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Genetic and morphological characterization of the barley *uniculm2* (*cul2*) mutant

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Abstract Axillary meristem growth and development help define plant architecture in barley (*Hordeum vulgare* L). Plants carrying the recessive *uniculm2* (*cul2*) mutation initiate vegetative axillary meristem development but fail to develop tillers. In addition, inflorescence axillary meristems develop into spikelets, but the spikelets at the distal end of the inflorescence have an altered phyllotaxy and are sometimes absent. Double mutant combinations of *cul2* and nine other recessive mutations that exhibit low to high tiller number phenotypes resulted in a uniculm vegetative phenotype. One exception was the occasional multiple shoots produced in combination with *granum-a*; a high tillering mutant that occasionally produces two shoot apical meristems. These results show that the CUL2 gene product plays a role in the development of axillary meristems into tillers but does not regulate the development of vegetative apical meristems. Moreover, novel double-mutant inflorescence phenotypes were observed with *cul2* in combination with the other mutants. These data show that the wild-type CUL2 gene product is involved in controlling proper inflorescence development and that it functions in combination with some of the other genes that affect branching. Our genetic analysis indicates that there are genetically separate but not distinct regulatory controls on vegetative and inflorescence axillary development. Finally, to facilitate future positionally cloning of *cul2*, we positioned *cul2* on chromosome 6(6H) of the barley RFLP map.

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Introduction

Plant architecture is governed by the action of meristems. During vegetative development, the shoot apical meristem is responsible for initiating all of the above-ground structures including the nodes, internodes, leaves, axillary meristems and the inflorescence (Sussex 1989). In barley (*Hordeum vulgare* L.), the shoot apical meristem is made up of two layers, the L1 (tunica) and L2 (corpus) (Döring et al. 1999). Vegetative axillary meristems, which give rise to tillers, are established at the base of the internode by the leaf above and originate from both the L1 and L2 layers of the shoot apical meristem (Döring et al. 1999). The developing bud first produces the prophyll, a leaf enclosing the axillary meristem with a function very similar to that of the coleoptile in the main shoot (Sharman 1945; Kirby and Appleyard 1981). Axillary shoot development (tiller production) entails two general processes: the development of an axillary meristem and subsequent axillary bud growth. During inflorescence development in barley, axillary meristems produce the spikelet, the primary unit of the inflorescence composed of glumes, lemma, palea, pistil and style. Although complete descriptions of the morphology of shoot and inflorescence axillary development have been reported in barley (Bonnett 1935; Sharman 1945), the genetic control of axillary meristem development and outgrowth is largely unknown.

To examine axillary shoot development in plants, axillary meristem mutants have been identified and characterized in several species including maize, pea, petunia, tomato and *Arabidopsis* (Kerstetter and Hake 1997; Schmitz and Theres 1999). In general, there are two classes of axillary shoot mutants. One class of mutants produces excessive branches, characterized by the maize *teosinte branched1* (*tb1*) mutant (Doebley et al. 1995), the *supershoot* (*sps*) and *auxin insensitive* (*axr1-12*) mutants

in Arabidopsis (Stirnberg et al. 1999; Tantikanjana et al. 2001), the *ramosus* mutants in pea (Arumingtyas et al. 1992) and the *decreased apical dominance* (*dad*) mutants in petunia (Napoli and Ruehle 1996). The maize *TB1* and Arabidopsis *SPS* genes have been isolated and they encode a probable transcription factor and a cytochrome P450 gene, respectively (Luo et al. 1996; Doebley et al. 1997; Tantikanjana et al. 2001). The other class of mutants is characterized by a lack of or reduced branching, including the *lateral suppressor* (*ls*), *blind* and *torosa* mutants of tomato (Schmitz and Theres 1999), and the *revoluta* mutant in Arabidopsis (Talbert et al. 1995). The tomato *Ls* gene encodes a protein that belongs to the VHIID family of transcription factors (Schumacher et al. 1999).

