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SUPEROXIDE DISMUTASE ACTIVITY IN TRANSGENIC CANOLA

Superoxide dismutase (SOD) activity was investigated in leaves of transgenic canola plants which expressed heterologous genes of different origin, namely 1) herbicide resistance genes (bar and simultaneously bar and epsps); 2) DesC desaturase gene (desC) of cyanobacterium Synechococcus vulcanus; 3) human interferon α2b gene (huIFN-α2b); 4) esxA::fbpB$^{\Delta TMD}$ fused gene, encoding ESAT-6 and Ag85b Mycobacterium tuberculosis proteins, inducing immune response against tuberculosis; 5) cyp11A1 gene of cytochrome P450$_{SCC}$ from bovine adrenal cortex mitochondria. Introduction of herbicide resistance genes as well as desaturase gene of cyanobacterium and mycobacterium's genes did not change leaf SOD activity. At the same time it was shown that cyp11A1 and huIFN-α2b canola have increased leaf SOD activity up 58 and 33 %, respectively, compared with control ones in non-stress conditions. It may be a prerequisite for improved resistance of these plants to the stressors of different origin.

Key words: *Brassica napus, cyp11A1, desC, epsps, esxA::fbpB$^{\Delta TMD}$, huIFN-α2b, SOD.*

Introduction. Plant cells are continuously exposed to reactive oxygen species (ROS) generated as by-products of fatty acid β-oxidation, photorespiration, and photosynthesis. Environmental conditions such as extreme temperatures and/or water stress, especially in combination with high light intensities, and some pathogens can cause oxidative stress damage by overproduction of ROS. The first enzyme in the detoxifying process is superoxide dismutase (SOD, EC 1.15.1.1). It converts superoxide radicals to hydrogen peroxide.

In recent years, interest has increased in the study of the antioxidant enzyme activities, including SOD, at different stages of plant ontogenesis [1–3]. The specific features of SOD activity were also studied in different genotypes within one species [4, 5].

SOD activity was investigated in mutants and transgenic plants which expressed heterologous *sod* genes of different origin. Transgenic tobacco plants (*Nicotiana tabacum*) that expressed a chimeric gene which encoded chloroplast-localized pea Cu/Zn-SOD had 3-fold higher SOD activity than control ones [6]. It allowed for increased resistance to high light intensity under low temperature [7]. Alfalfa (*Medicago sativa*) transgenic plants which displayed the altered levels of SOD were created and identified as possessing enhanced freezing stress tolerance, enhanced drought resistance, and improved biomass production and persistence in field trials [8–10]. Rice (*Oryza sativa*) plants expressing pea MnSOD under the control of the oxidative stress-inducible *SWPA*2 promoter in chloroplasts demonstrated reduced electrolyte leakage compared to wild type leaf slices and exhibited less injury, measured by net photosynthetic rate, under drought stress induced by polyethylene glycol 6000 [11]. Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis* cv. Tropical Pride) plants which expressed maize Cu/ZnSOD in chloroplasts showed the levels of protection from SO_2 and salt stress that were moderately improved compared to wild-type plants [12]. The photosynthetic activity of *B. campestris* transgenic plants with two simultaneously expressing heterologous genes (Cu/Zn*SOD* and *CAT* (catalase)) decreased by only 6 %, whereas that of initial plants decreased by 72 % when they were exposed to the high NaCl salinity (200 mM) for 4 weeks [12]. Overexpression of wheat mitochondrial Mn superoxide dismutase (MnSOD3.1) enhanced transgenic canola (*Brassica napus*) heat, drought and cold tolerance both under artificial and in the field stress conditions [13]. Transgenic *Arabidopsis* plants, expressing cytosolic Cu/ZnSOD of *Potentilla atrosanguinea* showed an enhanced tolerance to salt stress during germination, seedling establishment and growth in terms of larger root length, larger rosette area and the higher number of leaves besides the high levels of SOD [14]. Thus, plants characterizing by higher SOD activity are more resistant to abiotic and biotic stresses. They also form larger biomass both in normal and stress conditions. Plants with improved SOD activity have shorter vegetative growth stage and bloom earlier allowing get the harvest in a shorter time.

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Still little is known about the influence of genetic transformation using other target genes on the SOD activity in transgenic plants.

Earlier we created transgenic canola plants with heterologous genes of different origin [15–20]. The aim of the present work was to estimate SOD activity in these plants for future stress testing.

