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Anthocyanins Participate in the Protection of Wheat Seedlings against Cadmium Stress

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Due to anthropogenic activity, the environment is contaminated with high levels of cadmium, which is a dangerous heavy metal. At very low concentrations, cadmium is bioaccumulative and toxic to animals and plants, generating reactive oxygen species (ROS) that are destructive to cells of organisms. Anthocyanin pigments are natural antioxidants produced in various plant tissues and play a protective role under different environments. In the present study, the putative role of anthocyanins that accumulate in the grains and shoots of bread wheat (*Triticum aestivum* L.) in response to cadmium-induced toxicity (25 and 50 μM CdCl_2) was studied at the seedling stage. For this purpose, a set of near-isogenic lines carrying different alleles of the *Pp* (purple pericarp) and *Rc* (red coleoptile) genes was used. The lines responded differently to Cd treatment. The observed changes in anthocyanin metabolism under stress conditions were dependent on the alleles of the *Rc* genes that determine coleoptile pigmentation and on CdCl_2 concentration. In less-colored line carrying the *Rc-A1* allele, the antioxidant system was unable to fully cope with oxidative stress and thus induced the synthesis of additional antioxidants, whereas in the most tolerant lines, which have dark-purple coleoptile pigmentation predetermined by *Rc-A1+Rc-D1*, the level of anthocyanins in the coleoptiles was independent of stress. A protective role of anthocyanins presented in the coleoptiles of wheat seedlings was observed under moderate Cd stress (25 μM), whereas anthocyanins seemed to be ineffective as protective compounds under heavier stress.

Keywords: *Triticum aestivum* L., heavy metal, intensity of stress, root and shoot lengths

Introduction

Heavy metals are major environmental pollutants. Heavy metals enter the soil, water, and air as a result of both natural processes (weathering of rocks, volcanic activity) and human economic activity (mining, metallurgy, chemical industry, transport, mineral fertilizing) (Kulaeva and Tsyganov 2011). Among heavy metals, Cd is one of the most dangerous and widespread contaminant elements. It is highly toxic to plants and animals even at very low concentrations.

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In plants, one of the harmful effects of Cd is the generation of reactive oxygen species (ROS), such as superoxide radical, hydrogen peroxide, and hydroxyl radical, that cause oxidative stress and disrupt the biochemical and physiological functions of cells (Azevedo et al. 2012).

To cope with ROS, plants have an antioxidant protective system. This system includes both enzymatic antioxidants, such as superoxide dismutase, glutathione reductase, catalase, glutathione and ascorbate peroxidases, that detoxify H₂O₂ and non-enzymatic antioxidants, such as ascorbate and glutathione, vitamins, flavonoids, alkaloids, and carotenoids (Ahmad et al. 2010). Among the non-enzymatic antioxidants, colored flavonoid compounds anthocyanins can also be considered. These compounds demonstrate a powerful antioxidant capacity that is almost four times greater than that of ascorbic acid and α -tocopherol (Bors et al. 1994; Wang et al. 1997). The intensification of anthocyanin biosynthesis has been observed under different types of stresses, including heavy metal stress (Chalker-Scott 1999; Landi et al. 2015b). Anthocyanins can chelate some heavy metal ions, preventing oxidative stress and the translocation of the harmful heavy metals ions (Landi 2015a).

Bread wheat can accumulate anthocyanins in vegetative and generative organs (Khlestkina 2012), but the protective functions of the pigments in wheat tissues with respect to the cadmium-induced toxicity have not been studied. The goal of the present study was to characterize the putative protective role of anthocyanins of wheat grains and coleoptiles in response to Cd stress. To investigate this issue, a precise genetic model – a set of near-isogenic lines (NILs) differing by the allelic state of genes conferring purple grain (*Pp*) and coleoptile (*Rc*) color – was used.