In barley, a large collection of single gene, recessive mutations are available which exhibit a variety of tillering and inflorescence phenotypes (Figs. 1 and 2; Bossinger 1992; Franckowiak 1996; J. Franckowiak, personal communication). Three classes of barley mutants are available that affect vegetative axillary development. Plants carrying the *uniculm2* (*cul2*) mutation do not produce tillers (Fig. 1B). Another class of mutants including *absent lower laterals* (*als*), *low number of tillers1* (*lnt1*), *uniculm4* (*cul4*), *intermedium spike-b* (*int-b*) and *semi brachytic* (*uzu*) produce significantly fewer tillers than wildtype (Fig. 1). The third class of mutants including *densonidosum6* (*den6*), *granum-a* (*gra-a*), *intermedium spike-m* (*int-m*), and *many noded dwarf1* (*mnd1*) produce significantly more tillers than wild-type plants. Two of these mutants, *den6* and *mnd1*, branch not only from leaf axils at the crown, but also from leaf axils at above-ground nodes. Additionally, since the spikelet (the primary unit of the barley inflorescence, or spike) is the result of axillary development in the spike, the *cul2*, *als*, *lnt1*, *int-m*, *int-b*, *den6*, *mnd1*, *uzu* and *gra-a* mutants also display mutant inflorescence phenotypes (Fig. 2). Therefore, these mutants can be used to dissect the genetic components of tiller and inflorescence development in barley.

In this paper, we describe the genetic and morphological characterization of the *cul2* mutant. We show that the wild-type *Cul2* gene plays an important role during vegetative and inflorescence axillary development in barley.

Materials and methods

Genetic stocks

The genetic stocks used in this research are summarized in Table 1. *uniculm2.b* (*cul2.b*) was found in a thermal neutron radiation mutagenesis of Kindred (Shands 1963). *cul2.b* is available in the Bowman genetic background. Bowman is a commercial 2-row barley cultivar where only the central spikelets are fertile and give rise to kernels. The *cul2.b rob1.a* genetic stock contains *orange lemma* (*rob1*), a single gene recessive mutation linked in cis two centimorgans from *cul2*, which colors the lemma orange. *cul2.b rob1.a* is available in the Bowman genetic background. All other barley mutants examined in this study are single gene recessive mutations and have been backcrossed to the Bowman genetic background. The vegetative and inflorescence phenotypes of each mutant are shown in Fig. 1 and Fig. 2, respectively, and described in Table 2. For observation, plant material was grown in the field at St. Paul, Minn. and the greenhouse, following common cultural practices. To ensure that all barley tillering mutants used in this study produce significantly greater or fewer tillers than wild-type plants, the total number of axillary branches (including tillers that form in the leaf axils at the crown and branches that form in the leaf axils at above-ground nodes in some mutants) were counted from a minimum of ten plants just prior to physiological maturity for each mutant and Bowman grown in two environments; the field during the summer of 2000 and greenhouse during the fall of 2000.

Population development for morphological analyses

An F_2 population segregating for *cul2* was developed in Bowman. Phenotypes, either "wild-type tillering" or "mutant tillering," were assigned to 150–200 individual plants of the segregating F_2 families; the genotype of the individual F_2 "wild-type tillering" plants was determined by observation of segregation patterns in the

Table 1 Genetic stocks

Genotype	Source	Genetic background	# Backcrosses to background	Chromosome location
Bowman	Franckowiak[a]			
absent lower laterals1.a (*als1.a*)	GSHO1990[b]	Bowman	7	3HL
densonidosum6 (*den6*)	GSHO2235	Bowman	7	ND[d]
granum-a.1 (*gra-a.1*)	GSHO1980	Bowman	7	3HL
intermedium spike b.3 (*int-b.3*)	Franckowiak	Bowman	6	5HL
intermedium spike-m.85 (*int-m.85*)	Franckowiak	Bowman	5	ND
low number of tillers1.a (*lnt1.a*)	GSHO1984	Bowman	8	3HL
low number of tillers (*int-1.81*)	GSHO1916	Bowman	6	3HL
many noded dwarf1.a (*mnd1.a*)	GSHO2038	Bowman	9	ND
uniculm2.b (*cul2.b*)	GSHO2074	Bowman	4	6HL
uniculm2.b rob1.a (*cul2.b rob1.a*)[c]	GSHO2075	Bowman	8	6HL
uniculm4 5 (*cul4.5*)	GSHO2261	Bowman	5	ND
uzu1.a or *semi brachytic* (*uzu1.a*)	GSHO1963	Bowman	7	3HL

