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ORIGINAL ARTICLE

Physiological Consequences of Genetic Transformation: Result of Target Gene Expression or Stress Reaction?

A.G. Enikeev, T.V. Kopytina, L.A. Maximova, Yu.V. Nurminskaya,

T.N. Shafikova, T.M. Rusaleva, I.V. Fedoseeva, S.G. Shvetsov

Siberian Institute of Plant Physiology and Biochemistry, Siberian Branch, Russian Academy of Sciences, Irkutsk, 664033, Russia

Email: enikeev@sifibr.irk.ru

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The transgenic and non-transgenic tobacco cell cultures were analyzed for resistance to abiotic and biotic stress. The different physiological reaction of cell culture depending on T-DNA structure (or transgen structure) was observed. The cell culture transformed by disarmed *Agrobacterium tumefaciense* A699 with pCNL 65 *nptII* demonstrated the same stress-resistance as non-transgenic control cell culture. The cell culture transformed by *Agrobacterium tumefaciense* LBA 4400 pBiCaMV *nptII* + *hsp101* showed a raised stress-resistance to high temperature, high KF concentration, and to the action of *Clavibacter michiganensis* ssp *sepidonicus*. Obviously, the expression of transferred arabodopsis gene *hsp101* provides protection properties of transgenic cell culture under the influence of various stress factors. Moreover, that agrobacterial transformation as previous stress-factor is supposed to make a contribution to formation of transgenic cell culture cross-resistance.

Key words: Nicotiana tabacum L., agrobacterial transformation, transgenic cell culture, physiological peculiarities, biotic and abiotic stressing factors, cross-resistance

Application of genetic engineering technologies to physiological studies contributed to significant enhancement of our knowledge of the functions of individual proteins (Bourque, 1995; Hewezi *et al*., 2006) and detailed description of various metabolism processes (Bock, 2001; Kuzovkina *et al*., 2004). Genetically modified plants become one of the most important objects of biotechnology fostering decision of the problems of formerly inaccessible complexity level (Ahmad *et al*., 2012; Eapen, D'Souza, 2005; Kos *et al*., 2009; Rashid, 2009; Liénard *et al*., 2011; Peleg *et al*., 2011). At the same time, there is no unambiguous answer to the question what transgenic plant is from physiological viewpoint. Reasons and mechanisms of appearance of multiple effects of transgenesis, silencing of transferred genes remain unidentified to a large extent (Filipecki, Maepszy, 2006; Rischer, Oksman-Caldentey, 2006; Sorochinsky *et al*., 2011). Absence of distinct vision of peculiarities of transgenic plant physiology may invoke wrong interpretation of the results acquired and, as a consequence, erroneous conclusions and inferences. Errors in creation of commercial transgenic plants my later end up in multi-million losses.

Fusion of T-DNA agrobacterium into the genome of recipient plant, as well as the attack of any phytopathogen, involves a cascade of reciprocal physiological responses resulting in significant changes in metabolism (Enikeev *et al*., 2008). Therefore, physiological consequences of transformation may to a large extent be determined by not only properties of the transferred target gene, but also by the reaction of plant cells to stress caused by the very fact of transformation. It is very difficult to differentiate these effects at the level of the whole plant due to mutual imposition of a large number of metabolism processes.

Cell cultivars are a convenient object for the cell level study of physiological-biochemical processes in the plant, and simplicity of modeling environmental conditions *in vitro* provides a possibility to a great variety of the experiment conditions (Bhojwani, Razdan, 1996). The use of transgenic plants cell cultivars, in its turn, assures access to the investigation of transgenesis effects.

The present work was aimed at the study of physiological response of normal and transgenic strains of tobacco cell cultivars acquired from the plants transformed by various genetic constructions to biotic and abiotic stress factors.

