The Genetic Basis of Composite Spike Form in Barley and 'Miracle-Wheat'

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ABSTRACT Inflorescences of the tribe *Triticeae*, which includes wheat (*Triticum* sp. L.) and barley (*Hordeum vulgare* L.) are characterized by sessile spikelets directly borne on the main axis, thus forming a branchless spike. 'Compositum-Barley' and tetraploid 'Miracle-Wheat' (*T. turgidum* convar. *compositum* (L.f.) Filat.) display noncanonical spike-branching in which spikelets are replaced by lateral branch-like structures resembling small-sized secondary spikes. As a result of this branch formation 'Miracle-Wheat' produces significantly more grains per spike, leading to higher spike yield. In this study, we first isolated the gene underlying spike-branching in 'Compositum-Barley,' *i.e.*, *compositum 2* (*com2*). Moreover, we found that *COM2* is orthologous to the *branched head*^{t} (*bh*^{t}) locus regulating spike branching in tetraploid 'Miracle-Wheat.' Both genes possess orthologs with similar functions in maize *BRANCHED SILKLESS 1* (*BD1*) and rice *FRIZZY PANICLE/BRANCHED FLORETLESS 1* (*FZP/BFL1*) encoding AP2/ERF transcription factors. Sequence analysis of the *bh*^{t} locus in a collection of mutant and wild-type tetraploid wheat accessions revealed that a single amino acid substitution in the DNA-binding domain gave rise to the domestication of 'Miracle-Wheat.' mRNA *in situ* hybridization, microarray experiments, and independent qRT-PCR validation analyses revealed that the branch repression pathway in barley is governed through the spike architecture gene *Six-rowed spike 4* regulating *COM2* expression, while *HvIDS1* (barley ortholog of maize *INDETERMINATE SPIKELET 1*) is a putative downstream target of *COM2*. These findings presented here provide new insights into the genetic basis of spike architecture in *Triticeae*, and have disclosed new targets for genetic manipulations aiming at boosting wheat's yield potential.

KEYWORDS 'Miracle-Wheat'; 'Wunder-Weizen'; 'Compositum-Barley'; inflorescence branching; yield potential

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INFLORESCENCES of the tribe *Triticeae*, containing wheat (*Triticum* sp. L.), barley (*Hordeum vulgare* L.), and rye (*Secale cereale* L.), display a raceme-like branchless shape and are therefore called a spike. Each spike is normally composed of spikelets arranged in two opposite rows along the main axis (rachis). Individual spikelets contain one or several florets, each producing one grain. Conventionally, in wheat, single spikelets arise from single rachis nodes (Figure 1A). Noncanonical spike forms showing ramified or branched wheat spikes (Figure 1, B–D), have been described as 'Miracle-Wheat' [*Triticum turgidum* convar. *compositum* (L.f.) Filat.] also recognized as 'Wunder-Weizen,'

'Blé de Miracle,' or 'Blé d'Osiris.' The branching-appearance of the 'Miracle-Wheat' inflorescence is evidently due to a naturally occurring mutation that has been known since ancient times (L'Obel 1591; Tschermak 1914; Sharman 1944). Spike-branching is of particular importance for enhancing sink capacity and boosting the yield potential of the crop, because in the case of wheat cultivars, current performance is generally thought to be sink restricted (Miralles and Slafer 2007; Lawlor and Paul 2014). The spike branching has been observed in diploid wheat ($2n = 2x = 14$, bh^m locus; Amagai *et al.* 2014), tetraploid wheat ($2n = 4x = 28$, bh^t locus; Klindworth *et al.* 1997), as well as barley [$2n = 2x = 14$; *compositum 2* (*com2*) locus], and rye [$2n = 2x = 14$; *monstrosum ear 1* (*mo1*) locus; Devries and Sybenga 1984]. The loci maintaining the branchless inflorescence form of the tribe *Triticeae* are all located in syntenic chromosome positions. This suggests that in *Triticeae*, the spike form (*i.e.*, branch repression) is controlled by a major orthologous gene. Defects in this gene result in lateral branch formation that, in its completely developed form, resembles a small-sized indeterminate spike (Figure 1, B–D). These lateral branches are distinct from the supernumerary spikelets (SS) phenotype, which comprises only additional spikelets per rachis node (Pennell and Halloran 1983). The underlying genetic factors for the SS phenotype can be diverse as it has been exemplified for the *multi-rowed spike* (*mrs*) locus (Dobrovolskaya *et al.* 2015) or paired spikelets phenotype (Boden *et al.* 2015). Moreover, a recent genome-wide QTL analysis in common wheat (*T. aestivum* L.) identified seven QTL regulating SS formation located on five chromosomes (2D, 5B, 6A, 6B, and 7B) (Echeverry-Solarte *et al.* 2014). Despite the long scientific scrutiny, "true spike-branching" in tetraploid wheat or barley, which represents the formation of laterally formed branch-like structures within the spike, has always remained elusive.

In the present report, we investigated the genetic and molecular basis of true spike-branching in 'Compositum-Barley' and tetraploid 'Miracle-Wheat.' Here we positionally cloned the gene *com2* underlying spike-branching in barley and found that it is orthologous to bh^t, which regulates spike branching in 'Miracle-Wheat.' Both genes possess orthologs with similar functions in maize *BRANCHED SILKLESS 1* (*BD1*) (Chuck *et al.* 2002), rice *FRIZZY PANICLE/BRANCHED FLORETLESS 1* (*FZP/BFL1*) (Komatsu *et al.* 2003; Zhu *et al.* 2003), and *Brachypodium distachyon MORE SPIKELETS 1* (*MOS1*) (Derbyshire and Byrne 2013). Moreover, bh^t is orthologous to *mrs* identified in hexaploid wheat (Dobrovolskaya *et al.* 2015). Sequence analysis of the bh^t locus in a collection of mutant and wild-type tetraploid wheat accessions revealed that a single mutation gave rise to the domestication of 'Miracle-Wheat.' As a result of branch formation, this mutant allele produces significantly more grains per spike, leading to higher spike yield.

Materials and Methods

Plant material

The 'Compositum-Barley' mutants were obtained from the Nordic Genetic Resource center, the National Small Grains Collection (US Department of Agriculture), and the Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) gene bank (Supporting Information, Table S1). For haplotype analysis, barley accessions from a previous report were used (Castiglioni *et al.* 1998) (Table S3). Mutant allele *com2.g*, its two-rowed progenitor Ackermann's Donaria, and Bowman near isogenic line (BW-NIL=BW 192) carrying *com2.g* were used for phenotypic descriptions and SEM analysis. Plant material used to generate mapping populations is reported in the corresponding section for genetic mapping. In the case of wheat, for allelism tests and genetic mapping in wheat, branched head wheat mutants were received from the National Small Grains Collection (US Department of Agriculture), and the IPK gene bank (Table S4 and Table S5).

Marker development

Barley chromosome 2H genome zipper (GZ) (Mayer *et al.* 2011) was utilized for initial marker development. Barley sequence information, the homologs of the rice genes ordered along the 2H-GZ was used for primer design (File S2). Publicly available wheat SSR markers (Röder *et al.* 1998) were used for genetic mapping. The barley and wheat orthologs of the rice *FZP/BFL1* gene sequence (*Os07g0669500*) were used for candidate gene marker development (File S2).

Genetic mapping

The barley F_2 mapping population was developed by crossing Bowman introgression line BW-NIL(*com2.g*) and barley cv. Haruna Nijo. For initial mapping, 286 individuals were analyzed (File S2). Two different wheat F_2 mapping populations (Tamaroi45 $\times$ TRI 27966; 279 F_2 individuals; (Tamaroi42 $\times$ TRI 19165; 159 F_2 individuals) were created. In both species, segregation between mutant and WT F_2 plants fitted a 3:1 ratio typical for a monogenic recessive gene. Linkage analysis of segregation data were carried out using the maximum likelihood algorithm of Joinmap 4.0. Kosambi mapping function was used to convert recombination fractions into map distances. High-resolution mapping was performed only in barley (File S2).

Targeting induced local lesions in genomes analysis

For identifying further mutant alleles of *COM2* in barley, two different targeting induced local lesions in genomes (TILLING) populations, including ethyl methanesulfonate (EMS)-treated population of cv. Barke and sodium azide-induced TILLMore population of cv. Morex, were screened (File S2). To identify the *TtBH-1* mutants in tetraploid wheat, an EMS-treated TILLING population of cv. Kronos was screened. In all cases, the open reading frame (ORF)

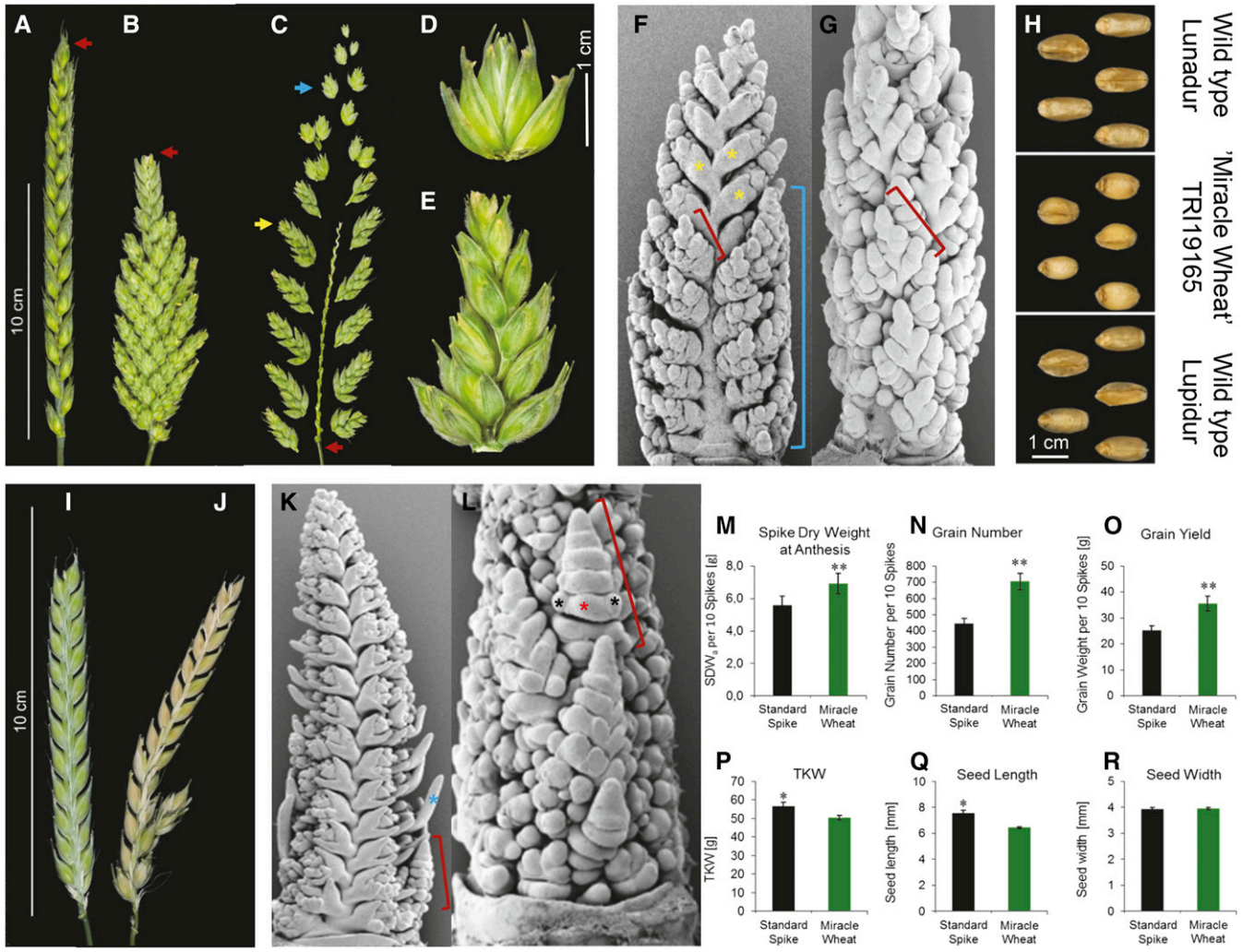

Figure 1 Spike morphology of wild-type and branched (mutant) genotypes in wheat and barley. (A) Hexaploid awnless wheat cv. 'Kanzler' with terminal spikelet (red arrowhead) and determinate spike. (B) 'Wunder-Weizen' accession TRI 1781 carrying the *bh*^{t} allele displays loss of terminal spikelet (red arrowhead) and indeterminate spike development; awns are removed for clarity. (C) Detached spike's main axis (red arrowhead), associated single spikelet (blue arrowhead), and multispikelet branch (yellow arrowhead indicates small-sized secondary spike; see also E). (D) Single spikelet containing multiple florets. (E) A multispikelet branch bearing fertile spikelets. (F) Lateral and (G) dorsal view on immature spike from 'Miracle-Wheat' TRI 27966 at the terminal spikelet stage showing ectopic branch-like structures emerging from glume primordia (GP, yellow asterisks); blue brackets enclose the branched region along the spike and red brackets delimits early small-sized secondary spike. (H) Seed shape differences (length and width) between two elite tetraploid durum wheat cultivars (top, 'Lunadur'; bottom, 'Lupidur') and 'Miracle-Wheat' accession TRI19165. (I) Wild-type barley spike cv. Bowman; awns clipped off for clarity. (J) A branched spike of *com2*-type barley in BW-NIL(*com2.g*). (K) Lateral and (L) dorsal view of immature BW-NIL(*com2.g*) mutant spike at the AP stage (AP, blue asterisk), red bracket delimits early developmental stage of small-sized secondary spike, and red and black asterisks in L represent central and lateral SMs, respectively, of secondary spike. (M–R) Comparison of yield components between elite durum wheat cultivars and 'Miracle-Wheat' accessions in the field. Data are based on averages of 200 spikes per phenotypic class. Asterisks indicate significant difference between pairwise comparisons as calculated by Student's *t*-test at 95% (*) and 99% (**) confidence intervals.

region of the corresponding gene was targeted for detection of causal SNPs (File S2).