[a] Gift from J. Franckowiak, Department of Plant Sciences, North Dakota State University, Fargo, N.D.
[b] GHSO obtained from USDA-ARS, National Small Grain Germplasm Research Facility, Aberdeen, Idaho
[c] *Uniculm2.b* linked in cis to *rob1.a*
[d] ND – not determined

F_3 generation. Genotypes of individual F_3 wild-type plants were determined in the same fashion. Comparisons were made between wild-type and *cul2* mutant siblings in the F_3 and/or F_4 generation(s).

Histology

Fresh tissue from the shoot apical meristem region of *cul2* and wild-type siblings in the Bowman genetic background was sampled from mature embryos and 1- and 2-week old seedlings. In addition, tissue from the shoot apical meristem region was sampled from 1- and 2-week old *gra-a* seedlings. From each of the developmental stages, at least ten samples from both wild-type and *cul2* mutant plants, and at least seven samples from both *gra-a* and additional wild-type plants, were examined. Tissue was fixed in FPA, a solution of 50% ethanol, 5% propionic acid and 10% formaldehyde (37%) (Ruzin 1992), passed through a graded ethanol/TBA series, and embedded in Paraplast Plus (Oxford, St. Louis, Mo.) paraffin wax. Ten-micrometer thick longitudinal serial sections through the apical meristem region were mounted on Fisher Probe-On Plus (Fisher Scientific, Pittsburgh, Pa.) microscope slides and stained with safranin and fast green. Using Permount (Fisher Scientific, Pittsburgh, Pa.), coverslips were mounted over the stained tissue sections. Tissue sections were subsequently viewed using light microscopy.

Scanning electron microscopy

Apical meristems were dissected from seedlings of *cul2* and wild-type siblings from the Bowman genetic background from 1- to 4-week old seedlings. A minimum of five apical meristems were observed from both wild-type and *cul2* mutant seedlings at each developmental stage. Samples were affixed to aluminum stubs using double-sided carbon tape and carbon paint, immediately frozen in liquid nitrogen, and directly viewed on a cold stage in the scanning electron microscope at 1.5 kV (Ahlstrand 1996).

Determination of genetic interactions

Crosses were made between *cul2-rob1* and nine other mutants to create F_2 populations segregating for double mutants. The genetic stocks used to make the crosses are summarized in Table 1; a description of the mutants is presented in Table 2, Fig. 1 and Fig. 2. To facilitate identification of the double mutants in each cross, the *cul2 rob1* stock used in these crosses contains *orange lemma* (*rob1*), a single gene recessive mutation linked in cis 2-centimorgans from *cul2*, which colors the lemma orange. F_2 plants exhibiting an orange lemma were classified as *cul2* and double mutants were identified based on phenotype. In addition to identifying the double mutants in the F_2 generation, double mutant phenotypes were confirmed in the F_3 generation by allowing the double mutant to segregate from F_2 plants homozygous for one of the mutations and heterozygous for the other mutation. To be termed an epistatic genetic interaction, the resulting double-mutant phenotype was the same as one of the single mutants used to make the cross. Genetic interactions more severe than either of the single mutations alone were termed synergistic interactions. The two mutations were said to be additive if they were acting independently of one another with the double-mutant phenotype showing aspects of both single mutations.

Mapping *cul2*

An F_2 population of 169 individuals segregating for *cul2* in the Bowman genetic background was developed. Homozygous and heterozygous wild-type plants were identified by examining the segregation of *cul2* in 15 to 20 F_3 progeny from individual wild-type F_2 plants. RFLP markers from chromosome 6(6H) were chosen to screen the Bowman and Bowman-*cul2* parents. DNA gel-blot analysis, hybridization and washing were conducted according to de la Peña et al. (1999). Linkage analyses were performed using Mapmaker, Version 2.0 (Lander et al. 1987).

