Theor Appl Genet (2013) 126:3049–3063
DOI 10.1007/s00122-013-2192-8

ORIGINAL PAPER

Copy number variation of chromosome 5A and its association with *Q* gene expression, morphological aberrations, and agronomic performance of winter wheat cultivars

Sebastian Förster · Erika Schumann · Mario Baumann · W. Eberhard Weber · Klaus Pillen

Received: 19 April 2013 / Accepted: 6 September 2013 / Published online: 28 September 2013

Abstract
Key message **Our investigations combine chromosome 5A copy number variation associated with relative 5A*Q* gene expression and morphological and agronomic data to characterize the occurrence of speltoid plants in winter wheat cultivars.**
Abstract The occurrence of speltoid aberrants in wheat breeding is a serious problem that may result in rejection of a candidate cultivar during licensing. The spear-shaped, hard threshing spike is caused by copy number reduction of the domestication gene *Q*, located on the long arm of wheat chromosome 5A. As a member of the *APETALA2*-like transcription factor family, the 5A*Q* gene is involved in flower development and pleiotropically controls other agronomic traits. In this report, a characterization of instability of chromosome 5A is given and effects due to the loss of the *Q* gene and other genes are discussed. Based on pyrosequencing, we correctly predicted the 5A*Q* copy number for 392 of 402 tested offspring plants (97.5 %) originating from single speltoid plants of eleven wheat cultivars. The findings indicate that the resulting speltoid plants were either reduced in chromosome 5A copy number or possessed a partial deletion of the distal end of chromosome arm 5AL. 5A*Q* specific real-time PCR analysis revealed varying transcription levels among cultivars. During early spike development, the relative transcription of the 5A*Q* gene was always lower in speltoids than in normal square headed wheat plants, most likely leading to the occurrence of the characteristic speltoid spike phenotype. The parallel analysis of 18 agronomic traits revealed pleiotropic effects governed by genes located on 5A. Our results demonstrate that through pyrosequencing one can identify aneuploidy or deletions within chromosome 5A to select against the occurrence of speltoid plants in wheat seedlings.

Communicated by A. E. Melchinger.

Electronic supplementary material The online version of this article (doi:10.1007/s00122-013-2192-8) contains supplementary material, which is available to authorized users.

S. Förster (✉) · E. Schumann · M. Baumann · W. E. Weber · K. Pillen
Institute of Agricultural and Nutritional Sciences, Martin-Luther-University Halle-Wittenberg, Betty-Heimann-Str. 3, 06120 Halle (Saale), Germany
e-mail: sebastian.foerster@landw.uni-halle.de

Introduction

Wheat (*Triticum aestivum* L., $2n = 6x = 42$ chromosomes) is the most important cereal in Europe. A prerequisite for the economic success of wheat breeding companies is the stability of the wheat cultivar candidates and the resulting new varieties under diverse environmental conditions. Registration of bread wheat cultivar candidates in Europe is determined by a test of distinctness, uniformity and stability (DUS), which has been further exacerbated in line of the harmonization of European implementing rules (EU CPVO 2008). In addition to distinctness and stability, a further important prerequisite for cultivar certification in wheat is the uniformity of the cultivar candidate. Since 2008, all visible aneuploid plants are counted as off-types, following the directive of the Community Plant Variety Office (CPVO). These plants are a major cause for the lack of the homogeneity criterion that defines a tolerance limit of ten off-types among 2,000 plants. Allopolyploid species like bread wheat tend to prevail meiotic instability more frequently than diploids caused by irregular homologous chromosome pairing (Comai 2005). Consequently, genome

or chromosome mutations like aneuploidy may occasionally occur. A well-known phenomenon appears when wheat plants are monosomic for chromosome 5A resulting in a speltoid phenotype. A spear-shaped spike and an elongated rachis are characteristics for this aberrant spike architecture. Although the B genome showed the highest sensitivity for aneuploidy in nascent allohexaploid wheat (Zhang et al. 2013), it still remains open, why chromosome 5A is frequently lost during meiosis leading to speltoid off-types.

Early analyses of cytogenetic stocks presumed a suppressor gene on wheat chromosome 5A which was designated as domestication gene *Q*, leading to a free-threshing spike morphology (Unrau et al. 1950; MacKey 1954; Sears 1954). Dosage dependent spike alterations of 5A*Q* could be observed by Huskins (1946) and Sears (1954) for the first time. The later author showed plants that were nullisomic, monosomic, disomic, trisomic and tetrasomic for chromosome 5A expressing a speltoid, semi-speltoid, square headed, subcompact and compact spike architecture, respectively. Also, awned speltoid spikes may arise due to the loss of *Q* and the awn inhibitor *B1* from wheat chromosome 5A (Sourdille et al. 2002). The *Q* gene pleiotropically influences plant height and maturity (Muramatsu 1963; Kato et al. 1999, 2003). Furthermore, radiation-induced mutations of the *Q* gene effected visible changes on spike length and spikelet size, threshability, maturity, fertility, and glume tenacity (Singh 1969).

Initially, the 5A*Q* gene could be physically located by submicroscopic deletion interval mapping (Endo and Gill 1996). The gene could be fine mapped (Faris and Gill 2002) and finally cloned and characterized as a member of the *APETALA2* (*AP2*)-like transcription factor family by Simons et al. (2006). In *Arabidopsis*, two putative DNA binding motives mediate DNA binding and it was shown that *AP2* plays an important role in determination of flower development (Theissen and Saedler 1999), flowering time (Yant et al. 2010) and seed development (Jofuku et al. 1994). However, pleiotropic effects also occur in a complex manner (Carrera et al. 2009). Less is known about mechanism and genetic pathways associated with the 5A*Q* gene. Putative yeast two hybrid interactors like protein kinases or a stress responsive protein indicate a possible interface in respect to abiotic stress responses (Tai 2007).

A conserved nucleotide polymorphism between 5A*Q* and its recessive wild relative 5A*q* was located in exon 8 by Simons et al. (2006), leading to the amino acid substitution from valine (5A*q*) to isoleucine (5A*Q*). The substitution causes a conformational change of the 5A*q* protein, resulting in a reduced homodimer formation that may be related to the emergence of free-threshing wheat genotypes during domestication. Asakura et al. (2009) used a cleaved amplified polymorphic sequence (CAPS) marker approach to genotype both alleles at this position. Also, a quantitative pyrosequencing assay was established to discriminate both alleles and to quantify the 5A*Q* gene copy number (Förster et al. 2012). The latter authors applied a real-time sequencing bioluminescence approach producing short DNA reads visualized as pyrograms. Quantitative pyrosequencing is applied in clinical and molecular studies, for instance as a diagnostic tool for prostate cancer in humans (Yoon et al. 2012) or to determine copy number variations of the *CYP2D6* gene during drug metabolism (Söderbäck et al. 2005). Recently, Zhang et al. (2013) developed a homoeolog-specific pyrosequencing approach to detect aneuploidy for each of the 21 wheat chromosomes and found the most lability for the B genome followed by the A genome in nascent allohexaploid wheat.

The 5A*Q* gene expression was investigated in several studies (Simons et al. 2006; Gil-Humanes et al. 2009; Zhang et al. 2011). Ectopic transcription of 5A*Q* in transgenic silencing and overexpression lines allowed studying pleiotropic effects of 5A*Q* on spike morphology and plant height. When measuring 5A*Q* gene transcripts in different tissues Simons et al. (2006) showed the highest level of expression during early spike development followed by root tips and young leaves in wheat variety 'Chinese Spring'. Gil-Humanes et al. (2009) reported on comparative expression analyses of *APETALA2*-like genes in wheat, barley and barley × wheat hybrids in different tissues and showed, like Simons et al. (2006), decreasing transcription of 5A*Q* gene from developing towards fully emerging spikes. Recently, the evolutionary origin, relative expression and function of the three homoeologous *Q* genes were described by Zhang et al. (2011). The authors showed that all three genes are transcriptionally active and that the expression levels of the three *Q* genes depend on each other. However, the 5A*Q* gene played the key role conferring the domestication-related free-threshing spike character, whereas 5B*q* became a pseudogene contributing indirectly to the speltoid phenotype. The 5D*q* gene seems to be sub-functionalized, thus, also effecting spike morphology variations, but to a much lesser degree than 5A*Q* (Zhang et al. 2011).

In this work, we investigated 402 single progeny plants in total, which originate from speltoid aberrant spikes selected from eleven wheat cultivars. Among those progenies, we aimed to predict the occurrence of speltoid aberrants due to copy number variations at the 5A*Q* gene. In addition, we studied the transcription level of 5A*Q* and morphological aberrations in selected normal and speltoid progeny plants predicted to be aneuploid due to the loss of chromosome 5A.

