EXPRESSION OF GENES INVOLVED IN SCLAREOL BIOSYNTHESIS IN *SALVIA SCLAREA* L.

Maria Duca[1] , Angela Port[1], Tatiana Şestacova[1], Rodica Martea[1] , Maria Gonceariuc[2]

[1]University Centre of Molecular Biology, UnASM
[2]Institute of Genetics, Physiology and Plant Protection of the ASM

Introduction

Sclareol is a labdane-type diterpene alcohol [9] with antibacterial, antifungal properties and growth regulating activity [4], thus being very useful for perfumery and flavoring [8, 14, 22].

This compound has been reported in four plant species from different botanical families: *Salvia sclarea* (*Lamiaceae*), *Cistus creticus* (*Cistaceae*), *Nicotiana glutinosa* (*Solanaceae*) and *Cleome spinosa* (*Brassicaceae*) [4].

Most of the commercially produced sclareol is derived from cultivated clary sage (*Salvia sclarea* L.), which is a pluriannual herb commonly cultivated for its essential oil [1, 6, 19, 11, 21]. The relatively easy farming of this plant and its high sclareol yield has encouraged clary sage producers to begin genetic improvement programs and expand *S. sclarea* L. plantations. According to European Herb Growers Association (Europam), the annual cultivated area of clary sage in France is 5 000 ha and 2 000 ha in Bulgaria and Moldova [16].

Despite the expanded cultivation area of clary sage, the annual production and the sclareol yield is very variable, which is caused by the different synthesis and acumulation capacities of this compound in inflorescences [3]. The success of increase of sclareol content by *S. sclarea* breeding programs could be ensured through ability to regulate the sclareol synthesis metabolic pathway.

It is known that, the biosynthesis of sclareol proceeds in two steps – from geranylgeranyl diphosphate (GGPP) to labda-13-en-8-ol diphosphate and sclareol. The first reaction is catalyzed by labda-13-en-8-ol diphosphate synthase and the second – by sclareol synthase (Figure 1) [4].
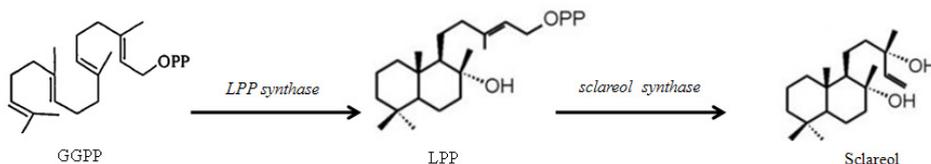
Fig. 1. Biosynthetic pathway of sclareol in Salvia sclarea L.
(GGPP - geranylgeranyl diphopsphate, LPP - labda-13-en-8-ol diphopsphate).

Sclareol synthase has been successfully cloned and functionally characterized and its synthesis pathway had been reconstructed in recombinant *E. coli*, using genetic engineering through overexpression for metabolic biosynthesis of sclareol [23].

Chemical synthesis would seem to be another obvious option for the obtaining of sclareol. However, due to its highly complex structure, an inexpensive synthetic process for this compound is still difficult [15]. So, the analysis of the transcriptional activity of genes involved in the biosynthesis of secondary metabolites, including sclareol, correlated with the selection of valuable parental forms is a current objective of molecular breeding.

The investigation of the molecular basis of the sclareol biosynthesis provides opportunities for further research in a wide range of areas, from structure – function to

genetics of disease resistance, via metabolic engineering of fragrance ingredient precursors.

Materials and methods

Plant material. Twenty-eight genotypes of clary sage, from Aromatic and Medicinal Plants Collection of the Institute of Genetics, Physiology and Plant Protection, ASM, including 13 hybrids and 15 parental forms were evaluated in this study (Table 1). Biological samples for each genotype were collected at 4-6 pairs of leaves stage from five independent plants, grown in field conditions and immediately frozen in liquid nitrogen for further analysis (Figure 2).

Fig. 2. Cultivation in field conditions of clary sage plant material.

Primer design for real-time PCR. The design of primers was performed using **PRIMER3** *web tool* (http://primer3plus.com/web_3.0.0/primer3web_input.htm).