Materials and methods. *Plant material.* Aseptic spring canola plants (*Brassica napus* L. var. *oleifera* DC.) with heterologous genes of different origin were analysed. They carry in their nuclear genomes such foreign genes as: 1) herbicide resistance genes (*bar* and *epsps*). Plants were obtained by using cv Kalinovskii (5/44/1, 5/44/2 lines) [15] and Exgold (15/133/9, 15/133/3 lines) [16]; 2) DesC desaturase gene (*des*C) of cyanobacterium *Synechococcus vulcanus*. Plants were created by using cv Obreey (18a, 18b lines) [17]; 3) human interferon α2b gene (*huIFN-α2b*). Plants were obtained by using cv Magnat (9/125/10, 9/125/20 lines) [18]; 4) *esxA::fbpB*$^{\Delta TMD}$ fused gene, encoding ESAT-6 and Ag85b *Mycobacterium tuberculosis* proteins, inducing immune response against tuberculosis. Plants were created by using cv Kalinovskii (5/67/4, 5/67/14 lines) [19]; 5) *cyp*11A1 gene of cytochrome $P450_{SCC}$ from bovine adrenal cortex mitochondria. Plants were created by using cv Mariia (12/93/1a, 12/93/2c lines) [20]. Due to *bar* gene expression all tested canola plants were resistant to BASTA herbicide treatment in greenhouse conditions. It was used in transformation cassettes as selective marker. Both control and transformed plants were propagated *in vitro* by grafting and were grown under cultivation conditions (16/8 light/dark photoperiod, +23 °C, 4000–5000 lux) during four weeks in the Sigma 25 × 150 mm test tubes with 15 ml agar-solidified MS medium [21].

The total soluble protein (TSP) content was measured using *Bradford* method [22]. The extracts from plant leaves were prepared in triple volume of 100 mM Tris/HCl buffer, pH 8.0. The optical density was determined by BioPhotomether Eppendorf, v.1.35 (Germany).

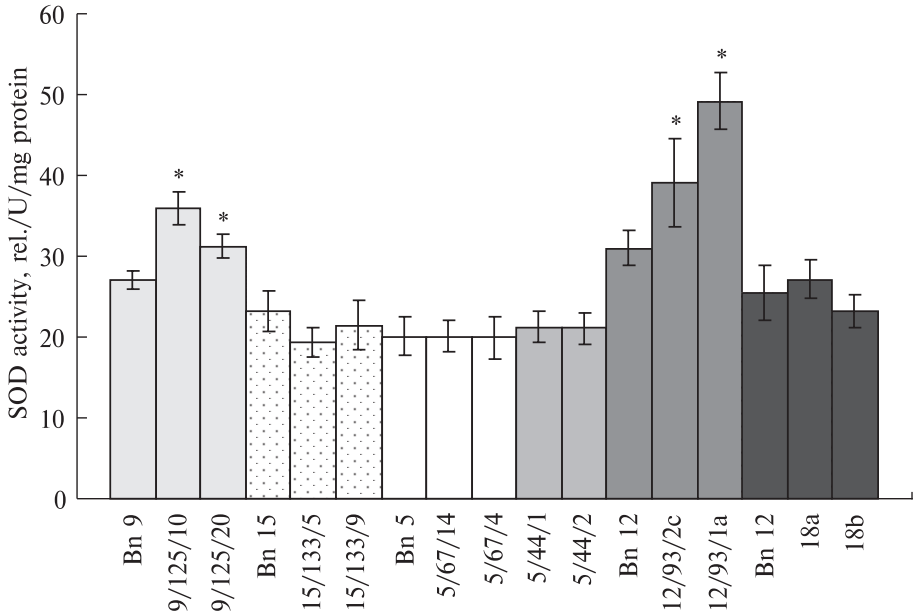
Superoxide dismutase activity. Photochemical oxidation of nitro blue tetrazolium (NBT) method was used for SOD activity determination [23]. Fresh plant material (100 mg) was pounded with 1 ml of Tris-HCl buffer (pH 8.0) in mortar and was centrifuged at 13 000 g (4 °C) for 15 min. The supernatant was used for analyses. Formation of formasan (violet color substance) reaction was held in Eppendorf tube (1.5 ml). One tube for each probe was retained in the dark. The others were illuminated with white light lamp (fluorescent lamp T5/G5, model ELI-230A-T5-8W) during 5 min in the thermostat at 23 °C. Null probe had no leaf extract in its composition. In this probe oxidation was complete. Plant extracts could inhibit formasan formation due to SOD activity. The optical density of illuminated probe solution was measured by BioPhotomether Eppendorf (Germany) versus the optical density of dark probe. SOD activity was expressed as relative unit/ mg protein.

Statistical analysis was performed according to Duncan multiple range test. Differences from control values were significant at $p \leq 0.05$. Three independent experiments were conducted in five replications. There were nine replications for formazan measurement.

Results and discussion. It was found that leaf SOD activity differed within the control (untransformed spring canola of five varieties) plants after four weeks growth in the sigma test tubes with agar-solidified MS medium (Figure). The highest SOD activity was detected for cv Mariia, it significantly differed from all the tested varieties. The differences in SOD activity were not detected among cvs Exgold, Kalinovskii, Obreey.