Materials and methods

Plant materials

Grains from speltoid spikes were taken from eleven bread wheat cultivars (Table 1). All speltoid spikes were awnless

Table 1 Phenotypic segregation and pyrosequencing-based *Q* ratio assessment of wheat plants originating from speltoid plants of wheat cultivars

Wheat cultivar	Registered in Germany since	Breeding company	Awnedness of speltoid spike	Number of investigated plants	Fully awned speltoid plants	Awnless speltoid plants	Normal plants[a]	Sub-compact plants	Segregation of the four phenotypes (in %)	Agreement between *Q* ratio and spike phenotype
Alitis	2004	Strube	Awnless	25	0	21	4 (1)	0	0:84.0:16.0:0	24 of 25 (96 %)
Anthus	2005	KWS Lochow	Awnless	59	1	27	31 (5)	0	1.7:45.8:52.5:0	54 of 59 (91.5 %)
Batis	1994	Strube	Awnless	29	1	24	4	0	3.4:82.8:13.8:0	29 of 29 (100 %)
Biscay	2000	KWS Lochow	Awned	30	30	0	0	0	100:0:0:0	30 of 30 (100 %)
Dekan	1999	KWS Lochow	Awnless	25	0	19	6 (1)	0	0:76.0:24.0:0	24 of 25 (96 %)
Kranich	2007	Lantmännen SW Seed	Awnless	59	0	40	19 (1)	0	0:67.8:32.2:0	58 of 59 (98.3 %)
Lars	–[b]	Nordsaat Saatzucht	Awnless	30	0	18	12	0	0:60.0:40.0:0	30 of 30 (100 %)
Mulan	2006	Nordsaat Saatzucht	Awnless	29	0	24	5	0	0:82.8:17.2:0	29 of 29 (100 %)
Plutos	2006	Strube	Awnless	56	1	44	11	0	1.8:78.6:19.6:0	56 of 56 (100 %)
Skalmeje	2006	KWS Lochow	Awnless	30	0	23	6	1	0:76.7:20.0:3.3	30 of 30 (100 %)
Tiger	2001	Pflanzenzucht Oberlimpurg	Awnless	30	0	24	6 (2)	0	0:80.0:20.0:0	28 of 30 (93.3 %)
Total				402	33	264	104 (10)	1	8.2:65.7:25.9:0.2	392 of 402 (97.5 %)

Q ratio is calculated as the allele dosages of 5A*Q* versus 5B*q* and 5D*q*

[a] Number of plants exhibiting contrasting results for spike phenotype and pyrosequencing-based *Q* ratio assessment is indicated in brackets

[b] Cultivar 'Lars' is not registered in Germany, but in several other European countries

with the exception of cultivar 'Biscay'. Between 25 and 59 grains originating from a speltoid parent spike were germinated and the resulting offspring plants were used for molecular and phenotypic investigations. In addition, the standard wheat variety 'Chinese Spring' (CS) and its nullisomic-tetrasomic lines (N5AT5D, N5BT5A, N5BT5D and N5DT5A) were obtained from the German gene bank at the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), Gatersleben, and used as controls to verify genome specificity of primers. 5A*Q* specificity of qPCR primers was tested using the fast neutron-induced speltoid deletion mutant 'CS fndel-143', containing no 5A*Q* alleles, but functional 5B*q* and 5D*q* homoeoalleles (Simons et al. 2006). For tissue specific expression studies we used selected monosomic 5A (*Q*−) and disomic (*QQ*) CS lines (Sears 1954).

Plant cultivation and phenotyping

Grains from cultivars were germinated on filter paper in January 2011. Seedlings were vernalized for 7 weeks at 4 °C in a climate chamber and, subsequently, planted in the field (March 15, 2011) in 3 m rows spanning 10 and 12.5 cm within and between rows, respectively. Fertilization and phytosanitary protection measures were carried out according to local practice. All single progeny plants of the eleven wheat varieties were classified according to spike type as normal, speltoid, or compact. The number of investigated plants for each cultivar refers to the classification in Table 1. In addition, heading (date of BBCH 55 of main spike in days after May 1), flowering time (date of BBCH 61 of main spike in days after May 1), tiller number, peduncle length (in cm), flag leaf length (in cm), flag leaf width (in cm), and plant height (in cm) were determined in the field. Spike morphology traits were evaluated after harvest. Main spike length (in cm) and spikelet number per spike were analyzed for each plant. To determine threshability (in %), a method according to Kerber and Dyck (1969) was applied. The main spikes of single plants were threshed twice in a rubber sleeve for 4 s. The number of threshed grains was compared to total grain number of the same spike. Subsequently, grain number per ear, spike density (in number of spikelets per cm spike length), fertility (in grain number per number of spikelets) and grain weight per ear (in g) were determined. For grain width (in mm), grain length (in mm), grain area (in mm^2) and thousand grain weight (in g) a digital image MARVIN analyzer (GTA Sensorik GmbH, Neubrandenburg, Germany) was used. To compare qPCR expression data during early spike development, we calculated the ratio of length of the harvested young spike and length of the main spike of the fully emerged plant and defined it as spike fraction length.

DNA extraction, PCR amplification and 5A*Q* copy number detection using quantitative pyrosequencing

Fresh leaf material was collected from each plant in 2 ml tubes, equipped with 5 mm tungsten carbide beads (Qiagen GmbH, Hilden, Germany) and frozen at −80 °C over night. Next day, frozen leaf material was mechanically homogenized (at 30 Hz for 1 min) using the TissueLyser II (Qiagen). DNA extraction was carried out using a CTAB protocol modified from Doyle and Doyle (1987). DNA concentration was determined with NanoDrop 2000c (PEQLAB Biotechnologie GmbH, Erlangen, Germany). A total amount of 50 ng DNA was used for each PCR reaction. PCR conditions and pyrosequencing assays for 5A*Q* gene copy number quantification were carried out according to Förster et al. (2012) using primers differentiating adenine (5A*Q*) and cytosine (5B*q* and 5D*q*) at the *Q* gene position SNP2299. Subsequently, peak heights were exported from Pyrosequencing Software and the *Q* ratio between 5A*Q* and 5B*q* and 5D*q* was calculated according to Förster et al. (2012). Expected *Q* ratios for one, two, three and four 5A*Q* gene copies are 0.25, 0.5, 0.75 and 1.0, respectively. Subsequently, *Q* ratios between 0 and 0.125, 0.126 and 0.375, 0.376 and 0.625 and above 0.625 were considered to indicate full awned speltoid, speltoid, normal, and subcompact or compact plants, respectively.

Sequence analysis of pyrosequencing and qPCR primer binding sites in exon 8

For 5A*Q* gene specific qPCR expression studies at least one genome specific primer is required. 5A*Q*, 5B*q* and 5D*q* sequences are known in CS (AY702956), *T. turgidum* ssp. *durum* (DQ123819) and *Aegilops tauschii* (EU350482), respectively (Simons et al. 2006; Ning et al. 2009, sequences available at http://www.ncbi.nlm.nih.gov/). We aligned sequences using Geneious Software 5.5.7 (Drummond et al. 2011) and designed 5A*Q*, 5B*q* and 5D*q* specific primers flanking the qPCR forward primer binding site located in exon 8 of 5A*Q*. Each primer pair was homoeoallele specific by creating 3′ overhanging ends for at least one primer in both homoeoalleles. The following primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany):

5A*Q*:	5A_seq01	5′GACTTGGATCTGCGGATGTCGCAACCC3′
	and 5A_seq02	5′ATGCACAGGCCACTGGGAC3′,
5B*q*:	5B_seq01	5′ACACCCAAGTCTTATCTCATTGATATCTTC3′
	and 5B_seq02	5′CTAGCTAGTGCTAAACATGATTAATTTAA3′,
5D*q*:	5D_seq01	5′CAATCACACCAAGTCTTATCTCG3′
	and 5D_seq02	5′AATTTTCTATTGGAGCAAATCTG3′

Genome specificity was tested on CS nullisomic-tetrasomic lines. A total volume of 50 µl PCR reaction contained: 200 µM of each nucleotide, 0.4 µM of forward and reverse primers, 1× reaction buffer, 1.25 units *Taq* DNA polymerase and 50 ng of genomic DNA. All PCR reagents were obtained from Qiagen, Germany. The following PCR conditions were used on a PTC-200 thermal cycler (Bio-Rad Laboratories GmbH, München, Germany): initial denaturation (95 °C, 2 min), 35 cycles of denaturation (95 °C, 30 s), annealing (62 °C for 5A_seq01/02, 58 °C for 5B_seq01/02 and 5D_seq01/02, respectively, 30 s), synthesis (72 °C, 30 s), followed by a final extension (72 °C, 2 min). 5 µl of each PCR product were checked on a 1 % agarose gel. Next, 45 µl PCR products were purified using QIAquick PCR Purification Kit (Qiagen). Subsequently, genomic regions of all three homoeologous genes were sequenced from bread wheat varieties tested in this study (Eurofins MWG Operon) and multiple aligned for analyzing primer hybridization sites of each homoeoallele upstream of *Q* gene position SNP2299.

RNA extraction, cDNA synthesis and 5AQ qPCR expression studies

Three to five independent biological replicates from different single plants were used for RNA sampling of young leaves (2 weeks old), young spikes (prepared from tillers with ~20 cm length) and stems (along with young spikes) from fndel-143, monosomic 5A and disomic CS lines, respectively. Subsequently, tillers of single plants from screened cultivars were taken at a length of approximately 20 cm, spikes isolated from the stem, measured for spike length, frozen in liquid nitrogen and stored at −80 °C until RNA extraction. For RNA extraction frozen plant material was equipped with RNAse-free 5 mm tungsten carbide beads (Qiagen), homogenized (30 Hz for 1 min) using the TissueLyser II (Qiagen) and rapidly frozen in liquid nitrogen. Extraction was carried out using a guanidinium thiocyanate phenol chloroform procedure according to Chomczynski and Sacchi (2006). RNA was precipitated in a volume of 500 µl ice-cold isopropanol and solved in 100 µl DEPC-treated water. RNA concentration was determined with the NanoDrop 2000c (PEQLAB Biotechnologie GmbH, Erlangen, Germany). One µg of RNA was treated with DNaseI (Roche Diagnostics GmbH, Mannheim, Germany) and cDNA was synthesized using RevertAid™ H Minus First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Roth, Germany) according to manual instructions. For qPCR the Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen, Darmstadt, Germany) and Mastercycler® ep *realplex* (Eppendorf, Hamburg, Germany) were used. For each sample 1:4, 1:16 and 1:64 dilutions (calculated as technical replicates) of cDNAs were prepared. The qPCR efficiencies and relative expression were calculated according to Pfaffl (2001) using the internal reference gene actin. The qPCR mix contained: 12.5 µl 2 × Platinum® SYBR® Green qPCR SuperMix-UDG, 0.4 µM of forward and reverse primer (0.5 µl of each), 2 µl of each cDNA template and 9.5 µl of DEPC-treated water with a total volume of 25 µl. The following qPCR primers were used:

5A*Q*_for 5′CCTGAATCGTCAACCACAATGA3′
5A*Q*_rev 5′ATGCACAGGCCACTGGGA3′
actin_for 5′TGTGCTCAGTGGTGGCTCAAC3′
actin_rev 5′ACTTCCTCTCAGGCGGTGCCA3′

To check for genomic DNA contamination a minus-reverse transcriptase control sample was included in the assay. The following qPCR conditions were: Uracil-DNA-glycosylase reaction (50 °C, 2 min), *Taq* polymerase activation (95 °C, 2 min), 40 cycles of denaturation (95 °C, 15 s), annealing and synthesis in one step (60 °C, 1 min), denaturation (95 °C, 1 min) followed by a melting curve analysis (ranging from 60 to 95 °C, 20 min).

