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ORIGINAL ARTICLE

Biochemical and genetic polymorphism of *Bromopsis inermis* populations under chronic radiation exposure

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Abstract

Main conclusion **For the subsequent assessment of the genetic mechanisms responsible for the resistance of plants to chronic irradiation, the analysis of RAPD-cDNA with the subsequent isolation, cloning, and sequencing of expressed polymorphic sequences is a promising technique.**

A study was conducted on *Bromopsis inermis* populations that have been growing for a long time in the EURT area. Using RAPD primers, we studied the genetic spectra of plants. In analysing the UPGMA algorithm, we identified two well-distinguishable clusters with a high level of bootstrap support (> 85%): background samples hit the first, and impact samples hit the second. Our data indicate a decrease in diversity in the most polluted population, as well as the appearance of new alleles in chronically irradiated samples of the *B. inermis*. Smooth brome seedlings were characterised by the content of anthocyanins, comparable with other types of cereals. In the gradient of chronic irradiation, the relative content of anthocyanins was not significantly changed. For the first time, the partial nucleotide sequences of the key genes of anthocyanin biosynthesis (*Chi* and *F3h*) in the brome were determined, these sequences were found to be 191 and 356 bp in length, respectively, and were cloned and sequenced. Three copies of the *Chi* gene were identified in the *B. inermis* genome. One copy (*BiChi-1*) clustered with the sequences of the *Aegilops tauschii* gene (*D* genome), and the other two copies (*BiChi-2* and *BiChi-3*) formed a separate cluster in the Pooideae subfamily adjacent to *Hordeum vulgare*. In the copy of *BiChi-1*, a complete deletion of intron 1 was detected. For the *F3h* gene, one copy of the *B. inermis* gene was obtained, which forms a separate branch in the subfamily Pooideae.

Keywords *Bromopsis inermis* (= *Bromus inermis*) · Ionising radiation · Low-level doses · Polymorphism · RAPD · Anthocyanins · *Chi* · *F3h*

Abbreviations

cDNA	Complementary DNA
CHI	Halcone isomerase
EURT	East Ural Radioactive Trace
F3H	Flavanone-3-hydroxylase
RAPD	Random amplified polymorphic DNA
UPGMA	Unweighted pair-group method using arithmetic averages

Introduction

Random amplified polymorphic DNA (RAPD) markers are widely used to conduct research in radiobiology (Danylchenko and Sorochinsky 2005; Atak et al. 2004; Dhakshanamoorthy et al. 2011; Lu et al. 2007; Turuspekov et al. 2002; Roy et al. 2006) and ecotoxicology (Conte et al. 1998;

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Mengoni et al. 2000; Penner et al. 1995; Yap et al. 2007). Variability of RAPD loci in *Bromopsis inermis* (=*Bromus inermis* Leyss.) populations has been extensively studied (Joachimiak et al. 2001; Sutkowska and Mitka 2008; Zhang et al. 2011; Diaby and Casler 2003).

Anthocyanins are water-soluble pigments of the flavonoid family with high biological importance. They play a key role in the adaptation of plants to biotic (Kordali et al. 2005; Franklin et al. 2009; Diaz-Vivancos et al. 2006) and abiotic (Chalker-Scott 1999; Treutter 2005; Khlestkina 2013; Gordeeva et al. 2013; Bandy and Bechara 2001) stress. After irradiation, the role of anthocyanins is particularly relevant (Treutter 2005), since with their help, peroxides and free radicals are partially utilised. The key enzymes of the early stages of flavonoid biosynthesis are chalcone isomerase (*CHI*, EC 5.5.1.6) and flavanone-3-hydroxylase (*F3H*, EC 1.14.11.9). The nucleotide sequences of these genes are well-studied in different plant species (Jez et al. 2000; Khlestkina et al. 2013; Shoeva et al. 2014; Winkel-Shirley 2001). A series of studies has shown a relationship between changes in the activity of these genes, the intensity of the colour of various plant organs, and the action of environmental factors (Andre et al. 2009; Lovdal et al. 2010; Lillo et al. 2008; Shoeva and Khlestkina 2015).

Previously, we studied the 7-year dynamics (Antonova et al. 2014) and the intra-annual variability (Antonova et al. 2015) of the viability, mutability, and radiosensitivity of seeds and the content of low-molecular antioxidants in seedlings of the smooth brome (*B. inermis* Leyss.) that has grown for a long time in the most impact area of the East Ural Radioactive Trace (EURT) and beyond.

