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 (bh) locus regulating spike branching in tetraploid ‘Miracle-Wheat’. Both genes possess orthologs with similar functions in maize BRANCHED SILKLESS 1 (BD1) and nice FRIZZY PANICLE BRANCHED FLORETLESS 1 (V2PIBFL1) encoding AP2/ERF transcription factors. Sequence analysis of the bh 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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do: 10.1534/genetics.115.176628
Manuscript received March 21, 2015; accepted for publication June 27, 2015; published Early Online July 7, 2015.
Available freely online through the author-supported open access option.
Supporting information is available online at www.genetics.org/lookup/suppl/doi:10.1534/genetics.115.176628/-/DC1.
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‘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’Oibel 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 Sláfer 2007; Lawlor and Paul 2014). The spike branching has been observed in diploid wheat (2n = 2x = 14, bh'-locus; Amagai et al. 2014), tetraploid wheat (2n = 4x = 28, bh' 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', 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' is orthologous to mrs identified in hexaploid wheat (Dobrovolskaya et al. 2015). Sequence analysis of the bh' 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 Resources 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 F2 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 F2 mapping populations (Tamaroi45 × TRI 27966; 279 F2 individuals; (Tamaroi42 × TRI 19165; 159 F2 individuals) were created. In both species, segregation between mutant and WT F2 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)
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 MicroKit (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 HindIII or NotI were used as templates to generate antisense (HindIII) and sense (NotI) 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 F₂ 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 fsp/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 weight 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 bbr, 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 bbr 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 F2 plants for recombination events. A total of 52 F2 recombinant plants and their corresponding F3 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 and exclusively present in 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, HORIZON14427 (a double mutant of com2/gooded 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 M2 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 M2 plants, and their corresponding M3 families, transmitted a branched spike. In contrast to this, two of the remaining four M2 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 M3 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 develops, 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.
Identification of the gene underlying the *bht* locus in 'Miracle-Wheat'

Since phenotypes of *com2.g* and *bht* 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 *bht* locus in 'Miracle-Wheat.' Sequence analysis of the *TtBH-A1* ORF revealed that the two *bht* mutant parents of the corresponding two mapping populations carried the same recessive allele (Figure S2B). This *bht* 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 F1 progenies further indicates that the same
L96P mutation at *bh1* 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 *M₄* 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* shows 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 (*HvRAMOS2A*), 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(vrs4.2) 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 vrs4.2 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; Zha *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 4D).
These include orthologs of the *Arabidopsis* genes cytochrome P450, *CYP78A9*, *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 4D) 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 b1 and rice mutant fsp/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 homoalleles 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 ‘Composite-Barley.’ This may explain our tetraploid wheat TILLING mutant, T4-2447 (G61S), in which we predominantly found additional spikelet formation but not spike branching.

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., bht) 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-day 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 meristem development and subsequently inflorescence architecture in grasses may provide valuable insights into the manipulation of yield-relevant traits in various crop plants.

Acknowledgments

We thank H. Bockelman (US Department of Agriculture–Agricultural Research Service) and M. Grau [Leibniz Institute of Plant Genetics and Crop Plant Research (IPK) gene bank] for providing initial mutant germplasm; M. van Slageren (Royal Botanic Gardens, Kew, UK) for useful discussion on the Lobel reference; the International Barley Sequencing Consortium for prepublication access to sequence information of chromosome 2H; H. Ernst for taking photographs; and M. Pürschel, A. Marlow, E. Miatton, V. Talamé, J. Simmonds, R. Voss, and C. Weissleder for excellent technical support. A.B. was supported by the Scientific and Technological Research Council of Turkey 2214/A International Research Fellowship Programme as a visiting scholar in the John Innes Centre. This work was partly supported by grants from the IPK Gatersleben and the German Federal Ministry of Education and Research, GABI-FUTURE Start Young Investigator Program grant 0315071 to T.S.

