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ADAPTIVE PLASTICITY IN OSMOTIC STRESS OF BIOTECH CANOLA (*BRASSICA NAPUS* L.) POSSESSING *CYP11A1* OR SIMULTANEOUSLY *DESC* AND *EPSPS* TRANSGENES

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In order to study the osmotic stress tolerance of canola (Brassica napus L.) plants constitutively expressing cyp11A1 or simultaneously desC and epsps genes that encode bovine cytochrome $P450_{SCC}$ or Δ9 desaturase from cyanobacterium Synechococcus vulcanus and bacterial enolpyruvat shikimat phosphate syntase, respectively, the influence of different osmotic pressures induced by mannitol addition on plant in vitro growth was estimated by evaluation of fresh weight (FW), total soluble protein content (TSP), and superoxide dismutase activity (SOD). Transgenic cyp11A1 lines formed higher FW and TSP (by 30% and 36%, respectively) as well as they had up to 1.65-fold higher SOD activity in comparison with initial plants under normal conditions. FW and SOD activity of cyp11A1 plants were higher and TSP was similar compared the control plants under osmotic stress. Biotech desC/epsps lines showed no differences compared with untransformed plants both in normal and stressful conditions. Adaptive plasticity to osmotic stress of cyp11A1canola was higher than ones of initial and desC/epsps plants. It may be due to increase in SOD activity.

Keywords: *Brassica napus; cyp11A1*; *desC*; mannitol; osmotic stress; SOD activity

Drought and soil salinity are the major environmental factors limiting plant agricultural productivity [26, 6]. Both drought and salinity cause osmotic stress to plants. Tremendous efforts of plant biotechnologists are focused on obtaining of new genotypes which would able to grow without loss of productivity in different stressful conditions [15, 2]. Transgenesis is successfully used for the improvement in biotic and abiotic stress tolerance of crop plants [14, 17].

Recently we have obtained canola lines bearing mammalian *cyp11A1* gene [21] and ones expressing simultaneously cyanobacterial *desC* and bacterial *epsps* genes [19]

in their nuclear genomes. Cytochrome $P450_{SCC}$ from bovine adrenal cortex mitochondria is encoded by *cyp11A1* gene. Heterologous cytochrome affected the biosynthesis of steroid compounds in transgenic tobacco (*Nicotiana tabacum* L.) [24]. SOD activity increase in leaf tissue of *cyp11A1* canola was detected in physiological conditions [20]. It may be the prerequisite for stress tolerance of different origin [10]. Plant resistance to phosphonomethyl glycine herbicides (Roundup) is provided by *epsps* gene and was proved for our transgenic canola *in vitro* and greenhouse [19]. Acyl-lipid fatty acid desaturase DesC catalyzes the transformation of a single bond between carbon atoms (C-C) in acyl chains into the double bond (C=C) in position C9 [11]. The increase in the unsaturation of fatty acid residues in cellular membrane is needed for sustaining the required membrane fluidity at low temperatures. Despite activity of heterologous *desC* gene transgenic canola plants had no differences in tolerance to low positive temperature compared untransformed ones [23].

The aim of the present work was to analyze the transgenic canola growth under osmotic stress *in vitro* for future testing in greenhouse under drought because the correlation was shown of osmotic tolerance *in vitro* with drought resistance *in vivo* [7, 8].

Materials and methods. *Plant material.* Spring canola plants (*B. napus* L.) cvs Mariia and Obreey were used as the controls because they were initial material for genetic transformation in the experiments with *cyp11A1* and *desC/epsps* genes, respectively. Transgenic homozygous T_2 generation lines bearing *cyp11A1* gene ($T_2$1a and $T_2$2c) were obtained by self-pollination of primary (T_0) transformants under greenhouse conditions [21] and analyzed in this study. These lines were chosen because of the best growth parameters in seed germination experiments under heat [18]. The best lines expressing *desC/epsps* genes (18b (T_0 generation) and 18b/25 (T_1 generation) [19] were also investigated. The control and transgenic plants were propagated *in vitro* by grafting and were grown under same cultivation conditions (16/8 light/dark photoperiod, +23°C, 4000 – 5000 lux) before osmotic stress experiments in the test Sigma 25×150 mm tubes with 15 ml agar-solidified MS [13] medium for four weeks. Then the plant shoots bearing one fully expanded leaf were

cut off and immersed into 5 ml liquid MS medium supplemented with mannitol in the same Sigma tubes. The slips of filter paper were placed at the bottom of the tubes for better shoot location. Mannitol was added to liquid MS medium (0, 100 mM, 200 mM, 500 mM) before autoclaving for osmotic stress induction. Plants were grown under the same cultivation conditions for two weeks. Fresh weight (FW) was measured using the scale Pioneer™ PA413C (Ohaus Corporation, USA).

