DOI: 10.32615/bp.2019.144 BIOLOGIA PLANTARUM 64: 167-177, 2020

Altered fatty acid composition of *Nicotiana benthamiana* and *Nicotiana excelsior* leaves under transient overexpression of the cyanobacterial *des*C gene

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Abstract

Transient heterologous gene expression in two model plant species, *Nicotiana benthamiana* and *N. excelsior,* has been used to study the localization of the heterologous Δ9 acyl-lipid desaturase (Δ9 desaturase) of *Synechococcus vulcanus* in different cell compartments and its functional activity in the cases of the cytosol, chloroplast, and endoplasmic reticulum (ER) localization. The functional activity and substrate specificity of the heterologous desaturase under the conditions of transient expression have been confirmed by comparison of fatty acid (FA) profiles. The Δ9 desaturase, responsible for the synthesis of oleic and palmitoleic acids, has also been shown to strongly promote the accumulation of polyunsaturated FAs. The results convincingly demonstrate that the Δ9 desaturase of the thermophilic cyanobacterium transiently expressed in two *Nicotiana* species considerably alters lipid metabolism in their leaves towards a higher FA unsaturation. The functional activity of Δ9 desaturase depends on both the model plant species, *N. benthamiana* or *N. excelsior,* and the cellular localization of the enzyme. The method of transient expression of heterologous genes in plants is highly effective, inexpensive, and not time-consuming, which makes it attractive for estimating the functional activity and/or substrate specificity of heterologous desaturases.

Additional key words: cellular compartments, Δ9 desaturase, oleic and palmitoleic acids, signal sequences.

Introduction

Fatty acids (FAs) and their derivatives constitute energy stores of plant seeds and play the key role in plant metabolism. They are the main structural components of membranes and can serve as the precursors of signal molecules involved in response to stress factors and during plant development (Dar *et al.* 2017).

It is currently believed that lipids are synthesized *via* the prokaryotic and eukaryotic pathways in plastids and endoplasmic reticulum (ER), respectively. Most plant FA desaturases involved in the formation of unsaturated FAs are also located in these cell compartments (Los *et al*. 2013, Wang *et al*. 2016, Dar *et al.* 2017). The eukaryotic and prokaryotic pathways interact with each other through a reversible exchange of FAs between the plastids and ER, in particular, in the case of sequential desaturation of FAs by desaturases.

Desaturases catalyze the transformation of single bonds between carbon atoms (C–C) in the acyl chains of FAs into double bonds (C=C) (Los *et al*. 2013), thereby converting saturated FAs into unsaturated ones. Solvable acyl-[acyl-carrier-protein] desaturases and membrane-bound acyl-lipid desaturases are responsible for FA desaturation in plant cells (Los *et al*. 2013). The Δ9 acyl-[acyl-carrier-protein] desaturase (Δ9 desaturase) plays the key role in the formation of unsaturated FAs necessary for maintaining the optimal liquid-crystal structure of plant cell membranes because it forms the first double bond in the FA chain (López Alonso *et al*. 2003). For example, Δ9 desaturase converts stearic acid (18:0) into oleic acid (18:1) by introducing a double bond between the ninth and tenth carbon atoms in the FA acyl chain (Troncoso-Ponce *et al.* 2016), thereby providing the substrate for other desaturases sequentially forming the second (position Δ12), third (Δ15), and subsequent double bonds

Submitted 2 August 2019, *last revision* 20 October 2019, *accepted* 21 November 2019

Abbreviations: DBI - double bond index; ER - endoplasmic reticulum; FA - fatty acid; FAME - fatty acid methyl ester; GC-MS - gas chromatography-mass spectrometry; GFP - green fluorescent protein; PDR - fatty acid C16:1 to C16:0 ratio; PUFA - polyunsaturated fatty acid; RuBisCO - ribulose-1,5-bisphosphate carboxylase/oxygenase; SDR - fatty acid C18:1 to C18:0 ratio; TLC - thin layer chromatography.

Acknowledgements: We are grateful to Dr. Roman Sidorov (K.A. Timiryazev Institute of Plant Physiology, the Russian Academy of Sciences) for his help in analyzing the fatty acid composition.

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(Napier 2007, Los *et al*. 2013, Wang *et al*. 2016, Dar *et al.* 2017). Thus, the desaturation of 18:0 to 18:1 catalyzed by Δ9 desaturase is one of the crucial stages controlling the content of unsaturated FAs in the cells.

Gene engineering approaches, such as constitutive expression of heterologous desaturase genes (Peng *et al.* 2018, Shi *et al.* 2018) and the RNA interference technology as a means to inhibit the expression of the target desaturase gene (Wang *et al*. 2010), are often used as convenient models to study the role of desaturases, including Δ9 desaturase, in plant physiology. Specifically, it has been convincingly demonstrated that constitutive expression of heterologous Δ9 desaturase genes in transgenic plants leads to effective conversion of stearic acid (18:0) into oleic acid (18:1), which is accompanied by considerable changes in the unsaturation of FAs, including polyunsaturated FAs (PUFAs) in membrane lipids (Orlova *et al.* 2003, Craig *et al.* 2008, Gerasymenko *et al.* 2015, Zhang *et al.* 2015).It is currently assumed that plant enzymes of this group are characterized by diverse lipid substrates, region-specific desaturation, and the capacity for functioning in different cell compartments, which deserves further study.

It should be noted that gene engineering approaches, which usually entail obtaining transgenic plants, are expensive and time-consuming. In this connection, researchers studying the functional activity, substrate specificity, and physiological role of the protein products of target genes switch from obtaining stable plant transformants to using systems of transient expression of heterologous genes in model plants, such as *N. benthamiana* and *N. excelsior* (Anwar *et al.* 2018, Ji *et al.* 2018, Sheludko *et al*. 2018, Thodberg *et al.* 2018, Gerasymenko *et al.* 2019, Li *et al.* 2019).

Here, we used transient expression of the *des*C gene from *Synechococcus vulcanus,* which encodes Δ9 desaturase, in two model plants, *N. benthamiana* and *N. excelsior,* to estimate the applicability of this method to studying the functional activity of desaturases, including the cases when the heterologous Δ9 desaturase was located in different cell compartments, namely, the cytosol, chloroplasts, and endoplasmic reticulum (ER).

Materials and methods

Engineering of plant expression vectors: Standard molecular cloning procedures and PCR protocols were used. Restriction endonucleases, T4 DNA ligase, Taq and Pfu DNA polymerases, and phosphatases were used according to the manufacturers' protocols (*Promega*, Madison, USA; *Fermentas*, Vilnius, Lithuania).

