

ANALYSIS OF SEED GENERATIONS OF BIOTECHNOLOGICAL WHEAT PLANTS AN ADDITIONAL COPY OF THE GENE ORGYTIN- δ -AMINOTRANSFERASE OF ALFALFA

Larysa BRONNIKOVA

Cultivation of biotechnological winter wheat progeny under water deficit and salinity conditions allowed to analyse the level of free proline and to relate it to grain productivity. Under stressful conditions, the superiority of free proline content in the vegetative organs of genetically modified wheat compared to the original forms was noted. It was shown that the main indicators of the yield structure of the seed generation of wheat with a functional transgene significantly exceeded the control variants under normal and stress conditions. The object of the study was T1–T4 variants of winter wheat, genotype UK-209, UK 322/17. The aim of this study was to determine the tolerance to water deficit of seed generations T1–T4 of genetically modified wheat with partially suppressed expression of the proline dehydrogenase (ProDH) gene based on physiological and biochemical parameters and economic characteristics of plants. We used the following research methods: determination of yield structure parameters and biochemical methods for determining L-proline (Pro).

The level of Pro- and analysed elements of productivity in the progeny of transgenic plants and their original forms under normal and insufficient water supply was investigated. The winter wheat plants of T1–T4 generations of genotype UK 322/17, UK 209 h were studied for resistance to water and salt stress. The responses to short-term salinity and water deficit associated with the accumulation of free proline, as well as the nature of recovery from stress were analysed.

Key words: proline, *Triticum aestivum* L., osmotic stress, drought, productivity, yield, resistance, transgenic plants. Department of Genetic Engineering, Institute of Plant Physiology and Genetics, National Academy of Sciences of Ukraine, Vasylykivska St., 31/17, Kyiv, 03022, Ukraine; email: Zlenko_lora@ukr.net

Аналіз насіннєвих поколінь біотехнологічних рослин пшениці з додатковою копією гена орнітин- δ -амінотрансферази люцерни

Броннікова Л.

Культивування нащадків біотехнологічних рослин пшениці озимої за дії водного дефіциту та засолення дозволило проаналізувати рівень вільного проліну та пов'язати з зерновою продуктивністю. За стресових умов відмічали перевагу вмісту вільного проліну у вегетативних органах генетично модифікованої пшениці порівнянні з вихідними формами. Показано, що за основними показниками структури врожаю, рослини насіннєвого покоління пшениці з функціональним трансгеном достовірно перевищують контрольні варіанти в умовах норми та стресу. Об'єктом дослідження були T1–T4 варіанти пшениці озимої, генотипу УК-209, УК 322/17. Метою даної роботи було встановлення толерантності до водного дефіциту насіннєвих поколінь T1–T4 генетично зміненої пшениці з частково пригніченою експресією гена проліндегідрогенази (ProDH) на основі фізіолого-біохімічних показників та господарських характеристик рослин. Нами було використано наступні методи дослідження: визначення показників структури врожаю та біохімічні методи визначення L-проліну (Pro).

Досліджено рівень Pro- та проаналізовані елементи продуктивності в нащадків трансгенних рослин та їх вихідних форм за нормального та недостатнього водопостачання. Досліджено рослини пшениці озимої T1–T4 поколінь генотипу УК 322/17, УК 209 h на стійкість до водного та сольового стресу. Проаналізовано реакції на дію короткострокових засолення і водного дефіциту, пов'язані із акумуляцією вільного проліну, а також характер відновлення після стресів.

Ключові слова: пролін, *Triticum aestivum* L., осмотичний стрес, посуха, продуктивність, врожайність, стійкість, трансгенні рослини.

Відділ генетичної інженерії, Інститут фізіології рослин і генетики НАН України, вул. Васильківська, 31/17, Київ, 03022, Україна; e-mail: Zlenko_lora@ukr.net

Introduction

In the last decade, the rapid increase in warming dynamics accompanied by a lack of precipitation has been of particular concern, which negatively affects crop productivity (De Melo et al. 2022; Kanwal et al. 2022). Therefore, the development of new cereal varieties with improved genetic backgrounds that are more tolerant to environmental stresses is one of the highest priority breeding objectives. Unlike resistance to abiotic stresses, which is mainly controlled by single genes, abiotic stresses are expressed by a multigene system, so controlling and engineering resistance to a particular negative impact is quite complex.

The development of plants resistant to abiotic stresses is based on the expression of genes involved in signalling and regulatory systems, in the process of triggering the synthesis of stress proteins, functional and structural metabolites.

