SCIREA Journal of Biology

http://www.scirea.org/journal/Biology

October 10, 2016

Volume 1, Issue1, October 2016

Healthy omega-3 enhancement in *Echium acanthocarpum* transformed hairy roots by overexpression of a 6-desaturase gene from *Primula vialli*

Rafael Zárate[1§], Elena Cequier-Sánchez[1,2], Nabil El Jaber-Vazdekis[3], Covadonga Rodríguez[4,5], Roberto Dorta-Guerra[6], Ángel G. Ravelo[1,2]

[1] Canary Islands Cancer Research Institute (ICIC), Ave. La Trinidad 61, Torre A. Arévalo, 7th floor, 38204 La Laguna, Tenerife, Spain.

[2] Bio-Organic University Institute A.G. González, Universidad de La Laguna, Ave. Fco. Sánchez, 38206 La Laguna, Tenerife, Spain.

[3] Institute of Microbiology CAS, Laboratory of Photosynthesis, Novohradska 237, Opatovicky mlyn. Trebon 379 81, Czech Republic.

[4] Animal Biology, Edaphology and Geology Dept. (Animal Physiology Unit), Faculty of Sciences, Universidad de La Laguna, Ave. Fco. Sánchez, 38206 La Laguna, Tenerife, Spain.

[5] Institute of Biomedical Technologies (ITB), Universidad de La Laguna, Campus de Ofra, 38071 La Laguna, Tenerife, Spain.

[6] Statistics and Computation Dept., Maths Faculty, Universidad de La Laguna, Ave. Fco. Sánchez, 38206 La Laguna, Tenerife, Spain

[§] Author to whom correspondence should be addressed, rzarate@icic.es.

Abstract:

Omega-3 long change polyunsaturated fatty acids in higher plants are limited with just a few plant genus showing the accumulation of stearidonic acid (SDA) being also the longest and more unsaturated omega-3 fatty acid present. *Echium acanthocarpum* has been proven to be an efficient and attractive producer of SDA. Improved production of this fatty acid was attained by overexpression of a *6-desaturase* gene from *Primula vialii* in transgenic *E. acanthocarpum* hairy roots. In this transgenic line, a drastic reduction of the substrates LA (linoleic acid) and ALA (-linolenic acid)(40 and 30%, respectively) was parallel to the dramatic increase in GLA (-linolenic acid) and SDA in the total fatty acids extracted. Especially, SDA reached a percentage of 4.7% of total fatty acids, demonstrating the successful manipulation of this biosynthetic pathway in *E. acanthocarpum* hairy roots by overexpression of this gene. The temperature per se, was also a highly influential factor governing the fatty acid profiles in this novel transgenic hairy root culture. In terms of absolute values, the data were even more evident, due to the significant increase in total lipid extracted from the transgenic hairy root. The amount of SDA and GLA was increased 7.5 and 3 fold, respectively, compared to the control. In this transgenic culture, decreasing the culture temperature influenced directly the increments of polyunsaturated fatty acids, but did not affect lipid classes except when this factor interacted with the overexpression of the *P. vialii* 6-desaturase gene. The activation of the transgene did modify significantly the phospholipids, phosphatidylglycerol and phosphatidylcholine, whose percentages were significantly higher in these cultures compared to the control.

Keywords: *Echium acanthocarpum*, fatty acids, hairy roots, omega-3, overexpression, stearidonic acid, polyunsaturated fatty acids.

1. Introduction

The importance of lipids and fatty acids has been largely reported, highlighting their functions as structural components of cell membranes, energy sources, eicosanoids precursors, and activators in signalling pathways; as well as their important roles in human health and nutrition [1-6].

Higher plants have limited capability of producing omega-3 or n-3 long chain polyunsaturated fatty acids (LC-PUFAs), with just a few genus from Boraginaceae, Primulaceae and Saxifragaceae families, particularly *Echium* (*Boraginaceae*) well represented in the Canary Islands with 23 endemic species, displaying the accumulation of SDA (stearidonic acid, 18:4n-3), as the longest carbon chain and most unsaturated n-3 fatty acid found in higher plants [7-10]. In the biosynthesis of PUFAs a crucial and frequent enzyme is the 6-desaturase (EC 1.14.19.3), which introduces a double bond in the carbon change by means of an electron dependent desaturation mechanism, between the carboxyl group and the present instauration of the fatty acid acting as a substrate. In the very few examples of higher plants displaying this enzyme, it only carries out the addition of a double bond in the 6 position of the two substrates, either the omega-3 alfa-linolenic acid (18:3n-3, ALA) or the omega-6 linoleic acid (18:2n-6, LA) resulting in the synthesis of SDA and -linolenic acid (GLA), respectively. Unlike animals, mainly fish, and algae where the substrate 24:5n-3 is present but not in higher plants, this is also a natural substrate of this 6-desaturase, recording competition between these two 6-desaturase enzymes [11], resulting after several biosynthetic steps in the formation of the attractive n-3 fatty acid DHA (22:6n-3, docosahexaenoic acid) (Sprecher ś shunt) [12].

Different *6-desaturase* genes from practically all kingdoms have been identified, and an attractive number of them have been expressed in plants or other organisms. For instance, from *Borago officinalis* [13] which has even been expressed in mammals' cells [14], *Anemone leveillei* [15], three species of the *Echium* genus [16, 17], *Marchantia polymorpha* [18], species of *Primula* genus [19, 20]; from fungi such as *Pythium splendens* [21], *Pythium irregulare* [22], and *Mortierella alpine* [23]; from phytoplankton species; *Phaeodactylum tricornutum* [24], *Emiliania huxleyi* [25], from mosses; *Ceratodom purpureus* [26] and *Physcomitrella patens* [27]; from algae: *Ostreococcus tauri* [28] and *O. lucimarinus* [29]; from fish: *Scatophagus argus* [30], *Oreochromis niloticus* [31].

Several studies report on the overexpression of this gene in plants for the production of SDA with various results as cited in this article [32, 33], and accounts of the successful accumulation of fish oils in plants [34]; nonetheless, to the best of our knowledge, there are no accounts on the manipulation of 6-desaturase in hairy roots of higher plants for the improvement of SDA yields.

SDA is gaining increasing attention and its commercial demand is on the rise. It could be used in the treatment of several diseases i.e. alleviation of treatment of acne, skin drying, alcoholism, inflammation, thrombosis, rheumatoid arthritis, hypertriglyceridemia, atopic dermatitis, and prostate and other cancer types [35]. Moreover, following SDA feeding increased the level of EPA in red blood cells albeit the reduced ability of our biochemical machinery to synthesize EPA from shorter carbon change and low unsaturated n-3 fatty acids such as ALA, conversion that seemed to be more efficient when SDA, a closer precursor within the pathway, was fed [36, 37].

In previous reports, we have described the establishment of *E. acanthocarpum* hairy roots as an amenable culture system for fatty acid studies and production of the particularly unusual PUFAs; stearidonic (SDA; 18:4n-3) and -linolenic acids (GLA; 18:3n-6) of increasing pharmacological interest, mimicking and boosting their accumulation in this higher plant [38, 39]. Here we have made a further attempt to direct its lipid metabolism and boost the accumulation of SDA by establishing a transgenic hairy root culture overexpressing a *6-desaturase* gene from *Primula vialii*. This desaturase displays a strong marked preference, almost exclusively for its n-3 substrate i.e. -linolenic acid (ALA), unlike the rest of the characterised 6-desaturases from plants, which indistinguishable employ either the n-6 substrate linoleic acid (LA) or LA, resulting naturally in the production of GLA or SDA, respectively. It has been suggested that this n-3 substrate preference could be due to the presence of an additional sequence of 221 base pairs located before the histidine boxes highly conserved in all 6-desaturases [19]. Surprisingly, this additional sequence did not contain the typical sites for restriction and insertion, but 10 identical flanking base pairs, suggesting that it appeared to be a mobile genetic element.

This particular preference for its n-3 substrate ALA has been exploited here, as a very attractive characteristic to divert the fatty acids biosynthetic route towards the desired n-3 branch, and thus boosting the production of SDA in this novel transgenic hairy root system as presented in this report.

2. Materials and methods

2.1 Plant Material

The transgenic *E. acanthocarpum* hairy root culture was established following a similar protocol as described previously [38, 39] employing seedlings germinated *in vitro* from surface sterilised seeds donated by Jardín Botánico Viera y Clavijo (Gran Canaria, Spain). Treated seeds were germinated *in vitro* on a solid B5 medium [40], supplemented with 3% sucrose, 3-4 mg/L GA_3 (gibberelic acid), and solidified with 0.7% agar, with the pH adjusted to 6.0 prior to autoclaving (115 ℃, 1 atm. pressure, 15 min.), contained in Petri dishes (90 mm diameter), and cultured in the dark until beginning of germination. After germination, plants were transferred to the same solid nutrient medium (25mL) without the addition of GA_3, contained in translucent glass jars covered with a plastic lid (175 mL capacity) (SIGMA-ALDRICH, St. Louis, MO, USA), which were placed under light conditions (16 h photoperiod and irradiance of 35 mmol m^2s^{-1}, supplied by cool-white fluorescent tubes), and a temperature of 25 ± 2 ℃ to allow further plant growth.

Subsequently, 50-60 day old *in vitro* growing plants were employed for guided infection with *Agrobacterium rhizogenes* strain LBA1334 harbouring the plasmids pSoup and pGreen- 6des construct containing the *6-desaturase* encoding gene from *P. vialii* by repeatedly stabbing the internodal stem areas of the plants with a fine needle containing bacteria in the presence of or absence of acetosyringone (25 M) [38, 41]. After 25-30 days, hairy roots of 3-4 mm in length had developed which were aseptically excised, and transferred to B5 liquid medium, containing the antibiotics cefotaxime (200 mg/L) to remove the *A. rhizogenes*, as well as 1% of the antioxidant polyvinylpyrrolidone (PVP) for several subcultures. Finally, actively growing bacterium-free transgenic hairy roots (0.25-0.30 g) were cut into small segments and routinely cultured and refreshed in Erlenmeyer flasks (250 mL), containing 30 mL of sterile B5 liquid medium supplemented with 3% sucrose, 1% of PVP and kanamycin for selection of transformants, from 10 to a final concentration of 30 mg/L for several subcultures, sealed with a double layer of aluminium foil, and placed on an orbital shaker at 95 rpm, at a temperature of 15±2 ℃ in the dark.

2.2 Construction of transformation vectors and cloning of 6-desaturase gene from Primula vialii

The *P. vialii 6-desaturase* gene sequence 1,368 base pairs (GenBank accession number AY234127) was synthesized by GENSCRIPT CORP (New Jersey, US) following gene

sequence optimization for its expression in plants based upon the relative appearance of codons in its amino acids' sequence, inserted in a pUC57 plasmid and served.

The binary vector employed for the introduction of this transgene, first in *Agrobacterium rhizogenes* strain LBA1334, and subsequently in the plant tissue was a modification of the pGreen0029 plasmid which requires the helper plasmid pSoup for its replication in *Agrobacterium* following codification of a replicase (RepA) (John Innes Centre, Norwich, UK). For the construction of the modified pGreen0029, first a 35S-CaMVpolyA cassette of 672 bp was obtained following digestion with EcoRV of a pGreen plasmid harbouring the cassette. Then it was inserted in pGreen0029 at the sites EcoRV and SmaI. This asymmetric digestion allowed the removal of the restriction site EcoRI from pGreen0029, which would interfere with subsequent cloning steps, thus obtaining plasmid pGreen0029-1A (Figs. 1-3). The *E. coli* transformants containing this construct were selected on LB medium supplemented with the antibiotic kanamycin and X-gal substrate for distinguishing blue colonies. Since the insertion was performed with blunt ends, the correct insertion orientation was checked by partial digestion with the restriction enzymes NotI, EcoRI and HindII followed by gel electrophoresis, as well as after sequencing using the primers pGreen0029-Fw and pGreen0029-Rv (Annex I) which allowed reading the point of insertion of the promoter and terminator, respectively.

Finally, the transformation of the *A. rhizogenes* strain LBA1334 with the pGreen0029-1A 6des construct was achieved after electroporation [42, 43]. Initially, *A. rhizogenes* competent cells were slowly defrosted in ice and 40 L taken for transformation by adding plasmid DNA from the two plasmids required for the pGreen system (200ng pSoup, 200ng pGreen00291A-6des) which was gently mixed and incubated in ice for 10 min. The mixture was aseptically transferred to a cold electroporation cuvette of 0,2 cm in length between the electrodes (BIO-RAD, CA, US), applying different pulses and times (2.5Kv, 4ms, 25 FD, 400W, once or twice) employing a MicroPulser Electroporator (BIO-RAD, CA, US).