No SOD activity differences were shown between the control and transformed (5/44/1, 5/44/2) plants expressing promotorless *bar* gene. The product of this gene is an enzyme which acetylates phosphinothricin or demethylphosphinothricin [24]. *Bar* gene expression is not accompanied by superoxide radical formation. This gene is often used as selectable marker during plant genetic manipulations. We found no data on changes in SOD activity in plants which expressed only *bar* gene proving plant resistance to herbicides (such as BASTA) with phosphinothricin as active agent.

We did not observe significant distinctions in SOD activity in canola plants (15/133/5, 15/133/9) expressing *bar* and *epsps* genes simultaneously. The latter encodes 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase which catalyzes the chemical reaction: phosphoenolpyruvate + 3-phosphoshikimate $\rightleftharpoons$ phosphate+5-enolpyruvylshikimate-3-phosphate (EPSP) [25]. Glyphosate-based herbicides, such as Roundup, target the shikimate pathway enzyme 5-enolpyruvylshikimate 3-phosphate (EPSP)



Leaf SOD activity of aseptic canola plants: Bn9, Bn15, Bn5, Bn12, Bn18 were untransformed plants, cvs Magnat, Exgold, Kalinovskii, Mariia, and Obreey, respectively; 9/125/10, 9/125/20 lines expressed human interferon α2b gene; 15/133/5, 15/133/9 lines have *epsps* gene; 5/44/1, 5/44/2 were lines with active promotorless *bar* gene; 5/67/4, 5/67/14 lines expressed *esxA::fbpB*$^{\Delta TMD}$ fused gene; 12/93/1a, 12/93/2c lines carried *cyp*11A1 gene; 18a, 18b expressed *des*C of cyanobacterium *Synechococcus vulcanus* and *epsps* genes. *Error bars* represent mean ± one standard deviation and *asterics*. * Indicates significant differences between experimental values compared with the control ones ($p \leq 0.05$)

synthase, the functionality of which is absolutely required for plant survival. Roundup Ready plants carry the gene coding for a glyphosate-insensitive form of this enzyme, obtained from *Agrobacterium* sp. strain CP4 [26]. Synthase (*epsps*) expression does not create conditions for O_2^- formation and SOD activity changes.

Transgenic plants with simultaneously *bar, epsps* and *des*C (18a, 18b lines) gene expression have leaf SOD activity similar to control plants (Figure). Fatty acid desaturases catalyze transformation of a single bond between carbon atoms in acyl chains (C—C) into the double bond (C=C) [27]. It was shown that *des*C expression in transgenic tobacco provides the advantages of these plants under low temperature and their SOD activities increased in stress higher than those of control plants. But under normal condition *des*C tobacco SOD activities did not differ in comparison with the wild type plants [28] as well as in our experiments with *B. napus*.

Canola plants expressing simultaneously *bar* and *esxA::fbpB*$^{\Delta TMD}$ fused gene, which encodes ESAT-6 and Ag85b *M. tuberculosis* proteins (5/67/4, 5/67/14 lines) have the same SOD activities that control plants (Figure). Antigen 85B is a myocolyc transferase in the myc pathway and catalyses the transfer of the fatty acid mycolate from one trehalose monomycolate to another, resulting in trehalose dimycolate and free trehalose and helping build the cell wall [29]. Both *esxA* and *fbpB* gene expression do not cause superoxide radical formation [30].

SOD activities in 12/93/2c (39.1 ± 5.5) and 12/93/1a (49.11 ± 3.53) lines were 26 and 58 % higher in comparison with the control one (31.02 ± ± 2.06). These lines expressed *cyp*11A1 gene encoding cytochrome $P450_{SCC}$ from bovine adrenal cortex mitochondria [20]. In animals cytochrome $P450_{SCC}$ catalyzes three step cholesterol oxidation with formation of pregnenolone [31, 32]. Superoxide radicals are formed during these reactions. Therefore, SOD activity in *cyp*11A1 canola can increase due to cytochrome $P450_{SCC}$ activity.

The SOD activity increase was also detected in the leaves of *huIFN-α2b* canola plants (Figure). It reached 35.96 ± 2.1 (9/125/10 plants), 31.24 ± 1.5

(9/125/20 line) and 27.02 ± 1.08 (control plants). In this group of transgenic canola plants the SOD activity rose up 1.33 fold. Exogenous interferon-alpha application is accompanied by superoxide radical formation and SOD activity increase in animal cells [33, 34]. There are no SOD activity data about the other transgenic plants expressing *huIFN* gene. We propose that exactly heterologous protein activity (human interferon-alpha) is the reason of the SOD activity increase in our transgenic canola plants.