The purpose of the current study was to analyse biochemical (anthocyanin content) and genetic (variability of non-specific loci) parameters in *B. inermis* populations, both growing under chronic radiation conditions and from background areas. Sequence analysis of key genes for the anthocyanin biosynthesis pathway in *B. inermis* is relevant since these compounds play an important role in the adaptation of plants to adverse environments, including man-made.

Materials and methods

Plant material

Seeds of the awnless brome (ITIS no. 40502, *B. inermis* Leyss. = *B. inermis* Leyss.) were harvested along the central axis of the EURT: impact area (10–12 km, 55°46′N, 60°51′E) and on the periphery of the trace: buffer (17 km, 55°50′N, 60°52′E). Two background plots were located outside the EURT: background-1 (112 km, 56°42′N, 61°02′E) and background-2 (125 km, 56°47′N, 61°18′E). Vegetation of the most impact area of the EURT is represented by a complex of synanthropic and semi-natural communities at various stages of degradation and restorative successions (Pozolotina et al. 2012). In all studied phytocenoses, the *B. inermis* is dominant or subdominant. The investigated *B. inermis* populations are represented by octoploid forms ($2n = 56$) (Antonova et al. unpublished).

RAPD analysis

DNA was isolated by standard methods (Plaschke et al. 1995). For the RAPD analysis, 15 random primers (length of 10–11 nucleotides) were used, which were selected earlier for studying representatives of the Poaceae family, in particular, wheat (Khlestkina et al. 1999). The PCR conditions are identical to those described previously (Röder et al. 1998), except for using 2.5 mM $MgCl_2$. All experiments were repeated twice. A total of 19 plants were investigated. Cluster analysis was performed using TFPGA v.1.3 (Miller 1997) based on the UPGMA algorithm. The bootstrap test used 1000 replicates.

Anthocyanin extraction

Seeds of 15–20 plants were collected from each populations and germinated for 3 weeks in a climate cell using a roll culture in distilled water at a temperature of + 23 °C and a regimen day/night for 12 h. For anthocyanin extraction, fresh coleoptile ($N = 4$–6, $m = 150$ mg) was homogenised in 1 ml of a 1% mixture of $HCl + CH_3OH$ at room temperature and incubated for 2 h at + 4 °C (Christie et al. 1994). The extract was centrifuged at 10,000*g* for 10 min. The relative content of anthocyanins was measured at $\lambda = 530$ nm on SmartSpec™Plus spectrophotometer (BioRad) in triplicate. Statistical hypotheses were tested by non-parametric *U*-tests (Mann and Whitney 1947) and *z*-tests for normally distributed data using Statistica v.10 (StatSoft Inc. 2011).

Cloning the *Chi* and *F3h* genes

The partial nucleotide sequences of the *Chi* and *F3h* genes of *B. inermis* were amplified using primers selected previously for the conserved regions of the corresponding *Triticum aestivum* genes (Himi et al. 2005; Shoeva et al. 2014). The PCR conditions have been described in detail previously (Röder et al. 1998). The obtained PCR fragments were separated on a 2% agarose gel, excised, and purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Purified PCR fragments were sequenced or ligated into the *pDrive* vector from the QIAGEN PCR Cloning Kit (QIAGEN, Hilden, Germany). Transformation of *Escherichia coli* (strain *XL-blue*) by the resulting plasmids was performed using calcium and rubidium chlorides (Maniatis et al. 1982).

Recombinant plasmid DNA was isolated by the alkaline lysis method (Maniatis et al. 1982). Sequencing was performed at the SB RAS Genomics Core Facility (http://www.niboch.nsc.ru/doku.php/sequest). Multiple alignments of cloned sequences were carried out in the program Multalin 5.4.1 (Corpet 1988). The search for homologous nucleotide sequences was performed using the BLAST algorithm (Altschul et al. 1990) in the NCBI database (https://www.ncbi.nlm.nih.gov/). Cluster analysis was performed in MEGA 7 (Kumar et al. 2016). The sequences have been submitted to the NCBI database (MK052712, MK052713, MK052714, MK052715).