The expression vectors were obtained in several steps. First, the nucleotide sequence of the *des*C gene encoding the Δ9 acyl-lipid desaturase of *Synechocystis vulcanus* was obtained by means of PCR using the pQE-desC plasmid (Maali *et al.* 2007) as a template, the GGGCCCACATCATCCTTAGAA sequence as a forward primer (F1), and the GAATTCGGACAACGCTTTGGG sequence as a reverse primer (R1), with the *ApaI* and *EcoRI* restriction sites introduced into the forward and reverse primers, respectively. The PCR product was cloned into the pPGG vector (Vyacheslavova *et al.* 2012) preliminarily hydrolyzed at the *ApaI* and *EcoRI* sites to obtain the pPGG-D9 vector. The *des*C-*egfp* hybrid gene was constructed in the following steps. Firstly, the nucleotide sequence of the *egfp* gene encoding green fluorescent protein (GFP) was obtained by means of PCR using the pQE-egfp plasmid as a template, the GAATTCGTGAGCAAGGGCGAG sequence as a forward primer (F2), and the CCCGGGCTTGTAGTACAGCTCGT sequence as a reverse primer (R2), with the *EcoRI* and *SmaI* restriction sites introduced into the forward and reverse primers, respectively (Piruzian *et al.* 2002). The resultant PCR fragment was cloned into the pPGG-D9 plasmid preliminarily hydrolyzed at the *EcoRI* and *SmaI* sites to obtain the pPGG-D9E plasmid. The correctness of the hybrid gene construction was confirmed by sequencing.

The pVIG-D9 plant expression vector carrying the *des*C gene was obtained by cloning the SacI-XbaI fragment of the pPGG-D9 plasmid into the pVIG-T 1A vector (Vyacheslavova *et al.* 2012) preliminarily hydrolyzed at the *ApaI* and *SmaI* sites. The pVIG-D9E plant expression vector carrying the *des*C-*egfp* hybrid gene was obtained by cloning the ApaI-SmaI fragment of the pPGG-D9E plasmid into the pVIG-T 1A vector (Vyacheslavova *et al.* 2012) preliminarily hydrolyzed at the *ApaI* and *SmaI* sites.

The plant expression vector carrying the *des*C gene fused with the sequence for the transit peptide of the small chain of RuBisCO (Lch) of *Arabidopsis thaliana* (gene *ats1A*, *NCBI*, X13611) was obtained in several steps. At the first step, the Lch sequence encoding the transit peptide of the RuBisCO small chain was obtained by means of PCR using the pVIG-S2B plasmid (Vyacheslavova *et al.* 2012) as a template, the GGTACCATGGCTTCTATGATATC sequence as a forward primer (F3), and the GGGCCCCTGATATTCAACTATAT sequence as a reverse primer (R3), with the *KpnI* and *ApaI* restriction sites introduced into the forward and reverse primers, respectively. At the second step, the PCR product was cloned into the pVIG-D9 vector preliminarily hydrolyzed at the *KpnI* and *ApaI* sites to obtain the pVIG-Lch-D9 vector. The pVIG-Lch-D9E carrying the d*es*C-*egfp* hybrid gene fused with the sequence for the transit peptide of the small chain of RuBisCO (Lch) was obtained by cloning the ApaI-SmaI fragment of the pVIG-D9E plasmid into the pVIG-Lch-D9 vector.

The plant expression vector carrying the *des*C gene fused with the sequences ensuring protein transport in the endoplasmic reticulum (ER) was obtained as follows. First, the LeB4 sequence encoding the transport protein was obtained by means of PCR using the pVIG-T 2D plasmid (Vyacheslavova *et al.* 2012) as a template, the GGTACCATGTCCAAACCTTTTCT sequence as a forward primer (F4), and the GGGCCCTGCTAAACATGTGCT sequence as a reverse primer (R4), with the *KpnI* and *ApaI* sequences introduced into the forward and reverse primers, respectively. The PCR product was cloned into the pVIG-D9 vector preliminarily hydrolyzed at the *KpnI* and *ApaI* sites to obtain the pVIG-LeB4-D vector. Then, the SmaI-XbaI fragment of

the pVIG-T 2D plasmid carrying the sequence encoding the signal of protein retention in the ER (SRKDEL) and the polyadenylation signal was cloned into the pVIG-LeB4-D9 vector preliminarily hydrolyzed at the *SmaI* and *XbaI* sites to obtain the pVIG-D9-ER vector. The pVIG-D9E-ER vector carrying the *des*C-*egfp* hybrid gene fused with the *LeB4* sequence encoding the transport peptide and the sequence encoding SRKDEL was obtained by cloning the ApaI-SmaI fragment of the pVIG-D9E plasmid into the pVIG-D9-ER vector.

The pVIG-E expression vector carrying the *egfp* gene of GFP obtained earlier was used as a control (Vyacheslavova *et al.* 2012).

The correctness of the fusion of the genes with the corresponding leader signals in the plant expression vectors pVIG-Lch-D9, pVIG-Lch-D9E, pVIG-D9-ER, and pVIG-D9E-ER was confirmed by sequencing.

Plant species, Agrobacterium strains, and agroinfiltration: *Nicotiana benthamiana* Domin and *Nicotiana excelsior* J.M. Black were grown hydroponically at a temperature of 22 ± 2 °C, a 8-h photoperiod, an irradiance of 100 μmol m^{-2} s^{-1}, and a relative humidity of ?? %. Knop's solution was used as a nutrient medium. The pVIG-E, pVIG-D9, pVIG-D9E, pVIG-Lch-D9, pVIG-Lch-D9E, pVIG-D9-ER, and pVIG-D9E-ER vectors were used to transform the *Agrobacterium tumefaciens* GV3101 strain as described earlier (Berestovoy *et al.* 2018). Transformed bacteria were selected on a medium containing kanamycin and designated according to the expression vector used for the transformation. The transformed agrobacteria were used for agroinfiltration into the abaxial epidermis of the leaves of six-week-old *N. benthamiana* and *N. excelsior* plants by means of a syringe without a needle as described earlier (Berestovoy *et al.* 2018). After the agroinfiltration, the plants were cultured under the same conditions for seven days. Agroinfiltrated leaf fragments were used in subsequent analysis.

Protoplast isolation and fluorescence microscopy: Protoplasts were isolated from the leaf fragments with fluorescent areas as described earlier (Tyurin *et al.* 2017, Berestovoy *et al.* 2018). The fluorescence in protoplasts was recorded using an *Axio Imager Z2* (*Zeiss*, Oberkochen, Germany) microscope equipped with an *AxioCam MR* digital camera and filters No. 38 (λ_{ex} BP 470 nm/40 nm and λ_{em} BP 525 nm/50 nm, where ex stands for fluorescence excitation and em for fluorescence emission) and No. 45 (λ_{ex} BP 560 nm/40 nm and λ_{em} BP 630 nm/75 nm) for recording the GFP fluorescence and chlorophyll autofluorescence, respectively. The fluorescence of intravital preparations was recorded using an *ApoTome* module (*Zeiss*), which reduces defocused fluorescence. The images were manually processed using the *AxioVision 4.8* software (*Zeiss*). About 100 protoplasts were analyzed for each localization of the target protein.

