Plant Physiology Preview. Published on March 27, 2015, as DOI:10.1104/pp.114.252882

The barley *Uniculme4* gene encodes a BLADE-ON-PETIOLE-like protein that controls tillering and leaf patterning

Elahe Tavakol[1], Ron Okagaki[2], Gabriele Verderio[1], Vahid Shariati J.[3,4], Ahmed Hussien[1], Hatice Bilgic[2], Mike J. Scanlon[5], Natalie R. Todt[5], Tim J. Close[6], Arnis Druka[7], Robbie Waugh[7], Burkhard Steuernagel[8], Ruvini Ariyadasa[8], Axel Himmelbach[8], Nils Stein[8], Gary J. Muehlbauer[2,9], and Laura Rossini[1,3*]

[*] Corresponding author: laura.rossini@unimi.it

[1] Università degli Studi di Milano, DISAA, Via Celoria 2, 20133 Milan, Italy; [2] Department of Agronomy and Plant Genetics, University of Minnesota, St. Paul, MN USA; [3] Parco Tecnologico Padano, Via Einstein, 26900 Lodi, Italy; [4] Present address: National Institute of Genetic Engineering and Biotechnology, 14965/161,Tehran, Iran. [5] Department of Plant Biology, Cornell University, USA; [6] Department of Botany and Plant Sciences, University of California, Riverside, California, USA; [7] The James Hutton Institute, Invergowrie, Dundee DD2 5DA, Scotland UK; [8] Leibniz Institute of Plant Genetics and Crop Plant Research (IPK), OT Gatersleben, Corrensstraße 3, 06466 Stadt Seeland, Germany; [9] Department of Plant Biology, University of Minnesota, St. MN USA.

Keywords: shoot branching, developmental boundary, BTB-ankyrin, *Hordeum vulgare*

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 it encodes a BTB-ankyrin protein closely related to Arabidopsis 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 development of the ligule, which separates the distal blade and proximal sheath of the leaf. As the first functionally characterized *BOP* gene in monocots, *Cul4* suggests 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, 2012). 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) grow 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 determine 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 orthologous GRAS genes *LATERAL SUPPRESSOR* (*LAS*) in Arabidopsis, *LATERAL SUPPRESSOR* (*LS*) in tomato and *MONOCULM1* (*MOC1*) in rice (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, 2, 3* and *BLIND* genes of Arabidopsis and tomato (Schmitz et al., 2002; Keller et al., 2006; Muller et al., 2006) are conserved in eudicots but have not been identified in monocot genomes (Keller et al., 2006; Muller et al., 2006).

A number of barley 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; Dabbert et al., 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 *Cul4* gene result in reduced tillering (Babb and Muehlbauer, 2003). In the current 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 homologue of the Arabidopsis *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. 1A, B) (Dahleen et al., 2007). The cultivars Bonus and Flare typically produced an average of 8.8±1 and 11.9±1.5 tillers, respectively, on fully mature glasshouse grown plants. In contrast, *cul4* mutants showed tillering defects of varying severity with *cul4.5* and *cul4.16* (both in Bonus background) and *cul4.24* (in Flare background) developing an average of 0.4±1, 6.3±1.7 and 4.6±2 tillers, respectively (Fig. 1B). In contrast to 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 (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 (Bowman-*cul4.5*) and its recurrent parent Bowman. At ten days after planting, shoot apices from Bowman-*cul4.5* and Bowman were very similar (Fig. 2A-B), except that fewer leaf axils had developed buds in the mutants compared to wild-type plants. Axillary buds were typically observed in two or three leaf axils in wild-type plants (Fig. 2C-D), whereas in the *cul4.5* mutant only one to two leaf axils contained axillary buds (Fig. 2E). In contrast to wild-type, transverse sections through the Bowman-*cul4.5* mutant shoot apex revealed two axillary buds in the same leaf axil (compare Fig. 2F and G). Scanning electron microscopy clearly revealed a single axillary bud developing in leaf axils from Bowman seedlings (Fig. 2H), while occasionally leaf axils developing two axillary buds were observed in Bowman-*cul4.5* seedlings (Fig. 2I). To better dissect the effect of *Cul4* on axillary development, we compared the number of active leaf axils (harbouring 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 to 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 four in *cul4* mutants and no secondary buds were observed. In addition, most *cul4* axillary buds turned into leafy side-shoots rather than developed tillers (Fig. S1). In agreement with previous histological analyses, formation of two or multiple axillary buds from a single axil was observed in some *cul4* plants (Fig. S1). Leafy side shoots were associated with such multiple axillary buds (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 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 off-set from the 2) 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. 1C, 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. 1D, F, and 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. 1J, K, and 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 Bowman-*cul4.5* x Morex located *Cul4* to a 0.55 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*, sorghum 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 *Brachypodium,* rice and sorghum. Partial genomic sequences were obtained exploiting Expressed Sequence Tag (EST) information and available barley genomic reads (Feuillet et al., 2012; The International Barley Genome Sequencing Consortium, 2012) and mapped using Single Nucleotide Polymorphisms (SNPs) identified between Bowman-*cul4.5* and Morex. A GRAS candidate gene (highly related to Bradi2g60750) was mapped ≥0.38 cM from the *cul4* locus and was excluded from further analysis. The second candidate gene, encoding a BTB (Broad-complex, Tramtrack, Bric-à-brac)-ankyrin protein (highly related to Bradi2g60710), showed co-segregation with *cul4* in all recombinants identified in the target interval. The two flanking genes in *Brachypodium* (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 Bacterial Artificial Chromosome (BAC) clone (HVVMRXALLeA0131P08, 22x coverage assembled using 454 reads) was identified as matching the co-segregating BTB-ankyrin gene and the ortholog of the proximal Brachypodium gene Bradi2g60705 (Fig. 3B). Physical and genetic mapping yielded new flanking markers 0.02 cM distal 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 pentatricopeptide repeat (PPR-like)-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 cDNA sequences isolated from Morex seedlings (and consistent with 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 (ORF) of 1,542

bp encoding a 513 amino acid protein of ~54 kDa containing a BTB/POZ (for Broad-complex, Tramtrack, Bric-à-brac/POx virus and Zinc finger) domain and ankyrin repeats (Fig. 3C). Sequence comparison of the *cul4.5* mutant allele with the Bonus background revealed a 3,141 bp deletion spanning most of exon 1 and 5' upstream region. The *cul4.5* mutant allele showed no expression in Reverse Transcription (RT)-PCR using a primer designed on the exons junction (downstream of the deletion site) with a primer on exon 2 (Fig. 3D; table S3). 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 Bonus and Flare backgrounds, respectively. One non-synonymous substitution was uncovered in the *cul4.16* allele changing leucine 354 to glutamine in the ankyrin repeat region; allele *cul4.24* carries a non-conservative substitution of leucine 420 to glutamine in a region conserved across highly related genes previously characterized in Arabidopsis, pea, *Medicago truncatula* and tobacco (see below), as well as the substitution of methionine 441 to threonine (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 program, $P \leq 0.01$). Notably, ankyrin repeats are known to mediate interactions between BTB-ankyrin protein NPR1 (Non-expressor of Pathogenesis-Related genes 1) and TGA (TGACG-sequence specific binding) transcription factors to regulate defense responses in Arabidopsis (Zhang et al., 1999; Després et al., 2000; Zhou et al., 2000; Després et al., 2003), highlighting the functional relevance of these motifs.

Recovery of distinct mutations in three independent *cul4* alleles indicates this gene is responsible for the *cul4* phenotype. In addition, we observed that severity of the *cul4* mutations was consistent with the reduction in tiller numbers (Fig. 1B), while all mutant alleles displayed similar leaf phenotypes (Fig. 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 (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 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 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% aminoacid identity and, as for *BOP* paralogs in other monocot species, fall in 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 loss of one paralog in an ancestor of sampled dicots, and orthology of one of MLOC_61451.6 or *Cul4* with *BOP1/2*. However, available data exclude individual orthology of pairs of barley and Arabidopsis genes.

The best characterized members of this gene 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 *bop1bop2* double mutants are characterized by ectopic outgrowth of blade tissue on the petiole (Ha et al., 2003; Ha et al., 2004; Hepworth et al., 2005; Ha et al., 2007). 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 *Medicago truncatula* (Couzigou et al., 2012). Phenotypic defects in dicot *bop* mutants and *cul4* (Fig. 1E-G) indicate that the

corresponding genes are required for 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-seq data show that, whileexpression 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 (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**

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-day 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 detected in leaves at the first leaf stage, while it was lower in roots (Fig. 4A). At the 4-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* hybridizations 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 and 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 development of the axillary bud and the ligule, respectively.