Total soluble protein (TSP) content was measured using Bradford's method [3]. Extracts from the upper plant leaves (100 mg) were prepared in triple volume of 50 mM Tris/HCl buffer, pH 8.0. The probe optical density was determined at 595 nm by BioPhotomether Eppendorf, v.1.35 (Germany). Bovine serum albumin (Fermentas) was used as standard for calibration curve.

Superoxide dismutase (SOD) activity was detected using the method based on photochemical oxidation of nitro blue tetrazolium [1]. Fresh plant material (100 mg) was pounded with 1 ml of 50 mM Tris-HCl buffer (pH 8.0) in a mixer mill Retsch MM 400 (Germany) and centrifuged at 13000 g (4°C) for 15 min. The supernatant was used for analyses. Formazan formation was held in an Eppendorf tube (1.5 ml) as described in [20].

Three independent experiments were conducted in five replications. Data were reported as means ± SD and calculated using Microsoft® Office Excel 2003 (Microsoft Corporation) standard functions. Data were analyzed statistically by the Student's *t*-test. Results were considered statistically significant when $p < 0.05$.

Results and discussion. Influence of *in vitro* osmotic stress on canola growth was investigated by evaluating such indexes as FW, TSP content, and SOD activity in the wild-type and biotech plants. $T_2$1a line bearing *cyp11A1* transgene produced FW up to 1.3-fold higher than the control plants in physiological conditions, similar to the control in medium with 100 mM mannitol, 55% and 100% above the control plants in media with 200 and 500 mM mannitol, respectively (fig.1). Thus, the expression of bovine *cyp11A1* gene in canola plants led to improvement tolerance to osmotic stress *in vitro* in term of higher FW production. Similar results were obtained under drought stress with transgenic canola that overexpressed wheat Mn SOD3.1 [7], and common

wheat lines that were transgenic for the *betA* gene encoding choline dehydrogenase from *Escherichia coli* [9]. There were no differences in FW between *desC/epsps* and wild-type plants both in normal conditions and osmotic stress (fig.1).

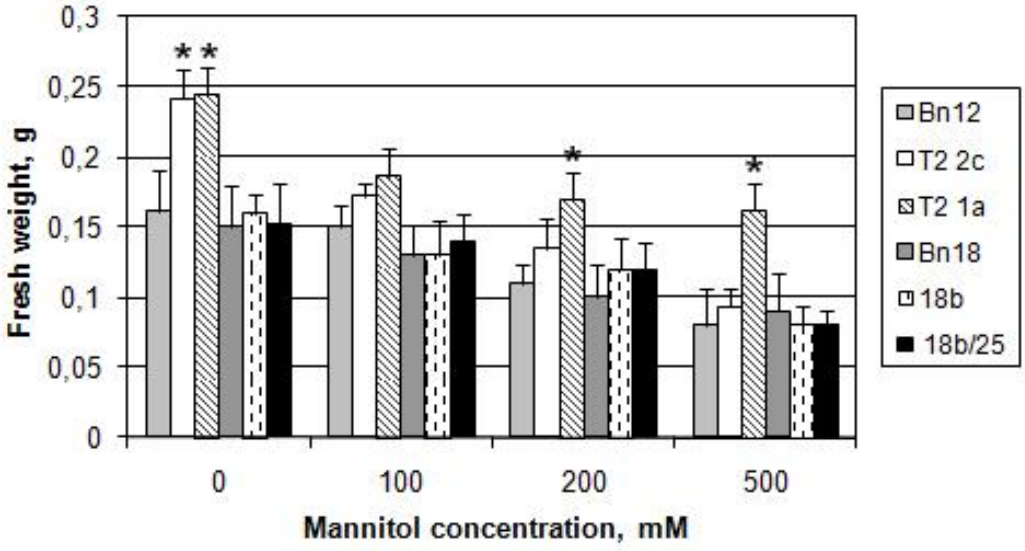

Fig.1. Fresh weight of control and biotech canola plants in osmotic stress growth. Here and in fig.2 and fig.3 *error bars* represent mean±one standard deviation and *asterisk* * indicates significant differences between experimental value compared with control ones ($p \leq 0.05$)

