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ORIGINAL PAPER

Variation in genome composition of blue-aleurone wheat

Veronika Burešová · David Kopecký · Jan Bartoš · Petr Martinek · Nobuyoshi Watanabe · Tomáš Vyhnánek · Jaroslav Doležel

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Abstract
Key message **Different blue-aleurone wheats display major differences in chromosome composition, ranging from disomic chromosome additions, substitutions, single chromosome arm introgressions and chromosome translocation of *Thinopyrum ponticum*.**
Abstract Anthocyanins are of great importance for human health due to their antioxidant, anti-inflammatory, antimicrobial and anti-cancerogenic potential. In common wheat (*Triticum aestivum* L.) their content is low. However, elite lines with blue aleurone exhibit significantly increased levels of anthocyanins. These lines carry introgressed chromatin from wild relatives of wheat such as *Thinopyrum ponticum* and *Triticum monococcum*. The aim of our study was to characterize genomic constitutions of wheat lines with blue aleurone using genomic and fluorescence in situ hybridization. We used total genomic DNA of *Th. ponticum* and two repetitive DNA sequences (GAA repeat and the *Afa* family) as probes to identify individual chromosomes. This enabled precise localization of introgressed *Th. ponticum* chromatin. Our results revealed large variation in chromosome constitutions of the blue-aleurone wheats. Of 26 analyzed lines, 17 carried an introgression from *Th. ponticum*; the remaining nine lines presumably carry *T. monococcum* chromatin undetectable by the methods employed. Of the *Th. ponticum* introgressions, six different types were present, ranging from a ditelosomic addition (cv. Blue Norco) to a disomic substitution (cv. Blue Baart), substitution of complete (homologous) chromosome arms (line UC66049) and various translocations of distal parts of a chromosome arm(s). Different types of introgressions present support a hypothesis that the introgressions activate the blue aleurone trait present, but inactivated, in common wheat germplasm.

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V. Burešová · D. Kopecký (✉) · J. Bartoš · J. Doležel
Institute of Experimental Botany, Centre of the Region Haná for Biotechnological and Agricultural Research, Šlechtitelů 31, 78371 Olomouc, Czech Republic
e-mail: kopecky@ueb.cas.cz

P. Martinek
Agrotest Fyto, Ltd., Havlíčkova 2787, 767 01 Kroměříž, Czech Republic

N. Watanabe
College of Agriculture, Ibaraki University, 3-21-1 Chuo, Ami, Inashiki, Ibaraki 300-0393, Japan

T. Vyhnánek
Mendel University in Brno, Zemědělská 1, 613 00 Brno, Czech Republic

Introduction

Anthocyanins are a group of intensely colored water-soluble pigments responsible for most of red, blue and purple colors of fruits, vegetables, flowers and other tissues. They are abundant in red, blue and purple-colored berries and their products (derivatives) such as red wine, and in seeds of some species (Mazza and Miniati 1993). Over 400 anthocyanins have been described so far. Of these, six are the most abundant in plant kingdom and are classified based on the number and position of hydroxyl and methoxyl groups on the flavan nucleus: cyanidin, the most widespread anthocyanidin in nature), delphinidin, pelargonidin, peonidin, petunidin and malvidin (Mazza 2007). Their presence in plants is beneficial as they attract animals, and thereby assist in pollination and seed dispersal (Harborne and Williams 2001) as well as offer protection against the ultraviolet-induced damage (Mazza and Miniati 1993).

Anthocyanins display a range of biological activities some of which are significant in human diet and health, such as antioxidant (Wang et al. 1997), anti-inflammatory (Wang and Mazza 2002), anti-microbial (Pisha and Pezzuto 1994) and anti-cancerogenic activities (reviewed in Wang and Stoner 2008; Bowen-Forbes et al. 2010), improvement of vision (Matsumoto et al. 2003; Lila 2004), induction of apoptosis (Katsube et al. 2003) and neuroprotective effects (Youdim et al. 2000). According to some reports, intake of anthocyanins may have protective effect against coronary heart disease, the leading cause of death in most developed countries (Anderson et al. 2000; Rechner and Kroner 2005). An intriguing question is the uptake of anthocyanins in humans after their ingestion. Their levels in human blood are far below the levels required to exhibit anti-cancerogenic effects in vitro (Wang and Stoner 2008). Thus, large and long-term intervention trials are needed for a definite proof of the potential human health benefits of these compounds (Mazza 2007). Anthocynanins can also serve as natural food colorants to prevent or decrease the usage of synthetic colors (Gao and Mazza 1994). Blue and purple corn grains are used for making blue and pink tortillas, and red rice is commonly used as a food colorant in bread, ice cream, and liquor (Yoshinaga 1986).