DISCUSSION

In the current 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. *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. Related genes Arabidopsis *BOP1* and *BOP2* function redundantly to regulate 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; Ha et al., 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 wild-type, although a single axil can sometimes produce two axillary buds. Compared to wild-type, the 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 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, is controversial (Arber, 1918; Kaplan, 1973), it is intriguing to note 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 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 (Bolduc et al., 2012). Among them, *LIGULELESS2* (*LG2*) encodes a TGA basic leucine zipper (bZIP) transcription factor (Walsh et al., 1998), which was proposed to link proximal-distal leaf patterning signals and 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 TGA factor

PERIANTHIA (PAN) 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 tiller development that - combined with the expression patterns in the developing ligule and leaf axil- suggest *Cul4* functions to define developmental boundaries. Comparison with Arabidopsis and pea suggests that *BOP* genes share conserved functions in 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 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 specification and patterning of inflorescence architecture (reviewed in 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 the cultivar Bonus and the *cul4.16* and *cul4.24* alleles were derived from fast-neutron mutagenesis of Bonus and Flare, respectively (Table S1). The *cul4.5* mutant allele was backcrossed five times into Bowman, a two-rowed spring feed barley (Franckowiak et al., 1985), to obtain the *cul4.5* near-isogenic line (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 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, Bonus and Flare, single plants were grown in 1.5 L pots in a glasshouse in a completely randomized design with nine replicates, under natural photoperiod and temperature in Lodi (Italy) during December 2011 to June 2012. Phenotyping data were analysed in SAS ver. 9.1.3.

Histological analysis of the shoot apex from ten-day old seedlings of Bowman and the Bowman-*cul4.5* mutant were performed to examine tiller development. Tissue from six to ten 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 in Ruzin (Ruzin, 1999).

For scanning electron microscopy (SEM), a minimum of five shoot apices were dissected from seedlings of Bowman and 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 in the scanning electron microscope and images were taken at 1.8 to 2.3 kV (Ahlstrand, 1996).

To further examine effects of *cul4* mutations on tiller development, the numbers of leaf axils harboring axillary buds, tillers or leafy side shoots were recorded in 7-9 seedlings of the Bowman-*cul4.5*, *cul4.16* and *cul4.24* mutant stocks, and the corresponding wild-type backgrounds Bowman, Bonus and Flare, respectively, from 2 to 5 weeks after planting (Fig. 2J, 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 6 segregating populations (Table S2) were genotyped using the Illumina Goldengate assay (Fan et al., 2003): starting from 8 SNPs previously identified as linked to *cul4* by comparison of the Bowman-*cul4.5* and recurrent parent 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 F3s from the Bowman-*cul4.5* x Morex cross was generated by selfing 72 F2 plants heterozygous for the *cul4* region: KASPar genotyping (KBioscience, UK) with SNPs 8919-758 and 2825-1609, which flank *cul4*, identified 174 recombinants. Phenotyping was conducted in the same conditions as above during 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 progenies were phenotyped for discrimination of homozygous *Cul4* and heterozygotes. The identified recombinants were then genotyped with SNP U35_6520_551 (co-segregating with *cul4* in the initial 266 F2s of Bowman-*cul4.5* x Morex mapping population).

To refine the location of the recombination events nearest to *cul4* and evaluate colinearity with Brachypodium chromosome 2 and rice 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 (The International Barley Genome Sequencing Consortium, 2012): specific primers were designed (Table S3) and genomic PCR amplicons were sequenced in parents Morex and Bowman-*cul4.5* using the Sanger method at the Genomics Platform, Parco Tecnologico Padano (Lodi, Italy). 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 F3s were genotyped allowing us to determine their position and better resolve colinearity with reference genomes.

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

A Bacterial Artificial Chromosome (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. A BAC clone HVVMRXALLeA0131P08 was sequenced using Roche/454 Genome Sequencer FLX (GS FLX) technology and assembled after removal of short sequences, adapter and vector trimming and assembly using a previously described procedure (Steuernagel et al., 2009). The Triannot pipeline gene prediction programme (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 (ISBP) markers (Paux et al., 2010) using primers listed in Table S3.

For allelic comparisons, genomic PCR and resequencing of the *Cul4* gene was carried out in the three available allelic mutant stocks *cul4.*5 (GenBank: KF151193), *cul4.16* (KF151195) and *cul4.24* (KF151196) and the respective backgrounds Bonus (KF151192) and Flare (KF151194) using primers described in table S3 and the amino acid substitutions impact on the 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-day old seedlings when first leaf just emerging through the coleoptile (GRO:0007059; www.gramene.org); root, crown and leaves, at one leaf stage (GRO:0007060); 1 cm ligular region, the distal half of the blade from the third leaf at the 4-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 complementary DNA (cDNA) was synthesized from 1.5 µg of total RNA using SuperScript III Reverse Transcriptase (Invitrogen). After reverse transcription, cDNA samples were diluted 4-fold, and 2 µl were used for further analysis. Quantitative analyses were carried out with three biological and technical replications on a 7300 Real-time PCR System (Applied Biosystem) using primers reported in Table S3 and SYBR Green Master Mix according to the manufacturer's instruction. Normalization was carried out using the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*; Acc. no. EF409629) and ubiquitin (*UBQ*) (16) genes and ΔΔct method (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 Bonus plants grown in a growth chamber under 16 h light and 8 h dark photoperiod with day/night temperatures of 20°C/17°C, respectively.

For comparison of expression of *Cul4* and MLOC_61451.6, publicly available RNAseq data (The International Barley Genome Sequencing Consortium, 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 (FPKM) data for three biological replicates.

RNA *in situ* hybridization

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

Supplemental Materials

Supplemental Materials and methods

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

Fig. S2. Liguleless phenotype of *cul4* mutants.

Fig. S3. Ectopic auricle tissue formation in *cul4* mutants.

Fig. S4. Evolutionary relationships of CUL4 with members of the NBCL (Couzigou et al., 2012) clade of plant BTB-ankyrin proteins.

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

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

Table S1. Genetic materials.

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

Table S3. List of primers.

***Author contributions:** E.T. conducted fine mapping and positional cloning, gene characterization, expression and bioinformatics analysis and wrote the manuscript; R.O. conducted detailed analysis of axillary development in mutants and wild-types; G.V. helped with fine mapping; V.Sh.J. helped with bioinformatics analysis; A.H. performed

phylogenetic analysis; H.B. conducted the histology and SEM; M.J.S., N.R.T. conducted in situ expression analyses; T.J.C., A.D., R.W. provided SNP markers for mapping prior to publication; B.S., R.A., A.R.H., N.S. provided the BAC contig and sequence information; G.J.M. conceived and designed microscopy analyses, edited the manuscript, and shares senior authorship with L.R.; L.R. conceived and designed positional cloning, validation and characterization of the gene, wrote the manuscript and shares senior authorship with G.J.M.

ACKNOWLEDGEMENTS

The research leading to these results has received funding from the European Community's Seventh Framework Programme (FP7/ 2007-2013) under grant agreement n°FP7-212019, TriticeaeGenome project to LR and NS, the United States Department of Agriculture-CSREES-NRI Plant Growth and Development Program grant #2004-03440 to GJM and the GABI-FUTURE program of the German Ministry of Education and Research (BMBF grant Barlex-0314000A) to NS. We thank F. Salamini and C. Pozzi for providing *cul4* crosses with Proctor and Nudinka, E. Paux for help in designing ISBP markers, K. Smith for providing field space, T. Fusca for helping in development of initial mapping populations, B. Bucciarelli and G. Celio for assistance with histology and for SEM.

† **Data deposition:** The sequences reported in this paper have been deposited in NCBI database (Accession nos. KF151192, KF151193, KF151194, KF151195 and KF151196).

