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GETTING PLANTS *NICOTIANA TABACUM* THAT SIMULTANEOUSLY EXPRESS HETEROLOGOUS GENE OF TWO ACYL-LIPID DESATURASES CYANOBACTERIUM *DES*C AND *DES*A

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Abstract
An important role in plant resistance to low temperature plays a composition of membrane lipids. With the increase of unsaturated fatty acids (FA) in the membranes of cells decreases the transition temperature of the gel phase in liquid crystal phase. Desaturases are enzymes that contribute to the formation of double bonds in the crystal and thus turn the FA with a saturated in unsaturated. The paper used the plant Nicotiana tabacum, expressing the gene of Δ9 acyl-lipid desaturase (desC) cyanobacterium Synechococcus vulcanus. These plants were transformed with a vector-based pBISN with selective nptII gene, under the control of the 35S cauliflower mosaic virus, that carrying the targeted gene desA: licBM3 by Agrobacterium-mediated transformation. Spend regeneration on Murashige-Skooge medium with the addition of BAP, NAA, cefotaxime, kanamycin. Finally we obtained transgenic plants carrying the two genes heterologous desaturases cyanobacterium.

Key words: *acyl-lipid desaturases, fatty acid, transgenic plants*.

INTRODUCTION

The study of plant resistance to abiotic stress is very important nowadays. Structure of membrane lipids has a significant impact on the adaptation of plants to the action of the stressor. Adaptations of organisms to low temperatures and frost temperature dependent phase transition from gel phase in crystalline phase (Los, D.A. et al., 2013).

Due to the shift range of FA saturated in unsaturated membrane fluidity is increasing and decreasing the temperature of transition from liquid state to phase crystallization. Desaturases are enzymes that contribute to the formation of double bonds between carbon atoms and thus transforming saturated fatty acids into unsaturated.

There are several types desaturases: acyl-lipid, acyl-CoA desaturase, acyl-APB. The classification is due to the substrate specificity of these enzymes. Acyl-CoA using FA desaturases joining coenzyme A, acyl-APB-desaturases - FA with bound acyl-portable protein acyl-lipid desaturases used as the substrate crystal, which are composed of lipids (Maali R., et al. 2007). In plants there are two kinds of enzyme, acyl-lipid and acyl-APB (Los, D.A. and Murata, N., 1998). This paper used the acyl-lipid desaturases cyanobacterium *Synechococcus vulcanus* and *Synechocystis sp.* PCC 6803.

Desaturases cyanobacterium characterized by the fact that the desaturation of their participation is in strict sequence, initially formed double connections in the provisions of Δ9, Δ12 then, and then goes to the provisions desaturation Δ6 and ω3 (Maali-Amiri R., et al 2007). That is, first the reaction involving mono FA and then diene. The main goal of this work is to create a tobacco plant that will simultaneously express two genes heterologous acyl-lipid Δ9 desaturase cyanobacterium Δ12 and that should provide further changes in the membranes and reduced sensitivity transformants to cold.

MATERIALS AND METHODS

The paper used gene *des*C (Δ9) cyanobacterium *Synechococcus vulcanus* and gene *des*A (Δ12) *Synechocystis sp*.PCC 6803. Genetic transformation performed by plants *Nicotiana*

tabacum (*cv. Wisconsin*), expressing the gene of Δ9 acyl-lipid desaturase (Gerasymenko I.M., et al., 2010).
The substrate of the enzyme is in the chloroplast so the 5'-end section of the gene *des*C sequence was attached encoding the transit peptide of the small subunit RTP plant *Arabidopsis thaliana* (gene *ats*1A, NCBI, XI36II) for providing targeting. These genes are in the same reading frame of the reporter gene protein licBM3. The gene RTP::desC::licBM3 was cloned under the control of the constitutive 35S promoter in the binary vector of selective gene *bar*. Hybrid gene *des*A was previously cloned into the expression vector based pBISN with selective gene *nptII,* under control of the 35S cauliflower mosaic virus. This gene is located in one of the reading frame reporter protein lichenase *lic*BM3 thermostable bacterium *Clostridium thermocellum.* (Abdeev R.M., et al., 2009). As used control plant *Nicotiana tabacum* wild type and transform *Nicotiana tabacum*, expressing the gene *gfp* :: *lic*BM3 (Gerasymenko I.M., et al., 2015).