The primer pairs for the studied genes were designed based on mRNA sequences: JN133922.1 *Salvia sclarea* (clary) sclareol synthase; JQ478434.1 *Salvia sclarea* (clary) *labd-13-en-8-ol diphosphate synthase* [22] (Table 2). The clary sage actin (NCBI: HM231319.1) was used as a reference gene (Table 2) [25].

RNA isolation. Total RNA was extracted from a bulk of five leaves of each genotype using TriReagent (Ambion, Applied Biosystems), according to the manufacturer's instruction.

These samples were treated with dsDNase (Thermo Scientific) to remove the residual DNA. First-strand cDNAs were synthesized from 0,6 µg total RNA using Oligo(dT18), random hexamer primers (Thermo Scientific) and RevertAid Reverse Transcriptase (Thermo Scientific).

Real Time PCR analysis. qRT-PCR was performed with gene-specific primers (Table 2) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Scientific) on a DTprime Real-time cycler (DNA-Technology). The amplification program used for qRT-PCR was: 95 °C for 10 min, 950 C for 15 s and 60 0C for 30 s, 40 cycles of 95° C for 15 s, 60° C for 30 s, and 72° C for 30 s, followed by a melting curve cycle from 60°C to 90° C. All samples were analyzed in three replicates performed in three different runs.

Primer specificity was assessed by agarose gel electrophoresis as well as from melting curve analysis of every reaction. The relative expression was calculated via the $2^{-\Delta Ct}$ method [16].

Quantitative determination of the sclareol by HPLC. Extraction of essential oil from S. sclarea L. was carried out according to the method specified in the European Pharmacopoeia [12, 25]. The fresh plant samples were subjected to hydrodistillation in Ginsberg equipment and kept in freezer compartment prior to be used in the study.

The samples of essential oil were analyzed by HPLC (Gilson: 303 pump series, 803C manometric, 231 injector, RI 131 refractive index detector, Kipp & Zonen BD41 recorder), with the hexane solvents gradient system for elution with 1.0% (v/v) isopro-

pyl alcohol. It was used a column type SGX CN (Prague) 150 x 3,5 mm, mobile phase at a flow rate of 0,7 ml/min and operating pressure of 18 Bar. Detection was done at 270 nm.