LITERATURE CITED

Agusti J, Greb T (2012) Going with the wind - Adaptive dynamics of plant secondary meristems. Mech Dev **130:** 34-44

Ahlstrand GG (1996) Low-temperature low-voltage scanning microscopy (LTLVSEM) of uncoated frozen biological materials: a simple alternative. . *In* G Bailey, J Corbett, R Dimlich, J Michael, N Zaluzec, eds, Proceedings of Microscopy Microanalysis. San Francisco Press, San Francisco, pp 918–919

Arber A (1918) The phyllode theory of the monocotyledonous leaf, with special reference to anatomical evidence. Ann. Bot. os. **32:** 465–501

Babb S, Muehlbauer GJ (2003) Genetic and morphological characterization of the barley *uniculm2 (cul2)* mutant. Theor Appl Genet **106:** 846-857

Barton MK (2010) Twenty years on: the inner workings of the shoot apical meristem, a developmental dynamo. Dev Biol **341:** 95-113

Bolduc N, Connor D, Moon J, Lewis M, Hake S (2012) How to Pattern a Leaf. Cold Spring Harbor Symposia on Quantitative Biology **77:** 47-51

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao S, Varshney RK, Szucs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics **10:** 582

Couzigou JM, Zhukov V, Mondy S, Abu el Heba G, Cosson V, Ellis TH, Ambrose M, Wen J, Tadege M, Tikhonovich I, Mysore KS, Putterill J, Hofer J, Borisov AY, Ratet P (2012) *NODULE ROOT* and *COCHLEATA* maintain nodule development and are legume orthologs of *Arabidopsis BLADE-ON-PETIOLE* genes. Plant Cell **24:** 4498-4510

Dabbert T, Okagaki RJ, Cho S, Boddu J, Muehlbauer GJ (2009) The genetics of barley low-tillering mutants: absent lower *laterals* (*als*). Theor Appl Genet **118:** 1351-1360

Dabbert T, Okagaki RJ, Cho S, Heinen S, Boddu J, Muehlbauer GJ (2010) The genetics of barley low-tillering mutants: *low number of tillers-1* (*lnt1*). Theor Appl Genet **121:** 705-715

Dahleen L, Franckowiak J, Lundqvist U (2007) Descriptions of Barley Genetic Stocks for 2007. Barley Genetic Newsletter **37:** 188-301

Després C, Chubak C, Rochon A, Clark R, Bethune T, Desveaux D, Fobert PR (2003) The Arabidopsis NPR1 Disease Resistance Protein Is a Novel Cofactor That Confers Redox Regulation of DNA Binding Activity to the Basic Domain/Leucine Zipper Transcription Factor TGA1. The Plant Cell Online **15:** 2181-2191

Després C, DeLong C, Glaze S, Liu E, Fobert PR (2000) The Arabidopsis NPR1/NIM1 Protein Enhances the DNA Binding Activity of a Subgroup of the TGA Family of bZIP Transcription Factors. The Plant Cell Online **12:** 279-290

Donald CM (1968) The breeding of crop ideotypes. Euphytica **17:** 385-403

Druka A, Franckowiak J, Lundqvist U, Bonar N, Alexander J, Houston K, Radovic S, Shahinnia F, Vendramin V, Morgante M, Stein N, Waugh R (2011) Genetic dissection of barley morphology and development. Plant Physiol **155:** 617-627

Fan JB, Oliphant A, Shen R, Kermani BG, Garcia F, Gunderson KL, Hansen M, Steemers F, Butler SL, Deloukas P, Galver L, Hunt S, McBride C, Bibikova M, Rubano T, Chen J, Wickham E, Doucet D, Chang W, Campbell D, Zhang B, Kruglyak S, Bentley D, Haas J, Rigault P, Zhou L, Stuelpnagel J, Chee MS (2003) Highly parallel SNP genotyping. Cold Spring Harb Symp Quant Biol **68:** 69-78

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. . Evolution **39:** 783-791

Feuillet C, Stein N, Rossini L, Praud S, Mayer K, Schulman A, Eversole K, Appels R (2012) Integrating cereal genomics to support innovation in the Triticeae. Funct Integr Genomics **12:** 573-583

Franckowiak JD, Foster AE, Pederson VD, Pyler RE (1985) Registration of 'Bowman' barley. Crop Sci **25:** 883

Ha CM, Jun JH, Nam HG, Fletcher JC (2004) *BLADE-ON-PETIOLE1* encodes a BTB/POZ domain protein required for leaf morphogenesis in Arabidopsis thaliana. Plant Cell Physiol **45:** 1361-1370

Ha CM, Jun JH, Nam HG, Fletcher JC (2007) *BLADE-ON-PETIOLE 1* and *2* control *Arabidopsis* lateral organ fate through regulation of LOB domain and adaxial-abaxial polarity genes. Plant Cell **19:** 1809-1825

Ha CM, Kim GT, Kim BC, Jun JH, Soh MS, Ueno Y, Machida Y, Tsukaya H, Nam HG (2003) The BLADE-ON-PETIOLE 1 gene controls leaf pattern formation through the modulation of meristematic activity in Arabidopsis. Development **130:** 161-172

Ha CM, Kim GT, Kim BC, Jun JH, Soh MS, Ueno Y, Machida Y, Tsukaya H, Nam HG (2003) The *BLADE-ON-PETIOLE 1*gene controls leaf pattern formation through the modulation of meristematic activity in *Arabidopsis*. Development **130:** 161-172

Hepworth SR, Zhang Y, McKim S, Li X, Haughn GW (2005) BLADE-ON-PETIOLE-dependent signaling controls leaf and floral patterning in Arabidopsis. Plant Cell **17:** 1434-1448

Hussien A, Tavakol E, Horner DS, Muñoz-Amatriaín M, Muehlbauer GJ, Rossini L (2014) Genetics of Tillering in Rice and Barley. Plant Gen. **7**: 1-20

International Barley Genome Sequencing Consortium (2012) A physical, genetic and functional sequence assembly of the barley genome. Nature **491:** 711-716

Janssen BJ, Drummond RS, Snowden KC (2014) Regulation of axillary shoot development. Curr Opin Plant Biol **17:** 28-35

Juarez MT, Kui JS, Thomas J, Heller BA, Timmermans MC (2004) microRNA-mediated repression of *rolled leaf1* specifies maize leaf polarity. Nature **428:** 84-88

Jun JH, Ha CM, Fletcher JC (2010) BLADE-ON-PETIOLE1 coordinates organ determinacy and axial polarity in arabidopsis by directly activating ASYMMETRIC LEAVES2. Plant Cell **22:** 62-76

Kaplan DR (1973) The problem of leaf morphology and evolution in the monocotyledons. Q Rev Biol **48:** 437–457

Kebrom TH, Spielmeyer W, Finnegan EJ (2013) Grasses provide new insights into regulation of shoot branching. Trends in Plant Science **18:** 41-48

Keller T, Abbott J, Moritz T, Doerner P (2006) *Arabidopsis REGULATOR OF AXILLARY MERISTEMS1* controls a leaf axil stem cell niche and modulates vegetative development. Plant Cell **18:** 598-611

Khan M, Xu H, Hepworth SR (2014) BLADE-ON-PETIOLE genes: Setting boundaries in development and defense. Plant Science **215-216**: 157-171

Khan M, Xu M, Murmu J, Tabb P, Liu Y, Storey K, McKim SM, Douglas CJ, Hepworth SR (2012) Antagonistic interaction of BLADE-ON-PETIOLE1 and 2 with BREVIPEDICELLUS and PENNYWISE regulates Arabidopsis inflorescence architecture. Plant Physiol **158:** 946-960

Leroy P, Guilhot N, Sakai H, Bernard A, Choulet F, Theil S, Reboux S, Amano N, Flutre T, Pelegrin C, Ohyanagi H, Seidel M, Giacomoni F, Reichstadt M, Alaux M, Gicquello E, Legeai F, Cerutti L, Numa H, Tanaka T, Mayer K, Itoh T, Quesneville H, Feuillet C (2012) TriAnnot: A Versatile and High Performance Pipeline for the Automated Annotation of Plant Genomes. Front Plant Sci **3:** 5

Li X, Qian Q, Fu Z, Wang Y, Xiong G, Zeng D, Wang X, Liu X, Teng S, Hiroshi F, Yuan M, Luo D, Han B, Li J (2003) Control of tillering in rice. Nature **422:** 618-621

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods **25:** 402-408

Mascher M, Jost M, Kuon JE, Himmelbach A, Assfalg A, Beier S, Scholz U, Graner A, Stein N (2014) Mapping-by-sequencing accelerates forward genetics in barley. Genome Biol **15:** R78

Mayer KF, Martis M, Hedley PE, Simkova H, Liu H, Morris JA, Steuernagel B, Taudien S, Roessner S, Gundlach H, Kubalakova M, Suchankova P, Murat F, Felder M, Nussbaumer T, Graner A, Salse J, Endo T, Sakai H, Tanaka T, Itoh T, Sato K, Platzer M, Matsumoto T, Scholz U, Dolezel J, Waugh R, Stein N (2011) Unlocking the barley genome by chromosomal and comparative genomics. Plant Cell **23:** 1249-1263

Mohapatra PK, Panda BB, Kariali E (2011) Plasticity of Tiller Dynamics in Wild Rice Oryza rufipogon Griff.: A Strategy for Resilience in Suboptimal Environments. . International Journal of Agronomy **2011**

Muller D, Schmitz G, Theres K (2006) *Blind homologous R2R3 Myb* genes control the pattern of lateral meristem initiation in *Arabidopsis*. Plant Cell **18:** 586-597

Ng PC, Henikoff S (2001) Predicting deleterious amino acid substitutions. Genome Res **11:** 863-874

Norberg M, Holmlund M, Nilsson O (2005) The *BLADE ON PETIOLE* genes act redundantly to control the growth and development of lateral organs. Development **132:** 2203-2213