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

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SUPPORTING INFORMATION

The genetic basis of composite spike form in barley and ‘Miracle-Wheat’

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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 bht 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 Os07g. 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 (bht) locus in two different tetraploid wheat F2 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.
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).
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-NILcom2.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 ≤ 0.05, p ≤ 0.01 and p ≤ 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.
### 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/A/ 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 probably 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 substitution | 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 | Heterozygote | 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 | Heterozygote | G → S           | -              | -           |
| 11023-1   | Barke      | 541          | G→A | Heterozygote | G → S           | -              | -           |
| 11359-1   | Barke      | 572          | C→T | Heterozygote | A → V           | -              | -           |
| 4913-1    | Barke      | 587          | G→A | Heterozygote | S → N           | -              | -           |
| 13679-2   | Barke      | 605          | G→A | Heterozygote | S → N           | -              | -           |
| 6872-1    | Barke      | 631          | A→T | Heterozygote | S → C           | -              | -           |
| 9662-1    | Barke      | 662          | G→A | Heterozygote | S → N           | -              | conserved region |
| 6893-1    | Barke      | 695          | G→A | Heterozygote | 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 | Heterozygote | 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     | G     | C     | C     | C     | A     |       |       |       |
| GM 1E Nudinka      | G     | C     | C     | G     | C     | C     | C     | A     | A     | I     |       |
| GM713 Morex        | G     | C     | C     | G     | C     | C     | C     | A     | A     | I     |       |
| GM712 Donaria      | G     | C     | C     | G     | C     | C     | C     | A     | A     | I     |       |
| Haruna Nijo        | G     | C     | C     | G     | C     | C     | C     | A     | A     | I     |       |
| Barke              | G     | C     | C     | G     | C     | C     | C     | A     | A     | I     |       |
| 3167               | G     | C     | C     | G     | C     | C     | C     | A     | A     | I     |       |
| 3906               | G     | C     | C     | G     | C     | C     | C     | A     | A     | I     |       |
| GM 504 vulg. hyb.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 505 vulg. hyb.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 506 vulg. para. | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 507 vulg. para. | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 508 vulg. hyb.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 511 vulg. hyb.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 513 vulg. hyb.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 516 vulg. subv. | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 527 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 528 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 529 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 530 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 531 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 532 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 533 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 537 dist. erct. | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 542 vulg. hyb.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 543 vulg. wis.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 550 fap1 2158 L | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 558 dist. glab. | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 561 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 562 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 563 dist. nut.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM1050C brc1.5     | G     | C     | C     | G     | C     | C     | A     | A     |       |       |       |
| GM1116 com2.g      | G     | C     | C     | G     | C     | C     | A     | A     |       |       |       |
| GM1118 com2.g intro | G     | C     | C     | G     | C     | C     | A     | A     |       |       |       |
| GM570 Optic        | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 500 Wild agri.  | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |
| GM 518 vulg. trifi. | G     | C     | C     | G     | C     | C     | C     | A     |       |       |       |


| GM 545 fap1 0008a | G | C | C | C | G | C | C | C | A | G | III |
| GM 548 fap 1 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 |
| GM21 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 naktgerste | 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 elles | 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 manschurei | 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 aegytpische | 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. nigoibericum 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 steudelli 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 F₁ 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 \textit{bhf} locus.