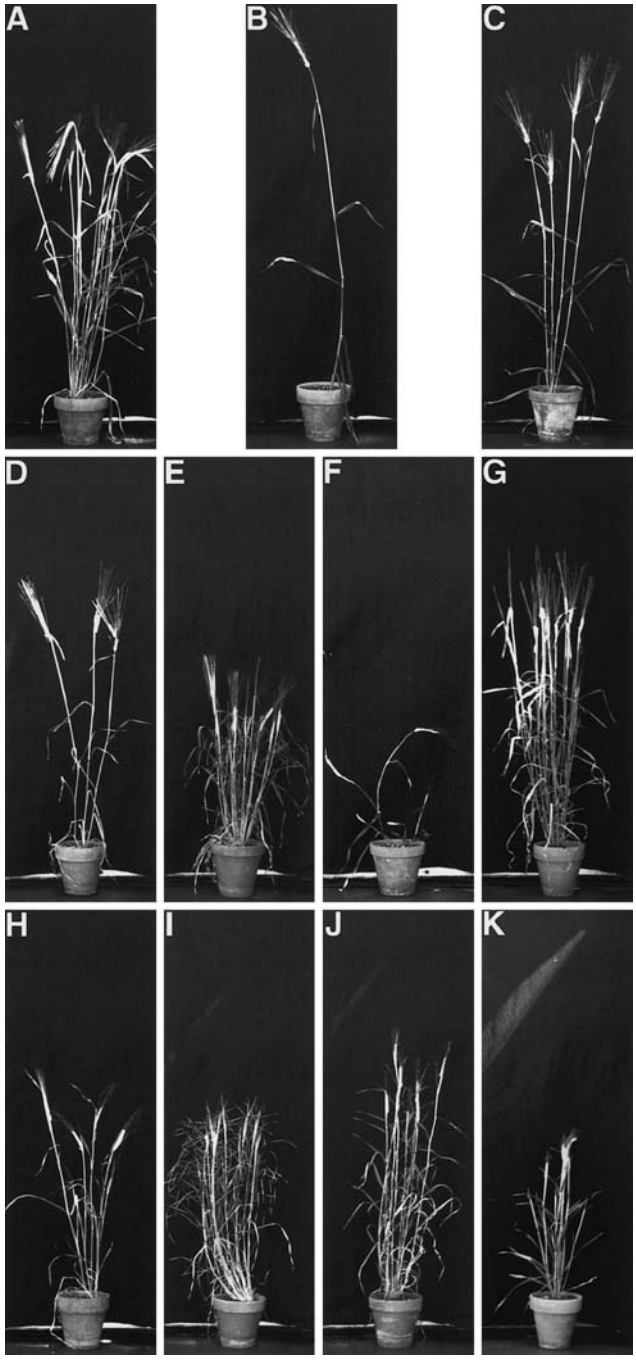

Fig. 1A–K Wild-type and mutant vegetative phenotypes. **A** Bowman (wild-type), the genetic background to which all of the tillering mutants have been backcrossed. **B** *cul2*. **C** *als*. **D** *lnt1*. **E** *gra-a*. **F** *cul4*. **G** *int-m*. **H** *int-b*. **I** *mnd1*. **J** *den6*. **K** *uzu*

