The Barley *Uniculme4* Gene Encodes a BLADE-ON-PETIOLE-Like Protein That Controls Tillering and Leaf Patterning[1][OPEN]

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Tillers are vegetative branches that develop from axillary buds located in the leaf axils at the base of many grasses. Genetic manipulation of tillering is a major objective in breeding for improved cereal yields and competition with weeds. Despite this, very little is known about the molecular genetic bases of tiller development in important Triticeae crops such as barley (*Hordeum vulgare*) and wheat (*Triticum aestivum*). Recessive mutations at the barley *Uniculme4* (*Cul4*) locus cause reduced tillering, deregulation of the number of axillary buds in an axil, and alterations in leaf proximal-distal patterning. We isolated the *Cul4* gene by positional cloning and showed that it encodes a BROAD-COMPLEX, TRAMTRACK, BRIC-À-BRAC-ankyrin protein closely related to Arabidopsis (*Arabidopsis thaliana*) BLADE-ON-PETIOLE1 (BOP1) and BOP2. Morphological, histological, and in situ RNA expression analyses indicate that *Cul4* acts at axil and leaf boundary regions to control axillary bud differentiation as well as the development of the ligule, which separates the distal blade and proximal sheath of the leaf. As, to our knowledge, the first functionally characterized *BOP* gene in monocots, *Cul4* suggests the partial conservation of *BOP* gene function between dicots and monocots, while phylogenetic analyses highlight distinct evolutionary patterns in the two lineages.

Tillering or vegetative branching is one of the most important components of shoot architecture in cereals because it contributes directly to grain yield (Kebrom et al., 2013; Hussien et al., 2014) and is involved in plant plasticity in response to environmental cues and stresses (Mohapatra et al., 2011; Agusti and Greb, 2013). The shoot apical meristem initiates a series of repetitive units called phytomers, each consisting of a leaf, a node, an internode, and an axillary meristem (AXM) located in the axil between the leaf and the shoot axis (Sussex, 1989). Visually, AXM development and branching can be divided into three stages: (1) establishment of the AXM in the leaf axil; (2) initiation of multiple leaf primordia to form an axillary bud, which may remain dormant; or (3) growth into a branch through expansion of the branch internodes and differentiation of the axillary leaves (Schmitz and Theres, 2005). In grasses, tillers are lateral branches (i.e. culms) that grow from nodes of unelongated internodes at the base of the plant, affecting important agronomical features such as competition with weeds and ease of harvesting (Donald, 1968; Seavers and Wright, 1999). Although sharing some key steps in their development, tillers differ from lateral branches in eudicots in that they can produce adventitious roots and grow independently from the main plant shoot. Primary tillers arising from the main culm initiate new axillary buds that may, in turn, develop into secondary tillers and so on in a reiterative pattern (Hussien et al., 2014).

A complex combination of differential gene expression in conjunction with hormonal signaling and responses and environmental cues determines the number and location of axillary branches (Hussien et al., 2014). A number of evolutionarily conserved genetic pathways control axillary branching in both monocots and eudicots (Kebrom et al., 2013; Janssen et al., 2014). For example, reduced-branching mutant phenotypes are conferred by mutations in the orthologous GRAS (for *GIBBERELLIC ACID-INSENSITIVE, REPRESSOR of GA1*, and *SCARECROW*) genes *LATERAL SUPPRESSOR* in Arabidopsis (*Arabidopsis thaliana*), *LATERAL SUPPRESSOR* in tomato (*Solanum lycopersicum*), and *MONOCULM1* in rice (*Oryza sativa*; Li et al., 2003). However, the molecular mechanisms that control branching in eudicots are not completely conserved with tiller development in grasses (Kebrom et al., 2013; Hussien et al., 2014; Waldie et al., 2014). For example, the reduced-branching mutations in the *REGULATOR OF AXILLARY MERISTEMS1*, *REGULATOR OF AXILLARY MERISTEMS2*, *REGULATOR OF AXILLARY*

MERISTEMS3, and *BLIND* genes of Arabidopsis and tomato (Schmitz et al., 2002; Keller et al., 2006; Müller et al., 2006) are conserved in eudicots but have not been identified in monocot genomes (Keller et al., 2006; Müller et al., 2006).

A number of barley (*Hordeum vulgare*) tillering mutants have been identified, and their characterization has provided insight into the genetic mechanisms of vegetative axillary development and tillering in this important crop plant (Babb and Muehlbauer, 2003; Dabbert et al., 2009, 2010). Despite some recent progress (Dabbert et al., 2010; Mascher et al., 2014), most genes underlying tillering in the Triticeae await identification.

Recessive mutations in the barley *Uniculme4* (*Cul4*) gene result in reduced tillering (Babb and Muehlbauer, 2003). In this study, we show that *cul4* mutations affect multiple aspects of branch development and also cause specific defects in leaf patterning. We identified the *Cul4* gene by positional cloning and show that it encodes a homolog of the Arabidopsis *BLADE-ON-PETIOLE1* (*BOP1*) and *BOP2* genes acting at boundary regions to regulate axillary development and leaf morphogenesis.

RESULTS

Recessive *cul4* Mutations Reduce Tiller Number and Disrupt Leaf Patterning

Recessive mutations at the *Cul4* gene result in reduced tiller number (Fig. 1, A and B; Dahleen et al., 2007). The cv Bonus and cv Flare typically produced an average of 8.8 ± 1 and 11.9 ± 1.5 tillers, respectively, on fully mature greenhouse-grown plants. In contrast, *cul4* mutants showed tillering defects of varying severity, with *cul4.5* (Supplemental Table S1) and *cul4.16* (both in the cv Bonus background) and *cul4.24* (in the cv Flare background) developing an average of 0.4 ± 1, 6.3 ± 1.7, and 4.6 ± 2 tillers, respectively (Fig. 1B). In contrast with the wild type, *cul4* tillers were often bent and distorted, possibly as a result of difficulties in emergence from the leaf sheaths that enclosed them (Dahleen et al., 2007); this was frequently observed in *cul4.16* plants. In addition, *cul4* mutant plants developed some leafy side shoots (Supplemental Fig. S1) that were not considered proper tillers, as they did not elongate to produce lateral culms.

To further examine the defect in tiller development in the *cul4* mutant, histological analyses were carried out to compare the number of axillary buds in a near-isogenic line carrying the *cul4.5* mutant allele (cv Bowman-*cul4.5*) and its recurrent parent cv Bowman. At 10 d after planting, shoot apices from cv Bowman-*cul4.5* and cv Bowman were very similar (Fig. 2, A and B), except that fewer leaf axils had developed buds in the mutants compared with wild-type plants. Axillary buds were typically observed in two or three leaf axils in wild-type plants (Fig. 2, C and D), whereas in the *cul4.5* mutant, only one to two leaf axils contained axillary buds (Fig. 2E). In contrast to the wild type, transverse sections through the cv Bowman-*cul4.5* mutant shoot apex revealed two axillary buds in the same leaf axil (compare Fig. 2, F and G). Scanning electron microscopy clearly revealed a single axillary bud developing in leaf axils from cv Bowman seedlings (Fig. 2H), while occasionally leaf axils developing two axillary buds were observed in cv Bowman-*cul4.5* seedlings (Fig. 2I). To better dissect the effect of *Cul4* on axillary development, we compared the number of active leaf axils (harboring axillary buds, tillers, or leafy side shoots) in different *cul4* mutant alleles and the respective backgrounds over the critical period of tiller development (i.e. 2–5 weeks after planting). Our results indicated that the numbers of active axils were significantly lower in all *cul4* mutants compared with their wild-type backgrounds (Fig. 2J). Consistent with the reiterative pattern of tiller formation, new axils continued to become active in wild-type plants with secondary buds forming. In contrast, few axillary buds emerged after week 4 in *cul4* mutants, and no secondary buds were observed. In addition, most *cul4* axillary buds turned into leafy side shoots rather than developed tillers (Supplemental Fig. S1). In agreement with previous histological analyses, the formation of two or multiple axillary buds from a single axil was observed in some *cul4* plants (Supplemental Fig. S1). Leafy side shoots were associated with such multiple axillary buds (Supplemental Fig. S1).

Taken together, these results show that the *Cul4* gene is required for promoting axillary development in the barley shoot and controlling the number of leaf axils that form AXMs as well as the number of AXMs formed in a single leaf axil. In addition, *Cul4* activity is critical for the

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E.T. conducted fine mapping and positional cloning, gene characterization, expression, and bioinformatics analysis and wrote the article; R.O. conducted a detailed analysis of axillary development in mutants and the wild types; G.V. helped with fine mapping; V.S.J. helped with bioinformatics analysis; A.Hu. performed phylogenetic analysis; H.B. conducted the histology and SEM; M.J.S. and N.R.T. conducted in situ expression analyses; T.J.C., A.D., and R.W. provided SNP markers for mapping prior to publication; B.S., R.A., A.Hi., and N.S. provided the BAC sequence information; G.J.M. developed the segregating populations used for fine mapping, conceived and designed the microscopy and morphological analyses of tiller and axillary bud numbers, and edited the article; L.R. conceived and designed positional cloning, validation, and characterization of the gene and complemented the writing of the article.