Haplotype analysis

Genomic DNA from a diverse set of barley accessions (Table S3) was PCR amplified using specific primers to amplify full coding sequence of the barley *COM2* gene (File S2).

Microarray hybridization and data analysis

Total RNA was isolated from spike meristems collected at glume, stamen, and awn primordium stages from mutants BW-NIL(*com2.g*) and respective wild type cv. Bowman using the RNA-queous MircroKit (Invitrogen). A detailed description of the genes present on the array and the experimental procedure are described in Koppolu *et al.* (2013). Microarray hybridizations were performed in three biological replications per stage.

Quantitative RT-PCR

Purelink RNA mini kit (Invitrogen) was applied to extract total RNA from immature spike tissues (double ridge, triple mound, glume primordium, lemma primordium, stamen

primordium, and awn primordium stages) followed by removal of genomic DNA contamination using RNAse-free DNAse (Invitrogen). RNA integrity and quantities were analyzed via Agilent bioanalyzer and nanodrop (peq lab), respectively. QuantiTect reverse transcription kit (Qiagen) was utilized for cDNA synthesis using 1 μg of total RNA. Real-time PCR was performed using QuantiTect SYBR green PCR kit (Qiagen) and the ABI prism 7900HT sequence detection system (Applied Biosystems). qRT-PCR results were analyzed using SDS2.2 tool (Applied Biosystems). The reference housekeeping gene used in all cases was *HvActin*.

mRNA in situ hybridization

A portion of *COM2* gene segment (444 bp in length; starting from CDS nucleotide position 888 toward 3′ UTR) was amplified using cDNA isolated from immature spikes of cv. Bonus with specific primers (Table S6). The PCR product was cloned into pBluescript II KS (+) vector (Stratagene, La Jolla, CA). Linearized clones by *Hin*dIII or *Not*I were used as templates to generate antisense (*Hin*dIII) and sense (*Not*I) probes using T3 or T7 RNA polymerase. *In situ* hybridization was conducted as described previously (Komatsuda *et al.* 2007).

Scanning electron microscopy

Scanning electron microscopy (SEM) was performed on immature spike tissues at five stages including triple mound, glume, lemma, stamen, and awn primordium from greenhouse-grown plants. SEM was conducted as described elsewhere (Lolas *et al.* 2010).

DNA preparation

DNA was extracted from leaf samples at the three-leaf stage. Plants for which the DNA was prepared included all genotypes of F_2 plants of wheat and barley, fine mapping population of barley, diverse wheat and barley genotypes used for haplotype analysis, and the wheat and barley TILLING lines.

Sequence information and analysis

Unpublished sequence information for the two BAC contigs (44575 and 47813; spanning the interval between M1 and M2) was made available from the international barley sequencing consortium (through Nils Stein). This sequence information was analyzed for gene annotation (File S2).

Data availability

Mutant plants from wheat and barley TILLING analysis are available upon request. File S3 contains the reference DNA sequence information of the TtBH and COM2 of some wheat and barley cultivars, respectively.

Results

Inflorescence form in 'Miracle-Wheat' and 'Compositum-Barley'

'Miracle-Wheat' and 'Compositum-Barley' display altered, branched inflorescence architecture (Figure 1, A–E, I, and J). Branch formation is more pronounced at the basal part of the spike. In 'Miracle-Wheat,' spikes show an indeterminate pattern of growth due to the loss of terminal spikelet formation (Figure 1, compare A to B). In wheat and barley, the inflorescence meristem (IM) progressively initiates lateral meristems acropetally, which give rise to the spikelet meristems (SMs). The SMs develop florets along the rachilla (spikelet axis). Wheat and barley mutants show a normal inflorescence development until the glume primordium (GP) stage at which the SM begins to differentiate. At this stage, predominantly in the basal part of the spike, the SMs revert to branch- or IM-like meristems (Figure 1, F, G, K, and L). This is demonstrated by the failure to initiate florets, and instead, indeterminately produce further spikelets in a distichous manner (Figure 1, compare D to E). It thus seems that in 'Miracle-Wheat' and 'Compositum-Barley' SMs have acquired an IM-like identity that potentially is able to produce a small-sized indeterminate spike in the form of a branch-like structure (Figure 1, F, G, K, and L). Similar branched-spike phenotypes are also found in the *bd1* (Chuck *et al.* 2002) and *fzp/bfl1* mutants of maize and rice (Komatsu *et al.* 2003; Zhu *et al.* 2003), respectively. 'Miracle-Wheat' and 'Compositum-Barley' may thus undergo identical developmental defects while acquiring SM determinacy with 'Miracle-Wheat' also losing the spike determinacy as it fails to produce a terminal spikelet (Figure 1, A and B). Two years of field experiments with 12 tetraploid 'Miracle-Wheat' landrace accessions showed a significant increase in spike dry weight at anthesis, grain number, and grain yield per spike as compared to the canonical spike forms of tetraploid elite durum wheat cultivars (Figure 1, M–O). Though seed width remained almost unaltered between both groups of wheats, there was a slight decrease in thousand kernel weight (TKW) and seed length in 'Miracle-Wheats' (Figure 1, P–R). In contrast, *com2* mutants of diploid barley usually show similar or slightly lower spikelet and floret fertility (Figure S1). For instance, barley plants carrying the more severe *com2* mutant allele *irregular spike 25* display drastically reduced fertility and seed set (Figure S1).

Positional cloning of the barley spike-branching allele com2.g

Low-resolution genetic mapping was performed in both tetraploid wheat and barley (File S2). Linkage maps localized the bh^t and *com2.g* phenotypes genetically to an interval on the short arm of wheat chromosome 2A (2AS) and barley chromosome 2H (2HS), respectively, at a similar region (File S2 and Figure S2, A and B). The genetic maps were established by including a genetic marker derived from a candidate gene; the wheat and barley orthologs of maize *BD1* and rice *FZP/BFL1* known to be located in this syntenic chromosome region (Rossini *et al.* 2006) (for marker details see File S2). The phenotype cosegregated with the candidate gene-based markers, confirming previous findings of bh^t and *com2.g* genetic positions (Klindworth *et al.* 1997; Rossini *et al.* 2006). Considering the lower complexity of

the barley genome, fine mapping was performed in barley by screening 1750 F_2 plants for recombination events. A total of 52 F_2 recombinant plants and their corresponding F_3 families were analyzed whereby six families initially showed a discrepant phenotype compared to the corresponding genotypic score. (See File S2 for more details on deviant plants). *com2.g* was ultimately mapped into an interval on 2HS, flanked by M1 and M2 CAPS markers (Figure 2, A–D and Table S6). Overlapping BAC clones (~190 kb) between markers M1 and M2 were sequenced. After annotation, 11 gene fragments and five putative complete gene models were identified, including the barley ortholog of rice *FZP/BFL1* (*Os07g0669500*) (Figure 2C). This candidate gene represents a putative transcription factor consisting of a single exon, encoding a protein of 307 amino acids containing an ethylene-responsive element DNA binding factor (*i.e.*, AP2/ERF) (Figure 2, E–G). Sequence analysis of the barley mutant parental allele [Bowman Near Isogenic Line of *com2.g* =BW192; *i.e.*, BW-NIL(*com2.g*)] (Druka *et al.* 2010) revealed a single amino acid substitution of serine to arginine at position 221 (S221R) in a highly conserved region of the ORF (Figure 2G, File S2, and Figure S3). Nonfunctional forms of this gene in rice and maize result in the conversion of determinate SMs to indeterminate branch meristems reminiscent of the *com2.g* phenotype (Chuck *et al.* 2002; Komatsu *et al.* 2003). The barley ortholog of the *BD1/FZP/BFL1* gene, *COM2*, was thus identified as an eligible candidate for the *com2.g* allele.

More com2 mutants and natural sequence diversity in barley

Resequencing a set of available barley spike-branching mutants revealed that four of them shared the same mutation (S221R) as found in the BW-NIL(*com2.g*) mutant, *i.e.*, *brc1.5*, *com.k*, Freak, HOR14427 (a double mutant of *com2*/hooded spike; see Table S1), while one showed a different amino acid substitution (L228H; *irregular spike 25*) (Figure 2G, File S2, and Figure S3). These *com2* mutants were the result of both induced or natural mutations and are affected in highly conserved nucleotide and protein regions outside of the AP2/ERF domain (Figure 2G). This high level of nucleotide similarity among diverse grass genera might suggest a post-transcriptional regulation of *COM2* transcripts. Such regulation seems in line with our observations that the *com2* phenotype can vary between different genetic backgrounds (*e.g.*, S221R substitution in several accessions; see Table S1) and/or due to environmental conditions.

We also screened two different barley TILLING populations from cv. Barke (two rowed) and cv. Morex (six rowed). Of these populations, 16 M_2 plants (11 homozygous and 5 heterozygous) revealed amino acid substitutions in the ORF region (Table S2). Neither homozygous nor heterozygous lines with mutations outside of the AP2/ERF domain in 12 M_2 plants, and their corresponding M_3 families, transmitted a branched spike. In contrast to this, two of the remaining four M_2 plants (TILLMore48 and TILLMore5865) carrying mutations inside the AP2/ERF domain did transmit a branched spike as was revealed by the phenotypes of the corresponding M_3 plants (Figure 2G, Table S2, and Figure S4). This observation further confirmed that the *COM2* gene is underlying *com2.g*.

To evaluate natural variation at the ORF of *COM2*, the respective region was sequenced and analyzed in a set of 85 diverse barley accessions (Castiglioni *et al.* 1998) (Table S3). Sequence analysis revealed a low level of *COM2* natural sequence variation. Nevertheless, we identified 10 different SNPs that resulted in seven different haplotypes, including two major and five minor groups. None of the groups could be assigned toward a particular geographical region. Among the 10 nucleotide changes, 4 caused amino acid substitutions and the remaining 6 resulted in silent mutations. Of the four amino acid substitutions, two were in conserved regions and two were in nonconserved regions. Of all the identified SNPs, amino acid substitutions, and haplotypes, only S221R (haplotype II) was associated with spike branching and exclusively present in *com2.g* and *brc1.5* mutant stocks (Table S3). The natural variation for *COM2* in barley further supports the uniqueness of all causal mutations detected for the *com2* locus. However, no allelism test among barley spike-branching mutants was performed.

Expression pattern of COM2 during barley spike development

The mRNA *in situ* hybridization experiments, performed in two-rowed barley (cv. Bonus), revealed that *COM2* expression starts early during spikelet development at the triple mound (TM) stage, when spikelet primordia differentiate (Figure 3A). Expression is initially localized at the boundary between central (CS) and lateral spikelets (LS). In the less developed lateral spikelets, expression is first detected in the apical region of the lateral SM (Figure 3A). When GP develop, expression shifts to the area between the SM and the emerging GP (Figure 3B). This resembles the expression pattern of *BD1* (maize) and *FZP* (rice) mRNA (Chuck *et al.* 2002; Komatsu *et al.* 2003). Signals for *COM2* mRNA expression were consistent along the longitudinal axis of the wild-type spike (Figure 3C, blue triangle). Since *COM2* is expressed very early in SM differentiation, it may be involved in mediating SM identity. *COM2* expression was also measured between the barley mutants BW-NIL(*com2.g*), *brc1.5*, and the wild-type cv. Optic at TM, GP, LP (lemma primordium), and AP (awn primordium) stages. We found no differences in *COM2* expression between the mutants and the wild type tested, indicating that branch formation is probably caused by changes at the protein level. In both wild-type and mutant plants, a slight elevation of *COM2* transcripts was observed toward lemma primordium stage, while the SM continued to enlarge (Figure 3D). Moreover, in earlier stages (TM and GP) the wild type shows slightly greater expression than the mutants.