Table 2 Vegetative and inflorescence phenotypes of mutants used in this study

Genotype	Vegetative phenotype			Inflorescence phenotype[c]
	Tiller number[a]	Tiller number[b]	Vegetative phenotype[c]	
Bowman (wild-type)	27	13		
absent lower laterals1.a (*als1.a*)	4**	5**	Large, rigid stems	Lack of development of lateral spikelets at the base of the inflorescence
densonidosum6 (*den6*)	87**	22**	Thin stems and leaves, axillary branches can develop from the crown as well as from upper nodes on the plant	Inflorescence about one half the length of wild-type
granum-a.1 (*gra-a.1*)	53**	17*	Short plant with thin stems and leaves	Inflorescence about one half the length of wild-type
intermedium spike-b.3 (*int-b 3*)	12**	7**	Stems same size as wild-type	Irregularities in lower portion of inflorescence, including formation of awns by lateral spikelets
intermedium spike-m.85 (*int-m.85*)	33	26**	Stems same size as wild-type	Short inflorescence, base of inflorescence more dense than upper portion, fused or fasciated terminal spikelets
low number of tillers1 (*lnt1.a* and *int-1.81*)	2**	2**	Thick stems and dark, green leaves	Lower portion of the inflorescence often more compact, irregular rachis internode lengths
many noded dwarf1.a (*mnd1.a*)	49**	27**	Short plant, axillary branches can develop from the crown as well as from nodes higher up the plant, thin leaves	Very short inflorescence, branching can occur in the inflorescence
uniculm2.b (*cul2.b*)	0**	0**	Thick culm with dark, green leaves	Disorganized arrangement of spikelets at the top half of the inflorescence
uniculm4.5 (*cul4.5*)	2**	2**	Thick culms with dark, green leaves	Long, wild-type-like inflorescence
uzu1.a or *semi brachytic* (*uzu1.a*)	16**	9*	Reduced height and length of leaves	Compact inflorescence with shortened rachis internodes and awns

*,** significantly different from Bowman at the 0.05 and 0.01 level, respectively

[a] Mean number of axillary branches (includes tillers that form in the leaf axils at the crown and branches that form in leaf axils at higher nodes in some mutants) counted from a minimum of ten plants of each tillering type grown in the field during the summer of 2000

[b] Mean number of axillary branches (includes tillers that form in the leaf axils at the crown and branches that form in leaf axils at higher nodes in some mutants) counted from a minimum of ten plants of each tillering type grown in the greenhouse during winter of 2000

[c] Data from Franckowiak, (1996) and observations in the field and greenhouse

Results

Axillary meristem development in wild-type and *cul2* mutant siblings

In multiple field and greenhouse plantings at St. Paul, Minn., mutant *cul2* plants did not produce tillers (Fig. 1; Table 2). The failure of *cul2* plants to develop tillers could be because axillary meristems are not initiated or development of axillary meristems is arrested following initiation. To determine the cause of the lack of tiller development, we examined axillary development in *cul2* and wild-type siblings using traditional histological techniques.

In wild-type plants, the first tiller bud was present in the embryo (Fig. 3A, D) and 1-week old seedlings in the axil of the first leaf (Fig. 3G, J). In wild-type, 2-week-old seedlings, the first, second and third tiller buds were present in the axils of the first, second and third leaves, respectively (Fig. 3N, P, Q, R). The coleoptile tiller bud was present in some wild-type 1- and 2-week-old seedlings (data not shown). In *cul2* mutant plants, a tiller bud was present in the axil of the first leaf of the mature embryo. However, unlike the first tiller buds present in mature wild-type embryos (Fig. 3A, D), *cul2* first tiller buds consisted of only a meristematic dome with no leaf initiation (Fig. 3B, F). In 1-week-old *cul2* mutant seedlings, the cells of the first tiller bud were enlarged and did not appear meristematic (Fig. 3H, L, M). In 2-week-old seedlings, the first tiller bud was no longer present (Fig. 3O). Therefore, as *cul2* plants develop, the enlarged cells from the non-differentiated *cul2* first tiller bud appear to become incorporated into the internode above. Although the first tiller buds were no longer observed in *cul2* 2-week-old seedlings, the second (Fig. 3T) and third (Fig. 3S) tiller buds were evident. These data indicate that vegetative axillary meristems are initiated in *cul2* plants, but fail to continue to develop into tillers.