Following electroporation, 1 mL of cold yeast mannitol broth (YMB) growth medium without antibiotics was gently added and transferred to a sterile 1.5 mL Eppendorf tube and kept at room temperature for 3-4 min. Then the tube containing the transformed solution was incubated at 25℃ for 3-4 hours in the dark without shaking. Finally, the transformed cells incubated mixture was spread on a 90mm Petri dish containing 20mL

of agar solidified YMB medium supplemented with different antibiotics for selection of the correct transformed cells (i.e. rifampicin (20mg/L) and spectinomycin (100mg/L) for the selection of *A. rhizogenes* LBA1334, tetracycline (10mg/L) for selecting the presence of the pSoup helper plasmid, and kanamycin (30mg/L) for selecting the presence of the pGreen- 6des plasmid), at 25°C in the dark for 2-3 days. Then the presence of the correct construct and the *6desaturase* gene in the transformed *A. rhizogenes* was determined by means of PCR, using the corresponding primers ie. Prom 35S Fw and Prom 35S Rv (Annex I), and ready for its used for hairy root induction as described above.

2.3 Extraction and quantification of RNA by RT-PCR and cDNA synthesis from Echium acanthocarpum hairy roots overexpressing 6 desaturase.

For gene expression studies *in vitro* grown hairy roots were collected taking 100mg for RNA extraction initially freezing by immersion in liquid nitrogen and ground with a sterile mortar and pestle. The powdered material was extracted employing an RNeasy Plant kit (QIAGEN, Hilden, Germany) following the manufacturer instructions. Extracted RNA was stored at -80 ℃ until use. Two negative controls were also RNA extracted from *E. acanthocarpum* leaf tissue taken from *in vitro* growing plants, and another hairy root line not overexpressing this *6-desaturase* gene [39].

In order to obtain complementary DNA (cDNA), the synthesis of first strand cDNA was performed using the commercial kit RetroscriptTM (Ambion Austin, USA), starting from 1 g of total RNA from *E. acanthocarpum* hairy roots overexpressing *6-desaturase*. The random sequence primers used were supplied by the kit (random decamers). The obtained cDNA was used as template in RT-PCR amplifications. These were performed using the RT-PCR iCycler iQ detection system, employing the iQ SYBR Green mix (BIO-RAD, CA, USA) and the primers RT-Fw2 and RT-Rv2 for the *P. vialii 6-desaturase* gene. In order to correct the possible variations in the total cDNA amount used in each reaction, the internal control housekeeping gene *gapdh* from *Arabidopsis thaliana* (AT1613440) encoding the enzyme GAPDH (glyceraldehyde-3-phosphate-dehydrogenase), was used together with the primers GADPH-Fw and GADPH-Rv (Annex I).

Considering that the inserted *6-desaturase* gene in the hairy roots does not contain introns and in order to avoid potential gene amplification of the native *E. acanthocarpum 6-desaturase*, primers were designed such that the amplification

should correspond to an area of the gene with the smallest possible similarity between homologous genes. Furthermore, it was experimentally proved that the primer pair was not able to amplify any product using as template genomic DNA or cDNA from leaf or control hairy roots. Likewise, to confirm that amplification did not occur due to possible contamination of the samples by genomic DNA, a PCR of each studied sample was performed with the primers RT-Fw2 and RT-Rv2, with 1 g RNA as a template, under the same conditions as for real-time PCR. Pretreatment with DNAse I was avoided, given the low concentration of RNA samples obtained from the harvested 6-desaturase *E. acanthocarpum* hairy roots (5-12 g).

The relative amounts of mRNA were calculated by the Ct method from the average of three independent estimates of the threshold cycle or Ct of each amplified gene. Finally, to confirm the specificity of the amplification reactions, the products obtained by PCR or by RT-PCR were analyzed by gel electrophoresis in 2% agarose.

2.4 Lipid extraction and transesterification of lipids

Harvested hairy roots were separated from the liquid nutrient medium by vacuum filtration through filter paper, weighed and lyophilised at -80 ℃ for 24h using a freeze-dryer (Christ Alpha 2-4, Osterode, Germany). Each sample was powdered using a mortar and pestle with liquid nitrogen. After homogenisation, total lipid was extracted and quantified following the method previously described [38, 44, 45].

Subsequently, acid catalyzed transesterification was performed in lipid aliquots (2 mg) with 1mL toluene, employed to ensure that the neutral lipids were dissolved, plus 2mL of a mixture of MeOH/1% H_2SO_4, and incubated in a capped glass test tube at 50 ℃ for 16 h [46]. Prior to transmethylation, heneicosanoic acid (21:0) (2.5% of the total lipid analysed, 50 μg), was added as internal standard to the lipid extracts. Transesterification was conducted as previously described [38, 44, 45]. Silica gel G-25 glass sheets (MACHEREY-NAGEL, Germany) were employed for preparative thin layer chromatography, developed with a solvent system composed of hexane/diethyl ether/acetic acid 97.7% (90:10:1, by vol), and visualized after brief sublimation of iodine with slight heat, conducted for the isolation and purification of the fatty acids methyl esters (FAMEs). These moved close to the solvent front, and were then scrapped off the glass sheet and extracted with 10mL of the mixture hexane/ethyl ether (1:1, v/v) and dried under nitrogen. Finally, the samples were dissolved in 0.5-1.0mL hexane, and kept under nitrogen in sealed glass vials at -20 ℃ until analysis.

2.5 Gas chromatography of FAMEs

A Shimadzu GC-14A apparatus (SHIMADZU, Japan), equipped with a flame ionization detector (250°C), a Supelcowax™ 10 fused silica capillary column (30m × 0.32mm ID), employing helium as carrier gas was employed for analysis and quantification of FAMEs. Samples (0.6μL) were injected into the system by an on-column auto-injector (SHIMADZU AOC-17) at 50 ℃. A temperature program of 180 ℃ the first 10 min, followed by an increase of 2.5 ℃/min until reaching 215 ℃, was employed for separation of the compounds.

FAMEs were identified according to their RT compared with standards of individual commercial FAMEs (linoleic acid methyl ester, methyl -linolenate, methyl oleate, stearidonic acid methyl ester, and heneicosanoid acid), and a well-characterized fish oil mix. They were quantified according to the amount of 21:0 added as internal standard prior to transmethylation, and by comparison with a calibration curve created with the individual standards.

2.6 Statistical analysis

Results are present as the means and standard deviations of three replicates for each sampling time. The data were checked for normal distribution by one-sample Kolmogorov-Smirnoff test, as well as for homogeneity of the variance with the Levene test, and when necessary, Bartlett test was also applied. When variance was not homogeneous, Kruskal-Wallis and Games-Howel tests were conducted to assess statistical differences.

The effects of culture conditions and fatty acid levels were firstly determined using one-way ANOVA-test ($p<0.05$). The percentages and total amounts of fatty acids, particularly the contents of GLA and SDA in the different cultures were included as variables in a principal component analysis (PCA). Principal components were subsequently analysed by two-way ANOVA to study the combined effects of both factors, fatty acid profiles and stress conditions, as well as their interconnections. Statistical analyses were performed employing the SPSS software (versions 15.0 and 17.0, SPSS Inc, IL, USA).

3. Results

This work aimed at genetic engineering the biosynthetic pathway of PUFAs in a transgenic *E. acanthocarpum* hairy root system acting in one particular step by overexpressing the *6-desaturase* gene from *P. vialii* to boost the accumulation of SDA (Fig. 4). Initially, the cloning steps and *A. rhizogenes* transformation were conducted, followed by the establishment of this transgenic culture system.

After the cloning steps for the construction of the appropriate genetic plasmid, the successful transformation of *A. rhizogenes* LBA1334 strain was achieved following electroporation, and the resulting transformants were selected by the addition of the four chosen antibiotics described earlier. PCR characterization of these transformed *A. rhizogenes* using the primers Prom 35S-Fw and Prom 35S-Rv gave positive results in all seven transformants studied.

Regarding the induction of hairy roots, the best results were obtained after infection with *A. rhizogenes* clone 7.1 with the addition of acetosyringone (75%), and 57% when it was not added. Furthermore, clone 3.1 with the addition of acetosyringone and ultrasound treatment showed a 50% hairy root induction, but appearing more effective clone 7.1 (Table 1). Moreover, in some instances at the point of infection the formation of undifferentiated tissue was recorded, and in other instances even the death of plants occurred due to bacterial infection.

Following this induction, hairy roots were established and cultured in liquid medium assaying the successful insertion of the gene construct by PCR employing two primers set 6EcoRI-Fw and 6EcoRI-Rv, and nptII-Fw(b) and nptII-Rv(b) (Annex I) and as template plant genomic DNA extracted from these hairy roots which resulted in the amplification of the two expected products of 1,380 and 499 bp (data not shown). Transgenic hairy roots were grown in the above mentioned liquid B5 medium, at two different temperatures 15 and 25 ℃.

Depending on the growth temperature, cultures were allowed to grow to reach their stationary phase at different times; thus, the time course needed ranged from 25-30 days for cultures grown at 25 ℃ and 70-80 days for those ran at 15 ℃. The maximum hairy root biomass at the stationary phase of growth increased 6-fold compared to the initial inoculum (Table 2).

Lipid studies were also conducted with these cultures. Total lipid values were higher and statistically different for the transgenic culture at 15 ℃ compared to control at both temperatures. Furthermore, when comparing the data for the transgenic culture at both growth temperatures, there was not significant difference (Table 3)

Similarly, lipid class composition analyses also displayed significant differences when 6-desaturase was being overexpressed. The polar lipids phosphatidylcholine (PC), phosphatidylglycerol (PG) and phosphatidylethanolamine (PE) varied at the two sampling points, and with respect to the control cultures (Table 4).

In order to statistically analyse the distribution of lipid classes, data from the transgenic and control cultures obtained at different growth temperatures were studied to thus evaluate the effect of the temperature, as well as the overexpression of the transgene. A principal component analysis with the percentage values of lipid classes was conducted obtaining two components, PC1 and PC2 explaining 86.28% of the variance. PC2 (21.11% of the variance) was positively correlated with PS+PI and PE, while PC1 (65.17% of the variance) was positively correlated with PC, PG and polar lipids, but was negatively correlated with neutral lipids. This would also indicate that PC1 was correlated with the richness of PC and PG, while PC2 referred to the amounts of PS+PI and PE in the hairy roots (Table 4 and Fig. 5).

Following removal of the principal components PC1 and PC2, we conducted a two - way ANOVA test in order to determine whether the studied factors i.e. overexpression of *6-desaturase* gene and growth temperature, and their interaction, had influenced on the extracted new variables PC1 and PC2 (Table 6).

As shown in Table 6, only the first factor significantly affected PC1 and PC2, while the interaction of both acted significantly on PC2. The majority of the studied lipids had been influenced by the presence of the transgene or by the interaction of this factor with the growth temperature. As displayed in Table 4, the percentage of PC and PG (PC1) present in the transgenic hairy roots grown at 15 ℃ increased by about 50% compared to the control culture at the same temperature. On the other hand, the increase in PE (PC2) in the *6-desaturase* overexpressed culture was evident but less marked, increasing from 10% to 14%. Similarly, a significant decrease in neutral lipids was recorded for the transgenic sample compared to control cultures, unlike polar lipids, which increased in the former. Thus, when the data were plotted based on the

components and stratified according to the presence or absence of the transgene, a clear separation between the groups was observed (Fig. 6). However, when this representation was stratified by growth temperature (15 vs. 25 ℃), a large overlap between the groups was observed (data not shown).

In order to evaluate the effect of the overexpression of the *6-desaturase* gene on the absolute content and relative fatty acid profile, quantification by GC-MS was conducted. Among the saturated fatty acids in the transgenic culture, 16:0, 18:0, 20:0 and 24:0 were more abundant, these were larger compared to the control data except for 16:0, and with low amounts of 14:0 and 22:0. Moreover, relatively higher percentages of monounsaturated fatty acids (18:1n-9 and 18:1n-7) were registered in the control. The most characteristic fatty acids were those of the n-6 series, LA and GLA showed the largest percentages; LA was larger in the controls, contrarily GLA was higher in the transgenic hairy roots, ranging from 20-29%, almost doubling the amounts in the control at 25 ℃ and 1.5fold at 15 ℃. On the other hand, the n-3 PUFAs, ALA and SDA, accounted for approximately 5-6% of total fatty acids. ALA, the substrate of 6-desaturase enzyme, was higher in the control; moreover, SDA, the target n-3 fatty acids was much larger in the transgenic culture 2.2 fold (Fig. 7; Table 7).

There was a drastic drop in ALA, the substrate of the overexpressed 6-desaturase was metabolized by this enzyme and got converted into SDA, remaining only 33.8% of this substrate (Table 7). On the other hand, the total saturated fatty acids were higher in the transgenic culture (ca. 36-38%) due to a slight increase in some of the minor ones (18:0, 20:0, 22:0 and 24:0). The monoenes also decreased from 9-11% of total fatty acids to 4.1-5.4% in the transgenic culture (Table 7).

Both 6-des (n-6) and 6-des (n-3) indexes were higher for the transgenic culture accordingly to the larger content of GLA and SDA, influenced by the overexpression of this transgene (Table 7). Furthermore, the double bond index (DBI) values were similar for the two cultures (Table 7).

The amounts of fatty acids were also quantified to determine the effect of the overexpression on the accumulation of these compounds, particularly the SDA and GLA. In the transgenic culture, SDA reached 538.9-600.2 g/gDW of fatty acids when grown at 15 ℃ (Fig. 8a), which corresponded to a 7.5-8.5fold increase compared to control at 25 ℃ and 2fold when grown at 15 ℃. GLA was clearly abundant in the transgenic culture with values of 3,500 g/g DW when grown at 15°C and ca. 2,000

g/g DW at 25 ℃; moreover, the control line also showed high amounts of this fatty acid (Fig 8b).