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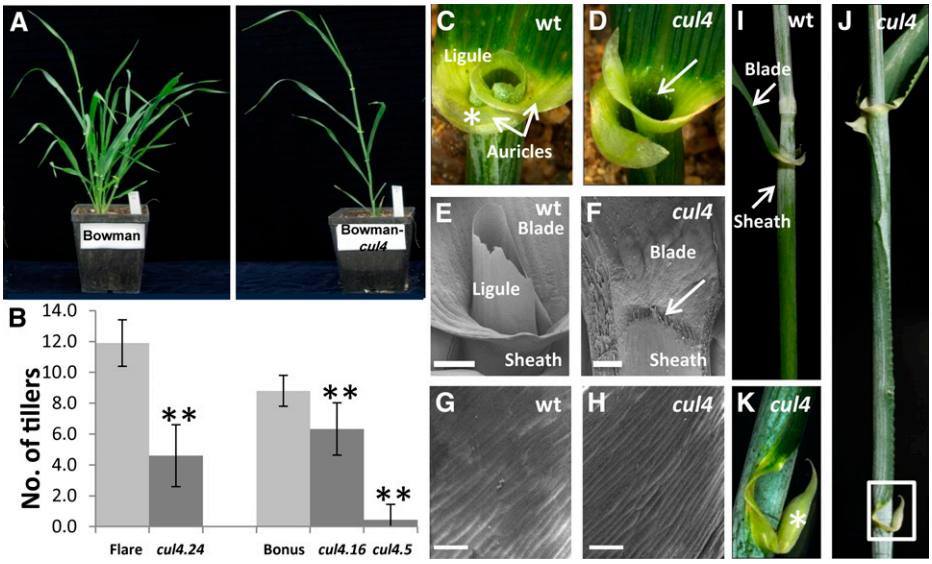

Figure 1. Shoot phenotypes of barley *cul4* mutants. A, Whole-plant phenotype of wild-type cv Bowman (left) and the cv Bowman-*cul4.5* mutant (right) at the vegetative stage. B, Comparisons between *cul4.24* and the wild-type cv Flare and between *cul4.16* and *cul4.5* and the wild-type cv Bonus for number of tillers in mature plants ($n = 9$). Error bars represent SD. Asterisks indicate significant differences ($P \leq 0.01$) relative to the wild-type background (Student's t test). C, Wild-type (wt) cv Bowman blade-sheath boundary region. D, cv Bowman-*cul4.5* mutant blade-sheath boundary region. The arrow points to the blade-sheath boundary where a ligule would normally develop. E, Scanning electron micrograph of the wild-type (cv Bowman) blade-sheath boundary region. F, Scanning electron micrograph of the cv Bowman-*cul4.5* mutant blade-sheath boundary region. The arrow indicates a fringe of tissue at the blade-sheath boundary. The auricle regions that wrap around the culm on both sides of the leaf were removed to take a scanning electron micrograph of the blade-sheath boundary. G, Scanning electron micrograph of wild-type (cv Bowman) auricle tissue from an equivalent position to the asterisk in C. H, Scanning electron micrograph of flap tissue from the cv Bowman-*cul4.5* mutant (from an equivalent position to the asterisk in K) exhibits auricle-like cells. I, Wild-type (cv Bowman) leaf. J, cv Bowman-*cul4.5* mutant leaf exhibiting a flap of tissue on the margin of the sheath. K, Closeup of flap (from the inset in J) on the sheath margin of a cv Bowman-*cul4.5* mutant plant. Bars = 1 mm (E and F) and 100 μm (G and H).

correct development of existing axillary buds into tillers and the formation of secondary buds on primary tillers.

Mutations at the *Cul4* locus also cause specific defects in leaf development. Grass leaves are organized in three distinct regions along the proximal-distal axis: (1) the proximal sheath is offset from (2) the distal blade by (3) a hinge-like structure comprising two wedge-shaped auricles, whereas an epidermal outgrowth called the ligule occurs on the adaxial leaf surface at the base of the auricles (Sylvester et al., 1990; Fig. 1, C and E). All *cul4* mutant alleles exhibited a liguleless phenotype, although the boundary between the sheath and blade remained intact with auricles observed at the proper location, and occasionally a fringe of tissue developed in place of the ligule (Fig. 1, D and F; Supplemental Fig. S2). However, ectopic flaps of auricle-like tissue often developed on the margins of *cul4* leaf sheaths, altering the proximal-distal development of the mutant leaf (Fig. 1, J and K; Supplemental Fig. S3). Scanning electron microscopy of *cul4* flap tissue (Fig. 1H) showed similar cells to those of wild-type auricle tissue (Fig. 1G), confirming that these outgrowths on the sheath margins are ectopic auricles. These phenotypes demonstrate that *Cul4* is required for ligule outgrowth and coordinating the proximal-distal patterning of the barley leaf.

Positional Cloning of the *Cul4* Gene

Previous mapping positioned *Cul4* on the distal end of chromosome 3HL (Pozzi et al., 2003; Druka et al., 2011). High-resolution mapping using 9,898 gametes from the cross cv Bowman-*cul4.5* × cv Morex located *Cul4* to a 0.55-centimorgan (cM) interval (Fig. 3). To further refine the barley *Cul4* region, additional markers were developed based on careful examination of the barley syntenic relationships with *Brachypodium distachyon*, sorghum (*Sorghum bicolor*), and rice from the virtual gene order map (genome zipper) of barley (Mayer et al., 2011; Fig. 3B).

Based on the current knowledge of genes involved in shoot development, two candidate genes were identified from annotated genes conserved among *B. distachyon*, rice, and sorghum. Partial genomic sequences were obtained exploiting EST information and available barley genomic reads (Feuillet et al., 2012; Mayer et al., 2012) and mapped using single-nucleotide polymorphisms (SNPs) identified between cv Bowman-*cul4.5* and cv Morex.

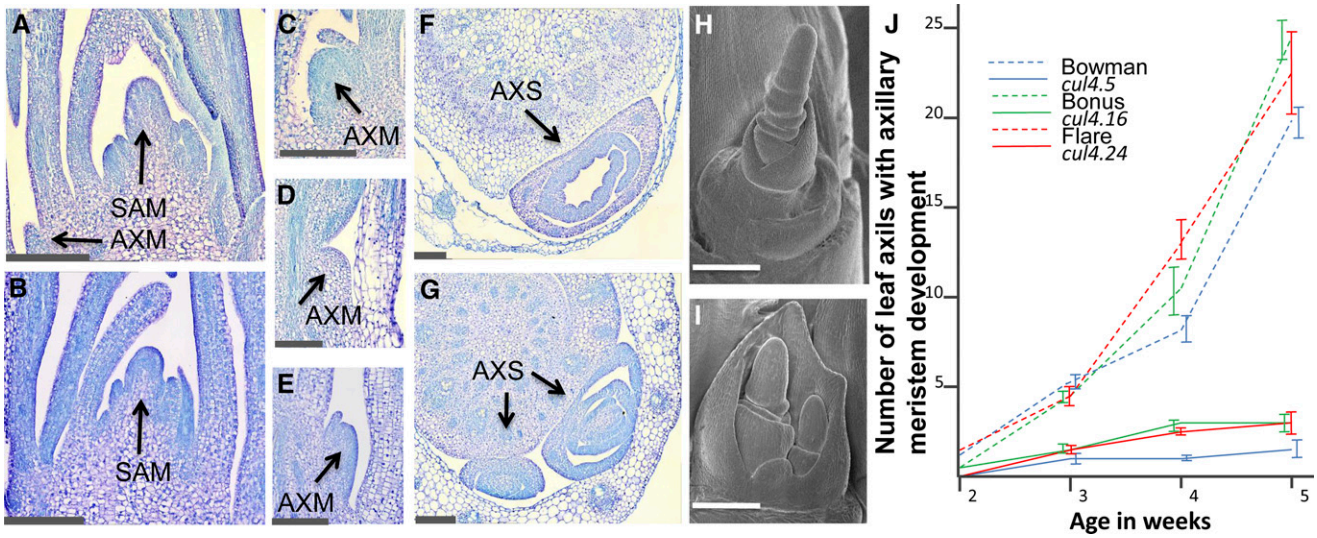

Figure 2. Axillary development in wild-type and *cul4* mutant plants. A, Longitudinal section through the shoot apical meristem (SAM) of a cv Bowman wild-type plant. B, Longitudinal section through the shoot apical meristem of a cv Bowman-*cul4.5* plant. C and D, AXMs in two successive leaf axils from the shoot apex shown in A captured in different sectioning planes. E, The only AXM from the same shoot apex shown in B. F, Transverse section through a single axillary shoot (AXS) in wild-type Bowman. G, Transverse section through two single axillary shoots in a single leaf axil in cv Bowman-*cul4.5*. H, Scanning electron micrograph of an AXM in a leaf axil from a wild-type cv Bowman plant. I, Scanning electron micrograph of two AXMs in a leaf axil from a cv Bowman-*cul4.5* mutant. J, Time course (2–5 weeks after planting) of the total number of axils containing axillary buds, side shoots, or tillers in *cul4* mutant alleles and the corresponding wild-type backgrounds. Values shown are means ± SE of biological replicates. Bars = 200 μm (A–G) and 250 μm (H–I).