Statistical analysis

Statistical analyses were performed with SAS Enterprise Guide 4.2 (SAS Institute 2008). Analysis of variance was carried out using the following general linear model:

$$Y_{ijk} = \mu + C_i + T_j + CxT_{ij} + \varepsilon_{ijk},$$

where μ is the general mean, C_i is the fixed effect of the ith cultivar, T_j is the fixed effect of the jth spike type, CxT_{ij} is the interaction between the ith cultivar and jth spike type and ε_{ijk} is the error of Y_{ijk}.

A Student's t test was carried out to identify significant deviations between normal and speltoid plants within a cultivar for phenotypic traits and expression data, respectively. Pearson correlation coefficients (r) were calculated between traits from means of cultivars using 'proc CORR' (SAS Institute 2008) for normal and speltoid plants separately. Further, correlation coefficients between cultivar means of normal and speltoid plants, respectively, were determined for each trait.

Results

Copy number variation of the 5AQ gene among wheat cultivars segregating for speltoid plants

The segregation of morphological aberrant spike phenotypes, which emerged from speltoid off-types of corresponding cultivars and *Q* ratio measurements are given in Table 1. Across 402 investigated plants originating from eleven wheat cultivars, a segregation of 8.2 % fully awned speltoids, 65.7 % awnless speltoids, 25.9 % normal and

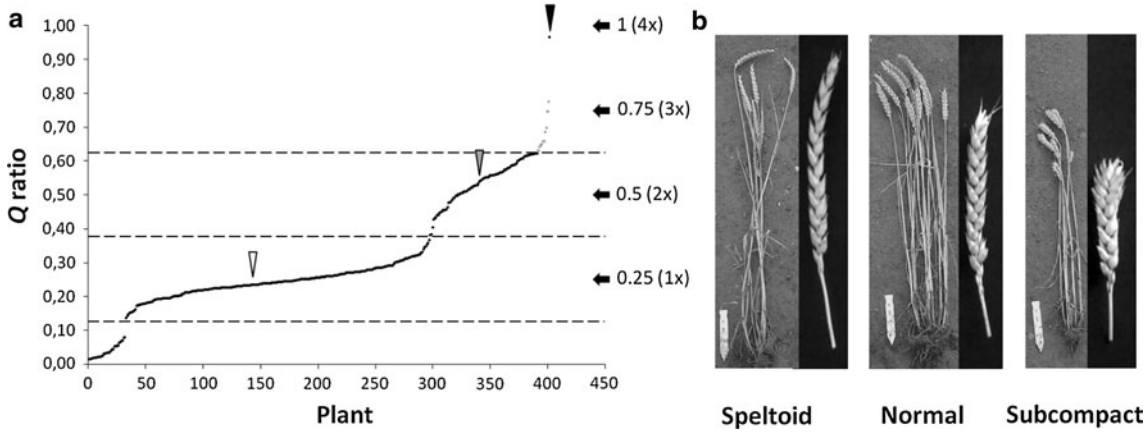

Fig. 1 *Q* ratio diagram estimated for 402 progeny plants (**a**) and stem and spike morphology of three contrasting progenies derived from one speltoid spike of cultivar 'Skalmeje' (**b**). **a** The *x* and *y* axis indicate 402 single offspring plants sorted from lowest to highest *Q* ratio and the calculated *Q* ratio, respectively, defined as ratio of 5A*Q* versus 5B*q* and 5D*q* peak heights for SNP2299 according to Förster et al. (2012). The *dashed horizontal lines* are three thresholds defined to predict the copy number of the 5A*Q* gene based on the *Q* ratio. The *gray dots* above the third threshold line indicate the ten single plants predicted to have more than two 5A*Q* alleles but expressing a normal spike type. *Black arrows* to the *right* indicate expected *Q* ratios for one (0.25), two (0.5), three (0.75), and four (1.0) 5A*Q* gene copies per plant. *White*, *gray*, and *black triangles* indicate the three single plants with varying *Q* ratios, which are illustrated in Fig. 1b. **b** Stem and spike morphology of three selected offspring plants marked with arrows in Fig. 1a in the same order

0.2 % compact plants were observed (Table 1). The phenotypic segregation varied between cultivars 'Batis' (86.2 % speltoid aberrant versus 13.8 % normal plants) and 'Anthus' (47.5 % speltoid aberrant versus 52.5 % normal plants) exhibiting the highest and lowest occurrence of speltoids, respectively (Table 1). 'Biscay' turned out to be a special case where all 30 progeny plants (100 %) exhibited an awned speltoid spike phenotype. Based on pyrograms, peak heights of 5A*Q* (nucleotide adenine) were compared to non-variable peaks from 5B*q* to 5D*q* (nucleotide cytosine) and calculated as *Q* ratio according to Förster et al. (2012). In total, the observed morphological spike phenotype of 392 out of 402 (97.5 %) tested single offspring plants could be correctly predicted by means of the *Q* ratio-based estimation of 5A*Q* copy number (Table 1; Fig. 1a). The *Q* ratio classification is given separately for each cultivar in Supplementary Fig. 1. The first spike group included 33 fully awned speltoid plants (Table 1) where no 5A*Q* allele (peak of nucleotide adenine) was detected via quantitative pyrosequencing, resulting in a *Q* ratio near zero (Fig. 1a below the first threshold line). Three fully awned plants originating from cultivars 'Anthus', 'Batis' and 'Plutos' exhibited a weak stature and a low fertility. The 30 offspring plants from cultivar 'Biscay' expressed a fertile, stable and awned phenotype like the parental spike. Genetic causes for both groups of fully awned speltoid plants having no 5A*Q* alleles are given in discussion. All 33 plants of this group were excluded from further phenotypic analysis, because either no control group was available (Table 1, 'Biscay') or the number of phenotypes was too small. Genotyping the 5A specific SSR marker *Xbarc56*, located on the short arm of chromosome 5A, for all 402 single plants revealed no PCR amplicon for the three single fully awned speltoid plants from 'Anthus', 'Batis' and 'Plutos' (exemplified for 'Batis' in Supplementary Fig. 2). In contrast, the residual 264 speltoid, 104 normal, and one subcompact plants revealed PCR amplifications for *Xbarc56* (data not shown). Using further 5A chromosome specific markers covering the whole chromosome indicated a deletion on the long arm of 5A including the *Q* and *B1* gene for progenies of cultivar 'Biscay' because no PCR product could be amplified for marker *Xwmc110* (Supplementary Fig. 3). The second spike group included 264 awnless speltoid individuals, which clustered around a *Q* ratio of 0.25 indicating to possess one copy of the 5A*Q* allele (Table 1; Fig. 1a). The third spike group with plants expressing a normal square headed phenotype predicted to have two copies of 5A*Q* clustered around a *Q* ratio of 0.5. For ten single plants a *Q* ratio >0.625 (third threshold level) was measured expressing a normal square headed phenotype (Table 1 and gray dots in Fig. 1a), which were also excluded from phenotypic analysis. Also, one subcompact plant with a *Q* ratio of 0.967 was identified that was derived from a speltoid spike of wheat cultivar 'Skalmeje' (Table 1; Fig. 1a). The spike and stem phenotypes of three progenies of wheat cultivar 'Skalmeje' expressing a speltoid, normal and subcompact spike phenotype, respectively, are shown in Fig. 1b.

Sequence alignment of exon 8 of the *Q* gene homoeoalleles

Amplifying the genomic region at SNP2299 revealed PCR products for the 5A*Q*, 5B*q,* and 5D*q* homoeologs with

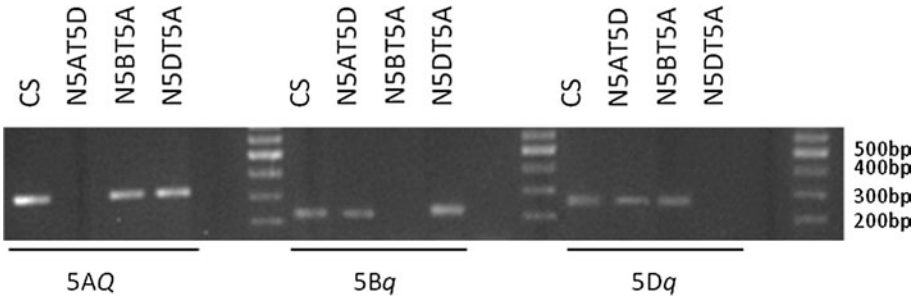

Fig. 2 Homoeoallele-specific PCR amplification of exon 8 in disomic and nullisomic-tetrasomic CS lines. Three primer combinations were tested on disomic 'Chinese Spring' (*CS*), nullisomic5A-tetrasomic5D (*N5AT5D*), nullisomic5B-tetrasomic5A (*N5BT5A*), and nullisomic5D-tetrasomic5A (*N5DT5A*) lines

fragment sizes of 328, 223, and 254 bp, respectively, for CS and their corresponding homoeoalleles in nulli-tetrasomics lines (Fig. 2). For N5AT5D, N5BT5A, and N5DT5A no PCR products were amplified using 5A*Q*_seq01/02, 5B*q*_seq01/02, and 5D*q*_seq01/02 primer combinations, respectively (Fig. 2). Sequence comparisons between ten licensed wheat cultivars and CS (Fig. 3) shows conserved nucleotides for *Q* when aligned to the reference sequence from CS (AY702956), *T. turgidum* ssp. *durum* (DQ123819), and *Ae. tauschii* (EU350482). At SNP2299, the 5A homoeoalleles differ in a single nucleotide (adenine) compared to 5B and 5D homoeoalleles (cytosine) for all cultivars (Fig. 3). The qPCR forward primer perfectly matches with the 5A*Q* allele, but not with 5B*q* and 5D*q* creating a 3′ mismatch in the latter cases. In contrast, the pyrosequencing primer binds to each of the three homoeoalleles (Fig. 3).