MATERIALS AND METHODS

The tobacco cell culture obtained from transgenic plants transformed by *Agrobacterium tumefaciens* A699 strain (pCNL 65 with *nptII* gene) and LBA 4400 strain pBiCaMV with *nptII* and *hsp101* (heat shock protein 101 from *Arabidopsis thaliana*) genes as sense and antisense orientation is using as object. The strain LBA4400 was kindly given by Vierling E., University of Arisona, USA (Queitsch *et al*., 2000). Transformation of plant was conducted by cocultivation leaf disks with agrobacterium culture as it written in handbook of (Dreiper *et al*., 1991). A selection of regenerants carried out by cultivation at

medium with kanamicin 100mg/ml. Transgenic status of To and T1 generation was proved PCR with primers to the *nptII* gene of two types:

1. Fo 5'ATG-ACT-GGG-CAC-AAC-AGA-CCA-TCG-GCT-GCT3'

Re 5'CGG-GTA-GCC-AAC-GCT-ATG-TCC-TGA-TAG-CGG3'

a fragment about 700 bp was amplified (Fig.1a);

2. Fo 5'TCG-GCT-ATG-ACT-GGG-CAC-AAC3'

Re 5' GAA-GGC-GAT-AGA-AGG-CGA-TGC 3'

a fragment about 670 bp was amplified (Fig.1b).

Transgenic plants were growing in climatic cameras of firms "Binder" and "C L F PlantClimatics" (Germany).

Biotic and abiotic stress. The increased temperature within the range of 37-53°C was used as abiotic stressor. The cell culture cultivated for 30 min after its vitality was evaluated at reaction deoxidization of 2,3,5-tripheniltetrazolium chloride (TTC) (Enikeev *et al*., 1995). Also the solution of KF was used as abiotic stressor in 0,1-10 mM concentration in cultural medium. After 7 days of cultivation the weight of cell culture was estimated at percent relation from control cell culture weight.

Pathogenic bacteria *Clavibacter michiganensis* ssp *sepidonicus* was used as biotic stressor. Tobacco cell culture was co-cultivated with bacterium at optical density 0,6 OD and titr 3x10 cell/ml. The optical density of bacterial suspension was detected on photometer "Biorad" at 655 nm. Co-cultivation was conducted at 26°C under jiggling.

Immunoblotting. The expression of arabidopsis heat shock protein 101 (HSP101) in transgenic lines of tobacco cell culture was defined by immunoblotting with antibodies against the HSP101 as written in standard protocols (Timmons *et al*., 1990; Sambrook *et al*., 1989). These antibodies were present kindly Vierling E., (University of Arisona, USA).

RESULTS

Confirmation of transgenic character of tobacco cells cultivars. The acquired cell cultivars were subjected to additional check for transgenic features. PCR-analysis confirmed the presence of amplificate 700 pare of base corresponding to *nptII* gene both in the strain transformed by disarmed construction A699 (Fig.1a), and in the strains transformed by the constructions carrying sense and anti-sense gene sequences *hsp101* (Fig.1b).

Presence of target gene expression is considered to be the final confirmation of successful transformation. Immunoblotting of proteins from the strains under study with antibodies HSP101 showed that under normal cultivation conditions (26°C) this protein is intensively expressed only in the cells of the strain transformed by sense sequence (Nt *hsp101*sense) of the respective gene, other strains demonstrated protein in trace quantities (Fig.2). Cultivation at 37°C (mild heat shock) caused HSP101 expression in all strains, including cells of the strain transformed by antisense sequence of *hsp101* (Nt*hsp101*antisense) gene. Therefore, despite the presence of antisense construction *hsp101* gene suppression did not take place.

Stressors impact on viability of tobacco cell cultures

High temperature impact. During incubation of cell cultivars at 43°C (moderate heat shock) cells of the strain transformed by disarmed agrobacterium Nt699 proved to be most sensitive to higher temperature (Fig.3). The strain Nt*hsp101*sense turned out to be the most sensitive to high temperature impact. Resistance of the strain Nt*hsp101*antisense was slightly lower, but at the same time considerably higher than in control strain Ntn.

