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PLANT INDUSTRY

Analysis of Obtained Chloroplast Transformants of Tobacco (*Nicotiana tabacum* L.) Plants with Marker Expression Cassette of *aadA*^{au} Gene, Changing the Color of Leaves

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Abstract—Transplastomic tobacco *Nicotiana tabacum* cv. Petit Havana plants were obtained by ballistic transformation. The modified expression cassette *aadA*^{au} was used as a marker, which confers the resistance to streptomycin and spectinomycin and causes the golden color of the leaf when planted in the soil after selection on selective medium. Plants were transferred into soil and screened for the golden leaf color. Molecular analysis was performed to confirm the transplastomic nature of the plants. The differences in plastid ultrastructure of mesophyll cells between the leaves of tobacco plants of wild-type (with green leaf fragments) and plastids with altered pigmentation (old leaves of wild-type and yellow pieces of the leaf with transformed chloroplasts) were analyzed by electron microscopy. It was shown that the ultrastructural organization of plastids of transplastomic plants with golden color had distinct differences from the chloroplasts of plants in the control group by the number and arrangement of thylakoids, the number and sizes of starch grains and plastoglobules. Decreased number of thylakoids and starch grain inclusions was marked in the mesophyll cells on a background of their increased number in the stroma and grana. There were also significantly more light areas of the stroma in transplastomic plants, which was typical for nucleoid areas.

Keywords: transplastomic plants, ultrastructure of chloroplasts, *pKMS8*, *aadA*^{au}, aurea phenotype, ballistic transformation, *Nicotiana tabacum*

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INTRODUCTION

One of the features of the plant cell is a large number of plastids, multifunctional organoids that contain their own genetic material and are able to perform proliferation. Although plastids posses identcal genetic material, they vary in morphology, number, and functions depending on the type of tissue [1]. Green leaves and stems contain photosynthetic plastids–chloroplasts. Cells of flowers, colorful leaves, fruits and roots contain plastids–chromoplasts which defy the distinct color of these organs due to the synthesis and accumulation of pigments–carotenoids. The stock-up function is performed by leucoplasts that synthesize and accumulate starch, fats, and proteins in tubers and other storage organs. Plastids ontogenesis provides the possibility of their conversion from one type to another, depending on the tissue type and stage of plant development [2]. Proplastids observed in meristematic cells during plant growth develop into plastids typical for the particular tissue [3]. All plastids are characterized by double membrane, genetic apparatus, protein-synthesizing system similar to prokaryotic, and the ability to divide within the plant cells [2]. Plastid DNA is a double-stranded closed circular molecule, organized as a nucleoid. Several such nucleoids are present in each plastid and every nucleoid carries multiple copies of the plastid genome. Hence, the plastid genome can reach very high levels of ploidy—up to 50000 copies in wheat cells, for example [4]. The genetic material of the chloroplast genome is inherited by offspring from the maternal line, and as a rule is not transferred from the plastids of pollen vegetative cells during the processes of pollination and fertilization, which greatly reduces the probability of using the pollen of any genes introduced into the plastid genome.

The transformation of chloroplast genome has several advantages for genetic engineering compared with the nuclear transformation: it provides a higher level of expression due to multicopy of the plastid genome [5]. In addition, the controllability of the transfer process at cross-pollination due to predominantly maternal inheritance is an important factor, as well as the absence of position effect due to site-specific integration of the transgene. Transplastomic engineering is the most promising for the overproduction of valuable therapeutic proteins, antigens, and vaccine components, including the so-called edible ones. It is, there-