Consumable anthocyanins are found in fruits and vegetables. Their content varies considerably and is affected by genes and environmental conditions (Horbowicz et al. 2008). The highest total anthocyanin content was found in blueberries, chokeberries, elderberries, grapes and eggplants, exceeding 5,000 mg kg^{-1} (Clifford 2000). Besides fruits and vegetables, anthocyanins may also be present in substantial amounts in cereals such as purple corn, red and black rice and wheat with purple pericarp or blue aleurone (Abdel-Aal et al. 2006). Blue-grained wheat genotypes are of particular interest due to their relatively high total anthocyanin content. Furthermore, anthocyanin pigments can be concentrated by dry milling and fractionation processes to produce fractions with high anthocyanin levels (Abdel-Aal et al. 2006). Another reason to place blue-aleurone wheat into the focus is the relative composition of anthocyanins. In the plant kingdom, the most abundant anthocyanidin is cyanidin-3-glucoside, which is the main anthocyanin in fruits such as various berries and black currant, vegetables, red and black rice, cob corn and purple pericarp wheat (Escribano-Bailon et al. 2004). On the other hand, the major anthocyanidin of the blue-aleurone wheat is delphinidin-3-glucoside (Trojan et al. 2014). It is the most potent angiogenic inhibitor among anthocyanins and may be helpful in cancer prevention and treatment (Lamy et al. 2006). Delphinidin is also said to be more effective in the inhibition of tumorogenesis, by blocking the activation of the mitogen-activated protein kinase (Hou et al. 2004). Additionally, Afaq et al. (2007) investigated the photo-chemopreventive effect of delphinidin on UVB-induced biomarkers of skin cancer development.

The biochemical pathway of anthocyanins is well known (Ficco et al. 2014). The early steps of their synthesis are regulated by a cascade of enzymes including chalcone synthase (CHS), chalcone-isomerase (CHI), flavanone 3-hydroxylase (F3H) and dihydroflavonol-4-reductase (DFR). In wheat, the genes for CHS, F3H and DFR were cloned and mapped to the proximal region of the long arm of the homoeologous group 3 (DFR) (Yang et al. 2004; Himi and Noda 2004). All these genes were identified in the parental genotypes of the blue grain wheat—standard wheat and *Th. ponticum*, which both do not express blue aleurone phenotype. This implies that there must be some regulatory gene(s) that control the expression of these genes in developing seeds of standard and blue-grained wheats, but the regulatory pattern in the blue-grained seeds may not be the same as that in standard wheat and *Th. ponticum* (Yang et al. 2004). This is in agreement with studies on maize and other flowering plants, where at least eight structural genes and two families of regulatory genes controlling the flavonoid biosynthesis were identified (Gao et al. 2000). Moreover, the situation is complicated by the effect of the environment, where the level of expression in blue-aleurone wheats is influenced by temperature, light intensity, pH and other factors (Zeven 1991). Different levels of anthocyanin concentration were found along the developmental process with the maximum peak observed during the mid-grain-development stage (Knievel et al. 2009; Trojan et al. 2014).

As indicated above, the expression of blue coloration of the aleurone layer (*Ba*) in blue-grained wheat is associated with the presence of a chromosome or chromosome segment introgressed from alien species. Three genes involved in regulating the expression of blue coloration of the aleurone in wheat have been identified. They originate from different species: *Ba1* (syn. *Ba(b)*) is a dominant gene originating from *Thinopyrum ponticum* ($2n = 10x = 70$, $StStStStE^eE^eE^bE^bE^xE^x$ (previously designated as Ag); syn. *Lophopyrum ponticum*; *Elytrigia pontica*; *Agropyron elongatum*) and was physically mapped to region 0.71–0.8 of the long arm of 4Ag from centromere (Zheng et al. 2006a), *Ba2* (syn. *Ba(a)*) is an incompletely dominant gene mapped close to the centromere on long arm of $4A^m$ and to $4A^{bo}$ in *Triticum monococcum* and *T. boeoticum*, respectively (Dubcovsky et al. 1996; Singh et al. 2007), while *BaThb* is expressed by introgression of *Th. bessarabicum* ($2n = 2x = 14$, E^bE^b = JJ) into wheat and was mapped to chromosome 4J between centromere and FL0.52 (William and Mujeeb-Kazi 1993). *Th. bessarabicum* is the probable donor species that contributed the E^b genome to many polyploid wheatgrasses including *Th. ponticum* (Zhang et al. 1996). Thus, *Ba* genes from *Th. bessarabicum* (*BaThb*) and from *Th. ponticum* (*Ba1*) may have a common origin.