TSP content in canola *cyp11A1* lines was significantly higher (up to 1.36-fold) than in the initial plants under non-stressed conditions (fig.2). TSP of *cyp11A1* plants decreased to 26±1.98 mg/g FW in $T_2$2c line and 28±3.36 mg/g FW in $T_2$1a line, and got be similar to the control one (27.5±1.38 mg/g FW) in growth on the culture medium with the lowest mannitol concentration (100 mM). TSP content was not influenced both in the control and transgenic lines when stress pressure arose (200 mM and 500 mM mannitol). Protein accumulation similar the control level was documented in *cyp11A1* leaves during osmotic stress growth. No effect of salt stress on leaf soluble proteins was observed in two canola cultivars Dunkeld (salt tolerant) and Cyclon (salt sensitive) [16]. Leaf TSP decreased in barley *Hordeum vulgare* L. [22] seedlings in drought, and in leaves and roots of maize *Zea mays* under PEG-induced osmotic stress [12], in medical water hyssop *Bacopa monnieri* L. plants in NaCl and mannitol stresses [4].

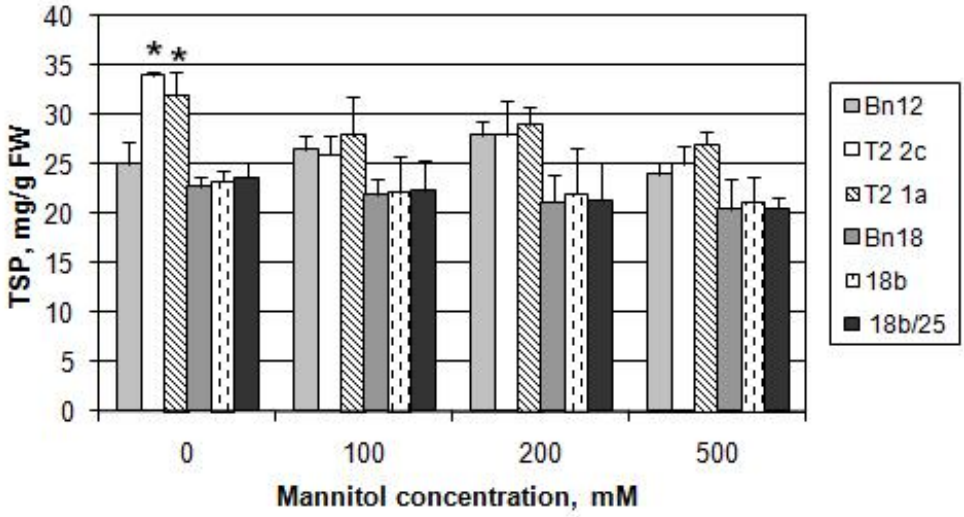

Fig.2. Total soluble protein content in leaves of control and biotech canola plants in osmotic stress growth.

In the absence of osmotic stress SOD activity in *cyp11A1* canola leaves was higher (51±3.53 U/mg protein for $T_2$1a line and 49±5.5 U/mg protein for $T_2$2c line) than in control ones (31±2.06) (fig. 3). When osmotic pressure rose (100 mM mannitol), SOD activity increased sharply in $T_2$2c transgenic line up to 1.7–fold comparing normal level, but it corresponded to the non-stressed level in the control and $T_2$1a plants. In $T_2$2c line SOD activity declined (62±1.5) and became comparable to the control (56±2.01) after mannitol increase in the medium up to 200 mM. At the same time SOD activity elevated markedly in $T_2$1a line by 51% (200 mM mannitol) and 100% (500 mM mannitol) above it in non-stressed conditions. This line was distinguished by SOD activity increase in hyperosmotic stress, while SOD activity lowered in the control (34±1.73 U/mg protein) and other transgenic *cyp11A1* line (53±1.96 U/mg protein). Changes in SOD activity were similar in *desC/epsps* canola and control plants under growth with- or without mannitol (fig.3).

