

The *Brassica napus* L. plants expressing antimicrobial peptide cecropin P1 are safe for colonization by beneficial associative microorganisms

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ABSTRACT

This work was aimed to study the opportunity of transgenic oilseed rape (*Brassica napus* L.), expressing the gene of antimicrobial peptide cecropin P1 (*cecP1*) to be inhabited with associative microorganisms *Methylobacterium mesophilicum* and *Pseudomonas aureofaciens*. Previously, resistance was demonstrated to the microbial pathogens: *Erwinia carotovora* B15, *Pseudomonas syringae*, *Fusarium oxysporum*, and *Botrytis cinerea*. Analysis of the fatty acid composition of transgenic plant seeds showed an increase in the proportion of unsaturated fatty acids. Plants studied showed increased photosynthetic activity in oxidative stress induced by paraquat instead of in oxidative stress and UV radiation compared to control ones. A study of the interaction of transgenic plants with the associative microorganisms showed that bacteria were located in all initially colonized plants, as well as in plants obtained after several passages of microproliferation. Accordingly, transgenic plants, containing the *cecP1* gene were actively colonized by associative bacteria, indicating their safety for associative bacteria, indicating their safety for beneficial microorganisms.

Abbreviations: AMP: antimicrobial peptide; *cecP1*: cecropin P1; CFU: colony-forming units; SOD: superoxide dismutase

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Introduction

In nature, plants exist in tight association with various colonizing endophytic bacteria inhabiting internal plant tissues, thus forming stable symbiotic communities assuring mutual benefits. Symbiotic relations occur within the intercellular spaces of plant tissues, which contain high levels of carbohydrates, amino acids, and other nutrients. Such bacteria can stimulate plant growth, develop photosynthetic activity, even resistance to unfavorable conditions, for better sustainable agriculture (Grover et al. 2011).

Some associative microorganisms supply plant with basic nutrients contribute the efficient consumption of minerals, examples include *R. leguminosarum* known for nitrogen fixation (Fischer 1994), *Azospirillum spp.* or *Rhizobia* for phosphate ions acquisition (Rodriguez et al. 2004; Qin et al. 2011), or *Pseudomonas sp.* supplementing the Fe³⁺ ions (Jin et al. 2006) by *Paenibacillus polymyxa* (Timmusk et al. 1999). Along with substances, such as cytokinins and gibberellins could be supplied or indoleacetic acid produced by *Pseudomonas putida* (Patten and Glick 2002). *Herbaspirillum frisingense* promotes root growth through ethylene signaling (Straub et al. 2013). Also, different processes of plant protection against phytopathogens with bacteria, including earthier niche occupation with *Sphingomonas sp.* and direct competition (Bacon and Hinton 2006; Innerebner et al. 2011) or action of antibiotics and lytic enzymes of

endophytic bacteria origin (Mazurier et al. 2009). Still, the actual mechanisms by which bacteria can influence plant growth differ among species and strains, so typically, there is no single way for promoting plant growth (Glick 2012; Kandel et al. 2017).

Earlier, we reported the production of transgenic plants expressing antimicrobial peptide cecropin P1 gene that resulted in increased resistance against certain pathogens and stress factors (Zakharchenko et al. 2013). Also, we successfully demonstrated diverse positive effects of tomato, sugar beet, white cabbage plants colonization with different symbiotic bacteria (Pigoleva et al. 2009, 2020).

Oilseed rape (*Brassica napus* L.) is an important oilseed and fodder crop (Peng et al. 2018; Hegelian et al. 2018), producing valuable biotechnological products (Ravanfar et al. 2017). Many transgenic herbicide-resistant *Brassica napus* plants (Nishizawa et al. 2016; Zhang et al. 2018), and plants resistant to insects (Wang et al. 2005), nematodes (Zhong et al. 2019), and certain diseases (Larkan et al. 2013; Ziaei et al. 2016; Aghazadeh et al. 2016) are also known.

One of the methods of increasing plant resistance to pathogens is the transformation of the plant by genes of antimicrobial peptides (AMPs) with a broad spectrum of antibiotic and fungicidal activity. This is a promising approach in plant genetic engineering (Kamo et al. 2015). AMPs are an essential supplement to the innate immune system of all multicellular organisms and have a wide range of bactericidal

and fungicidal activities (Choi et al. 2012). Antimicrobial peptides cause lysis of bacteria and fungal cells by disrupting the integrity of membranes or increasing their permeability. At a concentration of 0.1–5 μM , they exhibit lytic activity against various microorganisms (Mills and Hammerschlag 1993). The insect defensin sarcotoxin expressed in tobacco provides protection against *Pseudomonas syringae* pv. tabaci (Ohshima et al. 1999). The Rs-AFP2 radish defensin was expressed in tobacco and tomato, protecting them against *Alternaria longipes* (Terras et al. 1995). Studies of transgenic rape plants with heterologous gene DRR206 demonstrated resistance to fungus *Leptosphaeria maculans* (Wang and Fristensky 2001; Larkan et al. 2013). The cecropin A gene expressed in rice protected it against *Magneaporthe grisea*, *Fusarium verticillioides*, and *Dickeya dadantii* (Coca et al. 2006; Bundo et al. 2014). Mature cecropin P1 (cecP1), an antimicrobial peptide isolated from nematodes, consists of 31 amino acid residues that demonstrated high activity against various bacteria *Pseudomonas aeruginosa*, *Acinetobacter calcoaceticus*, *Salmonella typhimurium*, *Streptococcus pyogenes* (Pillai et al. 2005), and fungi *Sclerotinia sclerotiorum*, *Phytophthora infestans* (Zakharchenko et al. 2007). The artificial *cecP1* gene expressed in a cell-free system was active against *Escherichia coli* (Martemyanov et al. 1997). The expression of AMP in plants provided not only the resistance to pathogens, but also mitigated the oxidative stress (Goyal et al. 2013). Thus, broadening the spectrum of antimicrobial peptides expressed in plants can be an effective approach in plant biotechnology.

While the expression of AMPs has been a promising strategy, it is important to make sure that AMPs do not at the same time compromise symbiotic relationships with beneficial associative microorganisms.

The goal of the present work was to study the physiological and biochemical peculiarities of interaction between transgenic oilseed rape plants expressing cecP1 and associative bacteria.