Genomic constitution of blue grain wheat genotypes is largely unknown. This is mainly due to limited information passed from one breeder to another and because most of the breeders in the early blue-grained wheat breeding programs are no longer active. Moreover, it is possible that as a consequence of the exchange of breeding materials, the same or closely related accessions were used at several research programs (Zeven 1991). However, it is known that in case of *Ba1*, substitution lines were developed by replacing wheat homoeologous chromosomes 4B and 4D by *Th. ponticum* chromosome 4Ag (Cermeno and Zeller 1986; Arbuzova et al. 2012). Similarly, in *Ba2* wheat genotypes, 4A and 4B were replaced by $4A^{bo}$ chromosome from *T. boeoticum* or *T. monococcum* (Zeven 1991).

Segregation ratios indicate that *Ba* is controlled by a single dominant gene (Zeven 1991; Dubcovsky et al. 1996) and that at least the *Ba1* allele expresses a strong xenia effect when endosperm traits are influenced by genes from the male parent (Keppenne and Baenziger 1990; Knievel et al. 2009). As the aleurone is part of triploid endosperm tissue, four combinations of alleles are possible. Three doses of *Ba1* produce dark blue seed, two doses give medium-blue seed, one dose gives light blue seed, and the absence of the gene results in the lack of blue color. Thus, *Ba1* shows a clear dosage effect (Knott 1958).

Here, we summarize the results of a comprehensive study on the genomic constitution of almost all publically available genotypes of blue grain wheat. We combined GISH and FISH to detect introgressions of *Th. ponticum* chromosomes and chromosome segments and to identify wheat chromosome(s) involved in substitutions.

Materials and methods

Plant material

Seed samples of blue grain wheat genotypes were obtained from Prof. Adam J. Lukaszewski, University of California, Riverside, USA; Prof. C.O. Qualset, University of California, Davis, USA; Prof. F.J. Zeller, Technical University of München, Freising-Weihenstephan, Germany; Dr. Robert Metzger, Oregon State University, Corvallis, Oregon, USA; Prof. A. Börner, Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, Germany and from Genebank of the Crop Research Institute, Prague-Ruzyně, Czech Republic (Table 1).

In situ hybridization

Seeds were germinated on wet filter paper in Petri dishes, root tips were collected in ice water for 26–30 h and fixed in a mixture of absolute alcohol:glacial acetic acid (3:1) at 37 °C for 7 days. Cytological preparations and in situ hybridization with labeled DNA were made according to Masoudi-Nejad et al. (2002). In all experiments, genomic in situ hybridization (GISH) was done with a probe prepared from total genomic DNA of *Th. ponticum*. The probe was labeled with biotin by nick translation and detected with streptavidin-Cy3 using standard kits from Roche Applied Science following the manufacturer's instructions. The hybridization mix contained unlabeled genomic DNA of *T. aestivum* cv. Chinese Spring sheared to ca. 200–500 bp fragments at 1:150 ratio (probe:blocking DNA). Following the hybridization, preparations were counterstained with 4′,6-diamidino-2-phenylindole (DAPI) in VectaShield antifade (Vector Laboratories) and observed under Zeiss Axio Imager.Z2 microscope. For identification of individual chromosomes, two additional probes were employed: A digoxigenin-labeled probe for GAA microsatellites, prepared using PCR with $(GAA)_7$ and $(CCT)_7$ primers and wheat genomic DNA as a template, and a probe for a 260-bp fragment of the *Afa* family repeat, prepared and labeled by Texas Red using PCR with primers AS-A and AS-B on wheat genomic DNA according to Kubaláková et al. (2005).

Results

Chromosome constitutions

We found large variation among karyotypes of the blue grain wheat genotypes (Fig. 1; Table 1). 'Xiao Yan' is a homozygote for translocation of both arms of wheat chromosome 4D (Fig. 1b) where the distal about one halves of the arms of 4D were replaced by (probably) their homoeologues from *Th. ponticum* (4AgS and 4AgL). In UC66049 (Qualset et al. 2005) and its derivatives, the entire 4BL arm was replaced by an arm of a *Thinopyrum* chromosome. We can only speculate that this translocation is 4BS.4AgL, more so that tetraploid UC66049/LD222 (B_6F_4) produced by backcrossing of durum wheat LD222 to UC66049 was disomic for the same translocation and both lines were fertile. Two lines UC66049/RU440-4 (B_3F_2) were produced by backcrossing the blue aleurone RU440-4, a sib line of Skorpion (RU 440-6), to UC66049. These two lines were created to combine the *Ba1* and *Ba2* genes. The presence of monosomic or disomic chromosome arm substitution of *Thinopyrum* (4BS.4AgL) indicates introgression of *Ba1*. The presence of chromosome 4A from *T. boeoticum* carrying *Ba2* could not be detected using the probes employed here, but could be detected using aneuploid lines.