As for the n-6 substrate fatty acids of the 6-desaturase enzyme, as expected and although the overexpressed enzyme shows preferences for the n-3 substrate, LA achieved lower values in the transgenic culture compared to control, as their percentage decreased in the total set. Likewise, ALA only reached the amount of 152.53 to 252.98 g/g DW in the transgenic due to the activity of the transgene and in the control culture this amount was higher.

The differences between the absolute values of each fatty acid are due to the increase in total lipids, from 35 to 47-57 g/g DW (Table 7). This, in turn, was also reflected in the significant increase in polar lipids, which rose from constitute 20-35% of total lipids in control cultures to 41-53% range in the transgenic hairy root (Table 4).

With all these collected data, a comprehensive principal components analysis was performed in order to determine the influence of the overexpression of the *6 desaturase* gene in the transgenic hairy roots on mainly the production of SDA and GLA, analysing the variable (fatty acids) and growth temperature of both the control and transgenic cultures. Two principal components, PC1 and PC2, accounting for 85.21% of the variance, were obtained. PC1 (58.40% of the variance) was correlated with almost all the unsaturated fatty acids except with 18:1n-9, and with the two minor saturated fatty acids, 20:0 and 22:0 (Fig. 9, Table 8). Interestingly, PC1 was negatively correlated with the fatty acids substrates of the 6-desaturase enzyme, LA and ALA, and 18:1n-9, while it was positively correlated with its reaction products i.e. GLA and SDA, and with minor saturated fatty acids, 14:0, 20:0 and 22:0. Furthermore, PC2 which accounted for 26.80% of the variance, was positively correlated with the saturated fatty acids 16:0 and 24:0, and the monoene 18:1n-7.

Thus, PC1 was an image of the activity of the studied enzyme. Large values of PC1 indicated a high enzyme activity, reflected by an increase in the percentages of its products, GLA and SDA, and a drop in its substrates, LA and ALA. In addition, PC1 was associated with some saturated fatty acids, which also increased in the transgenic hairy root following the engineering and manipulation of this enzyme within the biosynthetic pathway (Table 8).

When the ANOVA test on the main components was applied, it was observed that there was a significant influence on both the type of hairy root (presence or absence of the transgene *6-desaturase*), and temperature, on the main components ($r \leq 0.05$) (Table 9). Thus, it was confirmed that the insertion of the transgene in the genome of *E. acanthocarpum* had significantly changed the fatty acids profile. This occurred both at the level of the target fatty acids (LA, GLA, ALA, SDA) represented by PC1, as well as the level of other fatty acids belonging to this metabolic pathway encompassed by PC2. When the hairy roots are plotted depending on the components (Fig. 10a) and stratified according to the growth temperature used, a very clear separation between the groups is obtained, as well as a division of the groups by the temperature (Fig. 10b).

Finally, regarding the gene expression of *6-desaturase* from *P. vialii*, after evaluating and confirming the activity of the gene and its effect on the fatty acids profiles of the transgenic cultures, RT-PCR was employed. The use of this technique took into account also the endogenous expression of the native *E. acanthocarpum* *6-desaturase* gene, whose gene sequence is unknown and would also contribute to the formation of SDA and GLA. Moreover, the manipulated gene from *P. vialii* did not contain introns, a feature that was exploited in the particular design of the set of primers utilized. For this, a less conserved region of the gene not corresponding to a characteristic domain of this transgene, as those rich in histidine (histidine boxes) [19] was selected using the Pfam software program, choosing the region of 255 to 423 base pairs of the gene corresponding to the amino-acids 85 to 141. Other *6-desaturase* gene sequences from *E. sabulicola* [47], *E. gentianoides* and *E. pitardii* [16] and *E. plantagineum* [17] available in GenBank database were studied, as well as their promoter regions [48]. Furthermore, a comparison of all these sequences was conducted with ClustalW2 software, locating primers in the regions with lower homologies among them to thus avoid amplification of the native *E. acanthocarpum* *6-desaturase* gene (Fig. 11).

Culture samples of the transgenic hairy roots were grown at 15 and 25˚C and harvested after 60 and 80 days, the control hairy roots were grown at 25˚C and collected after 20 days. Total RNA was extracted and first strand cDNA was synthesized, and used as template for two amplification reactions. The first reaction using the primers RT-Fw2 and RT-Rv2 (Annex I) (Ta 55 ℃, t_{ext} 30s) amplified a fragment of 136 bp, which corresponded to a region of the ORF of the *6-desaturase* transgene. The second

amplification employed the primers GAPDH-Fw and Rv-GAPDH (Annex I) (Ta 54 ℃, t_{ext} 30s), generating a fragment of 200 bp.

In both amplifications, the resulting cDNA fragments were checked in 2% agarose gel electrophoresis and also visualizing the –d(RFU)/dT curves (Fig. 12). The relative amounts of mRNA were calculated by the Ct method from the mean of three independent estimations of threshold cycle (Ct) of each amplified gene. The relative expression of *P. vialii 6desaturase* was quantified as the increase in the ratio of the mRNA levels of *6desaturase* and *gadph* in the samples with respect to the same ratio on the control hairy roots grown at 25 ℃.

In the studied cases, the ratio mRNA *6-desaturase/gadph* for the transgenic hairy root showed an increase in the first phase of culture when grown at 15 ℃ and sampled at day 60 which was 2 fold compared to the same hairy root but grown at 25 ℃ and sampled at day 20 (Fig. 13). After day 60, this ratio dropped to levels lower than the basal levels, corresponding to half of these basal levels.

4. Discussion

In previous reports, the fatty acid profiles of *E. acanthocarpum* hairy roots and the optimal culture conditions for the production of the *6-desaturated* fatty acids, GLA and SDA, studying the effect of different abiotic and biotic stress have been reported. These established the appropriate culture nutrient medium, and more importantly that lowering the growth temperature of *E. acanthocarpum* hairy roots culture from 25 to 15 ℃, clearly boosted the accumulation of SDA, demonstrating the activation of the native 6-desaturase enzyme [38, 39].

Here, the influence of the overexpression of the *6-desaturase* gene from *P. vialii*, a coding enzyme showing a marked preference for its n-3 substrate fatty acid, i.e. ALA, in a transgenic *E. acanthocarpum* hairy root culture was investigated. Phenotypically, there were not differences between the transgenic hairy roots and the control hairy roots, reaching also similar values of fresh weight for both cultures (Table 2), although the increase in fresh weight was slower in the transgenic culture, likely due to the presence of the antibiotic kanamycin in the nutrient medium as observed in other cultures of this species (data not shown), or for the insertion of the gene in its DNA, as reported by Zhang [49] expressing the *fad3* and *fad8* genes in tobacco.

Regarding the fatty acid profiles and the lipid composition of this *E. acanthocarpum* transgenic hairy root system, and in order to make direct comparisons with other genetically modified plant oils, it is important to consider that in this species, the roots are not the strict and major storage organ since larger amounts of fatty acids are present in its seeds and leaves. Nonetheless, in this report together with the cited work [38, 39] demonstrated the capacity and usefulness of these organs and hairy roots to synthesize and accumulate fatty acids, being an attractive and amenable model for studying the biosynthesis and metabolic pathway of these metabolites *in vitro* in this species, known for its rare capacity to further biotransform LA and ALA into GLA and SDA, respectively catalyzed by the enzyme 6-desaturase.

The data presented show the successful manipulation of the metabolic pathway following the overexpression of this transgene, with the enzyme substrates LA and ALA being clearly reduced by 40 and 30% with respect to the control. LA comprised 18-22% of total fatty acids, while ALA displayed 1.42-1.80% of total fatty acids (Table 7), similar to the overexpression of a 6-desaturase from *Pythium irregulare* in *Brassica juncea*, which resulted in the reduction of LA and ALA to 10 and 5%, respectively [22]. In *E. acanthocarpum* transgenic hairy roots, this decrease was parallel to an increase in the 6-desaturated products, especially SDA that reached 4.70% of total fatty acids, a 4-fold increase compared to non-transgenic hairy roots grown at 25 ℃ and 2-fold increase when cultured at 15 ℃ (Table 7).

Despite the fact that the used 6-desaturase from *P. vialii* displays preference for the n-3 substrate of the enzyme; surprisingly here, GLA, the n-6 product of the enzyme in the transgenic culture, increased to values of 20-29% of total fatty acids, corresponding to double the values of GLA in the control non-transgenic hairy roots cultured at 25 ℃, and 1.5 times when cultured at 15 ℃. When presented in absolute values, the increase in the target metabolites SDA, and to a lesser extent GLA, was 7.5 and 3-fold compared to the non-transgenic culture, respectively (Figs. 8a-b).

There are a number of records overexpressing 6-desaturase genes in different plant species, but those overexpressing this gene from *Borago officinalis* [13, 50-54], from various *Echium* species [16, 17], and from *P. vialii* [32], are more comparable to the data presented here. In all these studies, an increase in the 6-desaturated products was recorded, although none of these species harboured a native or endogenous 6-desaturase enzyme, except for the case in which this was cloned and overexpressed in

evening primrose, reaching considerable values of SDA, 11%; although GLA was still higher with 17% of total fatty acids [54]. In those instances in which the gene was cloned from *Echium*, it markedly increased the GLA content, with a maximum of 20.7% of total fatty acids and 9.4% of SDA in leaves of transgenic tobacco overexpressing this transgene from *E. plantagineum* [17], but with trace amounts when overexpressing the transgene from a Canary Island *Echium* species, likely due to the strong preference of this enzyme for the n-6 substrate [16, 47]. It was also suggested that the amount of ALA could also be a limiting factor for the synthesis of SDA [17], beyond the greater or lesser presence of a high and/or specific activity of the 6-desaturase enzyme. This was the case of the *6-desaturase* gene from *P. vialii*, given that in *E. acanthocarpum* transgenic hairy roots following the action of the manipulated 6-desaturase enzyme, ALA was reduced to baseline levels (1-2%), probably the minimum amounts left for the survival of the tissues.

This *P. vialii* transgene has also been expressed in *Arabidopsis thaliana* and *Linum usitatissimum* resulting in the highest levels of SDA, 7.1-15% of total fatty acids, in a genetic engineering strategy modifying only one single gene [32]. These values were larger than those obtained here in *E. acanthocarpum* transgenic hairy roots (4.7% of total fatty acids), but it has to be cited that these authors evaluated the accumulation of fatty acids in *L. usitatissimum* seeds, a specialized organ for lipid storage, especially in triacylglycerol form. Moreover, both species showed larger percentages of ALA, particularly flax, which seems to support our hypothesis that the lower pool of ALA in our system would be limiting a larger accumulation of SDA. Nonetheless, these authors attributed the limited accumulation of SDA in their system to a potential bottleneck in the metabolic pathway, since they observed pronounced differences between the amount of SDA accumulated in *Arabidopsis* and linseed, noting that when co-overexpressing the 15-desaturase enzyme, there was not accumulation of SDA, but only of its precursor, ALA, i.e. the -desaturase enzyme itself constituted the real brake on the accumulation of SDA, and not the amount of substrate. Unlike our data and those from Zhou et al. (2006), which suggest that SDA accumulation was affected by the limited amount of the enzyme substrate, despite the presence of the endogenous 15-desaturase in our hairy root system. It has to be repeated that these were hairy roots, and the tissue evaluated seems to affect the accumulation potential of SDA as reported following the overexpression of 6-desaturase from *E. plantagineum* in tobacco plants

[17], finding 9.4% of SDA in leaves and a surprising 2.6% in seeds; whereas, larger accumulation of GLA and SDA, was reported in leaves rather than seeds of *Lotus japonica*, or even the absence of SDA in seeds [55].

Analogously, the cloning and overexpression of 6-desaturase from *Borago officinalis* in linseed or *B. juncea* has been published, reporting larger amounts of GLA in roots compared to other plant organs [51]. In all discussed cases, the real availability of the substrates, LA and ALA, in the different organs could be speculated. For example, in seeds of *E. acanthocarpum* plants the larger presence of ALA allows the synthesis of higher quantities of SDA, displaying 24.23% of ALA and 7.45% of SDA [56, 57]. Furthermore, a larger amount of ALA (36.65%) present in *E. plantagineum* seeds led to 12.94% of SDA. Another factor likely influencing the accumulation of SDA could be the presence of larger amounts of LA, the n-6 substrate of the 6-desaturase enzyme, which competes with ALA in the activity of this enzyme for the production of 6-desaturated fatty acids, resulting in higher accumulation of GLA compared to SDA.

On the other hand, the bottleneck in the biosynthetic route reported in the literature could be due to the required transport of fatty acids within the different lipid pools requiring acyltransferases. In higher plants, the biosynthesis of extraplasmidic fatty acids, such as LA, ALA, GLA and SDA takes place through desaturation of the carbon change that esterifies in a phospholipid skeleton. For instance, the 6-desaturase enzyme from *B. officinalis* exclusively utilizes the sn-2 position of the phospholipids [58]; whereas in *A. thaliana* its 6-desaturase enzyme acts upon LA esterified in phosphocholine in the reticulum endoplasmatic [59], in accordance with the strong increase in this lipid class in our transgenic *E. acanthocarpum* hairy roots.

