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BIOCHEMISTRY, BIOPHYSICS AND MOLECULAR BIOLOGY

Identification of the *NtFZY* Gene Family in Tobacco (*Nicotiana tabacum*) Involved in the Tryptophan-Dependent Auxin Biosynthesis Pathway

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The main endogenous auxin—indole-3-acetic acid (IAA)—is involved in almost all processes of plant growth and development. This is one of the key plant hormones [1]. The biochemical pathways of IAA biosynthesis and, accordingly, the genes that control these processes are still poorly understood. To date, five different biochemical pathways of IAA synthesis (both tryptophan-dependent and tryptophan-independent) are known [2, 3]. However, none of these pathways has been studied comprehensively in terms of the enzymes and intermediates of biosynthesis and the genes that control them. Today, nothing is known about the genes that ensure the biosynthesis of auxin in the important model object tobacco (*Nicotiana tabacum* L.). Therefore, the identification and characterization of the tobacco *NtFZY* gene family, which ensures the tryptamine pathway of auxin biosynthesis, is of considerable theoretical and practical interest.

The novelty and priority of this work is that the genes involved in the biosynthesis of auxin, orthologous to the *YUCCA* genes of *Arabidopsis thaliana*, were identified in *N. tabacum* for the first time. Despite the fact that tobacco is widely used in various biochemical and genetic engineering studies, its genetics remains poorly studied. The *NtFZY1–NtFZY4* genes, identified by us, are the first identified tobacco genes that are involved in the tryptamine pathway of auxin biosynthesis and encode flavin monooxygenase-like proteins functioning at the initial stages of the tryptophan-dependent pathway of auxin biosynthesis. Identification of *YUCCA* genes in tobacco is promising not only in terms of broadening the possibilities of a detailed study of the tryptamine pathway of IAA biosynthesis in plants in general. These genes can later be used to create new genotypes of different agronomically important plants with altered auxin dynamics.

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It should be noted that not all IAA biosynthesis pathways are present in all plant families [4]. Two biosynthetic pathways, in which *CYP79B* and *YUCCA* genes play the key role, were most thoroughly studied in *A. thaliana*. The *CYP79B* gene is involved in the indole–acetaldoxime pathway, whereas the *YUCCA* gene functions in the tryptamine pathway [3]. The *FZY* gene encodes a monooxygenase-like protein, is an ortholog of the *YUCCA* gene of *A. thaliana* and was detected in petunia [5]. This fact suggests that the *YUCCA* pathway of the synthesis of IAA is represented in the Solanaceae family. Another confirmation of this assumption was the discovery of the *YUCCA* pathway and *FZY* orthologs in the genome of the tomato (*Lycopersicum esculentum* L.), which also belongs to this family [6]. The authors of [6] found that tomato genes *ToFZY* form a multigene family comprising five divergent copies (GenBank accession AM177499.1) and are expressed in tissues at different stages of plant development [6].

N. tabacum, as well as petunia and tomato, is a member of the Solanaceae family. To date, only one gene involved in the synthesis of IAA is known in tobacco. This is the *NtAMI1* gene [7], which functions at late stages of the indole–acetamide pathway of auxin biosynthesis [3].

Earlier, we obtained a transgenic *N. tabacum* line IF4/11, which differs from the control plants by the flower phenotype. In these plants, the pistil is much greater than the length of the stamens and the corolla (the so-called "longostily" phenotype) [8]. In the literature, there are examples of the influence of altered levels of phytohormones on the morphology of the flower [9]. For this reason, it was of interest to analyze the dynamics of IAA in flower tissues at different stages of development of control plants and IF4/11 plants with the longostily phenotype as well as to attempt to find orthologs of genes of the *ToFZY* family in tobacco, which could be involved in the observed morphological changes of the flower (pistil elongation). This was the goal of this study.

Oligonucleotide primers used in the study

Oligonucleotide name	Oligonucleotide sequence	PCR product length, bp
FZY2up	5'-AGATGGGTTGTTGTAAAGAGGAAGAA-3'	372
FZY2lo	5'-TCCAAAACCCACAGACTCCATCA-3'	
FZY3up	5'-GTCCTTGTTCACCACTAATGGTTCA-3'	340
FZY3lo	5'-CTGATCTCGAAGTTCTTGGCATAAG-3'	
FZY4up	5'-TGGCTAGCTTCAATGACCATGAT-3'	355
FZY4lo	5'-GCAAGTTGAACACACTCATTGAACA-3'	
FZY5up	5'-ACCTTCTCATCAGAACAAGATTCCG-3'	321
FZY5lo	5'-CGGGTTAATGTCAAAGTGTTTAGCA-3'	
FZY6up	5'-GTCAACTTCCTTTGTTTCCTTTTCC-3'	364
FZY6lo	5'-CCACAACCAACAACTAGGACATTTT-3'	

The study was carried out using two *N. tabacum* lines: SR1 line (control) and the transgenic line IF4/11 with the mutant phenotype, which was obtained on the basis of the SR1 line [8]. Plants were grown hydroponically in a greenhouse at a 25–16°C at a light/dark period of 16/8 h.