Paux E, Faure S, Choulet F, Roger D, Gauthier V, Martinant JP, Sourdille P, Balfourier F, Le Paslier MC, Chauveau A, Cakir M, Gandon B, Feuillet C (2010) Insertion site-based polymorphism markers open new perspectives for genome saturation and marker-assisted selection in wheat. Plant Biotechnol J **8:** 196-210

Pozzi C, di Pietro D, Halas G, Roig C, Salamini F (2003) Integration of a barley (Hordeum vulgare) molecular linkage map with the position of genetic loci hosting 29 developmental mutants. Heredity (Edinb) **90:** 390-396

Ruzin SE (1999) Plant Microtechnique and Microscopy. Oxford University Press, Oxford, New York

Schmitz G, Theres K (2005) Shoot and inflorescence branching. Curr Opin Cell Biol **8:** 506-511

Schmitz G, Tillmann E, Carriero F, Fiore C, Cellini F, Theres K (2002) The tomato *Blind* gene encodes a MYB transcription factor that controls the formation of lateral meristems. Proc Natl Acad Sci U S A **99:** 1064-1069

Schulte D, Ariyadasa R, Shi B, Fleury D, Saski C, Atkins M, deJong P, Wu CC, Graner A, Langridge P, Stein N (2011) BAC library resources for map-based cloning and physical map construction in barley (*Hordeum vulgare* L.). BMC Genomics **12:** 247

Seavers GP, Wright KJ (1999) Crop canopy development and structure influence weed suppression. Weed Research **39:** 319-328

Stam P (1993) Construction of integrated genetic linkage maps by means of a new computer package: Join Map Plant J **3:** 739-744

Steuernagel B, Taudien S, Gundlach H, Seidel M, Ariyadasa R, Schulte D, Petzold A, Felder M, Graner A, Scholz U, Mayer KF, Platzer M, Stein N (2009) De novo 454 sequencing of barcoded BAC pools for comprehensive gene survey and genome analysis in the complex genome of barley. BMC Genomics **10:** 547

Sussex IM (1989) Developmental programming of the shoot meristem. Cell **56:** 225-229

Sylvester AW, Cande WZ, Freeling M (1990) Division and differentiation during normal and *liguleless-1* maize leaf development. Development **110:** 985-1000

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol **28**: 2731-2739

The International Barley Genome Sequencing Consortium (2012) A physical, genetic and functional sequence assembly of the barley genome. Nature **491:** 711-716

Waldie T, McCulloch H, Leyser O (2014) Strigolactones and the control of plant development: lessons from shoot branching. The Plant Journal **79**: 607-622

Walsh J, Waters CA, Freeling M (1998) The maize gene liguleless2 encodes a basic leucine zipper protein involved in the establishment of the leaf blade-sheath boundary. Genes Dev **12:** 208-218

Wu XM, Yu Y, Han LB, Li CL, Wang HY, Zhong NQ, Yao Y, Xia GX (2012) The tobacco *BLADE-ON-PETIOLE2* gene mediates differentiation of the corolla abscission zone by controlling longitudinal cell expansion. Plant Physiol **159:** 835-850

Xu M, Hu T, McKim SM, Murmu J, Haughn GW, Hepworth SR (2010) Arabidopsis BLADE-ON-PETIOLE1 and 2 promote floral meristem fate and determinacy in a previously undefined pathway targeting *APETALA1* and *AGAMOUS-LIKE24*. Plant J **63:** 974-989

Zhang Y, Fan W, Kinkema M, Li X, Dong X (1999) Interaction of NPR1 with basic leucine zipper protein transcription factors that bind sequences required for salicylic acid induction of the PR-1 gene. Proceedings of the National Academy of Sciences **96:** 6523-6528

Zhou J-M, Trifa Y, Silva H, Pontier D, Lam E, Shah J, Klessig DF (2000) NPR1 Differentially Interacts with Members of the TGA/OBF Family of Transcription Factors That Bind an Element of the PR-1 Gene Required for Induction by Salicylic Acid. Molecular Plant-Microbe Interactions **13:** 191-202

Figure legends

Figure 1- Shoot phenotypes of barley *cul4* mutants. (*A*) Whole plant phenotype of wild-type Bowman (left) and the Bowman-*cul4.5* mutant (right) at vegetative stage. (*B*) Comparisons between *cul4.24* and the wild-type Flare, and *cul4.16* and *cul4.5* and the wild-type Bonus for number of tillers in mature plants (n = 9). Error bars represent standard deviation. Asterisks indicate significant difference ($P \leq 0.01$) relative to the wild-type background (Student's t-test). (*C*) Wild-type Bowman blade-sheath boundary region. (*D*) 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 (SEM) of the wild-type (Bowman) blade-sheath boundary region. (*F*) SEM of the 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 SEM picture of the blade-sheath boundary. (*G*) SEM of wild-type (Bowman) auricle tissue from an equivalent position to the asterisk in panel C. (*H*) SEM of flap tissue from the Bowman-*cul4.5* mutant (from an equivalent position to the asterisk in panel K) exhibits auricle-like cells. (*I*) Wild-type (Bowman) leaf. (*J*) Bowman-*cul4.5* mutant leaf exhibiting a flap of tissue on the margin of the sheath. (*K*) Close up of flap (from insert in panel J) on sheath margin of Bowman-*cul4.5* mutant plant. Scale bars in E and F represent 1 mm, and scale bars in G and H represents 100 μm.

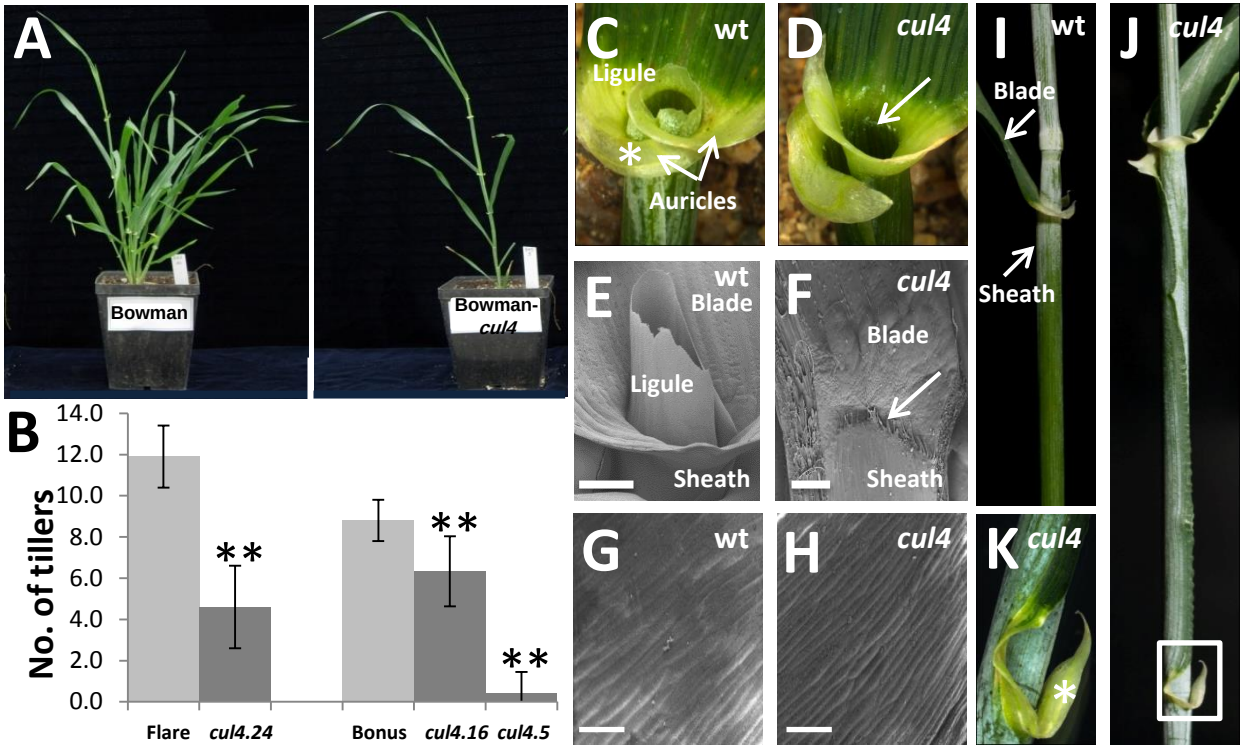
Figure 2- Axillary development in wild-type and *cul4* mutant plants. (*A*) Longitudinal section through the shoot apical meristem (SAM) of Bowman wild-type plant. (*B*) Longitudinal section through the SAM of Bowman-*cul4.5* plant. (*C* and *D*) Axillary meristems (AXM) in two successive leaf axils from the shoot apex shown in A captured in different sectioning planes. (*E*) The only AXM from the same shoot

