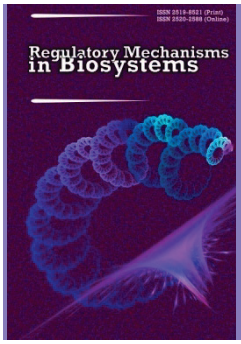

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Drought-tolerance of transgenic winter wheat with partial suppression of the proline dehydrogenase gene

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The global climate changes and the consequent increase in the number of soil and air droughts during the vegetation period of grain crops require the development of new strategies to adapt plants to those yield-decreasing stressors. A relevant way of increasing drought-tolerance of cereals is the use of biotechnological methods, particularly RNA interference, which can down-regulate the activity of plants' genes and increase concentration of stress metabolites that perform osmoprotective functions during drought. We studied the tolerance to soil moisture shortage in transgenic plants of winter wheat with partial suppression of the proline dehydrogenase gene, obtained using the technology of short interfering RNAs. We analyzed physiological and biochemical parameters and structural elements of yield productivity of 4 wild genotypes and their transgenic lines with reduced activity of proline dehydrogenase in the conditions of 7-day drought during the late booting–ear emergence. We determined that the presence of double-stranded RNA suppressor of the proline dehydrogenase gene in transgenic lines led to increase in the level of accumulation of free proline in flag leaves. At the same time, its concentration in transgenic lines was higher than in untransformed plants of the wild genotypes in both drought conditions and conditions of sufficient moisture. We found that against the background of water deficiency, the total chlorophyll content in leaves of plants of transgenic lines was significantly higher, and the ratio of carotenoids to chlorophyll was lower than in plants of the wild genotypes, suggesting mitigation of the negative impact of drought on the plants of transgenic lines. Lacking soil moisture, genetically altered lines of wheat had significantly higher parameters of the structure of grain yield compared with untransformed genotypes. At the same time, we observed genotypic difference according to grain productivity in biotechnological plants. Therefore, the results we obtained confirm the perspectives of using the technology of short interfering RNAs to increase tolerance of winter wheat to water deficiency.

Keywords: RNA-interference; water deficiency; proline; transgenic plants; chlorophylls; productivity.

Introduction

Wheat is one of the world's major food crops, cultivated in over 17% of arable lands and is consumed by around 40% of the world's population (Shewry, 2009). The wides distribution of this crop is due to its high biological flexibility regarding ecological conditions and high nutrition value of grains, from which many food products are made. Despite the general increasing tendency towards the global production of wheat, climate changes that are provoking significant temperature differences, unexpected precipitations and droughts, as well as emergence of new races of pathogens and pests, are significantly affecting wheat yield (FAO, 2020. Food outlook – biannual report on global food markets: http://doi.org/10.4060/ca9509en). Over recent decades, scientists have been especially concerned about the rapid spike in the dynamics of warming, accompanied by the absence of precipitations, which negatively impacts the yield productivity of this crop (El Sabagh et al., 2019; Hossain et al., 2021).

Drought is the main unfavoruable environmental factor that causes the greatest harm to fields of grain crops. On average, drought-caused losses of grain crops' yield in the main regions of cultivation accounted for about 14% over the recent two decades and have a tendency towards increase (Lesk et al., 2016). In Ukraine, droughts affect 10–30% of agricultural lands every two-three years and 50–70% every 10–12 years (Demyaniuk, 2015). Drought-related stress is the most harmful factor that negatively affects the yield through changes in growth, physiological and metabolic processes in the plant organism. The stressor provokes a complex of morpho-anatomical, physiological and biochemical changes, oriented at limiting water loss through stomata (Kapoor et al., 2020; Kiriziy & Stasik, 2022). The complexity and multi-component state of the reaction of plant organism to drought are significant challenges for the development of selective breeding drought resistance. Direct selection for productivity in the conditions of drought is complicated by the polygenicity of the trait, and therefore low hereditary, epistatic interaction of genes, significant dependence on genotype-environment interaction (Cattivelli et al., 2008).

The growing threats of the global climate changes and increasing frequency of extreme weather events require development of new strategies of plant adaptation to stresses. As of now, one such promising direction which can enhance the efficiency of creating new genotypes of cultivars that would be resistant to ecological stressors is the use of methods of biotechnology, in particular genetic engineering (Khan et al., 2015). Introduction of a small amount of heterologous genes into the genome of a recipient is a fast way to improve plants' tolerance (Hiei et al., 2014). Modern engineering strategies imply transferring of one or several genes that would encode either biochemical pathways or end points of the signal pathways (Joshi et al., 2017; Wang et al., 2017). Those genetic products provide a certain protection from ecological stresses, both direct and indirect.

Recently, cultivated crops have been genetically improved by means of biotechnologies using genes that control metabolism of "compatible" osmotically active substances – organic molecules that are able to concentrate in large amounts in plant cells during stress while having no toxic effect on growth and differentiation processes. Free proline is one of the most multi-functional stress metabolites in plants. Besides well-known

functions such as inert compatible osmoslith, proline – against the background of exposure to stress – carries out an array of other interrelated functions: membrane-protective, chaperone, antioxidant functions, and is involved in regulating the expression of some genes (Kolupaev et al., 2014; Meena et al., 2019; Ghosh et al., 2022). Likewise, it is an energy source and supports concentration of nitrogen and carbon contents (Sarker & Oba, 2020). The fundamental role of proline in osmotic regulation and increase in the ability of plants to oppose cell dehydration caused by salinization, drought or extreme temperatures have been studied quite well (Sripinyowanich et al., 2013; Hossain et al., 2014; Slama et al., 2015). Relatively low increase in the concentration of proline, typically at the beginning of stress action, performs regulatory and antioxidant roles, and higher concentration of it (at later stages of stress reaction) can carry out osmoprotective functions (Kolupaev et al., 2014).