The content of IAA was determined in flowers at different stages of development according to the classification of Goldberg [10] modified by Koltunow [11] after fixing in liquid nitrogen and subsequent lyophilization for 18 h in an Inei-6 device (Russia). Indole-3-acetic acid was extracted from the lyophilized flower tissues as described previously [12]. Quantification of IAA was performed by ELISA using an antibody kit (Farmkhiminvest, Russia) and IAA standards (Sigma, United States) [13]. The measurements were performed in triplicate or quadruplicate. Data are represented as the mean values and the standard errors of the mean.

To identify the genes of the *NtFZY* family, we performed PCR analysis of the DNA extracts isolated from tissues of flowers of lines IF4/11 and SR1 using the AxyPrep Multisource Genomic DNA Miniprep Kit (Axygen, United States) according to the protocol provided by the manufacturer and a set of primers that shared homology with tomato genes *ToFZY2–ToFZY6* (GenBank: AM177499.1; GenBank: AM177498.1; GenBank: GQ240890.1; GenBank: GQ240889.1; GenBank: GQ240888.1; GenBank: GQ240887.1; GenBank: GQ240886.1) [6]. The structure of the nucleotide sequences of primers is shown in the table.

Amplification was performed in 20 μL of the reaction mixture using the Taq PCR Master Mix Kit (Qiagen, Germany) in a Tertsik thermocycler. The amplification program was as follows: the first cycle at 95°C for 3 min; five cycles at 94°C for 20 s, at 58°C for 20 s, and at 72°C for 80 s; and twenty-five cycles of 94°C for 20 s, at 53°C for 20 s, and at 72°C for 80 s; and the last cycle at 72°C for 3 min. The amplified DNA fragments were separated by electrophoresis in 2% agarose gels in TAE buffer and then stained with ethidium bromide.

The results of comparative analysis of changes in the content of IAA in the tissues of flowers of line IF4/11 (with a modified flower structure) and the control line SR1 depending on the stage of flower development are shown in Fig. 1. It can be seen that the content of IAA in tissues of flowers of line IF4/11 at all stages of flower development (II–X) was lower than in the control plants, except for the final stage XII, in which the content of IAA in flowers of line IF4/11 exceeded the corresponding values in the control line (Fig. 1). In general, the dynamics of IAA in the control line in the course of the flower formation was characterized by a decrease in the IAA concentration, despite the slight increase in the IAA content at stage X. In contrast to the control, a similar IAA dynamics in line IF4/11 was observed only at stages II to VI; starting from stage VIII, the content of IAA increased (Fig. 1). It should be noted that it is at stages VIII–XII when

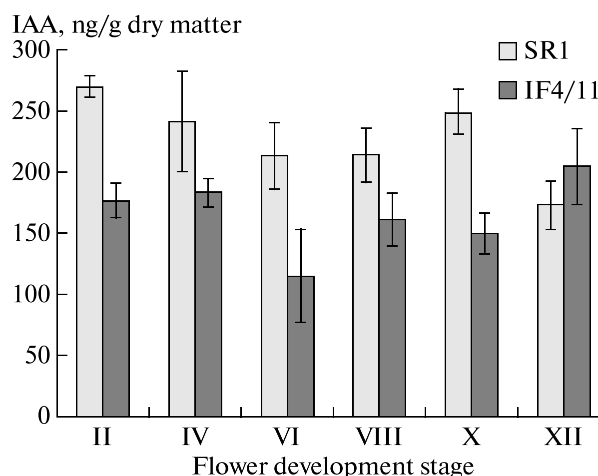

Fig. 1. Dynamics of changes in the concentration of IAA in the tobacco line with longostily and in the standard line depending on the stage of flower development.