Expression of the 5AQ gene in different tissues and cultivars varying for 5AQ copy number

The amplification of the reference gene actin could be detected for CS fndel-143 as well as the disomic CS line (Supplementary Fig. 4a, c). When using the 5A*Q* specific primer combination, a PCR product could only be amplified from young spikes of the disomic CS line but not from the CS deletion line fndel-143 (Supplementary Fig. 4b, d). Melting curve analyses of the reference gene actin (Supplementary Fig. 4e) and the 5A*Q* gene (Supplementary Fig. 4f) showed a single peak at ~84.6 and 80.2 °C, respectively. All qPCR products of 3–5 selected plants, possessing either one or two copies of the 5A*Q* gene, from ten investigated wheat cultivars melted at the same temperature for both genes (data not shown). Tissue specific transcription of the 5A*Q* gene revealed the highest level of mRNA transcript in young spikes of disomic CS for chromosome 5A (set to 1, Fig. 4a). Disomic CS plants exhibited a relative expression of 0.595 and 0.304 in stem and young leaves, respectively. Young spikes, stems, and young leaves from monosomic 5A CS plants expressed 0.395, 0.195, and 0.205 of 5A*Q* mRNA transcript, respectively (Fig. 4a). The 5A*Q* gene expression pattern in young spikes of ten investigated bread wheat cultivars indicated a lower relative expression level in single copy 5A*Q* speltoid plants than in disomic plants for all cultivars (Fig. 4b). Measuring relative transcription level revealed the highest and lowest value from normal plants for cultivars 'Anthus' (5.02) and 'Dekan' (1.55), respectively, relative to CS. Significant differences ($P = 0.05$) in 5A*Q* expression patterns were observed between normal and speltoid plants for wheat cultivars 'Anthus', 'Kranich', 'Lars', 'Mulan', and 'Tiger' (Fig. 4b). In addition, the cultivars themselves showed variations in transcription level. Across the ten cultivars a correlation coefficient of $r = 0.757$ was observed between the relative 5A*Q* expression of normal and speltoid plants (Fig. 5).

Phenotypic traits, Pearson correlation coefficients and analysis of variance for normal and speltoid plants

Phenotypic investigations of normal and speltoid plants were carried out to characterize pleiotropic effects across wheat progenies segregating for 5A*Q* gene copy number within each of the ten investigated cultivars (Fig. 6). Spike length of speltoids was significantly increased for cultivars 'Anthus', 'Kranich', and 'Tiger', whereas 'Lars' showed shorter spikes for speltoids (Fig. 6a). All speltoid plants were longer compared to normal plants (Fig. 6j) and had a tendency towards longer peduncles (Fig. 6k) and smaller flag leaves (Fig. 6l, m). In addition, speltoids produced fewer spikelets (Fig. 6p) and a reduced spike density (Fig. 6c), fewer grains (Fig. 6r) resulting in lower grain weight (Fig. 6b). Grain parameters of speltoids were characterized by more narrow and shorter grains (Fig. 6e, f) with reduced grain area (Fig. 6g) and lower TKW (Fig. 6h). Also, speltoids showed a delay in heading and flowering time (Fig. 6n, o; except for 'Mulan'). Threshability was significantly lower in speltoids than in normal plants and, in particular, 'Alitis' and 'Batis' showed a clearly reduced threshability with 58.93 and 46.73 %, respectively, compared to speltoid plants of the other eight cultivars (Fig. 6i). Comparing trait performance between 264 speltoid and 104 normal plants revealed significant differences for 13 of 18 traits with the exception of spike length, peduncle length,

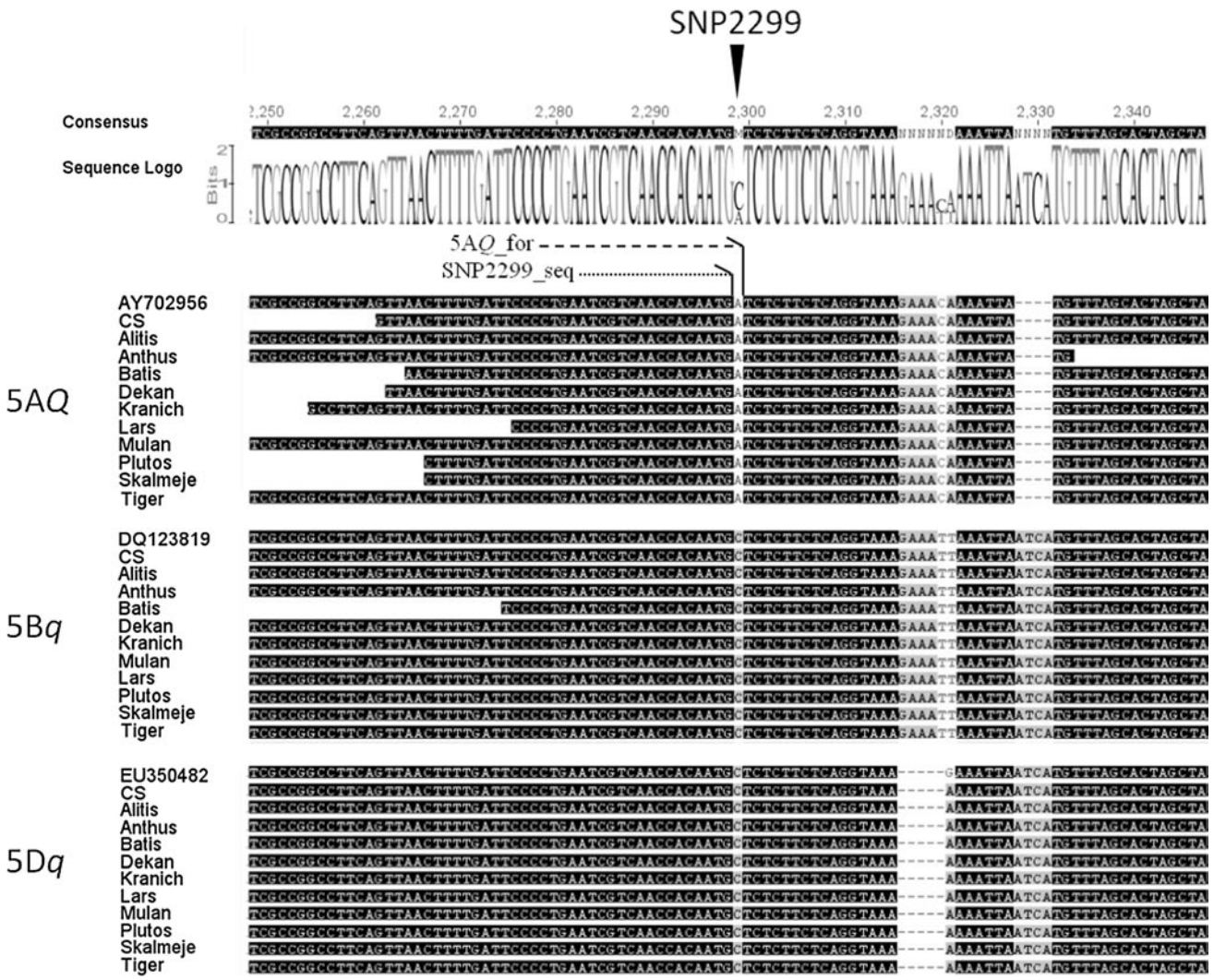

Fig. 3 Sequence alignment of the genomic region of exon 8 from the 5A*Q*, 5B*q*, and 5D*q* homoeoalleles of CS and ten investigated wheat cultivars. Each sequence was compared to complementary reference genome sequences of CS (AY702956), *T. turgidum* ssp. *durum* (DQ123819) and *Ae. tauschii* (EU350482). *Black*, *gray* and *white* bases indicate 100, 60–80 and <60 % similarity among all sequences, respectively. Primer binding sites of the 5A*Q* qPCR forward primer and the sequencing primer of the pyrosequencing assay at SNP2299 are indicated as *dashed* and *dotted lines*, respectively

number of tillers, grain length, and plant height (summarized in Table 2). The relative 5A*Q* gene expression was also significantly reduced in speltoids compared to normal plants (Table 2).