Material and methods

Plant material

The oilseed rape plant cultivar Galant used was obtained from V.S. Pustovoit All-Russian Research Institute of Oil Crops, Russia. The plants we produced earlier (Zakharchenko et al. 2005), were transformed with agrobacterial vector pGA482::cecP1 carrying the artificial gene of mature form of antimicrobial peptide cecropin P1 (NCBI accession number BAD89085.1 positions from 14 to 44) under control of 35S RNA promoter of cauliflower mosaic virus and selective kanamycin resistance *nptII* gene (Figure 1). In vitro plants were cultivated on a standard MS medium (Murashige and Skoog 1962) with 25 mg/l of kanamycin sulfate. Plants of six previously produced *cecP1* transgenic lines were grown in a greenhouse. Two homozygous lines were selected for subsequent study. Sterilized seeds of homozygous plants were grown in vitro on hormone-free MS media. Part of the rooted plants was further cultivated in a greenhouse to get seeds. Transgenic seedlings of F_1 progeny were self-pollinated for two generations to produce F_3 homozygous plants with one copy of the *nptII-cecP1* transgenes for further study.

Bacterial material

The experiments were carried out using the following phytopathogenic bacterial strains: *Erwinia carotovora* subsp. *carotovora* B15, from Horticulture Centre (Canada), *Pseudomonas syringae*, and the fungal phytopathogens *Fusarium oxysporum* f.sp. *lycopersici* and *Botrytis cinerea*, received from All-Russian Collection of Microorganisms (Pushchino, Moscow Region). The bacteria were grown on LB medium (Maniatis et al. 1982), and the fungal strains were grown on glucose medium containing 2.0 g/l NH_4NO_3 ; 20.0 g/l glucose; 0.1 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 3.0 g/l sucrose; 1.0 g/l KH_2PO_4 ; 1.0 g/l NaOH; 20.0 g/l agar in the dark for 3–4 days at 22–24°C (Fan et al. 2013). The following strains were used in colonization experiments: *Methylobacterium mesophilicum* Psm 140, kindly provided by A.A. Shirokikh, Rudnitsky Zonal North-East Agricultural Research Institute, Russian Academy of Agricultural Sciences (Shirokikh et al. 2005), and *Pseudomonas aureofaciens* BS1393(pBS216) (Anokhina et al. 2006) received from the Laboratory of Plasmids Biology, G.K. Skryabin Institute of Biochemistry and Physiology of Microorganisms, RAS. *M. mesophilicum* was grown at 37°C up to $\text{OD}_{600} = 1.5$ on the orbital shaker in K medium containing 2.0 g/l KH_2PO_4 ; 2.0 g/l $(\text{NH}_4)_2\text{SO}_4$; 0.5 g/l NaCl; 0.125 g/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$; 0.002 g/l FeSO_4 ; supplemented with 1% CH_3OH as a source of carbon and energy. *P. aureofaciens* was grown up to $\text{OD}_{660} = 1.0$ at 28°C on the shaker in LB medium.

Determination of copy number and localization of the cecP1 gene insertion

In the inverse PCR (Stefano et al. 2016), the determination of transgene copy numbers is based on the number of amplicons, obtained by amplification of plant gDNA, that was subjected to restriction digestion and self-circulation using ligase. Unlike conventional PCR, the primers for inverse PCR are directed ‘outward’ (Figure 1) to amplify sequences that flanks transgene insert, that was circularized by ligation. If there are several copies of the transgene in the plant genome, then several bands will be observed. This will happen due to the fact that the restriction site will be located at different distances from the transgene in different inserts. Nested reverse PCR was used in the work to overcome the low yield of PCR product.

Genomic DNA from transgenic and non-transgenic oilseed rape plants was isolated from young leaves by the method of Suman et al. (1999). For each experiment, 400 ng of genomic DNA was digested separately with restriction endonucleases *FspBI*, *AluI*, and *TaqI* (Thermo Fisher Scientific, USA) and digestion products were purified using GeneJET PCR Purification Kit (Thermo Fisher Scientific, USA), according to the manufacturer’s recommendations. The ligation of purified digested products with the formation of circular DNA molecules was carried out in 800 μl of reaction mixture containing 32 units of T4 DNA ligase (Thermo Fisher Scientific, USA) for 2 h at 22°C. The ligation products were purified and concentrated as described above. Subsequent reverse-PCR amplification was performed in 20 μl in $(\text{NH}_4)_2\text{SO}_4$ buffer using 50 ng of circular DNA molecules as a template, with 1 U of Taq DNA polymerase LC (Thermo Fisher Scientific, USA), 1.5 mM MgCl_2 , 100 μM of each dNTP, and 0.5 μM of each primer. The following primers

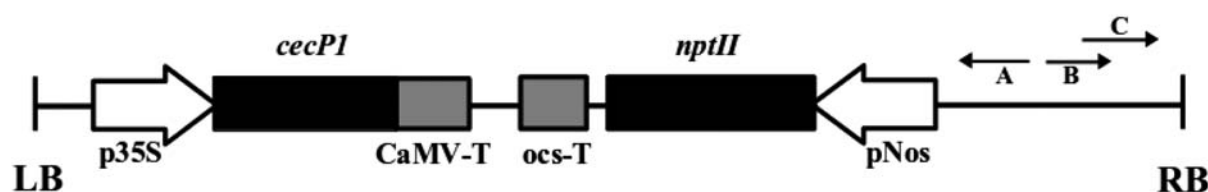


Figure 1. Structure of expression cassette of the plasmid pGA482 containing *cecP1* gene. p35S – promoter of cauliflower mosaic virus 35S RNA; pNos – promoter of nopaline synthase gene; CaMV-T and ocs-T – signals of polyadenylation of 35S cauliflower mosaic virus RNA and octopine synthase genes accordingly; *cecP1* – cecropin P1 gene; *nptII* – neomycin phosphotransferase II gene; LB, RB – left and right borders of T-DNA. Arrows show the location of the primary (A, B) and nested (A, C) primers for determination copy number of the insert.

were used: 5'-cggtgaaggagccactcagccg-3' (A) and 5'-tgcggttctgtcagttccaaacgtaa-3' (B). The reaction was conducted in thermal cycler TC-24/H, as follows: pre-denaturation (95°C, 2 min); 35 cycles: denaturation (95°C, 30 s), annealing (61°C, 30 s), elongation (72°C, 1.5 min); and final elongation (72°C, 5 min). PCR products were then diluted 1000 times and used for a semi-nested PCR as described above with primers 5'-cggtgaaggagccactcagccg-3' (A) and 5'-gttccaaacgtaa-3' (C), a number of cycles were 25 (Supplementary material). All primers were synthesized by 'Eurogen' (Moscow, Russia). Final PCR products were separated by electrophoresis in 1.3% agarose gel in 1×Tris-acetate buffer. For the sequencing procedure, resulting DNA fragments were isolated from the gel using a Gel DNA Recovery Kit (Zymo Research, USA).