Proline metabolism has a complex effect on growth of plants and reaction to stress. However, there are no doubts regarding the osmoprotective function of increasing their tolerance to abiotic stresses (Hossain et al., 2014; Kolupaev et al., 2014; Meena et al., 2019; Ghosh et al., 2022). Therefore, great attention is paid to identification and analysis of structural genes that control, among other things, synthesis and catabolism of proline. Over the recent years, a number of important scientific results have been obtained of both fundamental and practical significance in the sphere of transformation of a number of agricultural crops using genes of proline metabolism (Moiseeva et al., 2012; Mykhalska et al., 2014; Zhang et al., 2015; Komisarenko et al., 2016; Mykhalska et al., 2021; Anwar et al., 2021; Dubrovna et al., 2022).

Currently, it is possible to create genetically modified plants in such a way that the genetic construct would contain no protein-encoding transgenes. In this case, so-called genetic silencing is used, applied when there is a need to suppress or reduce the activity of one of a plants' own genes. This method is based on RNA-interference event, which is an effective natural mechanism of post-transcriptional modulation of gene expression. Discovery of short interfering RNAs-siRNAs (Hamilton et al., 2002) and recognition of their role in epigenetic silencing of genes gave principally new opportunities for genetic improvement of cultivated plants (Borsani et al., 2005; Fire, 2007; Maksimov et al., 2021). Understanding of the molecular basics of biogenesis of those short interfering RNAs, their functions as possible regulatory molecules, has brought new opportunities to regulate the processes of adaptation/tolerance of plants and led to development of a new direction of genetic engineering – siRNA technologies. Genetic silencing is of great practical significance because of the role of short RNAs as regulatory mechanisms of gene expression, for levels of their expression are in inverse relationship with the level of transcription of target genes. Molecular biotechnologies based on their use are of interest for the purposes of creating plants with heightened level of tolerance to abiotic stresses, including water shortage (Tishchenko, 2013; Dubrovna et al., 2022).

Level sof free L-proline could be increased by strengthening its synthesis or decreasing its degradation rates. The proline dehydrogenase gene (ProDH), associated with proline catabolism, has a great practical significance for genetic engineering, because partial suppression of its expression can increase proline concentration and, as a result, level of tolerance of plants to abiotic stresses (Tishchenko, 2013; Komisarenko et al., 2016; Ghosh et al., 2021). To suppress gene expression in plants using RNA interference, various genetic constructs are used. In particular, it was determined that a promising way to partially suppress ProDH gene is the use of vector constructs in which double-stranded RNA suppressor (dsRNA suppressor) is located as an inverse repeat fragment (Manavalan et al., 2012). Such a construct is assumed more to be effective for increasing the level of L-proline through RNA interference. Use of dsRNA suppressor of *Arabidopsis* ProDH gene produced increase in the level of proline accumulation (1.2–6.0-fold compared with non-transgenic control) in tobacco plants, characterized by heightened tolerance to salinization (Tateishi et al., 2005; Titov, 2008; Ibragimova et al., 2012). Positive experience of producing the construct containing double-stranded RNA suppressor of *Arabidopsis* ProDH gene was obtained for sunflower and maize, which as a result accumulated 1.5–9.0 times more proline and had higher tolerancesto water deficiency and salinization than control plants (Mykhalska et al., 2014; Tishchenko et al., 2014; Komisarenko et al., 2016). While the technologies of short interfering RNAs for dicotyledons have been quite successful, they have only recently begun to be applied to cereals, wheat in particular, and studies on this issue are quite rare (Mykhalska et al., 2021). The objective of our study was to evaluate the tolerance of transgenic plants of winter wheat with partial suppression of the proline dehydrogenase gene, obtained using technologies of short interfering RNAs under soil drought, based on comparative analysis of physiologic-biochemical parameters and agricultural characteristics of genetically modified and non-transgenic genotypes.

Materials and methods

The material for our studies was untransformed plants of new promising genotypes of soft winter wheat (Uk 065; Uk 095/17; Uk 209h; Uk 322/17), created in the Institute of Plant Physiology and Genetics of the National Academy of Sciences of Ukraine and transgenic lines obtained based on them. We studied 30 plants from each transgenic line and 30 plants of the wild genotype. Transgenic genotypes were obtained using the method of *Agrobacterium*-conditioned in planta transformation, carried out in the process of pollination using modified methods of Chumakov (Chumakov & Moiseeva, 2012). Anthers from flowers of the mother plant (castration) had been removed two-three days prior, after which pollen from father plants (pollination) was applied and inoculation with suspension of agrobacterial cells was done at the same time. Before and after applying suspension of cells of *Agrobacterium tumefacins*, the ear was isolated by parchment isolators, and pollination was performed using pollen of plants of therespective variety.

In the experiments with transformation, we used strain of *Agrobacterium tumefaciens* AGL0. Plants were transformed by binary vector pBi2E (Fig. 1a), which includes inverted repeat, comprising fragments of two copies of the first exon and intron of gene of *A. thaliana* proline dehydrogenase, and also selective gene of neomycin phosphotransferase II (nptII) of *E. coli*.