In Ukraine, *Triticum aestivum* L. is the most widely grown cereal crop. It occupies more than 6 million hectares, which is more than 22 % of all cereal crops (Komisarenko et al. 2020; Mykhalska et al. 2023). Among all the natural factors that negatively affect the physiological processes of wheat growth and development and lead to a decrease in yields is water deficit caused by drought (Komisarenko et al. 2021; Abrarm et al. 2022; Cordea, Borsai 2021).

The harmful effect of drought is dehydration and disruption of metabolic processes in plants, which leads to protein breakdown, changes in the colloidal and chemical composition of the cell cytoplasm and, as a result, to a decrease in the amount of organic matter accumulated by plants (Palivoda et al.; 2021; Seleiman et al. 2021).

Particular attention is paid to the directions and possibilities of using genes that control proline (*Pro*) metabolism in plant engineering. To date, a number of important scientific results have been obtained in the field of plant transformation. Most of the interest is focused on the role of proline in osmotic regulation and enhancing the ability of plants to withstand cell dehydration caused by salinity, drought or extreme temperatures. The widespread use of proline as a stress adaptor molecule indicates that it plays a fundamental biological role in the stress response (Khoma et al. 2021; Zhang et al. 2022).

Partial suppression of proline dehydrogenase gene expression can lead to an increase in the content of L-proline and, as a result, the level of plant tolerance to abiotic stresses. The use of vector constructs in which the elements forming the double-stranded RNA suppressor are arranged as a reverse repeat of two exons and a nitron of the ProDH gene of *Arabidopsis thaliana* is promising. Inhibition of proline catabolism

gene expression in genetically modified plants occurs at the transcriptional stage as a result of short siRNA formation (Moumita et al. 2019; Zheng et al. 2009).

The ornithine δ -aminotransferase (*oat*) gene encodes the enzyme OAT (EC 2.6.1.13), which catalyses the transfer of the delta amino group of ornithine to alpha ketoglutarate to form pyrroline 5 carboxymylase (P5CS) and glutamate. This reaction is part of the system of interconversion of amino acids such as arginine, ornithine, glutamate and proline. Their metabolism is associated with the fixation, storage and recombination of nitrogen, seed formation and germination, resistance to various abiotic stressors, and regulation of growth and development processes. Therefore, ornithine δ -aminotransferase can be a regulator of cellular metabolism, as the reaction it catalyses links several biochemical systems (Dubrovna, Slivka 2022; Mykhalska et al. 2023).

However, the created genetically modified plants require further research in generations both in control and laboratory conditions. Obtaining a seed generation of transgenic winter wheat plants and testing them *in vivo*, where they will be exposed to multiple stresses that can suppress the protective effects of the embedded gene, will allow determining their level of resistance to abiotic stress, which is an important area of research.

Materials and methods

The aim of this work was to determine the tolerance to water deficit of seed generations T1–T4 of genetically modified wheat with partially suppressed expression of the proline dehydrogenase (ProDH) gene based on physiological and biochemical parameters and economic characteristics of plants. We used natural methods of research: determination of yield structure indicators and biochemical methods for determining L-proline (*Pro*).

The object of the study was winter wheat plants UK 209h, UK 322\17, selected by the Institute of Plant Physiology and Genetics, as well as the seed generation of biotechnological plants created on their basis and obtained as a result of T1–T4 propagation as a result of *Agrobacterium*-mediated in planta transformation, the transgenic status of which was confirmed by molecular genetic analysis (Dubrovna, Slivka 2022; Komisarenko et al. 2020; Komisarenko, Mykhalska 2023). The transformation was carried out using the strain from *A. tumefaciens* LBA4404, which contained the plasmid pBi2E with the target gene, a double-stranded RNA suppressor of the Arabidopsis proline dehydrogenase (ProDH) gene.

The level of resistance of transgenic plants progeny (T1–T4) was analysed under osmotic stress conditions, and the content of free proline in vegetative organs was studied *in vitro* and *in vivo*.

The object of the study was 10-day-old seedlings. The seeds of transgenic plants and controls selected for the experiment were a group of seeds randomly selected from the total crop. Mature seeds were germinated for 10 days in a semi-diluted solution of microelements according to Murashige-Skoog. The nutrient solution was changed every two days. On day 10, seedlings were transferred to simulated stress conditions for three hours.

Osmotic stress was created by adding mannitol or seawater salts to the specified nutrient solution. Mannitol, concentration 0.8 M, modelled water stress; sea water salts (sea salt), concentration 25.0 g/l, modeled natural complex salinity.