Isolation and analysis of fatty acids from total lipids of N. benthamiana and N. excelsior leaf tissues: Samples of agroinfiltrated leaf areas were chopped up with a scalpel, heated to the boiling point for 30 min in isopropanol containing 0.001 % (v/v) of an antioxidant (butylated hydroxytoluen) and 1 mg of heptadecanoic acid as an internal standard (Sidorov *et al.* 2012), homogenized in a mortar, and boiled for 60 min in a 8 % (v/v) isopropanol solution of KOH. Isopropanol was evaporated and replaced with an equal volume of water; unsaponifiable lipids were removed using hexane. The solution was acidified to pH 2, and free FAs were extracted with hexane and converted into fatty acid methyl esters (FAMEs) by boiling in a mixture of 10 cm^3 of CH_3OH and 0.5 cm^3 of CH_3COCl for 1 h. FAMEs were purified by thin-layer chromatography (TLC) on a silica gel-coated plate and separated by gas-liquid chromatography (GLC) in a capillary column (Sidorov *et al.* 2012). The double bond index (DBI), C18:1 to C18:0 ratio (SDR), and C16:1 to C16:0 ratio (PDR) were calculated from the following equations: DBI = [(M)+2(D) + 3(T)]/100, where M is the sum of the mass fractions of monounsaturated FAs, D is the sum of the mass fractions of diunsaturated FAs, and T is the sum of mass fractions of triunsaturated FAs; SDR = (%18:1)/(%18:0 + %18:1), where %18:0 is the mass fraction of 18:0 FAs and %18:1 is the mass fraction of 18:1 FAs; PDR = (%16:1)/(%16:0 + %16:1), where %16:0 is the mass fraction of 16:0 FAs and %16:1 is the mass fraction of 16:1 FAs.

Statistical analysis: All experiments were performed in at least six independent replicates. The results were statistically treated using the *Statistica v. 9.0* software (Student's *t*-test was used, with differences considered significant at $P \leq 0.05$) and *Microsoft Office Excel 2007* software.

Results

Two series of expression vectors were constructed. In the first series comprising pVIG-D9E, pVIG-Lch-D9E, and pVIG-LeB4-D9E-ER, we used the *des*C-*egfp* hybrid gene obtained by fusing the *des*C gene encoding the cyanobacterial Δ9 desaturase and the *egfp* gene, whose protein product is GFP optimized for expression in eukaryotic cells (Fig. 1). Note that the *egfp* initiation codon was removed from the hybrid gene to avoid alternative translation. In the other series comprising the pVIG-D9, pVIG-Lch-D9, and pVIG-LeB4-D9-ER vectors, only the native cyanobacterial *des*C gene was used.

The native (*des*C) and hybrid (*des*C-*egfp*) genes in the pVIG-Lch-D9 and pVIG-Lch-D9E vectors, respectively, had a well-characterized signal in the 5' region ensuring the localization of the target proteins into chloroplasts (Fig. 1) (Gerasymenko *et al.* 2015). In the pVIG-LeB4-D9-ER and pVIG-LeB4-D9E-ER expression vectors, a sequence corresponding to the LeB4 signal peptide was cloned into the 5' regions of the target genes (*des*C and *des*C-*egfp*) for localizing the protein product to the ER, and the fragment encoding the signal of protein retention in the ER was introduced into the 3' region (Fig. 1) (Alanen *et al.* 2011, Vyacheslavova *et al.* 2012). In the pVIG-Lch-D9, pVIG-Lch-D9E, pVIG-D9-ER, and pVIG-D9E-ER vectors, the

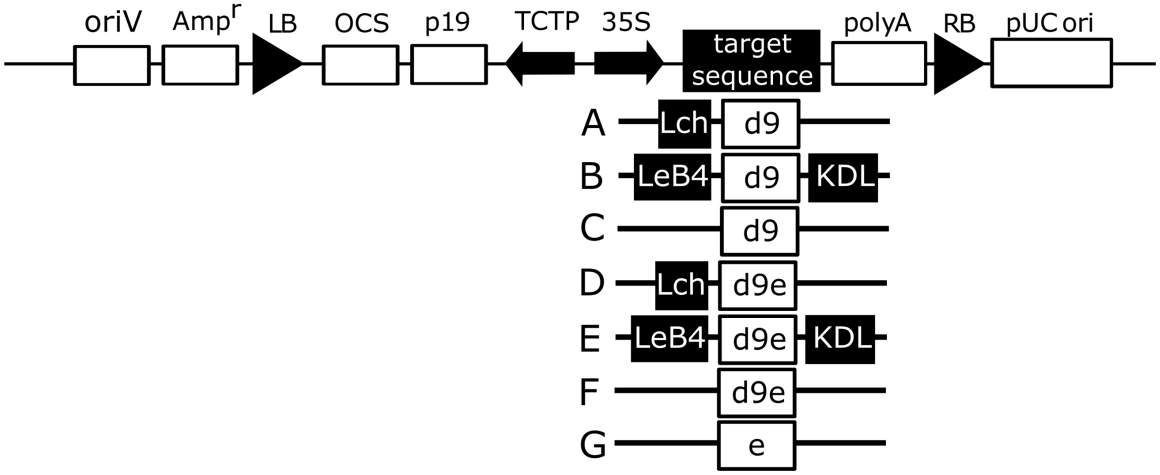

Fig. 1. A schematic representation of plant expression vectors. Each plant expression vector carries the *Cymbidium ringspot* virus gene (*CymRSV*), which encodes an RNA-silencing suppressor protein p19 under the control of the translationally controlled tumor protein (TCTP) promoter and the termination sequence of the *octopine synthase* (*OCS*) gene. oriV - replication start point for *Agrobacterium tumefaciens*; Ampr - ampicillin resistance cassette; LB and RB - left and right borders, respectively, of the T-DNA region; pUCori - origin of replication for *Escherichia coli*; 35S - CaMV 35S promoter; poly-A - polyadenylation signal; Target sequence - the *des*C gene sequence or the *des*C-*egfp* hybrid gene sequence; Lch - sequence for the transit peptide of *A. thaliana* RuBisCO small subunit (gene ats1A, NCBI, X13611); LeB4 - sequence for the signal peptide for protein localization into endoplasmic reticukum; KDL - endoplasmic reticulum-retention signal; d9 - *des*C gene sequence; d9e - *des*C-*egfp* hybrid gene sequence; Ø - absence of signal peptide for protein localization; e - green fluorescent protein gene sequence (*egfp*).

translation-initiation AUG codon was introduced into the 5' region of the sequence encoding the Lch and LeB4 signal peptides and removed from the target genes to avoid alternative translation.