Materials and Methods

Plant material

The following wheat genotypes differing in anthocyanin pigmentation of the pericarp and coleoptile were used: (1) cultivar ‘Saratovskaya 29’ (‘S29’), which has a light-red coleoptile controlled by the dominant *Rc-A1* allele and a non-colored pericarp predetermined by a combination of *Pp* alleles – *Pp-A1* + *pp-D1* + *pp3*; (2) near-isogenic lines (NILs) developed in the ‘S29’ background (Arbuzova et al. 1998) that have an additional *Rc* gene (*Rc-D1*) providing dark-red coleoptile pigmentation and two dominant complementary genes (*Pp-D1* and *Pp3*) that determine the dark-purple pigmentation of the pericarp, inherited from cultivars ‘Purple Feed’ (PF) and ‘Purple’ (P). These lines, designated i:S29*Pp-A1Pp-D1Pp3*^{PF} (‘iPF’) and i:S29*Pp-A1Pp-D1Pp3*^P (‘iP’), respectively, have been previously characterized using microsatellite markers (Tereshchenko et al. 2012; Gordeeva et al. 2015) and currently are maintained in the ICG collection “GenAgro”.

Experimental procedure

One-day-old germinated seedlings growing on filter paper at 20 °C under a 12-hour photoperiod in a “Rubarth Apparate” growth chamber (RUMED GmbH, Laatzen, Germany) were exposed to 0 (control), 25 or 50 µM CdCl₂. From the 3rd to the 7th day after germination, the lengths of shoots and roots were measured. The experiment was performed in triplicate for each concentration and each genotype, with ten seedlings per replicate. Seeds produced in the same year and under the same climatic conditions were used.

Anthocyanin extraction and measurement

For anthocyanin content evaluation in coleoptile of seedlings, three NILs were grown in the same conditions as described above (20 seedlings were grown per replicate). Every day from the 3rd to the 7th day after germination, coleoptiles from four seedlings were pooled and homogenized in 1% HCl/methanol (5 ml per 1 g of plant material). The mixture was incubated at 4 °C for 24 hours and was centrifuged at 10,000 g for 30 min at 4 °C. The collected supernatant was used to measure the relative anthocyanin content at a 530-nm wavelength (OD530) using a SmartSpec Plus spectrophotometer (Bio-Rad, USA).

The assessment of anthocyanin content in the grain was performed at the wax-ripeness stage. For each genotype, anthocyanins were extracted in triplicate. Seeds coats for each replicate were peeled from the grains of one spike. The anthocyanin extraction procedure was the same as that described above.

Statistical analysis

The data were recorded as the mean value ± standard deviation (n = 3). Significant differences between treated and control samples of the NILs were assessed using the non-parametric Mann–Whitney *U*-test, with $p \leq 0.05$ being significant. Changes in root length were calculated as follows:

$$\left(\frac{\text{root length under treatment}}{\text{root length without treatment}} - 1 \right) \times 100\%.$$

The changes in shoot length and OD530 values of anthocyanin extracts were calculated in the same manner. The non-parametric Kruskal–Wallis *H*-test was used to determine the influence of the factor ‘color’ on the changes of growth parameters of wheat seedlings. Spearman’s rank correlation coefficients between parameters were calculated using the software Statistica v. 6.1 (StatSoft, Inc.).

Results

Changes in shoot and root length

Under control conditions, the root lengths did not differ between the lines. The longest shoots were observed in ‘iP’ (from the 5th to the 7th day after germination) and in ‘iPF’ (on the 6th day) (Table 1). Cd affected morphological parameters. The lengths of shoots and