The content of free proline was determined separately in the aerial (seedling) and root parts on the 10th day of cultivation according to the standard method (Darm et al. 2016). Parameters measured under were considered as control parameters; ‘stress’ parameters were measured three hours after the onset of water or salt stress. The experiment was carried out in triplicate. The data were statistically processed.

Sampling for structural analysis of the crop was carried out during the period of full ripeness of grain

in 3 replicates. The experimentally obtained data were processed by mathematical statistics.

Results and discussion

In general, diagnosing the level of genotype viability at early stages of ontogeny is important for winter wheat breeding, as in Ukraine, periods of spring or autumn drought have become more frequent during grain germination.

When applying any biotechnology, the inheritance of a new trait in generations is always a necessary result. This is especially true when obtaining stress-resistant plants, as there are cases of meiosis disruption and elimination of the acquired characteristic (Mykhalska et al. 2022; Imran et al. 2021). Cultivation under water deficit will allow to establish the level of osmotolerance of plants, which depends on the functionality of the transgene and to link it to changes in the proline content, which will affect the crop yield (Komisarenko et al. 2023; Semiani et al. 2016).

For most higher plants, seeds are a stage of ontogeny that requires water for development, and a lack of water negatively affects germination. Winter wheat seeds were germinated under different conditions.

The addition of mannitol (lowering the osmotic pressure) caused a delay in germination of the grains, especially at the beginning of the experiment (day 5). This was probably due to the limited access to water,

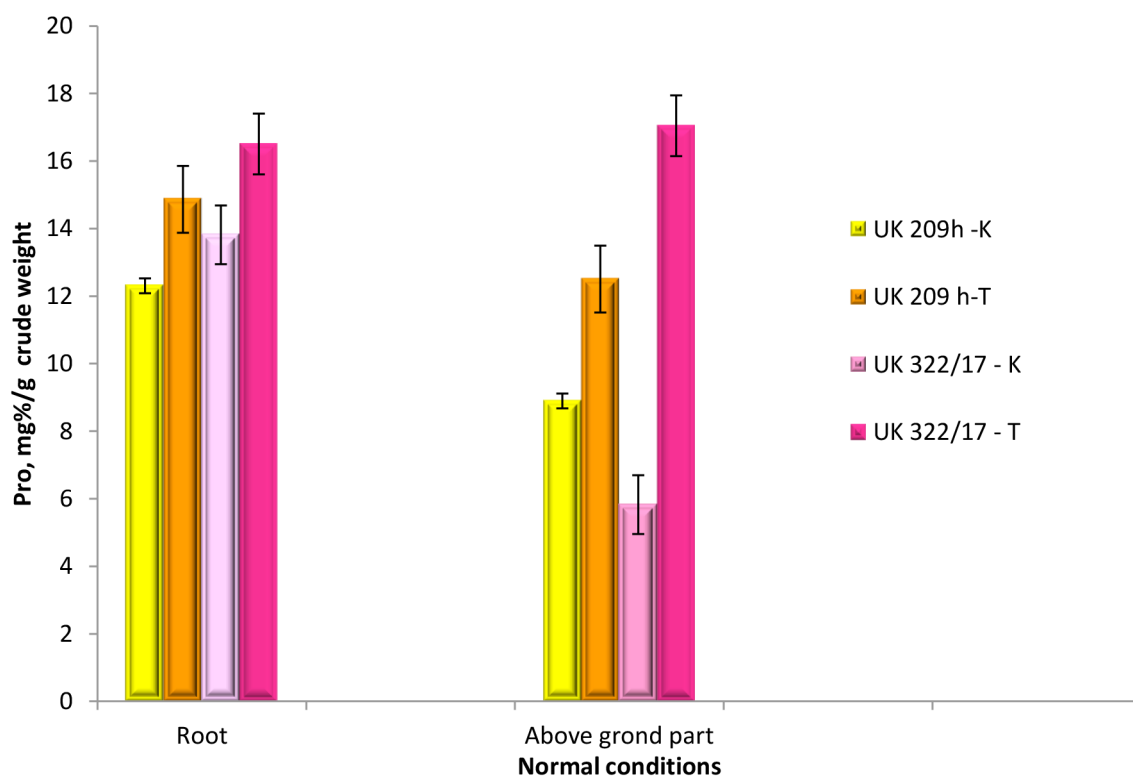


Fig. 1. Free proline (mg%/g crude weight) in vegetative organs of 10-day-old *Triticum aestivum* L. seedling under normal conditions

as it is known that mannitol can pull moisture from plant cells. This may be indicated by the indicators of the 7th day; the number of germinated grains increased. At the same time, the negative effect of stress was more significant in the genotype UK 322/17.

