ARTIFICIAL ASSOCIATIVE SYMBIOSES BETWEEN TOMATO PLANTS AND FUNGISTATIC *Rhizobium*

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Abstract

Biomethods in plant protection against pests and diseases considered the most prospective alternative to chemicals which pollute soil and water causing concern about public health. The possibility of creating an artificial association of nodule bacteria with plants to protect them from the adverse effects of pathogenic fungi can be realized using one of the specific mechanism of nodule bacteria attachment to the roots of leguminous plants by plant lectins, able to recognize and specifically bind with different carbohydrates, particularly polysaccharides of rhizobia cell wall. In our study we used the composite plants of tomato (*Lycopersicon esculentum*) Dubok variety and bacterial strains associated with the roots of wild legumes from the collection of *Institute of Biochemistry and Genetics* (IBG USC RAS). «Hairy rooted» tomato plants were obtained by treatment with *Agrobacterium rhizogenes* ATCC 15834, containing vehicle gene construction pCambia 1305.1 with inserted pea lectin gene *psl* under cauliflower mosaic virus 35S promoter. The antagonistic activity of bacteria towards pathogens was tested by dual culture study. The ability of microorganisms to produce siderophores and cyanide was analyzed. Few isolates were identified by sequencing of the 16S rRNA gene fragments. By screening of the collection of isolates from nodules of wild legume from tribe *Viceae* the candidate strains were detected, particularly *Rhizobium leguminosarum*, *Pseudomonas* sp. and *Stenotrophomonas rhizophila*, with fungistatic activity against *Fusarium solani*, *F. oxysporum*, *Fusarium* sp. and *F. oxysporum* f. sp. *lycopersici*. Production of siderophores was detected in two members of *Pseudomonas* genus, *S. rhizophila* and *R. leguminosarum*. Two *Pseudomonas* strains, the 14M and 103, and *S. rhizophila* were shown to produce cyanide. It was also found that treatment of roots transgenic by *psl* gene with *R. leguminosarum* 116 strain reduced the amount of hyphae of the pathogen *F. oxysporum* f. sp. *lycopersici* in the rhizosphere of tomato plant that could potentially contribute to plant defense against pathogenic fungi. Thus, the use of lectins as transgenes in roots allows us to obtain artificial association with rhizobia in non-symbiotic plants such as tomato, which in combination with the use of microorganisms possessing fungistatic activity can more effectively protect the plant root system against pathogens.

Keywords: rhizobia, phytopathogenic fungi, transgenic plants, lectins, associative symbiosis.

Phytopathogens in the agricultural industry are usually controlled using various pesticides, which leads to soil and water pollution. Moreover, accumulation of such compounds in plants is hazardous to human health. Biological methods of plant protection provide an alternative to chemical techniques. They include the use of the soil microorganisms exhibiting the protective effect, most of which belong to the genera *Bacillus* and *Pseudomonas*. The strains capable of inhibiting the growth of fungi were also found among bacteria of the family *Rhizobiaceae* [1-3], which is associated with various protection mechanisms, such as synthesis of toxic substances [4] and cyanide [5], induced systematic resistance of plants [6], release of siderophores [7] and some others [8].

Although (with rare exception) *Rhizobiaceae* are able to enter into endosymbiosis only with legumes, there are studies revealing their potential as associative microsymbionts for nonleguminous crops [9], including the tomato [10,

11]. The colonization of nonleguminous plant roots by rhizobia is improved by various methods, including the use of the transgenic plants that synthesize the substances involved in signaling at early stages of legume-rhizobia symbiosis. Such substances include lectins, the secreted proteins that can recognize and selectively bind to various carbohydrates [12], in particular, to polysaccharides on rhizobium cell walls, which provides the fixation of microorganisms on the root surface [13]. Earlier, several groups of scientists carried out works to change symbiosis specificity using lectins of leguminous plants [14-16]. Bacteria were found both on the external surface of roots and in intra- and intercellular space. Thus, the roots of the plants transgenic for lectin genes, potentially, may be specifically colonized only by the rhizobia which perform the functions useful for plants, for example, protection against phytopathogens.