Table 1. *Salvia sclarea* L. genotypes used in investigations

	♀ Form *		♂ Form *		Hybrid F1-2 **
1	S. s. Turkmen/N S_7	15	(K-36 x 0-41) F_2 x 0-19) F_1 x 0-22) B_4 x L-15) F_8	17	[S. s. Turkmen/N S_7 x (K-36 x 0-41) F_2 x 0-19)F_1 x 0-22) B_4 x L-15)F_8] F_1
1	S. s. Turkmen/N S_7	2	(S-1122 528 S_3 x K-50) F_1 x 0-48)F_6]	18	[S. s. Turkmen/N S_7 x (S-1122 528 S_3 x K-50) F_1 x 0-48)F_6] F_1
1	S. s. Turkmen/N S_7	4	(Rubin x S1122 9S3) F_1 x (0-56 x V-24) F_1) F_7	19	[S. s. Turkmen/N S_7 x (Rubin x S1122 9 S_3)F_1 x (0-56 x V-24) F_1) F_7] F_1
3	(V-24-86 809 S_3 x 0-33 S_6) F_7	4	(Rubin x S1122 9S3) F_1 x (0-56 x V-24) F_1) F_7	20	[(V-24-86 809 S_3 x 0-33 S_6) F_7 x (Rubin x S1122 9S_3)F_1 x (0-56 x V-24)F_1)F_7] F_1
3	(V-24-86 809 S3 x 0-33 S6) F_7	9	(S1122 528 S_3 x S. s. TianShan/sud) F_5 x S. s. Tian-Shan/sud) B_5	21	[(V-24-86 809 S_3 x 0-33 S_6) F_7 x (S1122 528 S_3 x S. s. Tian-Shan/sud) F_5 x S. s. Tian-Shan/sud) B_5] F_1
3	(V-24-86 809 S_3 x 0-33 S6) F_7	11	Cr. p. 160 S_{11}	22	[(V-24-86 809 S_3 x 0-33 S6) F_7 x Cr. p. 160 S_{11}] F_1
16	M-69 655 S_9	12	(S-1122 528 S_3 x (Rubin x S-786)F_1 x (0-33 S_3 x L-15) F_7) F_7	23	[M-69 655 S_9 x (S-1122 528 S_3 x (Rubin x S-786)F_1 x (0-33 S_3 x L-15) F_7) F_7] F_1
16	M-69 655 S_9	15	(K-36 x 0-41) F_{20-19} F_1 x L-15) B_5	24	[M-69 655 S_9 x (K-36 x 0-41)$F_{20-19}$$F_1$ x L-15)B_5] F_1
16	M-69 655 S_9	13	(M-69 429-82 S_3 x 0-40 S_5) F_7	25	[M-69 655 S_9 x (M-69 429-82 S_3 x 0-40 S_5) F_7] F_1
10	Cr. p. 11 S_{11}	1	S. s. Turkmen/N S7	26	[Cr. p. 11 S_{11} x (S. s. Turkmen/N S_7] F_1
15	(K-36 x 0-41) F_2 x 0-19)F_1 x 0-22) B_4 x L-15) F_8	7	(M-44S_4 x L-15)F_1 x L-15)] B_6	27	[(K-36 x 0-41) F_2 x 0-19)F_1 x 0-22) B_4 x L-15)] F_7 x (M-44S4 x L-15) F_1 x L-15)] B_6] F_2
14	(K-50) F5 x S 1122 (102+113) F_2 x K-43) F_4	6	(0-57S_5 x 0-21S_4) F_8	28	[(K-50)F_5 x S 1122(102+113)F_2 x K-43) F_4 x (0-57 S_5 x 0-21S_4) F_8] F_2
5	(M-55+130 S_4 x (K-44 x L-15) F_2 x 0-47) F_6	7	(M-44 S_4 x L-15)F_1 x L-15) B_6	29	[(M-55+130 S_4 x (K-44 x L-15) F_2 x 0-47) F_6 x (M-44 S_4 x L-15) F_1 x L-15) B_6] F_2
* 1 – 16 clary sage parental forms				***17 – 29 clary sage hybrids*	

Estimation of sclareol concentration in essential oil extracts from clary sage hybrids was performed by absolute calibration method, using as reference standards sclareol pure solutions. Sclareol concentration was estimated as the product of peak height and half-height length [5].

Data analysis. Statistical analysis of the experimental data was performed according to Dospehov [27]. For calculation of descriptive statistics (arithmetic mean, standard deviation etc.) and Pearson's coefficient of correlation was used Microsoft Excel spreadsheet program.

Table 2. The specific primers used for qRT-PCR

Access number	Nucleotide succession (5'- 3')	Tm	Amplicon, bp
EMBL: JN133922.1	F: GAGCACCAGCAGCGATTAT	56,7	133
	R: GAGAGTTGCTTAGGACGGATTT	58,4	
EMBL: JQ478434.1	F: GACTCCAGAAACAACCCACATT	58,4	138
	R: CCCAGACGACCCTCCACAAGA	63,7	
NCBI: HM231319.1	F: TGGATTTGCTGGAGACGATG	57,3	293
	R: CACGATTGGCCTTGGGATTA	57,3	

Result and discussions

Selection of parental forms is an important first step in any breeding program. The ability to assess accurately genetic differences between parents and subsequently to predict progeny performance could enhance the efficiency of breeding programs [2].

Actually there is a great temptation to use molecular genetics in plant breeding [7, 17]. But, as selection methods based on phenotypic data rely on accurate estimation of breeding values of individuals or groups, similarly, molecular techniques require the accurate estimation of breeding values of alleles from phenotypes [21]. So, molecular genetics does not provide a quick fix to breeders, but rather slightly increased response to selection in part of the genome at a great cost.

Because molecular techniques provide powerful tools to study genetics and physiology of crops, it has been investigated the transcriptional activity of LPPS and SS genes governing the biosynthesis of sclareol in clary sage.

The primary aim of this study was to assess expression levels of SS and LPPS genes in some parent lines and hybrid combinations. In case of the SS no specific product was identified and this gene was excluded from further investigations. Real-Time PCR results were considered only for one gene – LPPS. For this gene a specific DNA fragment of 133 bp was amplified (Figure 3).