Potassium fluoride impact. Even the minimal fluoride concentration - 0,1mM – used in the test provoked suppressing effect on the growth of normal strain (Fig.4). With KF concentration in the medium achieving 4,0 mM no culture growth took place. A similar regularity was revealed for the strain Nt699. At the same time, cells of the strain Nt*hsp101*sense possessed a much higher resistance to fluoride. Within the range of 0,1-4,0 mM a small growth stimulation was noticed. With further increase of KF concentration in the medium cultivar growth slowed down and at the concentration of 10,0 mM it completely stopped. By fluoride resistance, the cells of the strain Nt*hsp101*antisense were between the strains Ntn and Nt*hsp101*sense, total cessation of growth was not registered even at maximal KF concentration - 10,0 mM - used in the test.

Influence of pathogenic bacteria Clavibacter michigenensis. During co-cultivation of cell cultivars with bacterial pathogen *Clavibacter michigenensis* minimal resistance was found in Ntn strain (Fig.5). Unlike the impact of abiotic stressors the resistance of the strain Nt699 was slightly higher than in the strain Ntn. Maximal resistance was found in the strain Nt*hsp101*sense. The resistance of the strain Nt*hsp101*antisense was slightly lower.

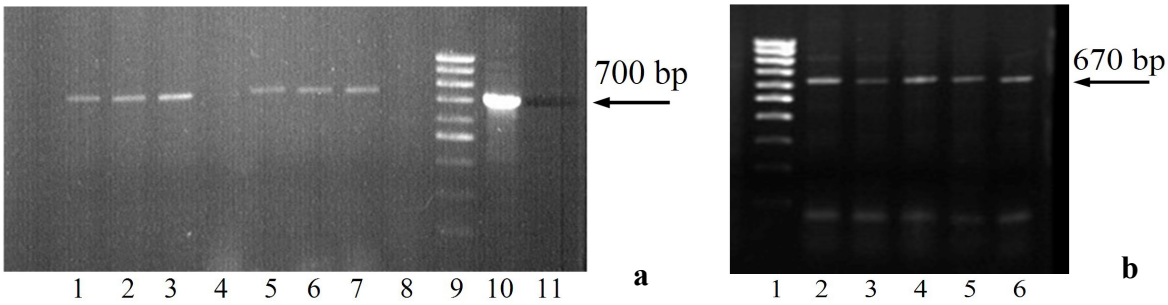

Figure 1: **a.** Amplification of *nptII* gene in the line of tobacco transformed by *A. tumefaciense* A699.
1-7 – tobacco transgenic lines; 8- control (non-transgenic tobacco); 9- marker-ladder 1000 b.p.; 10- positive control (plasmid DNA from *A. tumefaciense* A699), 11- negative control (sterile H_2O).
b. Amplification of *nptII* gene in the line of tobacco transformed by *A. tumefaciense* LBA 4400 (*hsp 101, nptII*).
1- ladder 1000 p.b.; 2-4- transgenic lines with *hsp101* sense; 5,6- transgenic lines with *hsp101* antisense.

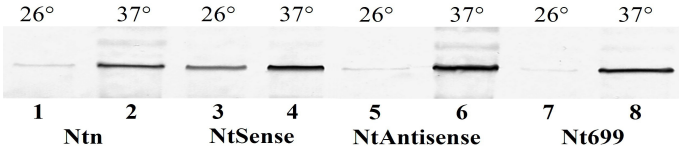

Figure 2: Immunoblotting with anti-bodies against HSP101. 1,2 – normal strain; 3,4 – strain with *hsp101* gene sense; 5,6 - strain with *hsp101* antisense; 7,8 –strain transformed by *A. tumefaciense* A 699