It was shown that SOD activity in transgenic *sod* plants increased up to 2.5 (rapeseed) [35], 3 (tobacco) [7], 4 (maize) fold [36] in without stress conditions. We have detected that *cyp*11A1 and *huIFN*α-2*b* gene expression can increase leaf SOD activity (up 1.58 and 1.33 fold, respectively). So we found that the SOD activity increase in transgenic plants may be not only due to heterologous *sod*, but also other target gene expression. Similar results were obtainted in experiments with tomato (*Solanum lycopersicum*) plants which expressed *ZAT12* gene from *Brassica carinata* encoding a C_2H_2 zinc finger transcription factor [37] and arabidopsis (*Arabidopsis thaliana*) plants expressing *TsRfBP* (riboflavin-binding protein) gene obtained from the soft-shelled turtle *Trionyx sinensis japonicus* [38]. The SOD activity was detected to be 1.69 and 1.1 fold higher in leaves of transgenic tomato and arabidopsis plants, respectively, under non-stress conditions in comparison with untransformed ones. Authors have proved drought resistance improvement of these plants in greenhouse [37, 38].

Plants with lower SOD activity were also observed among transgenic plants. It was shown that cherry tomato with *HbsAg* (hepatitic B virus) gene had 30 % lower leaf SOD activity in comparison with control one [39]. These plants had higher total soluble protein and relative water content in the leaves, and did not form fertile seeds.

Conclusions. Changes in SOD activity were not observed in *des*C and *esxA::fbpB*$^{\Delta TMD}$ transgenic canola. Leaf SOD activity has not also been affected by introduction of herbicide resistance genes (*bar* and *epsps*). However, it was shown that *cyp*11A1 and *huifn*-α2*b* canola plants have increased leaf SOD activity up 58 and 33 %, respectively, compared with untransformed ones, in non-stress conditions. Increased SOD activity may be a prerequisite for improved resistance of these plants to the stressors of different origin.

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АКТИВНОСТЬ СУПЕРОКСИДДИСМУТАЗЫ В РАСТЕНИЯХ ТРАНСГЕННОГО РАПСА

Исследована активность супероксиддисмутазы (СОД) в листьях трансгенных растений рапса, экспрессирующих гетерологичные гены различного происхождения, а именно: 1) гены устойчивости к гербицидам (*bar* и одновременно *bar* и *epsps*); 2) ген десатуразы DesC (*des*C) цианобактерии *Synechococcus vulcanus*; 3) ген α2b интерферона человека (*huIFN*-α*2b*); 4) слитый ген *esxA::fbpB*$^{\Delta TMD}$, кодирующий белки ESAT-6 и Ag85b *Mycobacterium tuberculosis,* которые индуцируют иммунный ответ против туберкулеза; 5) ген *cyp*11A1 цитохрома $P450_{SCC}$ митохондрий коры надпочечников быка. Введение генов устойчивости к гербицидам, а также гена десатуразы цианобактерии и генов микобактерии не изменяло активности СОД в листьях рапса. Вместе с тем показано, что в условиях без стресса у растений с трансгенами *cyp*11A1 и *huIFN*-α*2b* активность СОД повышена до 58 и 33 % соответственно в сравнении с контролем. Это может быть предпосылкой для увеличения их устойчивости к стрессорам различного происхождения.

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АКТИВНІСТЬ СУПЕРОКСИДДИСМУТАЗИ В РОСЛИНАХ ТРАНСГЕННОГО РІПАКУ

Досліджено активність супероксиддисмутази (СОД) в листках трансгенних рослин ріпаку, що експресують гетерологічні гени різного походження, а саме: 1) гени стійкості до гербіцидів (*bar* і одночасно *bar i epsps*); 2) ген десатурази DesC (*des*C) ціанобактерії *Synechococcus vulcanus*; 3) ген α*2b* інтерферона людини (*huIFN*-α*2b*); 4) злитий ген *esxA::fbpB*$^{\Delta TMD}$, що кодує білки ESAT-6 і Ag85b *Mycobacterium tuberculosis,* які індукують імунну відповідь проти туберкульозу; 5) ген *cyp*11A1 цитохрома $P450_{SCC}$ мітохондрій кори надниркових залоз великої рогатої худоби. Введення генів стійкості до гербіцидів, а також гена десатурази ціанобактерії та генів мікобактерії не змінювало активності СОД в листках ріпаку. Разом з тим встановлено, що у рослин з трансгенами *cyp*11A1 *і huIFN*-α*2b* в умовах без стресу активність СОД підвищена до 58 і 33 % відповідно в порівнянні з контролем. Це може бути передумовою для підвищення їхньої стійкості до стресорів різного походження.

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