As a result of germination of mature grains, young seedlings with formed vegetative organs were formed on day 10. The appearance of the seedlings was similar in all variants, which may indicate that T1–T4 plants do not have advantages in germination over the original forms under normal conditions. Such physiological characteristics of genetically modified plants have been reported (Zheng et al. 2009). Therefore, the parameters of metric evaluation of external morphological structures at n.u. cannot be reliable indicators of the impact of the introduced transgene.

Under normal conditions, the content of free proline (*L-pro*) was measured separately in the aerial parts and roots of 10-day-old winter wheat seedlings (Fig. 1).

In the control plants UK 209h and UK 322/17, the vast majority of the amino acid is located in the roots. It is highly likely that this was due to differential gene expression in vegetative organs. The reason for this phenomenon can be considered the fact of redistribution of free proline, since the n.u. cultivation was maintained (Seleiman et al. 2019; Wang et al. 2022). In absolute terms, the content of free *L-pro* in the root parts of all genotypes was identical, which may also indicate a normal (similar) course of metabolism.

At the same time, no organ-dependent accumulation of proline was observed in the T-forms. Thus, it can be concluded that under normal conditions, 10-day-old winter wheat plants (control and T-forms) already show a difference in the nature of *L-pro* distribution in vegetative organs. At the same time, no differences were observed between the arrays UK 209h-K, UK 209h-T, UK 322/17-K, and UK 322/17-T.

Young seedlings of winter wheat genotypes were exposed to severe osmotic stress (mannitol, sea water salts) for three hours, after which the level of free *L-pro* in the aerial and root parts was analysed (Table 1). Under stressful conditions, a wider range of genotypic features was observed. In absolute

value, the level of amino acid in the form of UK 209h practically does not differ from the control values, and in the form of UK 322/17 the changes were in plant parts. At the same time, in the line UK 209h, the accumulation of free proline prevailed in both studied plant parts under mannitol stress; at the same time, in the original form UK 322/17, a higher level of *L-pro* was observed under salt stress (factor dependence).

Such events could be the result of different genotypic sensitivity to the stressor. At the same time, in plants of both variants, the level of free proline was maintained due to its synthesis.

In T plants (T1–T4 generations), a number of discrepancies with the normal values were also noted. Thus, in the line UK 209h-T, the level of free *L-pro* in the roots increased in significant amounts under the influence of salinity; in the aerial part, the accumulation of the amino acid was moderate, the same in the presence of any stressor. In both parts of the plants of UK 322/17-T the level of amino acid was almost the same or slightly lower than the values measured at n.u. In general, the trend of free *L-pro* accumulation in the line UK 209h-T can be characterised as stabilisation/increase, while in the case of the line UK 322/17-T there is a stabilisation/decrease. Thus, the fact of genotypic differences in this case becomes clear.

Since T1–T4 plants of both genotypes were seed progeny of transformants obtained as a result of the introduction of a single construct, the question arises as to the contribution of the transgene to the overall functioning of the systems regulating the level of free *L-pro*. The nature of proline distribution in parts of T plants may indicate the absence of active delivery of the transport system. The interaction of oxidation synthesis systems comes to the fore.

It is a well-established fact that amino acid synthesis increases under the influence of stress factors. At the same time, this event is additionally facilitated by a decrease/termination of the activity of the degradation gene. This event is genetically programmed for both conventional plants and GM organisms. Therefore, in our particular case, it is impossible to establish the contribution of the introduced construct to the inhibition of PDH activity. At the same time, given the

Table 1. Free proline content (mg%/g crude substance) in vegetative organs of 10-day-old winter wheat seedlings under simulated osmotic stresses

Genotype	Root		Aerial part	
	Manitol	Sea water salts	Manitol	Sea water salts
UK 209h	13,81 ± 3,44	8,90 ± 1,11	12,71 ± 0,78	8,02 ± 0,20
UK 209h-T	18,37 ± 2,76	35,60 ± 3,48	17,25 ± 4,13	16,33 ± 3,15
UK 322/17	5,17 ± 1,09	21,95 ± 3,71	12,83 ± 3,36	18,82 ± 2,11
UK 322/17-T	11,54 ± 2,36	16,54 ± 0,99	13,87 ± 2,85	16,31 ± 2,11

different trend of free L-pro accumulation in the lines UK 209h-T and UK 322/17-T, it is possible to assume that transgenesis interacts with endogenous genes, which will affect the viability of the organism under stressful conditions. It is likely that by prolonging the period of stress exposure, this phenomenon can increase the stress resistance of GM plants, since this characteristic is known to be a polygenic trait.