Soil bacteria *Agrobacterium rhizogenes* carrying Ri-plasmids can cause the formation of transgenic «hairy roots» in many dicotyledon species. «Composite» plants with such roots, which carry target genes, are used in order to study plant interactions with microorganisms, fungi and nematodes [17]. In particular, the «hairy roots» of tomatoes, where a pea lectin gene is expressed [18], may become a good model to study the possibility of plant protection against pathogens through improvement of the efficiency of associative symbiosis with rhizobia.

The purpose of this work was to investigate the possibility of creating artificial targeted associations of bacteria with the roots of agricultural non-symbiotrophic plants in order to protect them against phytopathogenic fungi by the example of the tomato (*Lycopersicon esculentum*) and nodule bacteria *Rhizobium leguminosarum*.

Technique. The object of study was represented by the tomato (*Lycopersicon esculentum*) of variety Dubok. Plants were transformed using strain *Agrobacterium rhizogenes* ATCC 15834 taken from the collection of the All-Russia Research Institute for Agricultural Microbiology (St. Petersburg) with previously introduced vector pCambia 1305.1, where field pea lectin gene *psl* was incorporated under the control of 35S promoter of cauliflower mosaic virus [19]. This work also included the use of the strains of the bacteria associated with the roots of wild-growing legumes in the territory of the Republic of Bashkortostan (collection of the Institute of Biochemistry and Genetics of Ufa Scientific Centre of Russian Academy of Sciences).

Composite tomato plants were obtained using the method described by R. Collier et al. [17]. The seed surface was sterilized for 2 min in 70% alcohol and then for 15 min in 15% sodium hypochlorite solution with addition of several drops of Tween 20. The plant transformation experiment involved the use of 2-day cultures of *A. rhizogenes* (pCambia 1305.1-*psl*) and *A. rhizogenes* (initial strain) grown at 28 °C in TY liquid medium (0.1% yeastrel, 1.0% bactotryptone, 0.1% CaCl₂) with addition of kanamycin (100 mg/l) and acetocyringone (200 µM) in the first case and only acetocyringone in the second case.

Prior to relocation of plants with «hairy roots» into substrate (sterilized mixture of soil and sand) and 1 week after the relocation, the roots were histochemically analyzed for GUS activity [20]. Fragments were incubated in X-Gluc reagent containing 5-bromo-4-chloro-3-indolyl-β-D-glucoronide (1 mg/ml), 0.5% Triton X-100, Na₂EDTA (100 mM), methanol (20%), K₃Fe(CN)₆ (0.5 mM), K₄Fe(CN)₆ (0.5 mM) and Na-phosphate buffer (50 mM, pH 7.0) (Sigma-Aldrich, USA). The roots were held at 37 °C over night, and blue staining was detected.

In PCR analysis, DNA was extracted from «hairy roots» using the phenol-chloroform method. Lectin gene *psl* was detected in preparations using the primers (5'-ATAATGGCTTCTCTTCAA-ACCC-3' and 5'-GCAAAAAAACT-ATGCATCTGCA-3') flanking the site of this gene, as well as standard kits of reagents for amplification (Helicon, Russia). The PCR was carried out in a Tertsik MS2 amplifier (DNA-Technology, Russia) as per a protocol according to manufacturer's recommendations at optimal annealing temperature for the mentioned pair of primers. The positive control was represented by plasmid pCambia 1305.1-*psl*.

In order to identify symbiotic bacteria with fungistatic activity, isolates were screened using the test cultures of fungi *Fusarium solani*, *F. oxysporum* and *Fusarium* sp. from the collection of the Ufa State Petroleum Technological University, as well as *F. oxysporum* f. sp. *lycopersici* (F-140) from the All-Russian Collection of Microorganisms (G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms of Russian Academy of Sciences, Pushchino). The fungistatic activity of microorganisms was assessed using the dual-culture method. Bacteria were introduced in a cross pattern in the center of a Petri dish, dividing it into four sectors. A piece of agarized growth medium with fungus mycelium was put in the middle of each sector. The dishes were placed into a thermostat and incubated at 27 °C. After 3 days, fungal colony radius was measured in directions towards bacteria (R_1) and dish edge (R_2), and a degree of fungal growth inhibition was calculated by the formula [21]:

$$T = (R_2 - R_1)/R_2 \times 100 \text{ \%}.$$

The specific identity of the bacteria was determined based on the analysis of 16S rRNA gene sequence [22].