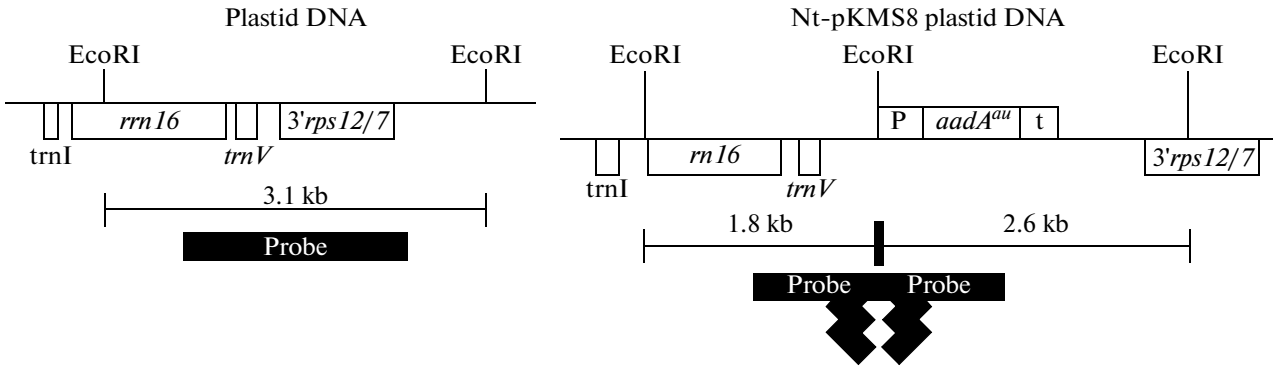

Fig. 1. Schematic maps of plastid DNA of control tobacco plants and transgenic plastid DNA containing pKMS8. It shows plastid genes in the area designated for targeted insertion of the transgene (*rrn16*, *trnV*, and *rps12/7*), as well as sites for EcoRI restriction enzyme, which were used to cut off the fragments of plastid DNA and to hybridize subsequently with the probe.

fore, important to study changes in the chloroplasts during the genetic manipulations.

The aim of this work was to obtain transplastomic tobacco plants and to identify the dependence between phenotypic and structural parameters of plastids using prospective visual marker *aadA*^{au} that confers the golden color to transplastomic plants. The task of the research was to find out the difference between the ultrastructural organization of chloroplasts in the mesophyll of transplastomic tobacco plants with golden color of the leaves and chloroplasts in the mesophyll of young and aging leaves of nontransgenic plants with green (middle layer) and yellow (lower layer) color.

MATERIALS AND METHODS

Vector for Transformation of Tobacco Chloroplasts

Plasmid pKMS8, provided by P. Maliga (Waksman Institute of Microbiology, Rutgers, State University of New Jersey) and containing an expression cassette with a selectable marker, gene *aadA*^{au}, was used—*aad* gene was flanked by the sequences encoding *rrn16* and *trnV* genes on the left and a sequence encoding tobacco *rps 12/7* gene of tobacco on the right [6] (see Fig. 1)—for the targeted insertion of a transgene into the chloroplast genome by homologous recombination. *AadA* gene, which confers resistance to the antibiotic spectinomycin, is under control of the hybrid promoter in the expression cassette. This hybrid promoter consists of *Prrn* promoter of the plastid operon that directs the transcription of plastid ribosomal RNAs [7], and *PcplP* promoter of *clpP* plastid gene [8]. The hybrid promoter is supplemented with 5'-UTR region of the *clpP* gene and a nucleotide sequence encoding the first 14 amino acids of the plastid protein subunit of *ClpP* protease. This nucleotide sequence is fused translationally to the sequence of *aadA* gene. Terminator in this cassette is this of chloroplast gene *psbA* of tobacco.

Plant Material

Sterile tobacco plants *Nicotiana tabacum* cv. Petit Havana were used; they were cultured at 26°C under a 16 hour photoperiod. Plants were grown on artificial nutrient media, which included macro and micro salt according to Murashige-Skoog, vitamins, plant hormones, and sucrose; with pH adjusted to 5.6–5.8 [9]. Medium size green leaves (excluding the leaves of the upper and lower layers) were used for the transformation.