The $T_2$1a *cyp11A1* line was characterized by the highest biomass production in the hyperosmotic stress induced by mannitol (fig. 1). At the same time it possessed

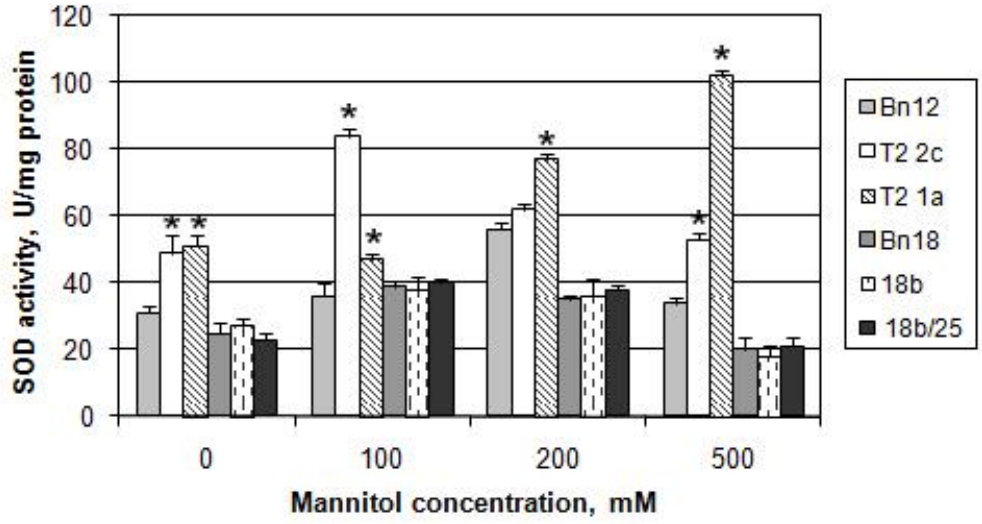

Fig. 3. SOD activity of the control and biotech canola plants in leaves under growth in osmotic stress.

the highest SOD activity (fig. 3) in these conditions. The similar pattern was observed in *Arabidopsis thaliana* seedlings expressing cytosolic Cu/Zn SOD of *Potentilla atrosanguinea* [5] in NaCl stress. It was suggested that SOD activity can be used as an indirect selection criterion for screening drought-resistant plant materials [25]. SOD activity increase due to heterologous gene expression often leads to plant growth improvement especially in unfavourable conditions. Adaptive plasticity of plants characterized by increased SOD activity is higher than one of plants with lowered SOD activity [5, 7, 10].

Conclusions. Physiological indexes such as FW, TSP, and SOD activity were higher in *cyp11A1* canola in non-stressed conditions. FW and SOD activity of these plants were higher and TSP was similar compared to the control plants under osmotic stress. Biotech *desC/epsps* lines showed no differences in comparison with untransformed plants both in normal and stressful conditions. Thus, adaptive plasticity to osmotic stress of c*yp11A1*canola was higher than ones of initial and *desC/epsps* plants in term of FW formation. Expression of heterologous cytochrome $P450_{SCC}$ in biotech plants led to increase in adaptive plasticity under osmotic stress, and activity of both alien DesC and EPSPS did not.

REFERENCES

1. *Beyer W.F., Fridovich I.* Assaying for superoxide dismutase activity some large consequences of minor changes in conditions // Anal Biochem. – 1987. – Vol.161, №2. – P. 559-566.
2. *Bhargava S., Sawant K.* Drought stress adaptation: metabolic adjustment and regulation of gene expression // Plant Breed. – 2013. – Vol.132, №1. – P. 21–32.
3. *Bradford M.M.* A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle to protein dye binding // Anal Biochem. – 1976. – Vol.72, №2. – P.248-254.
4. *Debnath M.* Responses of *Bacopa monnieri* to salinity and drought stress *in vitro* // J Med Plants Research. – 2008. –Vol.2, №11. – P.347–351.
5. *Gill T., Kumar S., Ahuja P.S., Sreenivasulu Y.* Over-expression of *Potentilla* superoxide dismutase improves salt stress tolerance during germination and growth in *Arabidopsis thaliana //* J Plant Genet & Transgenics. – 2010. – Vol.1, № 1. – P.1–10.
6. *Gusta L.* Abiotic stresses and agricultural sustainability // J Crop Improv. – 2012. – Vol.26, №3. – P.415-427.
7. *Gusta L.V., Benning N.T., Wu G. et al.* Superoxide dismutase: an all-purpose gene for agri-biotechnology // Mol Breed. – 2009. – Vol.24, № 2. – P.103–115.
8. *Hassanein A.M.A.* Establishment of efficient *in vitro* method for drought tolerance evaluation in *Pelargonium //* J Horticult & Ornament Plants. – 2010. – Vol.2, №1. – P.8–15.
9. *He C., Zhang W., Gao Q. et al.* Enhancement of drought resistance and biomass by increasing the amount of glycine betaine in wheat seedling // Euphytica. – 2011. – Vol. 177, № 2. – P.151-167.
10. *Jiang J., Su M., Chen Y. et al.* Correlation of drought resistance in grass pea (*Lathyrus sativus*) with reactive oxygen species scavenging and osmotic adjustment // Biologia. – 2013. – Vol.68, №2. – P.231–240.
11. *Los D. A., Murata N.* Structure and expression of fatty acid desaturases // Biochim. Biophys. Acta. –1998. – Vol.1394, № 1. – P. 3–15.