A GRAS candidate gene (highly related to Bradi2g60750) was mapped 0.38 cM or more from the *cul4* locus and was excluded from further analysis. The second candidate gene, encoding a BROAD-COMPLEX, TRAMTRACK, BRIC-À-BRAC (BTB)-ankyrin protein (highly related to Bradi2g60710), showed cosegregation with *cul4* in all recombinants identified in the target interval. The two flanking genes in *B. distachyon* (Bradi2g60705 and Bradi2g60720) defined a 0.22-cM interval flanking *cul4* (Fig. 3C). To verify gene content and identify the flanking genes within the corresponding barley genomic region, a sequenced BAC clone (HVVMRXALLeA0131P08; 22× coverage assembled using 454 reads) was identified as matching the cosegregating BTB-ankyrin gene and the ortholog of the proximal *B. distachyon* gene Bradi2g60705 (Fig. 3B). Physical and genetic mapping yielded new flanking markers 0.02 cM distal to and 0.07 cM proximal from *cul4*, confirming the genetic position of the locus within this BAC clone (Fig. 3B). Two other predicted genes annotated from this BAC (encoding a penta-tricopeptide repeat-containing protein and a hypothetical protein, respectively) showed recombination with the *cul4* locus, confirming the correspondence between the BTB-ankyrin candidate gene and the *cul4* mutant locus.

Cul4 Encodes a BTB-Ankyrin Protein Related to Arabidopsis BOP1 and BOP2

The *Cul4* candidate gene has two exons and one intron, as shown by comparison of genomic and full-length complementary DNA (cDNA) sequences isolated from cv Morex seedlings (and consistent with the published full-length cDNA sequences AK360734.1 and AK355716.1). The *Cul4* gene extends 2,632 bp from start to stop codon, with an open reading frame of 1,542 bp encoding a 513-amino acid protein of approximately 54 kD containing a BTB/POX VIRUS AND ZINC FINGER (POZ) domain and ankyrin repeats (Fig. 3C). Sequence comparison of the *cul4.5* mutant allele with the cv Bonus background revealed a 3,141-bp deletion spanning most of exon 1 and the 5′ upstream region. The *cul4.5* mutant allele showed no expression in reverse transcription (RT)-PCR using a forward primer designed on the exon junction (downstream of the deletion site) and a reverse primer on exon 2 (Fig. 3D). To gain further support for the correspondence between the candidate gene and the *cul4* locus, sequences of mutant alleles *cul4.16* and *cul4.24* were also compared with those from the cv Bonus and cv Flare backgrounds, respectively. One nonsynonymous substitution was uncovered in the *cul4.16* allele changing Leu-354 to Gln in the ankyrin repeat region; allele *cul4.24* carries a nonconservative substitution of Leu-420 to Gln in a region conserved across highly related genes previously characterized in Arabidopsis, pea (*Pisum sativum*), *Medicago truncatula*, and tobacco (*Nicotiana tabacum*; see below) as well as the substitution of Met-441 to Thr (Fig. 3C). The two amino acid substitutions L354Q and L420Q in *cul4.16* and *cul4.24*, respectively, are located in highly conserved regions among barley and other known genes in dicots (Fig. 3E) and are predicted to have a deleterious impact on the biological function of the protein (SIFT

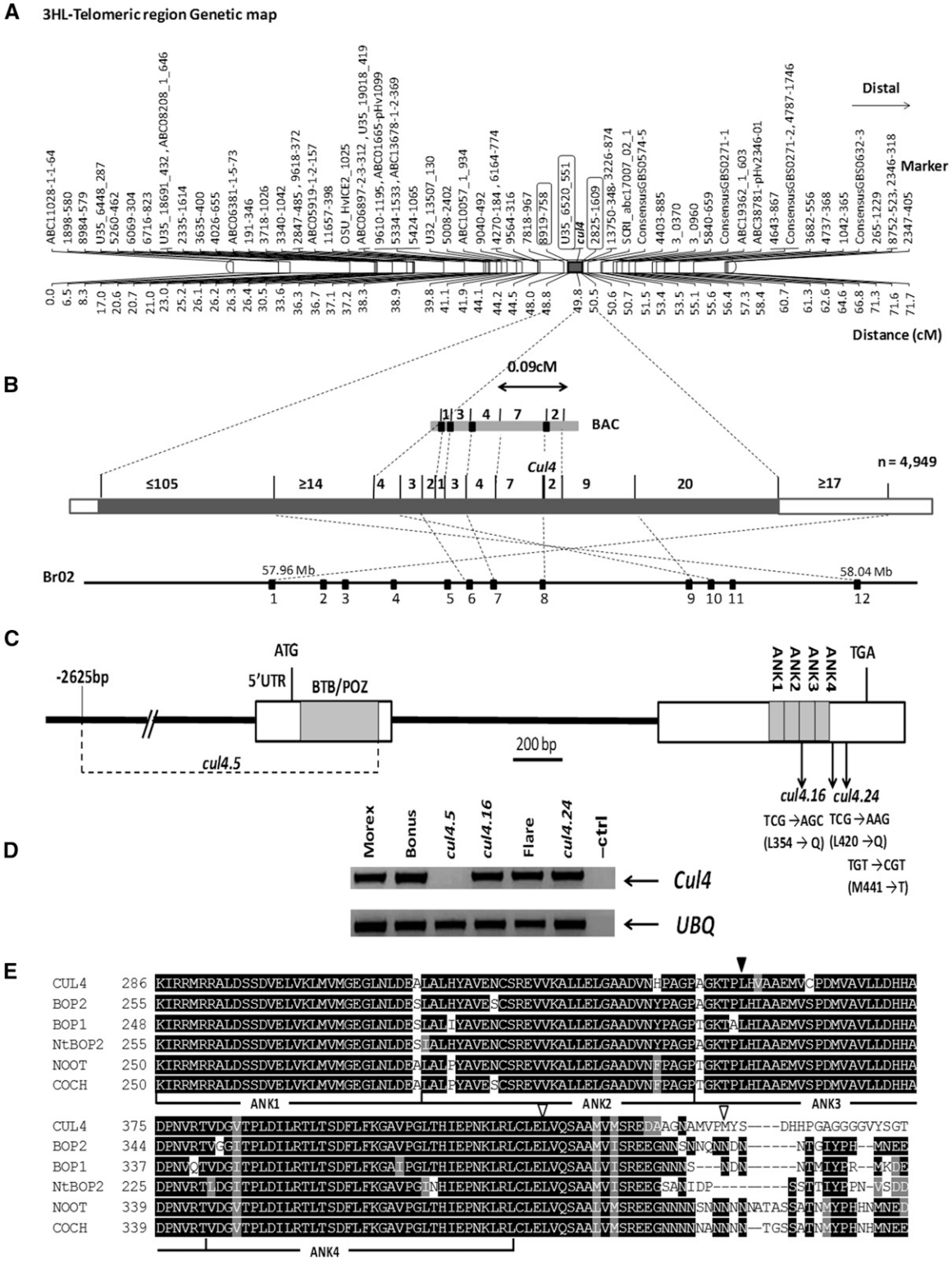

Figure 3. Map-based cloning, structure, and molecular characterization of the *Cul4* gene. A, Integrated map generated from an analysis of 386 F2 plants from crosses of the *cul4.5* mutant allele with six wild-type cultivars (Supplemental Table S2) using 61 polymorphic markers identified in the distal region of chromosome 3HL. Three tightly linked markers, including one cosegregating marker, are boxed. B, High-resolution linkage map of the *Cul4* region produced with 4,949 F3 plants from the cv Bowman-*cul4.5* × cv Morex cross derived from 72 F2 plants heterozygous in a small interval around *Cul4* (black bar in A). The number of recombinants between adjacent markers is indicated above the linkage map. Details of these markers can be found

program; $P \leq 0.01$). Notably, ankyrin repeats are known to mediate interactions between the BTB-ankyrin protein NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1 (NPR1) and TGACG sequence-specific binding (TGA) transcription factors to regulate defense responses in Arabidopsis (Zhang et al., 1999; Després et al., 2000, 2003; Zhou et al., 2000), highlighting the functional relevance of these motifs.

Recovery of distinct mutations in three independent *cul4* alleles indicates that this gene is responsible for the *cul4* phenotype. In addition, we observed that the severity of the *cul4* mutations was consistent with the reduction in tiller numbers (Fig. 1B), while all mutant alleles displayed similar leaf phenotypes (Supplemental Figs. S2 and S3).

Similarity searches show that the *Cul4* gene encodes a BTB-ankyrin domain protein sharing high similarity with Arabidopsis *BOP1* and *BOP2*, pea *COCHLEATA* (*COCH*), as well as numerous as yet uncharacterized monocot genes (Couzigou et al., 2012), including a paralogous gene in barley (MLOC_61451.6). In a recent survey in a range of species, most plant genomes were found to harbor two or three *BOP* genes (Khan et al., 2014). Phylogenetic analyses (Supplemental Fig. S4) reveal that the *BOP1* and *BOP2* genes of Arabidopsis, which mutually share 80% identity at the amino acid level, as well as the soybean (*Glycine max*) Glyma03g28440 and Glyma19g31180 genes (82% amino acid identity), both derive from independent, recent lineage-specific gene duplications within the dicots. This is consistent with the observed high degree of redundancy of Arabidopsis *BOP* genes at both the functional and expression pattern levels (Norberg et al., 2005; Ha et al., 2007; Xu et al., 2010). By way of contrast, *Cul4* and MLOC_61451.6 share 58% amino acid identity and, as for *BOP* paralogs in other monocot species, fall into distinct, highly supported clades deriving from a more ancient duplication. These observations are consistent with both the divergent gene expression patterns observed between barley *BOP* paralogs (see below) and the fact that single *cul4* mutated alleles present phenotypic defects in barley. It is unclear whether the inferred gene duplication occurred within monocots after their divergence from dicots, whereby both *Cul4* and MLOC_61451.6 should be considered as inparalogs of Arabidopsis *BOP1/2*, or before the monocot/dicot divergence, implying a loss of one paralog in an ancestor of sampled dicots and orthology of either MLOC_61451.6 or *Cul4* with *BOP1/2*. However, the available data exclude the individual orthology of pairs of barley and Arabidopsis genes.

