Russian Journal of Genetics, Vol. 41, No. 11, 2005, pp. 1187–1193. Translated from Genetika, Vol. 41, No. 11, 2005, pp. 1445–1452.

MOLECULAR GENETICS

Enhanced Resistance to Phytopathogenic Bacteria in Transgenic Tobacco Plants with Synthetic Gene of Antimicrobial Peptide Cecropin P1

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Received November 10, 2004; in final form, April 24, 2005

Abstract—Plasmids with a synthetic gene of the mammalian antimicrobial peptide cecropin P1 (*cecP1*) controlled by the constitutive promoter 35S RNA of cauliflower mosaic virus were constructed. Agrobacterial transformation of tobacco plants was conducted using the obtained recombinant binary vector. The presence of gene *cecP1* in the plant genome was confirmed by PCR. The expression of gene *cecP1* in transgenic plants was shown by Northern blot analysis. The obtained transgenic plants exhibit enhanced resistance to phytopathogenic bacteria *Pseudomonas syringae, P. marginata,* and *Erwinia carotovora.* The ability of transgenic plants to express cecropin P1 was transmitted to the progeny. F_0 and F_1 plants had the normal phenotype (except for a changed coloration of flowers) and retained the ability to produce normal viable seeds upon self-pollination. Lines of F_1 plants with Mendelian segregation of transgenic traits were selected.

INTRODUCTION

Antimicrobial peptides are the integral part of the innate immune system of all multicellular organisms, having a wide spectrum of bactericidal and fungicidal activity [1, 2]. To date, the structure of more than 100 such compounds, whose characteristic features are the total positive charge and amphiphility, has been established. Antimicrobial peptides lead to lysis of bacterial and fungal cells, causing disturbances in the integrity of membranes and increasing their permeability. At a concentration of 0.1–5 μM, they manifest lytic activity against various microorganisms, but not against animal and plant cells [3, 4]. Resistance of eukaryotic cells to positively charged antimicrobial peptides synthesized by these cells may be explained by the neutral charge of their membranes, whereas cellular membranes of microorganisms are negatively charged and easily interact with them. It should be noted that antimicrobial peptides synthesized by various eukaryotes exhibit high homology of amino acid sequences within their structural families [1]. Expansion of the spectrum of antimicrobial peptides in the plant cell is a promising strategy of plant defense against phytopathogenic microorganisms [5–8]. However, study of the effect of heterologous antimicrobial peptides on plant resistance to bacterial and fungal phytopathogens was conducted using a limited number of these compounds and produced contradictory results concerning the defense effect of the same peptide on various plant species and cultivars [9].

The aim of this work was to examine the effect of expression of synthetic gene cecropin P1 (*cecP1*), encoding a 31-mer amino acid sequence [10], on the resistance of transgenic tobacco plants to phytopathogenic bacteria.

MATERIALS AND METHODS

Plant material. Seeds of tobacco (*Nicotiana tabacum* L., cultivar Samsun) were grown (after surface sterilization in 1% solution of sodium hypochlorite) in vitro on nonhormonal agar medium MS containing a standard set of salts, and including 7 g/l agar and 30 g/l sucrose (pH 5.8) [11]. Plants were cultivated at 22–24°C during a 16-h-light day at illumination intensity of 2 kLx.

Strains of phytopathogens. We used strains *Erwinia carotovora* subsp. *carotovora* B15 from Horticulture Center (Canada), *Erwinia carotovora* subsp. *carotovora* ATCC 495, and strains of phytopathogenic microorganisms *Pseudomonas syringae* B-1546 and *Pseudomonas* sp. *marginata* B-1298 from the All-Union Collection of microorganisms (Institute of Biochemistry and Physiology of Microorganisms, Russian Academy of Sciences).