In the pVIG-D9 and pVIG-D9E expression vectors, the target genes (*des*C and *des*C-*egfp*) did not contain additional signal sequences in either 5' or 3' region, their expression was controlled only by the 35S RNA CaMV promoter, which did not entail any specific compartmentalization of the protein products of these genes (Fig. 1). All the vectors were based on the pVIG-T construct, which we developed earlier specially for effective transient expression of heterologous genes in plants (Vyacheslavova *et al.* 2012). The T-DNA region of this vector contains the sequence of the gene of the p19 tomato virus protein, a suppressor of posttranscriptional gene silencing under the control of the TCTP (translationally controlled tumor protein) promoter (Fig. 1) (Vyacheslavova *et al.* 2012). The first series of vectors was used to study the subcellular localization of the heterologous desaturase in plant cells, and the other one, to compare the modification of the FA composition of plant membrane lipids as dependent on the subcellular localization of the heterologous Δ9 desaturase.

We estimated the localization of the heterologous desaturase with the C-terminal fusion with the fluorescent protein (hereinafter referred to as D9E) in experiments where the signal sequences for target subcellular compartments were used. For this purpose, we used the first series of vectors, the method based on obtaining protoplasts in plant tissue agroinfiltrated with bacterial strains carrying different expression vectors, and fluorescence microscopy.

In the protoplasts obtained after agroinfiltration with the strain carrying the pVIG-Lch-D9E plasmid (Fig. 1), almost all chloroplasts displayed fluorescence in the yellow spectral region (Fig. 2*B*). This indicated the chloroplast localization of the D9E fusion protein, which was transferred to these compartments by means of the signal sequence (Lch) of the *A. thaliana* RuBisCO small chain. The fluorescence signal from D9E was detected in the ER of the protoplasts (Fig. 2*C*) obtained after agroinfiltration with the strain carrying the pVIG-D9E-ER vector (Fig. 1), where the N-terminal signal peptide LeB4 transferred the D9E protein across the ER membrane, and the C-terminal SRKDEL signal retained D9E in the ER. The protoplasts obtained from the leaves agroinfiltrated with the strain carrying the pVIG-D9E vector (Fig. 1) displayed the fluorescence signal of the D9E fusion protein in the cytosol (Fig. 2*A*). Thus, the results demonstrated that the signal sequences used ensured the localization of the heterologous Δ9 desaturase in the target compartments of the plant cell.

In the protoplasts obtained after agroinfiltration with the strain carrying the pVIG-Lch-D9E plasmid (Fig. 1), almost all chloroplasts displayed fluorescence in the yellow spectral region (Fig. 2*B*, Fig. 1 Suppl.). This indicated the chloroplast localization of the D9E fusion protein, which was transferred to these compartments by means of the signal sequence (Lch) of the *A. thaliana* RuBisCO small chain. The fluorescence signal from D9E was detected in the ER of the protoplasts (Fig. 2C, Fig. 1 Suppl.) obtained after agroinfiltration with the strain carrying the pVIG-D9E-ER vector (Fig. 1), where the N-terminal signal peptide LeB4 transferred the D9E protein across the ER membrane, and the C-terminal SRKDEL signal retained D9E in the ER. The protoplasts obtained from the leaves agroinfiltrated with the strain carrying the pVIG-D9E vector (Fig. 1) displayed the fluorescence signal of the D9E

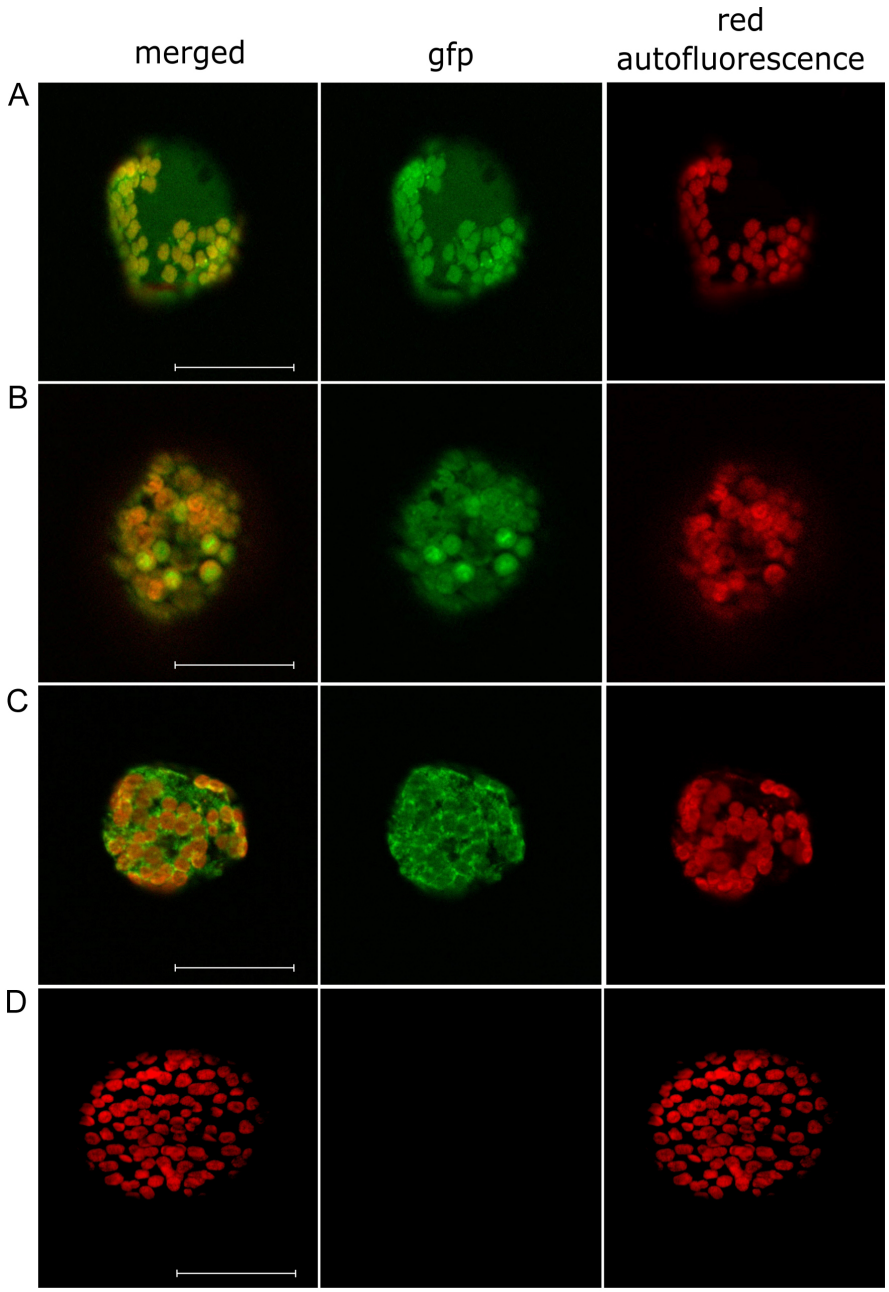

Fig. 2. Subcellular localization of green fluorescent protein (GFP) fusions in *Nicotiana benthamiana* protoplasts isolated from the cells of the agroinfiltrated leaf area. Transient gene expressions of plant expression vectors, with leader signal sequences providing their localization in various compartments of plant cells, demonstrate that the GFP fusions localized in cytosol (*A*), chloroplasts (*B*), endoplasmic reticulum (*C*), and non-transformed protoplasts (*D*). The *merged images* include the GFP channel (*green*) and the chloroplast autofluorescence channel (*red*). Each picture represents a single protoplast. All images obtained using the single-sectioned fixed focus plane produced by the *ApoTome* module (*Zeiss*). The s*cale bar* is 40 µm.