Evaluation of resistance of detached leaves and whole plants to phytopathogens

Non-transformed and *cecP1* rape plants were grown from sterile seeds cultivated in vitro for three weeks. Leaf petioles were infected with *E. carotovora* and *P. syringae* suspension (10^3 – 10^5 CFU/ml) or with mycelia of the fungi *F. oxysporum* and *B. cinerea*, then propagated on solid MS media at 24°C under a 16-h light-day photoperiod. The degree of damage was assessed after 1–14 days. Four leaves were infected for each experiment. The whole plants were infected into the internodes using a syringe needle moistened with the bacterial suspension. For fungal infection, a small piece of agar with mycelium was placed on axillary buds. Three plants were infected for each experiment.

Analysis of the antimicrobial activity of plant extracts

The antimicrobial activity of extracts from transgenic plants was determined by inhibition of the growth of plant pathogenic *Erwinia carotovora* B15, *Pseudomonas syringae*, and nonpathogenic *Pseudomonas aureofaciens* BS1393(pBS216), *Methylobacterium mesophilicum* Psm 140 bacteria using the agar diffusion method (Ohshima et al. 1999). The protein content in plant extracts was determined by the Bradford method (Bradford 1976). Bacterial suspensions ($100\ \mu\text{l}$, 10^5 CFU/ml) were applied on the surfaces of 14-cm Petri plates with 1.5% agar media. Wells with a diameter of 5 mm and a depth of 10 mm were pierced in the agar, and aliquots of plant extracts containing 500 μg of soluble protein were placed into the wells. The plates were incubated at 4°C for 8 h to provide the diffusion of extracts into agar, then the temperature was elevated to 25°C, and the incubation continued for 24 h. At the end of the incubation, the antibiotic

activity of the plant extracts was determined by the size of sterile zones around the wells.

Extraction of fatty acids and analysis of their content and composition in oilseed rape seeds

The conversion of triglycerides and phospholipids of the plant seeds to methyl ethers of the corresponding fatty acids was performed by the method of non-extraction methylation with sodium methoxide and boron trifluoride in methanol (Griffiths et al. 2010). Gas chromatography system VARIAN 3900 with the following process parameters was used: column 15 m × 0.2 mm × 0.2 μm with the polar phase of Supelkovaks-10; helium as a carrier gas (1.8 ml/min); sample input by dividing the gas flow in the ratio of 1:65; volume of injected liquid sample was 2.0 μl . The gas chromatograph was programmed for an initial temperature of 100°C for 0.5 min followed by an increase of 10°C/min to 245°C and maintained for further 5 min; temperatures of the evaporator and flame ionization detector were 260°C and 255°C, respectively (Zakharchenko et al. 2019).

UV irradiation

Plants were exposed to UV light (wavelength range 280–380 nm with maximum at 315 nm) for 30 min using lamps LE-30-1 (Russia). The intensity of UV radiation on the leaf surface was 12 W/m².

Gas exchange and transpiration studies

CO₂ exchange and transpiration were measured with a portable IR gas analyzer LCPro+ (ADC BioScientific, United Kingdom) connected to a leaf chamber (working area 6.25 cm²) supplied with the analyzer. The measurements were taken on leaves exposed to UV radiation after 24 h post-infection by *E. carotovora* pathogen.

Analysis of physiological effects of oxidative stress

To determine the plant resistance to oxidative stress, leaf discs (1 cm diameter) of non-transgenic and transgenic plants were incubated in 5 μM paraquat water solution or distilled water (control). Initially, the discs were kept in the dark for 1 h and then exposed to light for 20 h. The endogenous hydrogen peroxide was determined spectrophotometrically (Bellincampi et al. 2000). The supernatant absorption was measured at 560 nm. The changes in the rate of formazan production were detected spectrophotometrically for 5 min at 560 nm (Chaitanya and

Naithani 1994). SOD was measured using nitroblue tetrazolium (Beauchamp and Fridovich 1971). One unit of the SOD activity corresponded to the amount of the enzyme causing 50% inhibition of the photochemical reduction of NBT. The chlorophyll content was determined spectrophotometrically in 96% ethanol (Wintermans and Motts 1965).

Plant colonization by associative bacteria

Sterile one-month-old leaves of non-transgenic and transgenic oilseed rape plants were sprayed with cell suspension (10^3 – 10^5 CFU/ml) of *M. mesophilicum* Psm 140 and *P. aureofaciens* BS1393(pBS216) bacteria, placed into glass tubes with MS agar medium, and cultured in glass tubes at 22–24°C, 2 klx illuminance in a 16-h daylight photoperiod. After being rooted, bacteria-colonized plants were grown in the greenhouse. The presence of associative bacteria in tissues of the colonized plant was verified by method (Kandel et al. 2017). Roots, stems, or leaves were homogenized. Diluted homogenate was applied to the surface of solid medium LB or K in dishes and incubated at 22–24°C. After 2 days the number of colony-forming units (CFU) per 1 cm² was counted.

Resistance of colonized plants to naphthalene

Naphthalene (Chimmed, Russia) was dissolved in 96% ethanol (initial concentration, 100 mg/ml). Molten agarized MS medium was supplemented with naphthalene (1 mg/ml). Newly grafted plants were transferred into test tubes, and plant growth was observed for a month.

Biometric studies

The effects of associative bacteria on the growth and development of oilseed rape plants and on their adaptation to greenhouse conditions were determined in colonized plants transferred from sterile conditions to the greenhouse of the artificial climate station Biotron (Branch of the Institute of Bioorganic Chemistry, Russian Academy of Sciences, Pushchino, Russia). Biometrical measure the onset of phenological phases. The following parameters and phenological phases were monitored: roots length, plants height, the blooming of the first flowers, the appearance of pods.

Statistical analysis

The statistical evaluation of the data was performed using the Statistica 6.0 and Microsoft Excel 2007 software. The measurements were carried out in three analytical and biological replicates. The graphs and diagrams contain mean values and their standard deviations. The significance of differences between the values was assessed by the nonparametric Mann–Whitney U test.

Results

Previously, we obtained transgenic oilseed rape plants with the gene of the antimicrobial peptide cecropin P1 and carried out molecular genetic analysis of the plants received (Zakharchenko et al. 2013). In this paper, we conducted a

physiological and biochemical analysis of transgenic plants and studied their safety for beneficial associative bacteria.

Copy number analysis and localization of cecropin P1 gene in rape plant genome

The T-DNA copy number analysis demonstrated the presence of one single copy in the line studied since only one band of amplification product appears on a gel for all restriction enzymes used (Figure 2). Subsequent sequence analysis of all these products also confirmed the presence of single-copy insertion into the genome. Based on the available sequencing data, we can conclude that the insertion of T-DNA occurred on the C7 chromosome (NC_027773.2, position 44096892) (Supplementary material). All other physiological features of rape plants were studied using the homozygous transgenic line No 1 of the F₃ generation that contained one single copy cecropin P1 gene.