T-3 seeds were obtained from the transformed plants and used to study the osmotolerance of plants in a vegetation experiment. T-3 seeds were sown in 10-litre buckets filled with soil mixture. To simulate drought, the plants were switched to limited watering at the stage of tube emergence.

The duration of the artificial drought was three weeks. During the first week, soil moisture was maintained at 60 % of full moisture saturation, during the second – 50 %, and during the third – 40 %. Plant growth under water deficit conditions, free L-proline content and structural yield parameters were analysed.

The higher resistance to water deficit of T3 plants compared to the original ones was reflected in their growth pattern. Under normal irrigation, the average height of plants of the original variety and transformants was the same and averaged 66 cm. Under conditions of water stress at the stage of tube emergence, the average height of the original plants was approximately 45 cm, and the resistant plants had an average height of 55–60 cm.

The transgenic plants of the lines under osmotic stress were slightly inferior in terms of yield to the control plants grown under optimal conditions. However, the yield of most transformed lines was higher than that of non-transformed plants under soil moisture deficit (Table 2).

Thus, the use of the vector construct pBi2E containing a double-stranded RNA suppressor of the *pdh* gene is effective for the creation of transgenic durum wheat plants with an increased level of resistance to water deficit.

It was found that transgenic plants under normal conditions do not differ from control plants in morphological parameters and developmental

time, while under stress conditions the initial forms prevailed in terms of the main yield parameters. A positive correlation between the level of free L-proline and resistance of transgenic wheat plants to osmotic stress was found, which may be due to the influence of *L-proline* on the expression of other plant stress response genes or to the positive effect of increased content of this amino acid on resistance in the early stages of stress development.

Conclusions

1. Cultivation of control forms and progeny of biotechnological winter wheat plants under water deficit and salinity allowed to analyse the level of free proline and grain productivity. Under stressful conditions, the growth of free proline in the vegetative organs of winter wheat was observed. In the control forms UK 209 h and UK 322, the accumulation of the amino acid in the roots under the influence of mannitol was 13.81 mg/g of fresh weight and 5.7 mg/g of fresh weight, respectively. In T-plants UK-209 h and UK 322/17, the level of L-proline in the root part was 18.37 and 11.54 mg/g of fresh weight, respectively.

2. Under the influence of salinity, the accumulation of *Pro* in the aerial part of the control forms of UC 290 h was 8.02 mg/g of fresh weight, in UC 322/17 the level of amino acid increased to 18.82. In biotechnological plants in the aerial part, the level of *L-proline* in UK 290 h was 17.25 mg/g of fresh weight, in UK 322/17-16.31.

3. It was found that plants with reduced proline dehydrogenase activity are characterised by a significantly higher content of free L-proline compared to the control.

4. A positive correlation between the level of free *L-proline* and the resistance of transgenic wheat plants to osmotic stress was found.

5. It was shown that the main indicators of the yield structure, plants of seed generation T-3 of wheat with a functional transgene significantly exceed the control variants under normal and stress conditions.

Table 2. Indicators of yield structure of T3 and control plants under osmotic stress

Option	PH, cm	LMS, cm	GWMS, g	GWPP, g	WTG, g
Control (-)	92,2 ± 2,1	9,8 ± 0,7	1,4 ± 0,2	2,4 ± 0,3	29,6 ± 0,3
Control (+)	59,2 ± 6,0*	6,6 ± 1,2*	0,8 ± 0,1	1,7 ± 0,2*	23,4 ± 1,8
UK 322/17	85,2 ± 5,2*	9,7 ± 0,9	1,2 ± 0,1	2,2 ± 0,2	26,9 ± 0,7
UK 209 h	86,8 ± 4,0*	9,3 ± 0,7	1,2 ± 0,1	2,1 ± 0,2	26,8 ± 0,4

Note: PH – Plant height, LMS – Length of the main spike, GWMS – Grain weight in the main spike, GWPP – Grain weight per plant, WTG – Weight of a thousand grains, Control (-) – non-transformed plants grown without osmotic stress, Control (+) – non-transformed plants grown under osmotic stress conditions; * the difference between control and experience is significant when $p \leq 0,05$

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