The best characterized members of this family are Arabidopsis BOP1 and BOP2, which act as complexes with transcription factors (Hepworth et al., 2005) to control leaf development and floral organ determination. Arabidopsis *bop1 bop2* double mutants are characterized by the ectopic outgrowth of blade tissue on the petiole (Ha et al., 2003, 2004, 2007; Hepworth et al., 2005). Morphological alterations of the stipules located at the base of the leaf were also observed in loss-of-function mutants of *BOP* orthologs in pea and *M. truncatula* (Couzigou et al., 2012). Phenotypic defects in dicot *bop* mutants and *cul4* (Fig. 1, E–G) indicate that the corresponding genes are required for the correct morphogenesis of the proximal region of the leaf. This suggests at least a partial conservation of *BOP* gene function in leaf development of barley and eudicots. In Arabidopsis, *BOP1* and *BOP2* have highly redundant functions and near-identical expression patterns (Norberg et al., 2005; Ha et al., 2007; Xu et al., 2010). In contrast, publicly available barley RNA sequencing data show that, while expression patterns partially overlap, *Cul4* has significantly higher expression in the embryo of germinating grains (where axillary buds are present) and MLOC_61451.6 exhibits highest expression in the developing inflorescence (Supplemental Fig. S5). In agreement with phylogenetic analyses, these results are consistent with functional divergence between *Cul4* and its barley paralog.

Cul4 Expression Is Associated with Axillary Bud and Ligule Formation

The expression of *Cul4* in wild-type plants was further analyzed by quantitative RT-PCR and RNA in situ hybridization. Consistent with a role in tiller and leaf development, *Cul4* transcripts were detected in 3-d-old seedlings and highly expressed in the crown at the first leaf stage, when axillary buds and leaf primordia develop (Fig. 4A). Transcript accumulation was also

Figure 3. (*Continued.*)
in Supplemental Table S3. At the top, the light-gray bar indicates the bacterial artificial chromosome (BAC) clone HVVMRXALLeA0131P08, and the positions of predicted genes are indicated as black boxes. At the bottom, *B. distachyon* genes are indicated as black boxes, and anchored genes are connected with dashed lines to the barley genetic map. *B. distachyon* genes are annotated as follows: 1, Bradi2g60650; 2, Bradi2g60660; 3, Bradi2g60670; 4, Bradi2g60680; 5, Bradi2g60690; 6, Bradi2g60700; 7, Bradi2g60705; 8, Bradi2g60710; 9, Bradi2g60720; 10, Bradi2g60730; 11, Bradi2g60740; and 12, Bradi2g60750. C, Exon-intron structure of the *Cul4* gene. Two exons are represented as boxes, with the BTB/POZ domain and ankyrin repeats (ANK) as gray boxes, and the intervening intron is represented as a black line. Mutant alleles of *Cul4* show a deletion in *cul4.5* and radical amino acid substitutions in *cul4.16* and *cul4.24* compared with their progenitor backgrounds. D, RT-PCR analysis of *Cul4* transcripts in mutant alleles and their corresponding wild-type backgrounds using the primers reported in Supplemental Table S3 (34 PCR cycles). *UBIQUITIN* (*UBQ*) was used as an internal control (25 cycles). E, Alignment of predicted amino acid sequences at the ankyrin repeats region of CUL4 with Arabidopsis BOP2 (AT2G41370) and BOP1 (AT3G57130), tobacco NtBOP2 (EF051131), *M. truncatula* NODULE ROOT (JN180858), and pea COCH (JN180860). Ankyrin repeats are indicated by black lines (Wu et al., 2012). The positions of amino acid substitutions in *cul4.16* and *cul4.24* are represented by black and white arrowheads, respectively.

detected in leaves at the first leaf stage, while it was lower in roots (Fig. 4A). At the four-leaf stage, *Cul4* was strongly expressed in the ligular region of the fully expanded leaf, while it was less expressed in the leaf blade. RNA in situ hybridization showed *Cul4* expression in the leaf axil preceding AXM development. *Cul4* signal was also observed as a distinct clear pattern in the developing axillary bud, followed by a more diffuse pattern in the more mature bud (Fig. 4B). In addition, *Cul4* transcripts were detected in the leaf axil derived from an axillary bud (Fig. 4C). Examination of a cross section of the shoot apex shows that *Cul4* is expressed in a crescent of cells on the stem side of the leaf axil (Fig. 4D). Expression was also evident in developing ligules of two successive leaf primordia (Fig. 4B; Supplemental Fig. S6). No signal was detected in the *cul4.5* deletion mutant (Fig. 4E). These data indicate that the *Cul4* gene is specifically expressed at the leaf axil and at the blade-sheath boundary to guide the development of the axillary bud and the ligule, respectively.

DISCUSSION

In this study, we have shown that *Cul4* is required for tiller development and leaf patterning in barley. Multiple lines of evidence provide conclusive proof that *Cul4* encodes a BTB-ankyrin protein highly related to Arabidopsis BOP1 and BOP2 (Norberg et al., 2005), including (1) cosegregation with the phenotype in 9,898 gametes; (2) physical mapping and recombination with adjacent genes identified within the BAC clone spanning the *Cul4* locus; (3) identification of three independent mutant alleles confirming that *Cul4* mutations account for recessive *cul4* phenotypes of different severity; and (4) gene expression in the boundary regions at the leaf axil and ligule coincident with the alterations in morphology in *cul4* mutants. To our knowledge, *Cul4* is the first *BOP* gene functionally characterized in monocots.

Our results revealed the involvement of the *Cul4* gene in the control of tiller development, ligule formation, and proximal-distal leaf patterning. The related Arabidopsis genes *BOP1* and *BOP2* function redundantly to regulate the growth and development of lateral organs and are expressed at the leaf/meristem boundary governing leaf proximal-distal and adaxial-abaxial patterning (Ha et al., 2003, 2004; Hepworth et al., 2005; Norberg et al., 2005; Barton, 2010; Jun et al., 2010; Xu et al., 2010; Khan et al., 2012). In addition, Arabidopsis *bop1 bop2* double mutants showed partial reduction in the number of rosette paraclades (Khan et al., 2012), although this phenotype was not well characterized. Here, we observed that *cul4* mutants develop fewer axillary buds than the wild type, although a single axil can sometimes produce two to three axillary buds. Compared with the wild type, the

