

Detection of changes in the defence factors of *Nicotiana Tabacum* plant under the influence of insertion and expression of heterologous transgenes (*desA*, *desC*, *HuINF α -2b*)

T. Kyrpa^{1,*}, A. Potrokhov¹, M. Kharkhota² and M. Kuchuk¹

¹Institute of Cell Biology and Genetic Engineering of NAS of Ukraine, Akademika Zabolotnoho Str.148, UA03143 Kyiv, Ukraine

²Institute of Microbiology and Virology named after D.K. Zabolotny, Str. Akademika Zabolotnoho, 154, UA03143 Kyiv, Ukraine

*Correspondence: t-kirpa@ukr.net

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Abstract. Genetically modified plants may have some changes in physiological and biochemical reactions depending on the type of transgene. In this study, we present the results of the analysis of tobacco plants with the insertion and expression of the genes for human interferon alpha (*HuINF α -2b*), Δ 12-acyl-lipid desaturase (*desA* of the cyanobacterium *Synechocystis* sp. PCC 6803) and Δ 9-acyl-lipid desaturase (*desC* of the cyanobacterium *Synechococcus vulcanus*). Wild-type tobacco plants were used as a control. The level of accumulation of polyfructans and changes in the fatty acid spectrum in the leaves of plants under normal physiological conditions and after exposure to low temperatures were tested. It was found that all transgenic plants had some changes in the composition of fatty acids, however, only plants with the *HuINF α -2b* gene insertion had an increased content of polyfructans. These data may indirectly indicate a difference in the two defense strategies of the plant organism depending on the insertion and expression of the transferred gene.

Key words: abiotic stress, polyfructans, acyl-lipid desaturases, adaptation, fatty acids.

INTRODUCTION

Adaptation of plants to various stress factors requires the existence and functioning of certain protect mechanisms (Mareri et al., 2022). This can include the storage of essential substances (such as sugars, polysaccharides, etc.), as well as changes in the qualitative composition of certain plant cell structures (for example, changes in the spectrum of fatty acids in phospholipid membranes) (Riseh et al., 2021; Vahalová & Cifra, 2023). Genetic engineering helps accelerate the acquisition of new quantitative or qualitative traits by plant organisms (Dash & Osborne, 2023). Typically, biotechnological approaches reduce the time it would take to produce the same plants through breeding (Bigini et al., 2021). The insertion of a transgene into a plant organism can affect the expression of host genes or create competition for substrate-enzyme bonds, causing the expression of a new protein. Thus, not all aspects of the transformation event

itself remain sufficiently covered in terms of intracellular physiological and biochemical processes. (Müller, 2024). First of all, the phenomenon of transformation events, namely the insertion and expression of a transgene, can affect the accumulation of organic substances that affect metabolism and adaptive characteristics of plants, such as carbohydrates (Hafeez et al., 2023). They make up 85–90% of the substances that make up the plant (Kutzli et al., 2021). Carbohydrates are formed in the plant body as a result of the photosynthetic apparatus and are one of the main aspects in the food chain of humans, animals and microorganisms (Wani et al., 2023). In plants, carbohydrates are found as a support material; substances dissolved in cell sap; reserve deposits; and components of compounds that play an important role in metabolism (van Bel, 2021). The positive role of increasing the amount of sugars in plant tissues under the influence of various adverse environmental factors (salinity, drought, high temperatures, low temperatures, etc.) is known (Mehdi et al., 2024). Sugars increase the resistance of proteins to various physical and chemical influences that cause their coagulation (Ng et al., 2022). Therefore, the important role of sugars in frost, drought, gas and salt resistance is associated with the stabilization of protein molecules (Jahed et al., 2023). One of the main reserve compounds of higher plants is fructans (Márquez-López et al., 2022) (Benkeblia, 2022). There are several main types of fructans: inulin (mainly in dicots), levan, neo-inulin and neo-levan (in monocots), depending on the structure of the polymer molecule (Verma et al., 2021). Another important factor that plays an important role in maintaining the integrity of the cell and its interaction with the surrounding factors is the fatty acid spectrum (Rawat et al., 2021). First of all, fatty acids in membrane phospholipids affect the properties of the membrane itself (Martin & Douliez, 2021). With an increase in the proportion of unsaturated fatty acids in membrane phospholipids, the viscosity and plasticity of membranes increase, which prevents mechanical degradation due to cell desiccation (Kaur et al., 2022). In addition, an increase in the proportion of unsaturated fatty acids in membrane phospholipids reduces the freezing point, which leads to an increase in the adaptive potential of plants to low temperatures and frosts (Zhao et al., 2024). Desaturases are enzymes that promote the formation of double bonds in fatty acids and thus convert them from saturated to unsaturated (Kazaz et al., 2022). These groups have been broadly classified into two evolutionary unrelated groups of soluble acyl-acyl carrier protein (ACP) and membrane-bound desaturases (Halim et al., 2022). This natural mechanism makes it possible to improve the quality of plant resistance to various abiotic stress factors (Xiao et al., 2022). However, the transfer, insertion and expression of transgenes can have both negative and positive effects (Sayed et al., 2022).