Chloroplast Transformation

Ballistic gun PDS-1000/He Bio-RAD (United States) was used for plastid transformation in accordance with the procedure developed by [2] tobacco explants and 0.6 micron gold particles, coated with plasmid DNA *pKSM8* [10], were prepared for shots step by step in accordance with the said method. Gold particles were coated with DNA directly before the gun shots. A total of 50 shots were performed, 0.3–0.2 mg of plasmid DNA were used each time (Figs. 2a–2g).

Transformants Growth and Selection

Two days after bombardment leaves were cut into of 1–1.5 cm^2 fragments and placed on MS medium supplemented with 500 mg/L streptomycin (Fig. 2a). After 5–6 weeks the resistant to antibiotic regenerants were formed on these explants (Fig. 2b). Leaf fragments of resistant to spectinomycin regenerants were transferred to a medium supplemented with 500 mg/L of streptomycin and 500 mg/L of spectinomycin, on which only the transformed cells should remain green and grow and the mutant cells should die (Fig. 2c). After that, survived regenerants were multiplied by cloning and transferred to MS medium supplemented with 3% sucrose (with no hormones) [10] to grow and to form roots (Fig. 2d). To test the aurea phenotype regenerants that passed the screening with two antibiotics and formed roots were planted into soil (soil :

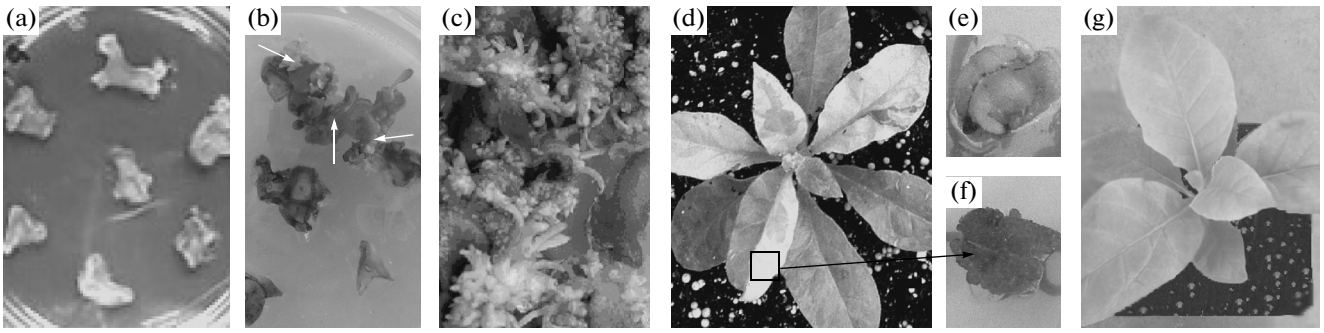

Fig. 2. Scheme of obtaining homoplastomic plants: (a) tobacco leaf explants on selective medium with the antibiotic spectinomycin, (b) regenerants resistant to the antibiotic (indicated by arrows), (c) regenerants resistant to two antibiotics, (d) transplastomic plant in the soil after one cycle of selection (plant with heteroplastomic set of chloroplasts), (e, f) the fragments of leaves of transplastomic tobacco on a selective medium with two antibiotics after their introduction into a sterile culture for selection of homoplastomic plants, (g) homoplastomic plant rooted in the soil.

perlite : peat 1 : 1 : 1). Plants phenotype was scored two weeks after transferred in soil.

Cloned transplastomic (geteroplastomic) tobacco plants, expressing *aadA*^{au} marker gene, selected on a selective medium with spectinomycin and streptomycin, were adapted to the soil substrate and grown for 2 weeks under 50 W/m light intensity at 25 ± 2°C and humidity of 60–70%. Then, the fragments of gold and variegated leaves were analyzed for the presence of *aadA*^{au} marker gene insertion in the plasmid DNA of plant chloroplasts.