Obtaining plasmid constructs. Cloning of DNA fragments into plasmid vectors and PCR were con-

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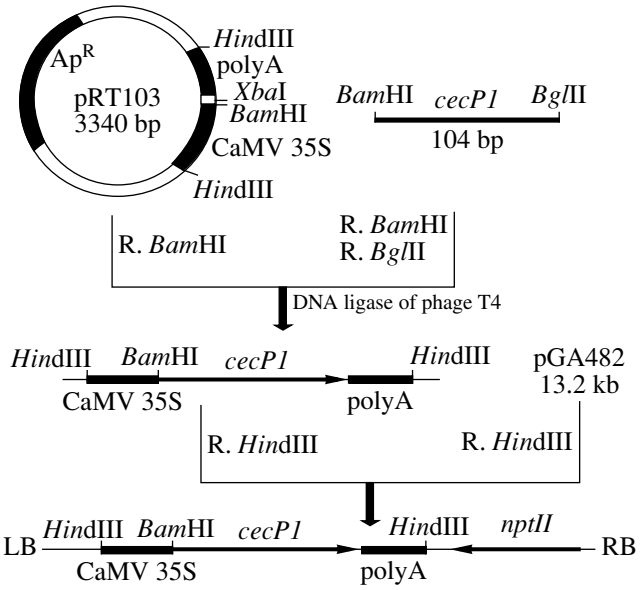

Fig. 1. Scheme of constructing plasmids for transformation of plants containing the cecropin P1 (*cecP1*) gene.

ducted by standard methods [12]. Restriction endonucleases, DNA ligase of phage T4, and *Taq* DNA polymerase were used according to recommendations of the supplier (Sibenzim, Russia). As a source of the target gene, we used the previously obtained vector plasmid pET21d that contained the structural part of the synthetic gene cecropin P1 (*cecP1*) encoding the amino acid sequence of the mature form of mammalian cecropin P1 [10]. Gene *cecP1* was resynthesized with primers 5'-CGGGATCCATGGCTCTTG-3' and 5'-CGAGATCTCTACTTAGCGCGGC-3' using plasmid DNA of pET21d-*cecP1*. The resulting *Bam*HI-*cecP1*-*Bgl*II PCR fragment was cloned into vector pRT103 [13], which had been preliminarily digested with *Bam*HI, producing plasmid pRT103-*cecP1*. Gene orientation relative to promoter CaMV 35S was tested via double digestion of pRT103-*cecP1* plasmid DNA with *Bam*HI and *Xba*I enzymes. A 104-bp fragment was evidence proving the sense orientation of *cecP1* relative to the promoter. For sequencing of the synthesized gene, a *Bam*HI–*Xba*I fragment was excised from plasmid pRT103-*cecP1* and cloned into specific plasmid BluescriptKS+. The 35S-*cecP1*-polyA cassette was cut from plasmid pRT103-*cecP1* with the help of *Hin*dIII enzyme and cloned into the binary vector pGA482 [14] that had been previously digested with *Hin*dIII. The scheme of constructing the vectors is shown in Fig. 1. Plasmids were transferred into strain *Agrobacterium tumefaciens* GV3101 (pMP90RK) [15] via conjugation [16]. Agrobacteria containing vector pGA482 with gene *cecP1* and selective kanamycin resistance gene *nptII* were used for plant transformation.

Development of transgenic plants. Plant transformation was accomplished by inoculating leaf disks with agrobacteria [17]. As explants, leaf fragments of 0.5–1.0 cm obtained from in vitro grown 3–4-week-old plants were used. Fifty explants were involved in each experiment. Regeneration of sprouts was observed two to three weeks after transformation. The resulting regenerants were implanted, analyzed, and then grown in a greenhouse in a sterilized soil mixture peat : sand (with the volume ratio 1 : 1) at 22–24°C, relative air humidity 60–70%, and illumination 4 kLx.

DNA isolation from plant leaves. DNA for PCR analysis was isolated from young leaves [18].

RNA–DNA hybridization. Total plant RNA was isolated from 5–6-week plants [19]. For analysis, 20 μg RNA was taken. RNA electrophoresis was conducted in a 4% polyacrylamide gel containing 6 M urea. RNA was then transferred to nylon membrane Hybond N^+ (Amersham, England) by electroblotting using a Transblot devise (Bio-Rad, USA) at 20 V overnight. Hybridization of RNA with a labeled α^{32}P-dATP DNA probe, a structural portion of cecropin P1 gene, was conducted as in [20].