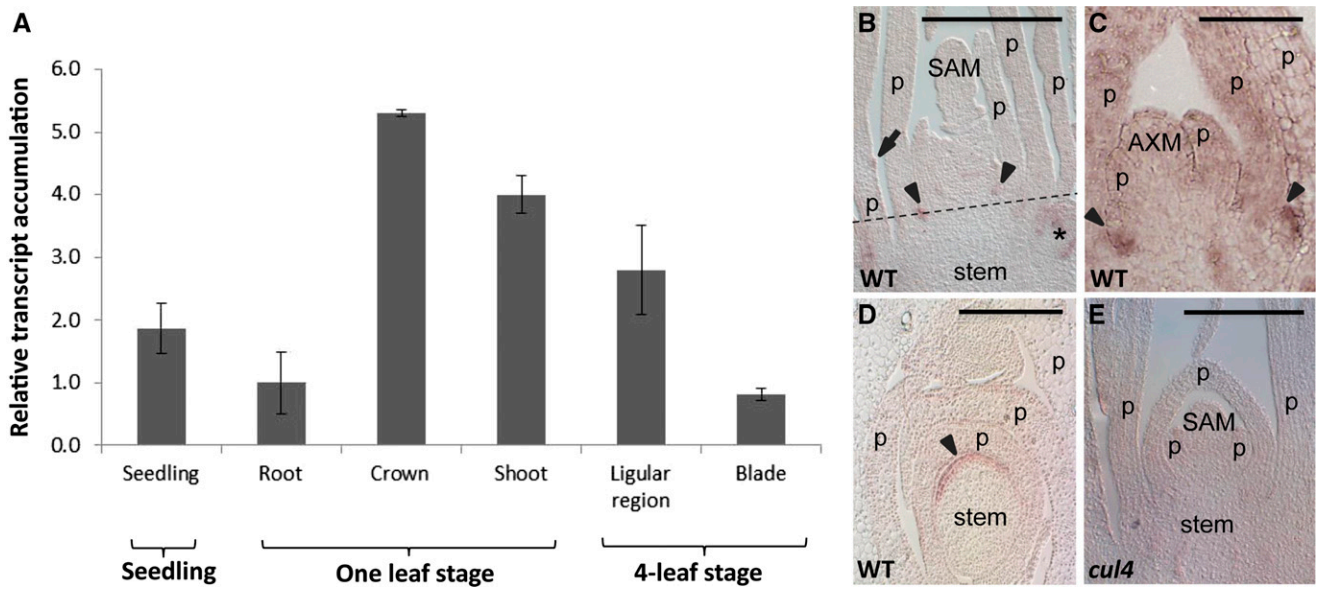

Figure 4. *Cul4* expression in cv Bonus wild-type plants using quantitative RT-PCR (A) and in situ RNA hybridization in the shoot apical region of cv Bonus (B–D) and the *cul4.5* mutant (E). A, Quantitative RT-PCR was performed using specific primers for *cul4* (Supplemental Table S3) on total RNA isolated from seedling (3-d-old seedling, when the first leaf was just emerging through the coleoptile); root, crown, and leaves at the one-leaf stage; and the 1-cm ligular region and the distal half of the blade from the third leaf at the four-leaf stage. The glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene was used to normalize the data. The data shown are averages of three biological replicates ± SD. B, Longitudinal section of the shoot apical region exhibiting *Cul4* hybridization in the leaf axils (arrowheads), axillary bud (asterisk), and ligule of a leaf primordium (arrow). C, Longitudinal section of an axillary bud; expression in leaf axils (arrowheads) is indicated. D, Cross section of the shoot apical region; *Cul4* hybridization on the stem side of the axil (arrowhead) is indicated. The sectioning plane corresponds to the dashed line in B. E, Shoot apical region in the *cul4.5* mutant showing no *Cul4* expression. p, Leaf primordium; SAM, shoot apical meristem; WT, wild type. Bars = 250 μm (B and E) and 125 μm (C and D).

overall reduction in *cul4* tiller numbers is linked to the formation of fewer active axils (i.e. axils that initiate axillary buds), the lack of secondary buds, and the formation of leafy side shoots instead of normal tillers. These results indicate that *Cul4* controls the number of leaf axils that develop an axillary bud and the number of axillary buds that develop in a single axil. The location of *Cul4* expression at the leaf axil preceding axillary bud development indicates that *Cul4* is involved in defining a boundary between an existing developmental axis (i.e. the main culm) and a new axis of lateral growth. In addition, *Cul4* function is required for tiller outgrowth, as some buds formed in *cul4* mutants developed into leafy side shoots rather than elongated lateral culms. Together, these results show that *Cul4* acts to control tiller development at multiple levels.

Cul4 is also responsible for proper proximal sheath cell fate and the location of distal auricle cells, as shown by the development of ectopic auricle-like tissue on the sheath margins in *cul4* mutants: this is reminiscent of the laminar outgrowths that form on petiolar edges of Arabidopsis *bop1 bop2* double mutants (Hepworth et al., 2005; Norberg et al., 2005). Although the homology of the leaf sheath in grasses, and its relationship to the petiole of eudicot plants, are controversial (Arber, 1918; Kaplan, 1973), it is intriguing that these ectopic outgrowths occur following the loss of function of homologous genes in both barley and Arabidopsis. In pea, loss-of-function mutations in the *BOP*-like *COCH* gene result in the reduction and absence of stipules in basal leaves (Couzigou et al., 2012), suggesting a conserved role of *BOP*-like genes in proximal-distal leaf patterning. In addition, in *cul4* mutants, the boundary between the sheath and blade is preserved, but the ligule does not develop, indicating that *Cul4* is required for outgrowth of the ligule but does not play a role in demarcating the separation between blade and sheath. Gene expression in the developing ligule is consistent with a specific role for *Cul4* in the differentiation of this structure. Other genes required for ligule development have been identified from genetic analyses in maize (*Zea mays*; Bolduc et al., 2012). Among them, *LIGULELESS2* (*LG2*) encodes a TGA basic Leu zipper transcription factor (Walsh et al., 1998), which was proposed to link proximal-distal leaf patterning signals and the induction of ligule development (Bolduc et al., 2012). Interestingly, BOP and NPR1 proteins have been shown to bind TGA transcription factors to regulate different processes (Khan et al., 2014). In particular, BOP1 and BOP2 interact with the TGA factor PERIANTHIA to pattern Arabidopsis floral meristems (Hepworth et al., 2005), suggesting the possibility that CUL4 controls leaf patterning in barley through interaction with yet unknown TGA factors possibly related to maize LG2.

Taken together, *cul4* mutants exhibit a range of phenotypes including proximal-distal leaf patterning defects, lack of ligule outgrowth, and defective tiller development that, combined with the expression patterns in the developing ligule and leaf axil, suggest that *Cul4* functions to define developmental boundaries. Comparison with Arabidopsis and pea suggests that *BOP* genes share conserved functions in the patterning of monocot and dicot leaves. In addition, *Cul4* plays a major role in the control of tillering in barley through the regulation of AXM formation and outgrowth.

In contrast to the largely redundant activities of BOP1 and BOP2 in Arabidopsis, phenotypic defects of *cul4* single mutants and phylogenetic analysis indicate that *Cul4* plays a specific and distinct function from its barley paralog MLOC_61451.6. While functional characterization of MLOC 61451.6 will tell if some level of redundancy with *Cul4* is maintained, expression data show that *Cul4* is more active in germinating embryos where axillary buds are developing, while the paralogous gene is highly expressed in developing inflorescences and might play a function in reproductive development, similar to the role of Arabidopsis BOPs in the specification and patterning of inflorescence architecture (for review, see Khan et al., 2014).

In conclusion, identification of the *Cul4* gene opens new opportunities for the genetic dissection and manipulation of shoot branching in Triticeae species. As tillers contribute directly to grain yield, competition with weeds, and plant plasticity in response to environmental conditions and stress, such knowledge can be applied in breeding for more adaptable and productive crops.

MATERIALS AND METHODS

Plant Materials

The *cul4.5* allele was derived from x-ray mutagenesis of barley (*Hordeum vulgare* 'Bonus'), and the *cul4.16* and *cul4.24* alleles were derived from fast-neutron mutagenesis of cv Bonus and cv Flare, respectively (Supplemental Table S1). The *cul4.5* mutant allele was backcrossed five times into cv Bowman, a two-rowed spring feed barley (Franckowiak et al., 1985), to obtain the *cul4.5* near-isogenic line (cv Bowman-*cul4.5*). Detailed information about *cul4* mutant stocks, the corresponding wild-type backgrounds, and the segregating populations used in this work can be found in Supplemental Tables S1 and S2.

Morphological Analysis

For quantitative phenotyping of mutant stocks *cul4.5*, *cul4.16*, and *cul4.24* and their corresponding wild-type backgrounds, cv Bonus and cv Flare, single plants were grown in 1.5-L pots in a greenhouse in a completely randomized design with nine replicates under natural photoperiod and temperature in Lodi, Italy, from December 2011 to June 2012. Phenotyping data were analyzed in SAS version 9.1.3.

Histological analysis of the shoot apex from 10-d-old seedlings of cv Bowman and the cv Bowman-*cul4.5* mutant were performed to examine tiller development. Tissue from six to 10 plants was fixed, passed through an ethanol dehydration series, and embedded in paraffin wax. Ten-micrometer-thick longitudinal and transverse sections through the apical meristem region were obtained and stained with Toluidine Blue following the protocols described by Ruzin (1999).

For scanning electron microscopy, a minimum of five shoot apices were dissected from seedlings of cv Bowman and cv Bowman-*cul4.5*. Tissue samples were attached to aluminum stubs using double-sided carbon tape and/or carbon paint and immediately frozen in liquid nitrogen. Frozen tissue samples were viewed on a cold stage of the scanning electron microscope, and images were taken at 1.8 to 2.3 kV (Ahlstrand, 1996).

To further examine the effects of *cul4* mutations on tiller development, the numbers of leaf axils harboring axillary buds, tillers, or leafy side shoots were recorded in seven to nine seedlings of the cv Bowman-*cul4.5*, *cul4.16*, and *cul4.24* mutant stocks and the corresponding wild-type backgrounds, cv Bowman, cv Bonus, and cv Flare, respectively, from 2 to 5 weeks after planting (Fig. 2J; Supplemental Fig. S1).

Linkage Mapping and Positional Cloning

To identify SNPs tightly linked to the *cul4* locus and select the most appropriate cross for high-resolution mapping, initially, 386 F2 plants from six segregating populations (Supplemental Table S2) were genotyped using the Illumina Goldengate assay (Fan et al., 2003). Starting from eight SNPs identified previously as linked to *cul4* by comparison of the cv Bowman-*cul4.5* and recurrent parent cv Bowman (data not shown), a total of 96 EST-derived SNP markers covering the interval 120.6 to 173.2 cM in the 3HL telomeric region (Close et al., 2009; Barley HarvEST database; http://harvest.ucr.edu/) were examined. An integrated genetic linkage map was constructed from the six initial mapping populations using JoinMap 4.1 (Stam, 1993).

A population of 4,949 F3 plants from the cv Bowman-*cul4.5* × cv Morex cross was generated by selfing 72 F2 plants heterozygous for the *cul4* region; KASPar genotyping (KBioscience) with SNPs 8919-758 and 2825-1609, which flank *cul4*, identified 174 recombinants. Phenotyping was conducted in the same conditions as above from December 2009 to June 2012. Plants exhibiting defective tillering and liguleless phenotype were classified as homozygous *cul4*. Wild-type F3 individuals harboring recombination events in the vicinity of the locus were propagated, and F4 progeny were phenotyped for discrimination of homozygous *Cul4* and heterozygotes. The identified recombinants were then genotyped with SNP U35_6520_551 (cosegregating with *cul4* in the initial 266 F2 plants of the cv Bowman-*cul4.5* × cv Morex mapping population).