Molecular Biological Analysis

DNA of transformed plants was analyzed by Southern blot hybridization according to the protocol described in [11]. Plant DNA was extracted from leaf fragments (200 mg) of pKMS8 clones and subclones as described in CTAB protocol [12] and was treated with EcoRI restriction enzyme. DNA fragments were separated by electrophoresis in 0.8% agarose gel, and transferred to nylon membrane (GE Healthcare, United States). Hybridization with the probe took place overnight at 65°C in buffer RHB (GE Healthcare). Double-stranded DNA probe was obtained by labeling with 32P by random priming. APAI/BamHI-fragment of native tobacco plastid DNA, including transgene targeting site, a part of *rrn16* gene and part of *rps12/7* gene, served as matrix for the probe (Fig. 1). The membrane was further subjected to autoradiography.

Analysis of the Plastids Ultrastructure in the Leaf Mesophyll Cells

Fragments of the middle part of the third leaf (transversal pieces of 1–3 mm in width) of control and transplastomic plants of golden color, of old (yellowing), and young leaves of untransformed plants were fixed in 2.5% solution of glutaraldehyde (Merck, Germany) in 0.1 M Sorensen phosphate buffer (pH 7.2) supplemented with 1.5% sucrose. The plant material was washed from the fixing mixture and fixed with 1% solution of tetroxid osmium (Sigma, United States), dehydrated in ethanol with rising concentrations (30, 50, 70, 96, and 100%) in propylene oxide (Fluka, Germany), and embedded in the mixture of epon araltide epoxy resins. Ultrathin sections were made with a ultramicrotome LKB-V (LKB, Sweden). Sections were contrasted with uranyl acetate and lead citrate according to Reynolds [13]. Sections were studied under H-300 and H-500 electron microscopes (Hitachi, Japan) at an accelerating voltage of 75 kV. Obtained photographs were digitized (scanned with an EpsonPerfection 3170) with a resolution of 600 dpi. Processing and arrangement of images were performed in Microsoft Photo Editor and CorelDRAW.

RESULTS AND DISCUSSION

To obtain transplastomic plants ballistic, transformation of sterile leaves of tobacco variety Petit Havana was carried out. The leaves were incubated for two days after bombardment, and then leaf segments were transferred for the regeneration to the selective medium supplemented with spectinomycin (Fig. 2a). Spectinomycin blocks the greening of leaves of regenerants if plastids in photosynthetic tissues were not transformed. Leaves of formed green regenerants (Fig. 2b) were cut off and transferred to the medium supplemented with spectinomycin and streptomycin in concentrations of 500 mg/L (Fig. 2c). Expression of *aadA* gene confers the resistance to both antibiotics and allows the formation of green callus. Two plant lines were obtained after 50 shots as a result of selection for resistance to two antibiotics; they were propagated by cuttings in vitro, acclimatized, and transferred to soil. On the first stage, during the visual assessment of golden phenotype (Fig. 2a), a greater part of the plants had a chimeric nature characterized by the presence of variegated color. Such plant con-