When bacterial strains were checked for siderophore synthesis, minimal medium with CAS reagent (blue agar) was prepared as described [23]. The bacteria grown on YM medium were relocated to blue agar and grown within 5 days. The change of agar color to yellow, orange or pink was indicative of siderophore release. In order to reveal cyanide synthesis, bacteria were grown in Petri dishes within 1 day on YM medium with addition of glycine (4.4 g/l). Then, filter paper impregnated with 0.5 % aqueous solution of picric acid with 2 % Na_2CO_3 was put on the covers of the Petri dishes. The dishes were wrapped with Parafilm and incubated for 4 days at 28 °C. In case of cyanide release, paper color changed from yellow to orange or brown due to picrate formation [24].

For joint inoculation of plants by bacteria and fungi, suspension of spores of *F. oxysporum* f. sp. *lycopersici* was obtained. The fungus was grown in a Petri dish with YM medium for 5 days. Then, it was poured with 20 ml of sterile water and put to a refrigerator over night. The number of washed-off spores was counted in a Goryaev chamber. *R. leguminosarum* bacteria were accumulated at 28 °C within 1 day in YM liquid medium to titer of 10^7 CFU/ml. Plant roots were placed into the obtained bacterial suspension for 1 day. Then, they were washed out in sterile water; the plants were relocated to soil containing 10 ml of fungus spore suspension (10^5 pcs/ml) and grown within 3 days. After that, the plant roots were washed out and stained with toluidine blue for 1 hr (fungus hyphae changed color to violet, and plant cells to blue), and then washed out again in citrate buffer [25] and examined using an Axio Imager M1 microscope (Carl Zeiss AG, Germany).

Results. After plant treatment with *A. rhizogenes*, «hairy roots» began to form after 10-12 days on 90 % of plants (Fig. 1). Adventitious roots emerged during the first week; they were removed using a scalpel.

Fig. 1. Composite tomato (*Lycopersicon esculentum*) plants of variety Dubok after transformation with *Agrobacterium rhizogenes* ATCC 15834 carrying pCambia 1305.1 with incorporated field pea lectin gene *psl* under the control of 35S promoter of cauliflower mosaic virus: A — plants in mineral wool, B — «hairy roots» after 10-day growth, C — «hairy roots» after 3-week growth.

Fig 2. The PCR analysis of the field pea lectin gene *psl* in DNA from the «hairy roots» of composite tomato (*Lycopersicon esculentum*) plants of variety Dubok, obtained using pCambia 1305.1: 1 — «hairy roots», 2 — pCambia 1305.1; M — molecular weight marker 100 bp + 1.5 Kb + 3 Kb (Sibenzyme, Russia).

After 2 weeks, the roots were checked for GUS activity, and the plants were relocated to sterile mixture of soil and sand and grown for 1 more week. GUS-stained roots were found in 53% of the plants. The PCR analysis of DNA from these roots revealed the presence of the lectin gene (Fig. 2).

Earlier, we obtained completely transgenic tobacco plants, as well as the chimeric rape and tomato plants expressing the pea lectin gene [26-28]. On such plants, we found the number of *R. leguminosarum* 1078 bacteria increased 37-, 14- and 10-fold, respectively, as compared to the non-transgenic plants. This fact gave evidence of interaction of rhizobia with lectin on the surface of transgenic roots. The colonization of roots by bacteria with fungistatic activity with the increase in the number of the latter ones could potentially protect plants against pathogens. In nature, the plants belonging to the tribe *Vicea* are the most frequent symbionts with *R. leguminosarum*. Because rhizobia with fungistatic activity were found earlier in the nodules

of some wild-growing plants [29], we have screened the bacterium collection obtained on the wild vegetation belonging to the mentioned tribe [30] in order to reveal strains with such properties. A total of 568 isolates were investigated. Fungistatic activity with regard to the studied fungi was found in seven strains (Table).