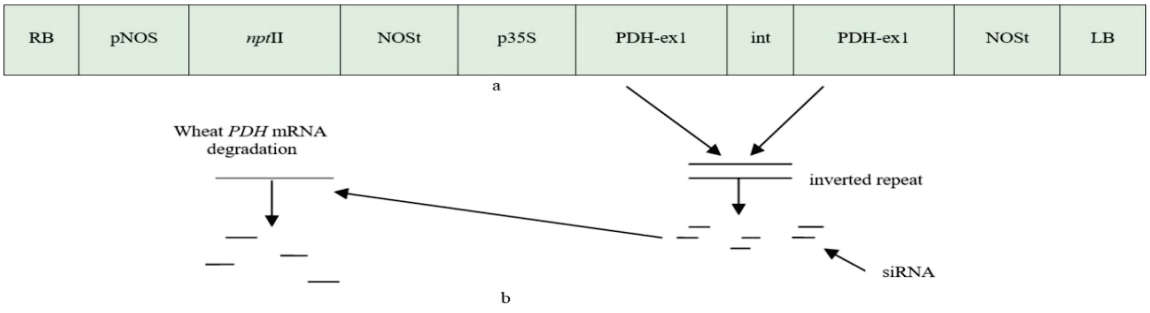

Fig. 1. Schematic image of T-DNA of genetic construct of pBi2E: pNOS – promoter of nopaline synthase gene; p35S pro – promoter of 35S RNA of virus of mosaic of cauliflower (CaMV); PDH-ex1 – first exon of pdh gene (construct contains two fragments, located as inverse repeat); int – fragment of first intron of pdh gene; ntpII – neomycin phosphotransferase of II *E. coli*; NOSt – gene terminator of nopaline synthase, polyadenylation signal; LB – left border) of T-DNA, RB – right border of T-DNA (*a*), predicted mechanism of partial suppression of endogenic ProDH genes of wheat by post-transcription silencing of RNA through formation of siRNA (*b*)

Its encoding part has areas that have a significant level of homology to ProDH genes of cultivated plants. As a result of their complementation, they interact with a copy of the target gene, which is a signal for enzymes to cut it by enzymes into short fragments that would be unable to provide synthesis of complete protein. This results in formation of short interfering RNAs (siRNAs), leading to partial fragmentation of endogenic ProDH genes of wheat (Fig. 1b).

Genetic transformation was carried out in the conditions of vegetative experiment, and seeds were collected at the end of vegetation. Genetically modified variants were selected according to marker sign, adding 100 mg/L of kanamycin antibiotic to the growth medium, blocking neomycin phosphotransferase and causing whitening of leaves of non-transgenic variants. Through self-pollination of obtained T1 transgenic plants, plants of T3 grain generation were obtained. Integration of the elements of vector construct was determined using the PCR method according to presence of fragments of exon and intron of the ProDH1 *Arabidopsis* gene and selective gene of neomycin phosphotransferase – *npt*II.

Plants that had been selected for the analysis for presence of the construction were put into 10 L vegetative pots, which were then filled with soil mixture (soil : sand = 3 : 1) and cultivated at a special plot. In one half of the pots, wild and transgenic forms (15 plants of each wild genotype and 15 plants of each transgenic line) were cultivated in the conditions of normal water supply – 70% of field capacity. In the other half of the pots (15 of transgenic and non-transgenic plants of each genotype), the soil moisture was reduced to 30% of the field capacity at flag leaf sheath opening (GS 47) by terminating watering (Zadoks et al., 1974) and this condition was maintained for 7 days. Soil moisture was controlled by gravimetric method. After the drought, plants of the variants with 30% of the field capacity were watered together with the control until the phase of complete grain ripeness.

The biochemical parameters and content of photosynthetic pigments in theflag leaves were determined on the 7th day after the beginning of the drought. The average sample for determining the biochemical parameters and content of pigments was formed of flag leaves of the main and lateral shoots. The activity of the enzyme, concentrations of proline, chlorophyll, carotenoids were determined in 5 replicates.

The activity of proline dehydrogenase was evaluated using the method (Mattioni et al., 1997) according to the rates of expenditure of NAD+ for proline oxidation, measuring increase in NADH concentration that had formed per unit time. The concentration of free proline in leaves was determined using the method based on formation of colour substance through interaction of L-proline and ninhydrin (Bates et al., 1973).

The chlorophyll *a*+*b* and total carotenoids content were determined by non-maceration method of extraction of pigments from leaves using dimethyl sulfoxide according to the method of Wellburn (1994). To analytically determine the content of pigments, we extracted pigments using 10 mL of dimethyl sulfoxide (USA, 2020) from the general sample of finely fragmented leaves of 100 mg of leaves. Then, the samples were put in a water bath (the water temperature of 60 ºC) for 4 h. After cooling to the room temperature, 0.5 mL of the obtained extracts was diluted using 4.5 mL of dimethyl sulfoxide. Optical density of this solution was determined using a spectrophotometer (Specord 200, AnalyticJena, Germany, 2012) at the wavelengths of 480, 649 and 665 nm. The content of photosynthesis pigments was calculated taking into account the volume of the solution, amount of dimethyl sulfoxide for dilution and amount of optical density. Re-calculation of the content of pigments to g of dry weight was carried out taking into account all dilutions and weight of leaves.

The structural parameters of yield productivity were evaluated according to amount of grain and grain weight from the main ear, and also from plants and weight of thousand grains. We used 10 plants for each variant of the analysis of structural yield parameters. The obtained data were statistically analyzed using ANOVA and the Tukey HSD Test. The results are presented as mean values and standard deviation (m ± SD). Difference between the data was considered significant at $P < 0.05$.