The analysis of LPPS transcriptional activity for 15 parental genotypes and 13 hybrid combinations showed that relative expression ranged from 0,2 to 5,8 conventional units. The quantity of transcripts in parental forms was relatively lower compared to clary sage hybrids.
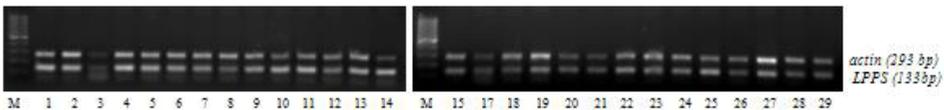
Fig. 3. Gene expression profile obtained using primers for LPPS gene and actin.
(M – 1000 bp Ladder, parental forms: 1 – 15, hybrids: 17 – 29).

In the case of studied hybrids the highest activity of gene was revealed for *([M-69 655 S9 x (M-69 429-82 S3 x 0-40 S5)F7]F1,* followed by *[Cr. P. 11 S11 x (S. s. Turkmen/N S7]F1, [S. s. Turkmen/N S7 x (Rubin x S1122 9S3)F1 x (0-56 x V-24) F1)F7] F1* and *[M-69 655 S9 x(K-36 x 0-41)F20-19)F1 x L-15)B5]F1* but the lowest values were recorded for *[(V-24-86 809 S3 x 0-33 S6)F7 x (S1122 528S3 x S. s. Tian-Shan/sud) F5 x S. s. Tian-Shan/sud)B5]F1* and *[(V-24-86 809 S3 x 0-33 S6)F7 x (Rubin x S1122 9S3) F1 x (0-56 x V-24)F1)F7]F1* (Figure 4, A).

For the parental forms the highest transcript accumulation level of LPPS gene was detected in S. s. Turkmen/N S7, followed by *(S-1122 528 S3 x K-50) F1 x 0-48) F6* while the lowest quantities were observed in case of *Rubin x S1122 9 S3) F1 x (0-56 x V-24) F1 and Cr. P. 11 S11* (Figure 4, B.).
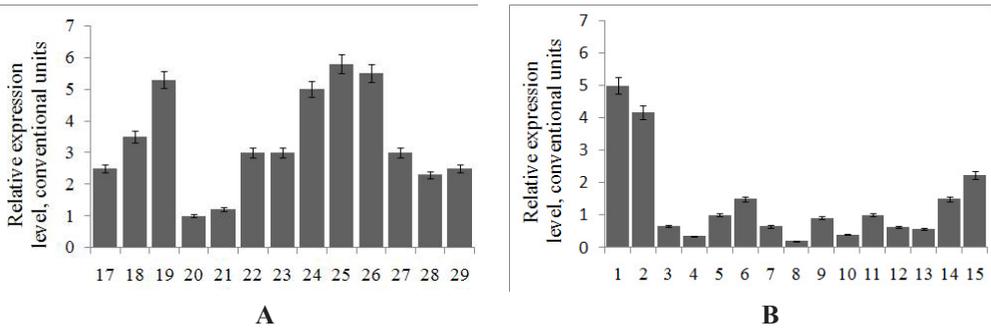
Fig. 4. Relative expression of LPPS gene for hybrids (A) and parental forms (B) .

Results obtained for genetic groups have revealed an association between transcriptional activity of LPPS gene and heterosis effect. Thus, 11 (19-29) of the 13 investigated hybrids have shown quantitative values of the transcriptional activity higher than such in parental forms. In six cases (22 hybrids, 24, 25, 27, 28 and 29) observed differences were significant (Figure 5).

Hybrid vigor is substantial and important for most commercial traits in plants [10, 13, 14]. At genetic level heterotic groups in clary sage have not been well studied or described. The characterization of genetic variability and an estimation of the genetic relationships among varieties are essential to clary sage breeding programs. Thus, these findings could represent a substantial advantage to predict the heterosis expected from crosses at all levels.