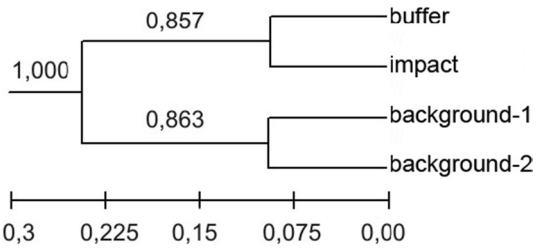

Fig. 1 UPGMA dendrogram of genetic distance constructed for *Bromus inermis* populations from the EURT area and from unexposed samples. 1000 permutations were performed. The bootstrap values are located above the axes

Results and discussion

Detailed radioecological descriptions of the investigated sites and dose calculations for mother plants and seed germs have been provided in our previous articles (Karimullina et al. 2018; Molchanova et al. 2014; Antonova et al. 2014, 2015). Note that the absorbed dose rate for brome under the pollution gradient is 1.5–19 times higher than the background level. These values do not exceed the limits of low-level doses for plants. We assessed the effects of low-level radiation on *B. inermis* according to the variability of the population genetic structure and the level of anthocyanins, and furthermore determined the relationship of the *Chi* and *F3h B. inermis* sequences to cultural cereals.

Hypothesis 1: genetic variability in chronically irradiated *B. inermis* populations is higher than in background samples

Using RAPD analysis, the most polymorphic spectra with primers *R_057* (1722-05) and *R_160* (311-04) were identified. The remaining primers gave monomorphic spectra or PCR reactions with their participation completely inhibited. The *PIC* values (with the Bayes correction) characterising the level of informativeness of polymorphism at the locus *R_057* varied from 0.869 to 0.889 in background samples and from 0.897 to 0.873 in impact samples. For the locus *R_160*, the values were higher (0.931–0.939 and 0.943–0.893, respectively). This indicates that these loci are highly informative for population studies, but the variability in the most polluted population was minimal. In total, 137 alleles were found in the background populations, and 92 alleles were found in the chronically irradiated population.

Cluster analysis using the UPGMA method (Nei 1972) identified two groups. Both background samples were in the first cluster, and impact samples were in the second (Fig. 1). Since the bootstrap support levels were high (> 0.85%), unexposed samples ($D_N = 0.093$) were genetically closer to each other than to impact plants ($D_N = 0.241$), also with high affinity within the cluster ($D_N = 0.092$). This is probably due to the fact that the locus *R_160* has a 230 bp allele found only in background samples, while the 203 bp and 176 bp alleles were found only in impact populations.

The level of genetic variation in each population may equally be associated with neutral mutations, isolation, migration, gene drift, and the founder effect (Hedrick 2011). High levels of variability may be due to the wide variation of the ecological niche (Babbel and Selander 1974; Prentice et al. 1995). Under environmental pollution, an increase in variability may be associated with an increase in the incidence of rare alleles, as was shown in *Centaurea scabiosa* (Lysenko et al. 1999), *Stellaria graminea* (Pozolotina et al. 2010), and *Pinus sylvestris* (Geras'kin and Volkova 2014); with the advent of unique alleles (Karimullina et al. 2016) that were absent in unexposed samples of *Silene latifolia*; and in the case of RAPD, with the formation of new bands (Roy et al. 2006; Conte et al. 1998). An increase in genetic diversity has also been noted in chronically irradiated populations of *Hordeum bogdanii* and *Agropyron pectinatum* growing on the Semipalatinsk nuclear test site (Turuspekov et al. 2002). However, the reasons for increased diversity are often not provided by the authors. In some studies, RAPD markers associated with a low level of Cd accumulation of plants have been identified (Penner et al. 1995).

Our data indicate a decrease in diversity in the most polluted sample from the EURT, which disproves our hypothesis. This may be due to low migration and gene drift (Soule 1973; Hoffmann and Blows 1994), as well as the bottleneck effect (Nei et al. 1975). Similar results were obtained in *Lychnis flos-cuculi* populations (Dulya and Mikryukov 2016), *Sedum alfredii* (Deng et al. 2007), and *Deschampsia cespitosa* (Bush and Barrett 1993), which grow under chronic chemical pollution, and also *Plantago major* from a radioactive contamination area (Pozolotina et al. 2005). The loss of genetic diversity under anthropogenic stress is called "genetic erosion" (van Straalen and Timmermans 2002). In addition, our data indicate the emergence of new alleles in chronically irradiated samples of *B. inermis*. Similar data

were obtained earlier in Cu-resistant *Silene paradoxa* populations (Mengoni et al. 2000), as well as using the model species *Arabidopsis thaliana* (Conte et al. 1998). Such changes may be the result of structural changes in DNA (mutations), such as breaks, translocations, or deletions (Danylchenko and Sorochinsky 2005; Atienzar and Jha 2006).