12. *Mohammadkhani N., Heidari R.* Effects of drought stress on soluble proteins in two maize varieties // Turk J Biol. – 2008. – Vol.32, №1. – P.23–30.

13. *Murashige T., Skoog F.A.* A revised medium for rapid growth and bioassays with tobacco tissue cultures // Physiol Plant. – 1962. – Vol.15, №3. – P.473-497.

14. *Park J.R., McFarlane I., Phipps R.H., Ceddia G.* The role of transgenic crops in sustainable development // Plant Biotechnol. J. – 2011. – Vol.9, № 1. – P. 2–21.

15. *Peterhansel C., Niessen M., Kebeish R.M.* Metabolic engineering towards the enhancement of photosynthesis // Photochem. Photobiol. – 2008. – Vol.84, №6. – P. 1317–1323.

16. *Qasim M., Ashraf M., Ashraf M.Y. et al.* Salt-induced changes in two canola cultivars differing in salt tolerance // Biol Plantarum. – 2003. – Vol.46, № 4. – P.629–632.

17. Reguera M., Peleg Z., Blumwald E. Targeting metabolic pathways for genetic engineering abiotic stress-tolerance in crops // Biochim Biophys Acta. – 2012. – Vol.1819. – P.186–194.

18. *Sakhno L.O.* Seed germination features of canola plants expressing mammalian cytochrome P450SCC *cyp11A1* gene // The Bulletin of Vavilov Society of Geneticists and Breeders of Ukraine.–2011. – Vol.9, №2.– P.253-259.

19. *Sakhno L.O., Gerasymenko I.M., Komarnitsskii I.K. et al*. Creation of glyphosate-resistant *Brassica napus* L. plants expressing DesC desaturase of cyanobacterium *Synechococcus vulcanus* // Biopolym Cell. – 2012. – Vol.28, №6. – P.449–455.

20. *Sakhno L.O., Slyvets M.S.* Superoxide dismutase activity in transgenic canola // Cytol Genet. – 2014. –Vol.48, №3. – P.145-149.

21. *Sakhno L.O., Morgun B.V., Kvasko O.Y., Kuchuk M.V.* Transformed canola plants expressing mammalian *cyp11A1* gene of cytochrome $P450_{SCC}$. Biotechnol Acta. – 2010. – Vol.3, № 5. – P.74–82.

22. *Sinoh T.N., Paleo L.G., Aspinall D.* Stress metabolism. I. Nitrogen metabolism and growth in the barley plant during water stress // Aust J biol Sci. – 1973. – Vol.26, №1. – P.45–56.

23. *Slyvets M.S, Sakhno L.O., Sheludko Y.V.* Expression of cyanobacterium *des*C transgene does not change canola plant cold resistance // The Bulletin of Vavilov Society of Geneticists and Breeders of Ukraine.–2013. – Vol.11, №2.– P.267-275.

24. *Spivak S.G., Berdichevets I.N., Litvinovskaya R.P. et al.* Some peculiarities of steroid metabolism in transgenic *Nicotiana tabacum* plants bearing the *CYP11A1* cDNA of cytochrome $P450_{SCC}$ from the bovine adrenal cortex // Russ J Bioorganic Chem. – 2010. – Vol.36, №2. – P.224–232.