Reports indicate that it can be assumed that once synthesized, ALA would be transferred to another lipid class by acyltransferases [23, 60]. Moreover, when a 6-desaturase from *P. irregulare* was overexpressed in *B. juncea*, GLA was almost totally accumulated in triacylglycerol [22]. This fact was not likely taking place in our transgenic hairy root system, since the neutral lipid content significantly dropped in favour of polar lipids (Table 4). Indeed, the 6-desaturase enzyme from *P. vialii* possesses a substrate specificity on sn-2 position of the phosphatidycholine where SDA is accumulated in *Arabidopsis*; whereas, in linseed this enzyme accumulated SDA on sn-2 and sn-3 positions of the triacylglycerols, with the participation of acyltransferases, results demonstrating the large differences that would appear depending on the

expression system utilized i.e. *Arabidopsis* versus linseed [32]. Here in our transgenic culture, it could be suggested that the acyltransferases were not fully operational or were functioning in a low mode, facts that could prevent a further accumulation of SDA. For clarifying these scenarios, further studies of the percentage determination of fatty acids in each of the lipid classes, as well as conducting position analyses for the determination of fatty acids esterified on positions sn-1 or sn-2 of phospholipids, or on the three union sites (sn-1, sn-2 and sn-3) in triacylglycerols, would be required.

The effect of the culture temperature on the lipid classes and fatty acid profiles, in particular PUFAs was studied. Interestingly and contrary to what was observed in non-transgenic *E. acanthocarpum* hairy roots, the ANOVA two-way analysis made to components PC1-1 and PC2-1, extracted from the percentages of lipid classes of transgenic hairy roots cultured at 15 or 25 ℃ (Table 6), showed that the temperature did not significantly influence the lipid classes profile. Just when the factor temperature interacted with the overexpression of the gene, its influence on phosphatidylethanolamine and phosphatidylserine plus phosphatidylinositol as reflected by component PC2-1 was observed (Table 6).

There seemed to exist a trend in which phosphatidylglycerol increased in transgenic hairy roots grown at 15 ℃, as compared to those grown at 25 ℃, as also observed with the control hairy root (Fig. 6, Table 6). In turn, the content of phosphatidylglycerol was significantly higher in cultures overexpressing the 6-desaturase gene than control, as it occurred with other polar lipids, except phosphatidylserine and phosphatidylinositol, but this result was independent of the growth temperature. Likewise, temperature was a highly influential factor on the fatty acid profiles in both cultures, and it was reinforced by the overexpression of the gene (Figs. 10a-b, Table 9).

Regarding the qRT-PCR experiments, these demonstrated that the gene was transcribed correctly in the hairy roots, resulting in a high enzyme activity, especially in the first phase of culture (Fig. 13). Thus, one could assume that the availability of the substrate itself (ALA), rather than the enzyme activity would be the limiting factor, as discussed above, although in the design of our approach it was estimated that it could lead the pathway to the n-3 series (Fig. 4). The data, in turn, correlate strongly with the percentages obtained of SDA in the transgenic culture grown at 15°C and the 6-desaturation indices (n-6 and n-3), which showed maximum values in the first sampling

point (Table 7). Undoubtedly, these results are attributed to the increased expression of the *P. vialii 6-desaturase* gene (Fig. 13).

All presented data suggest that the expression of this gene in this culture is primarily responsible for the strong modification of the fatty acid profiles, especially the 6-desaturated ones. On the other hand, the increased expression of the *P. vialii 6-desaturase* gene in the first stages of growth can be explained assuming the adaptive mechanism of any species to an abiotic stress, although it should be taken into account that this expression depends on a strong constitutive promoter as practiced here. Nonetheless, it has been reported that the CaMV35S promoter is not strictly constitutive, and can be partially regulated [51, 61]. Furthermore, it has been determined that after the expression of the *B. officinalis 6-desaturase* gene in tobacco controlled by the CaMV35S promoter, lower amounts of GLA accumulated in mature seeds compared to young seeds [62]. This was attributed to the fact that this promoter showed a plateau activity in the early stages of the transgenic seeds development, or due to a preference of the acyltransferases by endogenous products, rather than newly formed ones, and that for example, transferring the substrates LA and ALA from the phosphatidylcholine, a site of desaturation, to triacylglycerols so rapidly provoked that desaturation did not take place. It was also observed when expressing the same gene under the same promoter in evening primrose, showing that its efficiency decreased in seeds [54].

Based on the positive results presented here, the manipulation strategy conducted seemed appropriate, based primarily in the unusual richness of the Canary Islands *Echium* species in ALA and LA, and choosing the *6-desaturase* gene from *P. vialii* that displays a marked preference for the n-3 substrate, ALA; although there exist other genes from algae displaying also this preference albeit weaker [63, 64], although in a more recent report, *Micromonas pusilla*, a type of marine microalgae whose FADS6 (MpFADS6) appears to function as an acyl-CoA desaturase, has the highest preference for the n-3 substrate (ALA) compared to other plants and fungi [65]. Moreover, in order to further direct the pathway to the SDA end product, another possible genetic engineering approach would have been the simultaneous overexpression also of a -*desaturase* gene, which would likely result in a larger accumulation of the substrate ALA, and hence a higher production of SDA (Fig. 4). This approach has been successfully performed in *B. napus*, where the high levels of 18:1n-9 (oleic acid) made it advisable to divert the metabolic pathway to the n-3 series, achieving 16 to 23% of

SDA, and a favorable change in the ratio n-3/n-6 [66]. Also in soy, a *6-desaturase* gene from *B. officinalis* and a *15-desaturase* gene from *A. thaliana* were overexpressed, obtaining around 30% of SDA and 60% of n-3 fatty acids [67]. Likewise, another plant oil that could be modified in a similar fashion would be *Camelina sativa* since 50% of its oil is composed of LA, and mainly ALA (35-40%) [68, 69]. Moreover, the overexpression of these two transgenes was also reported in the cyanobacterium *Synechocystis* sp which markedly increased accumulation of ALA and SDA and decreased accumulation of LA and GLA [70]. Nonetheless, some authors suggested that the success of this approach might be limited since there is a competition between the enzymes for the LA substrate [71]. In another instance, it was reported that this approach only managed to accumulate ALA but failed to accumulate SDA [32].

Here, before attempting to overexpress these two genes in *E. acanthocarpum*, it would be adequate to previously study the expression of the endogenous *15-desaturase* gene in the presence and absence of the *P. vialii* *6-desaturase* gene to thus determine the suitability of this double approach. Finally, another possible and less studied alternative, would be the introduction in the pathway of genes capable of inter converting fatty acids of the n-6 series into fatty acids of the n-3 series, such as the *17-desaturase* gene [72].

Acknowledgements

The authors acknowledge funding from European Union PCT-MAC program project MAC/3/C217. ECS also acknowledges a scholarship from "Programa de Formación de Profesorado Universitario-FPU" MEC, Spain. NJV was supported by National Programme of Sustainability I of The Czech Ministry of Education, Youth and Sports (ID:LO1416).

FIGURES and TABLES

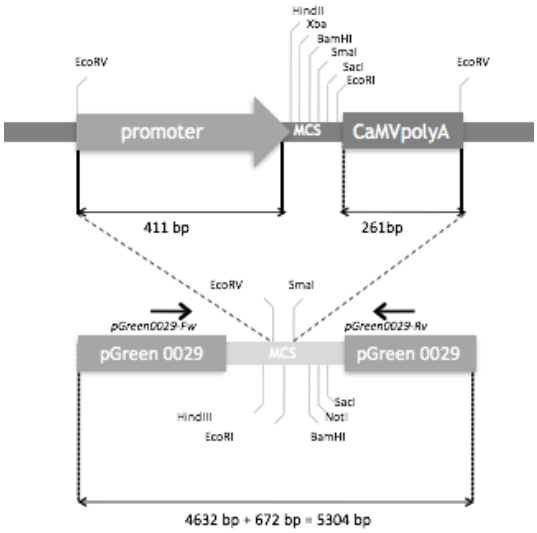

Fig. 1.- Plasmid pGreen0029 and 35S-CaMVpoly A cassette utilized for the construction of modified plasmid pGreen0029-1A with different restriction sites present and used.

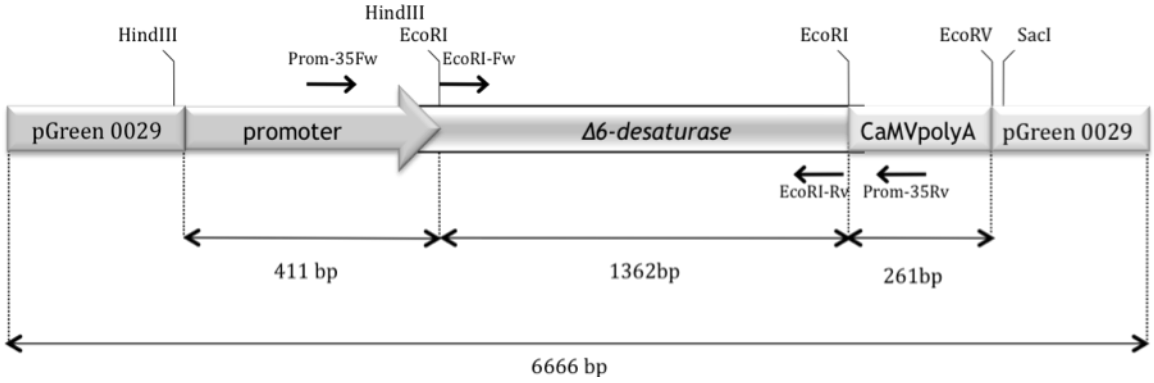

Fig. 2.- Modified pGreen0029-1A Δ6-desaturase employed for the transformation of transgenic *Echium acanthocarpum* hairy roots showing the localization of the primers targets employed for the characterization of the transformants *E. coli* in the cloning strategy of this transgene.

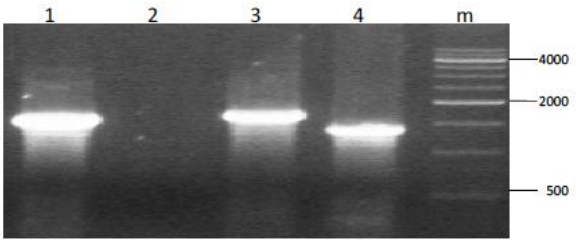

Fig. 3.- Agarose gel electrophoresis of the PCR products of the *Agrobacterium rhizogenes* LBA1334 transformed with the gene construct pGreen00291A- 6des. 1: PCR reaction conducted with the primers set Prom 35S-Fw and Prom 35S-Rv (1,532 bp product); 2: negative control amplified with the primers Prom 35S-Fw and 6EcoRI-Fw; 3: amplified with the primers Prom 35S-Fw and Prom 35S-Rv (1,643 bp product); 4: positive control amplified with the primers set 6EcoRI-Fw and 6EcoRI-Rv (1,362 bp product).

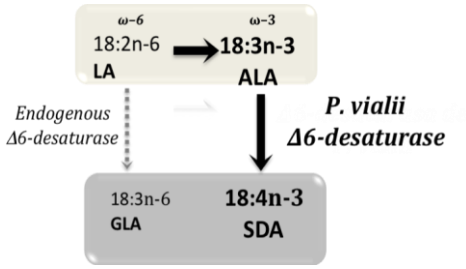

Fig.4.- Biosynthetic steps of fatty acids where 6-desaturase participates in *Echium acanthocarpum*, indicating in thicker arrows and lettering the genetic engineering strategy conducted by the overexpression of this gene from *Primula vialii*.

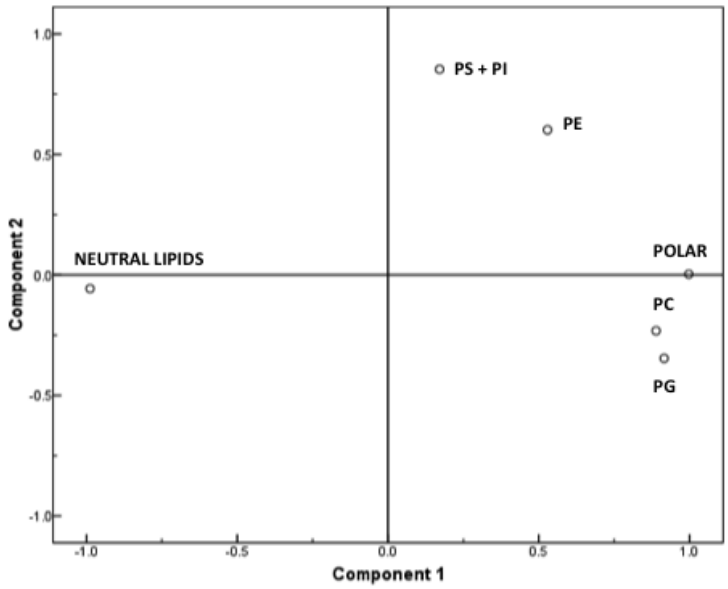

Fig. 5.- Plot of factor loadings obtained after performing a principal component analysis of the profiles of lipid classes in *Echium acanthocarpum* transgenic and control hairy roots.