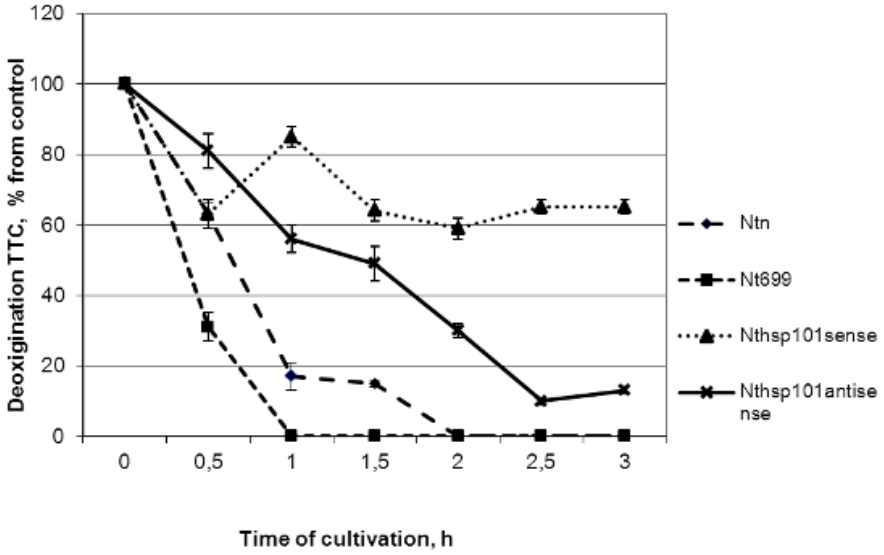

Figure 3: High temperature (43°) impact on viability of cell cultivars of normal and transformed tobacco strains. n=4.

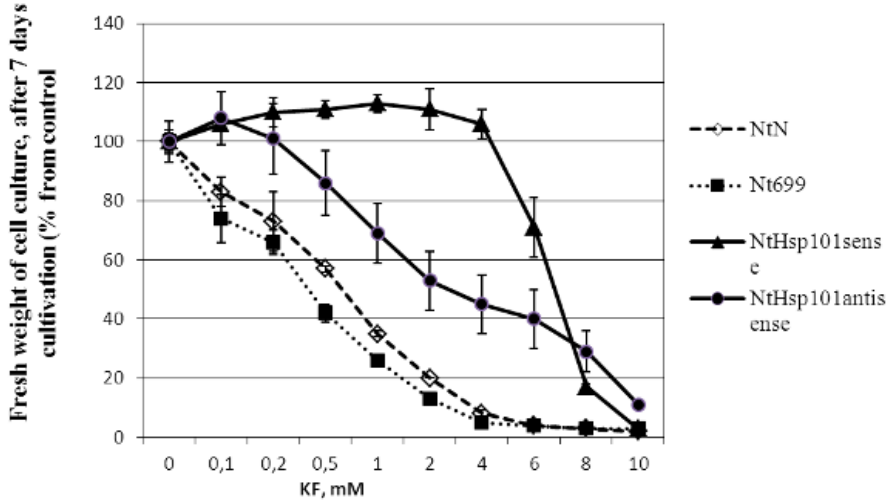

Figure 4: KF impact on the tobacco cell culture growth. n=4.