The two-factorial linear model analysis revealed significant effects ($P = 0.05$) of the fixed factor cultivar for all traits except peduncle length and of the fixed factor spike type except for spike length, peduncle length, and tiller number (data not shown). The cultivar × spike type interaction was significant for ten traits at $P = 0.05$ (data not shown). These were spike length, spikelet number, spike density, grain number, fertility, grain weight, threshability, flag leaf length, tiller number, and flowering time. Pearson correlation between and within all investigated traits are shown in Supplementary Table 1 for speltoid and normal plants. Eighteen and 25 of 171 correlations for normal and speltoid plants between the traits were significant ($P = 0.05$), respectively. Eleven of 18 traits and 5A*Q* gene expression were significantly correlated between normal and speltoid plants ($P = 0.05$). Particularly, grain parameters like grain area and grain width were highly correlated ($r > 0.9$) with thousand kernel weight for normal and speltoid plants. For speltoid progeny plants significant positive correlations between the traits spike length and grain number, flag leaf length, flag leaf width, and plant height were observed (between $r = 0.64$ and $r = 0.66$). This correlation was not obtained (or somewhat weaker) for normal plants (Supplementary Table 1). A similar observation was found

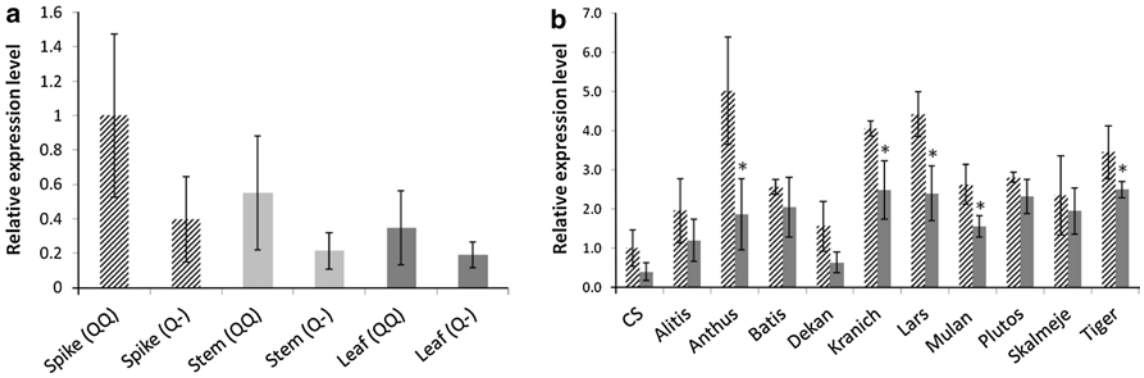

Fig. 4 Relative transcript levels of the 5A*Q* gene from different tissues of monosomic 5A (*Q*−) and disomic 5A (*QQ*) CS lines (**a**) and from young spikes of ten normal (*hatched*) and speltoid (*gray*) wheat cultivars (**b**). Calculation of relative transcription was carried out according to Pfaffl (2001) using the internal reference gene actin. Expression levels are represented as fold-difference over CS spike (*QQ*), which is set to 1. Each measurement includes 3–5 biological replicates. *Vertical bars* and *asterisks* represent standard errors and significant differences at $P = 0.05$ (Student's *t* test), respectively

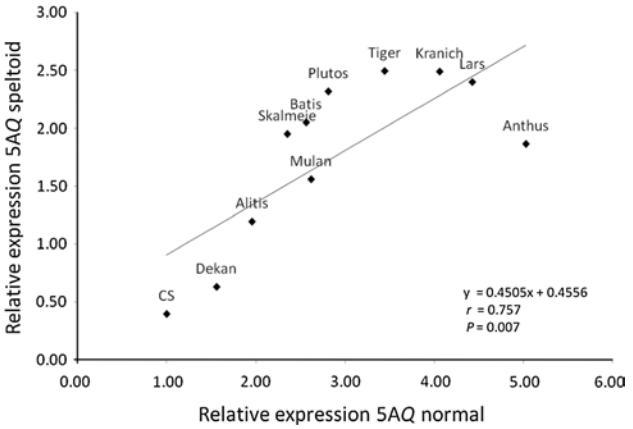

Fig. 5 Correlation plot of the relative 5A*Q* expression between normal and speltoid plants across ten wheat cultivars and CS. Regression line, linear equation (*y*), correlation coefficient (*r*), and significance value (*P*) are indicated in the diagram. Each measurement includes 3–5 biological replicates

for grain number and fertility, which were negatively correlated with grain parameters like thousand kernel weight, grain area, and grain width (significant for speltoid plants at $P = 0.05$ with a range from $r = -0.69$ to $r = -0.83$, whereas the effect of normal plants was weaker with a range from $r = -0.50$ to 0.57, Supplementary Table 1). Further, grain weight of normal plants was correlated highly significant with grain parameters like thousand kernel weight, grain area, grain width, and grain length (range from $r = 0.70$ to $r = 0.83$), whereas no significant correlation between these traits was found in speltoid plants (Supplementary Table 1). To connect the phenotype data with the relative transcription level of the 5A*Q* gene correlations between the traits and 5A*Q* transcription level were separately calculated for normal and speltoid plants across the ten cultivars (Supplementary Table 1). Only a negative significant correlation between number of tillers and relative *5AQ* expression was calculated for normal plants with $r = -0.69$ (Supplementary Table 1). Speltoid plants revealed a similar negative correlation tendency with $r = -0.45$ (Supplementary Table 1).

Discussion

Breeding applications using pyrosequencing and genetic causes for speltoids

Pyrosequencing is an advanced and highly reliable next-generation sequencing application based on sequencing-by-synthesis bioluminescence technique. Short PCR products can be analyzed for diagnostic and genotyping approaches. In barley it was already used for SNP genotyping to fine-map the *Rrs2* scald resistance gene (Hanemann et al. 2009) or to develop high-throughput SNP assays for grain and malting parameters (Matthies et al. 2012). In our previous work, we have developed a quantitative pyrosequencing assay for genotyping and copy number quantification of the 5A*Q* gene (Förster et al. 2012).

In this work, we applied the assay to detect (1) plants having no 5A*Q* gene due to a deletion event (Supplementary Fig. 3) and (2) plants segregating for the 5A*Q* copy number (Fig. 1a; Supplementary Fig. 1). In total, we could predict the occurrence of speltoid versus normal spike phenotypes based on the *Q* ratio with an accuracy of 97.5 % across 402 investigated plants, which were derived from 11 cultivars (Table 1). Only ten plants were not correctly predicted. In these cases the calculated *Q* ratio was above the threshold of 0.625 (Fig. 1), however, a normal spike phenotype was expressed. This finding may indicate possible

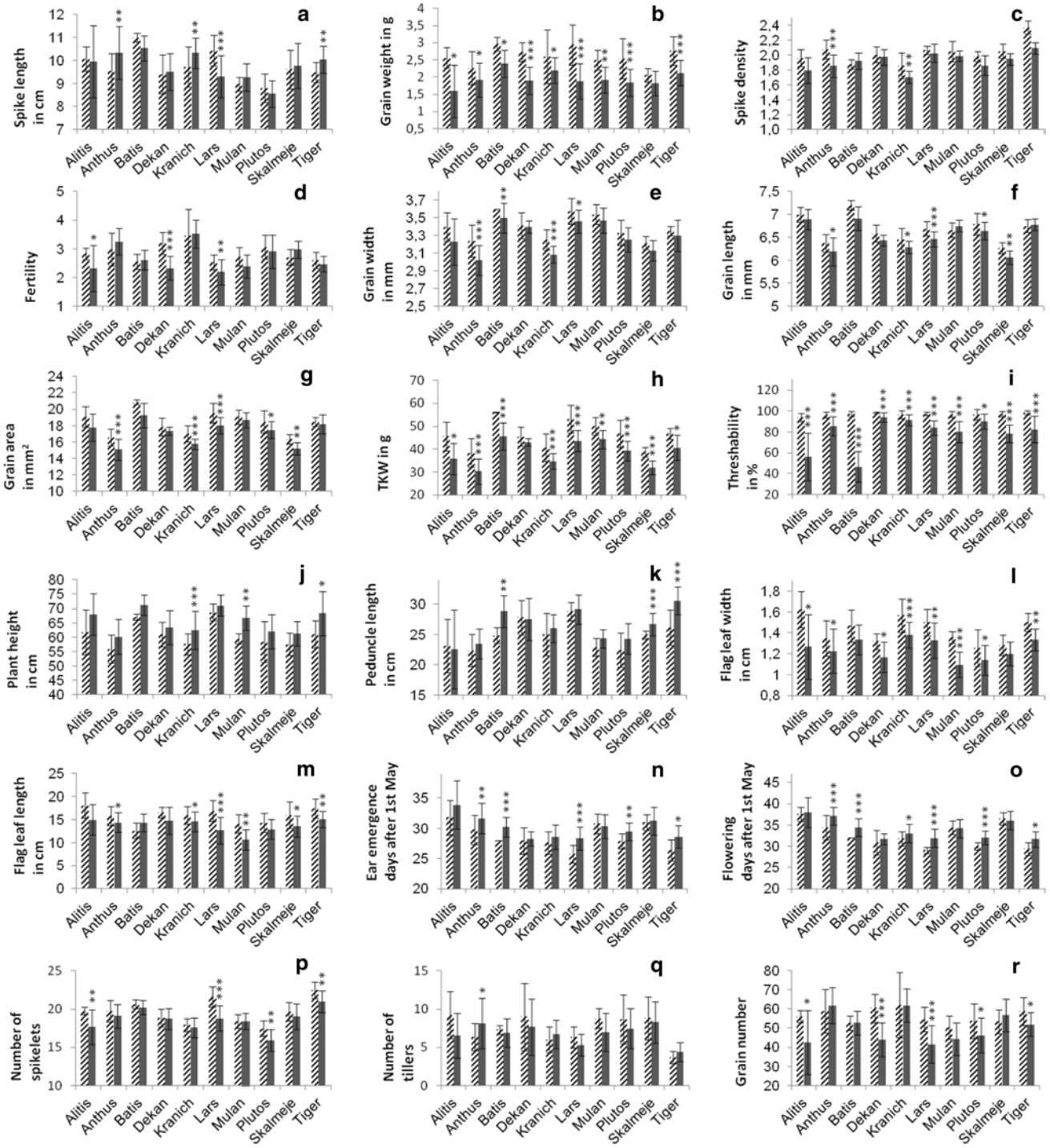

Fig. 6 Histograms with 18 morphological traits for normal (*hatched bars*) and speltoid plants (*gray bars*) within ten investigated wheat cultivars. **a** spike length in cm, **b** grain weight in g, **c** spike density (number of spikelets/cm spike length), **d** fertility (grain number/number of spikelets), **e** grain width in mm, **f** grain length in mm, **g** grain area in mm^2, **h** thousand kernel weight in g, **i** threshability in %, **j** plant height in cm, **k** peduncle length in cm, **l** flag leaf width in cm, **m** flag leaf length in cm, **n** heading time in days after 1st May, **o** flowering time in days after 1st May, **p** number of spikelets, **q** number of tillers and **r** grain number/spike. All spike and grain parameters are based on the main spike of each single plant. *Vertical bars*, *, **, and *** represent standard errors and significant differences at $P = 0.05$, $P = 0.01$, and $P = 0.001$ (Student's *t* test), respectively