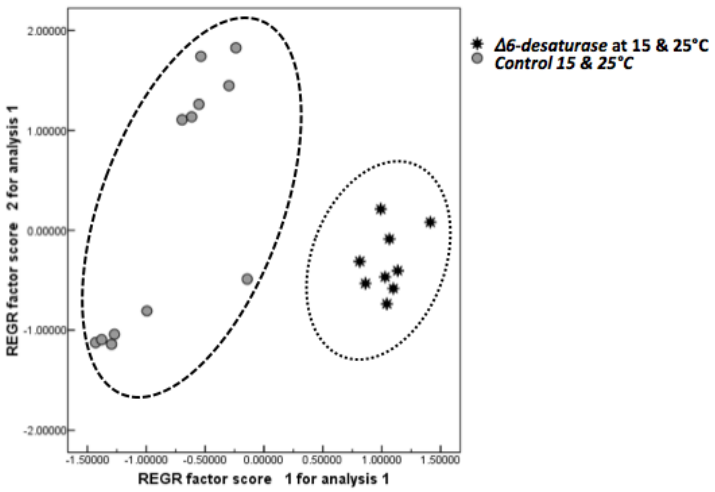

Figure 6.- Plotting of all hairy root samples data (transgenic and control) based on the principal components and stratified according to growth temperature.

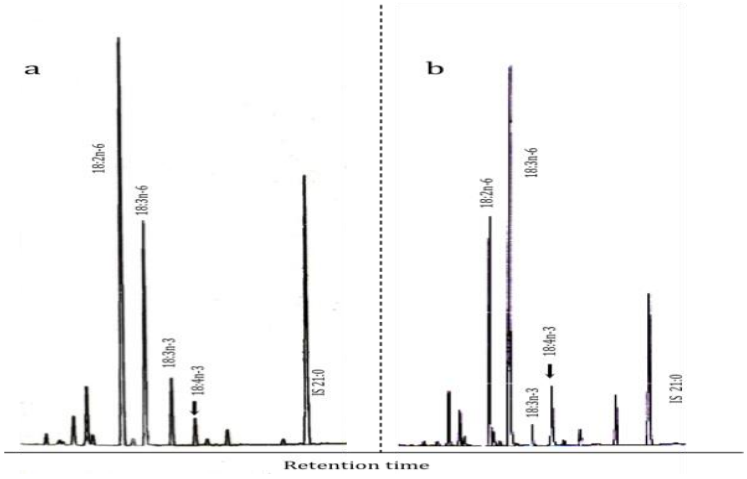

Figure 7.- Gas Chromatography chromatogram of FAMEs prepared from *Echium acanthocarpum* hairy roots a) control culture not overexpressing 6-desaturase cultured in a shaker at 15 ℃ in the dark; b) transgenic culture overexpressing this transgenes also cultured under the same conditions. Arrow indicates the Peak corresponding to SDA (18:4n-3, stearidonic acid), which increased 2-fold compared to control.

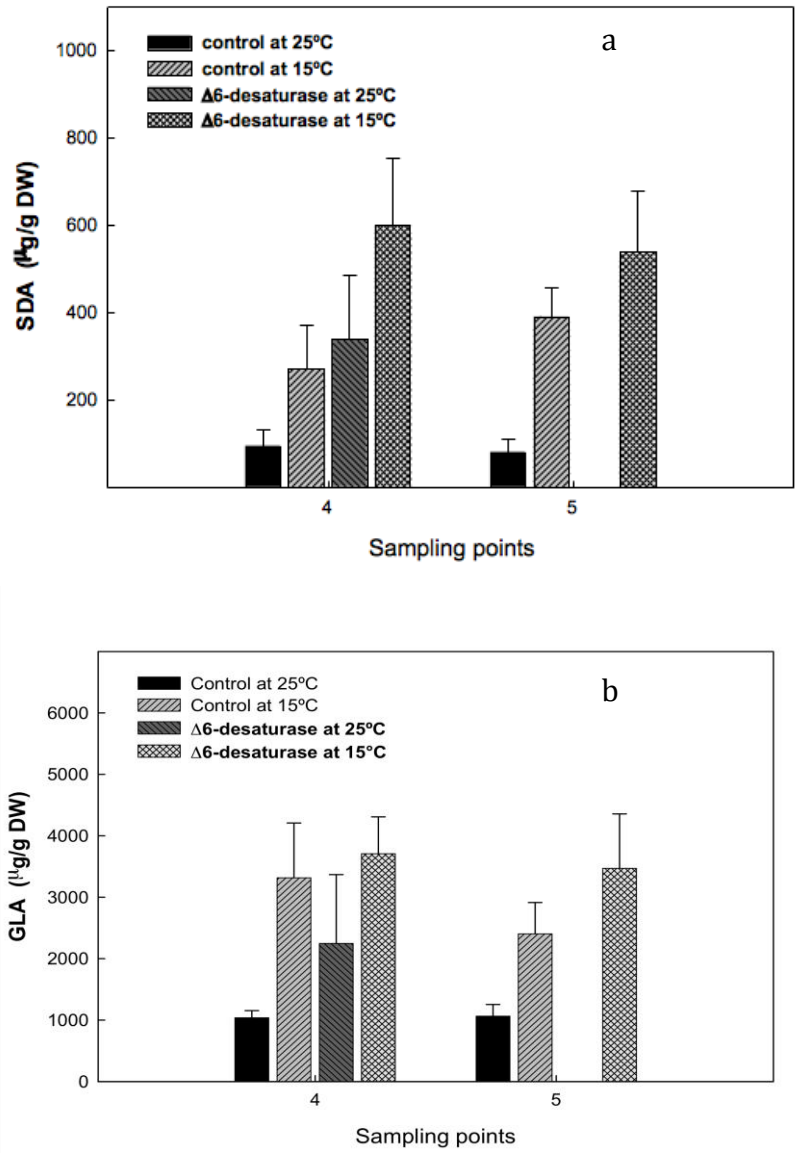

Figure 8.- Content of SDA (a) and GLA (b) (g/g DW) in transgenic hairy roots of *Echium acanthocarpum* overexpressing the 6-desaturase gene from *Primula vialii* at two sampling times (T4-T5) and control culture. Values represent the mean of 3 replicates ± SD.

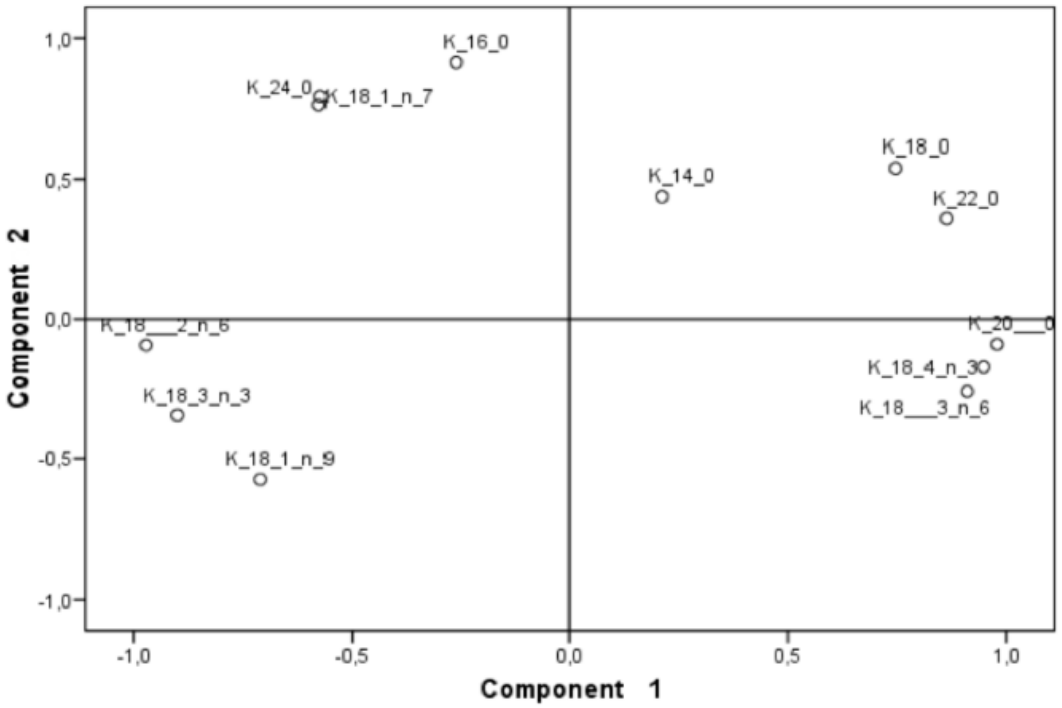

Figure 9.- Plot of factor loadings obtained after performing a principal component analysis of the percentage of the fatty acids of *Echium acanthocarpum* transgenic and control hairy roots cultured at 15 and 25 ℃.