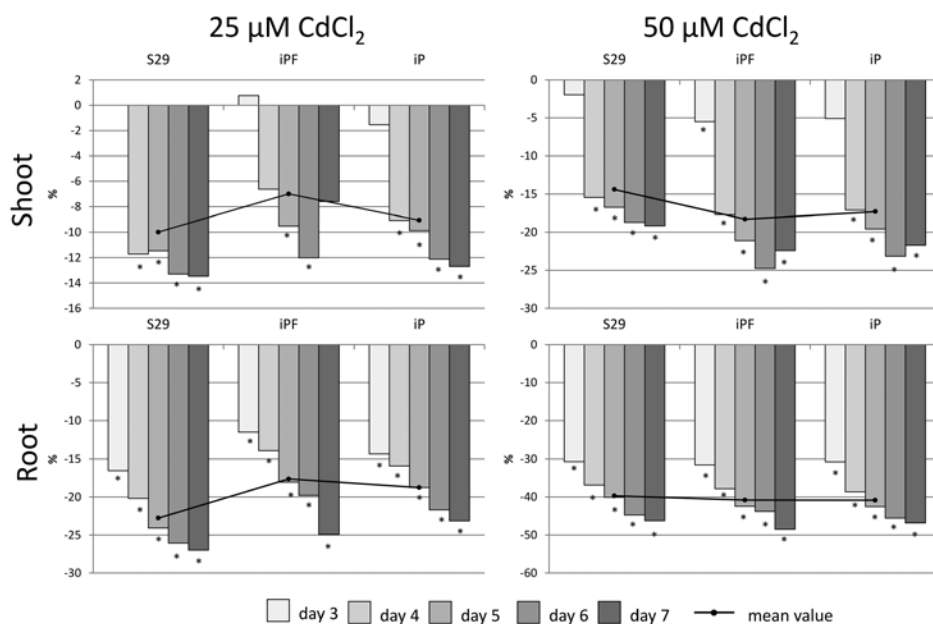


Figure 1. Changes in shoot and root length of 'S29', 'iPF' and 'iP' in response to cadmium chloride exposure relative to those of the control (%). *Significant differences between stressed and control samples at $p \leq 0.05$ (*U*-test)

roots of the lines decreased significantly after 25 and 50 µM CdCl₂ treatments in comparison with those of the untreated control samples (Fig. 1).

The level of change varied among lines. After 25 µM CdCl₂, the shoot length of 'S29' changed from 0.0 to -13.6% (-10.0% on average throughout the experiment); the shoot length of 'iPF' and 'iP' varied from +0.8 to -12.0% (-7.0%) and from -1.5 to -12.7% (-9.1%), respectively. A reduction in root length after 25 µM CdCl₂ occurred from -16.5 to -27.0% (-22.8%) in 'S29', whereas in 'iPF' and 'iP', root length varied from -11.5 to -24.9% (-17.6%) and from -14.3 to -23.1% (-18.8%), respectively.

After 50 µM CdCl₂ treatment, the shoot length changed from -2.0 to -19.2% (-14.4%) in 'S29', from -5.5 to -24.8% (-18.3%) in 'iPF', and from -5.1 to -23.1% (-17.3%) in 'iP'. The root length changed from -30.7 to -46.3% (-39.7%) in 'S29', from -31.6 to -48.5% (-40.8%) in 'iPF', and from -30.8 to -46.8% (-40.9%) in 'iP'.

The differences between the lines were more drastic after exposure to 25 µM CdCl₂ than after exposure to 50 µM CdCl₂.

There were negative correlations between the shoot length of control plants and the extent of the shoot length reduction under Cd stress ($r_s = -0.60$, $p \leq 0.05$) and between the root length of control plants and extent of the root length reduction ($r_s = -0.45$, $p \leq 0.05$).

Table 1. Shoot and root lengths of 'S29', 'iPF' and 'iP' and relative anthocyanin contents in coleoptiles under control and heavy metal stress