Results

In variants where the soil moisture was maintained at the level of 70% of field capacity, the activity of enzyme of proline dehydrogenase in flag leaves of wild genotypes varied 1.92 to 3.18 nmol of NAND/min x mg of protein, whereas plants of transgenic lines had somewhat lower values of this parameter – 1.40 to 2.36 nmol of NAND/min x mg protein (Fig. 2). Compared with the wild genotypes, their transgenic lines were characterized by somewhat lower activity of the enzyme. Functionality of the introduced construct and RNA interference of the ProDH gene were confirmed by average 20–28% decrease in the enzyme activity in the group of the examined genotypes.

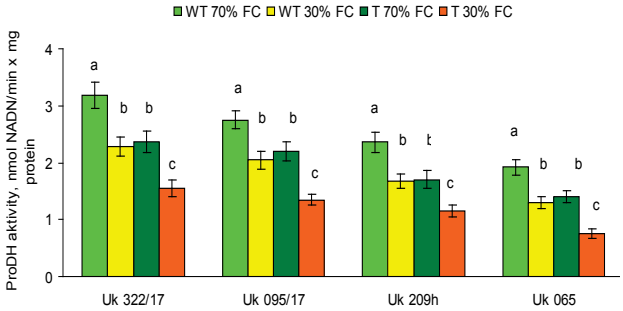

Fig. 2. The activity of proline dehydrogenase in the flag leaves of wild types (WT) of winter wheat and its transgenic (T) lines with reduced activity of the ProDH gene on the 7th day of drought (30% of field capacity, 30% FC) and under conditions of normal moisture supply (70% of field capacity, 70% FC): x ± SD, different letters in columns indicate values significantly different in the proline dehydrogenase activity for each genotype, Tukey test ($P < 0.05$), n = 5

Compared with the physiological conditions, the activity of proline dehydrogenase in plants of wild genotypes, as well as those obtained based on genetically altered lines, decreased during drought. Therefore, it accounted for 25.5–32.2% in plants of wild genotypes, and 32.4–46.4% in plants of transgenic lines. Thus, the transgenic lines were characterized by greater decrease in the activity of proline dehydrogenase, which manifested in both the physiological conditions and during stress, as evidenced by partial suppression of ProDH gene in plants of transgenic lines.

The content of L-proline in leaves of plants of the wild genotypes in the optimal conditions of watering ranged 2.14 to 3.03 μm/kg fresh weight and was 1.6–1.9 times higher in the transgenic plants, measuring 3.54–5.73 μm/kg fresh weight (Fig. 3). In the conditions of drought, the content of this aminoacid increased in both untransformed and transgenic plants. In transgenic lines that contained the suppressor of the ProDH gene, it varied 12.21 to 26.06 mg/kg fresh weight and exceeded such in plants of the wild genotypes by 2.3–3.0 times (6.23–8.88 mg/kg fresh weight).

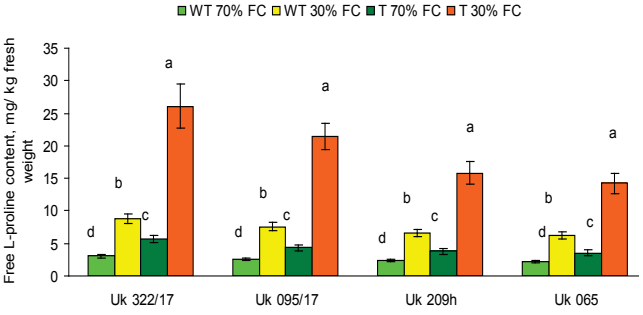

Fig. 3. The free L-proline content in the flag leaves of wild types (WT) of winter wheat and its transgenic (T) lines with reduced activity of the ProDH gene on the 7th day of drought (30% of FC) and under conditions of normal moisture supply (70% of FC): symbols – see Fig. 2

Under the drought, the free proline content in the leaves of the untransformed genotypes was 2.7–2.9 times greater than in the variety with normal moisture supply, while in genetically modified forms, it increased by 4.0–4.9 times.

Thus, transgenic plants were characterized by heightened content of L-proline in both the norm and under stress. Therefore, presence of the double-stranded RNA suppressor of the ProDH gene in transgenic plants leads to decrease in the activity of the enzyme, resulting in increase in the

level of accumulation of free L-proline in both close-to-optimal conditions and soil moisture shortage.

The content of the main photosynthetic pigment – chlorophyll – in flag leaves of plants of wild genotypes and their transgenic lines in the conditions of sufficient water supply remained within a narrow range. Chlorophyll *a*+*b* contents overall ranged 10.57 to 11.86 mg/g dry weight in the first of them and 10.38 to 11.76 mg/g dry weight in the second (Fig. 4).