Resistance of transgenic *Brassica napus* L. to phytopathogenic microorganisms

The transgenic plants with the *cecP1* antibacterial gene demonstrated the higher resistance to bacterial and fungal phytopathogenic microorganisms *E. carotovora*, *P. syringae*, *F. oxysporum*, and *B. cinerea*. Leaves of non-transgenic plants showed initial symptoms of necrosis when infected with bacteria *E. carotovora* and *P. syringae* in 24 h and 6–7 days, respectively. The non-transgenic leaves infected with the fungi *B. cinerea* and *F. oxysporum* showed symptoms of damage (leaf yellowing) after 7–10 days. The leaves of transgenic plants remained green and free from any damage for the same time periods. The infection of the whole plants led to identical results: the non-transgenic plants died within one month, while the signs of damage

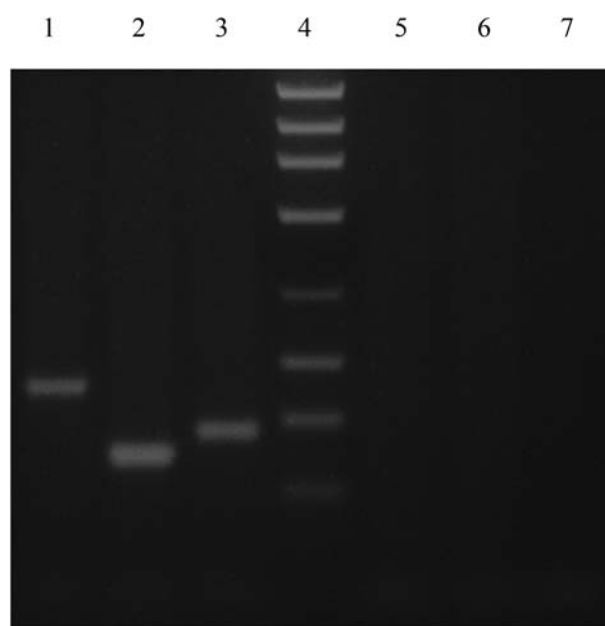


Figure 2. Inverse PCR to determine the copy of T-DNA in transformed plants (line No 1). 1 – Genomic DNA of the transformed plant, hydrolyzed *FspBI*; 2 – Genomic DNA of the transformed plant, hydrolyzed *AluI*; 3 – Genomic DNA of the transformed plant, hydrolyzed *TaqI*; 4 – Marker (3000, 2500, 2000, 1500, 1000, 700, 500, 300 bp); 5 – native genomic DNA of the transformed plant; 6 – native genomic DNA of the control (nontransformed) plant; 7 – water.

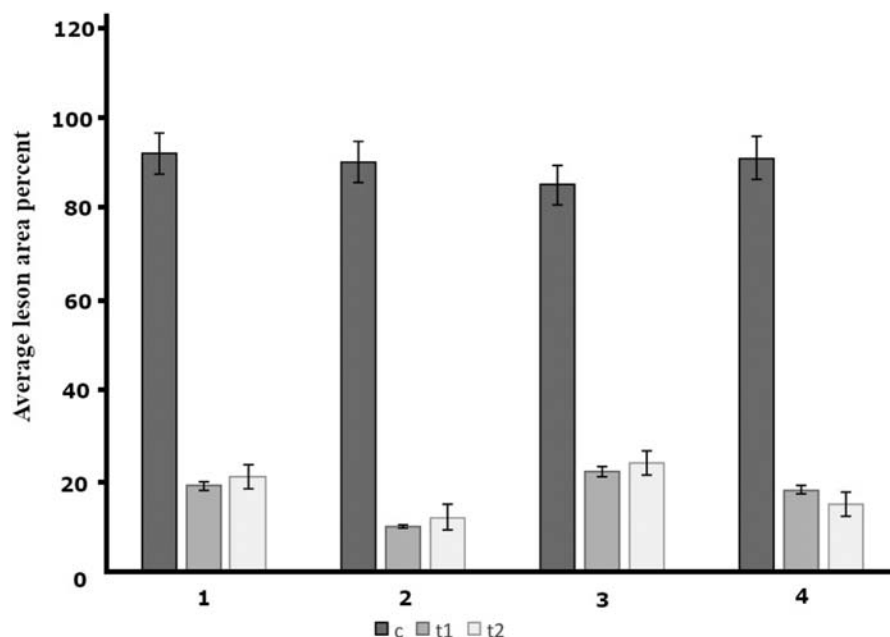


Figure 3. Survival of transgenic cecP1 rape plants infected with phytopathogenes after 10 days of inoculation. C – non-transgenic control plants; t1 – transgenic plants line No 1; t2 – transgenic plants line No 2; 1 – *Erwinia carotovora*, 2 – *Pseudomonas syringae*, 3 – *Botrytis cineria*, 4 – *Fusarium oxysporum*.

on transgenic plants were insignificant (Figure 3, supplementary material).

The antibacterial activity of *B. napus* L. extracts was evaluated by the method of diffusion of the plant extract in agar blocks. The extracts and solution of synthetic cecropin P1 showed significant antibiotic activity against phytopathogenic *E. carotovora*, *P. syringae*, and nonpathogenic bacteria. The extracts of the control (non-transgenic) plants and colonized control plants had almost no antibacterial activity. The diameter of a clear zone around the wells containing extracts of transgenic plants was about 2.0 cm (Table 1, Figure 4). The clear zone around the wells with extracts of cecP1-transgenic plants was approximately the same as the lysis area produced by extract of the non-transgenic plant with 0.25 µg of synthetic cecropin P1 added. Each slot contained plant extract with about 500 µg of total soluble protein. Thus, content of cecropin P1 in transgenic plants could be evaluated as 0.05% of the total soluble protein of plant leaves.

Table 1. Antibiotic activity of extracts from transgenic *Brassica napus* plants against the bacterium.

No	Bacterial strains	Growth inhibition zone, cm ^a			
		TR extract, 500 µg		CP extract, 500 µg	Cecropin P1, 0.25 µg
1	<i>Erwinia carotovora</i> B15	2.3 ± 0.12	2.0 ± 0.13	0.5	2.1 ± 0.25
2	<i>Pseudomonas syringae</i>	1.9 ± 0.20	1.8 ± 0.31	0.5	1.8 ± 0.09
3	<i>Methylobacterium mesophilicum</i> Psm 140	2.2 ± 0.18	2.1 ± 0.88	0.5	2.0 ± 0.16
4	<i>Pseudomonas aureofaciens</i> BS1393(pBS216)	1.7 ± 0.15	1.6 ± 0.19	0.5	1.9 ± 0.74

^aAverage diameter of a bacterial growth inhibition zone (including the well diameter (0.5 cm)). TR – transgenic plants; CP – control plants (nontransformed).