Growth parameter	Day	Control			25 µM CdCl ₂			50 µM CdCl ₂		
		S29	iPF	iP	S29	iPF	iP	S29	iPF	iP
Shoot length, mm	3	20.5±0.7 ^a	21.9±1.1 ^a	21.7±1.1 ^a	20.5±0.8 ^a	22.0±1.0 ^a	21.4±0.2 ^a	20.1±0.2 ^a	20.7±0.2 ^b	20.6±1.0 ^{ab}
	4	44.0±1.0 ^a	45.8±2.4 ^a	45.7±1.9 ^a	38.8±1.6 ^a	42.7±2.3 ^b	41.6±1.2 ^b	37.2±0.9 ^a	37.7±1.3 ^a	37.9±2.4 ^a
	5	68.6±1.0 ^a	72.2±2.5 ^{ab}	72.6±1.3 ^b	60.8±1.9 ^a	65.3±2.8 ^b	65.4±0.9 ^b	57.1±0.8 ^a	56.9±1.7 ^a	58.4±2.8 ^a
	6	92.9±1.4 ^a	98.0±2.1 ^b	98.7±1.6 ^b	80.5±2.1 ^a	86.3±3.4 ^b	86.7±2.3 ^b	75.5±1.8 ^a	73.8±1.7 ^a	75.9±4.2 ^a
	7	115.4±1.2 ^a	117.7±9.3 ^{ab}	123.4±2.9 ^b	99.8±3.1 ^a	108.7±2.5 ^b	107.7±3.6 ^b	93.3±1.3 ^{ab}	91.3±2.8 ^a	96.6±3.1 ^b
	3	35.6±1.0 ^a	36.3±1.6 ^a	36.3±1.0 ^a	29.7±1.5 ^a	32.2±1.6 ^a	31.1±1.0 ^a	24.6±2.8 ^a	24.9±2.0 ^a	25.1±3.0 ^b
	4	58.2±0.3 ^a	58.2±0.9 ^a	57.3±2.1 ^a	46.4±1.4 ^a	50.1±3.0 ^b	48.2±1.5 ^{ab}	36.7±3.1 ^a	36.2±2.2 ^a	35.1±3.4 ^a
Root length, mm	5	73.6±2.0 ^a	74.7±2.0 ^b	74.1±3.0 ^a	55.9±1.2 ^a	61.2±2.9 ^b	60.2±2.6 ^b	44.1±4.5 ^a	43.0±3.8 ^a	42.6±4.9 ^a
	6	87.4±2.6 ^a	85.2±4.9 ^a	85.9±3.3 ^a	64.6±2.5 ^a	68.3±0.9 ^a	67.2±3.5 ^a	48.3±5.2 ^a	47.9±3.3 ^a	46.8±5.8 ^a
	7	99.9±1.2 ^a	101.1±12.2 ^a	98.9±4.4 ^a	73.0±3.1 ^a	75.9±2.6 ^a	76.0±4.6 ^a	53.7±5.6 ^a	52.1±3.8 ^a	52.6±6.1 ^a
	3	0.55±0.03 ^a	2.27±0.19 ^b	2.27±0.43 ^b	0.61±0.05 ^a	2.13±0.15 ^b	2.09±0.13 ^b	0.62±0.09 ^a	2.48±0.67 ^b	1.62±0.26 ^c
	4	0.45±0.03 ^a	1.56±0.25 ^b	1.54±0.03 ^b	0.51±0.07 ^a	1.84±0.24 ^b	1.75±0.13 ^b	0.58±0.07 ^a	1.94±0.08 ^b	1.53±0.12 ^c
	5	0.43±0.08 ^a	2.03±0.33 ^b	1.58±0.21 ^b	0.53±0.03 ^a	1.87±0.15 ^b	2.10±0.30 ^b	0.54±0.13 ^a	1.84±0.16 ^b	1.52±0.11 ^c
	6	0.42±0.01 ^a	1.71±0.25 ^b	1.80±0.31 ^b	0.52±0.08 ^a	1.59±0.25 ^b	1.45±0.48 ^b	0.50±0.01 ^a	1.36±0.22 ^b	1.39±0.17 ^b
7	0.49±0.02 ^a	1.56±0.20 ^b	1.46±0.23 ^b	0.55±0.02 ^a	1.76±0.28 ^b	1.42±0.31 ^b	0.46±0.06 ^a	1.36±0.42 ^b	1.48±0.21 ^b	

Values are means of three replications ± standard deviations. The same superscript letter indicates the absence of statistically significant differences between the lines.