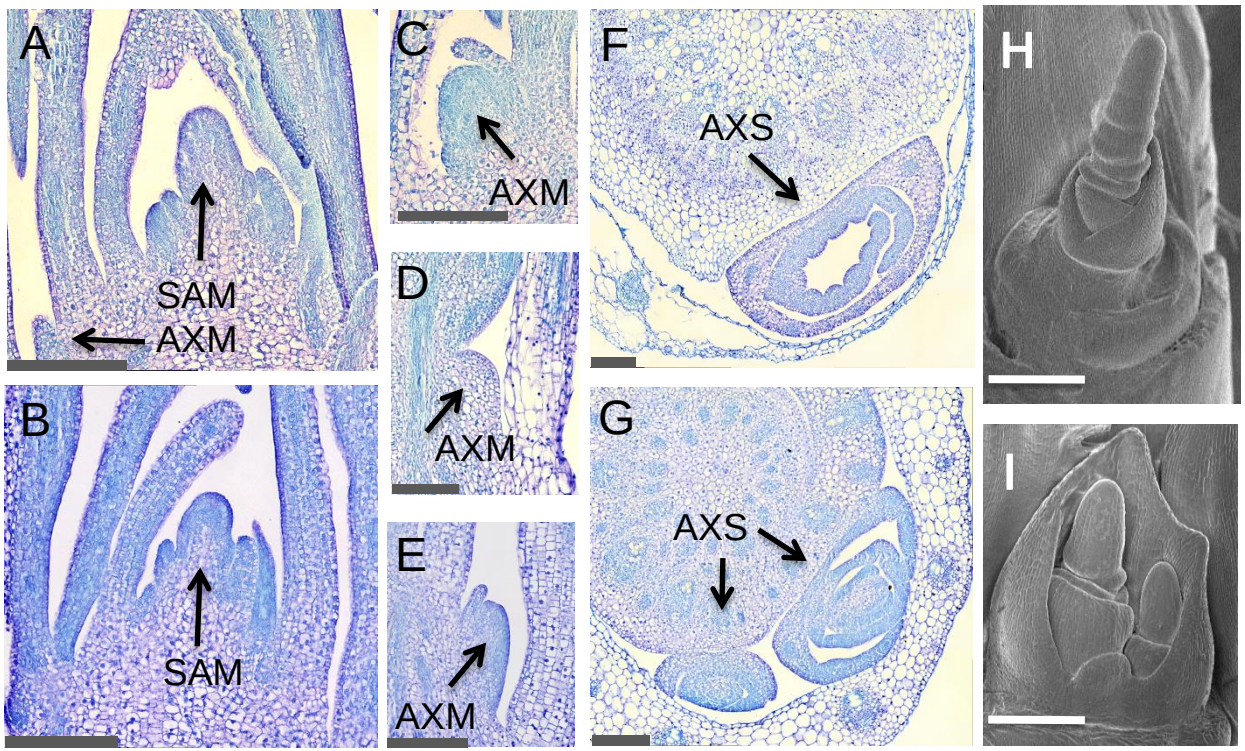
apex shown in B. (*F*) Transverse section through a single axillary shoot (AXS) in wild-type Bowman. (*G*) Transverse section through two AXSs in a single leaf axil in Bowman-*cul4.5*. Bars represent 200 µm. (*H*) Scanning electron micrograph (SEM) of an AXM in a leaf axil from a wild-type Bowman plant. (*I*) SEM of two AXMs in a leaf axil from a Bowman-*cul4.5* mutant. Bars represent 250 µm. (*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. Mean ± SE of biological replicates. WT, wild-type.

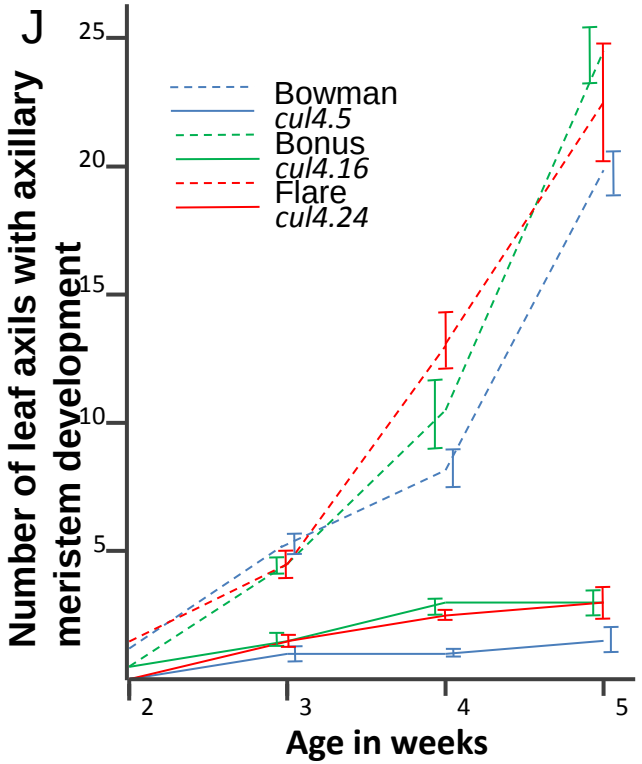
Figure 3- Map-based cloning, structure and molecular characterization of the *Cul4* gene. (*A*) Integrated map generated from analysis of 386 F2 plants from crosses of the *cul4.5* mutant allele with 6 wild-type cultivars (Table S2) using 61 polymorphic markers identified in the distal region of chromosome 3HL. Three tightly-linked markers including one co-segregating marker are boxed. (*B*) High resolution linkage map of the *Cul4* region produced with 4,949 F3 plants from the Bowman-*cul4.5* x Morex cross derived from 72 F2s heterozygous in a small interval around *Cul4* (filled bar in A). The number of recombinants between adjacent markers is indicated above the linkage map. Details of these markers can be found in Table S3. At the top, the light grey filled bar indicates the BAC clone HVVMRXALLeA0131P08 and the position of predicted genes indicated as black boxes. At the bottom, *Brachypodium* genes are indicated as black boxes and anchored genes are connected with dashed lines to the barley genetic map. *Brachypodium* genes are annotated as follow: (1) Bradi2g60650 (2) Bradi2g60660 (3) Bradi2g60670 (4) Bradi2g60680 (5) Bradi2g60690 (6) Bradi2g60700 (7) Bradi2g60705 (8) Bradi2g60710 (9) Bradi2g60720 (10) Bradi2g60730 (11) Bradi2g60740 (12) Bradi2g60750. (*C*) Exon-intron structure of the *Cul4* gene. Two exons are represented as boxes with BTB/POZ domain and Ankyrin repeats (ANK) as grey boxes and the intervening intron is represented as a black line. Mutant