Hypothesis 2: the intensity of the anthocyanin synthesis increases under chronic irradiation

The *B. inermis* seedlings had an average content of anthocyanins in different populations, which varied from 0.52 to 1.62 (Fig. 2). These data are located in the range of values typical for *T. aestivum* "Pyrothrix 28" (*Hordeum marinum*) of the substituted chromosome line 7Hm(7D) and *Secale cereale* of the variety Onokhoyskaya (Khlestkina et al. 2011). Along the gradient of chronic irradiation, the relative content of anthocyanins in the seedlings was not significantly different (Kruskal–Wallis test, $H_{3;\,21}=3.85$; $p=0.278$). The greatest variability of this parameter was seen in background samples (CV = 24.2–39.2%; with CV = 12.0–16.9% in EURT populations).

Anthocyanins (belonging to the group of flavonoids) are considered to be non-specific protectors, the synthesis of which increases under abiotic and biotic stress (Chalker-Scott 1999; Diaz-Vivancos et al. 2006; Franklin et al. 2009; Kordali et al. 2005; Treutter 2005; Gordeeva et al. 2013; Khlestkina 2013; Bandy and Bechara 2001). A series of investigations has shown that the content of flavonoids increases at low temperatures (Carrao-Panizzi et al. 1999; Gordeeva et al. 2013), under water (Shoeva et al. 2017) and salt stress (Shoeva and Khlestkina 2015), as well as after acute gamma irradiation (Gordeeva et al. 2018). When using exact genetic models such as wheat near-isogenic lines, differing in alleles of the genes that determine the accumulation of anthocyanins in the grain and coleoptile, the role of pigments has been demonstrated under the action of various types of stress of low or moderately intensity. However, under severe stress, anthocyanins are apparently not effective protective molecules (Gordeeva et al. 2013, 2018; Shoeva et al. 2017; Shoeva and Khlestkina 2018). The content of low molecular weight antioxidants has been positively correlated with the parameters of growth and development of smooth brome seedlings and negatively with the proportion of seedlings that have any developmental anomalies (necrosis of various organs, changes in the shape of cotyledons, etc.) (Antonova et al. 2015). It has been shown that, under salt stress, *T. aestivum* changes the expression of key flavonoid biosynthesis genes (*Chi* and *F3h*) (Shoeva and Khlestkina 2015). In *Lemna minor*, low radiation doses trigger altered flavonoid biosynthesis gene expression (*COMT1*, *PAL*, *CHS*) (Van Hoeck et al. 2017).

Our data on the anthocyanin content in smooth brome seedlings indicate the absence of differences between background and chronically irradiated populations. This may be due, on the one hand, to the fact that cyclicity is characteristic of any biological system (Nagata et al. 2003; Antonova et al. 2015). On the other hand, alternative ways of maintaining homeostasis in cells under stress are possible, for example, due to the intensive synthesis of other types of low molecular weight antioxidants (Antonova et al. 2014) or the activation of enzyme systems (Shimalina et al. 2018).

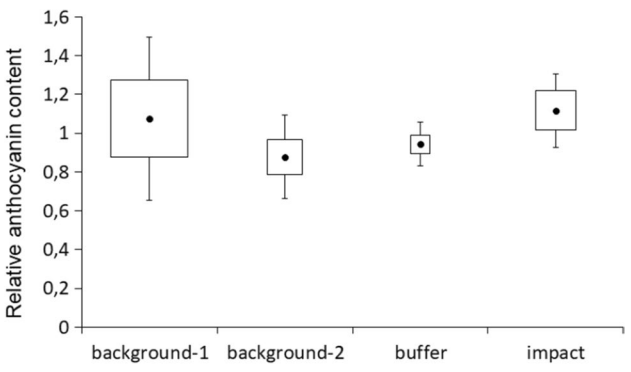

Fig. 2 The content of anthocyanins in the *B. inermis* coleoptile from the EURT and unexposed (background) populations. Black dots in the figure indicate average values, white squares are the standard errors of mean, bars are the standard deviations

Hypothesis 3: the sequences of the key genes of anthocyanin biosynthesis (*Chi, F3h*) are conserved and correspond to cultural cereals in the smooth brome

For PCR in *B. inermis*, primers selected for amplification of *T. aestivum* genes were effective. For the first time, partial sequences of the genes *Chi* and *F3h* in *B. inermis* (191 and 356 bp, respectively) were cloned and sequenced (Fig. 3).