Transformation and receiving plants
The method of cultivation of leaf discs with suspension *A.tumefaciens* conducted genetic transformation of tobacco plant *Nicotiana tabacum cv. Wisconsin* (Draper J., et al., 1991). For transformation of plant material genetic constructs used method of "leaf disks." Night *A.tumefaciens* culture built up in LB liquid medium with the addition carbenicillinum-dinatrlcum (50 mg / l) and rifampicin (50 mg / l) at 100-150 rpm and 26ºS in the dark.
The suspension of bacteria was centrifugated at 5000g for 5 minutes. Residue was stirred in a liquid nutrient medium MS, acetocyringone added at a concentration of 100 mM and this suspension was kept in the dark at 25ºS for 1.5 hours in the dark to induce *vir*-region *Agrobacterium*. As a source of great leaf discs used, normally shaped leaves of plants aged 1-1.5 months:1.5. Leaf blade cut into explants (1-1.5sm² square) and placed into the bacterial suspension. The suspension was cultured for one hour in an incubator at 25ºS in the dark. Then take explants, liberated them from drops suspension was transferred to MS medium and cultivated of *Agrobacterium* for two days at 25ºS (before the appearance *Agrobacterium*).
After culturing explants were transferred to agar nutrient medium Murashige-Skooge (MS) with the addition of phytohormones BAP - 1 mg / l and NAA – 0.1 0.1 mg / l. To stop the growth of *Agrobacterium* was added 700 mg / l cefotaxime as selective marker and kanamycin 100 mg / l. Within 2-3 weeks observed the regeneration potential of transgenic tobacco plants. Seedlings kept in cultivation in vitro under conditions 1ºS 25+, with 16-hour photoperiod, lighting of 100 mkM photons / (m2s).
In areas with intensive regeneration occurs greening seedling establishment and later. Lines plants that remain after the selection were grown in selective medium prior to PCR analysis for the presence of transgene.

Molecular biological analysis
To confirm availability of lines of transgenic *Nicotiana tabacum* regenerants obtained, analyzed the total plant DNA was extracted by CTAB (Berdichevets, I.N., et al., 2010) PCR using the appropriate primers:
desA 949 b-sense
GTTGACACCAACGGTAACGCC,
desA 949 b-antisense
CCAGTTAAAGGTGCGCTCGTAA,
desC 777 b-sense
CCTCAATTGGGGCTTTGTCTTC,
desC 777 b-antisense
AACTGTACCTTGGCGGCAAGA, licBM3 291 b-sense AATACGCCTTTTGTTGCAGTGTTT,
licBM3 291 b-antisense
GTCCGAAGGTCCTGTATAAGTGAAGA

Using techniques developed multiplexed PCR analysis of plants obtained after transformation of *Nicotiana tabacum* italic for the transferred T-DNA binary vector (gene fragments *des*C and *lic*BM3). After electrophoretic separation on agarose gel fragments are observed that correspond to the length gene fragment *des*C, *des*A, *lic*BM3. With 16 lines that were transformed vector pNPB14 (desA:: licBM3), 14 lines showed the presence of the transgene in the genome copies and contained no *Agrobacterium* contamination.

Thus was obtained lines of transgenic plants in the genome which proved the presence of hybrid genes *des*A :: *lic*BM3 and previously transformed *des*C :: *lic*BM3 :: RTP.

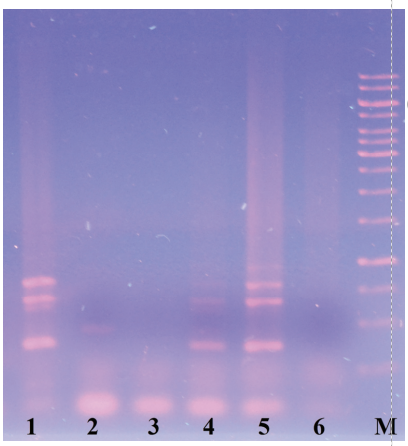

Figure 1. Multiplex PCR analysis of transgenic *N. tabacum* plants for detection of recombinant *lic*BM3, *des*A, and *des*C genes.
1 – *Nicotiana tabacum,* with gene desC :: licBM3 :: RTP;
2 – control *vir*D1;
3 – control H2O;
4 – non transgenic plant;
5 - transgenic plant with genes desC :: licBM3 :: RTP and desA :: licBM3;
6 – non transgenic plant;
M – 100 bp DNA ladder.
*(des*A - 949 bp, *des*C - 777 bp, *lic*BM3 – 642 bp, actin – 351, *vir*D1 – 432 bp.)

