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Transgenic Plants as Genetic Models for Studying Functions of Plant Genes

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Abstract—Transgenic plants are widely used for the investigation of functions of particular genes and for reconstruction of complex gene networks controlling plant morphology, biochemistry, and physiology during different development stages and in response to various external stimuli. Gene engineering instruments for the design of transgenic plants with either an elevated or suppressed expression of the target genes are discussed. Genetic constructs for protein synthesis or antisense RNA/self-complementary double-stranded RNA transcription are described. Transgenic plants with elevated or decreased levels of expression of S-like ribonucleases and a decreased expression of the proline dehydrogenase gene are considered as examples. It was believed that S-like RNase functions concern mainly phosphate remobilization from senescent plant organs. However, the expression patterns of some genes coding for S-like RNases were similar to some pathogen-responsive genes (both local and systemic induction after damage or pathogen inoculation). In addition, some pathogenesis-related proteins (PR-4 family) possess RNase activity and can inhibit the growth of pathogenic fungi. Investigation of transgenic tobacco plants revealed that high ribonuclease activity in the apoplast is correlated with increased resistance against the tobacco mosaic virus. Thus, S-like RNases may have a new function as a part of a plant's basal antiviral defense mechanism. Another set of transgenic plants carries an antisense suppressor of the proline dehydrogenase gene (PDH) constructed with an *Arabidopsis* target gene segment. Tobacco, maize, and sunflower plants with this heterologous suppressor were characterized by a moderate decrease in PDH activity and a moderate (1.5–3-fold) increase in the proline content under normal conditions. It was also found that these plants were more tolerant to various abiotic stresses (drought, NaCl, cold, and toxic heavy metals), which may result from the protective proline effect early in exposure to stress, preventing the cellular gene expression machinery from damage by stress-generated free radicals.

Keywords: plants, genetic engineering, pathogen resistance, stress tolerance, proline, ribonucleases
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The first lines of transgenic plants were obtained more than thirty years ago (Bevan et al., 1983; Murai et al., 1983) using a vector system based on *Agrobacterium tumefaciens* (Chilton, 1983). Over the past three decades, technologies for obtaining transgenic plants have become a common way of studying functions of individual genes and their systems and have paved the way for obtaining cultivars with new valuable properties for agriculture and biotechnology production (Kamthan et al., 2016; Nogue et al., 2016). The use of transgenic forms for research can be indirectly assessed by the number of scientific publications whose title or annotation, along with the name of the plant, contain the terms transgene or transgenic. This approach has been widely used for work not only with classical model plants (*Arabidopsis thaliana*, *Nicotiana tabacum*) but also with the most important crops:

Plant species	Number of papers*
Any plant (the term plant)	26222
Arabidopsis thaliana	7983
Nicotiana tabacum (tobacco)	6443
Solanum tuberosum(potato)	1450
Triticum aestivum (wheat)	1016

* Annotated in the PubMed system (http://www.ncbi.nlm.nih.gov/pubmed/), August 2016.

The classical scheme for obtaining transgenic plants is shown in Fig. 1. Planning the creation of transgenic plants involves the development of a scheme of a genetic construct whose structure is determined by the plant species and the way of transforma-