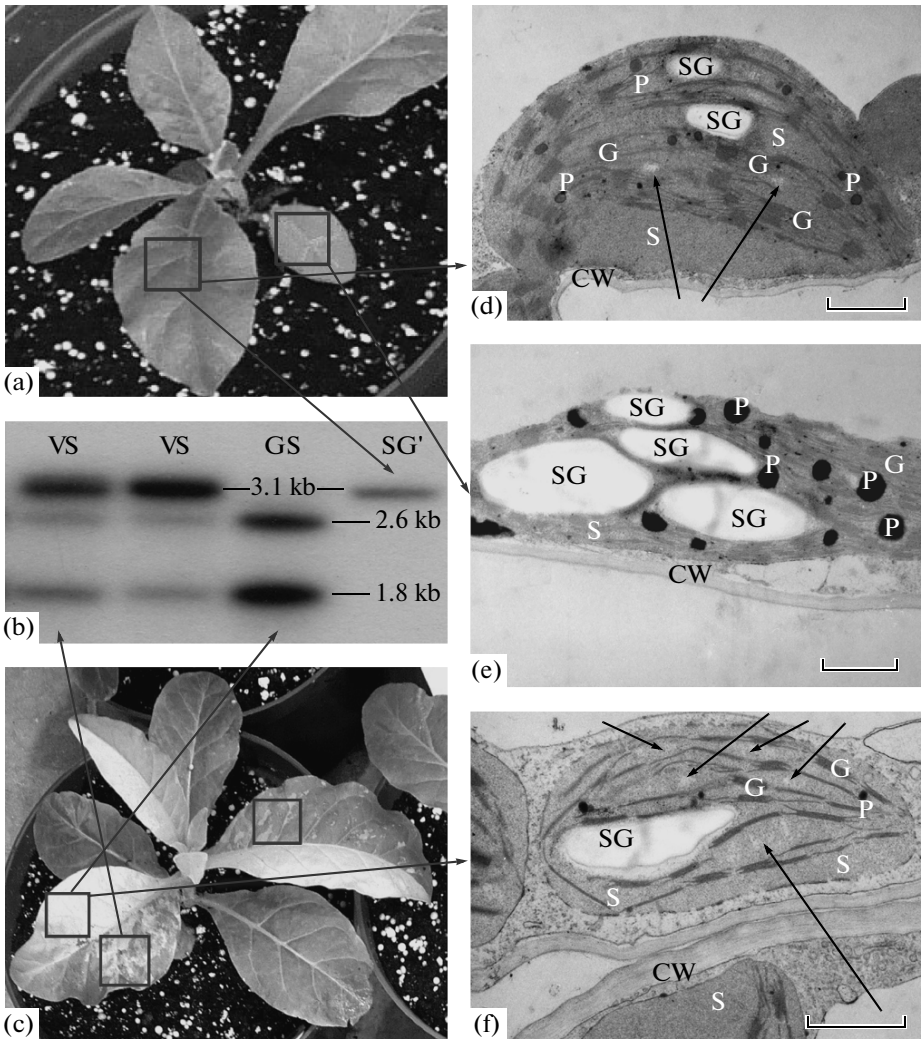

Fig. 3. (a) Control tobacco plants; (b) results of blot hybridization of chloroplast DNA by Southern taken from leaf sections with different colors: (VS) variegated sections, (GS) golden sections of pKMS8 transformed plants, (K') leaf fragments of control tobacco plants; (c) transformed pKMS8 tobacco plants; (d) structure of chloroplasts in the mesophyll of green young leaf of the middle layer: (SG) starch grains, (P) plastoglobules, (G) grana, (CW) cell wall, (S) stroma; (e) structure of aging yellowing leaf from the lower layer and (f) golden leaf fragment of transformed pKMS8 tobacco plants.

tains areas of green, golden, and variegated colors with some small areas of both green and golden colors.

After the first cycle of screening on the antibiotic medium purifying regeneration, the shoots had only small transplastomic sectors that were visually detected on the laminas of seedlings grown in the soil. Five plants of each line that passed the first step screening were rooted and transferred to soil in order to evaluate the screenable marker and to perform tests by external traits (golden phenotype). Homoplastomic zones of golden color were clearly visible on the laminas 2 weeks after planting the plants in soil due to the changes in transformed chloroplasts, which were located in these zones; zones with variegated color located in the cells with both transformed chloroplasts and plastids with the original plastid DNA were also observed also, as well as green areas with untransformed chloroplasts (Fig. 2d).

To confirm transplastomic nature of the selected plants, leaf sections with different colors were cut off in order to verify the presence of the insertion in the plastid genome of transformed plants with changed leaf color (Fig. 3c). DNA for Southern blot hybridization analysis was isolated from them, which confirmed that the control green plants and green leaf sections of transformed plants contained unmodified plastid DNA (Fig. 3b, K'). Variegated leaves of transformed plants contained both original and modified plastid DNA (with insertion of *PrrnPclpPLclp :: aadA :: TpsbA* cassette) (Fig. 3b, VS). At the same time, leaf sections with golden color (i.e., with transplastomic chloroplasts) contained plastid DNA with the insertion only (Fig. 3b, GS). Golden fragments were reintroduced

into the culture in order to obtain homotransplastomic plants (see Figs. 2e, 2f) and regenerants without chimeric fragments, i.e., containing only modified plastid DNA (see Fig. 2g) in the plastid genome, were obtained after selection in the presence of two antibiotics.