Changes in anthocyanin content

Under non-stress conditions, ‘S29’ (a carrier of the *Rc-A1* gene) had approximately 4-fold lower OD530 values (relative anthocyanin content) in coleoptiles in comparison with those of intensively colored NILs (‘iPF’ and ‘iP’ – the carriers of the *Rc-A1* + *Rc-D1* genes) (Table 1). ‘iPF’ and ‘iP’ coleoptiles had similar anthocyanin contents (Table 1), while their grains contained significantly different amounts of these pigmented substances. The OD530 of ‘iPF’ grain extracts was 2.3 ± 0.2 , while that of ‘iP’ was 3.5 ± 0.3 . Anthocyanins were absent in the grains of ‘S29’.

Under cadmium chloride exposure, the anthocyanin level in coleoptiles changed differently in different lines (Fig. 2). A statistically significant increase in anthocyanin content in the coleoptile was recorded in ‘S29’ on the 6th day under $25 \mu\text{M CdCl}_2$ and on the 4th and 6th days under $50 \mu\text{M CdCl}_2$. The anthocyanin content in coleoptiles did not change in ‘iPF’ under both concentrations of cadmium chloride; however, the anthocyanin content increased in ‘iP’ (on the 4th day) under $25 \mu\text{M CdCl}_2$ but decreased (on the 3rd day) under $50 \mu\text{M CdCl}_2$ (Fig. 2).

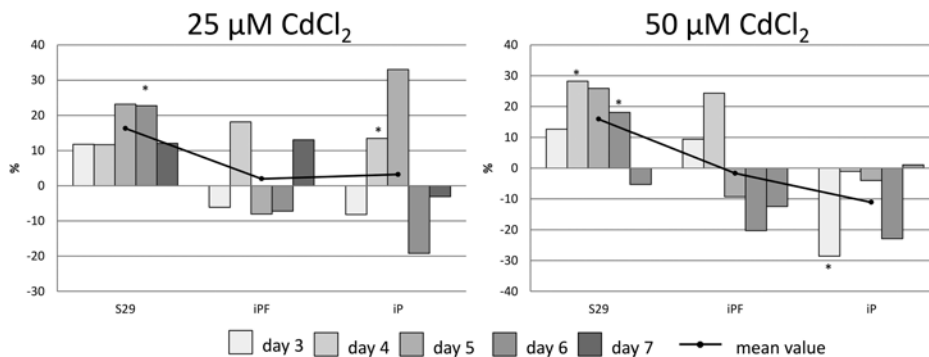


Figure 2. Changes in anthocyanin contents in coleoptiles of ‘S29’, ‘iPF’ and ‘iP’ in response to cadmium chloride exposure relative to those of the control (%). *Significant differences between stressed and control samples at $p \leq 0.05$ (*U*-test)

Influence of the coleoptile and grain color on growth parameters

One-way ANOVA on ranks was run to evaluate the effects of color on growth parameter changes in wheat seedlings under Cd stress. As summarized in Table 2, two groups of samples (‘non-colored’ vs ‘colored’) were significantly different with respect to anthocyanin content in coleoptiles and grains under control conditions, in changes in shoot length under $25 \mu\text{M CdCl}_2$ and in changes in anthocyanin content in coleoptiles under $50 \mu\text{M CdCl}_2$. On the other hand, the other parameters, including root and shoot length under control conditions, were not different.

Table 2. Kruskal–Wallis H -test analysis. Group size: ‘non-colored’ group has five values of traits of ‘S29’, ‘colored’ group has ten values of traits of ‘iPF’ and ‘iP’; degrees of freedom (df): 1

Trait	Group	Group size	Sum of ranks	H	p -Value
0 μM CdCl ₂					
Anthocyanin content in the coleoptile (in control)	‘non-colored’	5	15	9.38	0.0022
	‘colored’	10	105		
Anthocyanin content in the grains	‘non-colored’	5	15	10.5	0.0012
	‘colored’	10	105		
Root length (in control)	‘non-colored’	5	40	0.00	1.0000
	‘colored’	10	80		
Shoot length (in control)	‘non-colored’	5	35	0.38	0.5403
	‘colored’	10	85		
25 μM CdCl ₂					
Changes in the anthocyanin content in the coleoptile	‘non-colored’	5	51	1.82	0.1779
	‘colored’	10	69		
Changes in root length	‘non-colored’	5	25	3.38	0.0662
	‘colored’	10	95		
Changes in shoot length	‘non-colored’	5	24	3.85	0.0498
	‘colored’	10	96		
50 μM CdCl ₂					
Changes in the anthocyanin content in the coleoptile	‘non-colored’	5	58	4.86	0.0275
	‘colored’	10	62		
Changes in root length	‘non-colored’	5	44	0.24	0.6242
	‘colored’	10	76		
Changes in shoot length	‘non-colored’	5	53	2.54	0.1113
	‘colored’	10	67		