fusion protein in the cytosol (Fig. 2*A*, Fig. 1 Suppl.). Thus, the results demonstrated that the signal sequences used ensured the localization of the heterologous Δ9 desaturase in the target compartments of the plant cell.

It should be noted that that transient gene expression in plants has not been used before to estimate the functional activity of desaturases. This method entails wounding, if only slight, of the leaves during agroinfiltration. It is known that the expression of plant FA desaturases, including Δ9 desaturase, may be activated in response to wounding (Dar *et al.* 2017, Xue *et al.* 2018). Therefore, we first attempted to determine whether the FA profile was altered after the agroinfiltration and, hence, whether the transient gene expression method could be used to subsequently estimate the functional activity of desaturase.

For this purpose, we compared the FA compositions

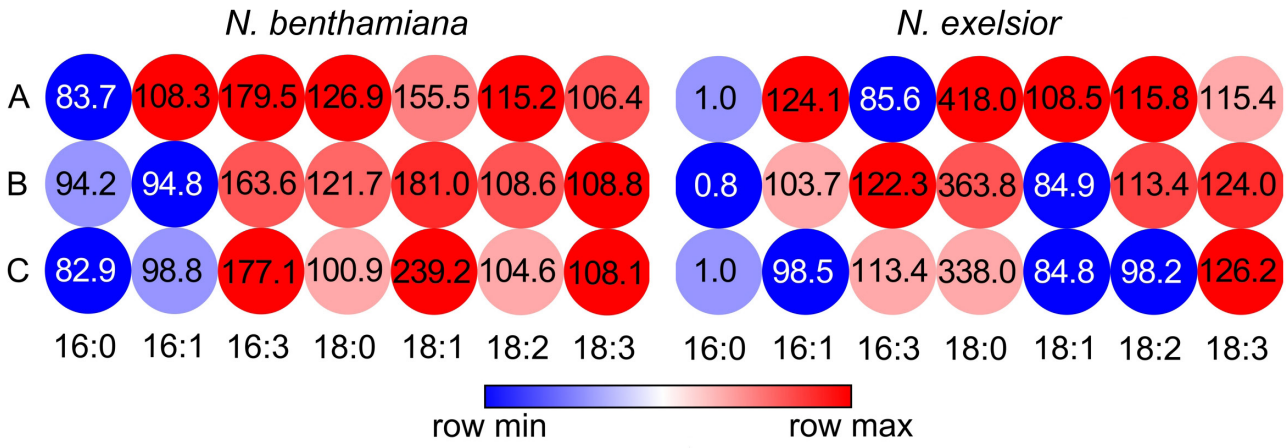

Fig. 3. A heat map of changes in the content of major fatty acidss of *Nicotiana benthamiana* and *N. excelsior* in percentage of those in the control plants (pVIG-E expression) 7 d after transformation. Δ9 desaturase localization: *A* - cytosol; *B* - plastid; *C* - endoplasmic reticulum.

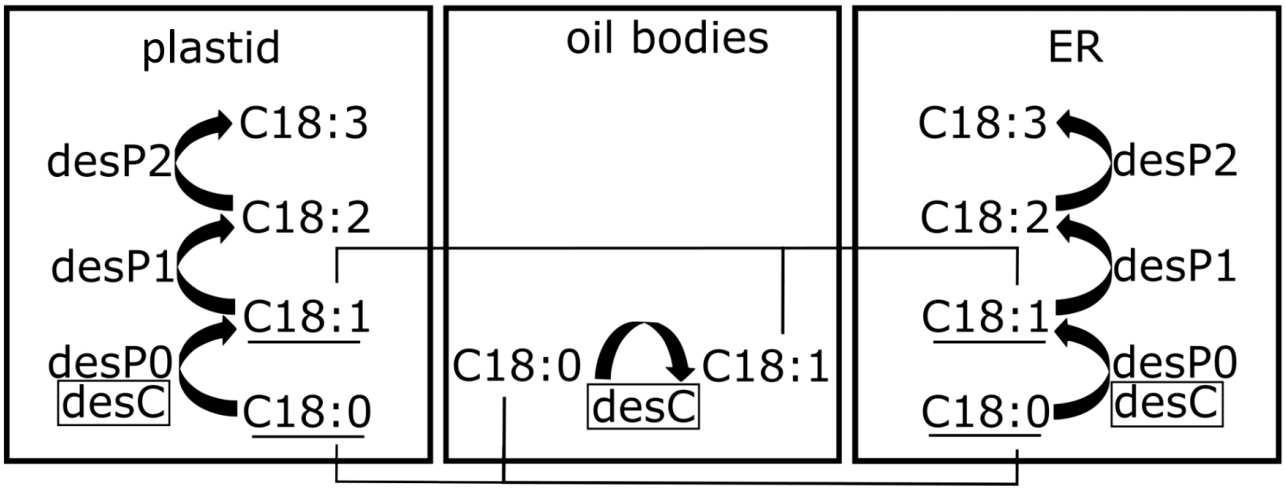

Fig. 4. The putative mechanism of functioning the heterologous Δ9-desaturase in different compartments of a plant cell. PL - plastid; ER - endoplasmic reticulum; OB - oil body; desC - Δ9-desaturase; desP0,P1,P2 - plant desaturases (Δ9, Δ12, Δ15); C18:0, C18:1, C18:2, C18:3 - fatty acids.

and calculated the DBI, SDR, and PDR values in the control *N. benthamiana* and *N. excelsior* plants (hereinafter referred to as wild-type plants), as well as the *N. benthamiana* and *N. excelsior* plants that had been agroinfiltrated with the *Agrobacterium* strain carrying the pVIG-E expression vector with constitutive expression of the reporter *egfp* gene (hereinafter referred to as agroinfiltrated plants).

The FA compositions of control and agroinfiltrated *N. benthamiana* leaves were measured. After agroinfiltration, only the content of the 18:1 and 16:3 FAs and SDR were significantly decreased (Table 1 Suppl.).