To exclude the assumptions about yellowing leaves as a result of plastid modification of photosynthetic cells, which is typical for damaged or aging leaves, the structural organization of chloroplasts from the fragments of young and aging leaves of control nontransgenic plants (see Fig. 3a) and of the fragments of transplastomic sections of transformed plants with the golden color was studied (Fig. 3c). The cloroplasts structure from the mesophyll of the control plants was typical for the chloroplasts in the mesophyll of tobacco true leaves [14]. Plastids (Fig. 3d) had a lenticular shape, a large number of stroma thylakoids, as well fully-developed actively photosynthesizing tissues with a large number of grana, formed by 4–10 thylakoids. Small starch grains, plastoglobules, and light areas were located in the interior of the plastids (zones of plastid DNA location, nucleoids, are indicated by arrows) on the background of dense stroma (Fig. 3d). Plastids in the mesophyll of old yellowing leaf of the lower layer (Fig. 3e) had typical changes in the structural organization, expressed in the appearance of larger starch granules, increase in the number and area of the sections of plastoglobules by 3–7 times, enlightenment of stroma, and the absence of clearly identifiable light areas with the nucleoids (Fig. 3e). The number of grana thylakoids and stroma was somewhat reduced—grana contained from three to eight compartments.

Chloroplasts in the mesophyll of the heteroplastomic tobacco plants were significantly different from the plastids of control plants: plastids were 1.5–2 times smaller then in control, the number of thylakoids in grana was reduced to 2–5, and the amount of light areas of the stroma typical for the location of nucleoids was increased (Fig. 3f); uneven scurfs of osmiophil material in the intermembrane space were marked on the surface of the chloroplast.

Changes in the structural organization of plastids have been observed previously, in transgenic plants, with altered nuclear genome in both cases: when the using the signal sequence, targeting into a plastid, was used or when transgene lacked such signal sequence [15, 16]. For this reason, changes in the ultrastructure of plastids may have different consequences, and its plastic compensation is possible due to the increase in plastids number or due to the changes in the operation of various systems, providing the functioning of both photosystems.

Thus, the reason of the changed color of transplastomic plants is not premature aging of the leaf but the modification of chloroplasts, resulting in a decrease in plastids area and the number of thylakoids in grana. This evidences the reduction of the photosynthetic activity, and probably corresponds, to the loss of color under low light. Similar changes in the structure of plastids in the cells of photosynthetic tissues were described in the study of the action of several genes in the plants mutant for these genes [17, 18]. At the same time, an increse number of nucleoids in transplastomic plants indicates the activation of this process that is typical for the young tissues, and an active plastid division. This is partly confirmed by the presence of smaller chloroplasts in the leaves of the transplastomic plants. Since older transplastomic plants lose the golden color, one can assume that plastids form a denser stroma during the formation of normal leaves of adult plants and the number of grana increases without increase in the number of thylakoids, or these plants form a larger number of thylakoids in the grana at a later stage of development. The latter option is more likely, since the slow greening of leaves is common in plants with mutations in several genes. For example, the younger leaves of rice plants with mutant gene *OsDG2* had fewer thylakoids in a stack, than more mature leaves [17].

Although the marker of visual selection by the golden color *aadA (aadA^{au})* [6, 19] allows one to facilitate the obtaining and selection of transplastomic plants and guarantees the deliverance from the plants with chimeric plastome during the double selection, it can influence ambiguously on the formation of photosynthetic organoids, affecting indirectly the efficiency of photosynthesis and, consequently, plant productivity. In connection with detected changes in the photosynthetic apparatus of plastids, the features of physiological effects of *aadA^{au}* marker require further studying, taking into account its reliability and prospects of use.

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