Shoot apical meristem development in wild-type and *cul2* mutant siblings

The structures present in a mature wild-type barley plant arise from a series of developmental changes occurring at

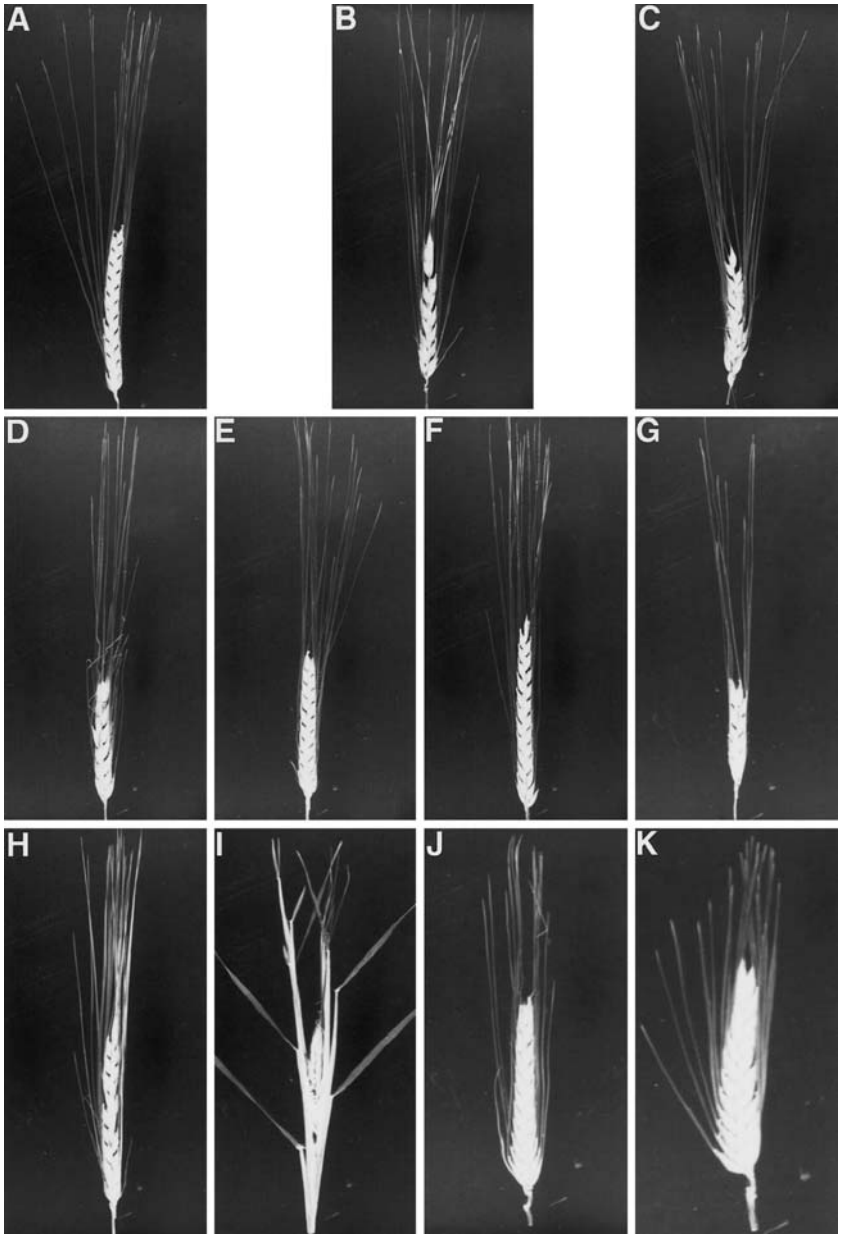

Fig. 2 Wild-type and mutant inflorescence phenotypes. **A** Bowman (wild-type), the genetic background to which all of the mutants have been backcrossed. **B** *cul2*. **C** *als*. **D** *lnt1*. **E** *gra-a*. **F** *cul4*. **G** *int-m*. **H** *int-b*. **I** *mnd1*. **J** *den6*. **K** *uzu*