Analysis of the nucleotide sequences of the *Chi* gene, obtained by sequencing the plasmid DNA of nine individual colonies, revealed three individual copies corresponding to different subgenomes combined in the polyploid genome of the *B. inermis*. In the copy of *BiChi-1*, a complete deletion

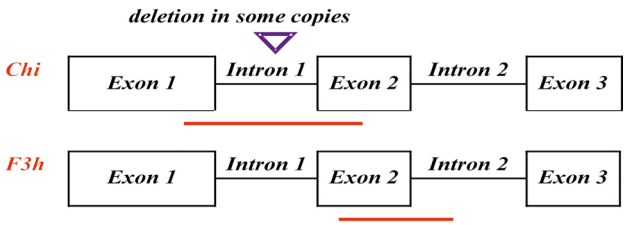

Fig. 3 A scheme for structure of the *Chi* and *F3h* genes and corresponding fragments isolated from *Bromus inermis* genome (the red line below each scheme)

of intron 1 was noted. The nucleotide sequences of *BiChi-2* and *BiChi-3* differed from each other by one substitution in the coding region and 15 substitutions and insertions/deletions of 9 nucleotides in the intron. The differences between *BiChi-2* and *BiChi-3* versus *BiChi-1* amounted to 10–11 substitutions in the coding region (Fig. 4).

Comparison of the isolated sequences of the *Chi* gene of the *B. inermis* with the plants represented in GenBank (cultivated cereals and *A. thaliana*) revealed two clades: the first includes members of the subfamily Panicoideae (*Sorghum bicolor* and *Zea mays*) and Oryzoideae (*Oryza sativa*), and the second includes representatives of the subfamily Pooideae (Fig. 5; Supplementary Materials, Fig. S1). At the bootstrap level of 63%, the closeness of *BiChi-1* of the *B. inermis* to *A. tauschii* (*D* genome) is shown. The second and third copies of the *Chi* gene of the *B. inermis* form a separate cluster in the subfamily Pooideae. Thus, none of the *Chi* sequences is related to maize, sorghum, rice, or *A. thaliana*. At the same time, due to the low bootstrap support, it is not possible to determine the relationship of the nucleotide sequences *BiChi-2* and *BiChi-3* to any member of the Pooideae subfamily (for example, to the rye or barley).

One copy of the *F3h* gene was obtained for the *B. inermis*. Comparison of the *F3h* coding sequences of *B. inermis* with cultivated cereals and *A. thaliana* (Fig. 6) showed that *Bromus* forms a separate branch in the subfamily Pooideae, which is localised with *Hordeum vulgare* (31% of bootstrap replicates). These sequences differ from each other by at

Fig. 4 Nucleotide sequences of three partial copies of the *Chi* gene of the *B. inermis*. In the copy of *BiChi-1*, the intron is absent, for *BiChi-2* and *BiChi-3* the intron is underlined. Blue highlighting indicates substitutions and insertions