In Sebesta Blue 3 (SB3) and four other genotypes, chromosome arm 4BL carries a *Thinopyrum* introgression (Fig. 1) covering about two-thirds of 4BL. Thus, these

Table 1 Genomic constitution and color intensity of blue aleurone genotypes

Accessions	Karyotype	Type of introgression	Provided by
Xiao Yan	$2n = 6x = 42$	Disomic substitution on 4DS and 4DL	F.J. Zeller
UC66049	$2n = 6x = 42$, 4BS.4AgL	Disomic chromosome arm substitution of 4BL	C.O. Qualset
UC66049/RU440-4 (B_3F_2)	$2n = 6x = 42$, 4BS.4AgL	Disomic chromosome arm substitution of 4BL	N. Watanabe
RU440-4/UC66049 (B_3F_2)	$2n = 6x = 42$, 4BS.4AgL	Monosomic or disomic chromosome arm substitution of 4BL	N. Watanabe
UC66049/LD222 (B_6F_4)	$2n = 4x = 28$, 4BS.4AgL	Disomic chromosome arm substitution of 4BL	N. Watanabe
EF02-54-9 (Sebesta Blue 3)	$2n = 6x = 42$, 4BS.4BL.4AgL	Disomic introgression on 4BL	Martinek (Šebesta)
H90-35-1 (Metzger Blue3)	$2n = 6x = 42$, 4BS.4BL.4AgL	Disomic introgression on 4BL	Martinek (Metzger)
M90-41	$2n = 6x = 42$, 4BS.4BL.4AgL	Disomic introgression on 4BL	Martinek (Metzger)
M90-41-1 (Metzger Blue8)	$2n = 6x = 42$, 4BS.4BL.4AgL	Disomic introgression on 4BL	Lukaszewski
M90-99-2[a] (Metzger Blue9)	$2n = 4x = 28$, 4BS.4BL.4AgL $2n = 4x = 28$	Disomic, monosomic or no introgression on 4BL	Martinek (Metzger)
EF02-5426-3 (Sebesta Blue 1)	$2n = 6x = 44$	Introgression on two pairs of *T. aestivum* chromosomes	Martinek (Šebesta)
EF02-5430-2 (Sebesta Blue 2)	$2n = 6x = 44$	Introgression on two pairs of *T. aestivum* chromosomes	Martinek (Šebesta)
48 M	$2n = 6x = 44$	Introgression on two pairs of *T. aestivum* chromosomes	Martinek (Woś)
H90-15-1 (Metzger Blue1)	$2n = 6x = 44$	Introgression on two pairs of *T. aestivum* chromosomes	Lukaszewski
H90-15-2 (Metzger Blue2)	$2n = 6x = 44$	Introgression on two pairs of *T. aestivum* chromosomes	Martinek (Metzger)
Blue Baart	$2n = 6x = 44$	Disomic addition of *Th. ponticum* chromosome (4 J?)	Martinek (Lukaszewski)
Blue Norco[a] (Metzger Blue5)	$2n = 6x = 42 + 2t$ $2n = 6x = 42 + 1t$ $2n = 6x = 42$	Monosomic or disomic addition of telosomic *Th. ponticum* chromosome	Martinek (Lukaszewski)
1066/91 amphiploid (Metzger Blue7)	$2n = 6x = 42$, 34T.a. + 8Th.p.	Eight *T. aestivum* chromosomes replaced by their *Th. ponticum* counterparts	Lukaszewski
Skorpion (RU 440-6)	$2n = 6x = 42$	Not detected	Martinek (Škorpík)
Tschermaks Blaukörniger Sommerweizen	$2n = 6x = 42$	Not detected	Martinek (Börner)
Barevna 9	$2n = 6x = 42$	Not detected	Martinek (Škorpík)
Barevna 11	$2n = 6x = 42$	Not detected	Genebank Ruzyně
Barevna 17	$2n = 6x = 42$	Not detected	Genebank Ruzyně
Barevna 23	$2n = 6x = 42$	Not detected	Genebank Ruzyně
Barevna 25	$2n = 6x = 42$	Not detected	Martinek (Škorpík)
H83-952-1	$2n = 6x = 42$	Not detected	Martinek (Metzger)

[a] Variation in genome composition has been detected in these genotypes

genotypes were developed by backcrossing of UC66049 to *T. aestivum*, where homoeologous recombination between 4AgL of UC66049 and 4BL of *T. aestivum* took place. Tetraploid M90-99-2 was probably produced by crossing of SB3 or its close relative with durum wheat. This genotype was unstable in genomic constitution with two, one, or no translocated segments present in individual plants (Fig. 2).