Gene expression by the reporter protein is not checked, as used in the plant *Nicotiana tabacum*, which were previously transformed gene *des*C :: *lic*BM3.

RESULTS AND DISCUSSIONS

The method of *Agrobacterium*-mediated transformation of plant lines were obtained *Nicotiana tabacum*, simultaneously expressing two genes acyl-lipid desaturases *des*C cyanobacterium *Synechococcus vulcanus* and *des*A *Synechocystis sp*.PCC 6803.

This was proven by polymerase chain reaction (PCR) which allows the insertion of genes to confirm the presence amplicon *des*A – 949 bp, *des*C - 777 bp, *lic*BM3 - 642 bp (Fig. 1) On track number 1 contains DNA of the plant *Nicotiana tabacum,* with gene *des*C :: *lic*BM3 :: RTP; track 2 control that contains DNA GV3101; track 3 contains a set of primers used to identify hybrid genes with the addition of H2O; track 4 contains DNA of the plant *Nicotiana tabacum*, with gene *des*C :: *lic*BM3 :: RTP held the transformation, but contains one gene desaturase; track 5 contains the DNA of a plant carries 2 genes of desaturases (*des*A and *des*C); track 6 contains DNA of the plant *Nicotiana tabacum* wild type; M – 100 bp DNA marker.

Regenerated plants from which the material seized were grown in vitro on MS selective medium (with the addition of a selective agent kanamycin). After selection all selected clones are transferred to regeneration medium with the same selective agents. After 2-3 months on of selected callus have been arising intensely green cell regeneration, including during further cultivation of seedlings been arising. With 16 lines was confirmed that the insertion of genes in 14 lines. It should be noted that the obtained regenerated not different from control plants phenotype (Fig 2.).

It should be noted that the obtained regenerated not different from control plants phenotype. From previous studies we can conclude that plants that synthesize additional enzymes desaturases thus changing the composition of membrane lipids.

This is done by increasing the proportion of unsaturated fatty acids. In plants expressing the gene of Δ9 acyl-lipid desaturases an increase in the proportion of oleic acid as a substrate of the enzyme is stearic acid. A plant gene expressing Δ12-acyl lipid desaturases observed increase in linoleic acid as a substrate of the enzyme is oleic acid. A very important factor is consistency desaturation FA, which is in strict sequence.

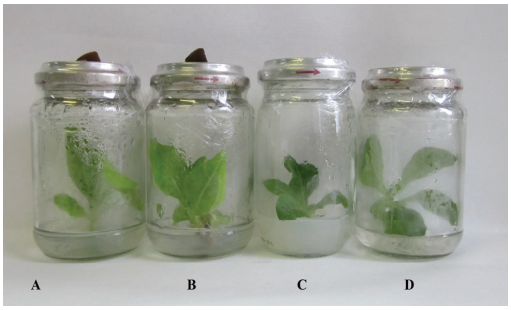

Figure 2. A- control plant *Nicotiana tabacum*,
B – control plant with gene *GFP::licBM3,*
C,D – plants with gene *desA::licBM3* and gene *RTP::desC::licBM3*

First, dual connections are formed between atoms of carbon (C = C) in position Δ9, Δ12 then in position, and then in the provisions of Δ6 and ω3. The formation of an acid depends not only on the level of expression of the corresponding enzyme, but also on the availability of substrate needed for this reaction, so very important to the adjustment of membrane lipids is available substrates in anticipation providing appropriate functioning of enzymes. As for our work, we used tobacco plants expressing the gene targeting *des*C the signal in chloroplasts, the gene for additional *des*A cyanobacterium *Synechocystis* sp.PCC 6803 has been provided with a substrate reaction desaturation (oleic acid). We can assume that in these plants observed shift range of FA rich in unsaturated oleic due to the increase in the share and linolenic acids.

You can also assume that plants that express both genes Δ9 and Δ12-acyl lipid desaturases is more likely providing desaturases other substrate (Δ6 and ω3).

CONCLUSIONS

As a result *Agrobacterium*-mediated transformation was obtained plants *Nicotiana tabacum*, which also carry two genes *des*C and *des*A desaturase cyanobacterium *Synechococcus vulcanus Synechocystis sp*.PCC 6803 respectively. These genes are characterized by different substrate specificity, and Δ9 acyl-lipid substrate provides desaturase work Δ12-acyl lipid desaturase. This leads to an increase in the proportion of unsaturated FA composed of lipids membrane tobacco plants, and thus increase plant resistance to abiotic stresses.

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