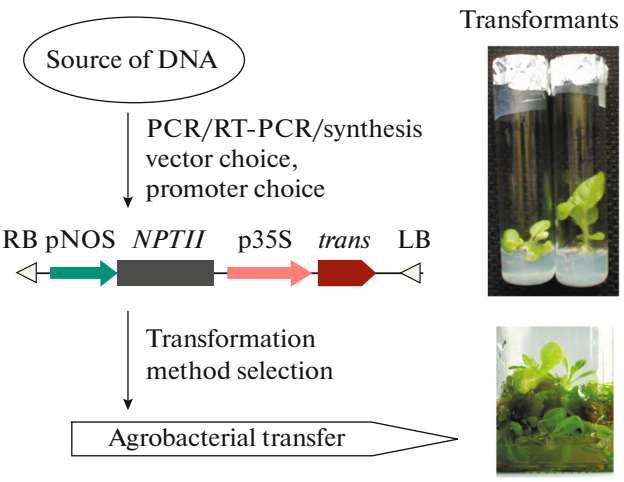


Fig. 1. Classical scheme for production of transgenic plants. PCR, polymerase chain reaction; RT, reverse transcription; synthesis, synthetic segments of DNA; RB, LB, terminal repeats bordering T-region in binary vector; *NPTII*, gene of neomycin transferase II of *E. coli*, one of variants of reporter gene that provides selection of transgenic plants on selective media; pNOS, promoter of gene for nopaline synthase of *A. tumefaciens*; p35S, promoter of 35S RNA gene of cauliflower mosaic virus; *trans*, transgene or antisense segment/inverted repeat for suppressing target gene by RNA interference. Agrobacterial transfer is carried out with help of "disarmed" strains of *A. tumefaciens* whose T-regions of Ti-plasmid are deleted, and T-region from binary vector is transferred to genome of plant cell. As example, photographs of kanamycin-resistant transformants of *Nicotiana tabacum* are given.

tion. The efficiency of the transformation depends not only on the plant species but also the genotype and can vary significantly in different cultivars (Altpeter et al., 2016). Transformation of dicotyledonous plants is usually implemented using "disarmed" strains of *Agrobacterium tumefaciens*; however, the agrobacterial transformation of many monocotyledonous plants is complicated, and in this case other methods are used, for example, bombardment by particles containing the DNA of the genetic structure sorbed on them, followed by the selection of calluses on selective backgrounds and the regeneration of transgenic plants. Agrobacterial transformation employs binary vectors that can replicate in both *Escherichia coli* and *Agrobacterium*. *A. tumefaciens* transfers a DNA segment (T-region) located between the terminal repeats (LB, RB) into the plant genome (Bourras et al., 2015; Peyret and Lomonossoff, 2015). In the structure of the T-region of the genetic construct, two key elements can be identified (see Fig. 1): a reporter gene for selecting transformants on selective media and a target genetic element for expression in plant cells, for the sake of which an experiment on obtaining and analyzing transgenic forms is carried out. As such an objective element, one can employ protein-coding genes, miRNA genes, and segments of the target genes for the synthesis of antisense or self-complement (double-stranded) RNAs. Service elements (promoter, poly(A)-signal, translational enhancers) are important here, as well as adaptation of the structure of the foreign DNA for correct expression in plant cells (translation initiation and termination sites, the secondary structure in the 5'-untranslated region, in some cases optimization of the codon composition to maximize expression levels) (Egelkrout et al., 2012; Smirnova et al., 2012; Kochetov et al., 2014; Gerasimova et al., 2016).

The most important stage in genetic construct planning is the choice of a promoter. At the early stages of development of genetic engineering, a limited range of viral or agrobacterial-derived promoters (35S, pNOS, pMAS), as well as promoters of highly expressed genes (actin, ubiquitin, and tubulin) were used. However, in order to solve the experimental tasks, expression of a transgene restricted to a specific tissue, developmental period, or external conditions (inducible promoters) may be required. The accumulation of information about the transcripts and patterns of expression of plant genes (including transgenes under the control of various promoter regions in transgenic plants) has significantly expanded the possibilities for the design of genetic constructs (Smirnova et al., 2012). A number of lines of transgenic plants were obtained at the Institute of Ecology and Genetics of the Siberian Branch, Russian Academy of Sciences to study the functions of individual genes and mechanisms for the genetic control of the phenotypic characteristics some of which are described below.