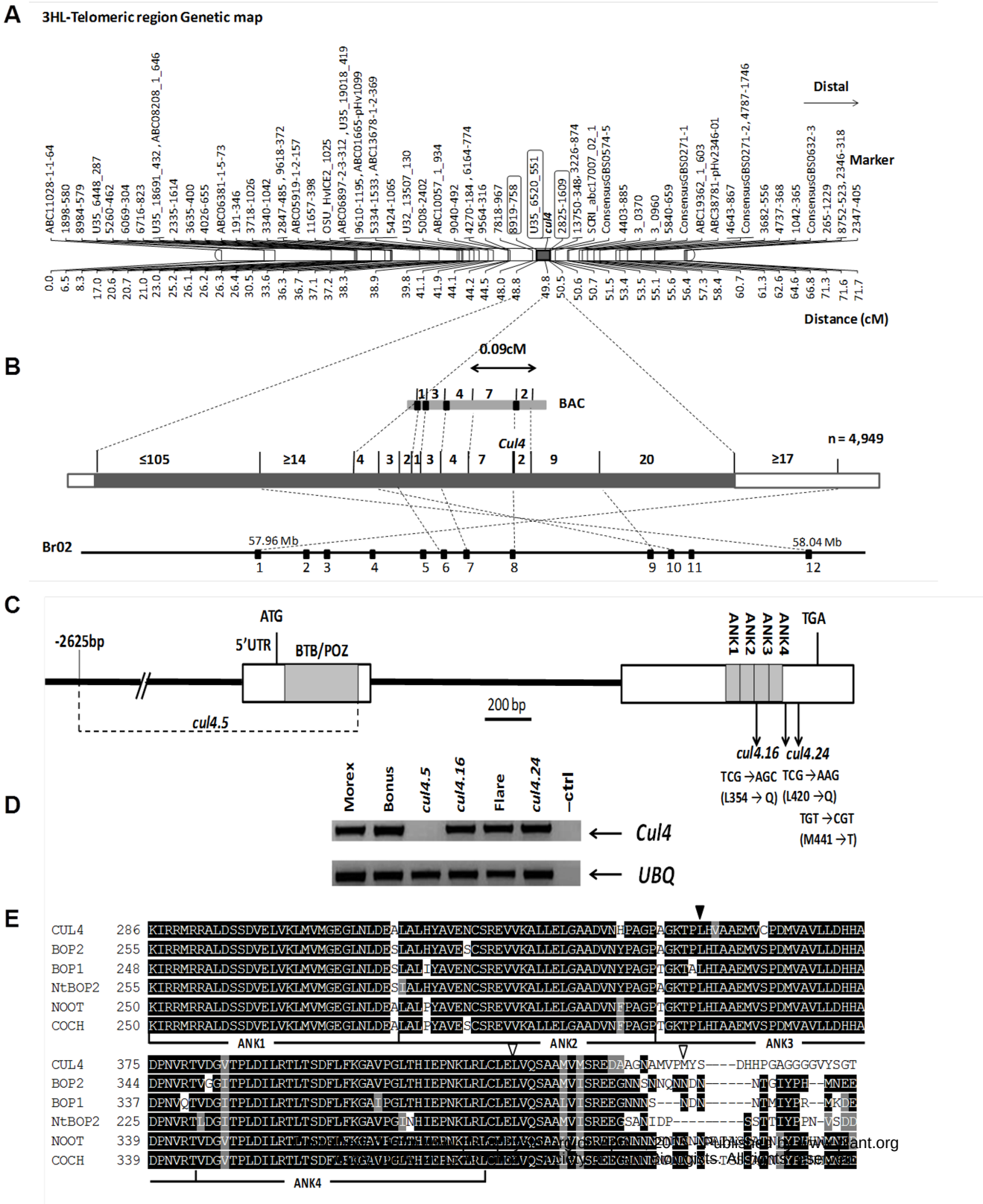
alleles of *Cul4* show a deletion in *cul4.5* and radical amino acid substitutions in *cul4.16* and *cul4.24* compared to their progenitor backgrounds. (*D*) RT-PCR analysis of *Cul4* transcripts in mutant alleles and their corresponding wild-type backgrounds using primers reported in table S3 (34 PCR cycles). *Ubiquitin* (*UBQ*) was used as an internal control (25 cycles). (*E*) Alignment of predicted amino-acid sequence at the ankyrin repeats region of CUL4 with Arabidopsis BOP2 (AT2G41370) and BOP1 (AT3G57130), tobacco NtBOP2 (EF051131), Medicago NOOT (JN180858) and pea COCH (JN180860). Ankyrin repeats are indicated by black lines (Wu et al., 2012). The position of amino acid substitutions in *cul4.16* and *cul4.24* are represented by filled and blank arrowheads, respectively.

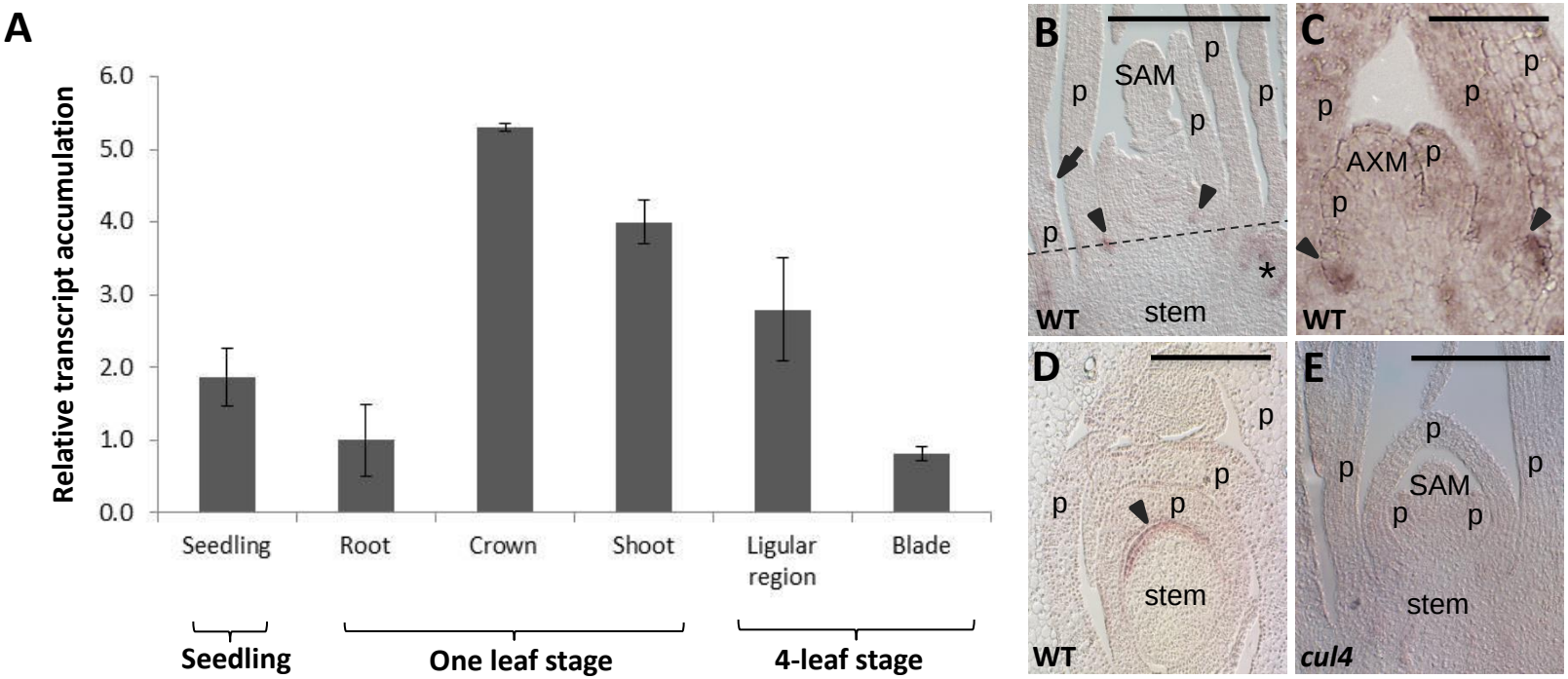
Figure 4- *Cul4* expression in Bonus wild-type plants using qRT-PCR (*A*) and *in situ* RNA hybridization in the shoot apical region of Bonus (*B-D*) and *cul4.5* mutant (*E*). (*A*) Quantitative RT-PCR was performed using specific primers for *cul4* (Table S3) on total RNA isolated from seedling (3-day old seedling when first leaf just emerging through coleoptile); root, crown and leaves at one leaf stage; 1 cm ligular region and the distal half of the blade from the third leaf at the 4-leaf stage. The *GAPDH* gene was used to normalize the data. The data shown here are the average of three biological replicates and their standard deviation. (*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 shoot apical region; *Cul4* hybridization on the stem side of the axil (arrowhead) is indicated. The sectioning plane corresponds to the dashed line in panel B. (*E*) Shoot apical region in *cul4.5* mutant showing no *Cul4* expression. WT, wild-type; SAM, shoot apical meristem; AXM, axillary meristem; p, leaf primordium. Bar in B and E= 250 µm; bars in C and D= 125 µm.








A
3HL-Telomeric region Genetic map
Distal
Marker
Distance (cM)
B
0.09cM
BAC
Cul4
n = 4,949
≤105
≥14
≥17
Br02
57.96 Mb
58.04 Mb
C
ATG
5'UTR
BTB/POZ
TGA
ANK1
ANK2
ANK3
ANK4
-2625bp
200 bp
cul4.5
cul4.16
cul4.24
TCG→AGC (L354→Q)
TCG→AAG (L420→Q)
TGT→CGT (M441→T)
D
Morex
Bonus
cul4.5
cul4.16
Flare
cul4.24
–ctrl
Cul4
UBQ
E
CUL4
BOP2
BOP1
NtBOP2
NOOT
COCH
ANK1
ANK2
ANK3
ANK4