The second aim of this study was to estimate correlations between a series of proposed heterotic groups and hybrid performance for sclareol content in clary sage.
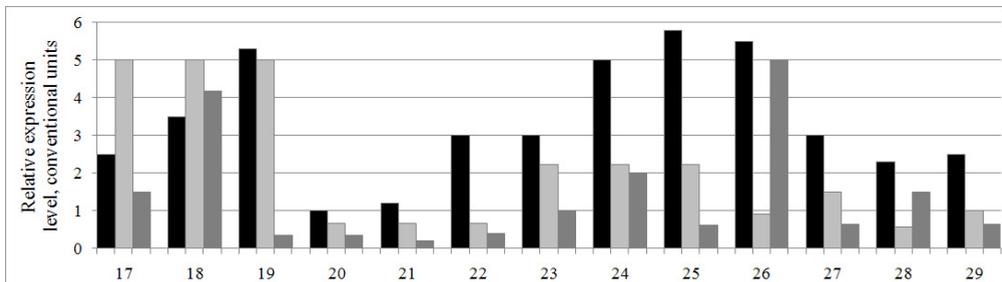
Fig. 5. Relative expression of LPPS gene for clary sage genetic groups
(*the genotypes order on the diagram is as follow: hybrid F1/ F2, ♀ form and ♂ form*)

The results obtained for the biosynthetic capacity show high intrapopulational variability of S. sclarea. Thus, the content of sclareol has varied from 1,9 % (hybrid no. 29) to 10 % (hybrid no. 25) (Figure 6).

The comparative analysis of data obtained for clary sage hybrids, in most cases ascertained a positive correlation (Pearson`s correlation coefficient r = 0,7) between the quantitative parameters which were studied – sclareol content in essential oil and LPPS transcriptional activity (Figure 7).

As example, the highest values of LPPS transcript concentration for the M-69 655 S9 x (M-69 429-82 S3 x 0-40 S5) F7] F1 hybrid correlates with biochemical data. Also, the data for the *[(M-55+130 S4 x (K-44 x L-15) F2 x 0-47) F6 x (M-44 S4 x L-15) F1 x L-15) B6] F2* hybrid, which showed the lowest amount of sclareol in essential oil correlates with the decreased level of transcriptional activity of the analyzed gene (Figure 7).

Summarizing, the obtained data support the hypothesis that LPPS gene transcriptional activity level could be useful in sclareol content prediction in clary sage.

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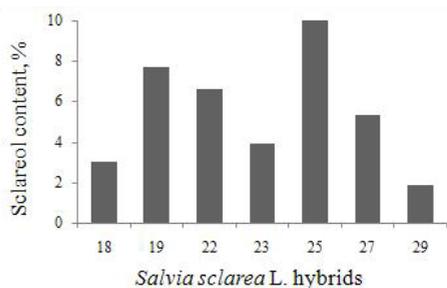
Fig. 6. Sclareol content for clary sage hybrids.
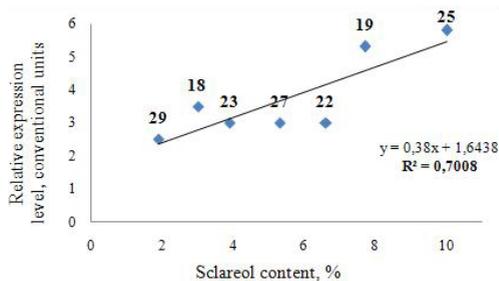
Fig. 7. The correlation between sclareol content and LPPS gene transcript level.

Conclusions

The level of relative expression of the LPPS gene was determined and its involvement in the biosynthesis of sclareol in *Salvia sclarea* L. was demonstrated. Quantitative RT-PCR results revealed six heterotic groups (no. 22, 24, 25, 27, 28, 29), for which the level of LPPS gene transcriptional activity was correlated with heterosis effect.

The results revealed that the highest values of LPPS transcript concentration for the *[M-69 655 S9 x (M-69 429-82 S3 x 0-40 S5) F7] F1* hybrid positively correlates with biochemical data – sclareol content in essential oil. Thus, such hybrid combination can be succesfully used in breeding programs.

The performed study ascertained the opportunity to predict heterosis effect based on the information about the levels of relative expression of genes, involved in secondary metabolites biosynthesis.

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