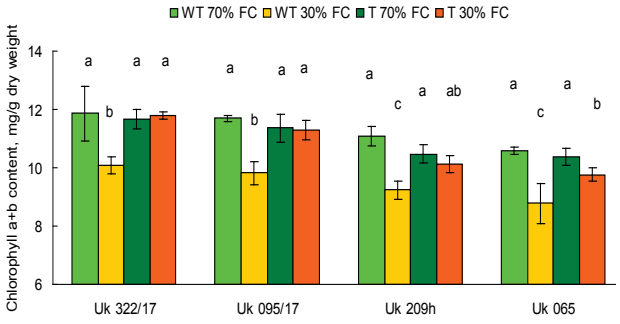

Fig. 4. Chlorophyll *a*+*b* content in flag leaves of wild types (WT) of winter wheat and its transgenic (T) lines with reduced activity of the ProDH gene on the 7th day of drought (30% of FC) and under conditions of normal moisture supply (70% of FC): symbols – see Fig. 2

Drought caused decrease in the content of total chlorophyll in flag leaves of the wild genotypes to 83–85%, compared with the physiological conditions. Its concentration in leaves of three genetically modified lines was at the level of respective variants with sufficient water supply; in the transgenic line Uk 065, we observed the tendency towards decrease: 9.76 ± 0.23 mg/g dry weight, compared with optimal water supply, measuring 10.38 ± 0.68 (Fig. 4). Also, we found that after the 7-day drought, the content of total chlorophyll in flag leaves of the wild genotypes was lower than in transgenic lines (Fig. 4). After stress, the greatest difference between plants of the wild genotype and transgenic line – 17% was observed in line Uk 322/17, 15% difference was seen in Uk 095/17 and 10–11% – in two other lines.

By the content of additional photosynthetic pigments – carotenoids – in the conditions of sufficient water supply, the wild genotypes and their transgenic lines Uk 322/17 and Uk 095/17 had no significant differences, while transgenic lines of genotypes Uk 209 and Uk 065 were characterized by lower (by 5–6%) content of overall carotenoids compared with non-transgenic plants (Fig. 5). Under stress, total carotenoid content in leaves of plants of the wild genotype Uk 322/17 was 16% lower than in the variant with sufficient water supply, while such a difference in the other three genotypes was insignificant. Under the drought, this parameter in leaves of transgenic plants of Uk 322/17 was higher than in the wild genotype, and, by contrast, was lower in line Uk 065, and insignificantly different from the wild lines in Uk 095/17 and Uk 209.

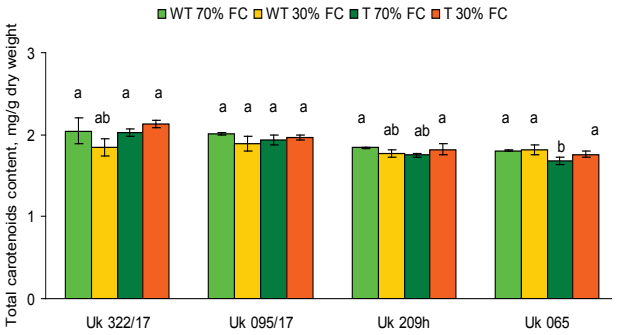

Fig. 5. Total carotenoids' content of flag leaves of wild types (WT) of winter wheat and its transgenic (T) lines with reduced activity of the ProDH gene on the 7th day of drought (30% of FC) and under conditions of normal moisture supply (70% of FC): symbols – see Fig. 2

Despite the fact that the carotenoids to chlorophyll *a*+*b* ratio depends mostly on the light conditions (Demmig-Adams, 1996), this parameter is also used to evaluate the physiological condition, photosynthetic activity, changes in the development (aging) and reaction of plants to stress (Merzlyak et al., 1999; Zhouet et al., 2019; Gitelson, 2020; Souahi, 2021; Zeng et al., 2021). We determined that plants of the wild genotypes and transgenic lines were not significantly different according to ratio of content of carotenoids to chlorophyll in the conditions of sufficient moisture, while under the drought this ratio was lower (Table 1).

Table 1

The carotenoids to chlorophyll *a*+*b* ratio in flag leaves of plants of wild genotypes and transgenic lines with reduced activity of the ProDH gene on the 7th day of drought (30% of FC) and under conditions of normal moisture supply (70% of FC) (x ± SD, n = 5)

Variant	Genotype	Uk 322/17	Uk 095/17	Uk 209 h	Uk 065
70% of FC	wild type	0.172 ± 0.002^{bc}	0.172 ± 0.001^{b}	0.166 ± 0.005^{ab}	0.171 ± 0.001^{bc}
	transgenic line	0.173 ± 0.007^{b}	0.170 ± 0.004^{b}	0.167 ± 0.003^{ab}	0.175 ± 0.007^{b}
30% of FC	wild type	0.189 ± 0.004^{a}	0.193 ± 0.001^{a}	0.191 ± 0.005^{a}	0.192 ± 0.003^{a}
	transgenic line	0.181 ± 0.003^{b}	0.174 ± 0.002^{b}	0.180 ± 0.008^{a}	0.180 ± 0.003^{b}

Note: different letters indicate statistically significant differences (Tukey test, $P < 0.05$) in the carotenoids to chlorophyll ratio for each genotype.

Therefore, we determined that after 7-days drought during the critical period of the development of wheat, the content of total chlorophyll in flag leaves of transgenic lines with decreased activity of the ProDH gene was significantly higher (by 10–17%) than in plants of the wild genotypes. Lower values of the carotenoids to chlorophyll ratio may indicate that transgenic lines retained higher photosynthesis activity while exposed to stress. The analysis of the yield structure elements of the main shoot of plants of the wild genotypes and plants of T_3 generation of genetically modified lines of winter wheat in the conditions of sufficient moisture revealed that differences between them were insignificant (Table 2). They were also insignificantly different according to height of the main shoot.