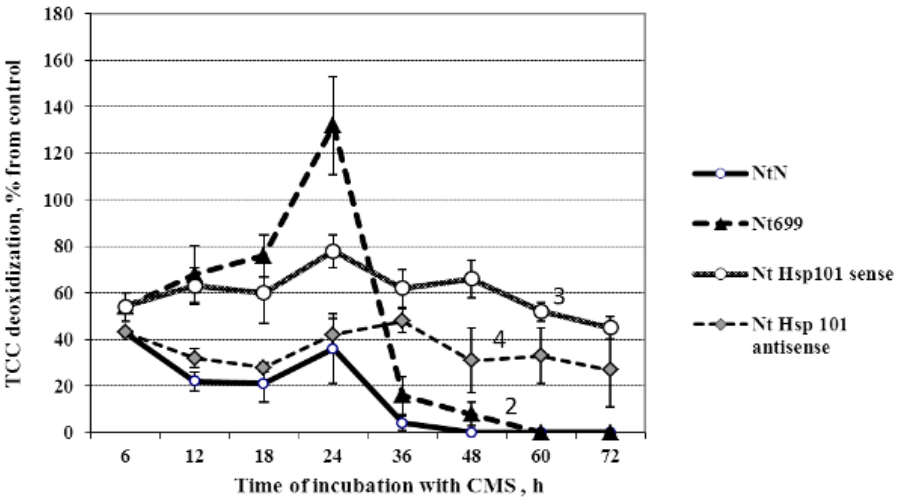

Figure 5: Impact of bacterial pathogen *Clavibacter michiganensis* on viability of cell cultivars of normal and standard tobacco strains. n=4.

DISCUSSION

The choice of evaluation criteria for physiological consequences of plant genetic transformation is a complex problem, which has not been resolved yet. According to modern views, each individual transgenic organism is a fundamentally new system, which is significantly different from the initial form. Variations of physiological reactions are known among transgenic plants acquired under identical conditions and through the use of the same genetic constructions (Bhat, Srinivasan, 2003). These might be caused by the accidental character of T-DNA insertion place and damage of host genes (Baudo *et al*., 2006; Day *et al*., 2000; Latham *et al*., 2006). This assumption is nevertheless disputable. Methodical basis of genetic engineering is plant-agrobacterial symbiosis, the parameters of which have been developed through millions of years of co-evolution, which, in its turn, presupposes presence of certain interaction mechanisms. In present day understanding, joint evolution of host and parasite is aimed to develop compatibility mechanisms via increase of the host resistance and selection of the least pathogenic forms of parasite. At the same time organisms-symbionts form specific systems of metabolism regulation targeted at preservation of common homeostasis (Roitman, Be'er, 2008).

The fundamental difference of natural transformation from artificial transgenesis is in the fact that artificially formed vectors, besides agrobacterial genes, carry genetic elements isolated from other organisms. Thus, multitude of effects is conditioned not so much by accidental character of the insertion spot, but by the properties of the transferred gene.

Maximal resistance, regardless of the nature of stressor, was found in the strain of Nt*hsp101*sense, which confirms universal character of HSP101 as a chaperon protein, which, via protein desagregation and reactivation (Queitsch *et al*., 2000), ensures plant cell protection from damages caused by the impact of unfavourable factors (Ogawa *et al*., 2007; Shafikova *et al*., 2013). In keeping with the currently prevailing views, absence of expression of in-built anti-sense sequence of the target gene witnesses unsuccessful transformation, and physiological consequences of the transformation will be analogous to those of transformation by disarmed construction. However, the results we acquired do not prove this idea. It appears more probable that along with protective function of HSP101 there is general increase of resistance resulting from response to transformation as a complex biotic stressing factor (Enikeev *et al*., 2010, 2012). Different characters of stress response in the strain transformed by the construction without target genes and strains carrying sequences of the gene *hsp101 Arabidopsis thaliana* L., apparently reflect diverse levels of stress response.

Alien gene *hsp 101* (in this case the one from *A. thaliana, Brassicaceae* family) is perceived by tobacco (*N. tabacum*, *Solanaceae* family) cells as a pathogen, which triggers additional protective mechanisms. In compliance with the concept of various transgenity levels (Nielsen, 2003), the probability of transgenesis by-effects is directly proportional to systematic remoteness of gene donors and recipient. Nevertheless, organisms-symbionts

could have developed other interaction mechanisms. As a result, plant response to in-building of agrobacterial genes in its genome takes place at the level of mechanisms, similar to interaction of closely related genes (cisgenic organisms), whereas response to *Arabidopsis* gene in-building into tobacco genome takes place according to the scenario for remote species of the same kingdom (line-genic organisms). A specific reaction of plant cells to in-building of *Agrobacterium* genes is confirmed by the fact of multiple identification of the latter in the genomes of plants of *Nicotiana* genus (Intrieri, Buiatti, 2001; Suzuki *et al*., 2002; Tanaka, 2008; Joshua *et al*., 2010), which is apparently due to horizontal genes transfer during formation and evolution of the mechanisms of plant-agrobacterial symbiosis. Recent studies established the presence of agrobacteria genes in the genomes of other plant species (Matveeva *et al*., 2012).