Sebesta Blue 2 (SB2) and its relatives, and Sebesta Blue 1 (SB1), have the most complicated karyotypes. SB2 and its relatives have 44 chromosomes of which 40 are normal wheat chromosomes with a chromosome pair (likely 4D) missing. We detected two pairs of translocated chromosomes involving wheat and *Th. ponticum* chromatin. One pair of these translocated chromosomes has the centromere and pericentromeric parts of *Th. ponticum* and a segment

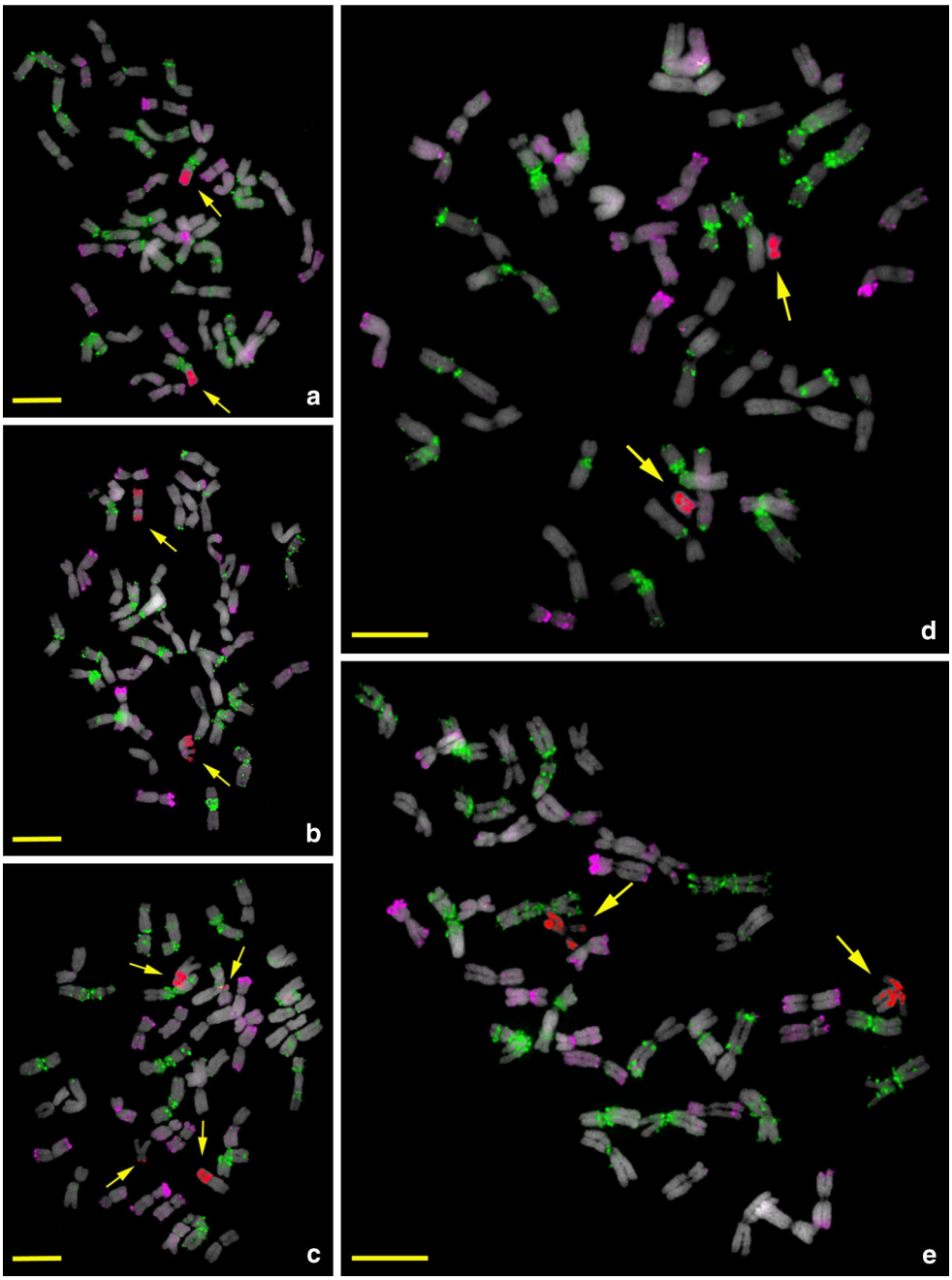

Fig. 1 Cytological analysis of blue-aleurone wheat genotypes. In situ hybridization on **a** M90-41, **b** Xiao Yan, **c** H90-15-2. **d** Blue Norco and **e** Blue Baart was performed using GAA (*green* color), *Afa* repeat (*purple* color) and total genomic DNA of *Th. ponticum* (*red* color) as probes. Chromosomes were counterstained by DAPI (*grey pseudocolor*). *Bar* 10 μm