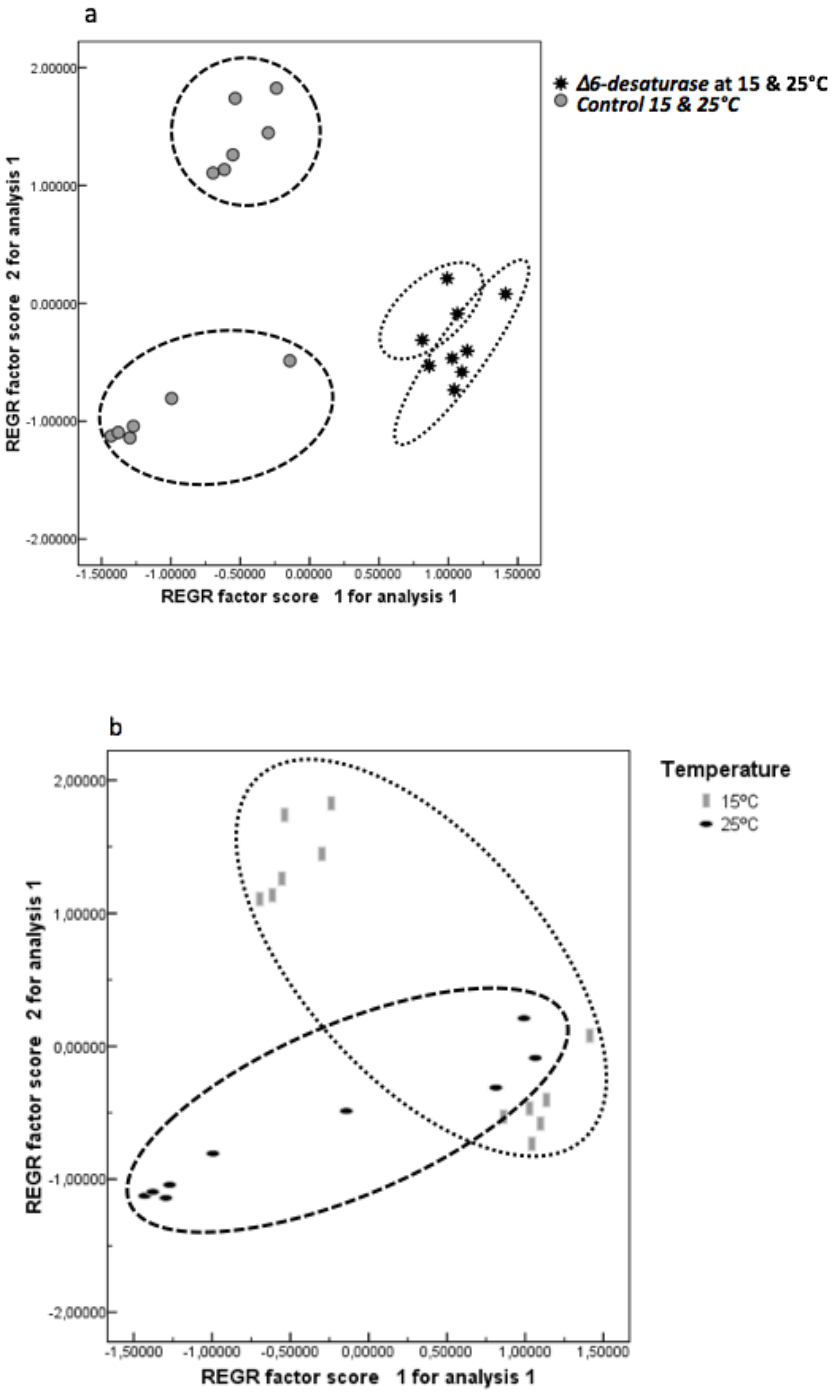

Fig. 10.- Plotting of data of each hairy root culture of *Echium acanthocarpum* based on the principal components stratified according to culture type (a) and temperature (b).

```
E.sabulicola        ACTTGGAAGCTTCTTGAAAAATTCTTCACTGGTTATTACCTTAAAGATTACTCTGTTTCA 261
E.plantagineum      ACTTGGAAGCTTCTTGAAAAATTCTTCACTGGTTATTACCTTAAAGATTACTCTGTTTCT 354
E.gentianoides      ACTTGGAAGTTTCTTGATAGTTTCTTCACTGGCTATTATCTTAAAGATTACTCTGTTTCT 261
E.pitardii          GCATGGCAAAATCTCGATGAATTCTTTAATGGTTATTATCTTAAAGATTACTATGTTTCT 263
P.vialii            ACTGCAAGACTTTTGCCTCCATTGTCAACTAATCTTTTGTTGCAAAACCATTCAGTGTCT 276
                    .*:  .... :* *  .:  :** *  *.*..  :**:  * .**.*  * *.:** **:

E.sabulicola        GAGGTGTCCAAAGATTACAGGAAGCTTGTGTTTGAGTTTAATAAAATGGGGTTGTTTGAC 321
E.plantagineum      GAGGTGTCCAAAGATTACAGGAAGCTTGTGTTTGAGTTTAATAAAATGGGCTTGTTTGAC 414
E.gentianoides      GAGGTGTCCAAAGATTACAGGAAGCTTGTGTTTGAGTTTAATAAAATGGGGTTGTTTGAC 321
E.pitardii          GATGTGTCCAAAGATTACAGAAAGCTTGTGTCTGAGTTTTCTAAGATGGGTTTGTTTAAA 323
P.vialii            CCTACAAGTTCTGATTATAGAAAATTGTTGCACAATTTCCATAAGATTGGTATGTTTAGA 336
                     . . .:  :.:***** **.**. *  **   .* **  .***.** ** :*****...

E.sabulicola        AAAAAGGGTCATATTGTTCTTGTGACTGTGTTGTTTATAGCTATGCTTTTTGCTATGAGT 381
E.plantagineum      AAAAAGGGTCATATTGTTCTTGTGACTGTCTTGTTTATAGCTATGTTGTTTGGTATGAGT 474
E.gentianoides      AAAAAGGGTCATATTGTGCTTGTGACTGTGTTGTTTATAGCTATGATGTTTGCTATGAGT 381
E.pitardii          AAAAACGGTCATACCGTGTTTGTAACTTTGTCCCTTATTGCTATGTCGTTTGCTATGAGT 383
P.vialii            GCAAGGGGTCACACTGCTTACGCTACATTTGTTATTATGATTGTTATGTTCTTGACATCT 396
                    ..**. ***** *  *   : *  **: *     **** . *.*    **    * .: *

E.sabulicola        GTTTATGGGGTTTTGTTTTGTGAGGGTGTTTTGGTACATTTACTTTCAGGGGGGTTGATG 441
E.plantagineum      GTTTATGGGGTTTTGTTTTGTGAGGGTGTTTTGGTACATTTGCTTGCTGGGGGGTTGATG 534
E.gentianoides      GTTTATGGGGTTTTGTTTTGTGAGGGTGTTTTGGTACATTTGCTTGCAGGGGGGTTGATG 441
E.pitardii          GTGTATGGGGTTTTGTTTTGTCAGGGTGTTTTGGTGCATTGGCTTTGTGGGTGTTTAGTG 443
P.vialii            GTGACAGGTGTTTTGTGCTCTGATTCTGCATGGGTGCACCTTGCTTCTGGAGCAGCTATG 456
                    ** :.:** *******  * * *   ** :* ***.**      *  :**.      .**
```

Figure 11.- Alignment of nucleotide sequences of *6-desaturase* genes from *Echium* species. The optimized *6-desaturase* sequence of *Primula vialii* was aligned with other phylogenetically close *6-desaturases* and related to *E. acanthocarpum* belonging to *E. sabulicola* (1,455 bp, DQ067612.1); *Echium plantagineum* (1,814 bp, AY952780.1); *Echium gentianoides* (1,478pb, AY055117.1) and *Echium pitardii* (1,450 bp, AY055118.1). In blue the position of the primers used in RT-PCR generating a 136 bp product specific for the *6-desaturase* gene of *P. vialii*.

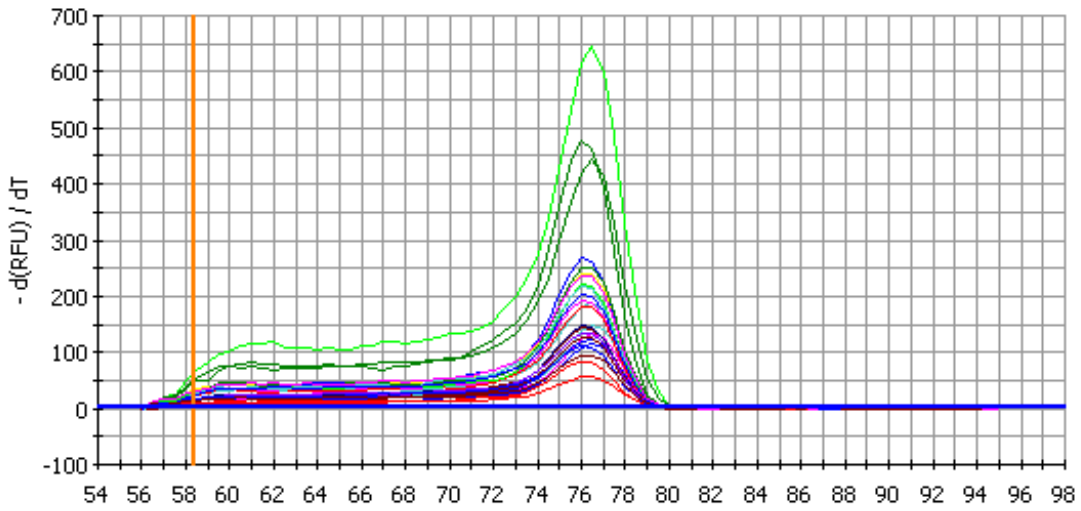

Fig. 12.- Different melting curves of PCR products corresponding to the 136 bp amplicons corresponding to a region of the ORF of the overexpressed *6-desaturase* transgene from *Primula vialii*.

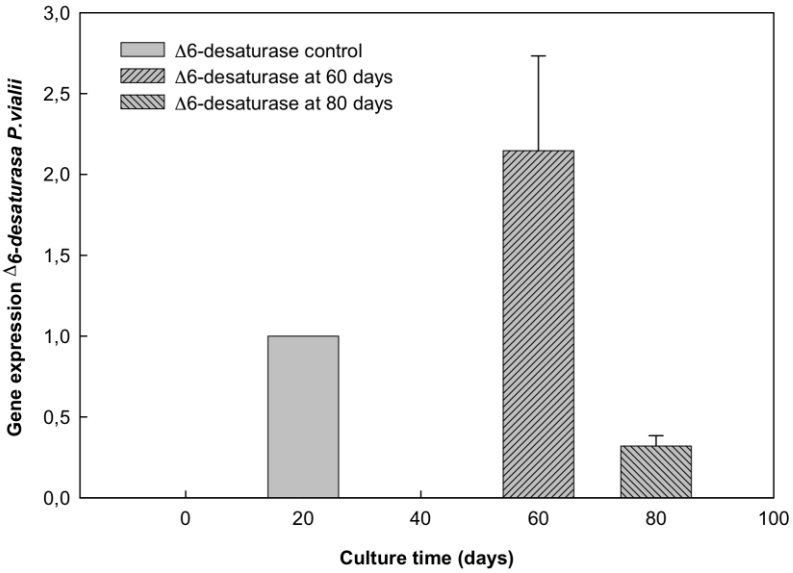

Figure 13.- Gene expression of *6-desaturase* from *Primula vialii* in transgenic *Echium acanthocarpum* hairy roots grown at different temperature 25 ℃ (control) and 15 ℃. At each sampling time RNA was extracted and the mRNA levels of *6-desaturase* were quantified by qRT-PCR. Values are expressed as the increase in the ratio between mRNA of *6-desaturase* versus *mRNA* of the *gadph* gene (house keeping gene) corresponding to the mean and SD of three independent estimations of each three replicates at the indicated sampling times.

Infection	OD	Acetosyr.	Ultrasound	Num. infected plants	Result (%)
Clone 3.1	2.74	No	No	8	25
Clone 3.1	2.74	Yes	No	3	33
Clone 3.1	2.74	Yes	Yes	2	50
Clone 7.1	2.79	No	No	7	57
Clone 7.1	2.79	Yes	No	4	75
Clone 7.1	2.79	Yes	Yes	3	-

Table 1.- Results of the induction of transgenic *Echium acanthocarpum* hairy roots using *Agrobacterium rhizogenes* LBA1334 harbouring the construct pGreen00291A-6des pSoup. OD= optical density of *A. rhizogenes* culture measured at 600nm; Acetosyr.=acetosyringone (25M).

	Fresh Weight (g)			
Sampling	6-des. hairy root culture		Control hairy root culture	
	15 ℃	25 ℃	15 ℃	25 ℃
T4	0.69 ± 0.15^{b}	$0.77 \pm 0.13^{a,b}$	$1.23 \pm 0.30^{a,b}$	1.43 ± 0.21^{a}
T5	1.64 ±0.33	-	1.08 ±0.40	2.76 ±1.21

Table 2.- Fresh weight variation of *Echium acanthocarpum* hairy roots overexpressing a 6-desaturase gene from *Primula vialii* and control culture grown at different temperature (15 and 25 ℃). Values are the mean of 3 replicates ± SD.

	Total Lipid (mg lipid/g DW)			
Sampling	6-des. hairy root culture		Control hairy root culture	
	15 ℃	25 ℃	15 ℃	25 ℃
T4	57.11 ±3.89	47.42 ±11.47	33.41 ±3.66	41.67 ±2.45
T5	40.81 ±8.84	-	35.00 ±5.40	35.58 ±7.33

Table 3.- Total lipid content (mg lipid/g DW) of *Echium acanthocarpum* hairy roots overexpressing a 6-desaturase gene from *Primula vialii* and control culture grown at different temperature (15 and 25°C). Letters a, b indicate significant differences ($p \leq 0.05$), and homogeneous subsets given by the Tukey's test when comparing data at different sampling points. Values are the mean of 3 replicates ± SD.

Main Lipid Class	6-des. hairy root culture			Control hairy root culture		
	15 ℃ (T4)	15 ℃ (T5)	25 ℃ (T4)	15 ℃ (T4)	15 ℃ (T5)	25 ℃ (T5)
TL	57.11 ±3.89	40.81 ±8.84	47.42 ±11.47	33.41 ±3.66	35.00 ±5.40	35.58 ±7.33
PC	13.25 ±2.43	15.10 ±5.46	13.14 ±1.47	9.30 ±1.37	8.53 ±1.34	7.19 ±1.06
PS+PI	3.66 ±0.85	4.37 ±0.55	3.01 ±0.95	6.17 ±0.67	4.51 ±4.09	2.80 ±2.09
PG	17.66 ±0.88	19.64 ±6.51	16.86 ±3.18	3.48 ±1.42	8.25 ±1.70	5.12 ±0.70
PE	13.33 ±1.33	14.42 ±2.86	8.17 ±1.16	10.75 ±0.62	12.18 ±1.49	8.79 ±1.59
Polar	47.91 ±0.41	53.54 ±10.00	41.19 ±4.83	29.70 ±2.77	33.49 ±5.93	23.91 ±5.42

Main Lipid Class	6-des. hairy root culture			Control hairy root culture		
	15 ℃ (T4)	15 ℃ (T5)	25 ℃ (T4)	15 ℃ (T4)	15 ℃ (T5)	25 ℃ (T5)
Neutral	45.38 ±4.16	31.73 ±12.56	56.37 ±4.78	64.05 ±5.67	59.59 ±5.34	71.97 ±7.95
Unknown	6.70 ±2.25	14.73 ±3.92	5.42 ±0.67	6.25 ±2.92	6.92 ±2.21	4.11 ±2.53

Table 4.- Total lipid (mg/gDW) and lipid class profiles (% of total lipid) of samples from *Echium acanthocarpum* transgenic and control hairy roots grown at 15 and 25 ℃ and harvested at two sampling times (T4, T5). TL=total lipid; PC= phosphatidylcholine; PS+PS= phosphatidylserine, phosphatidylinositol; PG= phosphatidylglycerol; PE= phosphatidylethanolamine. Values are the mean of 3 replicates ± SD.

Components matrix	Components		Communalities
	PC1	PC2	Extraction
	(65.17%)	*(21.11%)*	
PC	0.889	-0.232	0.844
PS+PI	0.171	0.853	0.757
PG	0.916	-0.346	0.958
PE	0.529	0.602	0.642
Polar	0.997	0.003	0.995
Neutral	-0.989	-0.057	0.980

Table 5.- Principal components (PC1, PC2) of lipid classes of *Echium acanthocarpum* transgenic and control hairy roots grown at 15 and 25 ℃. Factor loadings and communalities are shown. Factor loading of the variable correlated with PC1 are shown in red, and those correlated with PC2 are shown in blue. PC= phosphatidylcholine; PS+PI= phosphatidylserine, phosphatidylinositol; PG= phosphatidylglycerol; PE= phosphatidylethanolamine.