Thus, the results presented allow us to make a number of suppositions to be considered in further studies in the domain of transgenic plant physiology.

1. Confirmation of the transformation event exclusively on the basis of the expression of transferred target gene is methodologically wrong. Target gene silencing may be a consequence of protective reactions chain in response to “pathogen attack”. Therefore, lack of target gene expression does not mean absence of transformation fact.

2. The use of transgenic objects for the study of functions of individual proteins (particularly with the application of anti-sense strategy) is justified only in the case of close systemic affinity of donor and receptor. The transgenity level should not exceed cysgenic level, otherwise the results may be intensively distorted by the imposition of stress reaction.

3. Assessment of physiological consequences of transgenesis with control plants presented by plants transformed with disarmed strains as compared to the plants transformed by target genes, should be performed with particular caution, as here we mean fundamentally different systems. If the process of transformation with disarmed strains is affine to natural agrobacterial transformation, where plant and bacterium are co-adapted during millions of years of evolution, introduction of a construction with alien gene results in the formation of much less stable system.

REFERENCES

Ahmad P., Ashraf M., Younis M., Hu X., Kumar A., Akram N.A, Al-Qurainy F. (2012) Role of transgenic plants in agriculture and biopharming. *Biotechnology Advances*. **30(3)**. 524–540.

Bhojwani S.S., Razdan M.K.(1996) Plant tissue culture: theory and practice, a revised edition. – Elsevier, Amsterdam – Lausanne - New York – Oxford – Shannon - Tokyo. 767.

Bock R. (2001) Transgenic Plastids in Basic Research and Plant Biotechnology. *Journal of Molecular Biology*. **312(3).** 425-438.

Bourque J.E. (1995) Antisense strategies for genetic manipulations in plants. *Plant Science.* **105(2).** 125-149.

Draper J., Scott R., Armitage Ph., Dury G., Jacob L., Walden R., Kumar A., Jefferson R., Hamil J. (1991) Plant Genetic Transformation and gene expression: A Laboratory Manual. Moscow. Mir. 408. [In Russ.]

Eapen S., D'Souza S.F. (2005) Prospects of genetic engineering of plants for phytoremediation of toxic metals. Biotexnology Advances. 23(2). 97-114.

Enikeev A.G., Vysotskaya E.F., Leonova L.A., Gamburg K.Z. (1995) Viability assay with 2,3,5-tripheniltetrazolium chloride in plant cell cultures].- *Russ. J. Plant Physiol*. **42(3).** 372-375.

Enikeev A.G., Kopytina T.V., Semenova L.A., Natyaganova A.V.,Gamanetz L.V., Volkova O.D. 2008 Agrobacterial transformation as complex biotical stressing factor *J. of Stress Physiology & Biochemistry.* **4(1).** 11-19. [In Russ]

Filipecki M., Malepszy S. (2006) Unintended consequences of plant transformation: a molecular insight. *J. Appl. Genet.* **47(4).** 277–286.

Gelvin S.B., Liu C-N. (1994) Genetic manipulation of *Agrobacterium tumefaciens* strains to improve transformation of recalcitrant species. - In: Gelvin, S.B., Schilperoort R.A. (ed): Plant Molecular Biology Manual. pp. B4/1–B4/13. 2nd ed., Kluwer Academic Publishers, Dordrecht - Netherlands.

Hewezi T., Mouzeyar S., Thion L., Rickauer M., Alibert G., Nicolas P., Kallerhoff J.(2006) Antisense expression of a NBS-LRR sequence in sunflower (*Helianthus annuus*L.) and tobacco (*Nicotiana tabacum* L.): evidence for a dual role in plant development and fungal resistance. *Transgenic Research.* **15(2).** 165-180.