Analysis of fatty-acid composition of plant seeds

Comparison of the fatty acid composition of cecP1 F₃ plant seeds and the non-transgenic plant seeds showed an increased content of unsaturated linoleic and linolenic fatty acids in the transgenic lines 1 and 2 (Table 2).

Analysis of photosynthetic activity of the transgenic plant infected with phytopathogen *E. carotovora* and exposed to UV radiation

The analysis of plants showed that after infection with *E. carotovora*, photosynthetic activity decreased after 24 h by 20,9% instead of 7.7% for the transgenic plants and by

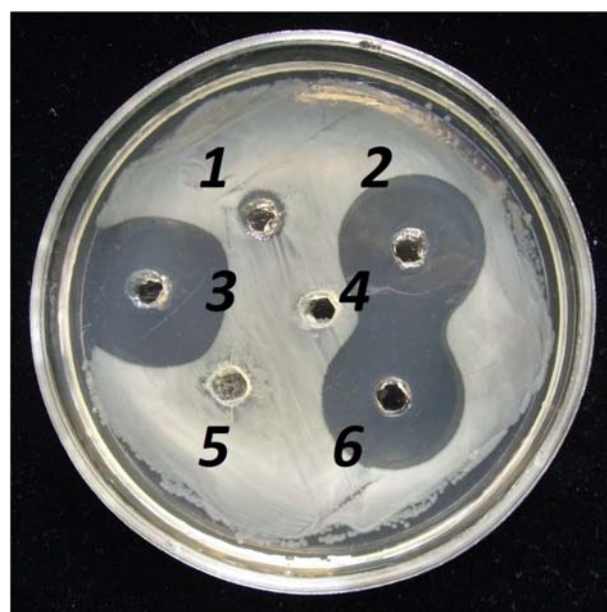


Figure 4. Antibiotic activity of *Brassica napus* extracts against *Erwinia carotovora*. 1 – extract from control (nontransformed) plant colonized by *Pseudomonas aureofaciens* BS1393; 2 – extract from transgenic plants line No 1; 3 – synthetic cecropin P1 (0.25 µg); 4 – extraction buffer; 5 – extract from control (nontransformed) plant; 6 – extract from transgenic plants line No 2.

Table 2. Composition and proportion, %, of fatty acids in seeds of control and transgenic rape plants (*Brassica napus* L.).

Fatty acids	Initial variety (control)	Transgenic rape seeds line no 1	Transgenic rape seeds line no 2	Normal
Myristic acid (Tetradecanoic acid) (C _{14:0})	0.15	0.16	0.21	Up to 0.3
Palmitic acid (C _{16:0})	7.90	7.90	7.90	2.5–6.5
Palmitoleic acid (cis-9-hexadecenoic, 16:1-cis-9)	0.36	0.43	0.39	Up to 0.6
Stearic acid (C _{18:0})	1.16	2.60	5.03	0.8–2.5
Oleic (cis-9-octadecenoic) acid (C _{18:1})	63.70	52.60	48.20	50–65
Linoleic (octadecadienic) acid (C _{18:2} – ω6)	18.10	24.60	24.70	15–25
α-Linolenic (octadecatrienic) acid (C _{18:3} – ω3)	7.20	10.10	11.70	2.0–15.0
Arachidic (eicosanoic) acid (C _{20:0})	0.95	0.97	1.08	0.1–2.5
Gondoic (cis-11-eicosenoic) acid (C _{20:1})	0.04	0.07	0.06	0.1–4.0
Behenic (docosanoic) (C _{22:0})	0.27	0.30	0.39	Up to 1.0
Erucic (cis-13-docosenoic) acid (C _{22:1})	0.17	0.18	0.30	Up to 5.0

Note: Arithmetic averages of three analytical measurements of three biological repetitions are shown. In all cases, the value of the standard deviation did not exceed 3% of average.

Table 3. The rates of photosynthesis and transpiration, the efficiency of water use of the rape transgenic line No 1 as an effect of UV and *E. carotovora* infection.

Variant	Apparent CO ₂ exchange, μmol CO ₂ /(m ² s)	Transpiration, mmol H ₂ O/(m ² s)	CO ₂ /H ₂ O exchange, μmol CO ₂ /mmol H ₂ O
Untransformed plant leaves (control)	7.8 ± 0.4	4.0 ± 0.2	1.95
Control plant leaves infected with <i>E. carotovora</i>	5.5 ± 0.3	3.9 ± 0.2	1.64
Leaves of <i>cecPI</i> -transformed plant	9.1 ± 0.6	2.9 ± 0.1	3.14
Leaves of <i>cecPI</i> -transformed plant infected with <i>E. carotovora</i>	7.2 ± 0.3	2.7 ± 0.1	2.66
Control plant leaves + UV	6.2 ± 0.2	4.2 ± 0.2	1.48
Control plant leaves infected with <i>E. carotovora</i> + UV	3.9 ± 0.1	2.6 ± 0.1	1.5
Leaves of <i>cecPI</i> -transformed plant + UV	7.6 ± 0.3	3.6 ± 0.2	2.11
Leaves of <i>cecPI</i> -transformed plant infected with <i>E. carotovora</i> + UV	6.4 ± 0.3	3.8 ± 0.2	1.68

Notes: The preliminary measurements showed that difference between the photosynthesis rates in control and *cecPI*-transformed plants are within the experimental error. In the subsequent experiments, the gas exchange was compared 24 h after leaf detachment from plants, their subsequent infecting with *E. carotovora* and UV. Uninfected leaves grown at the same conditions were used as controls. The light intensity during the measurement was 100 μE/(m² s). The mean values of measurements on 3–4 detached leaves from 2 to 3 plants and their standard errors are given.

29.5% for non-transgenic ones (Table 3). Exposure to ultra-violet radiation led to a decrease in photosynthetic activity of non-transgenic plants by 37.1% instead of 21% and *cecPI*-transformed plants by 15.8% instead of 3%. Transgenic plants which were infected with pathogen and simultaneously exposed to UV radiation showed better rate of photosynthesis and transpiration than non-transgenic plants under similar conditions (Table 3).

Analysis of transgenic plants resistance to the herbicide paraquat

For analysis, young leaves were treated with 5 μM paraquat. The non-transgenic plants showed vitrification patches observed during the first 24 h of the herbicide action. By the end of the second day, the non-transgenic plant leaves were fully vitrified, while the transgenic plant leaves had quite insignificant areas of damage. By the fourth day, all leaves on non-transgenic plants died, and the transgenic plant leaves had initial signs of damage, yellowing leaf tips, and vein. Before stress, the superoxide content in the non-transgenic plant leaves (3.4 conventional units/(min per g dry wt)) was pretty close to that in the *cecPI* plants (4.0 conventional units/(min per g dry wt)). After 24 h the superoxide radical production in non-transgenic plants increased by 152%, but in the transgenic plants dropped by 10% (Figure 5(a)). Before stress, in the *cecPI* plants, the SOD activity was higher by 29% than in non-transgenic plants. After stress, enzyme activity increased in both groups; however, it was higher by 27% in *cecPI*-plants than in non-transgenic ones (Figure 5(b)). The paraquat-induced oxidative stress caused the elevation of the hydrogen peroxide level in non-transgenic plants by 42%, whereas, in the transgenic plants, the hydrogen peroxide level remained almost unchanged (Figure

5(c)). A decrease in the chlorophyll level in non-transgenic plant leaves under stress caused by 5 μM paraquat, was 34%, while in transgenic plants, the chlorophyll content changed insignificantly (Figure 5(d)).