Under the drought, the number of grains from the main shoot of plants of the wild genotypes and transgenic lines did not differ, and at the same time, we observed a difference in their number between various genotypes: it was the highest in Uk 322/17 and the lowest in Uk 065 (Table 2). However, weight of grains and weight of 1,000 grains of transgenic lines was higher than in wild genotypes. Therefore, the weight of grains from the main shoot ear of transgenic lines Uk 322/17, Uk 095/17 and Uk 209 h accounted for 118–123% of the corresponding wild genotype. Weight of 1,000 grains in plants of transgenic lines exceeded such in the wild genotypes by 8–24%. The main shoots of plants of genetically modified lines of wheat were also 4–5 cm higher than in the wild genotypes. Over the 7-day drought, we observed decrease in the analyzed parameters of yield in all genotypes, though it was higher in the wild genotypes. Their grain productivity of the ear of the main shoot accounted for 71–76%, compared with the variant with 70% of field capacity, whereas in transgenic plants, it equaled 78–88%, and the weight of 1,000 grains decreased to 71–76% and 79–89%, respectively.

In the conditions of sufficient moisture, the differences according to the elements of the yield structure between plants of the wild genotypes and genetically modified lines of winter wheat were insignificant (Table 3). At the same time, in the conditions of drought, the number of grains from plants of transgenic lines Uk 322/17 and Uk 095/17 was 10–11% higher than in the respective wild genotype. Weight of grains from plants and weight of 1,000 grains in all transgenic lines was significantly higher (respectively by 0.4–0.6 and 3–6 g), compared with the wild genotypes. Drought caused decrease in all those parameters of the wild genotypes (Table 3). Therefore, the number of grains from those plants accounted for 89–92% of such in the variant with 70% of field capacity, weight of grain – 76–81%, compared with the variant with 70% of field capacity, weight of 1,000 grains – 82–92%. In transgenic lines Uk 322/17 and Uk 095/17, decrease in all the elements of the yield structure, caused by the action of the 7-day drought in the critical period, was insignificant compared with the control variant (Table 3). During the drought, biotechnological plants were characterized by comparatively higher grain yield of plants.

Table 2
Height and elements of the yield structure of the main shoot of plants of wild genotypes and genetically modified lines of winter wheat under conditions of sufficient moisture supply (70% of FC) and drought (30% of FC) (x ± SD, Tukey test, n = 10)

Traits	Variant	Genotype	Uk 322/17	Uk 095/17	Uk 209 h	Uk 065
Number of grains, pcs.	70% of FC	wild type	48.10 ± 4.20[a]	45.70 ± 4.74[a]	42.40 ± 4.97[a]	39.40 ± 3.98[a]
		transgenic line	48.80 ± 4.80[a]	47.00 ± 3.39[a]	43.80 ± 4.92[a]	41.20 ± 4.98[a]
	30% of FC	wild type	41.90 ± 4.33[a]	41.30 ± 4.45[a]	40.10 ± 3.84[a]	37.80 ± 4.26[a]
		transgenic line	46.30 ± 5.87[a]	43.40 ± 2.59[a]	40.20 ± 4.21[a]	38.50 ± 3.14[a]
Grain weight, g/ear	70% of FC	wild type	2.22 ± 0.19[a]	2.08 ± 0.19[a]	1.90 ± 0.24[a]	1.59 ± 0.17[a]
		transgenic line	2.29 ± 0.18[a]	2.17 ± 0.13[a]	1.98 ± 0.23[a]	1.77 ± 0.21[a]
	30% of FC	wild type	1.66 ± 0.16[b]	1.58 ± 0.11[c]	1.41 ± 0.12[b]	1.25 ± 0.11[b]
		transgenic line	2.02 ± 0.26[a]	1.86 ± 0.14[ab]	1.73 ± 0.16[a]	1.38 ± 0.09[b]
Weight of 1,000 grains, g	70% of FC	wild type	46.21 ± 1.45[a]	45.61 ± 1.79[a]	44.70 ± 1.04[a]	40.39 ± 1.39[a]
		transgenic line	47.00 ± 1.58[a]	46.28 ± 1.61[a]	45.14 ± 1.15[a]	42.93 ± 1.40[a]
	30% of FC	wild type	39.57 ± 1.50[c]	38.34 ± 2.09[b]	35.11 ± 0.83[b]	33.24 ± 0.89[c]
		transgenic line	43.42 ± 1.46[ab]	42.79 ± 1.59[ab]	43.10 ± 0.96[a]	35.92 ± 1.06[b]
Height, cm	70% of FC	wild type	94.10 ± 9.48[a]	85.90 ± 10.24[a]	91.00 ± 8.04[a]	86.00 ± 6.67[a]
		transgenic line	98.80 ± 10.02[a]	89.60 ± 11.68[a]	96.30 ± 2.26[a]	90.60 ± 3.31[a]
	30% of FC	wild type	85.30 ± 4.16[b]	76.60 ± 3.69[ab]	88.00 ± 5.54[ab]	79.00 ± 4.62[ab]
		transgenic line	93.30 ± 2.98[a]	82.30 ± 6.93[a]	95.30 ± 2.11[a]	86.50 ± 4.38[a]

Note: different letters – see Table 1.