Table 2 Trait performance of 264 speltoid and 104 normal plants, calculated across ten cultivars segregating for 5A*Q* gene copy number

Trait	Normal plants		Speltoid plants		
	Mean[a]	SD[b]	Mean[a]	SD[b]	Difference[c]
Spike length in cm	9.6	0.9	9.7	1.1	−0.1
Number of spikelets	19.5	1.8	18.4	2.1	1.1***
Spike density	2.0	0.2	1.9	0.2	0.1***
Grain number	57.5	11.1	51.1	12.0	6.4***
Fertility	3.0	0.6	2.8	0.6	0.2*
Grain weight in g	2.5	0.6	2.0	0.5	0.5***
Threshability in %	97.0	3.1	80.5	17.8	16.5***
Flag leaf length in cm	15.9	2.3	13.8	2.6	2.1***
Flag leaf width in cm	1.4	0.2	1.2	0.2	0.2***
Peduncle length in cm	26.5	20.1	26.1	3.8	0.4
Number of tillers	6.9	2.5	6.9	2.7	0.0
Ear emergence in days after 1st May	28.5	2.5	30.0	2.7	−1.5***
Flowering days in after 1st May	32.5	3.2	33.8	3.0	−1.3***
Thousand kernel weight in g	43.7	7.7	38.6	6.7	5.1***
Grain area in mm^2	17.7	1.6	17.2	1.7	0.5**
Grain width in mm	3.4	0.2	3.3	0.2	0.1**
Grain length in mm	6.6	0.3	6.5	0.3	0.1
Plant height in cm	54.7	16.2	55.5	22.9	−0.8
Relative expression of the 5A*Q* gene	3.1	1.2	1.9	0.8	1.2***

Asterisks (*, **, and ***) represent significant differences at $P = 0.05$, $P = 0.01$, and $P = 0.001$ (Student's t test), respectively. Calculation of the 5A*Q* gene expression refers to 3–5 biological replicates per cultivar

[a] Average across 264 speltoid and 104 normal plants, respectively

[b] Standard deviation

[c] Average difference between normal and speltoid plants

pyrosequencing errors because genomic background noise at the 5A*Q* allele may be present (Fig. 1; Supplementary Fig. 1). All 297 speltoid plants possessing no or one 5A*Q* allele could be precisely predicted. Recently, molecular karyotyping via homoeolog-specific pyrosequencing was established for all 21 wheat chromosomes by Zhang et al. (2013). The authors could also distinguish with high accuracy between varying copy numbers of individual chromosomes. Our results and the results of Zhang et al. (2013) demonstrate that pyrosequencing can be very useful for wheat breeding and basic research programs to identify and select against the occurrence of aneuploids or speltoid plants. To verify our pyrosequencing results, all 30 awned and speltoid progenies of cultivar 'Biscay' were tested with five 5A specific SSR markers. The results suggest that a homozygous deletion on the long arm of chromosome 5A, including the 5A*Q* gene and the awn inhibitor gene *B1*, may explain the fully awned speltoid phenotype in progenies of 'Biscay' (Supplementary Fig. 3). The remaining 372 progenies of the other ten cultivars segregated 3:264:104:1 (i.e. 0.81:70.96:27.96:0.27 %) in fully awned speltoid, awnless speltoid, normal square headed, and subcompact plants, respectively (Table 1). Most likely, these plants are nullisomic, monosomic, disomic, and trisomic for chromosome 5A, respectively. Since no PCR amplicon was detected with SSR marker *Xbarc56* (exemplarily shown for cultivar 'Batis' in Supplementary Fig. 2) for the three awned speltoid plants (from cultivars 'Plutos', 'Batis and 'Anthus', Table 1) it must be assumed that these plants are indeed nullisomic for chromosome 5A. The remaining 264 speltoid plants (Table 1) are most likely monosomic for the entire chromosome 5A since *Xbarc56* was PCR amplified (exemplified in Supplementary Fig. 2 for cultivar 'Batis') and a 5A*Q* copy number of one was estimated simultaneously (Fig. 1). A segregation ratio in cultivar CS was observed by Singh and Rajlakshmy (1994) with 0.5 % double monosomics, 62.4 % monosomics, 36.6 % disomics, and 0.5 % nulli- and trisomics over a period of 18 years. Sears (1954) found differences in vitality of paternal and maternal gametes in monosomic lines of cultivar CS. Resulting progenies were expected to segregate in 3, 73, and 24 % for nulli-, mono-, and disomic plants, respectively. In our own previous studies, we already confirmed a connection between plants having a single 5A*Q* allele and the loss of one chromosome predicted to be 5A for four bread wheat cultivars (Förster et al. 2012). However, the observation of contrasting segregation proportions in normal and speltoid plants for different cultivars, e.g. 'Anthus' and 'Batis' (Table 1), may suggest the presence of unbalanced gametes in different genotypes. This phenomenon may be due to different maternal and paternal gamete vitalities between cultivars resulting in a shift of chromosomal distribution as mentioned by Sears (1954). Own field studies revealed a cultivar-dependent segregation of progeny plants originating from speltoids indicating a genetic control mechanism (unpublished data). In future, a genome-wide association mapping approach could be performed to identify QTLs controlling the underlying mechanism of losing wheat chromosome 5A.

Meiotic recognition and correct pairing of homologous chromosomes is still barely understood in allopolyploids like wheat. As reviewed by Comai (2005), one disadvantage of polyploidy is the propensity to mitotic and meiotic instability resulting in chromosome rearrangements. Autopolyploids tend to form multivalents during meiotic metaphase I (Singh 2003). This, in return, may promote incorrect distribution of chromosomes caused by non-disjunction during anaphase, potentially resulting in unbalanced gametes. Vega and Feldman (1998) reported on the wheat homoeologous pairing gene *Ph1*, which mediates homoeologous chromosome

pairing by affecting the centromere-microtubule interaction. In detail, *Ph1* suppresses *Cdk2*-type activities during premeiosis and meiosis, thus, mediating wheat replication and histone H1 phosphorylation in analogy to mammalians (Greer et al. 2012). It is well known that chiasma frequency and meiotic stability strongly depend on temperature (Bayliss and Riley 1972) and univalents in wheat can undergo transverse division resulting in unbalanced gametes (Sears 1952; Friebe et al. 2005). Further, Mestiri et al. (2010) reported on progeny-dependent meiotic behavior in synthetic allohexaploids that was associated with the occurrence of univalents. A recent study published by Zhang et al. (2013) focused on aneuploid detection in early generations of synthetic hexaploid wheat using a combination of pyrosequencing and cytogenetic methods. The authors found higher frequencies of aneuploids for a certain line in each generation indicating a genetic predisposition for the loss and gain of chromosomes. They also showed that the B genome seems to be prone to aneuploidy. Own field trials corroborate a predisposition of certain genotypes producing significantly more speltoid off-types (unpublished results) where aneuploidy of chromosome 5A is assumed to be the genetic cause. However, the genetic mechanism leading to spontaneous aneuploid formation is still widely undisclosed. It must be assumed that both, the genetic background and environmental conditions, influence genome stability. Using pyrosequencing combined with an association mapping approach may be supportive to clarify if other genes beside *Ph1* are involved in aneuploidy formation.

Sequence analysis and relative 5AQ gene expression

The sequencing primer of the assay developed by Förster et al. (2012) binds to each of the three homoeoalleles within all cultivars (Fig. 3). This enabled us to quantify 5A*Q* gene copy number and further calculate *Q* ratio via pyrosequencing. By designing a 3′ overhanging end of the qPCR forward primer on 5B*q* and 5D*q* homoeoalleles (Fig. 3), only 5A*Q* transcription levels could be measured in CS (Supplementary Fig. 4b, d) and therefore in breeding cultivars (Fig. 4b). Tissue specific transcription levels of disomic CS plants corresponded well with already existing tissue analysis from Simons et al. (2006) and Gil-Humanes et al. (2009). Highest transcription of 5A*Q* in young spikes implicated a regulatory mechanism during spike development processes. In *Arabidopsis*, flower organ identity was explained by a flower "ABC" model (Coen and Meyerowitz 1991), where *AP2* transcription factors were grouped into the A class and assumed to control floral transition processes (Jofuku et al. 1994). Post-transcriptional regulation of *AP2* due to microRNAs could be described by Aukerman and Sakai (2003) and Chen (2004). Here, we reported on different relative transcription levels of the *Q* gene, which is an *AP2* homolog. In addition, we always found higher 5AQ transcription levels in winter cultivars than in wheat control 'Chinese Spring' (Fig. 4b), even though young spikes were harvested at slightly different developmental stages (spike fraction length ranged from minimum 0.15 to maximum 0.28, data not shown). Simons et al. (2006) showed an approximate constant transcription level during this developmental stage. Our expression data clearly showed that single copy speltoid bread wheat cultivars expressed 5A*Q* at a lower level than plants having two copies of the 5A*Q* gene (Fig. 4b). This may indicate that the 5A*Q* transcription in young spikes determines the expression of speltoid spikes early on. It is unclear why the investigated winter wheat cultivars expressed the 5A*Q* gene on different levels (Fig. 4b) and it remains speculative how far regulating elements or genomic backgrounds are responsible for this phenomenon. Simons et al. (2006) showed conserved variations in promoter sequences in species having either 5A*Q* or 5A*q*, presumably effecting transcriptional differences among the two alleles. How far the genetic regulation of 5A*Q* may depend on variation in the promoter region, environmental influences or genetic background remains largely unknown. According to Zhang et al. (2011), the three homoeologous *Q* genes are subjected to mutual genetic regulation. The authors suggested that 5D*q* plays a more pronounced role in the expression of spike phenotype variations when 5A*Q* is absent. Possibly, the regulation of the relative 5A*Q* transcription is controlled by homoeologous genes from chromosomes 5B and 5D. This explanation should be validated in future by setting up and testing qPCR assays for all three *Q* gene homoeologs among breeding cultivars.