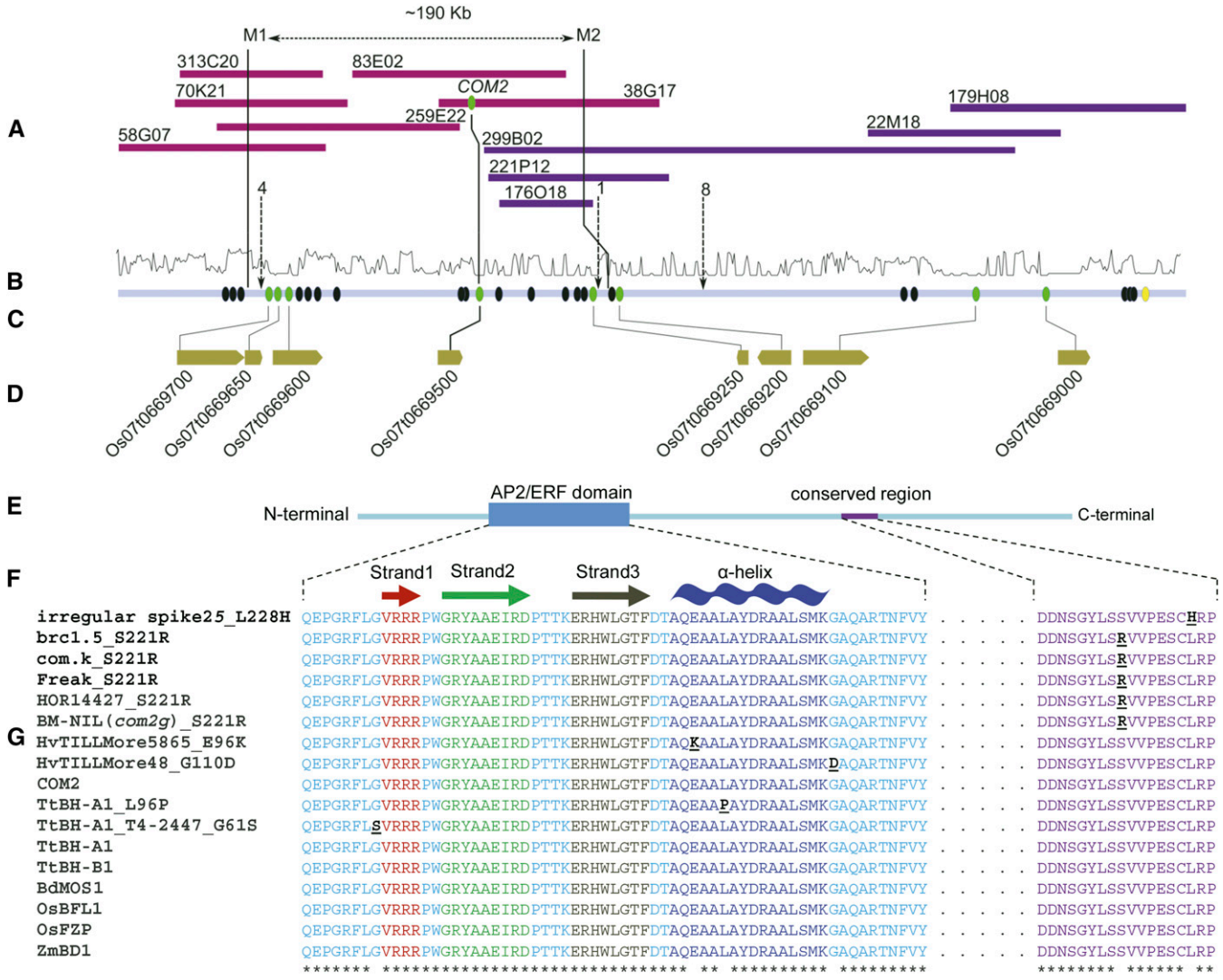

Figure 2 High-resolution genetic linkage map of *COM2* region on chromosome 2HS of barley and protein domain structure conservation among grasses. (A) Overlapped BAC clones (clones of the minimal tiling path) originated from two barley physical map contigs 44575 (purple) and 47813 (blue). The two contigs were merged as they showed significant edge sequence homology. (B) Depicts k-mer method-based repeat frequency (log-scaled; 0, 10). (C) Respective and predicted consensus sequence derived from BAC sequences in which circles represent either the Augustus gene model without sequence homology to *Brachypodium*, rice, sorghum, or barley genes (black), or with sequence homology to *Brachypodium*, rice, sorghum, or barley gene (green) as well as those with only sequence homology to predicted barley genes (yellow). The one-directional arrows connect the number of recombination to the corresponding position. (D) Rice genes syntenic to detected barley genes. (For corresponding low-resolution genetic mapping, see Figure S2 and File S2). (E) Protein sequence of COM2 with AP2/ERF DNA-binding domain and a highly conserved terminal region. (F) Position and structure of the AP2/ERF subdomains; including β-sheet (consists of strands 1, 2, and 3) and α-helix. (G) Alignment of AP2/ERF domain and conserved terminal region of COM2/ BH^t-A1 with other grass orthologs and mutant alleles in barley and wheat. (F and G) Functional amino acid substitutions are underlined and shown in black. Asterisks indicate no amino acid changes at the corresponding position. For phenotype of respective mutant wheat and barley, see Figure S4.

Identification of the gene underlying the bh^t locus in 'Miracle-Wheat'

Since phenotypes of *com2.g* and *bh^t* were mapped to the same chromosome group 2 of wheat and barley in syntenic regions (File S2 and Figure S2, A and B), *COM2* is likely the orthologous tetraploid wheat gene (*TtBH-A1*) underlying the *bh^t* locus in 'Miracle-Wheat.' Sequence analysis of the *TtBH-A1* ORF revealed that the two *bh^t* mutant parents of the corresponding two mapping populations carried the same recessive allele (Figure S2B). This *bh^t* allele contained a single amino acid substitution of leucine to proline at position 96 (L96P) within the AP2/ERF protein domain (Figure 2G). Three different 'Miracle-Wheat' landraces with naturally occurring branched phenotype were selected for allelism tests. Crosses among these lines always produced a spike-branching phenotype (Table S4). Resequencing *TtBH-A1* ORF from these lines revealed the identical L96P mutation as present in the parents of the mapping populations. Thus, lack of genetic complementation for spike branching in the F_1 progenies further indicates that the same

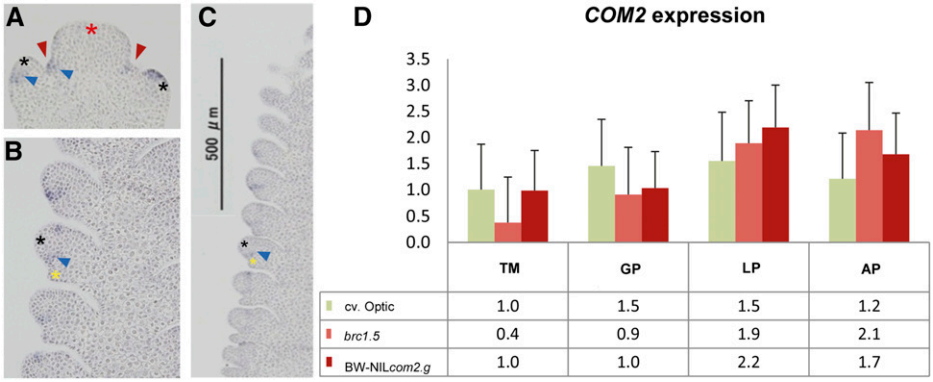

Figure 3 Expression pattern and quantification of *COM2* in two-rowed barley. (A–C) mRNA *in situ* hybridization of *COM2* in two-rowed wild-type barley cv. Bonus. (A) Transverse section at triple-mound stage shows *COM2* expression at sites of future glume primordia (GP) (red arrowhead). (B) Detail of immature spike with lateral spikelets (LSs) at GP stage reveal accumulation of *COM2* transcripts between LS and outer glume. (C) Immature spike with LS in the GP stage showing semicircular *COM2* expression between GP and LS primordia along the spike. Expression is clearer in the developmentally advanced basal part of the spike. Asterisks indicate central spikelet (CS) meristem (red), LS meristem (black), and GP (yellow), blue triangles point to the regions of gene expression. (D) *COM2* expression in mutants BW-NIL(*com2.g*) and *brc1.5* and wild type cv. Optic. LP and AP stand for lemma primordium and awn primordium stages, respectively. Mean ± SE of three biological replicates. Relative expression values are given at the bottom of the graph. The *y*-axis value shows the expression relative to *HvActin*, while genotype differences were tested at a significance level of $P > 0.05$.

L96P mutation at bh^t may be the casual factor for the branch phenotype. Further resequencing of *TtBH-A1* ORF in 30 wild-type accessions as well as 29 'Miracle-Wheat' landraces confirmed that all spike-branching accessions carried the L96P substitution (File S2 and Table S5). This suggests a monophyletic origin of this mutant during the domestication process of tetraploid wheat. To further confirm that *TtBH-A1* is the gene underlying spike branching in 'Miracle-Wheat,' a tetraploid wheat TILLING population derived from, cv. Kronos (Uauy *et al.* 2009) was screened. We found 40 mutant M_2 plants, with 28 of them leading to unique amino acid substitutions (10 homozygous and 18 heterozygous). Similar to our TILLING assay in barley, neither homozygous nor heterozygous lines with mutation outside of the AP2/ERF domain (26 lines) displayed a branched spike. Of the remaining two (one homozygous and one heterozygous) carrying mutations inside the AP2/ERF protein domain, plant T4-2447 (G61S) proved to confer mild spike branching (File S2, Table S7, and Figure S4). Furthermore, the same TILLING population was screened for mutations in the homeologous B genome copy of the gene (*TtBH-B1*) via which the TILLING plant T4-2432 was identified. This plant harbored a mutation giving rise to a premature stop codon at amino acid position 14 (Q14X, heterozygous form). Neither homozygous nor heterozygous progenies of this mutant plant showed any spike branching (Table S7), indicating that the *TtBH-B1* copy does not actively contribute to branch formation in 'Miracle-Wheat.'

COM2 is downstream of the spike architecture gene Six-rowed spike 4 (Vrs4); microarray analysis of com2.g reveals COM2 regulatory interactions

To identify potential downstream target genes of the putative barley transcription factor *COM2*, microarray analysis was performed in the barley spike-branching mutant BW-NIL(*com2.g*) and its respective wild type cv. Bowman (Figure 4A, Figure S5A, and File S1). Independent quantitative RT-PCR (qRT-PCR) analysis was performed to confirm the microarray data and to validate genes not present (Figure 4, B and E) on the array including the barley *Vrs4* (*HvRAMOSA2*), which controls SM determinacy and row type (Koppolu *et al.* 2013). Loss-of-function *vrs4* alleles promote lateral spikelet fertility as well as occasional branch formation, the latter trait resembling the *com2* phenotype (Figure S6). We tested *COM2* transcripts in the BW-NIL(*vrs4.k*) mutant and the corresponding wild type cv. Bowman by independent qRT-PCR at three spike developmental stages also used for the microarray experiment, and the triple mound stage. *COM2* transcripts were significantly down-regulated in the *vrs4.k* mutant (Figure 4B, blue column) compared to the wild-type barley cv. Bowman (Figure 4B, green). The significant down-regulation was observed at early spike developmental stages, *i.e.*, TM and GP, as well as in the late stage of AP (Figure 4B). This suggests that *Vrs4*, which has been reported to be highly expressed during early (TM to GP) barley spike development (Koppolu *et al.* 2013), may function upstream of *COM2*. Moreover, our observations in *COM2* mRNA *in situ* hybridization indicated that *COM2* and *Vrs4* are expressed in overlapping spikelet primordia domains (Figure 4C), supporting a possible interaction.

Among the genes significantly down-regulated in *com2.g* were those engaged in hormonal metabolism [barley cytokinin oxidase/dehydrogenase (*HvCKX2*) and barley phyB activation tagged suppressor 1 protein (*HvBAS1*)], spikelet determinacy, and floral organ differentiation and development (Figure 4A and Figure S5B). The low transcript levels of the barley *HvCKX2* (Figure S5B) may result in higher concentration of bioactive cytokinins in the *com2.g* inflorescence consistent with higher meristematic activity (Mok and Mok 2001; Zhu *et al.* 2013). The putative ortholog of maize *INDETERMINATE SPIKELET 1* (*IDS1*), the barley *HvIDS1* (an *AP2*-like gene), was also significantly down-regulated in the *com2.g* mutant (Figure 4E). In maize, the gene specifies a determinate SM fate and thereby limits the number of floral meristems (Chuck *et al.* 1998). This is in agreement with the loss of SM determinacy seen in *com2* mutant inflorescences.

Other genes down-regulated were mostly involved in floral organ development and fertility (Figure 4A and Figure

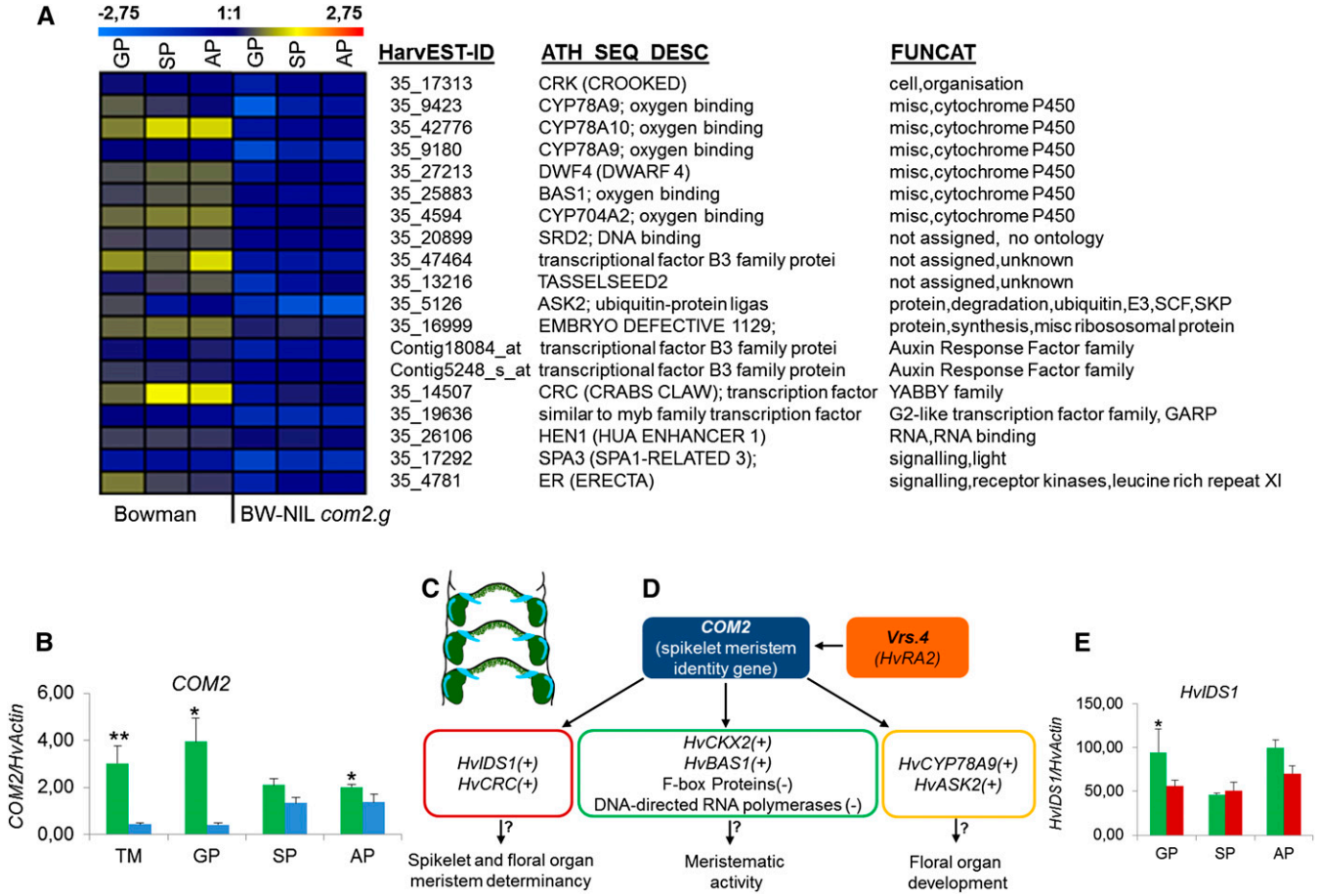

Figure 4 Transcriptome analysis of *com2.g* using microarray and independent qRT-PCR, as well as model of putative *COM2* interactions. (A) Heat map of genes conjointly down-regulated in the BW-NIL(*com2.g*) mutant as compared to corresponding wild type cv. Bowman. For up-regulated genes in the mutant, see Figure S5B. (Scale bar above heat map indicates transcript level between wild type and mutant with blue indicating down-regulation and red indicating up-regulation). (B) *COM2* expression in mutant BW 903 (*vrs4.k*) (blue) compared to corresponding wild type cv. Bowman (green). Mean ± SE of three biological replicates. (C) Schematic drawing of central and lateral SM at the triple mound stage. *Six-rowed spike 4* (*Vrs4*; green) and *COM2* (light blue) are expressed in overlapping domains of the lateral and central spikelets. (D) Model of putative wild-type *COM2* interactions. (+) and (−) indicate up- or down-regulation of the wild-type allele, respectively, in comparison to the mutant BW-NIL(*com2.g*). (E) *HvIDS1* expression in BW-NIL(*com2.g*) (red) as compared to corresponding wild type cv. Bowman (green). In both B and E, the mean ± SE of three biological replicates is shown. All expression values in both B and E were log10 transformed. Asterisks show the significance level calculated by Student's *t*-test, (no asterisk corresponds to $P > 0.05$. Single, double, and triple asterisks stand for $P \leq 0.05$, $P \leq 0.01$ and $P \leq 0.001$, respectively). Developmental stages include: TM, triple mound; GP, glume primordium; SP, stamen primordium; and AP, awn primordium. Genes including *HvIDS1*, *HvCKX2*, *and COM2* were not present on the array.