In this study, we investigated the content of polyfructans and changes in the proportion of fatty acids in experimental tobacco plants *Nicotiana tabacum* (as a model plant organism) expressing genes of different origin and characterised by different substrate specificity, since the effect of transferred genes on the functioning of a transgenic organism remains a very promising topic for research. In this study, we investigated plants with insertion and stable expression of cyanobacterial desaturase genes (*desA* (gene of $\Delta 12$ -acyl-lipid desaturase of cyanobacterium *Synechocystis* sp. PCC 6803), *desC* (gene of $\Delta 9$ -acyl-lipid desaturase of cyanobacterium *Synechococcus vulcanus*) and *HuINF α -2b* (gene coding for recombinant human interferon alpha-2b) on the level of polyfructans accumulation in leaves as one of the indicators of plant

adaptation to low temperatures and frosts and changes in the fatty acid spectrum as another strategy of plant adaptation to cold stress.

Acyl-lipid desaturases also function in the plant organism, so the desaturases that will be caused by transgene expression can be provided with a reaction substrate. Regarding the *HuINF α -2b* transgene, it expresses a protein that is not native to plants.

MATERIALS AND METHODS

Plant material

Transgenic plants of the model object genes *Nicotiana tabacum*, in which the insertion and expression of *desA* (gene of Δ 12-acyl-lipid desaturase of cyanobacterium *Synechocystis sp.* PCC 6803), *desC* (gene of Δ 9-acyl-lipid desaturase of cyanobacterium *Synechococcus vulcanus*) and *HuINF α -2b* (gene coding for recombinant human interferon *alpha*-2b) were confirmed, were taken from the collection of the Institute of Cell Biology and Genetic Engineering of the National Academy of Sciences of Ukraine. Plants were previously obtained as a result of *Agrobacterium tumefaciens*-mediated transformation. The initial genetic constructs were created on the basis of the pBISN vector with selective *nptII* gene (*desA*, *HuINF- α 2b*) and with selective *bar* gene (*desC*) under the control of 35S CaMV promoter. Transgene insertion was confirmed by PCR, and expression was demonstrated indirectly through the activity of the reporter gene for the thermostable lichenase *licBM3* in plants with insertion and expression of desaturase genes. A qualitative lichenase assay was performed for the initial detection of expression and a quantitative lichenase assay was performed to determine the level of transgene expression in the plant organism. (Gerasymenko et al., 2015). The expression of interferon in transgenic tobacco plants with human INF- α 2b gene was determined using ELISA method. Testing of extracts obtained from transgenic plants on cell culture of piglet inoculated textiles (PIT), which was infected with vesicular stomatitis virus (VSV) and have revealed interferon-like activity (Potrokhov et al., 2017). Wild-type tobacco *N. tabacum* plants were used as a control.

Microclonal plant propagation

Plants: *N. tabacum*, *N. tabacum* with insertion and expression of *desA* and *desC* and *HuINF α -2b* transgenes were grown and propagated on nutrient agar medium MS. All plants were cultured on standard MS medium, *in vitro* at 25 ± 1 °C and 16-h light period (100 quantum mmol m²s). To maintain the culture and increase plant biomass, transplants were carried out once a month. To do this, aseptic scalpels were used to cut off plants that had been cultivated for a month on agar medium and transferred to fresh medium. Plants after one month of cultivation were used for the study.