Two-way ANOVA	Expression *6-desaturase*		Temperature		Interaction	
	F-Value	*Sign.*	*F-Value*	*Sign.*	*F-Value*	*Sign.*
PC1	26.291	0.000	2.060	0.173	3.247	0.093
PC2	6.526	0.230	0.143	0.711	6.875	0.020

Table 6.- Two-way ANOVA (*6-desaturase* and temperature) of the two principal components PC1 and PC2 of recorded lipid classes in transgenic and control *Echium acanthocarpum* growing at 15˚C, Sign.=significance ($p \leq 0.05$)

	6-desaturase hairy roots			Control hairy roots	
	15 ℃ (T4)	15 ℃ (T5)	25 ℃ (T4)	15 ℃ (T5)	25 ℃ (T5)
TL (mg/gDW)	57.11 ±3.89	40.81 ±8.84	47.42 ±11.47	35.58 ±7.33	35.00 ±5.40
FAs (mg/gDW)	12.20 ±2.43	10.80 ±2.19	8.85 ±3.29	13.71 ±4.10	8.45 ±0.87
FAs (%)					
14:0	0.31 ±0.08	0.16 ±0.14	0.23 ±0.20	0.14 ±0.03	0.23 ±0.02
16:0	22.35 ±0.38	21.63 ±0.81	22.14 ±0.71	20.21 ±1.81	25.04 ±1.14

	6-desaturase hairy roots			Control hairy roots	
	15 ℃ (T4)	15 ℃ (T5)	25 ℃ (T4)	15 ℃ (T5)	25 ℃ (T5)
18:0	3.96 ±0.82	3.43 ±0.21	4.48 ±0.19	1.82 ±0.48	2.55 ±0.21
18:1n-9	3.80 ±0.14	3.11 ±0.53	4.37 ±0.17	8.79 ±2.43	6.77 ±1.10
18:1n-7	0.65 ±0.07	0.72 ±0.13	0.82 ±0.01	0.70 ±0.06	1.55 ±0.16
18:2n-6 (LA)	20.56 ±2.23	18.15 ±0.62	22.53 ±2.31	34.34 ±3.77	34.55 ±1.97
18:3n-6 (GLA)	26.46 ±1.18	29.49 ±1.64	20.74 ±1.37	17.51 ±1.77	11.77 ±1.63
18:3n-3 (ALA)	1.80 ±0.18	1.60 ±0.13	1.42 ±0.04	5.32 ±0.56	4.22 ±0.34
18:4n-3 (SDA)	4.63 ±0.54	4.70 ±0.39	3.47 ±0.31	2.12 ±0.19	0.88 ±0.21
20:0	1.15 ±0.06	1.16 ±0.07	1.08 ±0.05	0.64 ±0.30	0.27 ±0.04
22:0	3.87 ±0.40	4.18 ±0.43	4.27 ±0.17	2.42 ±1.50	2.51 ±0.35
24:0	4.72 ±0.75	6.39 ±0.67	0.22 ±0.19	0.21 ±0.02	2.08 ±0.60
unknown	5.45 ±1.56	4.95 ±1.06	6.22 ±0.42	2.23 ±0.46	6.08 ±2.68
GLA+SDA	31.09 ±1.72	34.19 ±2.03	24.21 ±1.68	19.63 ±1.96	12.65 ±1.84
∑ saturated FAs	36.35 ±2.44	36.94 ±2.05	38.43 ±0.59	27.15 ±4.25	32.68 ±2.21
∑ monoene FAs	4.76 ±0.04	4.16 ±0.35	4.37 ±0.17	11.32 ±2.42	9.54 ±1.29
n-9	3.80 ±0.14	3.11 ±0.53	4.37 ±0.17	10.40 ±2.41	7.59 ±1.15
n-6	47.02 ±3.29	47.65 ±2.23	43.27 ±0.17	51.85 ±2.00	46.32 ±3.60
n-3	6.42 ±0.70	6.30 ±0.52	4.89 ±0.35	7.44 ±0.42	5.09 ±0.53
6-des (n-6) index	0.73 ±0.13	0.62 ±0.02	0.48 ±0.04	0.34 ±0.05	0.25 ±0.02
6-des (n-3) index	0.49 ±0.19	0.34 ±0.01	0.52 ±0.17	0.29 ±0.04	0.17 ±0.02
DBI	1.49 ±0.10	1.53 ±0.08	1.31 ±0.02	1.57 ±0.05	1.30 ±0.08

Table 7.- Variation of total lipid (TL) content (mg/gDW), total fatty acid content (FAs) (mg/gDW) and percentage of each fatty acid (% of total FAs) of samples from *Echium acanthocarpum* transgenic and control hairy roots grown at 15 and 25 ℃ and harvested at two sampling times (T4, T5). 6-des (n-6) and 6-des (n-3) indexes, correspond to desaturation index of n-6 series and desaturation index of n-3 series. These were calculated as 18:3n-6/(18:3n-6+18:2n-6) and 18:4n-3/(18:4n-3+18:3n-3), respectively. DBI (double bond index) calculated as [(%18:1)+2*(%18:2)+3*(%18:3)+4*(%18:4)]/100. Values are the mean of 3 replicates ± SD.

Components matrix	Components		Communalities
	PC1	PC2	Extraction
	(58.40%)	*(26.80%)*	
14:0	0.361	0.326	0.237
16:0	0.107	0.944	0.903
18:0	0.897	0.213	0.850
18:1n-9	-0.874	-0.261	0.832
18:1n-7	-0.226	0.949	0.953
18:2n-6	-0.934	0.284	0.953
18:3n-6	0.742	-0.586	0.894
18:3n-3	-0.963	0.024	0.928

Components matrix	Components		Communalities
18:4n-3	0.810	-0.520	0.927
20:0	0.870	-0.457	0.965
22:0	0.935	0.005	0.873
24:0	-0.242	0.922	0.909

Table 8.- Principal components (PC1, PC2) of fatty acids of *Echium acanthocarpum* transgenic and control hairy roots grown at 15 and 25 ℃. Factor loadings and communalities are shown. Factor loading of the variable correlated with PC1 are shown in red, and those correlated with PC2 are shown in blue.

Two-way ANOVA	Expression *6-desaturase (culture)*		Temperature (stressing factor)		Interaction of both factors	
	F-Value	*Sign.*	*F-Value*	*Sign.*	*F-Value*	*Sign.*
PC1	174.033	0.000	7.162	0.016	2.740	0.116
PC2	14.505	0.001	60.930	0.000	115.782	0.000

Table 9.- Two-way ANOVA (culture and stressing factor) of the two principal components PC1 and PC2 obtained with the percentage values of fatty acids in culture of transgenic and control *Echium acanthocarpum* hairy root grown at 15 and 25˚C. Sign.=significance ($p \leq 0.05$).

Annex I

Different primers employed in this study for the performed PCR and sequencing steps. For primer design and calculation of Tm, the programs Primer 3.0 and Omiga 2.0 were used.

Name	**Sequence 5´— 3´**	**Tm (℃)**
pGreen0029Fw	TTGTAATACGACTCACTATAGGG	53.1
pGreen0029Rv	TGTTCTTTCCTGCGTTATCC	57.8
6EcoRI-Fw	CCGAATTCATGGCAAACAAGAGTCCTCC	72.5
6EcoRI-Rv	CCGAATTCTCATCCATGAGTGTG	63.9
nptII-Fw(b)	CTCTCAACTCGATCGAGG	54.1
nptII-Rv(b)	TTCGTCCAGATCATCCTG	55,3
Prom 35S-Fw	TCCAACCACGTCTTCAAAGC	61.2
Prom 35S-Rv	CTCAACACATGAGCGAAACC	59.3
RT-Fw2	TTGCCTCCATTGTCAACTAATC	59.1
RT-Rv2	ATGTAGCGTAAGCATGTGACC	57.8
GADPH-Fw	GGCTGCAATCAAGGAGGAA	61.3
GADPH-Rv	AAATCAATCACACGGGAACTG	59.8

References

[1] Oresic M, Hanninen VA, Vidal-Puig A. Lipidomics: a new window to biomedical frontiers. Trends Biotechnol. 2008; 26(12): 647-52.

[2] Siddiqui RA, Harvey K, Stillwell W. Anticancer properties of oxidation products of docosahexaenoic acid. Chem Phys Lipids. 2008; 153(1): 47-56.

[3] Calder PC. Omega-3 Fatty Acids and Inflammatory Processes. Nutrients. 2010; 2(3): 355-74.

[4] Horrobin DF. Nutritional and medical importance of gamma-linoleic acid. Prog Lipid Res. 1992; 31(2): 163-94.

[5] van Gool CJ, Thijs C, Henquet CJ, van Houwelingen AC, Dagnelie PC, Schrander J, et al. γ-Linolenic acid supplementation for prophylaxis of atopic dermatitis—a randomized controlled trial in infants at high familial risk. The American Journal of Clinical Nutrition. 2003; 77(4): 943-51.

[6] Schuchardt JP, Huss M, Stauss-Grabo M, Hahn A. Significance of long-chain polyunsaturated fatty acids (PUFAs) for the development and behaviour of children. Eur J Pediatr. 2010; 169(2): 149-64.

[7] Gunstone FD. Gamma linolenic acid - Occurence and physical chemical properties Prog Lipid Res. 1992; 31(2): 145-61.

[8] Tsevegsuren N, Aitzetmuller K. gamma-Linolenic and stearidonic acids in Mongolian Boraginaceae. J Am Oil Chem Soc. 1996; 73(12): 1681-4.

[9] Guil-Guerrero JL, Lopez-Martinez JC, Gomez-Mercado F, Campra-Madrid P. Gamma-linolenic and stearidonic acids from Moroccan Boraginaceae. Eur J Lipid Sci Technol. 2006; 108(1): 43-7.

[10] Ozcan T. Analysis of the total oil and fatty acid composition of seeds of some Boraginaceae taxa from Turkey. Plant Syst Evol. 2008; 274(3-4): 143-53.

[11] Portolesi R, Powell BC, Gibson RA. Competition between 24 : 5n-3 and ALA for D6 desaturase may limit the accumulation of DHA in HepG2 cell membranes. J Lipid Res. 2007; 48(7): 1592-8.

[12] Voss A, Reinhart M, Sankarappa S, Sprecher H. The metabolism of 7,10,13,16,19-docosapentanoic acid to 4,7,10,13,16,19-docosahexanoic acid in rat-liver independent of a 4-desaturase. J Biol Chem. 1991; 266(30): 19995-20000.

[13] Sayanova O, Smith MA, Lapinskas P, Stobart AK, Dobson G, Christie WW, et al. Expression of a borage desaturase cDNA containing an N-terminal cytochrome b(5)

domain results in the accumulation of high levels of Delta(6)-desaturated fatty acids in transgenic tobacco. Proc Natl Acad Sci U S A. 1997; 94(8): 4211-6.

[14] Chen Q, Nimal J, Li WL, Liu X, Cao WG. Delta-6 desaturase from borage converts linoleic acid to gamma-linolenic acid in HEK293 cells. Biochem Biophys Res Commun. 2011; 410(3): 484-8.

[15] Whitney HM, Michaelson LV, Sayanova O, Pickett JA, Napier JA. Functional characterisation of two cytochrome b(5)-fusion desaturases from Anemone leveillei: the unexpected identification of a fatty acid Delta(6)-desaturase. Planta. 2003; 217(6): 983-92.

[16] Garcia-Maroto F, Garrido-Cardenas JA, Rodriguez-Ruiz J, Vilches-Ferron M, Adam AC, Polaina J, et al. Cloning and molecular characterization of the Delta 6-desaturase from two Echium plant species: Production of GLA by heterologous expression in yeast and tobacco. Lipids. 2002; 37(4): 417-26.

[17] Zhou XR, Robert S, Singh S, Green A. Heterologous production of GLA and SDA by expression of an Echium plantagineum Delta 6-desaturase gene. Plant Sci. 2006; 170(3): 665-73.

[18] Kajikawa M, Yamato KT, Kohzu Y, Nojiri M, Sakuradani E, Shimizu S, et al. Isolation and characterization of Delta(6)-desaturase, an ELO-like enzyme and Delta(5)-desaturase from the liverwort Marchantia polymorpha and production of arachidonic and eicosapentaenoic acids in the methylotrophic yeast Pichia pastoris. Plant MolBiol. 2004; 54(3): 335-52.

[19] Sayanova OV, Beaudoin F, Michaelson LV, Shewry PR, Napier JA. Identification of Primula fatty acid Delta(6)-desaturases with n-3 substrate preferences. FEBS Lett. 2003; 542(1-3): 100-4.

[20] Sayanova O, Haslam R, Venegas-Caleron M, Napier JA. Identification of Primula "front-end" desaturases with distinct n-6 or n-3 substrate preferences. Planta. 2006; 224(6): 1269-77.

[21] Zhang RJ, Zhu YM, Ren L, Zhou PP, Hu JR, Yu LJ. Identification of a fatty acid a Delta(6)-desaturase gene from the eicosapentaenoic acid-producing fungus Pythium splendens RBB-5. Biotechnology Letters. 2013; 35(3): 431-8.

[22] Hong H, Datla N, Reed DW, Covello PS, MacKenzie SL, Qiu X. High-level production of gamma-linolenic acid in Brassica juncea using a Delta 6 desaturase from Pythium irregulare. Plant Physiol. 2002; 129(1): 354-62.