S5B). These include orthologs of the *Arabidopsis* genes cytochrome P450, *CYP78A9*, and *CYP78A10*, which control floral organ size and ovule integument development; *CRABS CLAW (CRC)*, involved in floral meristem determinacy; and *ARABIDOPSIS SKP1-LIKE2 (ASK2)*, which plays a role in embryogenesis and postembryonic development (Bowman and Smyth 1999; Liu *et al.* 2004; Sotelo-Silveira *et al.* 2013). Down-regulation of these genes is in accordance with the *com2.g* mutant phenotype, especially the low fertility associated in the more severe allele of mutant *irregular spike 25*.

In contrast to this, genes related to meristematic activity were up-regulated in *com2.g* mutant, including genes encoding F-box proteins (Figure S5A) that control degradation of cellular proteins (Jain *et al.* 2007). The increase in transcripts encoding for subunits of RNA polymersase I, II, and III (Figure S5A) hints at a connection between translational mechanisms and the high meristematic activity observed in *com2.g* (Figure 4D).

Discussion

COM2/TtBH-A1 confer a branchless spike in the tribe Triticeae

Among grasses, species from the tribe *Triticeae* have acquired a specific form of inflorescences in which spikelets, the actual building blocks of grass inflorescences, are directly attached to the main axis (or rachis). In contrast to other grass species like rice and maize, little is known about

the genetic determinants that regulate inflorescence specification in *Triticeae* (Zhang and Yuan 2014). Genes responsible for row types in barley (Komatsuda *et al.* 2007; Ramsay *et al.* 2011; Koppolu *et al.* 2013) and for the free-threshing character in wheat (Simons *et al.* 2006) are among the few that have been characterized so far. Here, we report on a gene in tetraploid wheat (*TtBH-A1*) and barley (*COM2*) containing an AP2/ERF domain that represses inflorescence branch formation. Across grass species, *TtBH-A1/COM2* shows 100% sequence conservation within the AP2/ERF domain and a highly conserved protein coding region. Nonfunctional forms of this protein always cause inflorescence branching, suggesting a consistent role in preventing formation of any ectopic branch-like meristems in grass inflorescences. In the maize mutant *bd1* and rice mutant *fzp/bfl1*, the SMs acquire indeterminate branched meristem (BM) identity through the reiterated formation of axillary meristems that prevent the transition of spikelet to floral meristem identity (Chuck *et al.* 2002; Komatsu *et al.* 2003; Zhu *et al.* 2003). A similar pattern of direct conversion of SMs to branch-like meristems was observed in barley and tetraploid wheat. The branch-like meristem resembles IM-like meristems that produce secondary spike-like structures. Since SS formation in hexaploid bread wheat appears to be under the control of *WFZP* (Dobrovolskaya *et al.* 2015), the ortholog of *TtBH*, it is possible that mild homoeoalleles of *wfzp-D* and/or *wfzp-A* cause a SS-like phenotype due to lost SM determinacy, but fail to initiate spike branching as seen in tetraploid 'Miracle-Wheat' or 'Compositum-Barley.' This may explain our tetraploid wheat TILLING mutant, T4-2447 (G61S), in which we predominantly found additional spikelet formation but not spike branching.

Polyploidy and its association with increased grain number per spike

In all diploid grass species in which noncanonical branch formation is the result of a mutation in branch repressor genes like *BD1*, *FZP/BFL1*, or *MOS1*, no increase in grain number was reported. The latter is often due to associated inferior floret fertility. Although we have not quantified the impact of spike branching on grain number or grain size in barley, the fertility penalty was clearly visible and most extreme in the mutant *irregular spike 25*. Here, the majority of spikelets failed to set seeds, a phenomenon also observed in severe mutant alleles in rice *FZP/BFL1* (Komatsu *et al.* 2003; Zhu *et al.* 2003). According to our microarray analysis, *COM2* is also engaged in pathway(s) related to spikelet/floret fertility, in addition to branch repression, suggesting that similar factors play a role in diploid barley. Interestingly, such negative pleiotropic effects on fertility were not observed in polyploid wheats. The recessive single-gene inheritance of the bh^t locus rather suggests that the A genome copy of *TtBH-A1* solely contributes to branch repression in tetraploid wheats, while the B genome copy appears inactive. This is in accordance with the very low transcript levels of *WFZP-B* in hexaploid wheat (Dobrovolskaya *et al.* 2015). In our study, we have not tested the expression pattern of the two homoeoalleles in tetraploid wheat. So, we do not (yet) know whether such transcript pattern is also maintained during later stages of floret development/fertility. It could well be that even low *TtBH-B1* transcript levels may still be sufficient to trigger expression of downstream genes involved in floral fertility, or that *TtBH-B1* transcripts are even elevated during later stages of floral development due to differential regulation. Future work has to resolve what role these different homoeoalleles of *TtBH/WFZP* play during the subsequent floral differentiation process for retaining fertility. This buffering effect of polyploidy on mutations might be the reason for the stable fertility in 'Miracle-Wheat,' resulting in improved grain number per spike and elevated sink capacity. It has to be realized, though, that such prospective sink capacity may depend on genetic background, phenology, and/or source capacity of the breeding line. So, the exploitation of the bh^t allele in combination with the genetic marker developed in this study may provide an interesting opportunity for introducing the spike-branching trait into modern varieties with conceivable implications for boosting wheat's yield potential.

Branched spike: a domestication-related trait in wheat

Our resequencing analysis of the *TtBH-A1* gene in wild-type and spike-branching tetraploid wheat accessions showed the presence of a single allele (*i.e.*, bh^t) in all 'Miracle-Wheat' accessions. This is clear proof of a single selection event during the domestication process of tetraploid wheat, which most likely took place in cultivated emmer (AABB; *T. dicoccum* L.) wheat. Domestication of emmer wheat from its wild progenitor *T. dicoccoides* L. was an important step in the evolution of modern polyploid wheat varieties (Salamini *et al.* 2002). This process includes emergence of traits, such as hulled seeds and nonbrittle rachis, and started >10,000 years ago (Salamini *et al.* 2002). However, timescale and site, and when and where the mutation underlying 'Miracle-Wheat' initially occurred remain unclear. 'Miracle-Wheat' has been cultivated under various names in different parts of the world, especially before the emergence of modern-days breeding activities (Ball and Leighty 1916). Evidently, this ancient trait captivated farmers and present-day scientists alike simply because of its magnificent appearance and promise of wealth. Introducing this genetic resource to modern wheat breeding may be a worthwhile endeavor.

The COM2 genetic framework of interactions to putative targets

Our microarray analysis provided novel insights into the transcriptional regulation and interaction of *COM2* in barley, suggesting that it may act downstream of the spike architecture gene *Vrs4* (*HvRA2*) (Figure 4D). The formation of spike branching in *vrs4.k*, accompanied by a lowered *COM2* expression and the presence of the *Vrs4 cis*-recognition motif 5′-GCGGCA-3′ in the 5′ UTR region (−44 bp to −39 bp of the start codon) of *COM2* all point in this direction (Husbands *et al.* 2007; Koppolu *et al.* 2013). This putative

interaction, however, is up to now unknown from maize (*i.e.*, between *ZmRA2* and *ZmBD1*; Chuck *et al.* 2002; Bortiri *et al.* 2006), suggesting that this pathway (*Vrs4–COM2*) appears to be tribe specific and related to inflorescence shape. In fact, a careful evaluation of *ZmBD1* expression in *ra2* mutants may provide a better understanding of branch repression pathways in different grass tribes. Additionally, *HvCKX2* is among the genes commonly down-regulated in both *vrs4.k* (Koppolu *et al.* 2013) and *com2.g* mutants. Mutation in rice *OsCKX2* and barley *HvCKX2* has already been reported to increase primary and secondary branches in rice as well as higher number of grains in barley (Ashikari *et al.* 2005; Zalewski *et al.* 2012; Li *et al.* 2013)

The down-regulation of the putative barley ortholog of maize *IDS1* (*HvIDS1*) remains intriguing. Although the role of *HvIDS1* in barley is still unknown, the putative ortholog of *IDS1* in wheat, the *Q* gene, confers the free-threshing character (Faris *et al.* 2003; Simons *et al.* 2006). Providing *HvIDS1* has a similar function as *IDS1* in maize, then lower expression of *HvIDS1* leads to the loss of SM determinacy in *com2* mutants. It thus seems that due to mutations in *COM2* the SM loses its identity and converts back to an IM-like meristem. The consequence is a small-sized and indeterminate spike, visible as a lateral branch. Elucidating more of the underlying genetic regulatory pathways related to meristematic development and subsequently inflorescence architecture in grasses may provide valuable insights into the manipulation of yield-relevant traits in various crop plants.

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SUPPORTING INFORMATION

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SI FIGURES

Figure S1 Additional mutant alleles of spike branching in barley. Different *com2* mutant alleles identified by resequencing of the *COM2* ORF region. The *irregular spike25* mutant shows a higher degree of spikelet infertility. *HOR14427* is a double mutant of *com2*/hooded, see Table S1.

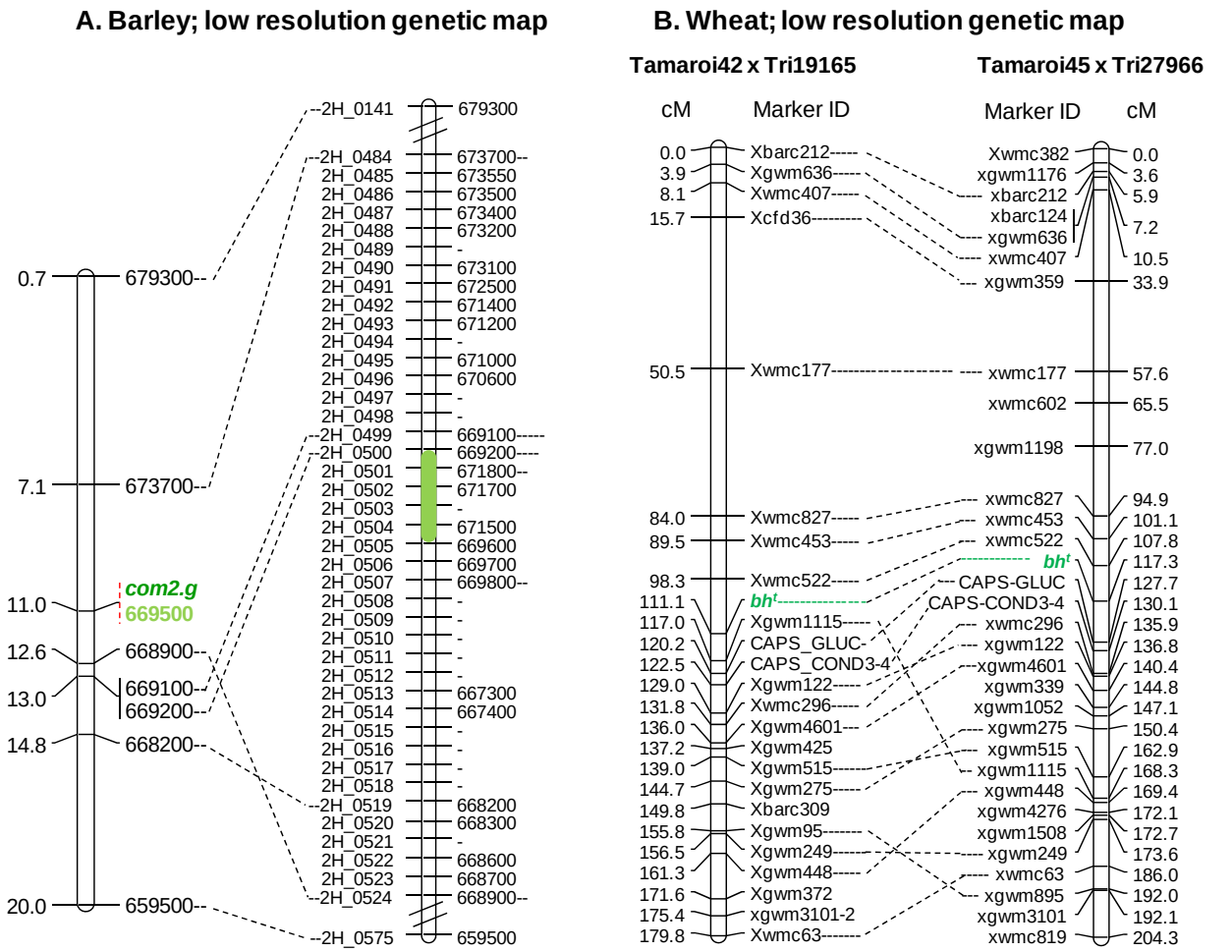

Figure S2 Low resolution linkage map of *com2.g* and *bh*^{t} *locus in* barley and tetraploid wheat. (A) Genetic linkage mapping of *com2.g* in barley. The genome zipper (GZ) model of barley chromosome 2H was considered as resource for marker development. Predicted order of rice genes along this barley chromosome is shown, all gene identifiers start originally with Os07g0. The virtual position for candidate gene ortholog *(Os07g0669500)* was not initially provided; a position (green area) was assumed for the *Os07g0669500* gene according to the gene identifier. Barley BW 192 (com2.g) and barley cv. Haruna Nijo were used as mutant and the wild type parents of the population, respectively. **(B)** Genetic linkage mapping of the *branched head*^{t} (*bh*^{t}) locus in two different tetraploid wheat F_2 mapping populations. Connected markers represent those used in both populations. cM stands for centiMorgan. Tetraploid wheat Tamaroi45 and Tamaroi42 were used as wild type while TRI 19165 and TRI 27966 were mutant parents of the corresponding population.