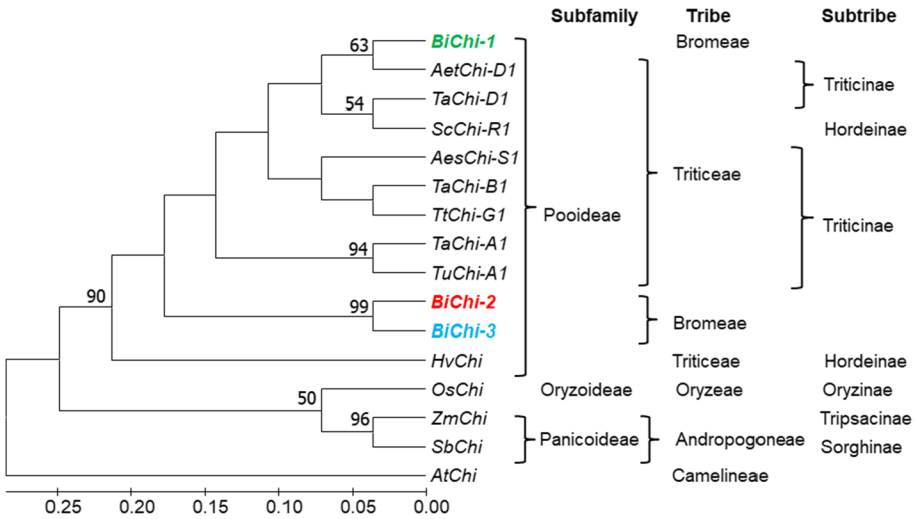

Fig. 5 Comparison of the partial nucleotide sequences of the *B. inermis Chi* gene obtained in the current study with the sequences of other plant species identified in the NCBI database: *Aegilops speltoides* (*S* genome) KF826811.1, *Aegilops tauschii* (*D* genome) XM_020323671.1, *Arabidopsis thaliana* NM_126020.2 (outgroup), *Hordeum vulgare* AK374952.1, *Oryza sativa* AF474922.1, *Secale cereale* (*R* genome) KC788192.1, *S. bicolor* XM_002463586.2, *Triticum aestivum* (*A* genome) JN039037.1, *Triticum aestivum* (*B* genome) JN039038.1, *Triticum aestivum* (*D* genome) JN039039.1, *Triticum timopheevii* (*G* genome) KJ000522.1, *Triticum urartu* (*A* genome) KF826812.1, and *Zea mays* NM_001150530.2. The dendrogram was inferred using the neighbour-joining method and two-parameter Kimura model nucleotide substitutions. The bootstrap consensus tree was inferred from 10,000 replicates. Branches corresponding to partitions reproduced in fewer than 50% bootstrap replicates are collapsed

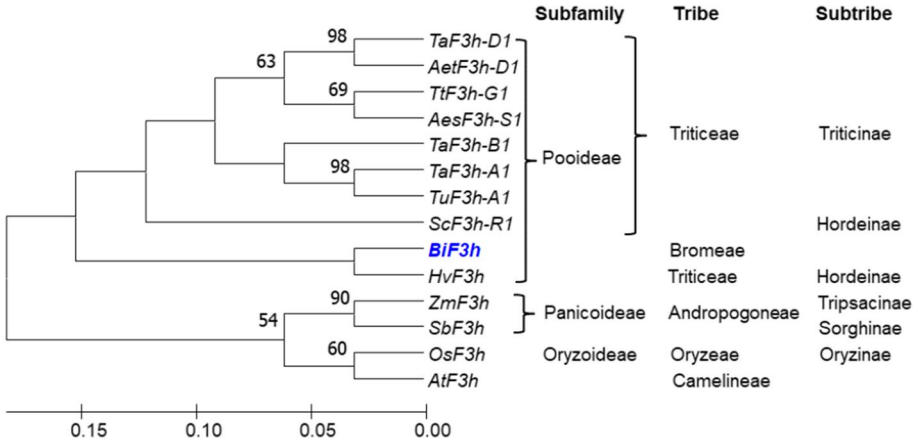

Fig. 6 Comparison of partial sequences of the *B. inermis F3h* gene obtained in the current study with the sequences of other plant species identified in the NCBI database: *Aegilops speltoides* (*S* genome) EU402963.1, *Aegilops tauschii* (*D* genome) DQ233637.1, *Arabidopsis thaliana* AF064064.1 (outgroup), *Hordeum vulgare* EU921438.1, *Oryza sativa* AK072222.1, *Secale cereale* (*R* genome) EU815625.1, *Sorghum bicolor* GU320740.1, *Triticum aestivum* (*A* genome) AB223024.1, *Triticum aestivum* (*B* genome) AB223025.1, *Triticum aestivum* (*D* genome) DQ233636.1, *Triticum timopheevii* (*G* genome) EU402960.1, *Triticum urartu* (*A* genome) EU402961.1, and *Zea mays* U04434.1. The dendrogram was inferred using the neighbour-joining method and two-parameter Kimura model nucleotide substitutions. The bootstrap consensus tree was inferred from 10,000 replicates. Branches corresponding to partitions reproduced in fewer than 50% bootstrap replicates are collapsed

least nine substitutions (Supplementary Materials, Fig. S2). Thus, the smooth brome does not belong to the Triticinae subtribe cluster, which includes various species of *Triticum* and *Aegilops*, as well as the subfamily Panicoideae (*S. bicolor* and *Z. mays*), Oryzoideae (*O. sativa*), and the dicotyledon *A. thaliana*. However, *B. inermis* is related to members of the Hordeinae subtribe (*H. vulgare* and *Secale sereale*).