Comparison of the control and agroinfiltrated *N. excelsior* leaves showed a significant decrease in the 18:1 and 16:3 FA levels and SDR, as well as a significant increase in the 16:1 FA level as a result of agroinfiltration (Table 2 Suppl.).

In general, these results suggest that neither agroinfiltration nor a possible damaging factor (wounding) activates dramatically the expression of plant desaturases, including Δ9 desaturase, because the linoleic acid (18:2) and linolenic acid (18:3) content, as well as DBI, remained unchanged, and the C18:1 content and SDR were significantly decreased after agroinfiltration in both *N. benthamiana* and *N. excelsior* leaves (Table 1, Table 2 Suppl.).

In summary, our data suggest that transient gene expression in plants upon agroinfiltration is eligible for estimating the functional activity of Δ9 desaturases and that agroinfiltration with the strain carrying the pVIG-E vector with constitutive expression of the reporter *egfp* gene can be used as a justified and reliable control.

Furthermore, we used the data on the FA profile in wild-type *N. benthamiana* and *N. excelsior* to compare these species in terms of the C18 to C16 ratio and found that they considerably differed from each other. This ratio was 3:1 in *N. benthamiana* and 1:1 in *N. excelsior.* Besides, these species differed in DBI and PDR, which were significantly higher in *N. benthamiana* than in *N. excelsior* (Table 1,2 Suppl.). Thus, the two species that we used as model plants for estimating the functional activity of the heterologous desaturase can be assigned to two different types according to the FA composition: *N. benthamiana,* C18 type; *N. excelsior,* C16 type.

Then, we estimated the functional activity of the heterologous Δ9 desaturase as dependent on its localization in the plant cell. For this purpose, we used *Agrobacterium* strains carrying the second series of vectors containing the heterologous Δ9 desaturase gene without a signal sequence (pVIG-D9; Fig. 1), or the gene fused with signal sequences (pVIG-Lch-D9, Fig. 1) served as a control.

We analyzed the desaturase functional activity by comparing the FA profiles. Comparison of the FA compositions, SDR, PDR, and DBI showed a significant increase only in the C18:2 and C18:3 content, as well as DBI, in agroinfiltrated *N. benthamiana* and *N. excelsior* compared to the control. The increase in the C18:3 content and DBI did not depend on the Δ9 desaturase localization in plant compartments, whereas the increase in the C18:2 was characteristic of the Δ9 desaturase localization in the chloroplasts and ER, but not in the cytosol. Furthermore, the C16:1 content remained unchanged in all agroinfiltrated variants of *N. benthamiana* and *N. excelsior,* in contrast to the C16:0 and C16:3 content. The C16:0 content was decreased significantly in all variants of plants except for the *N. benthamiana* variant with the plastid localization. It should be emphasized, however, that a substantial (on average, tenfold) decrease in the C16:0 content was characteristic of all variants of *N. excelsior.* The C16:3 content was increased significantly in all agroinfiltrated variants of *N. benthamiana,* but not *N. excelsior,* where its significant increase was observed only in the cases of cytosolic and chloroplast localizations of Δ9 desaturase, but not in the case of ER localization. Comparison of the C18:0 and C18:1 content in all agroinfiltrated variants of *N. benthamiana* and *N. excelsior* showed several differences. Specifically, the C18:0 content was increased significantly in all agroinfiltrated *N. benthamiana* and *N. excelsior* plants except for the *N. benthamiana* with the cytosolic localization of the heterologous Δ9 desaturase. However, all variants of *N. excelsior* were characterized by a significant increase in the C18:0 content (by a factor of more than three). The C18:1 content exhibited different trends in agroinfiltrated *N. benthamiana* and *N. excelsior*: it was significantly increased in all *N. benthamiana* plants, irrespective of the localization of the heterologous Δ9 desaturase, whereas no significant changes in the C18:1 content were found in any *N. excelsior* variant.

Comparison of the C16:1 to C16:0 (PDR) and C18:1 to C18:0 (SDR) ratios in the agroinfiltrated variants of *N. benthamiana* and *N. excelsior* also showed drastically different trends in the two species. In *N. benthamiana,* SDR was significantly increased in all variants of heterologous Δ9 desaturase localization except for the ER localization, whereas PDR was significantly increased only in the case of the ER localization. In *N. excelsior,* SDR was decreased, whereas PDR was increased substantially (on average, by a factor of ten) (Tables 1, 2).

Thus, our data show that the Δ9 desaturase of the thermophilic cyanobacterium transiently expressed in two species considerably alters the lipid metabolism in the leaves towards a higher unsaturation of fatty acids,

Table 1. Fatty acid (FA) composition (in percentage of the total FA content) of transformed leaves of *Nicotiana benthamiana* 7 d after transformation as dependent on the cellular localization of the heterologous Δ9 desaturase. Means ± SEs of six experiments. *Asterisks* indicate significant differences ($P \leq 0.05$) from the control (pVIG-E, constitutive expression of the reporter *egfp* gene).

	16:0	16:1	16:3	18:0	18:1	18:2	18:3	DBI	SDR	PDR
Control	22.16±1.74	2.40±0.35	3.27±0.68	3.15±0.25	0.79±0.17	7.42±0.25	52.74±1.54	1.94±0.03	0.22±0.01	0.10±0.01
Cytosol	18.36±0.9*	2.37±0.40	5.79±0.65*	3.19±0.81	1.91±0.28*	7.77±0.23	57.00±1.12*	2.05±0.03*	0.35±0.05*	0.12±0.01
Plastid	20.87±0.86	2.28±0.56	5.35±0.94*	3.84±0.17*	1.44±0.21*	8.07±0.13*	57.39±1.43*	2.02±0.02*	0.27±0.02*	0.11±0.02
ER	18.54±1.26*	2.60±0.57	5.87±0.55*	4.01±0.25*	1.24±0.12*	8.56±0.31*	56.10±1.16*	2.06±0.04*	0.22±0.04	0.13±0.01*

Table 2. Fatty acid (FA) composition (in percentage of the total FA content) of transformed leaves of *Nicotiana excelsior* 7 d after transformation as dependent on the cellular localization of the heterologous Δ9 desaturase. Means ± SEs of six experiments. *Asterisks* indicate significant differences ($P \leq 0.05$) from the control (pVIG-E, constitutive expression of the reporter *egfp* gene).

	16:0	16:1	16:3	18:0	18:1	18:2	18:3	DBI	SDR	PDR
Control	38.73±1.53	3.54±0.49	3.84±0.17	10.2±0.57	1.40±0.20	14.4±0.57	23.61±0.88	1.19±0.05	0.12±0.02	0.08±0.02
Cytosol	0.40±0.18*	3.48±0.41	4.35±0.12*	34.6±1.32*	1.19±0.18	14.1±0.63	29.80±0.87*	1.40±0.04*	0.03±0.02*	0.90±0.03*
Plastid	0.29±0.05*	3.67±0.80	4.70±0.27*	37.2±1.33*	1.19±0.28	16.3±0.55*	29.28±1.90*	1.33±0.04*	0.03±0.01*	0.92±0.05*
ER	0.38±0.07*	4.39±1.66	3.28±0.66	42.7±3.84*	1.52±0.66	16.6±0.70*	27.23±0.81*	1.37±0.06*	0.03±0.01*	0.92±0.02*

its functional activity depending on both the model plant species (*N. benthamiana* or *N. excelsior*) and the specific localization of Δ9 desaturase in the plant cell.