Lines of Transgenic Plants with Altered Activity of Apoplastic Ribonucleases

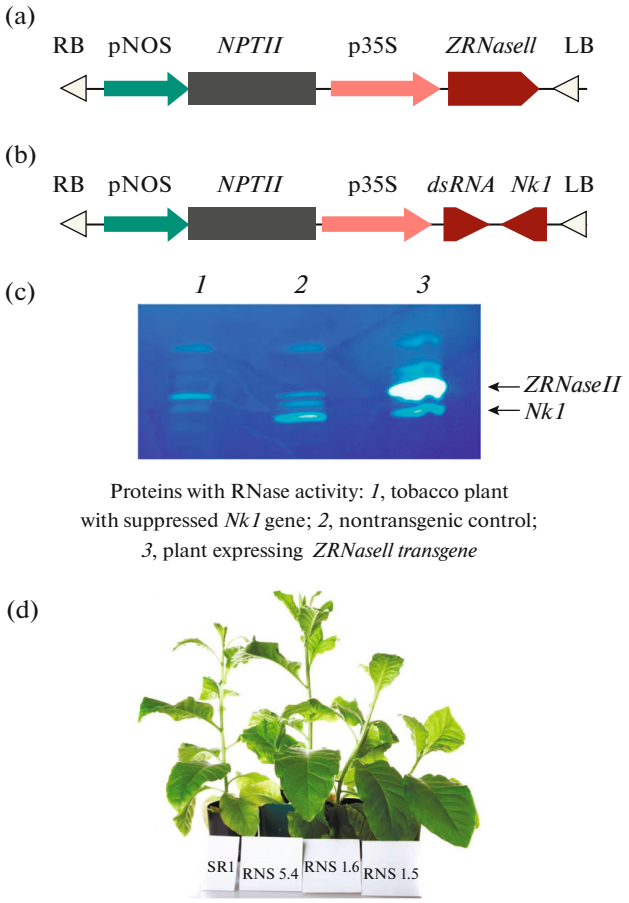
The genome of plants contains extracellular ribonuclease (RNase) genes whose functions have traditionally been associated with the remobilization of phosphates from the dying parts of plants (S-like RNases), as well as with resistance to phytopathogenic fungi (some proteins of the PR-4 family) (Filipenko et al., 2013; Jashni et al., 2015; Stigter and Plaxton, 2015). It has been suggested that apoplast RNAses might play a certain role in the resistance to viruses (the overwhelming majority of plant viruses contain RNA genomes that can be destroyed by ribonucleases). To test this assumption, lines of transgenic *Nicotiana tabacum* plants expressing the genes of pancreatic RNase of *Bos taurus*, S-like RNase of *Zinnia elegans*, as well as the tobacco line with the suppressed gene of its own S-like RNase *Nk1*, have been created (Sangaev et al., 2007, 2010; Trifonova et al., 2007, 2012).

Figure 2 shows the schemes of two genetic constructs: an extracellular RNase of plant origin is synthesized using the cDNA of the *ZRNaseII* gene of zinnia placed under the control of a strong constitutive CaMV 35S RNA promoter (see Fig. 2a); the *Nk1* gene of the S-like RNase of tobacco is suppressed using the segments of this gene arranged in the form of an inverted repeat (see Fig. 2b). It can be seen that the

expression of genetic constructs changes the range of active ribonucleases in plant protein extracts in comparison with the original (non-transgenic) forms in a given direction (see Fig. 2c). In this case, the transgenic plants and the initial tobacco cultivar had no apparent differences (see Fig. 2d).

To express the heterologous gene (mammalian secretory RNAse), another variant of the genetic construct was used, in which the cDNA of the *Bos taurus* pancreatic RNase was placed under the control of the 2' promoter of the mannopine synthase gene. Unlike the strong constitutive CaMV 35S promoter, this promoter was characterized by a lower level of normal expression, as well as a high level of local induction, in the area of damage to plant tissues (Trifonova et al., 2007). Since viruses often occur in plants with the help of phytophagous carriers, the transcription pattern of the 2' promoter was close to the natural variants of protective protein genes. Both the constructs provided high ribonuclease activity in the apoplast (8–15 times higher than in the control), where the transgenic plants were characterized by a significant delay in the development of the tobacco mosaic virus (Trifonova et al., 2007, 2012), as well as the cucumber mosaic virus, which belong to different taxonomic groups (Sugawara et al., in press). Hypothetical mechanisms of the antiviral effect of extracellular RNases can be based both on the vulnerability of the genomic RNA of the viruses at some stages of their penetration into the plant cell and on the simulation of the mechanism of programmed cell death: when the integrity of tissues is disturbed, the contents of the apoplast can penetrate the cytoplasm of damaged cells, in which case active RNases function as "killer" proteins that kill the cell and prevent replication of the genomic RNA of the virus that penetrated it.