Overall, the data demonstrated that the factor ‘dark-purple color’ had a significant impact on changes in growth parameters of wheat seedlings under exposure to low doses of cadmium chloride.

Correlations between anthocyanin levels and growth parameters

Moderate negative correlations were observed between anthocyanin contents in the coleoptiles of control plants and reductions of shoot ($r_s = -0.595$, $p \leq 0.05$) and root ($r_s = -0.600$, $p \leq 0.05$) length under exposure to 25 μM CdCl₂. No correlation was ob-

served between the parameters under 50 μM CdCl_2 or between the anthocyanin content in grains and changes in growth parameters.

Discussion

Genotype- and stress-dependent regulation of anthocyanin biosynthesis

Under cadmium stress, the accumulation of anthocyanins has been observed in different plant species. For example, enhanced anthocyanin content has been demonstrated in the leaves of *Lathyrus maritimus* grown in soil polluted with Cd (Maslennikov 2013) as well as in the seedlings of *Secale cereale* (Maslennikov et al. 2013) and *Oryza sativa* (Roychoudhury et al. 2012) under Cd stress. Under conditions of environmental cadmium pollution, positive correlations between Cd and anthocyanin content in the needles of *Picea abies* as well as the leaves of *Tilia cordata*, *Taraxacum officinale* and *Plantago major* have been established (Tchoupakhina et al. 2012). Cadmium-induced anthocyanin accumulation and increased transcript abundance of related genes encoding chalcone synthase (CHS) and dihydroflavonol reductase (DFR) have been shown in the aquatic fern *Azolla imbricata* (Dai et al. 2006, 2012a). The induced anthocyanins demonstrate powerful antioxidant ability against various free radicals as well as Cd^{2+} chelation ability (Dai et al. 2008, 2012b). In the leaves of *Populus deltoides*, increasing anthocyanin concentration and transcription of the related genes encoding chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), glycosyltransferase (UFGT) and DFR have been observed under 50 μM CdCl_2 , whereas under higher Cd concentration (100 and 150 μM), the anthocyanin content and gene transcription decrease (Zhang et al. 2014).

In the present study, the relationship between anthocyanin biosynthesis and cadmium stress was investigated using the wheat NILs differing by grain and seedling coloration, which is predetermined by the *Pp-1 + Pp3* and *Rc-1* genes, respectively (Khlestkina 2012). The three NILs respond differently to cadmium stress. In 'S29', which has light-red coleoptile coloration determined by *Rc-A1*, intensification of anthocyanin biosynthesis was observed under 25 and 50 μM CdCl_2 . In the 'iPF' line, with intense anthocyanin pigmentation of the coleoptile controlled by *Rc-A1 + Rc-D1*, anthocyanin content was not altered under either CdCl_2 concentration. 'iP', which has the dominant alleles *Rc-A1 + Rc-D1*, like 'iPF' seems to be more sensitive to stress than is the sister line (the anthocyanin level in 'iP' increased under 25 μM but decreased under 50 μM CdCl_2) (Fig. 2). 'iPF' and 'iP' have *Rc-D1* alleles from different donors (Gordeeva et al. 2015) and may have different *cis*-regulatory elements. Thus, the response of anthocyanin biosynthesis to cadmium stress is dependent on the alleles of the *Rc-1* genes. The genotype-dependent response of anthocyanin metabolism has been observed under different types of stresses in different cultivars and NILs of diverse plant species (Maekawa et al. 2001; Daneshmand et al. 2010; Basu et al. 2010; Borghesi et al. 2011; Roychoudhury et al. 2012; Ploenlap and Pattanagul 2015).