Table 3
Elements of the yield structure of whole plants of wild genotypes and genetically modified lines of winter wheat under conditions of sufficient moisture supply (70% of FC) and drought (30% of FC) (x ± SD, Tukey test, n = 10)

Traits	Variant	Genotype	Uk 322/17	Uk 095/17	Uk 209 h	Uk 065
Number of grains, pcs.	70% of FC	wild type	89.20 ± 7.47[a]	84.30 ± 9.42[a]	78.20 ± 9.51a	75.00 ± 8.76[a]
		transgenic line	89.30 ± 7.92[a]	85.90 ± 9.00[a]	82.10 ± 6.92[a]	78.30 ± 9.81[a]
	30% of FC	wild type	81.20 ± 9.99[a]	77.40 ± 7.79[a]	73.30 ± 7.66[a]	66.60 ± 6.20[a]
		transgenic line	90.30 ± 9.97[a]	85.20 ± 8.52[a]	76.80 ± 9.73[a]	70.10 ± 8.27[a]
Grain weight, g/plant	70% of FC	wild type	3.88 ± 0.26[a]	3.41 ± 0.42[a]	3.12 ± 0.34[a]	2.68 ± 0.35[a]
		transgenic line	3.84 ± 0.34[a]	3.59 ± 0.36[a]	3.26 ± 0.22[a]	2.99 ± 0.37[a]
	30% of FC	wild type	3.01 ± 0.35[b]	2.75 ± 0.30[b]	2.41 ± 0.27[b]	2.05 ± 0.19[b]
		transgenic line	3.57 ± 0.35[a]	3.37 ± 0.29[a]	2.79 ± 0.34[ab]	2.30 ± 0.30[b]
Weight of 1,000 grains, g	70% of FC	wild type	41.81 ± 1.87[a]	38.17 ± 4.30[a]	39.64 ± 3.15[a]	33.67 ± 3.29[a]
		transgenic line	42.40 ± 2.64[a]	40.90 ± 2.16[a]	40.47 ± 4.01[a]	37.34 ± 1.72[a]
	30% of FC	wild type	36.93 ± 1.41[b]	35.16 ± 1.55[ab]	32.58 ± 1.04[b]	30.51 ± 0.88[b]
		transgenic line	40.85 ± 4.66[a]	37.99 ± 3.91[a]	35.83 ± 2.46[a]	33.60 ± 3.55[ab]

Note: different letters – see Table 1.

Discussion

As is known, RNA interference is an efficient natural mechanism of post-transcriptional modulation of gene expression. It controls the activation of genes by forming short double-stranded RNAs and synthesizing special ribonucleases that induce the selective degradation of target RNAs or inhibition of their transcription or replication. The mechanism of RNA interference plays an important role in the regulation of development, epigenetic modification and response reactions of plants to impacts of various harmful natural factors (Lee & Carroll, 2018) and are currently used as a powerful tool to improve resistance of plants to biotic and abiotic stressors (Maksimov et al., 2021).

The process of RNA interference has started to be used in the modern genetic engineering of plants. Therefore, transgenic *Arabidopsis* plants with altered levels of ProDH activity were created (Mani et al., 2002), which accumulated 25% more proline than wild plants and were more tolerant to low temperatures and salinization. The analysis of tobacco plants, transformed using the vector construct, containing the double-stranded RNA suppressor of *Arabidopsis* ProDH gene, exhibited their heightened tolerance to salinization (Titov, 2008), which was accompanied by 1.2–6.0 times greater accumulation of proline than in non-transgenic control. Also, sunflower and maize plants with similar vector constructs accumulated 1.5–9.0 times more of this aminoacid and were different from non-transgenic by heightened tolerance to water deficiency and salinization (Moiseeva et al., 2012; Tishchenko et al., 2014; Komisarenko et al., 2016).

The studies were performed on transgenic plants of winter wheat of various genotypes that bore the double-stranded RNA suppressor of *Arabidopsis* ProDH gene also demonstrated a 2.3–3.0-fold increase in the level of free proline compared with untransformed forms and heightened tolerance to soil drought. The parameters of grain yield of biotechnological plants subject to stress significantly exceeded those of the wild genotypes, which could be related to higher content of proline in them, which plays a key role in increasing the tolerance of plants to abiotic stress and overcoming its consequences. This aminoacid is able to form hydrophilous colloids that imbibe water and protect plant proteins from destruction during drought, take part in chlorophyll synthesis and optimize water intake, its distribution and metabolism (Meena et al., 2019). Proline was shown to be essential for the normal development of anthers and seeds (Funck et al., 2012), a significant amount of it is transported to the reproductive organs of plants, where its accumulation is a signal to begin flowering (Kolupaev et al., 2014). Accumulation of this osmolyte can cause decrease in the formation of AOS, decrease in lipid peroxidation and preservation of integrity of membranes of thylakoids (Monirul et al., 2015; Rehman et al., 2016; Yu et al., 2017; Kaur et al., 2018), in particular by induction of intensification of antioxidant protection (Hayat et al., 2013; Abdelaal et al., 2020; Uhr et al., 2022). Positive effect of accumulation of the level of free proline on tolerance of transgenic plants to soil moisture deficiency could also be related to the effect of L-proline on the expression of other genes of stress response of plants or positive effect of heightened content of this aminoacid on resistiance at early stages of stress development, and also induction of antioxidant protection of chloroplast thylakoid membranes.

Because one of the links sensitive to water deficiency is photosynthesis apparatus, the physiological and biochemical parameters related to it may be indirect markers of drought tolerance. Therefore, during drought, there is a decrease in the amount or degradation of the main photosynthesis pigments (Liu et al., 2015; Kolupaev, 2016; Rehman et al., 2016). The important role of pigment apparatus in drought tolerance of wheat is indicated in a number of reports revealing positive dependence between chlo-

rophyll content in leaves and photosynthesis intensity (Liu et al., 2016; Sharifi et al., 2016). Furthermore, grain productivity of winter wheat on the 4th and 14th days of drought (30% of FC) was demonstrated to be closely associated with chlorophyll in flag leaf (Morgun et al., 2016).