| 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                                          |
| Variety | Country | Type | Mutant Status | Found Status |
|---------|---------|------|---------------|-------------|
| TRI 18959 | FRA | spring type? | Mutant | Found as Homozygote |
| CITr 13712 | USA | spring type | Mutant | Found as Homozygote |
| CITr 13713 | USA | spring type | Mutant | Found as Homozygote |
| PI 225308 | IRAN | spring type | Mutant | Found as Homozygote |
| PI 349056 | ARM | spring type | Mutant | Found as Homozygote |
| PI 438971 | KAZ | spring type | Mutant | Found as Homozygote |
| TRI 3411 | SU | spring type | Mutant | Found as Homozygote |
| TRI 4045 | EUR | spring type | Mutant | Found as Homozygote |
| TRI 4341 | EUR | spring type | Mutant | Found as Homozygote |
| TRI 5911 | IRAN | spring type | Mutant | Found as Homozygote |
| TRI 9548;W1420 | ARM | spring type | Mutant | Found as Homozygote |
| TRI 27966 | - | spring type | Mutant | Found as Homozygote |
| TRI 1781 | GER | winter type | Mutant | Found as Homozygote |
| TRI 1782 | GER | winter type | Mutant | Found as Homozygote |
| TRI 3365 | CHN | winter type | Mutant | Found as Homozygote |
| TRI 4270 | ITA | winter type | Mutant | Found as Homozygote |
| TRI 4446 | HUN | winter type | Mutant | Found as Homozygote |
| TRI 4461 | EUR | winter type | Mutant | Found as Homozygote |
| TRI 4653 | AUS | winter type | Mutant | Found as Homozygote |
| TRI 9628;W1529 | IND | winter type | Mutant | Found as Homozygote |
| TRI 19165 | - | winter type | Mutant | Found as Homozygote |
| TRI 19292 | FRA | winter type | Mutant | Found as Homozygote |
| TRI 28396 | ITA | winter type | Mutant | Found as Homozygote |
| TRI 24012 | - | winter type | Mutant | Found as Homozygote |
| TRI 4448 | EUR | winter type | Mutant | Found as Homozygote |
| TRI 9652;W1554 | CSR | spring type | Mutant | Found as Homozygote |
| PrimerID   | Orientation | 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        | GTGATCGGCGGCGATTGG            | 57.2    | -              | -                                             |
| HvCKX2    | Forward     | qRT-PCR        | GTTCGTGGAGCGGAGAGGAGG         | 60      | 107            | -                                             |
| HvCKX2    | Reverse     | qRT-PCR        | CCGGCGCAATCGATTACTCTGG        | 60      | -              | -                                             |
| HvBAS1    | Forward     | qRT-PCR        | AGACGCGATCATCACCTGTG          | 59.1    | 101            | -                                             |
| HvBAS1    | Reverse     | qRT-PCR        | ACATGGTCAATCCGCTGCTG         | 62.9    | -              | -                                             |
| HvIDS1    | Forward     | qRT-PCR        | CTTAGCTCTGTGTTAATCGG          | 60      | 134            | -                                             |
| HvIDS1    | Reverse     | qRT-PCR        | CATGGCTCGGCAATGTTAATCTCTCC   | 59.2    | -              | -                                             |
| HvASK2    | Forward     | qRT-PCR        | GTTGACTCTCACTGCACGGA          | 60.3    | 76             | -                                             |
| HvASK2    | Reverse     | qRT-PCR        | CGTTAACGGCTGCTCCAGG          | 60.9    | -              | -                                             |
| HvCYP78A9 | Forward     | qRT-PCR        | CCCATTGGCCTAAACGCGA          | 60.4    | 96             | -                                             |
| HvCYP78A9 | Reverse     | qRT-PCR        | AGAAACGTACAGCAGAGGGCG         | 60.1    | -              | -                                             |
| HvCRC     | Forward     | qRT-PCR        | ATGGATGTGCTCTGGTGTG          | 59.7    | 82             | -                                             |
| HvCRC     | Reverse     | qRT-PCR        | TGATGTCAGGCTGGTATGCG         | 59.1    | -              | -                                             |
| HvLUX1    | Forward     | qRT-PCR        | CAGAGTTGCAGAGAGTGTTGTGC      | 58.6    | 107            | -                                             |
| HvLUX1    | Reverse     | qRT-PCR        | TCTTGCCACCTGCGCAAATGGG       | 58.1    | -              | -                                             |
| HvERECTA  | Forward     | qRT-PCR        | TGAAGTGCAGAGGCTGACG          | 59.6    | 66             | -                                             |
| HvERECTA  | Reverse     | qRT-PCR        | GCGTCTTAATCGAGCAGCAGATCC     | 59.7    | -              | -                                             |
| HvTASSELSEE D2 | Forward     | qRT-PCR        | ACTGCGCTAGTGGTGTCGAG         | 57.4    | 97             | -                                             |
| HvTASSELSEE D2 | Reverse   | qRT-PCR        | TCCTGCACACTTCAACACCA         | 57.1    | -              | -                                             |
| com2_p11  | Forward     | ForRecombinantScreen_distant_proximal(ortholog of Os07g0673700) | CCGTCTTCGCAGTACGTCG          | 66      | 1147           | HpaII                                         |
| com2_p12  | Reverse     | ForRecombinantScreen_distant_proximal(ortholog of Os07g0673700) | CTGGTATTTCCAAAACCTGAAG      | 63      | -              | -                                             |
| com2_p19  | Forward     | ForRecombinantScreen_flank_proximal(ortholog of Os07g0673700) | GTCAACACACCCAGGCTCTCTC       | 64      | 968            | EcoRV                                         |
| Location | Type | Forward Reversi... | Primer Sequence | Tm | Length | Restriction Enzyme |
|----------|------|--------------------|-----------------|----|--------|-------------------|
| com2_p20 | Revers | ForRecombinantScreen_flank proximal (ortholog of Os07g0668900) | AGGCTATGGCCTCATGGAAAT | 60 | - | - |
| M1 (com2p31) | Forward Flank marker M1 (morex_contig_1566969 CAJW011566969 carma=2HS) | CGCTACCTGGCAGATTCTCA | 60 | 1041 | Hinfl |
| M1 (com2p32) | Reverse Flank marker M1 (morex_contig_1566969 CAJW011566969 carma=2HS) | CATTTGCTTTCGGGCTTTG | 60 | - | - |
| M2 (2HS_3D) | Forward Flank marker M2 (ortholog of Os07g0669200) | GATCCAACCTCCACTTGAAGCA | 60 | 1061 | Bsml |
| M2 (2HS_3D) | Reverse Flank marker M2 (ortholog of Os07g0669200) | CATGCAGTGCCTCAACTCA | 60 | - | - |
| In situ | Forward mRNA in-situ hybridization (cDNA isolated from cv. Bonus) | CAAAGGCTACTCACCtgtCA | 60.5 | 444 | - |
| In situ | Reverse mRNA in-situ hybridization (cDNA isolated from cv. Bonus) | GTGCGTACTACATTCTCGAG | 60.5 | - | - |
| TtBH-A1 | Forward tetraploid wheat TILLING screen | GCTAGGGGGGACGAGTAGTA | 60.8 | 2 | 1011 |
| TtBH-A1 | Reverse tetraploid wheat TILLING screen | GTGGGCACGGCAAGCAGCACC | 60.9 | 8 | - |
| TtBH-B1 | Forward tetraploid wheat TILLING screen | TCCCCCTCCCTACCCAAG | 59.2 | 1 | 1218 |
| TtBH-B1 | Reverse tetraploid wheat TILLING screen | TGGATCGTAAGGGTACGATCG | 59.4 | 9 | - |
| TILL_FZP_7_rev | Forward Barley TILLING screen | CAGTGGGAGGGAAGCTGAG | 60.5 | 1 | 1044 |
| TILL_FZP_7_for | Reverse Barley TILLING screen | GAAGCTCACAGCAACCACT | 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.
## Candidate gene specific marker development in barley and tetraploid wheat