Pleiotropic effects caused by the loss of 5A and the role of the domestication gene Q

Speltoids in bread wheat are the major cause for the lack of the homogeneity and may lead to rejection of a cultivar candidate during licensing. There is a major interest of breeding companies to analyze the genetic and phenotypical constitution of speltoid off-types to avoid their occurrence during the propagation process. In addition to copy number quantification of the 5A*Q* gene via pyrosequencing, we investigated 18 agronomic traits and the 5A*Q* gene transcription in developing spikes of current winter wheat cultivars. Several dosage dependent genes are located on chromosome 5A, which may contribute to the phenotypic variation between speltoid and normal wheat plants, predicted to be monosomic and disomic for chromosome 5A, respectively (Table 2; Fig. 6). We analyzed the 5A*Q* gene expression trying to explain the speltoid spike morphology due to a reduced transcription level of *Q* (Fig. 5a, b). In addition, possible pleiotropic effects, contributed by

other loci on chromosome 5A and possible interactions are discussed.

Aneuploid wheat was systematically investigated by Sears (1954) and monosomic analyses enabled locating major genes like *Q* on certain chromosomes. Genetic analyses of wheat chromosome 5A and its influence on important agronomic traits were also analyzed by Snape et al. (1985) using single chromosome recombinant lines. Chromosome 5A pleiotropic effects governed by *Q*, the awn inhibitor *B1* and the vernalization gene *Vrn-A1* on yield components were described. The authors found QTLs at the 5A*Q* locus affecting ear length, spikelet number, and ear emergence time for winter sowing. Further, they showed pleiotropic effects of *Vrn-A1* or closely linked genes on plant height, tiller number and spikelet number. Zhang et al. (2010) used a meta-QTL analysis approach and confirmed previously described yield and yield-related QTLs located close to the 5A*Q* and *Vrn-A1* loci.

In our study, we found that speltoid plants tended to produce smaller seeds and a lower thousand grain weight (Fig. 6e, f, g, h). Zhang (2008) did not observe any differences in thousand grain weight comparing the 'Bobwhite' mutant of the 5A*Q* gene with the wild type, indicating that 5A*Q* may not control this trait in spring wheat. Whether other loci than 5A*Q* are associated with yield parameters remains open. The reduced number of spikelets of speltoid plants (Fig. 6p) and the reduced grain number of the main spike (Fig. 6r) were consistent with the 'Bobwhite' 5A*Q* mutant (Zhang 2008) indicating that 5A*Q* may influence these traits in current winter wheat cultivars.

The delayed heading and flowering of speltoid plants (Table 2) seems to be governed by other genes on chromosome 5A since a reduced transcription of *Q* leads to early heading and flowering in 'Chinese Spring' as displayed by Zhang et al. (2011). A good candidate may be the vernalization gene *Vrn-A1*. Díaz et al. (2012) reported on copy number variation of *Vrn-A1* leading to altered flowering time. Due to a reduced copy number of the entire chromosome 5A, the *Q* gene may interact with *Vrn-A1* to cause a delayed heading time of speltoid plants. Another explanation may be the gene balance hypothesis. (Birchler and Veitia 2010) pointed on aneuploidy effects leading to unbalanced stoichiometric differences among members of macromolecular complexes and the interactome. Due to an altered regulatory balance, it must be assumed that aneuploids tend to reduce fitness, perhaps leading to delayed flowering when the entire chromosome 5A is lost. Zhang et al. (2013) reported on significantly lower seed-setting for aneuploidy of three chromosomes (3A, 3B and 5A) substantiating the gene balance hypothesis.

QTLs for glume toughness and threshability could be mapped to chromosome arms 2DS and 5AL using a recombinant inbred line (RIL) population (Jantasuriyarat et al. 2004). The 5A QTL corresponded to the 5A*Q* gene. All speltoid plants of investigated cultivars in our study were more difficult to thresh (Fig. 6i). Spikelets of cultivars 'Alitis' and 'Batis' were obviously tougher and harder to thresh (Fig. 6i) compared to normal plants of the same cultivars and to speltoid plants of the other eight cultivars indicating a genetic mechanism controlling this trait. The reduced 5A*Q* gene copy number in speltoid plants (Fig. 1), leading to lower relative expression of 5A*Q* (Fig. 4b), may explain the threshability effect (Fig. 6i). However, the level of transcription did not reflect the tenacity of spikelets since no correlation was found between these traits neither in normal nor in speltoid plants (Supplementary Table 1). Possibly, epistatic effects governed by 5B*q* and 5D*q* homoeoalleles or other genes may cause the reduced threshability. One example for the phenomenon is the 2DS QTL corresponding to the gene tenacious glume (*Tg*) that was mapped in a 8.1 cM interval by Sood et al. (2009). Kerber and Rowland (1974) reported that the recessive allele *tg* must be present for the expression of the free-threshing character, suggesting that *Tg* is epistatic to 5A*Q*. After *Tg* will be cloned, the genetic interaction between *Tg* and 5A*Q* in bread wheat may be studied in more detail in regard to the regulation of threshability.

A slight negative correlation between tiller number and relative transcription of 5A*Q* was obtained in our study (Supplementary Table 1). Interestingly, Kato et al. (2000) reported on a QTL closely linked to the 5A*Q* gene that was controlling tiller number. Although no variation for this trait was observed in CS fndel-143 and the disomic CS wild type (Zhang et al. 2011) a regulatory mechanism may effect variation in tiller number due to 5A*Q* because of a higher level of transcript in winter wheat cultivars (Fig. 4b). By testing an induced 5A*Q* mutant significantly fewer tillers and lower yield were observed compared to the wild type cv. 'Bobwhite' (Zhang 2008). The mutant line revealed a 5A*Q* transcript level that was reduced to approximately half of the 'Bobwhite' control, an observation, which corresponds well to our findings for 5A*Q* single copy plants (Fig. 4b). Significant effects of the factor cultivar (C_i) and the interaction between cultivar and spike type (CT_{ij}) on tiller number indicate that this trait is associated with the investigated genotypes. Our and previous studies (Kato et al. 2000; Zhang 2008) showed that the 5A*Q* gene could possibly be associated with the regulation of tiller number depending on the genomic background. This observation should be examined in more detail in future studies using a larger subset of winter wheat cultivars associated with 5A*Q* gene expression in different developmental stages.

The contrasting trait correlation between the two phenotypic groups, e.g. highly significant correlation of grain weight with other grain parameters for normal but not for speltoid plants (Supplementary Table 1), is rather difficult

to explain. Environmental conditions may favor trait correlations in one phenotypic group but not in the other, leading to a more pronounced association between traits. One example may be the flag leaf size in speltoid plants having an effect on spike length due to an increased assimilation rate. Further, it seems likely that altered trait correlations between normal and speltoid plants caused by the loss of chromosome 5A, and thus several dosage depended genes, are more phenotypically correlated due to gene balance hypothesis as mentioned before. Assuming that *Q* is the major gene controlling spike length, plant height and threshability on wheat chromosome 5A, the interacting effects might be more expressed in one of the two phenotypic classes (Supplementary Table 1) due to its pleiotropic character, but also due to altered epistatic effects with other major genes like *Vrn-A1*.

Conclusion and outlook

We reported on pyrosequencing as a highly accurate and reliable breeding approach to select against speltoids in young wheat seedlings varying for 5A*Q* gene copy number. The high degree of reproducibility makes the assay attractive for application in wheat breeding programs, for instance, to predict the occurrence of speltoids in breeding lines. Further, we suggest pyrosequencing as a useful tool to study meiotic disturbances of chromosome integrity in allopolyploid organisms like bread wheat.

The genome specific qPCR assay enables transcriptional analyses in breeding cultivars to investigate 5A*Q* copy number-dependent transcription levels in combination with pleiotropic effects of the 5A*Q* gene. The varying *Q* gene transcription levels among current winter cultivars seem to be subjected to varying control mechanism, perhaps leading to unequivocal pleiotropic effects of *Q*. Although, we only observed a weak correlation between tiller number and 5A*Q* gene expression, the extent of gene dosage in current breeding cultivars should be examined in more detail due its influence on agronomic traits like grain parameters. In future, additional plant developmental stages, breeding lines, and cultivation conditions like stress treatments should be studied to achieve further molecular insights into 5A*Q* dependent pleiotropic effects on wheat cultivation.

Acknowledgments We acknowledge Ms. Roswitha Ende for excellent technical assistance. Also, we thank Dr. Justin D. Faris and Dr. Andreas Börner for providing seeds of the CS fndel-143 deletion and CS nulli-tetrasomic lines, respectively. We are grateful to the Federal Plant Variety Office, Hannover, Germany, and the German breeding companies KWS Lochow GmbH, Bergen, Pflanzenzucht Dr. H.c.R. Carsten, Bad Schwartau, and Dr. Hermann Strube, Söllingen, for providing speltoid bread wheat material. This research was financially supported by the Federal Ministry of Economics and Technology, Berlin (BMWi, Code Number KF 2104501MD8) and the "FAZIT-STIFTUNG Gemeinnützige Verlagsgesellschaft mbH", Frankfurt am Main. The research project was administrated by the "Arbeitsgemeinschaft industrieller Forschungsvereinigungen e.V.", Köln (AiF) and the "Gemeinschaft zur Förderung der privaten deutschen Pflanzenzüchtung e.V.", Bonn (GFP).

Conflict of interest We certify that there is no conflict of interest with any financial organization. Further, we strictly followed ethical standards when conducting the reported research.