Discussion

This study was aimed at determining *1*) whether transient gene expression in model plants was in principle a suitable approach to studying the functional activity of desaturases, with heterologous Δ9 desaturase used as an example; *2*) whether any of the plant cell compartments studied (the cytosol, chloroplasts, or ER) was preferable in terms of the functioning of the heterologous Δ9 desaturase; and *3*) whether the functional activity of the heterologous Δ9 desaturase depended on the model plant species (*N. benthamiana* or *N. excelsior*).

We used the Δ9 desaturase of the thermophilic cyanobacterum *S. vulcanus* because *a*) cyanobacterial Δ9 acyl-lipid desaturases are known to be homologous to plant acyl-CoA desaturases (Chen and Thelen 2013, Los *et al*. 2013, Smith *et al*. 2013, Bryant *et al.* 2016); *b*) thermophilic Δ9 acyl-lipid desaturase constitutively expressed in transgenic *N. tabacum* and localized in the cytosol (Orlova *et al.* 2003) or chloroplasts (Gerasymenko *et al.* 2015) of leaf cells efficiently converts stearic acid (18:0) to oleic acid (18:1), causing a considerable increase in the degree of FA unsaturation in cell membrane lipids (Orlova *et al.* 2003, Gerasymenko *et al.* 2015); and *c*) thermophilic Δ9 acyl-lipid desaturase recognizes and desaturates two substrates, the 16:0 and 18:0 lipid-bound FAs (palmitic and stearic acids, respectively) (Kiseleva *et al.* 2000), with stearic acid being a preferable substrate and the specificity towards palmitic acid being 1 % of that towards stearic acid, as shown in experiments on Δ9 acyl-lipid desaturase expression in *N. tabacum* (Orlova *et al.* 2003, Gerasymenko *et al.* 2015).

Various gene engineering approaches, such as obtaining transgenic plants with an expression of target genes and silencing target genes using RNA interference, are used for studying the physiological roles of target genes and estimating the functional activities of their products. It should be noted that the systems of stable transformation of many plants are difficult to develop, and strategies based on ectopic expression, though applicable, are expensive and time-consuming even in model *A. thaliana* plants. On the other hand, highly efficient systems of transient expression have been developed for a number of *Nicotiana* species, including *N. benthamiana* and *N. excelsior* (Gerasymenko *et al.* 2019), and the functions of many plant proteins have been identified and clarified using the data on transient expression of target genes in these plants (Anwar *et al.* 2018, Ji *et al*. 2018; Sheludko *et al.* 2018, Thodberg *et al.* 2018, Li *et al.* 2019).

Plants have many FA desaturases, which are responsible for the synthesis of most PUFAs. Their important role in the variation of the FA composition of oilseeds, as well as in the vital activity of plants exposed to biotic and abiotic stress factors, has been confirmed experimentally (Orlova *et al.* 2003, Craig *et al.* 2008, Los *et al.* 2013, Smith *et al.* 2013, Zhang *et al.* 2014, Shi *et al*. 2018).

However, many representatives of this class of enzymes remain poorly studied in terms of functional activity, substrate specificity, and physiological role in plant cells. This is largely because the gene engineering approaches and manipulations used in these studies are labor- and time-consuming. Therefore, the question arises of whether the method of transient gene expression in plants, as a reliable and simple approach to studying the functions of gene products, can be used to estimate the functional activity of a heterologous desaturase.

Our data that the FA profile of *N. benthamiana* and *N. excelsior* leaves agroinfiltrated with pVIG-E remains about the same as that of wild-type plants (Tables 1, 2 Suppl.) led us to the conclusion that the method of transient gene expression in plants could be tested in experiments on the estimation of the heterologous Δ9 desaturase activity. Plants agroinfiltrated with a strain carrying the pVIG-E vector were concluded to be an appropriate control in these studies.

As noted above, thermophilic Δ9 acyl-lipid desaturase catalyzes the desaturation of two substrates, the 16:0 and 18:0 FAs (Kiseleva *et al.* 2000). The results of comparison of the FA profiles of wild-type *N. benthamiana* and *N. excelsior* gave grounds to assign these model plants to two different FA-composition types: *N. benthamiana* is a representative of the C18 type, and *N. excelsior,* of the C16 type. In this connection, the heterologous Δ9 desaturase in these model plants could be expected to have a preference for different substrates: the 16:0 FA in the case of *N. excelsior* and the 18:0 FA in the case of *N. benthamiana.*

In order to determine whether transient expression in model plants could be used to study the functional activity and/or substrate specificity of the heterologous Δ9 desaturase, we compared the FA compositions in the plants agroinfiltrated with the strains carrying the pVIG-E vector and with those carrying the target gene of the cyanobacterial Δ9 desaturase (referred to here as DesC plants) (Tables 1, 2). Detailed comparative analysis showed that the heterologous enzyme had different effects on the FA content and composition in different *Nicotiana* species. Specifically, the content of one of the major saturated FAs, 16:0, in *N. benthamiana* was decreased, whereas the content of another one, 18:0, was increased. The amount of the 16:1 monounsaturated FA remained unchanged in these plants, and the amount of the 18:1 FA was increased (Table 1, Fig. 3). In addition, the DesC plants displayed considerably increased content of major trienoic FAs, 16:3 and 18:3, as well as DBI (Table 1, Fig. 3). Therefore, transient expression of the heterologous Δ9 desaturase is likely to have resulted in accumulation of end products because of higher availability of intermediate products and/or direct activation of the enzymes responsible for the subsequent stages of desaturation. This phenomenon was earlier observed in transgenic plants with stable expression of heterologous Δ9 desaturases, including that of *S. vulcanus* (Orlova *et al.* 2003, Craig *et al.* 2008, Gerasymenko *et al.* 2015, Zhao *et al.* 2015, Bryant *et al.* 2016, Dar *et al*. 2017).