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Bowman                MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
Irregular spike 25    MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
Foma                  MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
Donaria               MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
Morex                 MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
Optic                 MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
Haruna Nijo           MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
brc1.5                MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
com2.k                MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
Freak                 MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
HOR14427              MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
BM-NIL (BW192)com2.g  MSIRSSSGGSGGGQTSQMMAFSEHSLPKPIAGHPQPQPSPPSSPSERPAPRGRRRAQEPG 60
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Bowman                RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
Irregular spike 25    RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
Foma                  RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
Donaria               RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
Morex                 RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
Optic                 RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
Haruna Nijo           RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
brc1.5                RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
com2.k                RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
Freak                 RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
HOR14427              RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
BM-NIL BW192com2.g    RFLGVRRRPWGRYAAEIRDPTTKERHWLGTFDTAQEAALAYDRAALSMKGAQARTNFVYA 120
                      ************************************************************

Bowman                HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHSHGGVGYHQQGPGAGA 180
Irregular spike 25    HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHSHGGVGYHQQGPGAGA 180
Foma                  HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHSHGGVGYHQQGPGAGA 180
Donaria               HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHTHGGVGYHQQGPGAGA 180
Morex                 HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHTHGGVGYHQQGPGAGA 180
Optic                 HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHTHGGVGYHQQGPGAGA 180
Haruna Nijo           HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHTHGGVGYHQQGPGAGA 180
brc1.5                HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHTHGGVGYHQQGPGAGA 180
com2.k                HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHTHGGVGYHQQGPGAGA 180
Freak                 HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHTHGGVGYHQQGPGAGA 180
HOR14427              HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHTHGGVGYHQQGPGAGA 180
BM-NIL BW192com2.g    HAAYNNYPPFLAPFHAQPAYASSTMPYGGQQHAGAAPPHIGSYHTHGGVGYHQQGPGAGA 180
                      *******************************************:****************

                                                                  (L228H)

Bowman                GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSSVVPESCLRPRGGDLQDARR 240
Irregular spike 25    GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSSVVPESCHRPRGGDLQDARR 240
Foma                  GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSSVVPESCLRPRGGDLQDARR 240
Donaria               GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSSVVPESCLRPRGGDLQDARR 240
Morex                 GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSSVVPESCLRPRGGDLQDARR 240
Optic                 GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSSVVPESCLRPRGGDLQDARR 240
Haruna Nijo           GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSSVVPESCLRPRGGDLQDARR 240
brc1.5                GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSRVVPESCLRPRGGDLQDARR 240
com2.k                GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSRVVPESCLRPRGGDLQDARR 240
Freak                 GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSRVVPESCLRPRGGDLQDARR 240
HOR14427              GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSRVVPESCLRPRGGDLQDARR 240
BM-NIL BW192 com2.g   GECSMPVPNAADHAASPMDVRSSGHDFLFPSADDNSGYLSRVVPESCLRPRGGDLQDARR 240
                      ************************************ ****** ************

                                                                  (S221R)

Bowman                YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
Irregular spike 25    YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
Foma                  YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
Donaria               YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
Morex                 YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
Optic                 YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
Haruna Nijo           YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
brc1.5                YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHXMVASSQGSDNGYS 287
com2.k                YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
Freak                 YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
HOR14427              YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
BM-NIL BW192com2.g    YSVSDADAYGLGLREDVDDLASMVAGFWGGADAAYGGFAPANGGGHDMVASSQGSDNGYS 300
                      ************************************************************

Bowman                PFSFLSH 307
Irregular spike 25    PFSFLSH 307
Foma                  PFSFLSH 307
Donaria               PFSFLSH 307
Morex                 PFSFLSH 307
Optic                 PFSFLSH 307
Haruna Nijo           PFSFLSH 307
brc1.5                PFSFLSH 307
com2.k                PFSFLSH 307
Freak                 PFSFLSH 307
HOR14427              PFSFLSH 307
BM-NIL BW192com2.g    PFSFLSH 307
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Figure S3 COM2 protein sequence alignment of different mutant alleles: Mutated positions between parents of the population BW-NIL(*com2.g*) and Haruna Nijo as well as other identified mutants that either shared the same mutation observed in the mutant parent of *com2.g* (S221R) (four mutants; *brc1.5*, *com.k*, Freak , HOR14427) or showed a different mutation (L228H) (one mutant; the *irregular spike 25*). The remaining cultivars represent the donor lines; see Table S1.

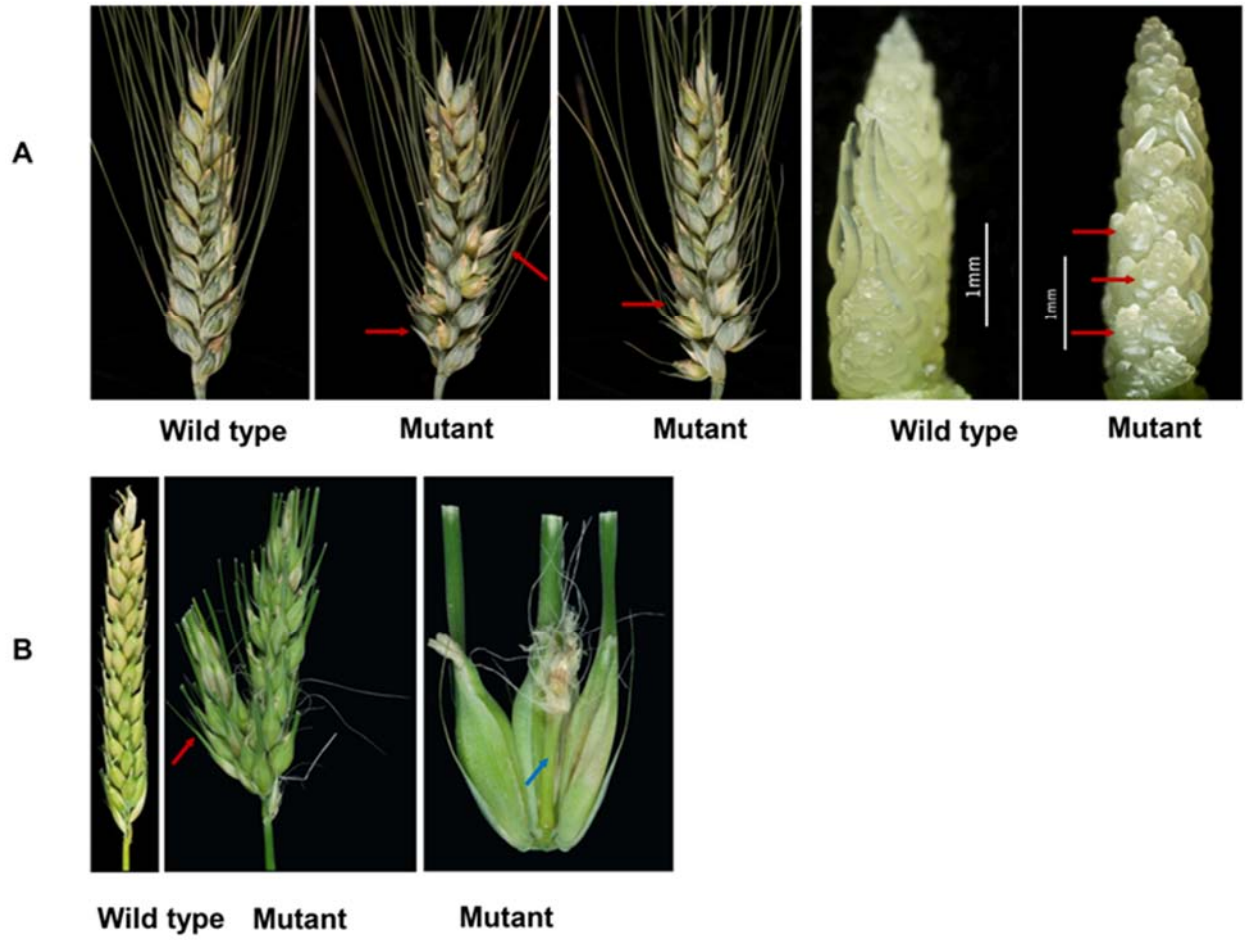
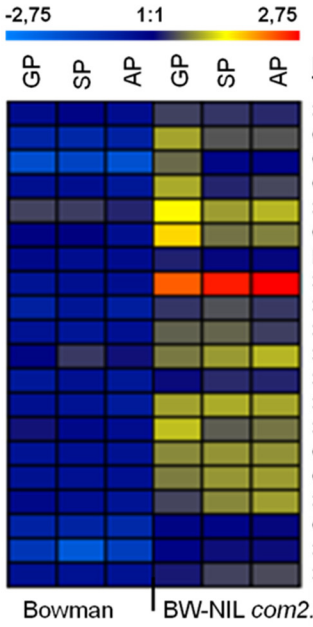


Figure S4 Phenotype of the tetraploid wheat and barley TILLING lines. (A). Supernumerary spikelet formation at the wheat homozygous TILLING plant T4-2447-7 (Mutant). The donor cultivar is tetraploid wheat cv. Kronos (Wild type). The images at the far right showed ectopic branch formation (red arrows) at early stage of development of cv. Kronos (Wild type) and the T4-2447-7 (Mutant). **(B)** Branch formation of the two barley TILLING mutant plants derived from barley cv. Morex (Wild type). Branch formation showed a range of severity from formation of a small-sized secondary spike (red arrows) to an extended rachilla at the central spikelet (blue arrows).

A



HarvEST-ID	ATH_SEQ_DESC	FUNCAT
35_4012	IAA8 (indoleacetic acid-induced protein 8); transcription factor	hormone metabolism,auxin,induced-regulated-responsive-activated
Contig10299_at	gibberellin 2-oxidase / GA2-oxidase (GA2OX2)	hormone metabolism,gibberelin,synthesis-degradation,GA2 oxidase
Contig602_at	sucrose synthase, putative / sucrose-UDP glucosyltransferase,	major CHO metabolism,degradation,sucrose,Susy
Contig481_at	sucrose synthase / sucrose-UDP glucosyltransferase (SUS1)	major CHO metabolism,degradation,sucrose,Susy
35_14480	sucrose synthase/ transferase, transferring glycosyl groups	major CHO metabolism,degradation,sucrose,Susy
Contig481_s_at	sucrose synthase, putative / sucrose-UDP glucosyltransferase,	major CHO metabolism,degradation,sucrose,Susy
HU05I05u_x_at	sucrose synthase type I	major CHO metabolism,degradation,sucrose,Susy
35_13498	no description	not assigned,unknown
35_49587	AGP9 (ARABINOGALACTAN PROTEIN 9)	not assigned,unknown
35_29657	F-box family protein (FBL10)	not assigned,unknown
35_35664	no description	not assigned,unknown
35_50591	zinc finger (MYND type) family protein / F-box family protein	not assigned,unknown
35_16272	BTB-POZ AND MATH DOMAIN 1; protein binding	protein,degradation,ubiquitin,E3,BTB/POZ Cullin3,BTB/POZ
35_4488	BRASSINOSTEROID-RESPONSIVE RING-H2; protein binding	protein,degradation,ubiquitin,E3,RING
Contig12668_s_at	E2F transcription factor-3 (E2F3)	RNA,regulation of transcription,E2F/DP transcription factor family
Contig12668_at	E2F transcription factor-3 (E2F3)	RNA,regulation of transcription,E2F/DP transcription factor family
35_20248	E2F3 (E2F TRANSCRIPTION FACTOR-3)	RNA,regulation of transcription,E2F/DP transcription factor family
Contig15953_at	F-box family protein / tubby family protein	RNA,regulation of transcription,TUB transcription factor family
35_15379	DNA-directed RNA polymerase II, putative	RNA,transcription
35_16422	DNA-directed RNA polymerase I, II, and III, putative	RNA,transcription