References

Asakura N, Mori N, Nakamura C, Ohtsuka I (2009) Genotyping of the *Q* locus in wheat by a simple PCR-RFLP method. Gen Genet Sys 84:233–237

Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2*-like target genes. Plant Cell 15:2730–2741

Bayliss MW, Riley R (1972) Analysis of temperature-dependent asynapsis in *Triticum aestivum*. Genet Res 20:193–200

Birchler JA, Veitia RA (2010) The gene balance hypothesis: implications for gene regulation, quantitative traits and evolution. New Phytol 186:54–62

Carrera J, Rodrigo G, Jaramillo A, Elena SF (2009) Reverse-engineering the *Arabidopsis thaliana* transcriptional network under changing environmental conditions. Gen Biol 10:R96

Chen XM (2004) A microRNA as a translational repressor of *APETALA2* in Arabidopsis flower development. Science 303:2022–2025

Chomczynski P, Sacchi N (2006) The single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction: twenty-something years on. Nat Protoc 1:581–585

Coen ES, Meyerowitz EM (1991) The war of the whorls: genetic interactions controlling flower development. Nature 353:31–37

Comai L (2005) The advantages and disadvantages of being polyploid. Nat Rev Genet 6:836–846

Díaz A, Zikhali M, Turner AS, Isaac P, Laurie DA (2012) Copy number variation affecting the *Photoperiod-B1* and *Vernalization-A1* genes is associated with altered flowering time in wheat (*Triticum aestivum*). PLoS One 7(3):e33234

Doyle JJ, Doyle JL (1987) A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull 19:11–15

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A, Duran C, Field M, Heled J, Kearse M, Markowitz S, Moir R, Stones-Havas S, Sturrock S, Thierer T, Wilson A (2011) Geneious v5.4, Available from http://www.geneious.com/

Endo TR, Gill BS (1996) The deletion stocks of common wheat. J Hered 87:295–307

EU CPVO. European Union (2008) Protocol for distinctness, uniformity and stability tests, *Triticum aestivum* L.: wheat. EU Community Plant Variety Office, p 40

Faris JD, Gill BS (2002) Genomic targeting and high-resolution mapping of the domestication gene *Q* in wheat. Genome 45:706–718

Förster S, Schumann E, Weber WE, Pillen K (2012) Discrimination of alleles and copy numbers at the *Q* locus in hexaploid wheat using quantitative pyrosequencing. Euphytica 186:207–218

Friebe B, Zhang P, Linc G, Gill BS (2005) Robertsonian translocations in wheat arise by centric misdivision of univalents at anaphase I and rejoining of broken centromeres during interkinesis of meiosis II. Cytogene Gen Res 109:293–297

Gil-Humanes J, Piston F, Martin A, Barro F (2009) Comparative genomic analysis and expression of the *APETALA2*-like genes from barley, wheat, and barley-wheat amphiploids. BMC Plant Biol 9:66

Greer E, Martin AC, Pendle A, Colas I, Jones AME, Moore G, Shaw P (2012) The *Ph1* locus suppresses Cdk2-type activity during premeiosis and meiosis in wheat. Plant Cell 24: 152–162

Hanemann A, Schweizer GF, Cossu R, Wicker T, Röder MS (2009) Fine mapping, physical mapping and development of diagnostic markers for the *Rrs2* scald resistance gene in barley. Theor Appl Genet 119:1507–1522

Huskins CL (1946) Fatuoid, speltoid and related mutations of oats and wheat. Bot Rev 12:457–514

Jantasuriyarat C, Vales MI, Watson CJW, Riera-Lizarazu O (2004) Identification and mapping of genetic loci affecting the free-threshing habit and spike compactness in wheat (*Triticum aestivum* L.). Theor Appl Genet 108:261–273

Jofuku KD, den Boer BGW, Van Montagu M, Okamuro JK (1994) Control of Arabidopsis flower and seed development by the homeotic gene *APETALA2*. Plant Cell 6:1211–1225

Kato K, Miura H, Sawada S (1999) QTL mapping of genes controlling ear emergence time and plant height on chromosome 5A of wheat. Theor Appl Genet 98:472–477

Kato K, Miura H, Sawada S (2000) Mapping QTLs controlling grain yield and its components on chromosome 5A of wheat. Theor Appl Genet 101:1114–1121

Kato K, Sonokawa R, Miura H, Sawada S (2003) Dwarfing effect associated with the threshability gene *Q* on wheat chromosome 5A. Plant Breed 122:489–492

Kerber ER, Dyck PL (1969) Inheritance in hexaploid wheat of leaf rust resistance and other characters derived from *Aegilops squarrosa*. Can J Genet Cytol 11:639–647

Kerber ER, Rowland GG (1974) Origin of free threshing character in hexaploid wheat. Can J Genet Cytol 16:145–154

MacKey J (1954) Neutron and X-ray experiments in wheat and a revision of the speltoid problem. Hereditas 40:65–180

Matthies IE, Sharma S, Weise S, Röder MS (2012) Sequence variation in the barley genes encoding sucrose synthase I and sucrose phosphate synthase II, and its association with variation in grain traits and malting quality. Euphytica 184:73–83

Mestiri I, Chagué V, Tanguy AM, Huneau C, Huteau V, Belcram H, Coriton O, Chalhoub B, Jahier J (2010) Newly synthesized wheat allohexaploids display progenitor-dependent meiotic stability and aneuploidy but structural genomic additivity. New Phytol 186:86–101

Muramatsu M (1963) Dosage effect of spelta gene *Q* of hexaploid wheat. Genetics 48:469–482

Ning SZ, Chen QJ, Yuan ZW, Zhang LQ, Yan ZH, Zheng YL, Liu DC (2009) Characterization of *WAP2* gene in *Aegilops tauschii* and comparison with homoeologous loci in wheat. J Sys Evol 47:543–551

Pfaffl MW (2001) A new mathematical model for relative quantification in real-time RT-PCR. Nucl Acids Res 29:e45

SAS Institute (2008) The SAS enterprise guide for windows, release 4.2. SAS Institute, Cary

Sears ER (1952) Misdivision of univalents in common wheat. Chromosoma 4:535–550

Sears ER (1954) The aneuploids of common wheat. MO Agr Exp Sta Res Bull 572:1–59

Simons KJ, Fellers JP, Trick HN, Zhang ZC, Tai YS, Gill BS, Faris JD (2006) Molecular characterization of the major wheat domestication gene *Q*. Genetics 172:547–555

Singh MP (1969) Some radiation induced changes at '*Q*' locus in bread wheat (*Triticum aestivum* L.). Caryologia 22:119–126

Singh RJ (2003) Plant cytogenetics. CRC Press, Boca Raton

Singh D, Rajlakshmy C (1994) Breeding behaviour of monosomics in hexaploid wheat. Wheat Info Serv 78:18–20

Snape JW, Law CN, Parker BB, Worland AJ (1985) Genetical analysis of chromosome 5A of wheat and its influence on important agronomic characters. Theor Appl Genet 71:518–526

Söderbäck E, Zackrisson AL, Lindblom B, Alderborn A (2005) Determination of *CYP2D6* gene copy number by pyrosequencing. Clin Chem 51:522–531

Sood S, Kuraparthy V, Bai G, Gill BS (2009) The major threshability genes soft glume (*sog*) and tenacious glume (*Tg*), of diploid and polyploid wheat, trace their origin to independent mutations at non-orthologous loci. Theor Appl Genet 119:341–351

Sourdille P, Cadalen T, Gay G, Gill B, Bernard M (2002) Molecular and physical mapping of genes affecting awning in wheat. Plant Breed 121:320–324

Tai YS (2007) The potential wheat signaling pathways in response to abiotic stress. Amer J Plant Physiol 2:295–302

Theissen G, Saedler H (1999) The golden decade of molecular floral development (1990–1999): a cheerful obituary. Dev Genet 25:181–193

Unrau J, Smith WE, McGinnis RC (1950) Spike density, speltoidy, and compactoidy in hexaploid wheat. Can J Res C 28:273–276

Vega JM, Feldman M (1998) Effect of the pairing gene *Ph1* on centromere misdivision in common wheat. Genetics 148:1285–1294

Yant L, Mathieu J, Dinh TT, Ott F, Lanz C, Wollmann H, Chen X, Schmid M (2010) Orchestration of the floral transition and floral development in *Arabidopsis* by the bifunctional transcription factor *APETALA2*. Plant Cell 22:2156–2170

Yoon HY, Kim SK, Kim YW, Kang HW, Lee SC, Ryu KH, Shon HS, Kim WJ, Kim YJ (2012) Combined hyper methylation of *APC* and *GSTP1* as a molecular marker for prostate cancer: quantitative pyrosequencing analysis. J Biomol Screen 17:987–992

Zhang Z (2008) Genomic analysis of *Q* domestication alleles and genes for susceptibility to *Stagonospora nodorum* in wheat, Dissertation Chapter III. NORTH DAKOTA STATE UNIVERSITY, US, pp 40–60

Zhang LY, Liu DC, Guo XL, Yang WL, Sun JZ, Wang DW, Zhang AM (2010) Genomic distribution of quantitative trait loci for yield and yield-related traits in common wheat. J Integr Plant Biol 52:996–1007

Zhang Z, Belcram H, Gornicki P, Charles M, Just J, Huneau C, Magdelenat G, Couloux A, Samain S, Gill BS, Rasmussen JB, Barbe V, Faris JD, Chalhoub B (2011) Duplication and partitioning in evolution and function of homologous *Q* loci governing domestication characters in polyploid wheat. PNAS 108:18737–18742

Zhang H, Bian Y, Gou X, Zhu B, Xu C, Qi B, Li N, Rustgi S, Zhou H, Han F, Jiang J, von Wettstein D, Liu B (2013) Persistent whole-chromosome aneuploidy is generally associated with nascent allohexaploid wheat. PNAS 110:3447–3452