Study of ecological safety of transgenic plants *Brassica napus* L.

Plant growth characteristics

To determine the characteristics of the growth of plants, non-transgenic and transgenic rape plants were grown in the greenhouse for three seasons. We studied the interaction of transgenic rape plants with *M. mesophilicum* methylobacteria and *P. aureofaciens* pseudomonades. In nature, these microorganisms and plants form an association that stably continues upon micropropagation. These microorganisms have a beneficial effect on the physiological parameters of plants, increasing their growth and development rate and the rooting and germination of seeds (Lugtenberg and Kamilova 2009).

One-month-old leaves of *Brassica napus* transgenic plants expressing the antimicrobial peptide cecropin P1 were treated with a liquid culture of *M. mesophilicum* and *P. aureofaciens* with a titer of 10³–10⁵. The presence of methylobacteria and pseudomonas on segments of leaves, stems, and roots were determined in 1–5 weeks (Table 4). To this purpose, various explants (leaves, stems, and roots) were homogenized; the extracts were placed on the surface of the nutrient medium in Petri dishes and incubated at 22–24°C for 2 days. It turned out that methylobacteria and pseudomonas presented in all initially colonized plants and also in plants obtained after several passages of micropropagation. During the first cycle of plant micropropagation, the

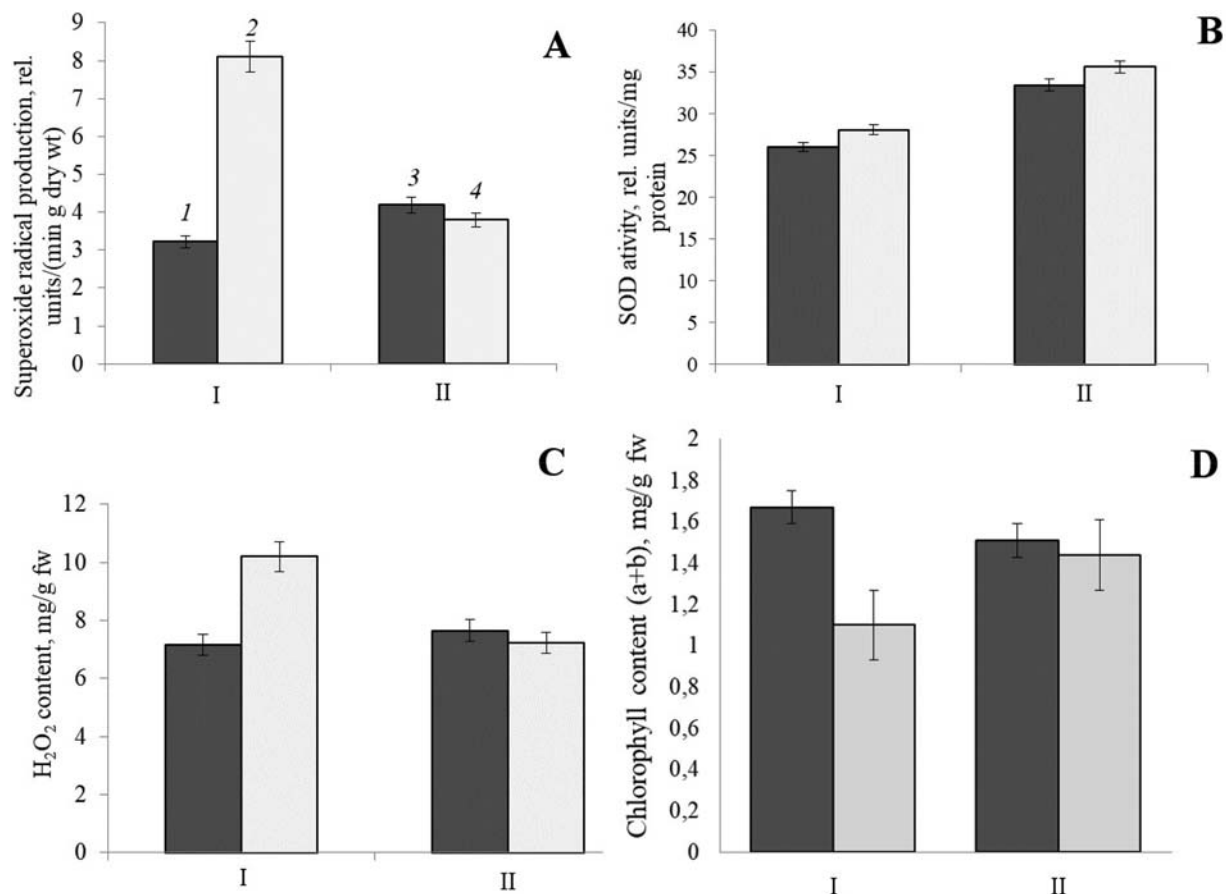


Figure 5. The effects of paraquat on superoxide anion production (a), SOD activity (b), hydrogen peroxide level (c), chlorophyll content in the leaves (d) in control (I) and transgenic rapeseed plants (line No 1) (II). (1, 3) water, (2, 4) paraquat.

number of colony-forming units (CFU) of methylobacteria was 1000–3000 CFU/cm² in the leaves, 200–300 CFU/cm² in stems, and 2000–3000 CFU/cm² in roots. During colonization with pseudomonade, the number of bacteria was 1000–2000 CFU/cm² in leaves, 200–300 CFU/cm² in stems, and 10,000–20,000 CFU/cm² in roots. Consequently,

transgenic plants bearing the gene encoding cecropin P1 were actively colonized by methylobacteria and pseudomonas. In successive cycles of micropropagation, the content of bacteria *M. mesophilicum* and *P. aureofaciens* in plants remained unchanged, indicating their strong association with plants.

Observed differences in the number of beneficial bacteria found in the leaves and roots correspond to their plant associative specificity. The bacteria *P. aureofaciens* are rhizosphere-inhabitant microorganisms, and that is why they inhabit roots. At the same time, *M. mesophilicum* are of endophyte nature and prefer to populate shoots and leaves but not roots. In subsequent cycles of micropropagation, the content of bacteria *M. mesophilicum* and *P. aureofaciens* in plant tissues remained unchanged, indicating the formation of stably lasting associative conjunctions with transgenic and non-transgenic plants.