from wheat chromosome (presumably 4DL) on one arm. The other translocated pair has one arm (presumably 4DS) and pericentromeric region of the second arm from wheat with a small terminal translocation from *Th. ponticum* (Fig. 1c). The karyotype of SB1 is even more complicated. It seems to have the same two translocations between wheat and *Thinopyrum* as SB2, but also 1–3 telocentrics from the B-genome of wheat.

Blue Baart is a disomic addition of a pair of *Th. ponticum* chromosomes (Fig. 1e), presumably 4Ag. Blue Norco is a ditelosomic addition from *Th. ponticum* (Fig. 1d). However, plants with only one *Thinopyrum* telocentric chromosome as well as plants without any *Thinopyrum* chromosome were also presented (see below). Amphiploid 1,066/91 has 42 chromosomes of which 34 are of *T. aestivum* and eight of *Th. ponticum* without any identifiable translocations (Fig. 3).

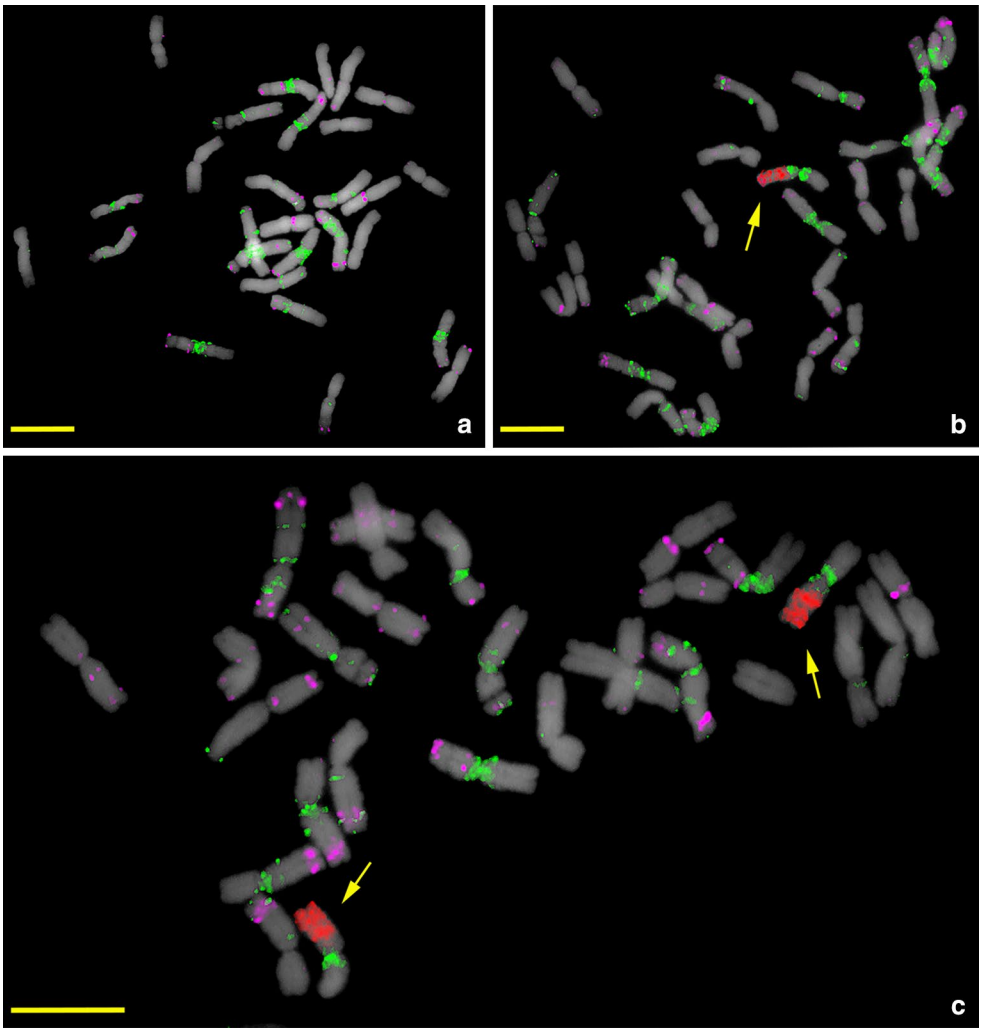

Fig. 2 Variation in genomic composition of M90-99-2 blue-aleurone wheat. In situ hybridization was performed using GAA (*green* color), *Afa* repeat (*purple* color) and total genomic DNA of *Th. ponticum* (*arrows*; *red* color) as probes. Chromosomes were counterstained by DAPI (*grey pseudocolor*). Intensity of *blue coloring* of seeds corresponds with the dose of *Th. ponticum* segment: seeds with standard (*red*) color had no *Th. ponticum* chromatin (**a**), monosomic substitution was detected in *light blue* seeds (**b**) and disomic substitution had seeds with *dark blue coloring*. *Bar* 10 μm

No detectable *Thinopyrum* chromatin was found in Skorpion, Barevna 9, Barevna 11, Barevna 17, Barevna 23, Barevna 25, Tschermaks Blaukörniger Sommerweizen and H83-952-1 and we can only speculate that the blue aleurone pigmentation is a consequence of a *T. monococcum* introgression and, therefore, represents *Ba2* locus. At least the first seven genotypes as listed have a common