Thus, the results of the analysis of the viral resistance of transgenic plants with a modified level of RNase activity in the apoplast have led to the conclusion that these proteins can form a "nuclease" barrier for RNA viruses and be considered as elements of a nonspecific defense system similar in the expression pattern and some functions to PR-proteins (pathogenesis-related proteins) (Trifonova et al., 2007). In addition to general scientific information, the data obtained can be used to develop new methods for obtaining cultivars of agricultural plants with increased nonspecific resistance to viruses. For this, it is possible to use transgenes of extracellular RNases, as well as genotypes with high RNase activity in the apoplast, selected after analysis of their genetic variability for the indicated trait (Sindarovska et al., 2014). At present, the increase in basic (nonspecific) resistance to phytopathogens in combination with the directed increase in specific resistance is considered as one of the most promising directions of breeding (Lee et al., 2016).



Proteins with RNase activity: *1*, tobacco plant with suppressed *Nk1* gene; *2*, nontransgenic control; *3*, plant expressing *ZRNasell transgene*

Fig. 2. Transgenic plants with modified levels of expression of extracellular ribonucleases. a, b, schemes of genetic construct: a, for expression of cDNA of gene of S-like RNase of *Zinnia elegans* (*ZRNaseII*); b, of dsRNA suppressor of gene of S-like RNase of *Nicotiana tabacum Nk1*; c, foregram of proteins with RNase activity (gel matrix contains RNA, special dye is used) (Sangaev et al., 2010); d, transgenic (RNS) and nontransgenic (SR1) plants of *N. tabacum*.

Lines of Transgenic Plants with Suppressed Expression of the Proline Dehydrogenase Gene

In the cells of many plant species, proline functions as a compatible osmolyte, and changes in its content are important for the rapid adaptation of plants to changes in the water supply regime. In addition, according to some data, proline is able to inactivate free radicals and protect proteins and membranes of plant cells from damage (Hayat et al., 2012). It was believed that proline can be synthesized from glutamate; the rate-limiting enzyme of its synthesis is pyrroline-5-carboxylate synthase (P5CS). Functions of the *P5CS* gene in various plant species have been actively investigated, including in the model of transgenic plants (Verdoy et al., 2006; Vendruscolo et al., 2007; etc.). However, functions of proline catabolism genes, in particular the rate-limiting gene of proline dehydrogenase (PDH), have been studied to a much lesser degree.