When planted in a greenhouse, the colonized rape plants showed better adaptation compared to non-colonized plants – notably in faster rooting (Table 5). Adaptation to in vivo is a difficult stage for plants after in vitro cultivation, especially for transgenic plants. Therefore, various ways are used to increase the number of well-adapted plants: reducing nitrates in nutrient environments, using adaptive ionit substrates, etc. One way to optimize the adaptation period may be to use plant colonization with beneficial associative microorganisms. In this work, colonization contributed to the intensification of the growth processes of the above-ground and root parts of plants. Expression of cecP1 gene in plants caused a delay in the flowering for 11 days and the extension of the vegetative phase. Due to prolonged vegetation period for transgenic

Table 4. Contents of microorganisms in roots, stems, and leaves of *Brassica napus* L., plants (lane no 1) colonized by *Methylobacterium mesophilicum* Psm 140 and *Pseudomonas aureofaciens* BS1393(pBS216).

Variant	Week after colonization	Roots	Stems	Leaves
<i>Methylobacterium mesophilicum</i> Psm 140				
A	1	NT	NT	2.17 ± 0.21
B	1	NT	NT	2.17 ± 0.21
A	2	2.23 ± 0.28	1.30 ± 0.85	2.69 ± 0.27
B	2	2.23 ± 0.30	1.30 ± 0.78	2.69 ± 0.25
A	3	2.39 ± 0.42	1.39 ± 0.62	3.04 ± 0.30
B	3	2.41 ± 0.13	1.41 ± 0.63	3.04 ± 0.28
A	4	2.46 ± 0.28	1.49 ± 0.52	3.39 ± 0.41
B	4	2.47 ± 0.38	1.50 ± 0.51	3.39 ± 0.43
A	5	2.54 ± 0.43	1.47 ± 0.78	3.46 ± 0.40
B	5	2.54 ± 0.33	1.47 ± 0.79	3.47 ± 0.35
<i>Pseudomonas aureofaciens</i> BS1393(pBS216)				
A	1	2.84 ± 0.25	1.63 ± 0.86	2.84 ± 0.26
B	1	2.84 ± 0.25	1.63 ± 0.73	2.84 ± 0.25
A	2	3.14 ± 0.40	1.76 ± 0.96	2.90 ± 0.26
B	2	3.17 ± 0.53	1.77 ± 0.86	2.90 ± 0.19
A	3	4.03 ± 0.64	1.77 ± 0.56	3.00 ± 0.35
B	3	4.04 ± 0.44	1.83 ± 0.26	3.04 ± 0.37
A	4	4.25 ± 0.52	1.84 ± 0.76	3.25 ± 0.48
B	4	4.25 ± 0.22	1.84 ± 0.76	3.25 ± 0.37
A	5	4.30 ± 0.39	1.84 ± 0.81	3.27 ± 0.53
B	5	4.30 ± 0.27	1.84 ± 0.81	3.30 ± 0.61

Notes: The numbers provided are common logarithms of CFU in roots, stems, and leaves. Acceptable values of deviations lie within a range of 0–1 log CFU g⁻¹ of wet weight. A – control plants; B – transgenic cecP1-plants; NT – not tested.

Table 5. Phenotype analysis of colonized cecP1 and control plants in *B. napus* species.

Variant	<i>M. mesophilicum</i> Mean (std error)	<i>P. aureofaciens</i> Mean (std error)	Non-colonized Mean (std error)
<i>Transgenic plants</i>			
Root length (mm)	459 (41)	450 (40)	410 (38)
Height (cm)	80 (2.3)	78 (1.8)	92 (1.5)
N days to opening of the first flower	95.1 (1.3)	98.3 (0.8)	105.7 (1.5)
N days to bolting	92 (1.6)	96 (1.7)	112 (1.2)
<i>Non-transgenic plants</i>			
Root length (mm)	468 (39)	435 (41)	430 (40)
Height (cm)	83 (0.9)	79 (0.8)	76 (0.7)
N days to opening of the first flower	92.4 (1.5)	91.6 (0.6)	93.8 (0.8)
N days to bolting	90 (1.1)	89 (1.3)	96 (1.4)

Notes: For each variant, plants were grown in the same conditions at the same time. Details of measurements are found in Materials and methods section.

plants, by the time the mature pods were formed, the plant height was 20% longer than of control one; though pod length, seed number, as well as flower color or other characteristics, did not differ compared to non-transgenic plants (Table 5). Our results are consistent with earlier data which showed that constitutive expression of the AMP *msrA3* gene in transgenic potato led to delayed development of floral buds and prolonged vegetative phase (Goyal et al. 2013). Colonization of transgenic plants by associative microorganisms led to the normalization of plant growth (Table 5).

Naphthalene resistance of the colonized plants

To study the growth of oilseed rape plants colonized with naphthalene-degrading strain *P. aureofaciens* BS1393 (pBS216), these plants were propagated on MS medium containing naphthalene (toxic aromatic oil component) at different concentrations. *P. aureofaciens* BS1393 carried plasmid pBS216 with the gene encoding naphthalene oxygenase that degraded naphthalene to less toxic compounds as salicylic acid and catechol. At 1 mg/ml naphthalene concentration in medium non-colonized plants died after 2–3 days (Figure 6). Oppositely, colonized plants grown on the same medium showed slow but steady growth and were green throughout the experiment (one month). Identical results were observed both for transgenic and non-transgenic plants.

Discussion

In this work, interactions between homozygous oilseed rape plants (*Brassica napus* L.) with *cecP1* gene and associative microorganisms were studied. Physiological and biochemical analysis of transgenic plants was held. Expression of antimicrobial peptide *cecP1* helps transgenic plants to become more resistant to phytopathogens such as *E. carotovora*, *P. syringae*, *F. oxysporum*, and *B. cineria*. Analysis of the fatty acid composition of seeds showed a higher content of linoleic and linolenic unsaturated fatty acids in transgenic lines, as compared to non-transgenic plants, while the general content of fatty acids was close to normal. The increased degree of unsaturation of fatty acids during leaf development is associated with biogenesis of chloroplasts in which thylakoid membranes are characterized by a high degree (85–90%) of unsaturation (Murphy 1986). The linoleic and linolenic acids are known as intermediate degradation products of plant lipids. Therefore, changing the ratio of linoleic and linolenic acids under stress can influence the plant adaptation efficiency (Tarchevsky 1992). In transgenic oilseed

rape plants expressing the antimicrobial peptide cecropine P1, increased levels of unsaturated fatty acids linoleic and linolenic were observed. At the same time, the number of associative bacteria colonizing both transgenic and non-transgenic plants was almost the same, which indicates the safety of elevated levels of unsaturated fatty acids for associative bacteria.

