanus cajan	XP_020224986.1
	Glycine soja	KHN44338
	Lupinus angustifolius[a]	XP_019415077.1
	Vigna radiata var. *radiata*	XP_022634568
	Glycine max	XP_006591509
	Lupinus angustifolius[b]	XP_019437626
	Phaseolus vulgaris	XP_007163644
	Vigna angularis var. angularis	BAT86520
	Brassica napus	XP_013678084
	Arachis ipaensis	XP_020969062
	Arachis duranensis	XP_020988640
	Arachis ipaensis	XP_020975499
	Trifolium subterraneum	GAU43653
	Medicago sativa	contig_65868

Table 2. Primers used to generate VIGS constructs and for quantitative real time RT-PCR assays

Primer	Sequence (5’ -- 3’)
US2F	TGTATTAAAGACATGGAGAGTGGAGTG
US2R	CTTAAAGAACGACCACAAGTACAG
MTtubulinf	GCAATGTTCCGTGGTAAGATG
MTtubulinr	TGTACCAATGCAAGAAAGCCTT
MTEFf	TGATTGAGAGGTCCACCAACCT
MTEFr	CCACCAATCTTGTAAACATC
MtSDP1fwd1	CTACTTCGGCAGATAGGTTCAT
MtSDP1rev1	CTATCGTAGTCATGACTTCGAC
MtSDP1fwd2	CGGTATAAGTAATGAAGCTTCC
MtSDP1rev2	ATATGCTAATTCAGCCCTTACC
MtAPS1fwd1	CAAAATCTTCCATTCTGTGGTCG
MtAPS1rev1	TCCACTAGGAATTAACGCATCC
MtTGD5fwd1	GGATTTGGTTTCGGTGTTGG
MtTGD5rev1	TTGTGGTTGGAGATTTCTGGAC
MtPXA1fwd1	TCGGTTGAATTATCTTCTAGAAAGG
MtPXA1rev1	TTGCTTGATCGAACGGAGTTGC
Qsdp1f	CTAGATGCCTTAACTACTTGACTTC
Qsdp1r	GCCATCAATTCTTGAGCCTC
Qaps1f	CACTGTGGCTGCATTGCCAATG
Qaps1r	TCGACCTTCATAGCTTGCAACTG
Qtgd5f	GTGGGTGTACTTGAAGGTGC
Qtgd5r	CGTACTGTCCTCTTTGATCAC
Qpxa1f	TGCTGAGTCCGTTGCTTTCTTTGGA
Qpxa1r	GTATGAGAAGGTCGCTGAATCTTG

Table 3. Fatty acid composition of total acyl lipids extracted from the vegetative tissue of plants exhibiting silencing of *SDP1*, *APS1*, *TGD5* or *PXA1*, as well as empty vector controls.

Treatment	Fatty acid composition + SE						
	16:0	16:1	18:0	18:1	18:2	18:3	20:0
$MgCl_2$	26.08 ± 0.73	1.50 ± 0.46	4.39 ± 0.18	3.23 ± 0.37	13.64 ± 1.03	48.88 ± 1.72	2.25 ± 0.21
EV	25.23 ± 0.73	1.43 ± 0.46	4.19 ± 0.18	2.98 ± 0.37	14.21 ± 1.03	49.68 ± 1.72	2.25 ± 0.21
SDP1	20.37 ± 0.73*	2.28 ± 0.46	2.42 ± 0.18*	2.01 ± 0.37	12.43 ± 1.03	60.42 ± 1.72*	1.03 ± 0.21*
APS1	23.03 ± 0.73	1.18 ± 0.46	2.83 ± 0.18*	1.81 ± 0.37*	12.60 ± 1.03	57.65 ± 1.72*	0.89 ± 0.21*
TGD5	23.46 ± 0.73	1.39 ± 0.46	3.61 ± 0.18*	2.3 ± 0.37	12.16 ± 1.03	55.22 ± 1.72*	1.83 ± 0.21
PXA1	21.01 ± 0.73*	1.55 ± 0.46	2.80 ± 0.18*	1.93 ± 0.37*	10.48 ± 1.03	61.29 ± 1.72*	0.91 ± 0.21*

Values represent the means ± standard error of three technical replicates of 4--6 silenced plants in each case. Asterisks indicate significant differences ($P < 0.05$) in fatty acid content between plants harbouring each of the four VIGS silencing vectors and the EV control.