Supplemental Materials

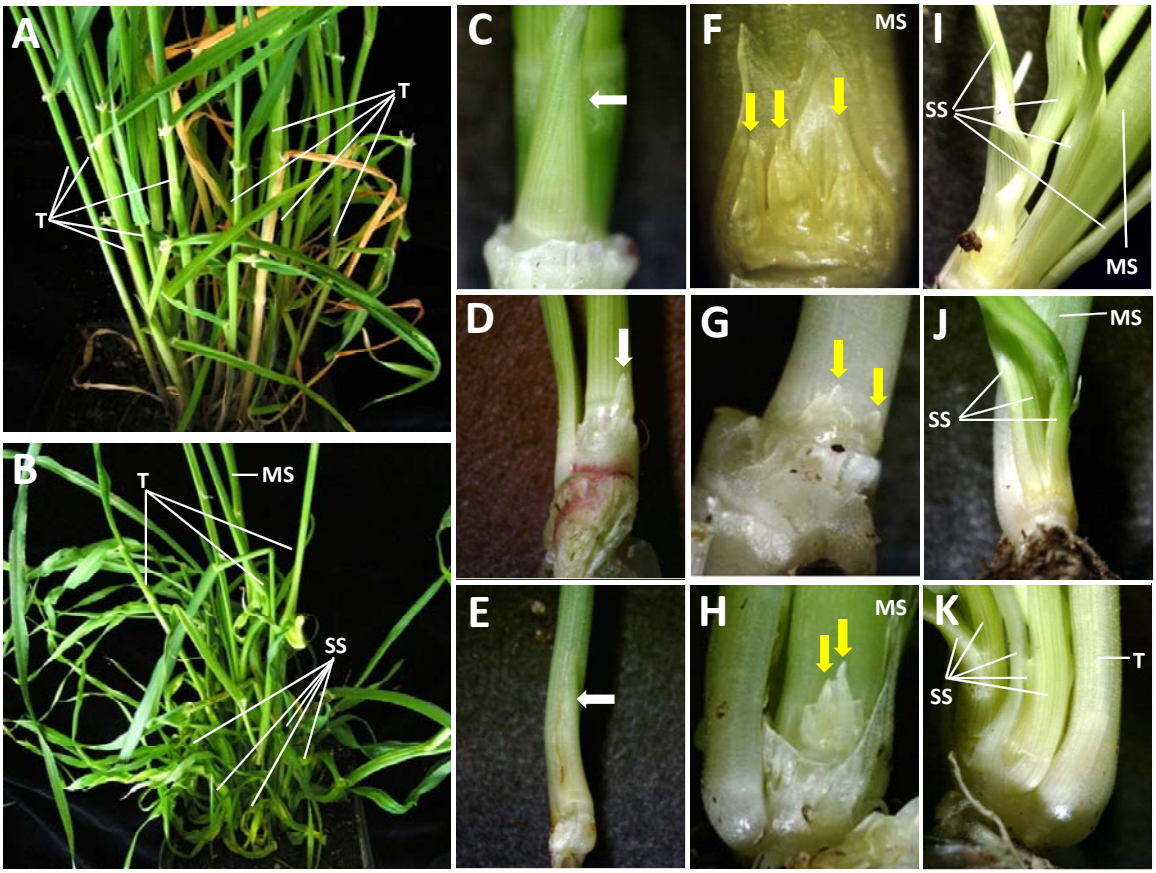


Fig. S1. Formation of double/multiple axillary buds and side shoots in *cul4* mutants. (A) Tillers (T) in wild-type plant. (B) Main stem (MS) and leafy side shoots (SS) in *cul4.16* mutant plant. Single axillary buds in wild-type: (C) Bowman 3-week old plant, (D) Bonus 5-week old plant, (E) Flare 3-week 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.

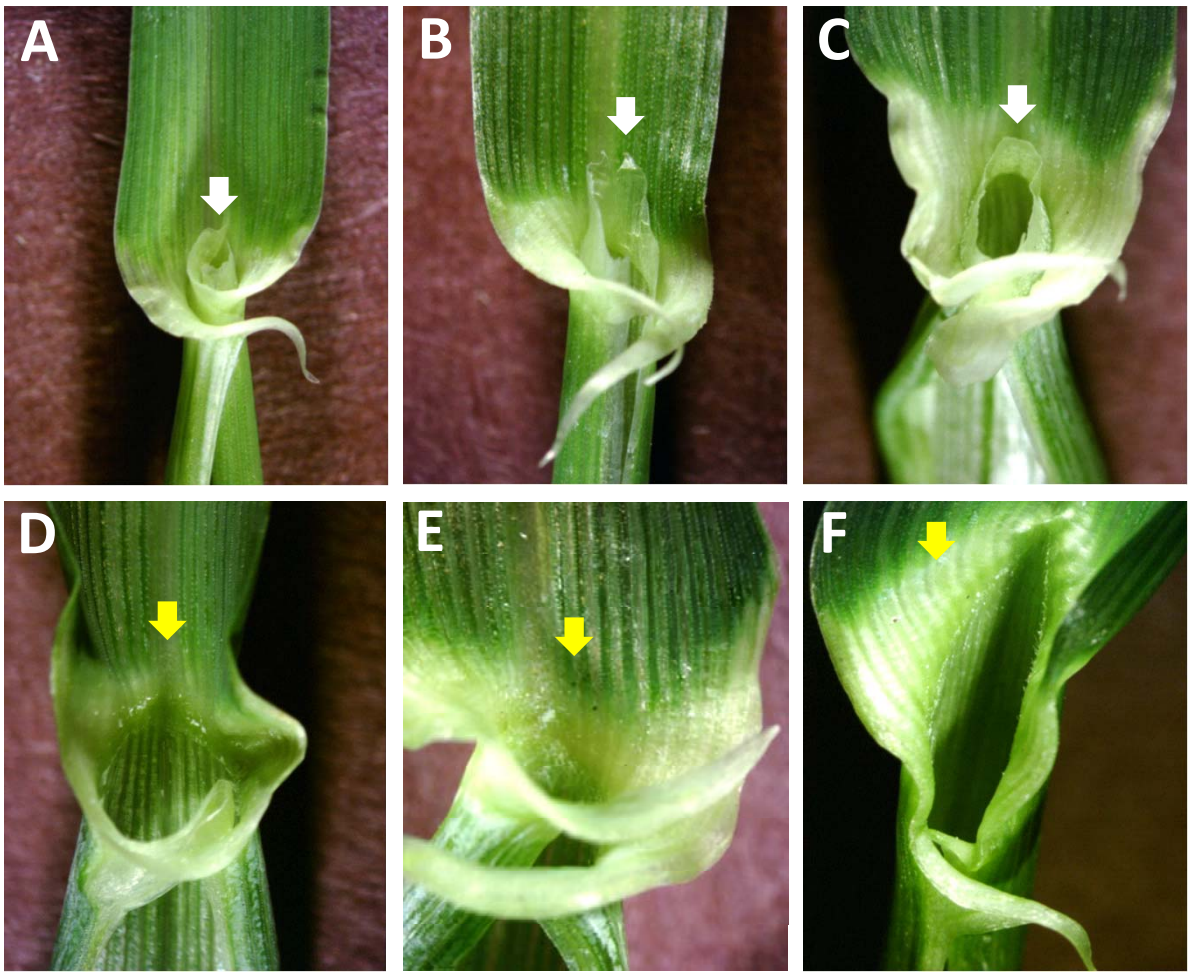


Fig. S2. Liguleless phenotype of *cul4* mutants. Ligules are indicated by white arrows in (A) Bowman, (B) Bonus and (C) Flare. Ligules are absent at the sheath-blade boundary (indicated by yellow arrows) in (D) Bowman-*cul4.5,* (E) *cul4.16* and (F) *cul4.24*.