ancestor and belong to the legacy of Erich von Tschermak. It is in agreement with results of Zeller et al. (1991) who concluded, based on C-banding patterns and meiotic chromosome pairing in crosses of several European blue-grained wheat strains with double ditelosomic lines and other aneuploid lines of Chinese Spring that the *T. aestivum* Blaukorn strains "Berlin", "Probstdorf", "Tschermak", and "Weihenstephan" were chromosome substitutions of chromosome 4A from diploid *T. monococcum* or *T. boeoticum* for 4A of *T. aestivum*.

Dosage effects

In two genotypes, M90-99-2 and Blue Norco, we observed significant differences in color intensities within each sample (Fig. 4a–c). Thus, we selected 10 seeds each from the dark blue color, light blue color and the standard red color of grain. For Blue Norco, the intensity of the blue color correlated with the dosage of a telocentric *Th. ponticum* chromosome. All plants originating from dark blue kernels had a pair of telocentric chromosomes. In the light blue kernels, only one telocentric *Th. ponticum* was present,

while red seeds had a standard wheat karyotype with 42 chromosomes and no detectable *Thinopyrum* chromatin. In M90-99-2, the situation was more complicated. Among 10 dark seeds, five were homozygous for the 4BS.4BL.4AgL translocation, while the other half were heterozygous. Among 10 light blue kernels, one was homozygous for the translocation and nine were heterozygotes. All 10 red seeds had no *Thinopyrum* chromatin (Fig. 2). Thus, in this genotype 20 % (6/30) seeds were misclassified based on the aleurone color.

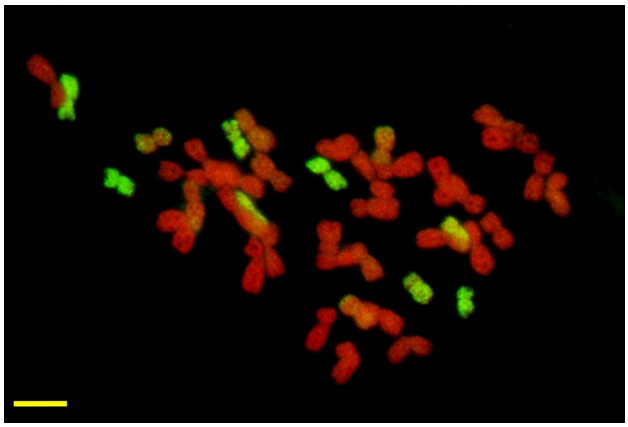

Fig. 3 Cytological analysis of 1066/91 amphiploid blue-aleurone wheat. Genomic in situ hybridization was performed using total genomic DNA of *Th. ponticum* (*green* color) as probe and blocking DNA of *T. aestivum*. Chromosomes were counterstained by DAPI (*red pseudocolor*). *Bar* 10 µm