B

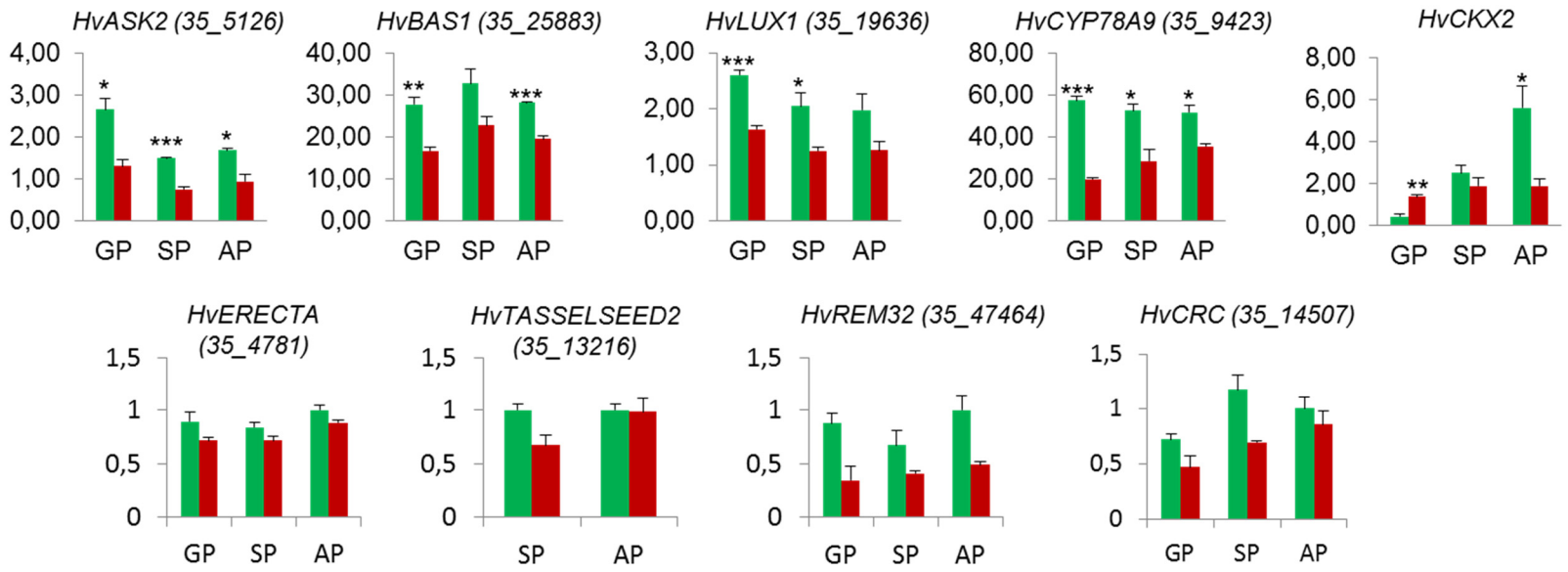


Figure S5 Transcriptome analysis of *com2.g* using microarray experiments and independent qRT PCR validations. (A) Heat map of genes conjointly up-regulated in the BW-NIL(*com2.g*) as compared to the corresponding wild type cv. Bowman. For down-regulated genes in the mutant; see Figure S5A. The scale bar at the top of the heat map indicates the transcript level of differentially regulated genes observed between wild type and mutant (blue color indicates down-regulation while red shows up-regulation). (**B**) qRT-PCR analysis performed for validation of down-regulated genes identified in the BW-NIL*com2.g* (red) as compared to the corresponding wild type cv. Bowman (green). Only highly relevant genes (9 genes) were picked up for qRT-PCR validation. Of these genes, five randomly selected genes were validated using three different biological replicates (B; upper panel) while the remaining four genes were validated using one biological, (with four technical replicates). The mean ± SE of three biological or technical replicates is shown. Expression values were log10 transformed. Asterisks show the significance level calculated by Student's t-test, (no asterisk corresponds to $p > 0.05$. While, single, double and triple asterisks stand for $p \leq 0.05$, $p \leq 0.01$ and $p \leq 0.001$, respectively). The Y-axis value shows the expression relative to *HvActin*. For the description of the genes; see Figure 4A and File S1. Corresponding unigene IDs or barley MLOC IDs are given in parentheses. Mean ± S.E of one biological replicate is shown. Y axis is the relative expression of the corresponding gene to *HvActin*. The developmental stages analyzed during microarray and qRT PCR experiments include TM: triple mound, GP: glume primordium, SP: stamen primordium and AP: awn primordium.

Figure S6 Branch formation in *vrs4* mutant (*mul1.a*). (**A**) Mature spike of wild type progenitor cv. Montcalm with determinate triple spikelet meristem. (**B-D**) Mature spikes of *vrs4* mutant MC (*mul1.a*) showing various levels of branch proliferation at the spike base and middle portion of the spike.

SI TABLES

Table S1 Complementary information of the BW-NIL(*com2.g*) allelic mutants.

Branched (*compositum*) Barleys	**origin**
BW-NIL BW 192 (*com2.g*)	EMS or neutrons; branched spike; pedigree: BOWMAN *8/4/ 7.1 / 3ND8670 // ND7015 / CIM; com2.g allele
HOR 14427	according to M. Stanca: double mutant mk: m=branched/k=hooded; *m* and *k* old symbols, have to be *mul* and *Kap*:
FREAK	dense branched ?? ; cv. Freak; 1961; FREAK is not a cultivar but a selection of CIMMYT collection.
com.k	spontaneous mutant probaly in Atlas (PI 539108) isolated by C.A. Suneson
Irregular spike 25	EMS; induced mutant in Foma (CIho 11333, NGB 14659) isolated by U. Lundqvist
brc1.5	A naturally occurring variant in barley from the Braunschweig seed collection

Table S2 Different barley TILLING plants and the corresponding positions of different amino acid substitution.

Mutant ID	population	SNP position	SNP	SNP allele	aa substitiution	Domain position	Conservation
10782-1	Barke	151	C→T	Heterozygote	R → C	-	-
10607-1	Barke	155	G→A	Heterozygote	G → D	-	-
12171-1	Barke	176	C→T	Heterozygote	P → L	-	conserved region
2723-1	Barke	308	G→A	Homozygote	R → H	within AP2/ERF domain	conserved region
3919-1	Barke	320	C→T	Heterozygote	S → F	within AP2/ERF domain	conserved region
6816-1	Barke	529	G→A	Homozygote	G → S	-	-
11023-1	Barke	541	G→A	Homozygote	G → S	-	-
11359-1	Barke	572	C→T	Homozygote	A → V	-	-
4913-1	Barke	587	G→A	Homozygote	S → N	-	-
13679-2	Barke	605	G→A	Homozygote	S → N	-	-
6872-1	Barke	631	A→T	Homozygote	S → C	-	-
9662-1	Barke	662	G→A	Homozygote	S → N	-	conserved region
6893-1	Barke	695	G→A	Homozygote	G → D	-	-
9624-1	Barke	748	G→T	Heterozygote	G → W	-	-
48	Morex	329	G→A	Heterozygote	G → D (110)	within AP2/ERF domain	conserved region
5865	Morex	286	G→A	Homozygote	E → K (96)	within AP2/ERF domain	conserved region
AP2/ERF domain				184 - 357 bp			
phylogenetically highly conserved domain				638 - 691 bp			

Table S3 Barley accessions used for *COM2* haplotype detection and the respective haplotype identified

Accession ID	216bp	300bp	414bp	494bp	536bp	642bp	663bp	696bp	822bp	873bp	Haplotype category
MUT2201 com2.f	G	C	C	C	G	C	**C**	C	A	MISSING	?
GM 1E Nudinka	G	C	C	C	G	C	C	C	A	A	I
GM713 Morex	G	C	C	C	G	C	C	C	A	A	I
GM712 Donaria	G	C	C	C	G	C	C	C	A	A	I
Haruna Nijo	G	C	C	C	G	C	**C**	C	A	A	I
Barke	G	C	C	C	G	C	**C**	C	A	A	I
3167	G	C	C	C	G	C	**C**	C	A	A	I
3906	G	C	C	C	G	C	**C**	C	A	A	I
GM 504 vulg. hybernum vikayarvi	G	C	C	C	G	C	C	C	A	A	I
GM 505 vulg. hybernum tystofte korsby	G	C	C	C	G	C	C	C	A	A	I
GM 506 vulg. parallelum montafon	G	C	C	C	G	C	C	C	A	A	I
GM 507 vulg. parallelum sechszeilige	G	C	C	C	G	C	C	C	A	A	I
GM 508 vulg. hybernum arrecife	G	C	C	C	G	C	C	C	A	A	I
GM 511 vulg. hybernum poliarnyj 14	G	C	C	C	G	C	C	C	A	A	I
GM 513 vulg. hybernum oberbrucker	G	C	C	C	G	C	C	C	A	A	I
GM 516 vulg. subviolaceum abessinien	G	C	C	C	G	C	C	C	A	A	I
GM 527 dist. nutans sarah	G	C	C	C	G	C	C	C	A	A	I
GM 528 dist. nutans loosdorfer	G	C	C	C	G	C	C	C	A	A	I
GM 529 dist. nutans proskowetz gerste	G	C	C	C	G	C	C	C	A	A	I
GM 530 dist. nutans triumf	G	C	C	C	G	C	C	C	A	A	I
GM 531 dist. nutans carbonera	G	C	C	C	G	C	C	C	A	A	I
GM 532 dist. nutans martonvasari	G	C	C	C	G	C	C	C	A	A	I
GM 533 dist. nutans saratov	G	C	C	C	G	C	C	C	A	A	I
GM 537 dist. erectum hokudai no. 1	G	C	C	C	G	C	C	C	A	A	I
GM 542 vulg. hybernum lyallpur	G	C	C	C	G	C	C	C	A	A	I
GM 543 vulg. wisconsin H42 (linie)	G	C	C	C	G	C	C	C	A	A	I
GM 550 fap1 2158 L	G	C	C	C	G	C	C	C	A	A	I
GM 558 dist. glabrierectum sanalta	G	C	C	C	G	C	C	C	A	A	I
GM 561 dist. nutans pfaelzer land	G	C	C	C	G	C	C	C	A	A	I
GM 562 dist. nutans szekacs linie II	G	C	C	C	G	C	C	C	A	A	I
GM 563 dist. nutans maiamana	G	C	C	C	G	C	C	C	A	A	I
GM1050C brc1.5	G	C	C	C	G	C	**A**	C	A	G	II
GM1116 com2.g	G	C	C	C	G	C	**A**	C	A	G	II
GM1118 com2.g introg bow	G	C	C	C	G	C	**A**	C	A	G	II
GM570 Optic	G	C	C	C	G	C	C	C	A	G	III
GM 500 Wild agriocrithon	G	C	C	C	G	C	C	C	A	G	III
GM 518 vulg. trifurcatum aegypten	G	C	C	C	G	C	C	C	A	G	III

GM 545 fap1 ooo8a	G	C	C	C	G	C	C	C	A	G	III
GM 548 fap1 2158 B	G	C	C	C	G	C	C	C	A	G	III
GM 564 hexastichon hybernum abarik	G	C	C	C	G	C	C	C	A	G	III
GM1087 Bowman	G	A	G	G	G	C	C	T	A	G	IV
GM702 Bowman	G	A	G	G	G	C	C	T	A	G	IV
GM2I Proctor	G	A	G	G	G	C	C	T	A	G	IV
GM 569 Golden Promise	G	A	G	G	G	C	C	T	A	G	IV
BM-NIL-flo-a.5 (BW369)	G	A	G	G	G	C	**C**	T	A	G	IV
Igri	G	A	G	G	G	C	**C**	T	A	MISSING	IV
GM 502 sp11 085–50	G	A	G	G	G	C	C	T	A	G	IV
GM 503 vulg. coeleste kleine nacktgerste	G	A	G	G	G	C	C	T	A	G	IV
GM 514 vulg. hybernum estanzuela	G	A	G	G	G	C	C	T	A	G	IV
GM 515 vulg. hybernum elses	G	A	G	G	G	C	C	T	A	G	IV
GM 517 vulg. hybernum marokkanische	G	A	G	G	G	C	C	T	A	G	IV
GM 519 vulg. hybernum algerian	G	A	G	G	G	C	C	T	A	G	IV
GM 522 vulg. himalayense tibet	G	A	G	G	G	C	C	T	A	G	IV
GM 523 vulg. horsfordianum weihenstephan	G	A	G	G	G	C	C	T	A	G	IV
GM 524 dist. nudiforcatum erfurt	G	A	G	G	G	C	C	T	A	G	IV
GM 525 dist. nutans kenia	G	A	G	G	G	C	C	T	A	G	IV
GM 526 dist. nutans spratt archer	G	A	G	G	G	C	C	T	A	G	IV
GM 534 dist. nutans swannek	G	A	G	G	G	C	C	T	A	G	IV
GM 535 dist.medicum anatolien	G	A	G	G	G	C	C	T	A	G	IV
GM 536 dist. nigricans mandschurei	G	A	G	G	G	C	C	T	A	G	IV
GM 538 dist. nutans australische fruche	G	A	G	G	G	C	C	T	A	G	IV
GM 541 vulg. hybernum aegyptische	G	A	G	G	G	C	C	T	A	G	IV
GM 546 dist. nutans bannerts	G	A	G	G	G	C	C	T	A	G	IV
GM 547 fap 1 0266C	G	A	G	G	G	C	C	T	A	G	IV
GM 554 ucnw c177	G	A	G	G	G	C	C	T	A	G	IV
GM 555 npc 0006	G	A	G	G	G	C	C	T	A	G	IV
GM 556 siglah	G	A	G	G	G	C	C	T	A	G	IV
GM 557 siglah	G	A	G	G	G	C	C	T	A	G	IV
GM 560 dist. nutans agio	G	A	G	G	G	C	C	T	A	G	IV
GM 565 hexastichon hybernum chilean	G	A	G	G	G	C	C	T	A	G	IV
GM 566 MPI 2	G	A	G	G	G	C	C	T	A	G	IV
GM 501 ucnw016	T	C	G	G	A	A	C	T	G	G	V
GM 509 vulg. nigroibericum otello	T	C	G	G	A	A	C	T	G	G	V
GM 549 fap 1 2158 H	T	C	G	G	A	A	C	T	G	G	V
GM 540 deficiens steudelii abessinien	T	C	G	G	A	A	C	T	G	G	V
GM 551 deficiens erythraeum foa II	T	C	G	G	A	A	C	T	G	G	V
GM 559 deficiens deficiens fehlgerste	T	C	G	G	A	A	C	T	G	G	V

GM 539 intermedium gymnanomalum	T	C	G	G	A	C	C	T	G	G	VI
GM 510 vulg. hybernum isthmos	G	C	G	G	G	C	C	C	A	G	VII
GM 512 vulg. rikotense brant	G	C	G	G	G	C	C	C	A	G	VII
GM 520 vulg. hybernum parallelum samsun	G	C	G	G	G	C	C	C	A	G	VII
GM 521 vulg. parallelum libanon	G	C	G	G	G	C	C	C	A	G	VII
GM 544 ucnwc72a	G	C	G	G	G	C	C	C	A	G	VII
GM 552 vulg. dundar-beyi nippon	G	C	G	G	G	C	C	C	A	G	VII
GM 553 intermedium horlani arlington	G	C	G	G	G	C	C	C	A	G	VII
AP2/ERF domain	184 - 357 bp										
phylogenetically highly conserved domain	638 - 691 bp										

Table S4 Tetraploid 'Miracle Wheat' used for allelism test and the corresponding F_1 phenotype.