Biotest with plant extracts. Leaves of analyzed plants were ground in the porcelain mortar containing liquid nitrogen with the addition of extraction buffer (10% glycerol, 40 mM EDTA, 150 mM NaCl, 100 mM NH_4Cl, 10.0 mM Tris-HCl, pH 7.5; 3.0 mg/ml dithiotreitol; 0.2 mg/ml leupeptine; 0.2 mg/ml trypsin inhibitor; 4 mM phenylmethyl sulfonyl fluoride; 2 mg/ml BSU). The prepared homogeneous suspension was centrifuged for 20 min at 10 000 g, and supernatant was used to determine its antibiotic activity by the method of radial diffusion [6]. The protein in plant extracts was determined as in [21]. The inhibitory effect of plant extracts on cell growth of phytopathogenic bacteria was assayed on Petri dishes containing 30 ml of 1.5% agar with corresponding bacterial strains (10^8 cells/ml). For this, extracts with equal quantity of protein from leaves of transgenic and two control plants (normal and transgenic plants transformed with an "empty" vector) were placed in slots 5 mm in diameter. Each experiment was conducted with one leaf from the middle part of the plant in three biological replicates. Petri dishes were incubated for 8 h at 4°C to attain extract diffusion into agar and then at 25°C for two days. The size of a free zone around the slot characterized bacterial growth arrest. Quantitation of cecropin P1 content in extracts of transgenic plants was done in comparison with control experiments, where known concentrations of synthetic cecropin P1 (Sigma, USA) were used as a standard.

Bioassays with isolated leaves and intact plants. To verify resistance of transgenic plants to bacterial phytopathogens, young leaves were inoculated with suspensions of bacteria *Erwinia carotovora* subsp. *carotovora* B15, *Erwinia carotovora* subsp. *carotovora* ATCC 495, *Pseudomonas syringae* B-1546, and *Pseudomonas* sp. *marginata* B-1298. We used three inoculation methods. In the first case, 10 tobacco leaf disks from each of various lines of transgenic and control plants were inocu-

lated with a suspension of phytopathogenic bacteria (10^6 cells/ml), placed in Petri dishes containing the nutrient MS medium, and tested for injuries after 1–14 days (depending on the pathogen type. According to the second method, the entire leaf with the injured surface of petiole only is inoculated. In each experimental variant, four leaves of each plant line were inoculated. A leaf (the third on top) of 8–10-week-old plants was kept in a suspension of examined bacteria for several seconds, dried on filter paper, transferred to Petri dish with MS medium, and incubated at 24°C during a 16-h-light day. The extent of leaf injury was determined 1–14 days after inoculation. The third method of inoculation involves three entire plants of each line growing in vitro or under conditions of closed ground. For this, plants grown in vitro or in vivo were sprayed with a suspension of phytopathogenic bacteria (10^6 cells /ml).

RESULTS AND DISCUSSION

As a gene source, we used plasmid pET21d-*cecP1*) containing the synthetic gene (*cecP1*), whose structure was inferred from the 31-mer amino acid sequence of the mature form of cecropin P1 [10]. This gene was again synthesized by PCR with the goal of flanking its ends by convenient sequences for cloning in vectors to be used in plant transformation. For this, the corresponding primers possessing *Bam*HI and *Bgl*II sites were synthesized. Analysis of nucleotide sequence of the obtained amplified gene revealed that this sequence does not differ from the original coding structure. The scheme of *cecP1* gene cloning is presented in Fig. 1. The gene was inserted into the intermediate vector pRT103 controlled by the strong constitutive promoter 35S RNA of cauliflower mosaic virus (CaMV35S). The cassette 35S-*cecP1*-polyA was then cloned into the binary vector for transformation of pGA482 plants, which was transferred to cells of *Agrobacterium tumefaciens* pGV3101 (pMP90RK). The resulting *Agrobacterium* strain was used for genetic transformation of tobacco plants.