To refine the location of the recombination events nearest to *cul4* and evaluate colinearity with *Brachypodium distachyon* chromosome 2 and rice (*Oryza sativa*) chromosome 1 genomic regions, markers were developed on the basis of revised genome zipper information (Mayer et al., 2011). Gene-based markers were developed exploring EST information and the available assembly of barley genomic reads (Mayer et al., 2012): Specific primers were designed (Supplemental Table S3), and genomic PCR amplicons were sequenced in parents cv Morex and cv Bowman-*cul4.5* using the Sanger method at the Genomics Platform, Parco Tecnologico Padano. The resulting polymorphic markers were mapped using the same method mainly on 55 recombinants between the *cul4* flanking markers U34_6520_551 and 2825-1609 (selected from 4,949 F3 plants); in the case of markers that were not mapped within this region, additional F3 plants were genotyped, allowing us to determine their positions and better resolve colinearity with reference genomes.

Candidate genes were considered based on annotated genes conserved among the three reference genomes of *B. distachyon*, rice, and sorghum (*Sorghum bicolor*) and current knowledge of genes involved in shoot development. Candidate genes were amplified from genomic DNA using the primers listed in Supplemental Table S3, and genomic sequences were compared in cv Bowman-*cul4.5* and cv Morex. Identified polymorphisms were used to map them as described for newly developed markers.

A BAC contig (FPcontig_460) of the barley physical map (Schulte et al., 2011) was identified by sequence homology search with *cul4* flanking markers to barley genomic sequence information. The BAC clone HVVMRXALLeA0131P08 was sequenced using Roche/454 Genome Sequencer FLX technology and assembled after the removal of short sequences, adapter and vector trimming, and assembly using a previously described procedure (Steuernagel et al., 2009). The Triannot pipeline gene prediction program (Leroy et al., 2012; http://urgi.versailles.inra.fr/Species/Wheat/Triannot-Pipeline) was used to annotate potential genes. Genomic markers were developed using the same method as above or insertion site-base polymorphism markers (Paux et al., 2010) using the primers listed in Supplemental Table S3.

For allelic comparisons, genomic PCR and resequencing of the *Cul4* gene were carried out in the three available allelic mutant stocks *cul4*.5 (GenBank accession no. KF151193), *cul4.16* (KF151195), and *cul4.24* (KF151196) and the backgrounds cv Bonus (KF151192) and cv Flare (KF151194) using the primers described in Supplemental Table S3. The amino acid substitution's impact on protein function was evaluated using http://sift.bii.a-star.edu.sg/www/SIFT_seq_submit2.html (Ng and Henikoff, 2001).

RNA Extraction, Expression Analysis, and Quantitative RT-PCR

Relative *Cul4* expression was measured in 3-d-old seedlings when the first leaf was just emerging through the coleoptile (GRO:0007059; www.gramene.org); root, crown, and leaves at the one-leaf stage (GRO:0007060); and the 1-cm ligular region and the distal half of the blade from the third leaf at the four-leaf stage (GRO:0007063). Total RNA was isolated using TRI-Reagent (Sigma-Aldrich) and treated with RNase-free DNase I (Invitrogen) according to the manufacturers' instructions. The concentration of RNA was determined using Agilent Bioanalyser 2100 (Agilent Technologies). First strand cDNA was synthesized from 1.5 μg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen). After RT, cDNA samples were diluted 4-fold, and 2 μL was used for further analysis. Quantitative analyses were carried out with three biological and technical replications on the 7300 Real-time PCR System (Applied Biosystems) using the primers reported in Supplemental Table S3 and SYBR Green Master Mix according to the manufacturer's instructions. Normalization was carried out using the *GAPDH* (accession no. EF409629) and *UBQ* (Osnato et al., 2010) genes and the $\Delta\Delta C_T$ method where $\Delta\Delta C_T = (C_{T,Cul4} - C_{T,GAPDH})_{tissue2} - (C_{T,Cul4} - C_{T,GAPDH})_{tissue1}$ (Livak and Schmittgen, 2001).

For expression analyses of *Cul4* in various tissues/stages, three biological replications were carried out, with each replication including samples from five cv Bonus plants grown in a growth chamber under a 16-h-light/8-h-dark photoperiod with day/night temperatures of 20°C/17°C, respectively.

For comparison of the expression of *Cul4* and MLOC_61451.6, publicly available RNA sequencing data (Mayer et al., 2012) were queried at the MorexGenes Barley RNA-seq database (http://ics.hutton.ac.uk/morexGenes/index.html) to obtain fragments per kilobase of exon per million fragments mapped data for three biological replicates.

RNA in Situ Hybridization

Two gene-specific fragments of 306 and 362 bp from the 5′ untranslated region and the 3′ end of the *Cul4* cDNA were PCR amplified with the primers shown in Supplemental Table S3. Samples from 14-d-old seedlings were fixed, processed, sectioned, and hybridized to both probes as described (Juarez et al., 2004).

Cul4 gene sequences reported in this article have been deposited in the National Center for Biotechnology Information database with accession numbers KF151192, KF151193, KF151194, KF151195, and KF151196. Sequences for BAC HVVMRXALLeA0131P08 have been deposited at the National Center for Biotechnology (accession no. AC256289; assembled contigs) and at the European Bioinformatics Institute's European Nucleotide Archive (accession no. PRJEB4166; raw data).

Supplemental Data

The following supplemental materials are available.

Supplemental Figure S1. Formation of double/multiple axillary buds and side shoots in *cul4* mutants.

Supplemental Figure S2. Liguleless phenotype of *cul4* mutants.

Supplemental Figure S3. Ectopic auricle tissue formation in *cul4* mutants.

Supplemental Figure S4. Evolutionary relationships of CUL4 with members of the NOOT/BOP/COCH like (Couzigou et al., 2012) clade of plant BTB-ankyrin proteins.

Supplemental Figure S5. Expression of *Cul4* and the barley paralogous gene MLOC_61451.6.

Supplemental Figure S6. RNA in situ hybridization analysis of *Cul4* expression in developing ligules.

Supplemental Table S1. Genetic materials.

Supplemental Table S2. Initial F2 mapping populations and corresponding *cul4* flanking SNP markers.

Supplemental Table S3. List of primers.

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Supplemental Materials

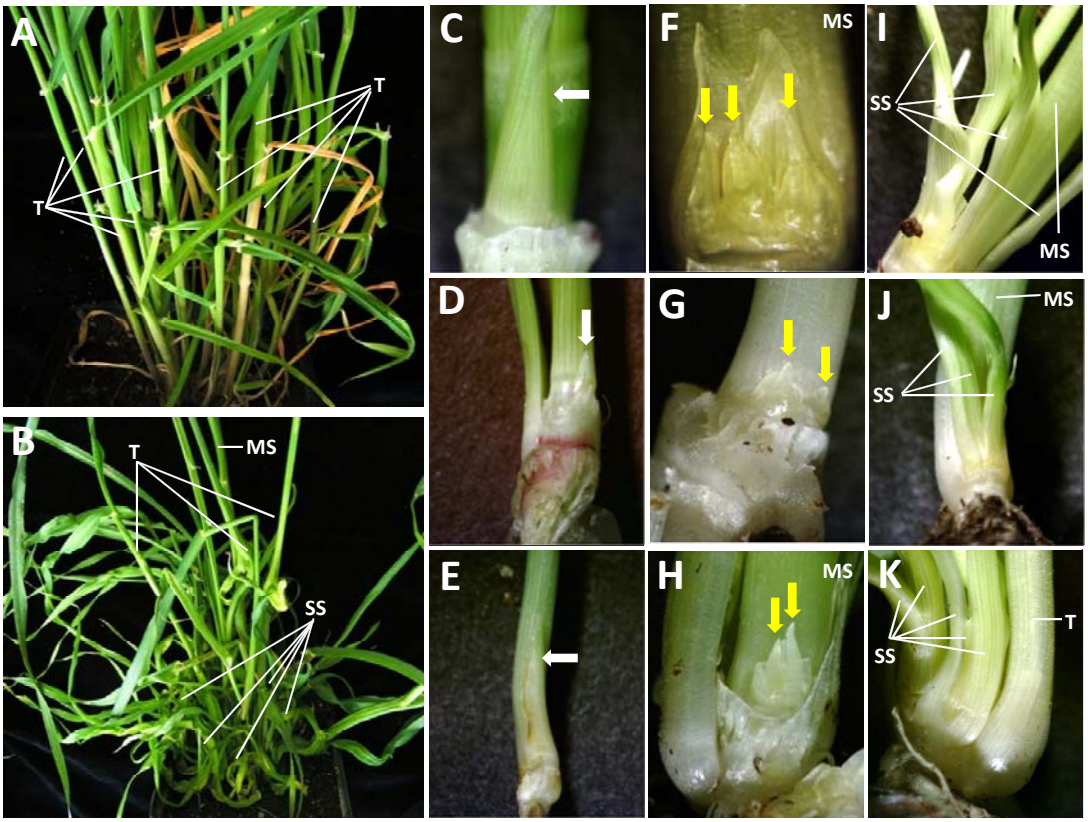


Fig. S1. Formation of double/multiple axillary buds and side shoots in *cul4* mutants. (A) Tillers (T) in wild-type plant. (B) Main stem (MS) and leafy side shoots (SS) in *cul4.16* mutant plant. Single axillary buds in wild-type: (C) Bowman 3-week old plant, (D) Bonus 5-week old plant, (E) Flare 3-week old plant. Double/multiple axillary buds in: (F) Bowman-*cul4.5* 2-week old plant, (G) *cul4.16* and (H) *cul4.24* 3-week old plants. Leaves and tillers were removed to expose axillary buds. Multiple leafy side-shoots (SS) arising from a leaf axil in (I) Bowman-*cul4.5* (J) *cul4.16* and (K) *cul4.24* 3-week old plants. Single and double/multiple axillary buds indicated by white and yellow arrows, respectively.