Analysis of the FA profile in *N. excelsior* showed that

transient expression of the heterologous Δ9 desaturase changed the amounts of major saturated FAs, the pattern of the changes differing from that in *N. benthamiana* (Fig. 3). The content of the 16:0 and 18:0 FAs was dramatically decreased and increased, respectively; however, whereas the amount of the 16:1 monounsaturated FA was significantly increased, that of the 18:1 FA remained at about the control level (Table 2, Fig. 3). In addition, overexpression of the heterologous Δ9 desaturase in *N. excelsior* was accompanied by a considerable change in the C18 to C16 ratio (1:3 in the wild-type and pVIG-E plants and 10:1 in the DesC plants). The increase in the C18 to C16 ratio, as well as the increase in the absolute amount of C18 FAs and the corresponding decrease in that of C16 FAs in the leaves of DesC plants may have resulted from an enhanced fatty acid synthase II activity, which was probably caused by an increased stearic acid utilization due to the high Δ9 desaturase expression rate (Orlova *et al.* 2003). Apparently, this regulatory mechanism is necessary for the homeostasis of the general level of unsaturation, which ensures the optimal fluidity of the plant cell membrane under normal environmental conditions (Los *et al.* 2013). Note that the content of other related unsaturated FAs, including the 16:3 and 18:3 ones, as well as DBI, were somewhat increased in the leaves of *N. excelsior* with overexpression of the heterologous Δ9 desaturase (Table 2, Fig. 3). These data indicate that the heterologous Δ9 desaturase, being responsible for the synthesis of oleic and palmitoleic acids, also strongly promotes PUFA accumulation.

Our data also demonstrate that, in general, the *S. vulcanus* Δ9 desaturase transiently expressed in plants exhibits substrate specificity for both 16:0 and 18:0 FAs in both *N. benthamiana* and *N. excelsior* (Tables 1, 2; Fig. 3), although it was earlier demonstrated that, under the conditions of stable expression in transgenic *N. tabacum* plants, this desaturase displayed preference for the 18:0 FA (Orlova *et al.* 2003, Gerasymenko *et al*. 2015).

Thus, taken together, the results of this study convincingly demonstrate that the functional activities and substrate specificities of heterologous Δ9 desaturases can be studied in detail using the model of transient expression of the target desaturase genes in *N. benthamiana* and *N. excelsior* plants.

As noted above, heterologous desaturases catalyzing the 18:0 to 18:1 conversion effectively function in plants in the cases of both cytosolic (Orlova *et al.* 2003, Zhao *et al.* 2015) and plastid (Craig *et al.* 2008, Gerasymenko *et al.* 2015) localizations, which eventually leads to the formation of PUFAs due to the activities of specific desaturases localized in the ER and plastids.

Here, we also attempted at determining whether different plant cell compartments (the cytosol, chloroplasts, and ER) had preferences for the functioning of the heterologous Δ9 desaturase, mainly in terms of the PUFA content and DBI. For this purpose, we determined the FA composition of *N. benthamiana* and *N. excelsior* leaves overexpressing the cyanobacterial *desC* gene with different localizations of the DesC protein. We used well-characterized signal sequences, namely, *1*) the N-terminal transit peptide of the RuBisCO small subunit, which ensures the localization of target proteins in the chloroplasts (Vyacheslavova *et al.* 2012, Tyurin *et al.* 2017); *2*) the N-terminal transit peptide of legumin B4 (LeB4) of *Vicia faba*; and *3*) the ER retranslation signal SRKDEL introduced into the C-terminal region of the target protein to ensure its transport to the ER and its retention there (Alanen *et al.* 2011, Vyacheslavova *et al.* 2012, Tyurin *et al.* 2017). We used the method of obtaining protoplasts from a plant tissue agroinfiltrated with bacterial strains carrying different expression vectors, which we developed and tested earlier, and fluorescence microscopy to demonstrate that the signal sequences ensured the localization of the heterologous Δ9 desaturase in the target subcellular compartments (Fig. 2).

Subsequent comparison of the FA composition of agroinfiltrated DesC plants with different localizations of the heterologous Δ9 desaturase and that of control plants (pVIG-E) showed that, in general, the *S. vulcanus* Δ9 desaturase transiently expressed in different plant cell compartments caused a significant increase in both the PUFA content and DBI (Tables 1, 2; Fig. 3) in the leaves of both *N. benthamiana* and *N. excelsior.* The significant increase in the PUFAs in the case of the plastid localization of the heterologous Δ9 desaturase is satisfactorily explained by the presence of stearic acid (18:0), a substrate of this enzyme, in plastids and formation of PUFAs through further desaturation of the Δ9 desaturase reaction product, oleic acid (18:1), by specific plant desaturases also localized in this compartment (Dar *et al.* 2017) (Fig.4).

In the ER, the heterologous enzyme desaturates the 18:0 FA transported as part of the FA-CoA pool from the cytosol (usually the oil body). The resultant 18:1 FA is also converted into PUFAs by specific plant desaturases localized in the ER (Los *et al*. 2013, Dar *et al.* 2017) (Fig. 4).

Regarding the localization and functioning of Δ9 desaturase in the cytosol, the following mechanism of the increase in the PUFA content can be supposed. Probably, the mechanism of FA exchange between the plastid and ER membranes is employed in this case; *i.e.*, the heterologous enzyme desaturates the 18:0 FA contained in the cytosolic FA-CoA pool, and the desaturation product, the 18:1 FA, is transported to the plastids and/or ER, where PUFA formation is catalyzed by specific desaturases contained in these compartments (Fig. 4).

The results of this study allow us to recommend both *N. benthamiana* and *N. excelsior* as model plants for estimating the functional activities and/or substrate specificities of not only the heterologous Δ9 desaturase studied here but also the desaturases that convert the 18:1 FA into PUFAs. Since the model plants used here, *N. benthamiana* and *N. excelsior,* are characterized by substantial differences in FA composition, including the C18 to C16 ratio, the choice of the model plant will certainly depend on the purpose of each particular study.

A specific advantage of the expression vectors for transient expression of target genes in plants that we engineered earlier (Vyacheslavova *et al*. 2012) should be specially noted. It is determined by an additional

expression cassette for the gene of the p19 tomato virus protein, a suppressor of posttranscriptional gene silencing controlled by the constitutive TCTP promoter, incorporated in the vectors. This makes it possible *a*) to overcome the mechanism of posttranscriptional silencing upon overexpression of heterologous genes in plants, which is usually triggered in the case of transient expression and interferes with effective expression of the heterologous gene, and *b*) to use a single bacterial strain for agroinfiltration, in contrast to the routine procedure of cotransformation with two *Agrobacterium* strains carrying different vectors, one with the p19 protein and the other with the target gene (Vyacheslavova *et al.* 2012, Tyurin *et al.* 2017).

Thus, we have proposed and tested a model of transient gene expression in plants that have been found applicable to the study of the functional activities and substrate specificities of heterologous desaturases. Transient expression is a simpler, less time-consuming, and less expensive approach than obtaining transgenic plants for both stable gene overexpression and gene silencing. The use of transient expression, in particular, for estimating the localization, functional activity, and substrate specificity of desaturases of different origins makes it possible to maintain molecular reproduction of new genotypes containing better combinations of target alleles and, combined, *e.g.*, with genome-editing technologies, lay the basis for a new generation of transgenes, which has important implications at the global level.

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