The orthologs of maize BD1 and rice F2P/BFL1 (Chow *et al.* 2002; Komatsu *et al.* 2003; Zhu *et al.* 2003) were considered as candidate genes underlying $bh^1$ 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 based on 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_SN2Ffor  | Forward     | AACTCGGGTT ACCTGAGCA | BtgZI | 327 bp | 219 bp; 108 bp | 327 bp | 327 bp; 215 bp; 112 bp | 60,66 |
| HN_SN2Frev  | Reverse     | CAGATCGGCC ATTAAGTGAG | Hpy99I | 207 bp | 10 bp; 31 bp; 168 bp | 10 bp; 31 bp; 59 bp; 108 bp | 10 bp; 31 bp; 59 bp; 108 bp; 168 bp | 57,7 |

Wheat marker information for the candidate gene underlying $bh^1$ 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 genome specific 1 | AGCCCACTCA CTTCACCTC | - | 946 bp | 946 bp | 946 bp | 946 bp |
| Tafzp_2A_Reverse 1 | A genome specific 1 | GAGCAATGCCA GCGGTCCGT | - | 946 bp | 946 bp | 946 bp | 946 bp |
| Tafzp_2A_Forward 2 | A genome specific 2 | CTAGGGCGGA GCAGTAGTA | - | 963 bp | 963 bp | 963 bp | 963 bp |
| Tafzp_2A_Reverse 2 | A genome specific 2 | AGCCCGTCGGTT TTCAGTTG | - | 963 bp | 963 bp | 963 bp | 963 bp |
| TdFZP2A_in_F | Internal for A.G. Specific 1 and 2 | GACCCGACCAC CAAAGGAG | BstNI | 147 bp | 61 bp; 86 bp | 147 bp | 61 bp; 86 bp; 147 bp |
| TdFZP2A_in_R | Internal for A.G. Specific 1 and 2 | GTAGGTGTGTT AGCGGCGT | - | 147 bp | 61 bp; 86 bp | 147 bp | 61 bp; 86 bp; 147 bp |
Genetic mapping in barley

To newly map the phenotype in barley, we developed an F2 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<sup>+</sup> 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 F2 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 F2 recombinants showed a discrepant phenotype. While genotypically heterozygous for COM2, they showed the supposedly recessive branched phenotype. F3 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 \( bh^3 \) in tetraploid wheat was established in parallel to genetic mapping of \textit{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 \( bh^3 \) genetic mapping (KLINDWORTH et al. 1997). Both mutant parents TRI 27966 and TRI 19165 carry the same recessive allele of \( bh^3 \) 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) (MAIER 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 (Iauy 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 Ceu1 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: GCATGCATGTCACCTGAACT (upstream of ATG) and Ptp #67: CTAGGCACCCAAACACG (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 ACCAACTTCTGCTACCGCA.
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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