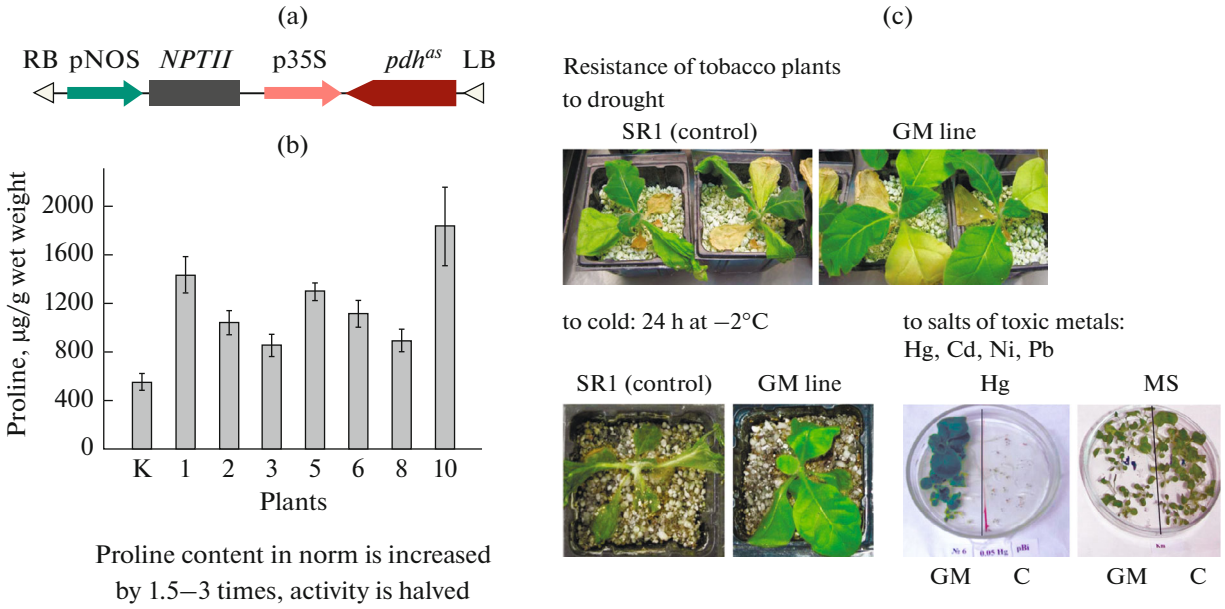


Fig. 3. Transgenic plants with suppressed levels of expression of proline dehydrogenase gene. a, scheme of genetic construct for partial suppression of plant proline dehydrogenase gene (*pdh*^{as} is antisense gene segment of *A. thaliana*); b, proline content in transformant leaves; c, resistance of transformants (GM plant lines) in relation to drought, cold, and heavy metal salts (Kochetov et al., 2004; Kolodyazhnaya et al., 2006, 2007; Ibragimova et al., 2012).

To determine the contribution of the PDH gene to the control of plant stress-resistance, transgenic forms of tobacco with a reduced level of its expression were developed (Fig. 3). As part of the genetic construct, a short antisense segment of the PDH gene of *Arabidopsis* was used (see Fig. 3a), which led to the partial suppression of the PDH gene, a decrease in the enzyme activity by half, and a moderate increase in proline activity in the norm (see Fig. 3b). Analysis of the morphophysiological characteristics of the plants showed that the transgenic forms had no apparent differences from the original cultivar *Nicotiana tabacum* SR1, but were characterized by increased resistance to drought, cold, and toxic heavy metal salts (see Fig. 3c). In general, the partial suppression of the PDH gene led to a marked shift in the normal response for traits of resistance to various abiotic stresses (Kochetov et al., 2004; Kolodyazhnaya et al., 2007; Ibragimova et al., 2012). The transgenic forms of sunflower and maize plants obtained with the same genetic construct also exhibited increased survival rates in conditions of water scarcity and salinity, which suggests a conservative role of the PDH gene in controlling stress resistance (Moiseeva et al., 2014; Tishchenko et al., 2014).

Apparently, the results obtained can be explained by the proline property of protecting protein molecules from damage. A sharp change in environmental conditions (water supply, higher or lower temperature, etc.) induces in plants the expression of stress response genes, triggering a complex of adaptive processes. In the case of intensive stress without an acclimatization period, proteins of the cellular expression machinery can be damaged, and the timely synthesis of protective proteins will not be started. Transgenic forms with suppressed PDH activity are characterized by a moderately high content of proline in the norm, which gives certain advantages at the earliest stages of stress, and in particular, protects the transcription and translation machinery factors and synthesis of protective proteins through which plant cells further adapt at the biochemical and physiological levels. Transgenic plants proved to be more resistant to salts of heavy metals whose toxic effects are largely related to the damage of protein molecules (see Fig. 3c), which can be regarded as confirmation of this hypothesis. In recent years, there has been a rethinking of the role of the metabolic chain of proline synthesis in the processes occurring in a plant, and more new data are published on the role of this amino acid in the formation of the gametophyte (Biancucci et al., 2015), control of plant aging (Zhang and Becker, 2015), protection from phytopathogens (Qamar et al., 2015), and other key processes. The lines of transgenic plants that we developed can also be in demand as genetic models for research in these directions.