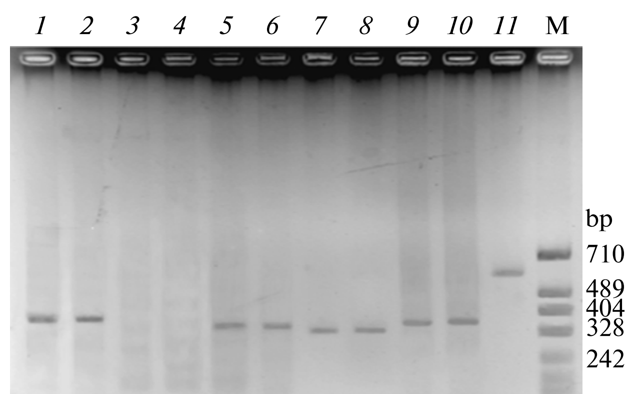

Fig. 2. The result of electrophoretic separation in 2% agarose gel of DNA fragments obtained by PCR with primers to tomato genes *ToFZY2–ToFZY6*. Designations: *1*, line SR1, primers FZY6up and FZY6lo; *2*, line IF4/11, primers FZY6up and FZY6lo; *3*, line SR1, primers FZY5up and FZY5lo; *4*, line IF4/11, primers FZY5up and FZY5lo; *5*, line SR1, primers FZY4up and FZY4lo; *6*, line IF4/11, primers FZY4up and FZY4lo; *7*, line SR1, primers FZY3up and FZY3lo; *8*, line IF4/11, primers FZY3up and FZY3lo; *9* line SR1, primers FZY2up and FZY2lo; *10*, line IF4/11, primers FZY2up and FZY2lo; *11, NtGA2* gene fragment from the tobacco genome; *M*, pBluescript/mspI marker, the length is shown on the right.

the long pistil formed in the plants of line IF4/11. The observed correlation between the change in the content of IAA and changes in the structure of the flower at the corresponding stages of development allowed us to assume that *FZY* genes may be involved in this process.

Figure 2 shows the electrophoregram of the products of amplification of genomic DNA, isolated from plants of lines SR1 and IF4/11, using respective pairs of primers selected on the basis of homology with tomato genes *ToFZY2–ToFZY6* (table). It can be seen that the length of the amplified fragment in lane *11* corresponds to a fragment of the *NtGA* gene from the tobacco genome. The detection of amplification products in lanes *1* and *2* (Fig. 2), whose size corresponds to the expected 372-bp fragment indicates the presence of an ortholog of the *ToFZY6* gene in the genomes of lines SR1 and IF4/11. The absence in lanes *3* and *4* of signals corresponding to the expected 321-bp fragment testifies to the absence of an ortholog of the *FZY5* gene in the genomes of the lines analyzed. The DNA fragments detected in lanes *5–10*, whose sizes correspond to the expected ones (table) confirm the presence of orthologs of *ToFZY4*, *ToFZY3*, and *ToFZY2* genes in the genomes of lines SR1 and IF4/11 (Fig. 2).

Thus, using the set of primers complementary to the sequences of genes of the *ToFZY* multigene family of tomato, we have identified four previously unknown genes (*NtFZY2, NtFZY3, NtFZY4*, and *NtFZY6*) in the *N. tabacum* genome that are homologous to tomato genes *ToFZY2, ToFZY3, ToFZY4*, and *ToFZY6*, respectively. An ortholog of the *ToFZY5* gene has not been identified in the tobacco genome. It can be assumed with a high probability that the coding regions of these genes were not affected in the process of transgenesis, because all the four genes of the *NtFZY* family of tobacco were found both in the control (nontransgenic) line SR1 and in the transgenic line IF4/11.

Flavin monooxygenase is the key enzyme involved in the IAA synthesis proceeding by one of the tryptophan-dependent pathways known to date—the tryptamine pathway [3]. It should be noted that the tryptamine pathway of IAA synthesis in the Solanaceae family, particularly in tomatoes, is not determinative [14]. Tryptophan-dependent and tryptophan-independent pathways of IAA biosynthesis differ greatly in their significance for response to changing environmental conditions and developmental stages in plant ontogeny. In normal vegetative growth of plants, IAA is synthesized primarily by the tryptophan-independent pathway. However, under conditions when high levels of IAA are required, tryptophan-dependent pathways are additionally triggered, which are able to provide higher rates of IAA biosynthesis at the expense of tryptophan deposited in the cell [15].

The group of genes of similar to *YUCCA* genes of *A. thaliana*, which includes the *FZY* genes of the family Solanaceae, encode flavin monooxygenase, which functions in the tryptophan-dependent pathway of IAA synthesis. These are usually multigene families including three to five diverged copies [6]. Since the tryptophan-dependent pathways of IAA biosynthesis function at full capacity only in the critical phases of plant ontogeny (for example, during flower formation upon the transition from the vegetative to the generative stage or as a result of a sudden change in environmental conditions), the disturbance of the balance in the expression of individual genes of this family may lead to significant changes in the IAA content. Such variations in the IAA content were observed at stages VIII–XII of flower formation in plants of the *N. tabacum* line IF4/11, which might have resulted in the disturbance of the morphology of the flower (longostily). It was shown earlier that tomato genes *ToFZY* are poorly expressed in young leaves; their expression is even weaker in mature leaves and is virtually absent in root tips. The maximum level of expression of *ToFZY* genes (an order of magnitude higher than in the young leaves) was detected in the developing and mature tomato flowers [6]. The identification of *FZY* genes in the *N. tabacum* genome is suggestive of their possible involvement in the formation of the longostily phenotype in the studied line IF4/11.

Further studies to continue this work will include a comparative study of the expression level of *NtFZY* genes in tobacco line SR1 and the transgenic line IF4/11 with the mutant phenotype by real-time PCR.

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