Direct regeneration of sprouts was observed after two weeks on transformed tobacco leaf explants cultivated on a regeneration medium. Sprouts 2–3 cm in length were transferred to MS tubes (3 × 20 cm) containing antibiotics at a concentration decreased two times (25 mg/l kanamycin and 250 mg/l cefotaxim). On this medium, plants were implanted well and grown to achieve the length of 10–15 cm. These plants were used in molecular genetic analysis after the preliminary test of their tissues for the absence of agrobacteria by plating them on a bacterial medium. For further study, eight transformants (of 25 isolated) with the best growth physiological characteristics were chosen.

The presence of gene cecropin P1 in genomes of transgenic tobacco plants was confirmed in PCR (Fig. 2). The expression of cecropin P1 mRNA was evaluated by Northern blot analysis of total RNA isolated from transgenic plants of all lines obtained. Hybridization with

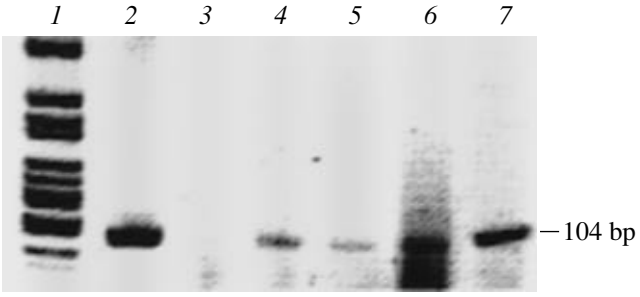

Fig. 2. PCR analysis of DNA from transgenic tobacco plants containing gene *cecP1* under control of promoter 35S RNA of cauliflower mosaic virus. (*1*) Reference DNA pBR322/*Msp*I; (*2*) DNA of plasmid containing gene *cecP1*; (*3*) DNA of nontransformed plant; (*4–7*) DNA of some lines of transgenic plants.

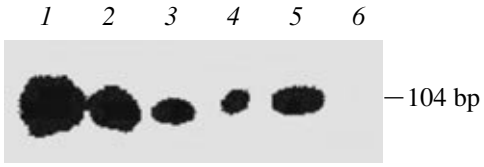

Fig. 3. Analysis of mRNA *cecP1* in transgenic tobacco plants by Northern hybridization. As a probe, labeled ^{32}P gene *cecP1* was used. (*1*) DNA containing *cecP1* gene; (*2–5*) RNA from some lines of transgenic tobacco plants containing gene *cecP1*; (*6*) RNA from nontransformed plant.

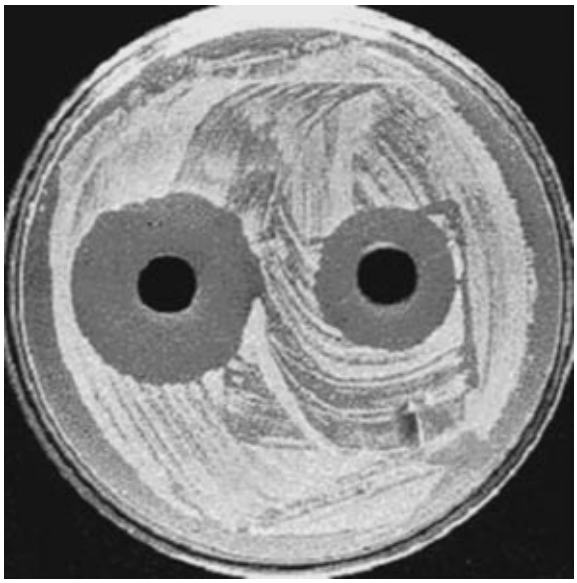

Fig. 4. Inhibitory effect of plant extracts from transgenic (left) and control (right) plants on the growth of pathogenic bacteria *Erwinia carotovora* B15.

labeled α^{32}P-dATP probe containing the coding part of gene *cecP1* demonstrated that synthesis of the corresponding mRNA occurred in all transgenic plants (Fig. 3). The difference in the intensity of mRNA signals