the shoot apical meristem. To determine the effect of the *cul2* mutation on shoot apical meristem development, scanning electron microscopy was used to examine the shoot apical meristems of developing *cul2* mutant and wild-type siblings. To provide a comparison to the *cul2* mutant, we provide a description of wild-type shoot apical meristem development. While in the vegetative stage, the apical dome initiates leaf primordia (Fig. 4A). The apical dome then elongates; and primordia are produced more quickly so that they accumulate on the shoot apex. The primordia present on the proximal portion of the apical dome will become leaves; however, the primordia present on the distal portion of the apical dome will initiate spikelet primordia. Once the primordia on the distal portion of the apical dome differentiate into two distinct ridges, termed the double ridge stage (Fig. 4C), the shoot apical meristem has transitioned from vegetative to reproductive. The spikelet primordium is the most distal ridge in each double ridge, and quickly differentiates into three mounds, the triple mound stage (Fig. 4E). Each mound represents a future spikelet; the center mound in each triplet corresponds to a central spikelet, while the flanking mounds will develop into lateral spikelets. In 2-row barley cultivars (pictured in Fig. 4), only the central spikelets are fertile and will produce kernels, while, in 6-row barley cultivars, both the central and lateral spikelets develop kernels. The first structures to differentiate from the spikelet primordia are the glumes, followed by the lemma, then the palea. Next, the stamen primordia develop (Fig. 4G, I). Within the stamen primordia, the carpel primordia begin to form. Finally, the awns begin to differentiate from the lemma (Fig. 4K).