25. *Zaefyzadeh M., Quliyev R.A., Babayeva S.M., Abbasov M.A.* The effect of the interaction between genotypes and drought stress on the superoxide dismutase and chlorophyll content in durum wheat landraces // Turk J Biol. – 2009. – Vol.33, №1. – P.1–7.

26. *Zlatev Z., Lidon F.C.* An overview on drought induced changes in plant growth, water relations and photosynthesis // Emir J Food Agric. – 2012. –Vol.24, №1. – P. 57-72.

АДАПТИВНАЯ ПЛАСТИЧНОСТЬ В УСЛОВИЯХ ОСМОТИЧЕСКОГО СТРЕССА БИОТЕХНОЛОГИЧЕСКИХ РАСТЕНИЙ РАПСА (*BRASSICA NAPUS* L.) С ТРАНСГЕНАМИ *CYP11A1* ИЛИ ОДНОВРЕМЕННО *DESC* И *EPSPS*

Л. А. Сахно

Для изучения толерантности к осмотическому стрессу растений рапса (Brassica napus L.), конститутивно экспрессирующих гены cyp11A1 или одновременно desC и epsps, которые кодируют бычий цитохром $P450_{SCC}$ или Δ9-десатуразу из цианобактерии Synechococcus vulcanus и бактериальную енолпируватшикиматфосфат синтазу, соответственно, при выращивании in vitro оценивали влияние различного осмотического давления, индуцируемого

маннитолом, на такие параметры, как сырая масса (СМ), суммарный растворимый белок (СРБ) и активность супероксиддисмутазы (СОД). В нормальных условиях линии с трансгеном cyp11A1 наращивали больше СМ и накапливали больше СРБ (на 30% и 36%, соответственно), а также обладали в 1,65 раза более высокой активностью СОД по сравнению с исходными растениями. В условиях осмотического стресса СМ и СОД активность растений с трансгеном cyp11A1 были выше и содержание СРБ было сравнимым с контролем. Биотехнологические линии с трансгенами desC/epsps не отличались от нетрансформированных растений и в условиях стресса, и без него. Адаптивная пластичность к осмотическому стрессу рапса с геном cyp11A1 выше, чем у исходных растений и линий с трансгенами desC/epsps. Это может обеспечиваться исходно повышенной СОД активностью.

***Ключевые слова**: Brassica napus; cyp11A1; desC; маннитол; осмотический стресс; СОД активность*

АДАПТИВНА ПЛАСТИЧНІСТЬ В УМОВАХ ОСМОТИЧНОГО СТРЕСУ БІОТЕХНОЛОГИЧНИХ РОСЛИН РІПАКА (*BRASSICA NAPUS* L.) З ТРАНСГЕНАМИ *CYP11A1* АБО ОДНОЧАСНО *DESC* І *EPSPS*

Л. О. Сахно

Для вивчення толерантності до осмотичного стресу рослин ріпака (Brassica napus L.), які конститутивно експресують гени cyp11A1 або одночасно desC і epsps, що кодують цитохром $P450_{SCC}$ бика або Δ9-десатуразу із ціанобактерії Synechococcus vulcanus і бактеріальну енолпіруватшикіматфосфат синтазу, відповідно, за умов вирощування in vitro оцінювали вплив різного осмотичного тиску, що його індукував маннітол, на такі параметри, як сира маса (СМ), сумарний розчинний білок (СРБ) і активність супероксиддисмутази (СОД). За нормальних умов лінії з трансгеном cyp11A1 нарощували більше СМ і накопичували більше СРБ (на 30% і 36%, відповідно), а також мали у 1,65 разів вищу активність СОД у порівнянні із вихідними рослинами. За умов осмотичного стресу СМ і СОД активність рослин з трансгеном cyp11A1 були вищими і вміст СРБ був подібний до контролю. Біотехнологічні лінії з трансгенами desC/epsps не відрізнялись від нетрансформованих рослин і в умовах стресу, і без нього.

Адаптивна пластичність в умовах осмотичного стресу ріпака з геном cyp11A1 вища, ніж у вихідних рослин і ліній з трансгенами desC/epsps. Це може забезпечуватися підвищеною СОД активністю, притаманною рослинам cyp11A1.

***Ключові слова**: Brassica napus; cyp11A1*; *desC*; маннитол; осмотичний стрес; СОД активність