Kos M., van Loon J.J.A., Dicke M., Vet L.E.M. (2009) Transgenic plants as vital components of integrated pest management. *Trends in Biotechology.* **27(11).** 621-627.

Kuzovkina I.N., Al'terman I.E., Karandashov V.E. (2004) Genetically Transformed Plant Roots as a Model for Studying Specific Metabolism and Symbiotic Contacts of the Root System. *Biology Bulletin of the Russian Academy of Sciences.* **3.** 255-261. [In Russ.].

Liénard D., Sourrouille C., Gomord V., Faye L. (2007) Pharming and transgenic plants. *Biotechnology Annual Review.* **13.** 115-147.

Matveeva T. V., Bogomaz D. I.,Pavlova O.A., Nester E.W., Lutova L.A. (2012) Horizontal Gene Transfer from Genus *Agrobacterium* to the Plant *Linaria* in Nature. *Molecular Plant-Microbe Interactions.* **25(12).** 1542-1551. [In Russ].

Meletzus D., Eichenlaub R. (1991) Transformation of the phytopathogenic bacterium *Clavibacter michiganense subsp. michiganense* by electroporation and development of a cloning vector. *J. of Bacteriology*. **173(1).** 184–190.

Nielsen K.M. (2003) Transgenic organisms: time for

conceptual diversification? *Nature Biotechnology.* **21(3).** 227-228.

Ogawa D., Yamaguchi K., Nishiuchi T. (2007) High-level over expression of the Arabidohsis Hsf A2 gene confers not only increased termotolerance but also salt/osmotic stress tolerance and enhanced callus growth. *J. of Experimental Botany.* **58(12).** 3373-3383.

Peleg Z., Walia H., Blumwald E. (2011) Integrating genomics and genetics to accelerate development of drought and salinity tolerant crops. In: Alman A ., Hasegawa P.M. (ed.): Plant Biotechnology and Agriculture. Academic Press, London. 271-286.

Queitsch C., Hong S.-W., Vierling E., Lindquist S.L. (2000) Heat Shock Protein 101 Plays a Crucial role in thermotolerance in *Arabidopsis. Plant Cell.* **12.** 479–492.

Rashid A. (2009) Introduction to genetic engineering of crop plants: aims and achievements. - I.K. International Pvt Ltd, New Delhi. 259.

Rischer H. Oksman-Caldentey K-M. (2006) Unintended effects in genetically modified crops: revealed by metabolomics? *Trends in Biotechnology.* **24(3).** 102-104.

Sambrook, J., Fritsch, E.F. and Maniatis, T.(1989) Molecular Cloning: A Laboratory Manual.. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. 447.

Shafikova T.N., Omelichkina Yu. V., Soldatenko A.S., Enikeev A.G., Kopytina T.V., Rusaleva T.M., Volkova O.D. (2013) Tobacco cell culture strans formed by hsp 101 gene exhibit on increased resistance to *Clavibacter micyiganensis ssp. Sepedonicus. Doklady Biological Sciences.* **450.** 165-167.

Sorochinskii B.V., Burlaka O.M., Naumenko V. D., Sekan A. S. (2011) Unintended effects of genetic modifications and methods of their analysis in plants. *Cytology and Genetics.* **45(5).** 324-332.

Suzuki K., Yamashita I., Tanaka N. (2002) Tobacco plants were transformed by *Agrobacterium rhizogenes* infection during their evolution. *The Plant Journal.* **32(5).** 775–787.

Tanaka N. (2008) Horizontal Gene Transfer . In: Tzfira T., Citovsky V. (ed.) Agrobacterium: From Biology to Biotechnology. Springer Science+Business Media, LLC. 623- 647.

Timmons T.M., Dunbar B.S. (1990) Protein Blotting and Immunodetection. *Methods Enzymol.* **182.** 679–701.

Transgenic plants: methods and protocols. (2005) In: Pena L. (ed): Methods in molecular biology. 286. Humana Press, Totowa - New Jersey. 437.