Cross			Resulted Progeny	plant number	spikes per plant	branched spikes
Tri 3261	*	Tri 9652	plant 1	XIX-2012-1	3	1
Tri 3261	*	Tri 9652	plant 2	XIX-2012-2	3	no branched spikes
Tri 3261	*	Tri 9652	plant 3	XIX-2012-3	2	2
Tri 3261	*	Tri 9652	plant 4	XIX-2012-4	4	3
Tri 3261	*	Tri 9652	plant 5	XIX-2012-5	2	2
Tri 3261	*	Tri 9652	plant 6	XIX-2012-6	2	2
Tri 3261	*	Tri 9652	plant 7	XIX-2012-7	4	4
Tri 3261	*	Tri 9652	plant 8	XIX-2012-8	3	3
Tri 3261	*	Tri 9652	plant 9	XIX-2012-9	3	3
Tri 3261	*	Tri 9652	plant 10	XIX-2012-10	3	3
Tri 9652	*	Tri 3261	plant 1	XIX-2012-11	4	4
Tri 9652	*	Tri 3261	plant 2	XIX-2012-12	2	2
Tri 9652	*	Tri 3261	plant 3	XIX-2012-13	3	3
Tri 9652	*	Tri 3261	plant 4	XIX-2012-14	3	2
Tri 9652	*	Tri 3261	plant 5	XIX-2012-15	3	3
Tri 9652	*	Tri 3261	plant 6	XIX-2012-16	3	3
Tri 9652	*	Tri 3261	plant 7	XIX-2012-17	4	4
Tri 9652	*	Tri 3261	plant 8	XIX-2012-18	2	2
Tri 9652	*	Tri 5283	plant 1	XIX-2012-22	2	2
Tri 9652	*	Tri 5283	plant 2	XIX-2012-23	2	2
Tri 9652	*	Tri 5283	plant 3	XIX-2012-24	3	3
Tri 9652	*	Tri 5283	plant 4	XIX-2012-25	4	3
Tri 9652	*	Tri 5283	plant 5	XIX-2012-26	4	4
Tri 9652	*	Tri 5283	plant 6	XIX-2012-27	3	3
Tri 9652	*	Tri 5283	plant 7	XIX-2012-28	3	3
Tri 9652	*	Tri 5283	plant 8	XIX-2012-29	3	3
Tri 9652	*	Tri 5283	plant 2	XIX-2012-31	3	2
Tri 9652	*	Tri 5283	plant 3	XIX-2012-32	3	3
Tri 9652	*	Tri 5283	plant 4	XIX-2012-33	3	3
Tri 3261	*	Tri 5283	plant 1	XIX-2012-34	3	3
Tri 3261	*	Tri 5283	plant 2	XIX-2012-35	3	3
Tri 3261	*	Tri 5283	plant 3	XIX-2012-36	5	5
Tri 3261	*	Tri 5283	plant 4	XIX-2012-37	3	3
Tri 3261	*	Tri 5283	plant 5	XIX-2012-38	3	3
Tri 984	*	Tri 5283	plant 1	XIX-2012-39	4	3

Table S5 Mutant and wild type Tetraploid 'Miracle Wheat' accessions used for resequencing of the *bh*^{t} locus.

				A genome	
Tt-WheatID	**Country of origin**	**Growth habit**	**Spike type**	**SNP (T287C; Tamaroi45 as Reference)**	**corresponding aa substitution (L96P; Tamaroi45 as Reference)**
KALKA	AUS	spring type	Wild type	Not Found	Not Found
BELLAROI	AUS	spring type	Wild type	Not Found	Not Found
TAMAROI	AUS	spring type	Wild type	Not Found	Not Found
FLORADUR	AUT	spring type	Wild type	Not Found	Not Found
WOLLAROI	AUS	spring type	Wild type	Not Found	Not Found
TRI11066	UZB	spring type	Wild type	Not Found	Not Found
TRI17236	TUR	spring type	Wild type	Not Found	Not Found
TRI2230	USA	spring type	Wild type	Not Found	Not Found
TRI3504	POR	spring type	Wild type	Not Found	Not Found
TRI758	TUR	spring type	Wild type	Not Found	Not Found
TD24	GER	winter type	Wild type	Not Found	Not Found
TD97	GER	winter type	Wild type	Not Found	Not Found
AURADUR	AUT	winter type	Wild type	Not Found	Not Found
LOGIDUR	AUT	winter type	Wild type	Not Found	Not Found
LUNADUR	AUT	winter type	Wild type	Not Found	Not Found
LUPIDUR	AUT	winter type	Wild type	Not Found	Not Found
ELSADUR	AUT	winter type	Wild type	Not Found	Not Found
TRI 13541	ITA	winter type	Wild type	Not Found	Not Found
TRI 1669	ALB	winter type	Wild type	Not Found	Not Found
TRI 19273	TUR	winter type	Wild type	Not Found	Not Found
TRI 3023	ALB	winter type	Wild type	Not Found	Not Found
TRI 3720	ESP	winter type	Wild type	Not Found	Not Found
TRI 4292	TUR	winter type	Wild type	Not Found	Not Found
TRI 4522	CHN	winter type	Wild type	Not Found	Not Found
TRI 4886	UK	winter type	Wild type	Not Found	Not Found
TRI 7021	POL	winter type	Wild type	Not Found	Not Found
TRI 7056	FRA	winter type	Wild type	Not Found	Not Found
TRI 9546	ARM	winter type	Wild type	Not Found	Not Found
TRI 9547	ARM	winter type	Wild type	Not Found	Not Found
TRI 9629	CSFR	winter type	Wild type	Not Found	Not Found
TRI 984	EUR	spring type	Mutant	Found as Homozygote	Found as Homozygote
TRI 3261	ESP	spring type	Mutant	Found as Homozygote	Found as Homozygote
TRI 5283	CHN	spring type	Mutant	Found as Homozygote	Found as Homozygote

TRI 18959	FRA	spring type?	Mutant	Found as Homozygote	Found as Homozygote
CITr 13712	USA	spring type	Mutant	Found as Homozygote	Found as Homozygote
CITr 13713	USA	spring type	Mutant	Found as Homozygote	Found as Homozygote
PI 225308	IRAN	spring type	Mutant	Found as Homozygote	Found as Homozygote
PI 349056	ARM	spring type	Mutant	Found as Homozygote	Found as Homozygote
PI 438971	KAZ	spring type	Mutant	Found as Homozygote	Found as Homozygote
TRI 3411	SU	spring type	Mutant	Found as Homozygote	Found as Homozygote
TRI 4045	EUR	spring type	Mutant	Found as Homozygote	Found as Homozygote
TRI 4341	EUR	spring type	Mutant	Found as Homozygote	Found as Homozygote
TRI 5911	IRAN	spring type	Mutant	Found as Homozygote	Found as Homozygote
TRI 9548;W1420	ARM	spring type	Mutant	Found as Homozygote	Found as Homozygote
TRI 27966	-	spring type	Mutant	Found as Homozygote	Found as Homozygote
TRI 1781	GER	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 1782	GER	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 3365	CHN	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 4270	ITA	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 4446	HUN	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 4461	EUR	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 4653	AUS	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 9628; ;W1529	IND	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 19165	-	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 19292	FRA	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 28396	ITA	winter type	Mutant	Found as Homozygote	Found as Homozygote
TRI 24012	-	winter type	Mutant	Found as Heterozygote	Found as Heterozygote
TRI 4448	EUR	winter type	Mutant	Found as Heterozygote	Found as Heterozygote
TRI 9652;W1554	CSR	spring type	Mutant	Found as Heterozygote	Found as Heterozygote

Table S6 Primer information

PrimerID	Orien-tation	Experiment	Sequence	Tm (◦C)	PCR product_bp	Restriction enzyme for CAPS marker development
HvCOM2	Forward	qRT-PCR	CGCACATTGGGTCGTACCA	57.9	107	-
HvCOM2	Reverse	qRT-PCR	GTGATCGGCGGCATTGG	57.2	-	-
HvCKX2	Forward	qRT-PCR	GTTCGTGGAGCGGAAGAGGAAG	60	107	-
HvCKX2	Reverse	qRT-PCR	CGGGGCCAATCGATTAACTTCGTG	60	-	-
HvBAS1	Forward	qRT-PCR	AGACGCCAGATCATCACCTGTG	59.1	101	-
HvBAS1	Reverse	qRT-PCR	ACATGGTCATTTCGGCCTGTCAT	62.9	-	-
HvIDS1	Forward	qRT-PCR	CTAGCTCTCTGCTTACTCCCATGGAC	60	134	-
HvIDS1	Reverse	qRT-PCR	CATGGCTCGGCAATGTTATCTCTCTC	59.2	-	-
HvASK2	Forward	qRT-PCR	GGTTGACTCTGCGACGCGA	60.3	76	-
HvASK2	Reverse	qRT-PCR	CGTTAACGGCTGCTCCCAGG	60.9	-	-
HvCYP78A9	Forward	qRT-PCR	CCCATTCGGCCTAAACGCGA	60.4	96	-
HvCYP78A9	Reverse	qRT-PCR	AGAAACGTACAGCAGCCAGCC	60.1	-	-
HvCRC	Forward	qRT-PCR	ATGGATGTGCTCCTGGGTGTG	59.7	82	-
HvCRC	Reverse	qRT-PCR	TGATGTCCCGGTGGATGATCG	59.1	-	-
HvLUX1	Forward	qRT-PCR	CAGAGTTGCAGAGAGTGTGTGC	58.6	107	-
HvLUX1	Reverse	qRT-PCR	TCTTGCCACTGCCCAAAATGG	58.1	-	-
HvERECTA	Forward	qRT-PCR	TGAAGTCGAATGGGCTGACCG	59.6	66	-
HvERECTA	Reverse	qRT-PCR	GCGTCTTAATCGACGAGCAGTCC	59.7	-	-
HvTASSELSEED2	Forward	qRT-PCR	ACTGCGCCTAGTTGTTCGAG	57.4	97	-
HvTASSELSEED2	Reverse	qRT-PCR	TCCTGCACACTTCAAACCACA	57.1	-	-
com2_p11	Forward	ForRecombinantScreen_distal proximal(ortholog of Os07g0673700)	CCCTTCCTCGTTAGGTACGTGTG	66	1147	HpaII
com2_p12	Reverse	ForRecombinantScreen_distal proximal(ortholog of Os07g0673700)	CTGGTGATTTCCCAAACCTGAAG	63	-	-
com2_p19	Forward	ForRecombinantScreen_flank proximal(ortholog of	GTCAACACACCAACGGGTCTTC	64	968	EcoRV

		Os07g0668900)				
com2_p20	Reverse	ForRecombinantScreen_flank proximal(ortholog of Os07g0668900)	AGGCCTATGGCATCATGGAAAT	60	-	-
M1 (com2g_p31)	Forward	Flank marker M1 (morex_contig_1566969 CAJW011566969 carma=2HS)	CGCTACTTGGCACATTCTCA	60	1041	HinfI
M1 (com2g_p32)	Reverse	Flank marker M1 (morex_contig_1566969 CAJW011566969 carma=2HS)	CAGTTAGCTTTCGGGCTTTG	60	-	-
M2 (2HS_3D)	Forward	Flank marker M2 (ortholog of Os07g0669200)	GATCCCACCTCCAGTAACGA	60	1061	BsmI
M2 (2HS_3D)	Reverse	Flank marker M2 (ortholog of Os07g0669200)	GATGCACTGCCTCAACTCAA	60	-	-
Insitu	Forward	mRNA in-situ hybridization (cDNA isolated from cv. Bonus)	CAACGGCTACTCACCCTTCA	60.5	444	-
Insitu	Reverse	mRNA in-situ hybridization (cDNA isolated from cv. Bonus)	GTGCGTACTACATTCTCGAG	60.5	-	-
TtBH-A1	Forward	tetraploid wheat TILLING screen	GCTAGGCGGGAGCAGTAGTA	60.82	1011	
TtBH-A1	Reverse	tetraploid wheat TILLING screen	GTGGGCACAGCAGACCAC	60.98	-	
TtBH-B1	Forward	tetraploid wheat TILLING screen	TCCCCTCCCCTACCCAAG	59.21	1218	
TtBH-B1	Reverse	tetraploid wheat TILLING screen	TGAGTACGTAAGAGGCTAAGATCG	59.49	-	
TILL_FZP_7_rev	Forward	Barley TILLING screen	CAGTGGGAGAGGAAGCTGAA	60.5	1044	
TILL_FZP_7_for	Reverse	Barley TILLING screen	GAAGCTCACAGCAACCACCT	60.5	-	

Available for download as Excel files at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.176628/-/DC1

Table S7 TILLING analysis in wheat

File S1 Detailed information of the up and down regulated genes in microarray experiment.