The coefficient of colony growth retardation (T, %) for *Fusarium* fungi in the presence of natural isolates of bacterial symbionts of *Vicea* wild plants

Microsymbiont	<i>Fusarium</i> sp.	<i>F. oxysporum</i> f. sp. <i>lycopersici</i>	<i>F. solani</i>	<i>F. oxysporum</i>
<i>Rhizobium leguminosarum</i> 116	17	41	65	70
<i>Pseudomonas</i> sp. 2	0	0	25	0
<i>Pseudomonas</i> sp. 102	40	29	53	53
<i>Pseudomonas</i> sp. 103	0	21	22	33
<i>Pseudomonas</i> sp. 15.2	0	13	17	19
<i>Pseudomonas</i> sp. 14M	0	25	19	25
<i>Stenotrophomonas rhizophila</i>	40	0	33	18

The specific identity of bacterial strains was confirmed by the analysis of

16S rRNA gene sequence (data are not presented).

The fungistatic activity of the bacteria may be caused by secretion of siderophores, the proteins capable of forming a complex with iron ions, making them inaccessible to fungi; also, some rhizobium strains release hydrogen cyanide (HCN), which has a negative impact on fungal growth [2, 5]. The microsymbionts studied by us had the following ability to synthesize these fungistatic metabolites (siderophores/cyanides): *Rhizobium leguminosarum* 116 — «+»/«-», *Pseudomonas* sp. 2 — «+»/«-», *Pseudomonas* sp. 102 — «+»/«-», *Pseudomonas* sp. 103 — «-»/«+», *Pseudomonas* sp. 14M — «-»/«+», *Stenotrophomonas rhizophila* — «+»/«+». I.e., among the studied strains, the ability to synthesize siderophores was found in two representatives of the genus *Pseudomonas*, *S. rhizophila* and *R. leguminosarum*, and cyanide was released by two strains of *Pseudomonas* (14M and 103) and *S. rhizophila*. Thus, strains with the highest fungistatic activity (*R. leguminosarum* 116 and *Pseudomonas* sp. 102) synthesized siderophores, but not cyanide. However, it should be noted that parameters for *S. rhizophila* secreting both substances were not the highest. It is possible that the fungistatic activity of the strains is associated with any other mechanisms (for example, with synthesis of antibiotics).

Strain *R. leguminosarum* 116 with the highest fungistatic activity with regard to *F. oxysporum* f. sp. *lycopersici*, which was found as a result of the studies, was used as a microsymbiont in further experiments to create artificial symbiotic associations.

Fig. 3. The joint treatment of tomato (*Lycopersicon esculentum*) plants of variety Dubok with bacterial symbionts exhibiting fungistatic properties (*Rhizobium leguminosarum*) and pathogenic fungi (*Fusarium oxysporum* f. sp. *lycopersici*): a — non-transgenic plants + *F. oxysporum* f. sp. *lycopersici*; b — plants with the roots transgenic for field pea lectin gene *psl* + *F. oxysporum* f. sp. *lycopersici*; c — non-transgenic plants + *F. oxysporum* f. sp. *lycopersici* + *R. leguminosarum*; d — plants with the roots transgenic for lectin gene *psl* + *F. oxysporum* f. sp. *lycopersici* + *R. leguminosarum*; e — noninfected non-transgenic plants (optical microscopy, Axio Imager M1, Carl Zeiss AG, Germany; staining with toluidine blue).

After inoculation of the composite tomato plants having the roots with the lectin transgene, with non-transgenic plants as a control, by the suspension of strain *R. leguminosarum* 116 and their relocation to the soil containing the spores of fungus *F. oxysporum* f. sp. *lycopersici*, optical microscopy examination confirmed (Fig. 3, a-e) that the treatment of the roots transgenic for gene *psl* with strain *R. leguminosarum* 116 reduces the number of the hyphae of pathogen *F. oxysporum* f. sp. *lycopersici* in the rhizosphere (see Fig. 3, d). The same effect, but to a far less extent, was observed for control plants with less effective rhizobium adsorption on their roots (see Fig. 3, c).

Earlier, it was shown in a number of studies that *Bradyrhizobium japonicum*, *Sinorhizobium meliloti* and *R. leguminosarum* are able to retard *F. solani*

growth in experiments with the sunflower and okra [1], and *S. meliloti* and *R. trifolii* can be used for biocontrol of *F. oxysporum* infecting the sunflower and tomato [31].

So, the use of legume lectin genes as transgenes for non-symbiotic plants, such as the tomato, makes it possible to create artificial root associations with the rhizobia exhibiting fungistatic activity. The obtained results can find application in creation of artificial associative symbioses for biocontrol of phytopathogens.

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