Thus, based on phylogenetic trees using the nucleotide sequences of the *Chi* and *F3h* genes, the *B. inermis* is close to *A. tauschii*. The other two copies (*BiChi-2* and *BiChi-3*) form a separate cluster in the Pooideae subfamily, to which *H. vulgare* is adjacent. A copy of the *F3h* gene together with *H. vulgare* forms a separate branch in the subfamily Pooideae. The results of our investigation are consistent with the data obtained when comparing the restriction sites of chloroplast DNA (cpDNA). It has been shown that the *B. inermis* (Bromeae tribe) is closer to the Triticeae tribe (Döring et al. 2007; Soreng et al. 1990; Kellogg 1992a), than to Aveneae (Pillay 1995). Within the Triticeae tribe, it is closer to *H. vulgare* (both species are members of the Triticodae supertribe) than to *S. cereale* (Pillay 1995), or to *T. aestivum* (Davis and Soreng 1993). The phylogenetic tree based on the nucleotide substitution data of DNA sequences (NFFA150) revealed similar results as obtained from SSR marker data. The genera *Bromus* and *Oryza* were placed in separate nodes, and *B. inermis* was placed close to Triticeae (Mian et al. 2005). At the same time, an analysis of 841 EST-SSR markers showed the proximity of barley and brome (Zeid et al. 2010). Thus, the tribe Bromeae is the sister group (closest relative) of Triticeae (Soreng et al. 1990; Kellogg 1992a, b). Most likely, the subgenomes of the *B. inermis* have different origins, with one of the genomes based on one copy of the *Chi* gene close to *A. tauschii* (*D* genome); while the other copies of the *Chi* gene form a separate cluster in the subfamily Pooideae.

In connection with the data presented above and the data obtained by us, the problem of the origin of the octoploid *B. inermis* again becomes important. Taking into account the genomic formula ($AAAAB_1B_1B_2B_2$), octaploid *B. inermis* is probably not a doubled form of the tetraploid *B. inermis* (Tuna et al. 2004). Most likely, it formed initially by the hybridisation of two species AAB_1B_1 and AAB_2B_2, followed by spontaneous doubling. One of the ancestors of *B. inermis* could be *B. pumpellianus* Scribn. (Armstrong 1980), and the second possible precursor candidate is *B. riparius* ($2x = 28$) (Armstrong 1991). Interspecific hybrids indicate that the *A* genome can come from *B. erectus* or *B. variegatus* ($2n = 4x = 28$), but their chromosomes are very different (Armstrong 1991; Walton 1980). If either species is a progenitor of *B. inermis*, significant chromosomal change should have occurred post-hybridisation and polyploidisation (Tuna et al. 2006). In general, the range of ribosomal DNA length phenotypes appearing in diploid, tetraploid, and octoploid *B. inermis* suggests that these plants share a common ancestry (Pillay 1996), while the tetraploid *B. inermis* is not an autopolyploid.

Conclusions

Thus, the analysis of genetic and biochemical diversity of the *B. inermis* showed a decrease in variability in the anthocyanin content and in the RAPD allele number in the impact population compared with background samples. At the same time, anthocyanin compounds, apparently, do not have a pronounced protective effect in brome under conditions of chronic irradiation, since interpopulation differences in their content were not found. For the subsequent assessment of the genetic mechanisms responsible for the resistance of plants to chronic irradiation, the analysis of RAPD-cDNA with the subsequent isolation, cloning, and sequencing of expressed polymorphic sequences is a promising technique.

Author contribution statement Conception and design: EKK and EVA. Collection and assembly of data: EVA. Analysis and interpretation of the data: EVA and OYS. Drafting of the article: EVA. Critical revision of the article for important intellectual content: EVA and OYS. Final approval of the article: EVA, OYS and EKK. Statistical expertise: EVA and OYS. Obtaining of funding: EVA and EKK.

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