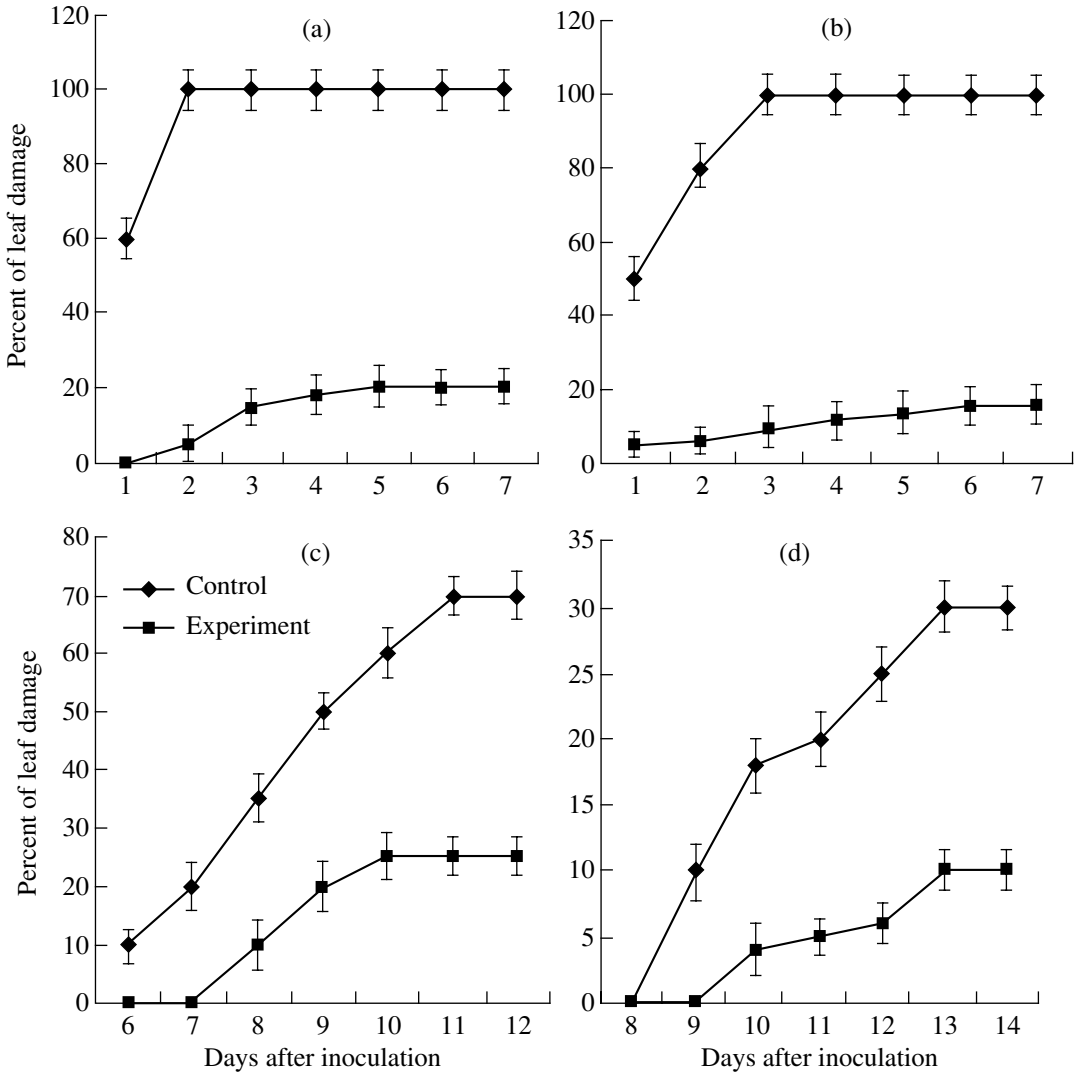

Fig. 5. Damage dynamics of leaves in control and transgenic tobacco plants inoculated with pathogenic bacteria. (a) *Erwinia carotovora* ATCC 495; (b) *E. carotovora* B15; (c) *Pseudomonas syringae*; (d) *P. marginata.* Charts represent mean arithmetic values and their standard deviations obtained in three experiments (4 biological repetitions in each of them).

between various lines of transgenic plants can be connected with varying expression of gene *cecP1* in various regions of its integration into the plant nuclear genome or with the degradation of the gene transcripts.