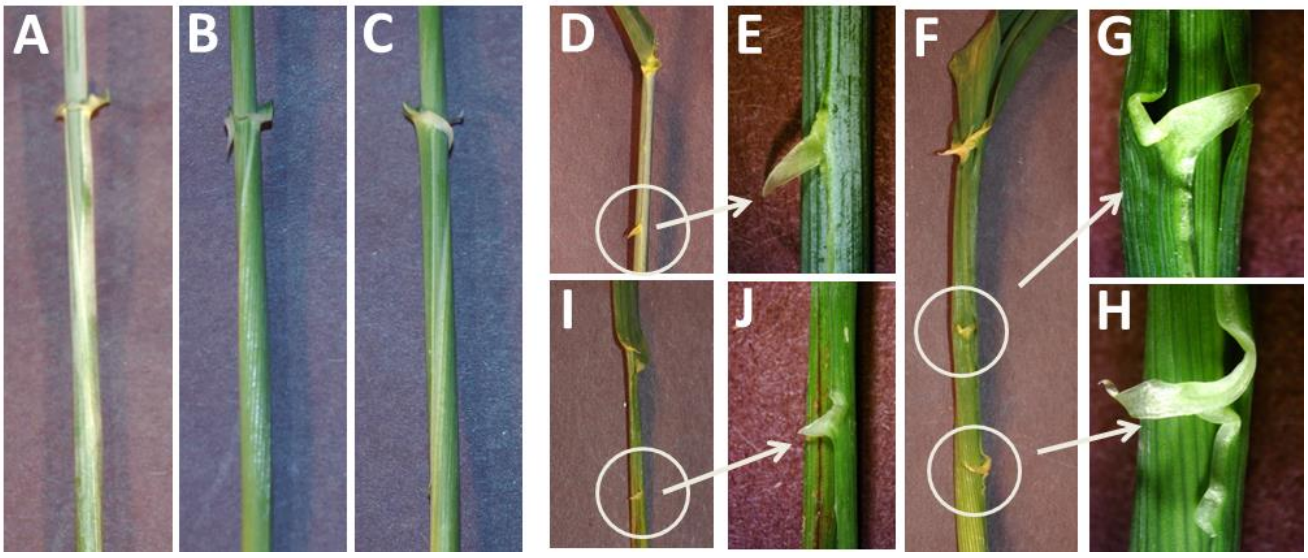
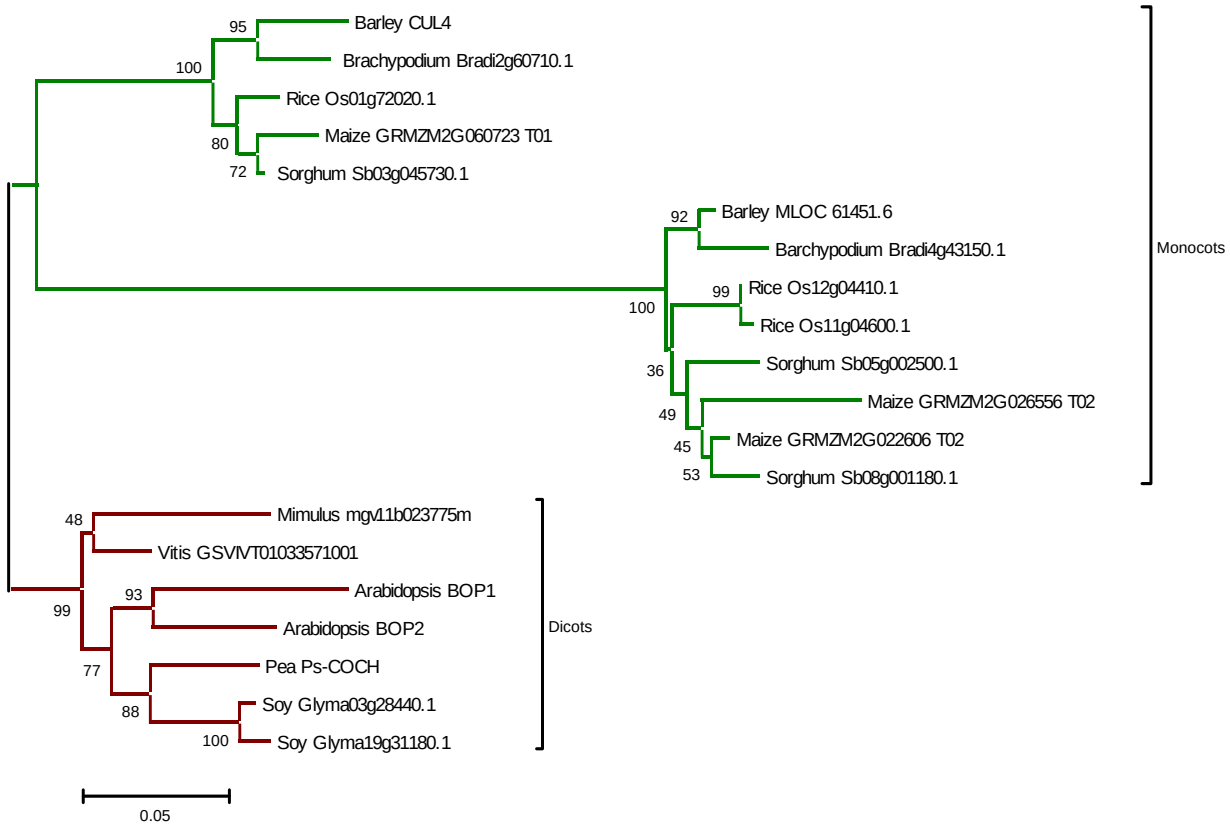


Fig. S3. Ectopic auricle tissue formation in *cul4* mutants. Auricles differentiate specifically at the sheath-blade boundary in wild type (A) Bowman (B) Bonus and (C) Flare plants. Ectopic flaps of auricle tissue form on leaf sheath margins in (D and close up in E) Bowman-*cul4.5,* (F and close ups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

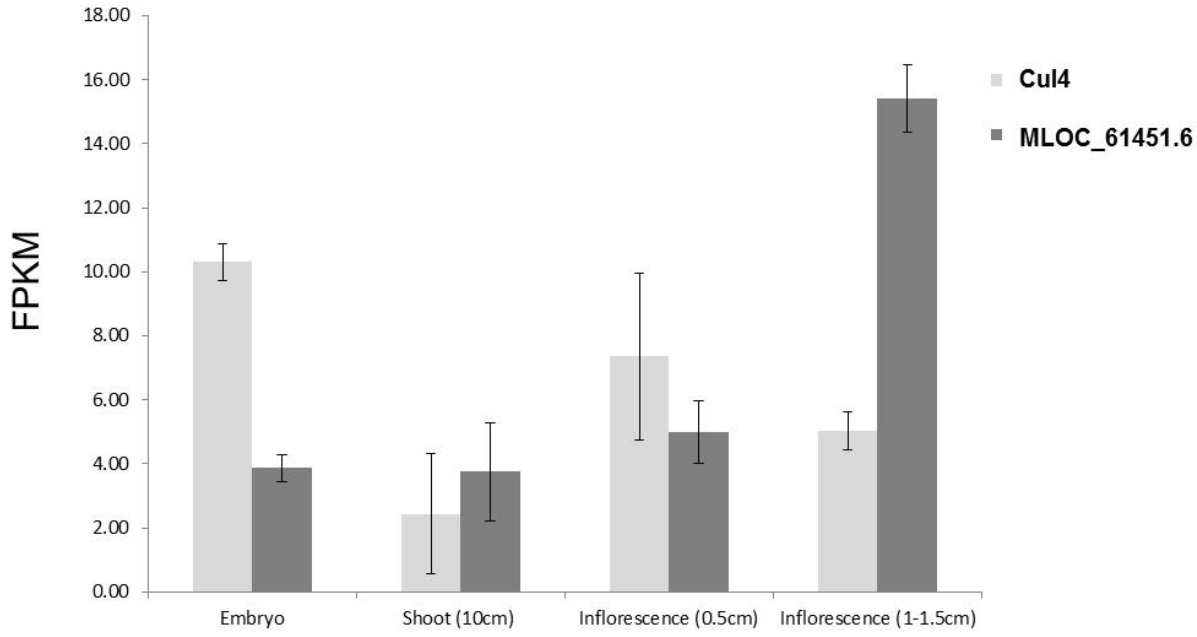


Fig. S5. Expression of cul4 and the barley paralogous gene MLOC_61451.6. Gene expression levels from RNA-seq data obtained from http://ics.hutton.ac.uk/morexGenes (IBSC, 2012). Tissues from left to right: 4-day old embryo dissected from germinating grain, seedling shoot (10 cm shoot stage), young developing 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
	RT_*cul4*****	CATGTACAGCGACCACCATC	TCACGTCCATCCCTAGGTTC
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 RT-PCR and quantitative RT-PCR.

References

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol **215:** 403-410

Close TJ, Bhat PR, Lonardi S, Wu Y, Rostoks N, Ramsay L, Druka A, Stein N, Svensson JT, Wanamaker S, Bozdag S, Roose ML, Moscou MJ, Chao S, Varshney RK, Szucs P, Sato K, Hayes PM, Matthews DE, Kleinhofs A, Muehlbauer GJ, DeYoung J, Marshall DF, Madishetty K, Fenton RD, Condamine P, Graner A, Waugh R (2009) Development and implementation of high-throughput SNP genotyping in barley. BMC Genomics **10:** 582

Couzigou JM, Zhukov V, Mondy S, Abu el Heba G, Cosson V, Ellis TH, Ambrose M, Wen J, Tadege M, Tikhonovich I, Mysore KS, Putterill J, Hofer J, Borisov AY, Ratet P (2012) *NODULE ROOT* and *COCHLEATA* maintain nodule development and are legume orthologs of *Arabidopsis BLADE-ON-PETIOLE* genes. Plant Cell **24:** 4498-4510

De Castro E, Sigrist CJA, Gattiker A, Bulliard V, Langendijk-Genevaux PS, Gasteiger E, Bairoch A, Hulo N (2006) ScanProsite: detection of PROSITE signature matches and ProRule-associated functional and structural residues in proteins. Nucleic Acids Res **34:** W362-W365

Felsenstein J (1985) Confidence limits on phylogenies: An approach using the bootstrap. . Evolution **39:** 783-791

Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG (2007) Clustal W and Clustal X version 2.0. Bioinformatics **23:** 2947-2948

Saitou N, Nei M (1987) The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol **4:** 406-425

Sigrist CJA, de Castro E, Cerutti L, Cuche BaA, Hulo N, Bridge A, Bougueleret L, Xenarios I (2012) New and continuing developments at PROSITE. Nucleic Acids Research **41:** D344-D347

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S (2011) MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Mol Biol Evol

The International Barley Genome Sequencing Consortium (2012) A physical, genetic and functional sequence assembly of the barley genome. Nature **491:** 711-716