In the transgenic lines of winter wheat which we examined, increase in the content of free proline was accompanied by a higher level of chlorophyll (10–17%). Similar results were obtained for genetically modified plants of sunflower using the same vector construct, in which the content of chlorophyll (*a*, *b*, *a*+*b*) and carotenoids exceeded such of untransformed forms (Komisarenko et al., 2016). A similar tendency – higher concentration of chlorophyll in genotypes with higher content of free proline – was observed in the studied reactions of pigment apparatus of modified plants that bore genes encoding transcription factors involved in the regulation of processes in resistance of plants to moisture deficiency. Therefore, it was revealed that proline in the flag leaf of transgenic (At-DREB1A) and T2 non-transgenic lines of Lasani-08 variety in the control did not differ, whereas in the conditions of a 15-day drought, it was significantly higher in transgenic plants (Noor et al., 2018). Chlorophyll *a* contents in those lines and in the control did no differ significantly, and chlorophyll *a* was higher in the transgenic line. In the conditions of 15-day drought, the concentrations of both forms of chlorophyll was significantly (1.9–2.4 times, compared with the non-transgenic line) higher than in transgenic plants (Noor et al., 2018). During two-year field experiments, transgenic lines of wheat of T5 and T7 generations, transformed by SeCs-pA genes, had higher content of proline and chlorophyll, compared with the wild line (Yu et al., 2017). At the same time, in the conditions of drought, the lines with higher concentration of proline were observed to have lower decrease in chlorophyll content (44–46%) than control plants (57%). Similar results were obtained after comparing plants of the wild variety of Fielder spring wheat and its modified lines bearing promoter of TaWRKY2 gene from drought-tolerant Xifeng 20 wheat: transgenic lines had higher content of chlorophyll and proline (Gao et al., 2018).

It has to be noted that such a tendency during drought – higher content of free proline accompanied by higher content of chlorophyll – was also found in non-transgenic plants. Therefore, leaves of seedlings of hard wheat with higher proline content were also observed to have higher concentration of chlorophyll (Othmani et al., 2019). Positive correlation between the content of proline and chlorophyll (with correlation coefficient ranging 0.331 to 0.535) was seen in each of the 3 variants of osmotic stress induced by polyethylene glycol (PEG 6,000) with concentrations equaling 5%, 10% and 15% and in the control variant, as revealed by the examination of seedlings of 15 genotypes of wheat according to morpho-physiological and biochemical parameters (Sharma et al., 2022). At the same time, relationship between proline content and intensity of photosynthesis was even closer ($r = 0.507–0.681$) (Sharma et al., 2022).

This assumption regarding improvement of the status of pigment apparatus in plants with heightened content of free proline may be confirmed by the data on increase in chlorophyll content after exogenous treatment of wheat with proline. In particular, when cultivating wheat in the dehydrated conditions (15% PEG-6000) and addition of proline to the solution for cultivation, the overall content of a and b chloropylls in the 3rd leaf of 4-week old seedlings was 1.4 times higher than in the control seedlings (without exogenous proline) (Bekka et al., 2018). Proline concentration in those variants differed by 1.2 times. In seedlings of the high-protein Beinong 9549 variety, cultivated under influence of another – cadmium – stressor, after exogenous treatment by three concentration of proline, the content of *a* and *b* chlorophylls in leaves exceeded such in the control (Song et al., 2013). Lower degradation of chlorophyll, compared with the control, was observed in leaves of 5-week old wheat of both varieties treated by proline against the background of cadmium stress. At the same time, such a treatment affected the chlorophyll *a* content to various degrees: difference between treated and control plants was insignificant in Millat-2011 variety, and significant in Punjab-2011 variety (Rasheed et al., 2014). The effect of exogenous proline treatment on content of chlorophyll *b* was significant for both varieties.

Based on the aforesaid, we may assume presence of interrelation between the content of free proline and chlorophyll. This is first of all related to the fact that accumulation of proline in leaves of tolerant genotypes in the conditions of stress is one of the adaptive reactions of drought tolerance. Its accumulation decreases the formation of free radicals and is associated with lipid peroxidation (Kaur et al., 2018). Secondly, heightened proline content can lead to mitigation of the negative impact of water shortage on functionality and structural changes in chloroplasts and can support the functional activity of photosynthesis apparatus (Monirul et al., 2015; Rehman et al., 2016).

Therefore, to provide stability in yield during impacts of unfavourable factors, including drought, it is important to determine metabolic strategies that would promote obtaining resistant genotypes by integration of recombinant DNA molecules to genome of cultivated plants, which would be able to control processes at genetic level. The results of such studies would be useful for selective breeding programs working on new varieties of wheat with heightened drought tolerance.

Conclusions

Compared with non-transgenic plants, we determined the effect of partial suppression of the proline dehydogenase gene on accumulation of proline in transgenic plants, conditions of sufficient moisture, as well as in drought conditions. Against the background of water shortage, higher content of total chlorophyll and lower ratio of carotenoids to chlorophyll in leaves of plants of transgenic lines – as compared with the wild genotypes – may suggest alleviation of negative impact of soil drought on plants of transgenic lines. Lacking soil moisture, structure of grain yield of genetically modified plants with lowered activity of the ProDH gene significantly exceeded such of untransformed plants. Biotechnological plants were observed to have genotypic difference according to grain productivity.

Complex analysis of physiological-biochemical characteristics and agricultural features of transgenic plants of winter wheat with double-stranded RNA suppressor of proline dehydrogenase gene, in comparison with non-transgenic genotypes, indicated increased tolerance to soil drought in biotechnological plants. Using the technology of short interfering RNAs proved to be efficient in creating transgenic plants of winter wheat with heightened level of tolerance to water deficit.

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