All 25 lines obtained in F_0 transgenic plants were placed in the greenhouse for flowering and kept there until the end of vegetation. Transgenic and control plants were virtually similar with respect to the development and growth rate, but flowers of plants transformed with a construct containing gene *cecP1* had a paler coloration than flowers of control plants (nontransformed and transformed with an "empty" vector).

We evaluated antibacterial activity of the tested extract estimating the size of the free zone on agar medium around the slot where plant extracts (2 mg of total protein) were placed. Data from one of these standard experiments are shown in Fig. 4. In parallel experiments, slots contained extracts from control plants with the addition of synthetic cecropin P1. Extracts from transgenic plants manifested much higher antibacterial activity than extracts from nontransgenic plants. This result was obtained for all bacterial strains used in this work: *Erwinia carotovora* subsp. *carotovora* B15, *Erwinia carotovora* subsp. *carotovora* ATCC 495, *Pseudomonas syringae* B-1546, and *Pseudomonas* sp. *marginata* B-1298. Some antibacterial activity expressed in extracts of control plants may be connected with the presence in these plants of endogenous defense compounds, including their own antibacterial peptides. Thus, the diameter of the free zone obtained for control extracts did not exceed 2 mm, whereas it was at least 5 mm in cases of transgenic plants (Fig. 4), which indicates enhanced antibacterial activity in plant cells expressing *cecP1* gene. The same size of pure zones was registered in both variants of

using the extracts: with the addition of cecropin P1 to extracts from control plants (34 ng) and with the addition of extracts from transgenic plants with gene *cecP1* containing 2 mg of total protein. Thus, the amount of cecropin P1 in transgenic plants was approximately 0.002% of total soluble protein in plant leaves.

Results of bioassays with plant leaves for resistance to the examined phytopathogenic bacteria are shown in Fig. 5. In all bioassays, transgenic plants with the expressed gene *cecP1* manifested greater resistance to the phytopathogens than control plants.

Bioassays for resistance in intact plants and their explants to *Erwinia* were most demonstrative. Immediately several hours after inoculation with *Erwinia*, the first signs of damage were observed on leaves of control plants in the region of the injured petiole surface. On the second day, virtually all leaf surface underwent necrosis; after a week, leaves acquired yellow color; and in a month, only a translucent cellulose skeleton was left. In leaves of transgenic plants carrying gene *cecP1*, only petiole and the adjacent portion of leaf plate were damaged. In such plants, the spread of the damaged zone was limited, and more than 80% of plant tissue retained green color during the experiment (Figs. 6a, 6b). When leaf disks were used as explants, the pathogen damaged the entire leaf disk and several hours later, leaf disks of control plants showed signs of damage, whereas leaf disks of transgenic plants carrying *cecP1* gene were mainly damaged in the region of the injured surface. Similar resistance to *Erwinia* was observed in intact transgenic plants carrying gene *cecP1*, which were grown in vitro (Figs. 6c, 6d) and under conditions of closed ground.