Determination of the total level of polyfructans by the colourimetric method

For colorimetric studies, extracts from the experimental control plants were obtained. The test material was weighed (100 μ g), homogenised with distilled water and centrifuged for 15 minutes at 15,000 g. After extraction, the required aliquot of the supernatant was taken for analysis. For the colorimetric analysis, 100 μ l of 0.1% resorcinol aqueous solution and 100 μ l of HCl (5:1) were added to 100 μ l of the extract. The resulting solution was heated in a water bath for 5 min at +80 °C. After heating for

5 min at +80 °C and the appearance of a characteristic cherry colour, the optical density was measured using an Eppendorf biofotometr plus automatic analyzer at 550 nm. For the blank sample, the reaction solution was used with distilled water 100 µl instead of sample extract.

Chromatographic determination of fatty acids in plants

The fatty acid (FA) spectrum was analysed by gas chromatography and mass spectrometry. The isolation of FACS and the formation of their methyl esters for gas chromatographic analyses was carried out in one step according to the method.

A weight of leaves (200 mg) was cut with grease-free scissors and transferred to glass test tubes with screw caps. Reaction mixture A was prepared from methanol:toluene:sulfuric acid in a volume ratio of 44:20:2. Reaction mixture B contained a solution of the internal standard hexadecanoic acid in heptane 10 mg mL⁻¹ and heptane at a ratio of 1:84.

To each tube was added 3.3 mL of reaction mixture (A), followed by 1.7 mL of reaction mixture (B). The tested tubes were tightly sealed with teflon gasketed lids and kept in a water bath at 80 °C for 2 hours. Cooled at room temperature, the mixture separated into two phases. The upper phase was selected, in which the methyl esters of fatty acids formed were concentrated. Selected 300 µl of the upper phase was transferred to a vial with a tightly closed lid. The internal standard was a 20% solution of hexadecanoic acid in heptane. The fatty acid spectrum was studied by GLPC MS using Agilent 6890N/5973inert instrument coupled to DBFFAP capillary column (30 m × 0.25 mm × 0.25 µm) (J&W Scientific, United States). The results were analysed according to the values of graphical absorption peaks calculated by the chromatograph software. FAMES were identified by comparison of obtained spectra with NIST 02 mass spectrum library entries and with spectra of standard mixture of bacterial FAMES (47080U, Supelco).

Statistical data processing

For processing of statistical data we used MC Exel 2019 programme package. The level of reliable probability Rndx was 0.95. The data obtained were statistically processed using Statistica 10.02. In of polyfructans studies, 1 analytical replicate and 9 biological replicates were used and in the study of the fatty acid spectrum1 analytical replicate and 6 biological replicates were used.

Plant cultivation, hypothermic stress, analysis of polyfructans, preparation of samples for gas chromatography and mass spectrometry analysis, calculation of the results were carried out at the Institute of Cell Biology and Genetic Engineering of NAS of Ukraine, gas chromatography and mass spectrometry analysis was carried out at the Institute of Microbiology and Virology named after D.K. Zabolotny

RESULTS AND DISCUSSION

Genetically modified organisms, in particular, genetically modified plants, are increasingly being used in agriculture and industry. At the same time, all factors that may affect the environment must be taken into account. While new quantitative or qualitative characteristics are being imparted to plants, the transferred genes can have a

negative impact on important intracellular physiological and biochemical processes of plants. For the study, tobacco plants of the model object *Nicotiana tabacum* were used. Wild-type and transgenic *Nicotiana tabacum* plants were used, in which the insertion and expression of the gene *desA* (encoding the $\Delta 12$ -acyl-lipid desaturase gene of the cyanobacterium *Synechocystis* sp. PCC 6803) or the *desC* gene (encoding the $\Delta 9$ -acyl-lipid desaturase gene of the cyanobacterium *Synechococcus vulcanus*). The cyanobacterial desaturase genes were fused in a same reading frame with the *licBM3* gene of the *Clostridium thermocellum* thermostable lychease reporter protein. It should be noted that the transgenic plants did not have any morphological differences with the control plants (Fig. 1).

Freezing temperatures and frosts are one of the most common factors that lead to plant damage or death, which negatively affects the yield of important crops. Therefore, research into mechanisms that can positively influence plant resistance to this type of stress is an important task. Plants were tested to hypothermic stress of 0 °C 20 min, -5 °C 60 min. The level of polyfructans and changes in the fatty acid spectrum were analysed under normal physiological conditions and after exposure to low temperature stress.

The research used transgenic plants tobacco in which the products of transgene expression were characterised by different substrate specificity. By studying the effect of the transferred genes on the accumulation of sugars in the plant organism, it was found that in plants expressing the human interferon gene, the level of this substance differed from tobacco plants expressing cyanobacterial desaturase genes under normal physiological conditions and under cold stress. At the usual ambient temperature, the content of polyfructans was higher in plants expressing the interferon gene, lower levels of sugars were found in non-transgenic tobacco plants, and the lowest levels were found in plants expressing desaturase genes.