There has been an increase in the content of erucic acid in the seeds of transgenic plants, but it is still in the ‘normal range.’ Breeders breed low-erucic rapeseed because erucic acid is toxic. In a certain range, the harmful effects of erucic acid can be balanced by the beneficial effects of unsaturated fatty acids of rapeseed oil.

Photosynthesis parameters characterize the viability and general condition of plants. The photosynthetic activity of



Figure 6. Rape plants on MS medium with naphthalene. A – non-transgenic plants. 1 – noncolonized plant, normal growth; 2 – noncolonized dying plant, containing 1 mg/l naphthalene; 3 – colonized plant, normal growth; 4 – colonized plant, containing 1 mg/l naphthalene, normal growth. B – transgenic plants line No 1. 1 – noncolonized plant, normal growth; 2 – noncolonized dying plant, containing 1 mg/l naphthalene; 3 – colonized plant, normal growth; 4 – colonized plant, containing 1 mg/l naphthalene, normal growth.

non-transgenic and transgenic plants of the F₃ generation exposed to biotic and abiotic stress factors was evaluated. Infection with *E. carotovora* was used as a biotic stressor and UV radiation as an abiotic stressor. The results obtained demonstrated a decrease in photosynthetic activity of both non-transgenic and transgenic plants when infected with the phytopathogen *E. carotovora* or exposed to UV radiation.

A significant reduction in the efficiency of water use was also demonstrated in infected non-transformed plants compared to infected cecP1-expressing plants. However, PSII of transgenic plants, both infected or uninfected, showed greater resistance to UV irradiation as compared to untransformed plants. Probably the primary photochemical processes of photosynthesis in transgenic cecP1-plants are more resistant to various stressors than in the wild type.

The primary reaction to the action of toxic xenobiotics is the induction of oxidative stress – quick and temporary production of reactive oxygen species (ROS) (Apel and Hirt 2004). As a result of changes in plant cells, ROS formation is predominant over the activity of the antioxidant protection system (Halliwell 2006). An increase in the rate of lipid peroxidation, the damage of membrane, proteins, and DNA are the consequences of the elevation in ROS level. Plant resistance to stress depends on the antioxidant system, which maintains ROS concentration in the cell at a fairly low level, and those plants that are more sustainable in stress conditions demonstrate higher activities of SOD and less oxidative damage (Mittova et al. 2003; Kreslavskii et al. 2012). Herbicides are a class of xenobiotics, which are usually used to control weeds. They have an adverse effect on the plant: suppressed germination of seeds, growth retardation, and impaired physiological functions (Boutin et al. 2014). The herbicide paraquat (methyl-viologen) induces oxidative stress in plants, inhibition of antioxidant protection and cause ultrastructural alterations of cell (Dodge 1994). After treatment with paraquat, transgenic cecP1 plants demonstrated an increased ability to neutralize ROS action as compared to non-transgenic plants. It was proven by the activity of SOD, along with decreased content of superoxide radical and peroxide in oxidative stress conditions. Increased antioxidant resistance of cec-P1 plants is observed not only in experiments in the effect of hers-P1 plants with herbicide paraquat, but also in the infection with phytopatogenes (Zakharchenko et al. 2015, 2019). Also, a substantial decrease in the chlorophyll content was found in non-transgenic but not in transgenic plant leaves. Our results are consistent with earlier data, which showed that constitutive expression of genes of antimicrobial peptide cecropin A in rice causes increased expression of a number of intrinsic genes of this plant and enhances antimicrobial and antioxidant protection (Campo et al. 2008). Also, transgenic potato with the AMP gene *msrA3* showed increased resistance to pathogens *Fusarium solani* and abiotic stress-induced by darkness, wounding, and high temperature (Goyal et al. 2013). It was shown previously that expression of AMP cecropin P1 increased the resistance of transgenic rape plant to pathogenic *E. carotovora* and *F. sporotrichioides* though contributed to some decrease in the rate of photosynthesis under infection (Zakharchenko et al. 2013).

The harmlessness of transgenic oilseed rape plants with the cecP1 gene for beneficial associative microorganisms was shown. In the natural conditions, various bacteria including pseudomonas and methylobacteria form stable

symbiotic associative conjunctions with plants. Associative microorganisms can have a beneficial effect on plants, causing an increase in the rate of their growth and development, as well as rooting and germination of seeds (Hassani et al. 2018). Aerobic methylophrophic bacteria use methanol secreted by the degradation of pectin components of plant cells, and are therefore usually associated with plants. In turn, plants get cytokinins, auxins, vitamins, and polysaccharides from methylobacteria (Doronina et al. 2000). The rhizosphere bacteria of the *Pseudomonas* genus are potential objects of agrobiotechnology, as they have a number of physiological and biochemical features necessary for biocontrol of phytopathogens and plants developments. Plants colonized by bacteria *P. aureofaciens* BS2013 (pBS216) were resistant to naphthalene and can grow steadily on the medium containing this compound. Thus, the colonization of rape plants by associative bacteria able to naphthalene biodegradation provides plant with resistance to this toxic xenobiotic. Study of inhabitation of beneficial associative microorganisms *M. mesophilicum* and *P. aureofaciens* within transgenic oilseed rape plants showed that the synthesis of antimicrobial peptide cecropin P1 does not affect their association with plants. It is possible that these microorganisms are able to colonize cecP1-plants, since the synthesized cecropin P1 is accumulated inside the cells while the associative microorganisms do not disrupt the cell membrane and reside in the intercellular space, and do not have direct contact with this antimicrobial peptide. This distinguishes them from phytopathogenic microorganisms, which disrupt cell membrane thus directly interact with cecropin P1 peptide, though, beneficial associative bacteria, as well as pathogenic bacteria, are sensitive to cecropin P1 too.

The results of this work demonstrate the safety of transgenic oilseed rape plants with the *cecP1* gene for methylobacteria and pseudomonas and the prospect of practical use as a microbiological aid to stimulate plant growth and protect them from biotic and abiotic stressors.

Conclusion

The endophytic bacterium, *M. mesophilicum* and *P. aureofaciens* proved its ability to colonize the transgenic plants (*Brassica napus*), expressing the gene of antimicrobial peptide cecropin P1 (*cecP1*). The colonization by methylobacteria led to their steady association with the plants, which led to increased growth speed, root formation, and adaptation to greenhouse conditions. CecP1-plants colonized by bacteria *P. aureofaciens* with resistance to naphthalene were able to grow steadily on medium containing this chemical compound.

We demonstrated that plant colonization with beneficial associative microorganisms can be used as an efficient way to improve plant adaptation to the conditions of their growth in soil.

Disclosure statement

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