Of great importance is the structure of the genetic construct in which a short segment of the cDNA of the *Arabidopsis* gene was used as an antisense suppressor. A heterologous antisense suppressor did not lead to a complete knockdown of the PDH gene in *N. tabacum* plants (and, possibly, maize); thus, the morphological characteristics and the development time of the transgenic forms in the norm did not differ significantly from the original cultivar, at least under the conditions

of the greenhouse and in experiments in vitro. The revealed patterns can be used to obtain new varieties of agricultural plants with increased nonspecific resistance to abiotic stresses, which can be done using both transgenesis methods and analysis of the population's genetic variability for the activity of the PDH gene/proline content in the norm followed by involving the selected genotypes in the breeding process using marker-oriented selection methods (Spoljarevic et al., 2011).

Other Genetic Engineering Methods for the Study of the Genetic Control of Plant Characteristics

In recent years, the range of genetically engineered technologies has expanded significantly. Among the most interesting technologies is virus-induced gene silencing (VIGS), which is based on the inclusion of the host plant gene segment into the viral genome for the induction of RNA interference and switching off the expression of the target gene at the posttranscriptional level (Zhirnov et al., 2015; Lacomme, 2015). The effect of using VIGS is similar to the effect observed in transgenic plants carrying genetic constructs with antisense or dsRNA suppressors (see Figs. 2b, 3a). However, the production of transgenic forms for certain plant species is a complex procedure. In addition, suppression of some genes can block plant development; thus, it is impossible to obtain such transgenic forms. VIGS makes it possible to turn off the expression of the target gene in most cells of an adult (nontransgenic) plant, which provides additional possibilities in the experiment. Technically, VIGS is induced using agrobacterial transfection (insertion of a suspension of *A. tumefaciens* into the leaf tissue). Under the control of the plant promoter, the T-region of the genetic construct contains a plant virus cDNA (usually a nontransmissive variant that infects most of the tissues of the experimental plant but that is not capable of being transmitted to another plant by the conventional method for the virus). The composition of this cDNA includes a target gene segment, which leads to the induction of RNA interference. One of the most frequently and widely used variants is a vector system based on the tobacco rattle virus (Zhirnov et al., 2015; Lacomme, 2015).

Examples of VIGS applications include recent studies on the transcription factors that control resistance to drought (Wang et al., 2016) and *Fusarium* (Kumar et al., 2016): analysis of the consequences of "switching off" the gene expression in the cells of adult plants is used as part of a comprehensive research process aimed at the comprehensive identification of the functions of the target genes.

Another very interesting genetic engineering approach is host-induced gene silencing (HIGS). This approach is based on the induction of RNA interference in a plant using transgenesis or VIGS, but using the antisense segments of not native plant genes but the genes of the organisms interacting with the plant, for example, pathogenic fungi or phytophages (Koch and Kogel, 2014). It turned out that siRNAs are able to penetrate phytophagous cells and trigger the evolutionarily conservative mechanism of RNA interference. Examples of the application of this technology are works on directed HIGS-mediated suppression of important genes of *Puccinia triticina* (Panwar et al., 2013), *Fusarium oxysporum* (Koch et al., 2013), nematodes (Dubreuil et al., 2009), and insects (Kumar et al., 2012).

Apparently, in the near future, new technologies for the genetic engineering of plants will be developed to directly affect phytophages or other organisms interacting with plants. Recently, there has been proposed an approach based on the synthesis of autonomous viral replicons in transgenic plants that are incapable of replication in plant cells but are capable of infecting the cells of phytophagous (alien replicon producing organisms, ARPO), which can provide new opportunities in the field of the biocontrol of natural populations of various organisms (Kochetov, 2014).

CONCLUSIONS

Methods and approaches of genetic engineering are necessary for conducting fundamental research in the field of plant genetics. Transgenic forms provide unique opportunities for identifying the functions of individual genes, intergenic interactions, and ultimately for the reconstruction of complex ensembles of genes that control the formation of the morphological, biochemical, and physiological characteristics of plants and mechanisms for adapting to changing environmental conditions.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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