File S2: SI TEXT

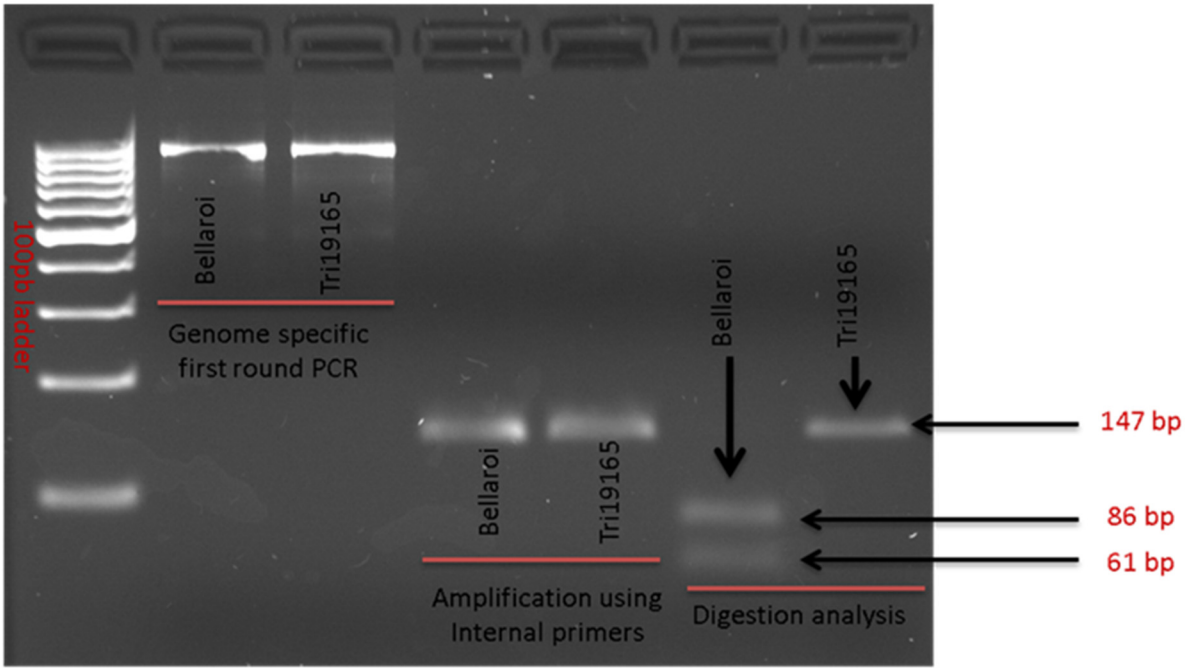
Candidate gene specific marker development in barley and tetraploid wheat

The orthologs of maize *BD1* and rice *FZP/BFL1* (CHUCK *et al.* 2002; KOMATSU *et al.* 2003; ZHU *et al.* 2003) were considered as candidate genes underlying *bh*^{t} and *com2.g* phenotypes in wheat and barley, respectively. To map the barley ortholog, the *COM2* gene, in barley the corresponding specific gene-based markers were developed. The two CAPS markers (table below) are both on the basis of SNP A873G found between the two parents of the population (Haruna Nijo x BW 192 *com2.g*). Barley marker information is provided in the table below. Each of the two markers could be utilized for mapping.

primer name	orientation	primer seq	Restriction Enzyme	Product size	Digested Product size_wild	Digested Product size_mutant	Digested Product size_heterozygote	Tm (°C)
HN_SNP2for	Forward	AACTCCGGGTACCTGAGCA	BtgZI	327 bp	219 bp; 108 bp	327 bp	327 bp; 215 bp; 112 bp	60,66
HN_SNP2rev	Reverse	CAGATCGGCCATTAAGTGAG						58,33
GW_3UTR_For3	Forward	GAGGACGTGGACGACCTG	Hpy99I	207 bp	10bp; 31bp; 168 bp	10bp; 31bp; 59 bp; 108 bp	10bp; 31bp; 59 bp; 108 bp; 168 bp	57,7
HN_SNP2rev	Reverse	CAGATCGGCCATTAAGTGAG						58,33

Wheat marker information for the candidate gene underlying *bh*^{t} is presented in the following table and exemplary image. This CAPS marker is developed on the basis of SNP T287C found between parents of the corresponding mapping populations. Two-step PCR reactions were followed. In the first round, any of the A genome specific primers 1 or 2 could be used. Second, the internal primer pair *(*TdFZP2A_in_F and TdFZP2A_in_R*)* was used to amplify a short fragment using the first round PCR product as template. This short fragment was used for CAPS marker development. At the image below, amplification of the candidate gene using genome-specific primers (left), amplification of region of interest using internal primers (middle), and digestion analysis (right) are depicted.

primer name	Product ID	primer seq	Restriction Enzyme	Product size	Digested Product size _ wild	Digested Product size _ mutant	Digested Product size _ heterozygote
Tafzp_2A_Forward 1	A geneome specific 1	AGCCAACCTCACTTCACTTC	-	946 bp	946 bp	946 bp	946 bp
Tafzp_2A_Reverse 1		GAGCAATGCCAGCGCGTCCGT					
Tafzp_2A_Foward 2	A geneome specific 2	CTAGGCGGGAGCAGTAGTA	-	963 bp	963 bp	963 bp	963 bp
Tafzp_2A_Reverse 2		AGCGCGTCCGTTTCAGTGG					
TdFZP2A_in_F	Internal for A.G. Specific 1 and 2	GACCCGACCACCAAGGAG	BstNI	147 bp	61 bp; 86 bp	147 bp	61 bp; 86 bp; 147 bp
TdFZP2A_in_R		GTAGTTGTTGTAGGCGGCGT					



Genetic mapping in barley

To newly map the phenotype in barley, we developed an F_2 mapping population comprising 286 individuals between the parental mutant BW 192 *com2.g* and barley cv. Haruna Nijo. The parental mutant BW 192 *com2.g* is the Bowman Near Isogenic Line of *com2.g* and was previously developed until BC7≤ generation (DRUKA *et al.* 2010). The segregation pattern of the phenotype among the corresponding F2 barley plants fitted well with a 3:1 ratio typical for a monogenic recessive trait. The syntenic information reported in the form of barley chromosome 2H Genome Zipper (MAYER *et al.* 2011) was explored to genetically localize the phenotype and to develop further markers surrounding the locus (Figure S2). This low resolution genetic mapping localized the *com2.g* phenotype in an interval of 5.5 cM along the barley chromosome 2H short arm. The interval was flanked by two barley gene based markers orthologous of rice genes Os07g0673700 (barley CAPS marker com2_p11/com2_p12) and Os07g0668900 (barley CAPS marker com2_p19/com2_p20), respectively. The two aforementioned flanking markers were used for screening a larger population consisting of 1750 F_2 plants from which 52 recombinant plants were identified.

While fine-mapping *com2.g*, we discovered that the observed genotypic alleles for the *COM2*-specific marker failed to fully match the *com2.g* phenotypic score since six out of the 52 F_2 recombinants showed a discrepant phenotype. While genotypically heterozygous for *COM2*, they showed the supposedly recessive branched phenotype. F_3 offsprings (17 to 28

progenies per F_2 totaling 128 F_3 plants) segregated for the respective spike-branching phenotype at both phenotypic and genotypic levels thus confirming the heterozygosity of the parental F_2 plants. However, also F_3 plants showed inconsistencies between genotypic and phenotypic data. While all 36 genotypically homozygous mutant plants showed clear spike-branching, 33 of the 70 heterozygous and three out of 22 homozygous wild type plants showed a faintly branched spike. The deviant F_3 plants were progenies from three out of six F_2 plants which suggest that additional genetic and/or environmental factors regulate spike-branching. This is supported by observations in maize that found an effect of genotypic background on the expressivity of the inflorescence branching (COLOMBO *et al.* 1998).

Genetic mapping in tetraploid wheat

A linkage map for *bh*^{t} in tetraploid wheat was established in parallel to genetic mapping of *com2.g* in barley. Two different mapping populations consisting of 279 and 159 F_2 plants were developed using a set of published microsatellite markers (RODER *et al.* 1998) (Figure S2), the phenotype could be genetically mapped to a region spanning ~20 cM on wheat chromosome 2A short arm (2AS) (Figure S2) that confirmed the previous finding of *bh*^{t} genetic mapping (KLINDWORTH *et al.* 1997). Both mutant parents TRI 27966 and TRI 19165 carry the same recessive allele of *bh*^{t} showing only a single amino acid substitution of leucine to proline at position 96 (L96P) as compared to the wild type. However, further genetic mapping for positional cloning of the gene was not followed in wheat.

SI MATERIALS AND METHODS

Marker development in barley

Barley chromosome 2H genome zipper (GZ) (MAYER *et al.* 2011) was utilized for initial marker development. Barley sequence information, the homologs of the rice genes ordered along the 2H-GZ was used for primer design. Barley sequence information was obtained from IPK Barley Blast Server (http://webblast.ipk-gatersleben.de/barley/viroblast.php). Corresponding rice genes were extracted from respective genome browser server (http://rice.plantbiology.msu.edu/cgi-bin/gbrowse/rice/). Rice gene sequences were blasted against the IPK barley blast server (http://webblast.ipk-gatersleben.de/barley/viroblast.php) to obtain barley sequences. Sequences were amplified in parental lines of the mapping population using primers designed to detect SNP polymorphisms. Identified SNPs were converted to restriction enzyme based CAPS (http://nc2.neb.com/NEBcutter2/). The barley ortholog of rice *FZP/BFL1* gene sequence (*Os07g0669500)* was used for candidate gene marker development. In case of wheat, publicly available SSR markers (RODER *et al.* 1998) were used for genetic mapping.

TILLING analysis

Barley

For identifying further mutant alleles of *COM2* in barley, a TILLING population consisting of 10,279 EMS (Ethyl methanesulfonate) treated plants of cv. Barke was screened. A primer combination (Table S6) was used to amplify the single exon of the *COM2* gene. The product was subjected to standard procedure of AdvanCE™ TILLING kit as described in (GAWRONSKI *et al.* 2014). Amplified products were digested with dsDNA cleavage kit followed by analysis via mutation discovery kit and gel - dsDNA reagent kit. These were performed on the AdvanCE™ FS96 system according to manufacturer's guidelines (advanced analytical, IA, USA). Amplified ORF was also re-sequenced by Sanger sequencing on all accessions carrying polymorphism identified to confirm SNPs. In addition to the Barke population, a different TILLING population, TillMore (in the cv. Morex) (TALAME *et al.* 2008) was also screened for additional mutant alleles of *COM2.* A set of TillMore lines with branched phenotype was screened. From these lines, the *COM2* single exon was re-sequenced for detection of causal SNPs.

Wheat

To identify *TtBH-1* mutants in wheat, a tetraploid TILLING population consisting of 1,139 EMS plants of cv. Kronos was screened (Uauy et al 2009). Homoeologue-specific primers were designed (Table S6) and tested for specificity; betaine was added at 1M final concentration to the PCR reactions. A 1,011 bp fragment of *TtBH-A1* and a 1,218 bp fragment of *TtBH-B1* were screened. Mutant detection was performed with *Cel*1 digestion followed by analysis on a capillary ABI3730 sequencer (Applied Biosystems, Foster City, California, USA) using published protocols (LE SIGNOR *et al.* 2009). Individual DNAs from positive pools were Sanger sequenced to identify the nature of the mutations.

Haplotype analysis

Genomic DNA was extracted using mixed alkyl trimethyl ammonium bromide method (SALLAUD *et al.* 2003) from a diverse set of barley accessions (Table S3) using standard protocols and the full coding sequence of the barley *COM2* gene was PCR-amplified using primers **Ptp #56:** GCATGCATGTCACTCGAACT (upstream of ATG) and **Ptp #67:** CTAGGGCACCGAAACAAGCC (downstream of stop codon). PCR reactions (15 μL) contained 40-50 ng genomic DNA, 0.3 μM of each primer, dNPT mix 0.5 mM, DMSO 5%, herculase buffer 1x, Herculase Hotstart DNA polymerase 0.74 U (Stratagene #600310). Thermal cycling protocol consisted of: 1 cycle (95°/5'), 4 cycles (95°/20", 65°/10" [with 0.5°reduction per cycle], 72°/45"), 2 cycles (95°/20", 62°/20",72°/45"), followed by 34 cycles (95°/20", 60°/30",72°/1') and the last step of 1 cycle (72°/10'). PCR products were purified using the ExoSAP-IT (Exo-nucleases) PCR clean-up protocol (Applied Biosystems®) following the manufacturer's instructions and sequenced using the Sanger method with BigDye™ Terminator v3.1 Matrix Standard Sequencing Kit (Applied Biosystems®, ABI PRISM® 3700 DNA Analyzer) using primers **Ptp** #56 and **Ptp** #23 ACCAACTTCGTCTACGCGCA.

Sequence annotation

Unpublished sequence information for the two BAC contigs (44575 and 47813; spanning the interval between M1 and M2) was made available from the international barley sequencing consortium (through Dr. Nils Stein). First, barley BAC contig overlap detection was performed by an all against-all alignment with megablast (ZHANG *et al.* 2000). This was to confirm the overlap between the two BAC contigs initially identified. The criteria were only BLAST hits longer than 2 kb and 99.5% sequence identity. Sequence annotation was performed as described by (MASCHER *et al.* 2014). Sequences were first subjected to k-mer-based repeat masking using Kmasker algorithm (SCHMUTZER *et al.* 2014). *Augustus* was implemented for structural gene annotation of repeat-masked contigs using the maize model. Finally, predicted protein sequences were functionally annotated with the AHRD pipeline. This included parses the description of BLASTP hits against the TAIR, Uniprot/trEMBL, and Uniprot/SwissProt databases as utilized by (ZHANG *et al.* 2000). Genes annotated as unknown proteins or transposable elements were excluded from further analysis.

File S3 TtBH and COM2 DNA sequence

Available for download at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.176628/-/DC1

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