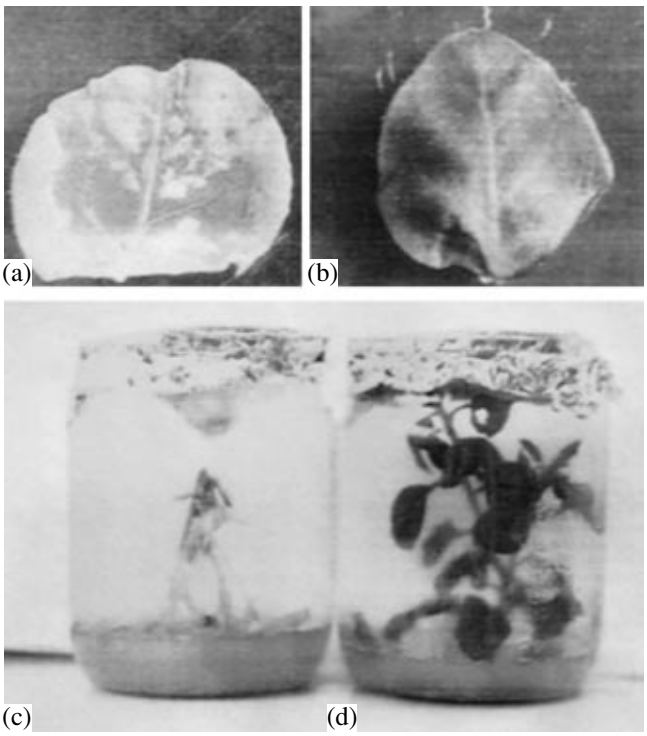

Fig. 6. Enhanced resistance of transgenic tobacco plants to phytopathogenic bacteria *Erwinia carotovora* B15. (a, b) Plant leaves on day 10 after inoculation (a) control plant: decoloration and appearance of zones of completely atrophied tissue; (b) transgenic plant with gene *cecP1*; the greatest part of leaf surface retains the original density and green color; (c, d) plants in vitro on day 10 after inoculation (c, control plant: complete turning yellow and atrophy; d, transgenic plant with gene *cecP1*: complete preservation of viability).

Rather a small size (approximately 800 bp) of the transgenic insertion in the genome, which contained not only gene *cecP1* but also selective marker *nptII*, suggests that independent segregation of these two genes is least probable. The segregation of transgenic characters was verified using the marker gene *nptII*. Seeds obtained from self-pollinated F_0 plants expressing the gene *cecP1* were transferred to MS medium with kanamycin sulfate (50 mg/l). A month later, some young plants grown from seeds manifested sensitivity to the antibiotic shown by the loss of color. The ratio of the number of kanamycin-resistant plants to the number of sensitive plants showed that the segregation with respect to this character among 25 lines of F_1 plants was 3 : 1 in four lines, which suggests the integration of one transgene copy into the nuclear genome. The values significantly exceeding Mendelian ratio 3 : 1 were obtained for the progeny of transgenic plants belonging to other lines, which points to the multiple transgene insertion. We did not use these plants in further work, since they possibly manifest the effect of transgene silencing via the mechanism of RNA interference induced by duplicated copies of transgenes [22]. Transgenic F_1 plants contained gene *cecP1* and retained enhanced resistance to the examined phytopathogenic bacteria in our bioassays (data not shown).

Transgenic plants capable of expressing mainly natural genes of antimicrobial peptides, insect defensins and cecropins, were obtained in [5, 7]. The contradictory data on the defense effect of examined peptides and their derivatives are explained by varying rates of their degradation with endogenous peptidases in cells of various plant species and cultivars [23]. The notion that some structures of antimicrobial peptides may be rather stable in plant cells cannot be excluded. We were the first to develop transgenic tobacco plants expressing the synthetic gene of cecropin P1 and to demonstrate substantial resistance of such plants to the examined phytopathogenic bacteria. The significant defense effect of the synthetic gene *cecP1* towards various phytopathogenic bacteria observed in this work possibly is connected with its structural features. Thus, in contrast to cecropins of insects, mammalian cecropin P1 can form one long α-helix involving virtually all amino acid residues [24].

The transgenic F_0 and F_1 plants with gene *cecP1* had less intense coloration of flowers, compared to flowers

of control plants. The reason for this phenomenon is still unknown. Note that transgenic plants with unexpected changes in flower color often manifest male sterility or nonviability of their seeds [25, 26]. However, in transgenic plants with the synthetic gene *cecP1* constructed in this work, we did not observe the correlation between pale coloration of flowers and male sterility: these plants retained the ability to produce normal viable seeds. In addition, it should be noted that gene *cecP1* responsible for resistance to phytopathogenic bacteria in transgenic plants is promising as a direct selective marker upon screening the corresponding transformants.

The obtained constructs with the synthetic *cecP1* gene can be further used in studies on the effect of this gene in various transformed plants.

ACKNOWLEDGMENTS

The authors are grateful to L.I. Evtushenko and S.V. Chernyshov for providing strains of pathogenic microorganisms.

This study was supported by the Program of the Presidium of Russian Academy of Sciences "Dynamics of Plant, Animal, and Human Gene Pools" and Russian Foundation for Basic Research (projects no. 04-04-48648 and Naukograd no. 04-04-97259).

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