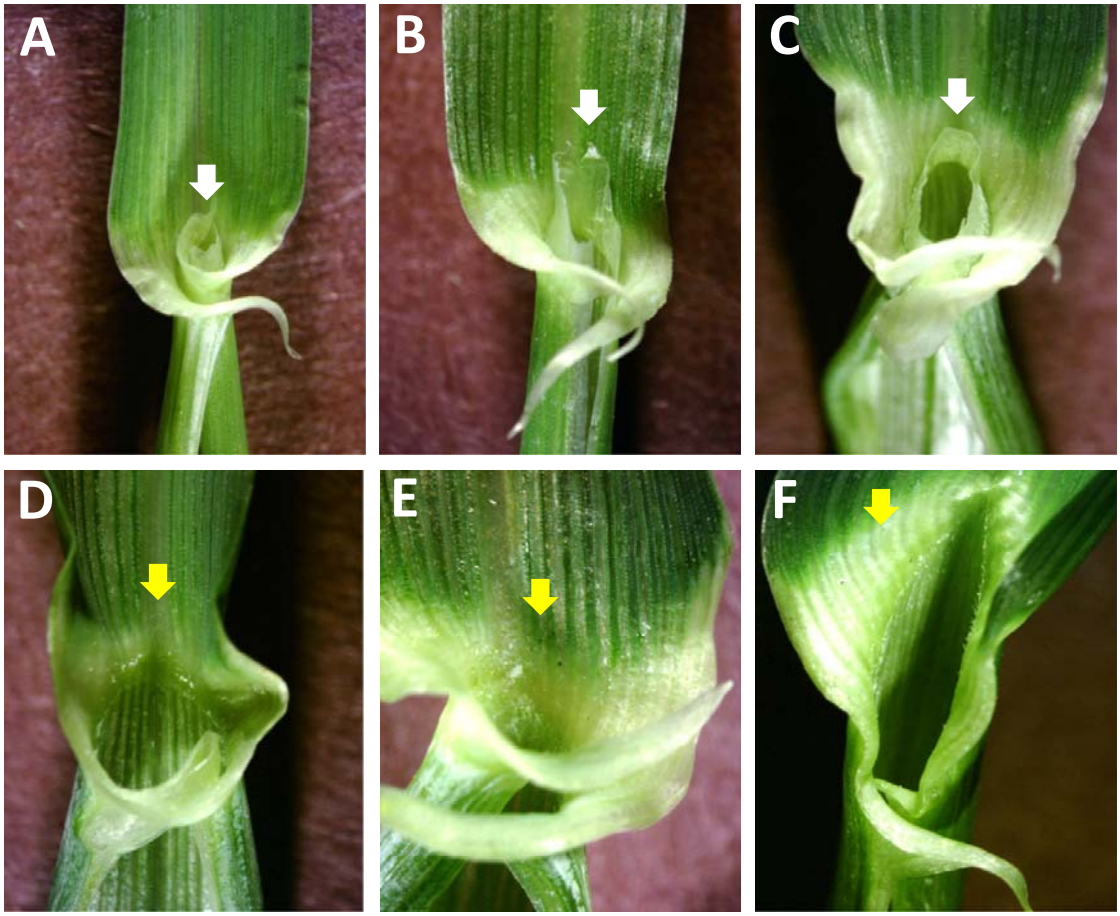
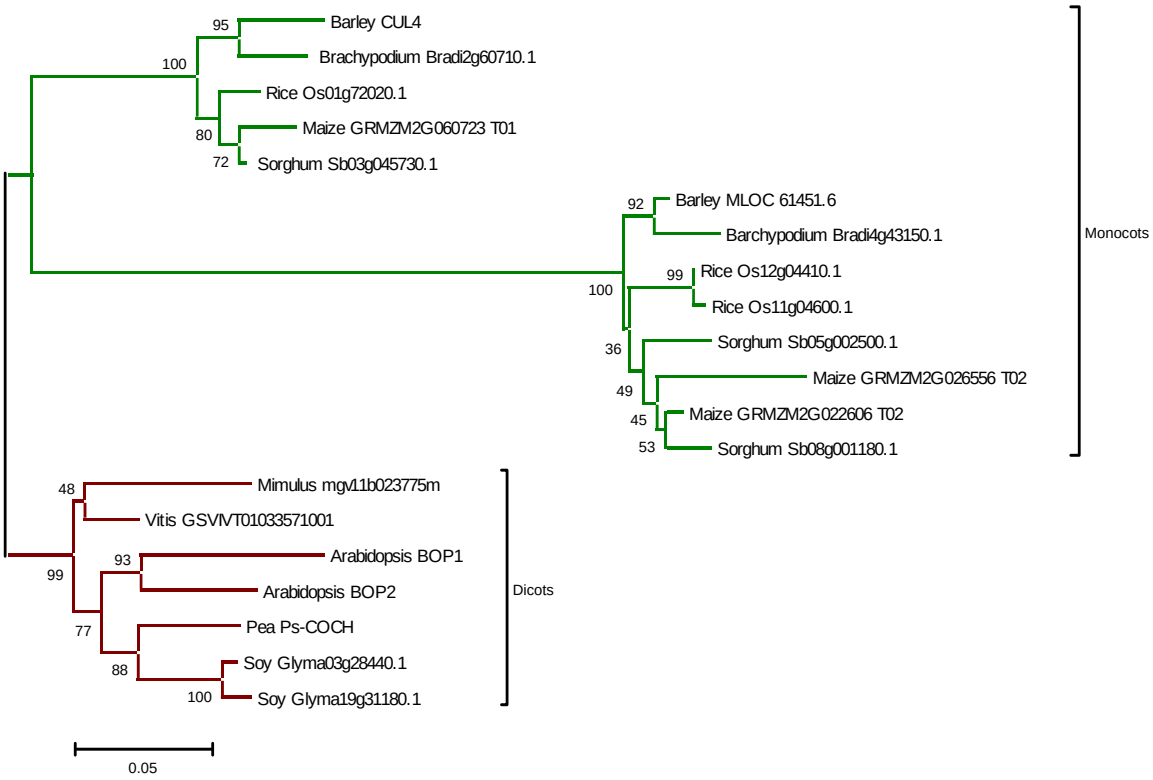


Fig. S2. Liguleless phenotype of *cul4* mutants. Ligules are indicated by white arrows in (A) Bowman, (B) Bonus and (C) Flare. Ligules are absent at the sheath-blade boundary (indicated by yellow arrows) in (D) Bowman-*cul4.5,* (E) *cul4.16* and (F) *cul4.24*.

Fig. S3. Ectopic auricle tissue formation in *cul4* mutants. Auricles differentiate specifically at the sheath-blade boundary in wild type (A) Bowman (B) Bonus and (C) Flare plants. Ectopic flaps of auricle tissue form on leaf sheath margins in (D and close up in E) Bowman-*cul4.5,* (F and close ups in G, H) *cul4.16,* and (I and close up in J) *cul4.24* plants.



Barley (*Hordeum vulgare*) CUL4 = KF151192
Pea (*Pisum sativum*) Ps-COCH = JN180860 (Couzigou et al., 2012)
Arabidopsis thaliana BOP1 = AT3G57130
Arabidopsis thaliana BOP2 = AT2G41370

Fig. S4. Evolutionary relationships of CUL4 with members of the NBCL (11) clade of plant BTB/ANK proteins. The CUL4 peptide sequence (513 amino acids) was used as query in BLASTP 2.2.22+ (Altschul et al., 1990) of Phytozome v9.1 (http://www.phytozome.net/) to recover similar sequences from *Arabidopsis thaliana, Brachypodium distachyon, Glycine max, Mimulus guttatus, Oryza sativa, Sorghum bicolor, Vitis vinifera, Zea mays*. *Pisum sativum* sequence JN180860 was retrieved from GenBank (Couzigou et al., 2012). Only one splicing form was considered based on highest EST support for each gene. The ScanProsite tool (De Castro et al., 2006) was used to scan each polypeptide (Sigrist et al., 2012) to ensure the presence of BTB and ankyrin domains. Alignment of protein sequences was carried out by CLUSTALW 2.0 (Larkin et al., 2007) in MEGA5 using Gonnet Protein Weight Matrix and manually refined by deleting ambiguous positions - final alignment was based on 440 unambiguously aligned positions (with gaps). Phylogenetic reconstruction was obtained using the Neighbour-Joining method (Saitou and Nei, 1987) in MEGA5. The optimal Neighbour-Joining tree with the sum of branch length = 0.94651914 is shown. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) are shown next to the branches ~~(Felsenstein, 1985)~~. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Evolutionary distances were computed using the Poisson correction method (Tamura et al., 2011) and are in the units of the number of amino acid substitutions per site.