After exposure to freezing stress, the level of sugar accumulation in wild-type tobacco plants and tobacco expressing the interferon gene increased. It was determined that in control plants the fructans content was $7 \pm 0.57 \text{ mg g}^{-1}$ wet weight before the onset of cold exposure, and after exposure to low temperatures the fructans content was $15 \pm 1.28 \text{ mg g}^{-1}$ wet weight. Similarly, in plants with the interferon gene, an increase in the level of fructans accumulation was observed from $15 \pm 0.63 \text{ mg g}^{-1}$ to $22 \pm 3.21 \text{ mg g}^{-1}$. However, in plants expressing cyanobacterial desaturase genes, the level of sugar accumulation remained without statistically significant changes (Fig. 2).



Figure 1. Plants used in the study: 1 – Control plant *N. tabacum* wild type; 2 – *N. tabacum* with gene coding for recombinant human interferon alpha-2b. 3 – *N. tabacum* with the gene of $\Delta 12$ -acyl-lipid desaturase of cyanobacterium *Synechocystis* sp. PCC 6803. 4 – *N. tabacum* with the gene of $\Delta 9$ -acyl-lipid desaturase of cyanobacterium *Synechococcus vulcanus*.

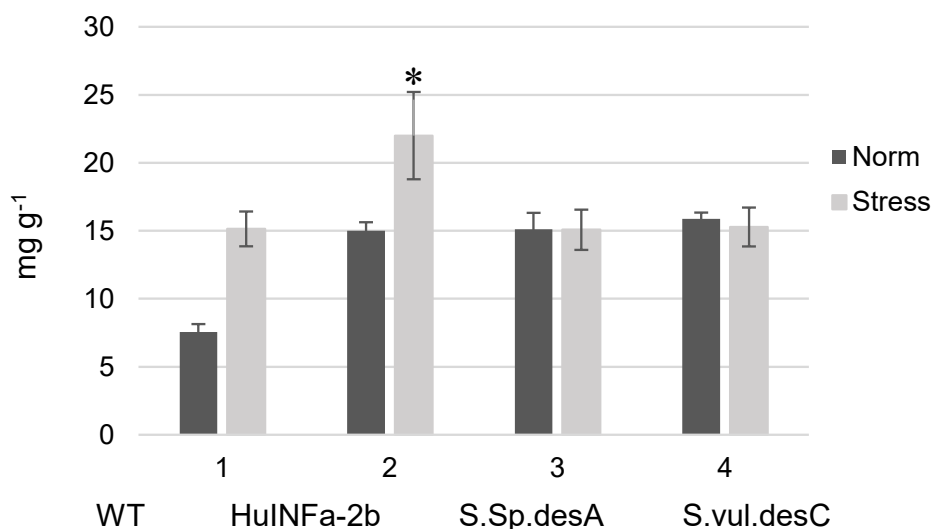


Figure 2. The level of sugars (polyfructans) after exposure to cold stress. WT – *N. tabacum* wild type; HuINFα-2b – *N. tabacum* with gene coding for recombinant human interferon alpha-2b. *S.sp.desA* – *N. tabacum* with the gene of Δ12-acyl-lipid desaturase from cyanobacterium *Synechocystis sp.* PCC 6803. *S. vul. desC* – *N. tabacum* with the gene for Δ9-acyl-lipid desaturase from the cyanobacterium *Synechococcus vulcanus*.

Since desaturases contribute to an increase in the proportion of unsaturated fatty acids in membrane phospholipids, the composition of the fatty acid spectrum was checked. The content of fatty acids in transgenic and control plants was analysed by gas chromatography and mass spectrometry. It was determined that under normal physiological conditions, wild-type tobacco had a predominant proportion of palmitic fatty acid (59.5%) in the composition of membrane phospholipids. Tobacco with insertion and expression of the human interferon gene had an increased proportion of palmitic acid (64.35%) with a reduced level of linoleic acid (12.3%) compared to wild-type tobacco (21.26%). Tobacco with the expression of the *desA* gene had an increased proportion of linoleic acid (35.09%) with a decrease in palmitic acid (46.81%) and tobacco with the expression of the *desC* gene had an increased proportion of palmitic acid (62.69%) compared to control non-transgenic tobacco (59.5%), but slightly lower than that of tobacco with the interferon gene (64.35%). These results are explained by the fact that certain proteins of the desaturase genes promote the formation of double bonds in the corresponding positions. The protein of the interferon gene has no direct substrate for binding in the plant organism, so the position of the insertion can affect the biochemical processes of plant metabolism.