The NILs ('S29', 'iPF', 'iP') exploited in the present study have been previously tested under cold, osmotic stress and ionizing radiation (Gordeeva et al. 2013, 2017; Shoeva et

al. 2017), which allows comparing the effects of different environmental stresses on changes in anthocyanin content in the same genotypes. Under cold stress, the anthocyanin content decreased in light-red coleoptiles of 'S29' but increased in the purple coleoptiles of 'iPF' and 'iP' (Gordeeva et al. 2013). After seeds were irradiated, a decrease in anthocyanin concentration was reported in the coleoptiles of 'S29', whereas in 'iPF' and 'iP', the anthocyanin content did not change (Gordeeva et al. 2017). Under osmotic stress, the mode of response was similar to that under Cd treatment: a higher intensification of anthocyanin biosynthesis was observed in 'S29' (Shoeva et al. 2017). Such patterns of anthocyanin metabolism under different types of stresses suggest that the regulation of anthocyanin biosynthesis is stress-dependent, although common mechanisms can be involved in the response to osmotic and cadmium stresses.

Protective role of anthocyanin pigmentation

Comparison of the sensitivity of the NILs under stress conditions allows revealing the protective role of pigments under various stress conditions (Gordeeva et al. 2013; Shoeva et al. 2017). In the present study, it was shown that anthocyanins produced in coleoptiles protect wheat seedlings against moderate Cd stress (25 μM). The more anthocyanins that are present in wheat seedlings in control conditions, the less reduction of shoot and root lengths under cadmium stress observed (Fig. 1, left part). The lines ranked by their tolerance to 25 μM CdCl_2 are as follows: 'S29' < 'iP' < 'iPF' (Fig. 1, left part), and the more sensitive lines ('S29' and 'iP') demonstrate a tendency toward enhanced anthocyanin accumulation under moderate Cd stress (Fig. 2, left part) in contrast to the most tolerant 'iPF', which has stress-independent anthocyanin accumulations in the coleoptile (Fig. 2). ANOVA demonstrated that the factor 'dark-purple color' determined by the allele combination *Rc-A1* + *Rc-D1* has a significant impact on the changes of growth parameters of wheat seedlings under 25 μM CdCl_2 . A similar role of this factor and associated genes was revealed for the same set of lines under osmotic stress (Shoeva et al. 2017). Furthermore, the most intense induction of anthocyanin synthesis was also observed in the coleoptiles of the most stress-sensitive line, suggesting similar mechanisms may be involved in Cd and osmotic stress responses in wheat seedlings. The observed protective role of the anthocyanins could be explained by their high antioxidant potential, while higher intensification of the anthocyanin biosynthesis in less-colored lines suggests that the antioxidant system is not able to fully cope with the oxidative stress, inducing the synthesis of additional antioxidants. The ability of anthocyanins to compensate for the deficiency of antioxidants was hypothesized by Zhu et al. (2013). In the work of those authors, the antioxidant systems of fully expanded and central leaves of *Saccharum officinarum* were compared under chilling stress. It was revealed that under a similar degree of chilling injury, the activity of antioxidant enzymes was higher in fully expanded leaves than in central ones, whereas anthocyanin concentration increased more in central leaves than in fully expanded ones.

In the present study, a protective role of anthocyanin pigments was observed under moderate (25 μM) CdCl_2 stress, whereas under heavier stress, the relationship between

pigmentation and decreased root and shoot lengths was not identified (Table 2; Fig. 2, right part). The obtained results confirmed the earlier observation of the protective role of the anthocyanins of wheat coleoptiles under low doses of ionizing radiation but not under higher doses (Gordeeva et al. 2017). It seems that anthocyanins are more effective under low or moderate stress but are not powerful enough under heavy stress.

Overall, the changes in anthocyanin metabolism under stress conditions and the level of stress sensitivity/tolerance both depend on the following three factors: (i) the alleles of genes that control anthocyanin synthesis, (ii) the type of the stress and (iii) the intensity of stress.

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