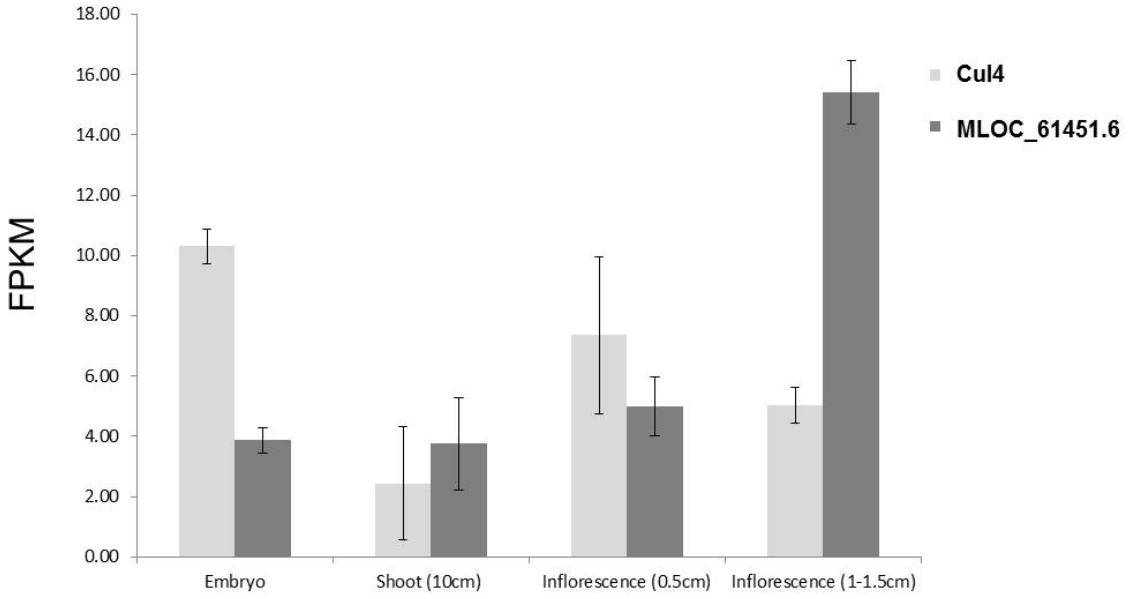


Fig. S5. Expression of cul4 and the barley paralogous gene MLOC_61451.6. Gene expression levels from RNA-seq data obtained from http://ics.hutton.ac.uk/morexGenes (IBSC, 2012). Tissues from left to right: 4-day old embryo dissected from germinating grain, seedling shoot (10 cm shoot stage), young developing inflorescence (0.5 cm), developing inflorescence (1-1.5 cm). Transcript levels are given in the form of fragment per kilobase of exon per million fragment mapped (FPKM). Bars represent standard error of three biological replicates.

Fig. S6. Expression of *Cul4* in developing ligules. *In situ* RNA hybridization on a longitudinal section of the shoot apical region from a 14-day-old wild-type (Bonus) plant showing *Cul4* expression in the developing ligules of two successive leaf primordia (arrows).

Table S1. Genetic materials. Accession numbers from the NordGen collection are indicated where applicable (http://sesto.nordgen.org/sesto/).

Genotype/ allele name	Accession number	Background	Mutagen and author
cul4.5	NGB115063	Bonus	An X-ray induced mutant isolated by U. Lundqvist
cul4.16	NGB115065	Bonus	An neutrons induced mutant isolated by U. Lundqvist
cul4.5		Bowman	Introgression line produced by 5 backcrosses of original *cul4.5* allele to Bowman recurrent parent
cul4.24	NGB119360	Flare	An neutrons induced mutant isolated by U. Lundqvist
Wild-type	NGB14657	Bonus	
Wild-type	NGB1518	Flare	
Wild-type	NGB20079	Bowman	
Wild-type	NGB23015	Morex	
Wild-type		Harrington	
Wild-type		Steptoe	
Wild-type		Nudinka	
Wild-type	NGB4663	Proctor	

Table S2. Initial F2 mapping populations and corresponding *cul4* flanking SNP markers. The total numbers of SNPs segregating in the selected region of 3HL is indicated for each cross. The cross Bowman-*cul4.5* x Morex showed markers tightly linked to the *cul4* locus and a high number of SNPs.

Allele	Genetic Back-ground	Cross	Population size	No. of Polymorphic markers	Proximal Marker(s)**	Distal Marker(s)**	Co-segregating Marker(s)**
*cul4.5**	Bowman	*cul4.5** x Harrington	24	7		3682_556 (166.2cM)	
	Bowman	*cul4.5** x Bowman	24	11	4643_867 (167.8cM)	3682_556 (166.2cM)	
	Bowman	*cul4.5** x Morex	266	37	8919_758 (151.2cM)	2825_1609 (154.3cM)	U35_6520_551 (152cM)
	Bowman	*cul4.5** x Steptoe	24	32	5008_2402 (141.5cM)	SCRI_abc17007_02_1 (155.9cM)	U35_6520_551 (152cM)
cul4.5	Bonus	*cul4.5* x Nudinka	24	11		4787_1746 (162.2cM)	
	Bonus	*cul4.5* x Proctor	24	8	9564_316 (145.9cM)	4403_885 (162.2cM)	

*Bowman-*cul4.5*

**Position in Close et al. (2009) map, as a reference map, indicated in parentheses.

Table S3. List of primers.

Primers used for the fine mapping reported in Fig. 3B*			
Marker name **	**Brachypodium orthologue/primers name**	**Forward primer**	**Reverse primer**
ET_umil0101	Bradi2g60750	ATGATCCACCAGGACGAAGG	CAGTTGAGCAGGAGGTGGAT
ET_umil0102	Bradi2g60730	AAGTTTGGACAGCCGAAGAA	TCCTGGATGCAAGTGACTGA
ET_umil0103	Bradi2g60700	GCAGCTCCTCAGATGCTCTC	TGTGTTTGCAGTAGCTGAAGG
ET_umil0104	Hypotetical protein	AGCTGCAGTGTCGTCTTCAG	CGCAAAGGGCTTTTATCTTG
ET_umil0105	PPR-like protein	TGTCGTGAAAGACCAAGGTG	CCGGCAAGTCTCTTTACTCG
ET_umil0106	Bradi2g60705	AACCCTGGCGATTACTTGTG	GTACCGTACGTCGGTCTCGT
EP_umil0107	ISBP1	TTTCCTTTCTTGCCAGCCTA	ACATCACGGGCATCACATAA
ET_umil0108	Bradi2g60710	AGCATGAACCTGAGCTTGGA	TGAATGTAGAGCCTAACGAACA
EP_umil0109	ISBP2	TTTATTCCGTTTGGACTCCG	AGGAGCCCAAGAAAATCGTT
ET_umil0110	Bradi2g60720	TTTCATGGCTGTGCTTTCAG	GGCAGCCAGTAATTTCGTGT
ET_umil0111	Bradi2g60650	TTGAAGGAAGCCAAGGAGAA	CTTCTGAACGTCTGCCATTG
Primers used to amplify the *Cul4* genomic region and the full length cDNA			
	Exon1	ACGGCTTCTTCCACTCCTCT	CGATCCCAACATAACCAACC
	Exon2	CGGTCTCTCCATGCCATATT	CATTCTCGTCGACCGATCTC
	Intron	GTGCTCCAGTTCCTGTACA	GAGGACGTGATGAAGGTGCT
	5'upstream-1	TTTGAGGTTGCAATGGCTCT	ATCAAAAGAGATCGGGCGAT
	5'upstream-2	CAGTCAAAGCATGGCACACT	CGATCCCAACATAACCAACC
	5'upstream-3	CAGTGAAGTCACGGCAAGAA	CGATCCCAACATAACCAACC
	Cul4-cDNA***	ACGGCTTCTTCCACTCCTCT	CATTCTCGTCGACCGATCTC
Primers used for RT-PCR and real-time RT-PCR			
	GAPDH	GTGAGGCTGGTGCTGATTACG	TGGTGCAGCTAGCATTTGAGAC
	UBI	AGCAGAAGCACAAGCACAAG	AAGCCTGCTGGTTGTAGACG
	Real-time RT_*cul4*****	CATGTACAGCGACCACCATC	TCACGTCCATCCCTAGGTTC
	RT_*cul4*****	CCCTCCTCACCCAGAAGCAG	AAGCTCAGGTTCATGCTCGT
Primers used for *Cul4 in situ*			
	in situ_5UTR	GGGGAGAGAAGAAGAAGTGGT	CACCAGCTTCTCGCTAATCC
	in situ_3end	GGTCATGTCCAGGGAGGAC	CATTCTCGTCGACCGATCTC

* Primers order here follows the proximal-distal order of markers in Fig. 3B.

** Markers names submitted to European TriticeaeGenome website: www.triticeaegenome.eu.

*** Used to amplify full length cDNA of *Cul4*

**** Forward and reverse primers were designed on the *Cul4* exons junction and on exon2, respectively and used for analysis of *Cul4* expression ~~both for~~in RT-PCR ~~and quantitative RT-PCR~~.

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