After exposure to freezing stress, certain differences in the composition of fatty acids in transgenic plants were found.

In general, the desaturation reaction can be enhanced by an increase in the level of free oxygen and free oxygen radicals in the cell, which arise as a result of negative influences and destructive changes in the cell. In this way, damage signalling is triggered to increase the expression of desaturase genes.

Table 1. *Analysis of fatty acid spectrum data under normal conditions (+) and after temperature stress (-)

No.	Plants	Conditions	Palmitic acid, $\mu\text{g mL}^{-1}$ (% of total fatty acid content)	Linoleic acid, $\mu\text{g mL}^{-1}$ (% of total fatty acid content)	Linolenic acid, $\mu\text{g mL}^{-1}$ (% of total fatty acid content)
1	<i>N. tabacum</i> Wild type (control)	+	80.8 ± 7.2 (59.5%)	$26.3 + 3.3$ (19.37%)	$28.7 + 4.2$ (21.26%)
		-	80.8 ± 7.3 (59.499%)	$26.3 + 1.3$ (19.37%)	$28.7 + 4.2$ (21.13%)
2	<i>N. tabacum</i> with <i>HuINFα-2b</i> transgene	+	$92.6 + 3.3$ (64.35%)	$33.6 + 1.3$ (23.35%)	$17.7 + 0.3$ (12.3%)
		-	$79.6 + 2.8$ (66.83%)	$26.9 + 1.96$ (22.586%)	$12.6 + 0.4$ (10.579%)
3	<i>N. tabacum</i> with <i>desA</i> transgene	+	$85.9 + 4.4$ (46.81%)	$29.9 + 1.5$ (16.29%)	$64.4 + 9.3$ (35.09%)
		-	$66.9 + 0.08$ (38.187%)	$8.7 + 0.9$ (4.966%)	$97.99 + 9.6$ (55.93%)
4	<i>N. tabacum</i> with <i>desC</i> transgene	+	$86.2 + 0.009$ (62.69%)	$29.5 + 0.1$ (21.45%)	$21.8 + 0.1$ (15.8%)
		-	$85.4 + 7.01$ (62.84%)	$26.6 + 1.4$ (19.57%)	$23.9 + 3.3$ (17.5864%)

Taking into account that desaturase enzymes have a reaction substrate in the plant organism, it can be assumed that the *desC* gene, which causes the expression of $\Delta 9$ -acyl-lipid desaturase, which promotes the formation of double bonds at the $\Delta 9$ position and converts palmitic acid into oleic acid, supplies the substrate for other desaturase species and thus triggers the desaturase cascade.

The protein of the *desA* ($\Delta 12$ -acyl-lipid desaturase) gene forms a double bond at the $\Delta 12$ position and converts oleic acid to linoleic acid. However, the results of the analysis show that there was also an increase in linolenic acid, which indicates the supply of a reaction substrate for further transformations.

As for the absence of changes in the control plants, it can be assumed that they had a different defence system based on an increase in the concentration of polyfructans and other defence mechanisms that were not analysed in this study. However, we did not study cold stress of greater severity. It is possible that with increasing stress levels, we can observe changes in the fatty acid spectrum of wild-type tobacco and/or changes in the level of polyfructans in tobacco with cyanobacterial desaturase gene expression.

CONCLUSION

The introduction and expression of transgenes is an interesting phenomenon that can have both negative and positive effects on the physiological processes of a plant organism. Sometimes it can affect defense systems and adaptation to environmental conditions. Tobacco plants with confirmed insertion and expression of transgenes with different substrate specificity may have some differences in the measurement of certain physiological indicators of adaptation. Desaturase genes (*desA* or *desC*) undoubtedly

enhance the protective properties of membranes (increasing their plasticity) against low temperatures and frost. In this case, we can consider the response to stress of two plant defense systems – an increase in the proportion of unsaturated fatty acids in membrane phospholipids and an increase in the accumulation of polyfructans (plants with the human interferon gene, *HuINF- α 2b* gene). It is likely that with increasing stress, plants expressing desaturases will tend to increase the accumulation of polyfructans. This is planned to be tested later.

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