Discussion

Blue-aleurone wheat is being heralded as a source of functional food due to high anthocyanin content. However, little is known on the variability of genomic constitution among and within various genotypes. Our results indicate that there are at least six different types of introgressions from *Th. ponticum* to bread wheat producing blue color of the aleurone layer. All of them appear to involve wheat chromosomes from the homoeologous group 4 (chromosomes 4B and 4D), or are disomic additions. Based on fertility of the analyzed lines, including tetraploid wheats, it can be assumed with some confidence that in all cases (translocations and additions), the *Th. ponticum* chromosome involved is its group-4 homoeologue. This is in agreement with previous reports. Jan et al. (1981) described UC66049 as 4BS.4AgL translocation, which was confirmed in our study. Similarly, complicated karyotypes were reported in the Sebesta Blue material by Morrison et al. (2004). Whelan (1989) described the karyotype of Blue Norco as disomic addition of a telocentric *Thinopyrum* chromosome. Here, we found variation in the blue color intensity of this genotype, which correlated well with the dosage of *Th. ponticum* chromosome (Fig. 4a–c). Dark blue kernels carry disomic addition of *Th. ponticum,* a monosomic addition generates light blue kernels, and the standard red kernels indicate the absence of any *Th. ponticum* introgression. A similar correlation has also been observed in M90-99-2 which exhibited variation in kernel coloration (data not shown).

Fig. 4 Seed samples of various blue-aleurone wheat genotypes. Note large variation in *blue* color intensity within cv. Blue Norco. Seeds with *dark blue* color were lately identified as disomic chromosome addition of *Th. ponticum* (**a**), *light blue colorizing* were in seeds with one chromosome of *Th. ponticum* (**b**), and in seeds with *red* color, we were unable to detect *Thinopyrum* chromatin. Similar variation was also found among genotypes with the same genomic constitution: **d** EF02-54-9 (Sebesta Blue 3), **e** M90-41 and **f** H90-35-1

Apart from the effect of chromosome instability, much variation in the blue color intensity was observed among the genotypes used in this study, ranging from dark blue seeds of Sebesta Blue genotypes to only slightly bluish kernels of Skorpion, Tschermaks Blaukörniger Sommerweizen and Barevna (data not shown). Generally, a lighter blue color was found among genotypes where no *Th. ponticum* chromatin could be detected and thus, probably carrying *Ba2* gene from *T. monococcum* or *T. boeoticum*. The exception was H83-952-1, which produces dark blue color kernels and has no detectable *Th. ponticum* chromatin. However, we cannot exclude a possibility that the introgression was too small to be detected by GISH. Some variation for color intensity was observed even among genotypes with the same confirmed chromosome constitution. The lines homozygous for the 4BS.4BL.4AgL translocation significantly vary in color from dark blue of Sebesta Blue 3 to light blue of H90-35-1 (Fig. 4d–f). This variation could be also due to different aging of seed samples donated for this study. Clear differences in color intensity between monosomic and disomic introgressions indicate a clear dosage effect. In this sense, color intensity (the amount of anthocyanin) could perhaps be further increased by a combination of two *Th. ponticum* introgressions on different wheat chromosomes.

This study reveals the locations of *Th. ponticum* introgressions and confirms earlier reports. What remains unclear is the sources of introgressions. We still have no information as to which chromosome from decaploid *Th. ponticum* is involved and even if it is the same chromosome in all cases. As mentioned above, all indications point to a group-4 homoeologue(s). However, because we do not have a detailed karyotype of *Th. ponticum*, the identity of the chromosome or chromosomes involved remains unanswered. A possible solution to identify and compare introgressions from different accessions of the blue-aleurone wheat would be to flow-sort chromosomes with *Th. ponticum* substitutions or additions forms (such as Blue Norco and Blue Baart), sequence them and analyze their gene content. This approach has been successfully applied to characterize *T. militinae* introgression in bread wheat (Abrouk et al. 2014). Our pilot experiments indicate that using FISHIS (Giorgi et al. 2013), it should be possible to flow-sort individual chromosomes with introgression from at least four distinct genotypes (data not shown). This should help to uncover the origin of the *Th. ponticum* introgressions in wheat. Based on the cytogenetic analysis, we suspect that there were various sources of *Th. ponticum* introgressions. If this hypothesis is confirmed, the introgressions probably serve as an activator of anthocyanin biosynthetic pathway in aleurone layer.

Most of the existing blue-aleurone wheats carry large blocks of *Th. ponticum* chromatin. This may preclude the use of blue-grained wheat in agriculture as negative effects on grain yield and nutritive characteristics are to be expected. Smaller segment translocations carrying the *Ba* gene must be developed as they ought to minimize the linkage drag. This cannot be accomplished by simple backcrossing, because *Th. ponticum* and *T. aestivum* homoeologues are not expected to pair. Compensating translocation chromosomes with small *Th. ponticum* introgressions can be produced in the presence of the *ph1b* mutation of Sears (1981). Alternatively, very small non-compensating introgressions with minimal adverse effect on grain yield can be produced by irradiation (Zheng et al. 2006b) or hybridization with a gametocidal line (Shi and Endo 1999). The development of lines with the segment of minimal length and still carrying *Ba* gene will increase the potential of blue-aleurone wheats in breeding and agricultural use.

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