[23] Sakuradani E, Shimizu S. Gene cloning and functional analysis of a second Delta 6-fatty acid desaturase from an arachidonic acid-producing Mortierella fungus. Biosci Biotechnol Biochem. 2003; 67(4): 704-11.

[24] Domergue F, Lerchl J, Zahringer U, Heinz E. Cloning and functional characterization of Phaeodactylum tricornutum front-end desaturases involved in eicosapentaenoic acid biosynthesis. Eur J Biochem. 2002; 269(16): 4105-13.

[25] Sayanova O, Haslam RP, Caleron MV, Ruiz-Lopez N, Worthy C, Rooks P, et al. Identification and functional characterisation of genes encoding the omega-3 polyunsaturated fatty acid biosynthetic pathway from the coccolithophore Emiliania huxleyi. Phytochemistry. 2011; 72(7): 594-600.

[26] Sperling P, Lee M, Thomas G, Zahringer U, Stymne S, Heinz E. A bifunctional Delta(6)-fatty acyl acetylenase/desaturase from the moss Ceratodon purpureus - A new member of the cytochrome b(5) superfamily. Eur J Biochem. 2000; 267(12): 3801-11.

[27] Girke T, Schmidt H, Zahringer U, Reski R, Heinz E. Identification of a novel Delta 6-acyl-group desaturase by targeted gene disruption in Physcomitrella patens. Plant J. 1998; 15(1): 39-48.

[28] Domergue F, Abbadi A, Zahringer U, Moreau H, Heinz E. In vivo characterization of the first acyl-CoA Delta(6)-desaturase from a member of the plant kingdom, the microalga Ostreococcus tauri. Biochem J. 2005; 389: 483-90.

[29] Petrie JR, Liu Q, Mackenzie AM, Shrestha P, Mansour MP, Robert SS, et al. Isolation and Characterisation of a High-Efficiency Desaturase and Elongases from Microalgae for Transgenic LC-PUFA Production. Mar Biotechnol. 2010; 12(4): 430-8.

[30] Xie DZ, Chen F, Lin SY, Wang SQ, You CH, Monroig O, et al. Cloning, Functional Characterization and Nutritional Regulation of Delta 6 Fatty Acyl Desaturase in the Herbivorous Euryhaline Teleost Scatophagus Argus. PLoS One. 2014; 9(3): 10.

[31] Tanomman S, Ketudat-Cairns M, Jangprai A, Boonanuntanasarn S. Characterization of fatty acid delta-6 desaturase gene in Nile tilapia and heterogenous expression in Saccharomyces cerevisiae. Comp Biochem Physiol B-Biochem Mol Biol. 2013; 166(2): 148-56.

[32] Ruiz-Lopez N, Haslam RP, Venegas-Caleron M, Larson TR, Graham IA, Napier JA, et al. The synthesis and accumulation of stearidonic acid in transgenic plants: a

novel source of 'heart-healthy' omega-3 fatty acids. Plant Biotechnol J. 2009; 7(7): 704-16.

[33] Abbadi A, Domergue F, Bauer J, Napier JA, Welti R, Zahringer U, et al. Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: Constraints on their accumulation. Plant Cell. 2004; 16(10): 2734-48.

[34] Napier JA, Usher S, Haslam RP, Ruiz-Lopez N, Sayanova O. Transgenic plants as a sustainable, terrestrial source of fish oils. Eur J Lipid Sci Technol. 2015; 117(9): 1317-24.

[35] Guil-Guerrero JL. Stearidonic acid (18 : 4n-3): Metabolism, nutritional importance, medical uses and natural sources. Eur J Lipid Sci Technol. 2007; 109(12): 1226-36.

[36] James MJ, Ursin VM, Cleland LG. Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. Am J Clin Nutr. 2003; 77(5): 1140-5.

[37] Burdge GC, Calder PC. Conversion of alpha-linolenic acid to longer-chain polyunsaturated fatty acids in human adults. Reprod Nutr Dev. 2005; 45(5): 581-97.

[38] Cequier-Sanchez E, Rodriguez C, Dorta-Guerra R, Ravelo AG, Zarate R. Echium acanthocarpum hairy root cultures, a suitable system for polyunsaturated fatty acid studies and production. BMC Biotechnol. 2011; 11: 14.

[39] Zarate R, Cequier-Sanchez E, Rodriguez C, Dorta-Guerra R, El Jaber-Vazdekis N, Ravelo AG. Improvement of Polyunsaturated Fatty Acid Production in Echium acanthocarpum Transformed Hairy Root Cultures by Application of Different Abiotic Stress Conditions. ISRN biotechnology. 2013; 2013: 169510.

[40] Gamborg OL, Miller RA, Ojima K. Nutrient requirements od suspension cultures of Soybean root cells Exp Cell Res. 1968; 50(1): 151-&.

[41] Zarate R, El Jaber-Vazdekis N, Medina B, Ravelo AG. Tailoring tropane alkaloid accumulation in transgenic hairy roots of Atropa baetica by over-expressing the gene encoding hyoscyamine 6 beta-hydroxylase. Biotechnology Letters. 2006; 28(16): 1271-7.

[42] Mattanovich D, Ruker F, Machado AD, Laimer M, Regner F, Steinkellner H, et al. Efficient transformation of agrobacterium of Agrobacterium spp by electroporation Nucleic Acids Res. 1989; 17(16): 6747-.

[43] Wise AA, Liu ZY, Binns AN. Three methods for the introduction of foreign DNA into Agrobacterium. Agrobacterium Protocols, Second Edition, Vol 1. 2006; 343: 43-53.

[44] Folch J, Lees M, Stanley GHS. A simple method for the isolation and purification of total lipids from animal tissues J Biol Chem. 1957; 226(1): 497-509.

[45] Cequier-Sanchez E, Rodriguez C, Ravelo AG, Zarate R. Dichloromethane as a solvent for lipid extraction and assessment of lipid classes and fatty acids from samples of different natures. J Agric Food Chem. 2008; 56(12): 4297-303.

[46] Christie WW. The isolation of lipids from tissues. The preparation of derivates of lipids. . In: Christie WW, editor. Lipid Analysis. 17-23. Canada: Pergamon Press Cnada Ltd.; 1982. p. 55-61.

[47] Garcia-Maroto F, Manas-Fernandez A, Garrido-Cardenas JA, Alonso DL. Substrate specificity of acyl-Delta(6)-desaturases from Continental versus Macaronesian Echium species. Phytochemistry. 2006; 67(6): 540-4.

[48] Garcia-Maroto F, Manas-Fernandez A, Garrido-Cardenas JA, Alonso DL, Guil-Guerrero JL, Guzman B, et al. Delta(6)-Desaturase sequence evidence for explosive Pliocene radiations within the adaptive radiation of Macaronesian Echium (Boraginaceae). Mol Phylogenet Evol. 2009; 52(3): 563-74.

[49] Zhang M, Barg R, Yin MG, Gueta-Dahan Y, Leikin-Frenkel A, Salts Y, et al. Modulated fatty acid desaturation via overexpression of two distinct omega-3 desaturases differentially alters tolerance to various abiotic stresses in transgenic tobacco cells and plants. Plant J. 2005; 44(3): 361-71.

[50] Beremand PD, Nunberg AN, Reddy AS, Thomas TL. Production of gamma-linolenic acid by transgenic plants expressing cyanobacterial or plant Delta(6)-desaturase genes. Williams JP, Khan MU, Lem NW, editors. Dordrecht: Kluwer Academic Publ; 1997. 351-3 p.

[51] Qiu X, Hong HP, Datla N, MacKenzie SL, Taylor DC, Thomas TL. Expression of borage Delta 6 desaturase in Saccharomyces cerevisiae and oilseed crops. Can J Bot-Rev Can Bot. 2002; 80(1): 42-9.

[52] Cook D, Grierson D, Jones C, Wallace A, West G, Tucker G. Modification of fatty acid composition in tomato (Lycopersicon esculentum) by expression of a borage Delta(6)-desaturase. Mol Biotechnol. 2002; 21(2): 123-8.

[53] Sato S, Xing AQ, Ye XG, Schweiger B, Kinney A, Graef G, et al. Production of gamma-linolenic acid and stearidonic acid in seeds of marker-free transgenic soybean. Crop Sci. 2004; 44(2): 646-52.

[54] de Gyves EM, Sparks CA, Sayanova O, Lazzeri P, Napier JA, Jones HD. Genetic manipulation of gamma-linolenic acid (GLA) synthesis in a commercial variety of evening primrose (Oenothera sp.). Plant Biotechnol J. 2004; 2(4): 351-7.

[55] Chen R, Tsuda S, Matsui K, Fukuchi-Mizutani M, Ochiai M, Shimizu S, et al. Production of gamma-linolenic acid in Lotus japonicus and Vigna angularis by expression of the Delta 6-fatty-acid desaturase gene isolated from Mortierella alpina. Plant Sci. 2005; 169(3): 599-605.

[56] Guil-Guerrero JL, Gomez-Mercado F, Garcia-Maroto F, Campra-Madrid P. Occurrence and characterization of oils rich in gamma-linolenic acid - Part I: Echium seeds from Macaronesia. Phytochemistry. 2000; 53(4): 451-6.

[57] Guil-Guerrero JL, García-Maroto F, Campra-Madrid P, Gómez-Mercado F. Occurrence and characterization of oils rich in γ-linolenic acid Part II: fatty acids and squalene from Macaronesian Echium leaves. Phytochemistry. 2000; 54(5): 525-9.

[58] Griffiths G, Stobart AK, Stymne S. Delta-6-desaturase and delta-12-desaturase activities and phosphatidic-acid formation in microsomal preparation from the developing cotyledons of common Borage (Borago officinalis). Biochem J. 1988; 252(3): 641-7.

[59] Somerville C, Browse J. Plants Lipids - Metabolism, Mutants and Membranes Science. 1991; 252(5002): 80-7.

[60] Sakuradani E, Kobayashi M, Shimizu S. Delta 6-fatty acid desaturase from an arachidonic acid-producing Mortierella fungus - Gene cloning and its heterologous expression in a fungus, Aspergillus. Gene. 1999; 238(2): 445-53.

[61] Yang NS, Christou P. Cell type specifc expression of a CAMV 35S-GUS gene in transgenic soybean plants. Dev Genet. 1990; 11(4): 289-93.

[62] Sayanova O, Davies GM, Smith MA, Griffiths G, Stobart AK, Shewry PR, et al. Accumulation of Delta(6)-unsaturated fatty acids in transgenic tobacco plants expressing a Delta(6)-desaturase from Borago officinalis. J Exp Bot. 1999; 50(340): 1647-52.

[63] Hoffmann M, Wagner M, Abbadi A, Fulda M, Feussner I. Metabolic engineering of omega 3-very long chain polyunsaturated fatty acid production by an exclusively acyl-CoA-dependent pathway. J Biol Chem. 2008; 283(33): 22352-62.

[64] Petrie JR, Shrestha P, Mansour MP, Nichols PD, Liu Q, Singh SP. Metabolic engineering of omega-3 long-chain polyunsaturated fatty acids in plants using an

acyl-CoA Δ6-desaturase with ω3-preference from the marine microalga Micromonas pusilla. Metab Eng. 2010; 12(3): 233-40.

[65] Shi HS, Chen HQ, Gu ZA, Song YD, Zhang H, Chen W, et al. Molecular mechanism of substrate specificity for delta 6 desaturase from Mortierella alpina and Micromonas pusilla. J Lipid Res. 2015; 56(12): 2309-21.

[66] Ursin VM. Modification of plant lipids for human health: Development of functional land-based omega-3 fatty acids. J Nutr. 2003; 133(12): 4271-4.

[67] Eckert H, LaVallee B, Schweiger BJ, Kinney AJ, Cahoon EB, Clemente T. Co-expression of the borage Delta(6) desaturase and the Arabidopsis Delta(15) desaturase results in high accumulation of stearidonic acid in the seeds of transgenic soybean. Planta. 2006; 224(5): 1050-7.

[68] Leonard EC. Camelina oil : Alpha-linolenic source Champaign, IL, ETATS-UNIS: American Oil Chemists' Society; 1998. 6 p.

[69] Lu CF, Kang JL. Generation of transgenic plants of a potential oilseed crop Camelina sativa by Agrobacterium-mediated transformation. Plant Cell Reports. 2008; 27(2): 273-8.

[70] Chen G, Qu SJ, Wang Q, Bian F, Peng ZY, Zhang Y, et al. Transgenic expression of delta-6 and delta-15 fatty acid desaturases enhances omega-3 polyunsaturated fatty acid accumulation in Synechocystis sp PCC6803. Biotechnol Biofuels. 2014; 7: 10.

[71] Petrie JR, Singh SP. Expanding the docosahexaenoic acid food web for sustainable production: engineering lower plant pathways into higher plants. AoB PLANTS. 2011; 2011: plr011.

[72] Kinney A, Cahoon E, Damude H, Hitz W, Liu ZB, Kolar C. Production of very long chain polyunsaturated fatty acids in oilseed plants. Google Patents; 2004.