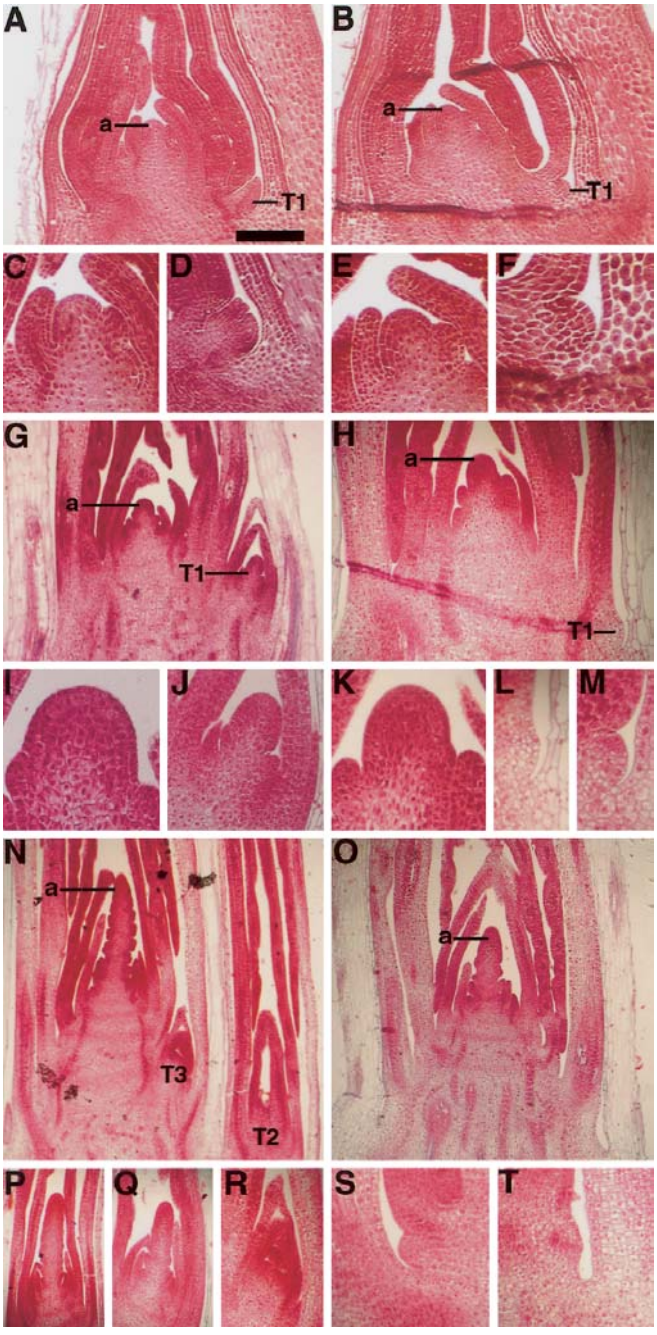

Fig. 3A–T Histological sections of wild-type and *cul2* mutant shoot apices. Shoot apices of **A** wild-type and **B** *cul2* mutant embryos. **C** Wild-type embryonic shoot apical meristem and **D** first embryonic tiller bud. **E** *cul2* embryonic shoot apical meristem and **F** axillary development under the first embryonic leaf. **G** Wild-type and **H** *cul2* shoot apices 1 week after planting. **I** Wild-type shoot apical meristem and **J** first tiller bud at 1 week. **K** *cul2* shoot apical meristem and **L** axillary development in the axil of the first leaf of the plant displayed in **H** at 1 week. **M** *cul2* axillary development in the axil of the first leaf of another plant. **N** Wild-type and **O** *cul2* shoot apices 2 weeks after planting. Wild-type tiller buds in the axils of the **P** first leaf, **Q** second leaf, and **R** third leaf, at 2 weeks. *cul2* axillary development under the **S** third leaf and **T** second leaf. Scale bar in **A** equals: **A, B** 200 μm; **C** 97 μm; **D** 171 μm; **E** 99 μm; **F** 80 μm; **G** 57 μm; **H** 149 μm; **I** 50 μm; **J** 94 μm **K** 55 μm; **L** 94 μm; **M** 105 μm; **N** 282 μm; **O** 261 μm; **P** 236 μm; **Q** 220 μm; **R** 173 μm; **S** 78 μm; **T** 91 μm. *a* apex; *T1*, *T2* and *T3* developing tiller buds under the first, second, and third leaves, respectively

No structural differences were observed in vegetative shoot apical meristems of the 8-day old *cul2* mutant and wild-type seedlings (Fig. 4A, B). Once the shoot apical meristem transitioned from vegetative to reproductive, however, variation in timing of different developmental stages between mutant and wild-type plants was observed. The *cul2* mutant reached the double ridge stage 1-day later than wild-type shoot apical meristems (Fig. 4C, D) and the triple mound stage 3-days later than wild-type (Fig. 4E, F). Once the spikelets began to develop, both the *cul2* mutant and wild-type shoot apices developed at the same rate (Fig. 4G–L). Flowers of *cul2* mutants emerged from the boot 2-days before wild-type siblings.

The *cul2* mutation also affects the structural development of the reproductive shoot apical meristem. During the double ridge stage, the ridges of the *cul2* mutant were less defined than those of wild-type siblings (Fig. 4C, D). Once the spikelet primordia began to differentiate, variation in the spikelet arrangement in the upper portion of the developing spike was evident. Most commonly, the spikelets near the distal end of the developing *cul2* spike were disorganized, forming a mixed clump of central and lateral spikelets (Fig. 4G, H, I and J). Furthermore, unlike wild-type developing spikes, entire spikelets were sometimes missing near the distal end of the developing *cul2* spike (Fig. 4K, L). Both of these disorganized forms of the *cul2* mutant spike were observed in the mature spike (Fig. 4N). In addition, rachis (the central axis of the spike) nodes without spikelets were often observed near the distal end of mature *cul2* spikes.

Genetic interactions between *cul2* and nine other barley mutants

Nine other single gene recessive barley mutants that exhibit a variety of tillering phenotypes (Table 2 and Fig. 1) were examined in combination with *cul2*. *als*, *lnt1*, *cul4*, *int-b* and *uzu* produce fewer tillers than wild-type. *den6*, *gra-a*, *int-m*, and *mnd1*, however, produce many more tillers than wild-type. Furthermore, two of these mutants, *den6* and *mnd1*, not only develop tillers from the crown, but also branch from above-ground nodes. The mutants examined in this study also display a wide range of unique inflorescence phenotypes (Table 2 and Fig. 2). The mutant spike phenotypes include differences in rachis length, rachis internode length, awn length, and spikelet development. Therefore, these mutants affect both vegetative and inflorescence axillary development. Crosses were made between *cul2* and the nine other mutants to examine genetic interactions between these mutant genes during tiller and inflorescence development. Resultant homozygous double-mutant phenotypes are shown in Fig. 5 and summarized in Table 3. Inflorescence axillary development was defined as the ability to develop spikelets and spikelet position on the rachis.

cul2; *int-b*. All *cul2*; *int-b* double mutants were uniculm (Fig. 5A). Growth of two of the double mutants ceased during internode elongation, generating plants

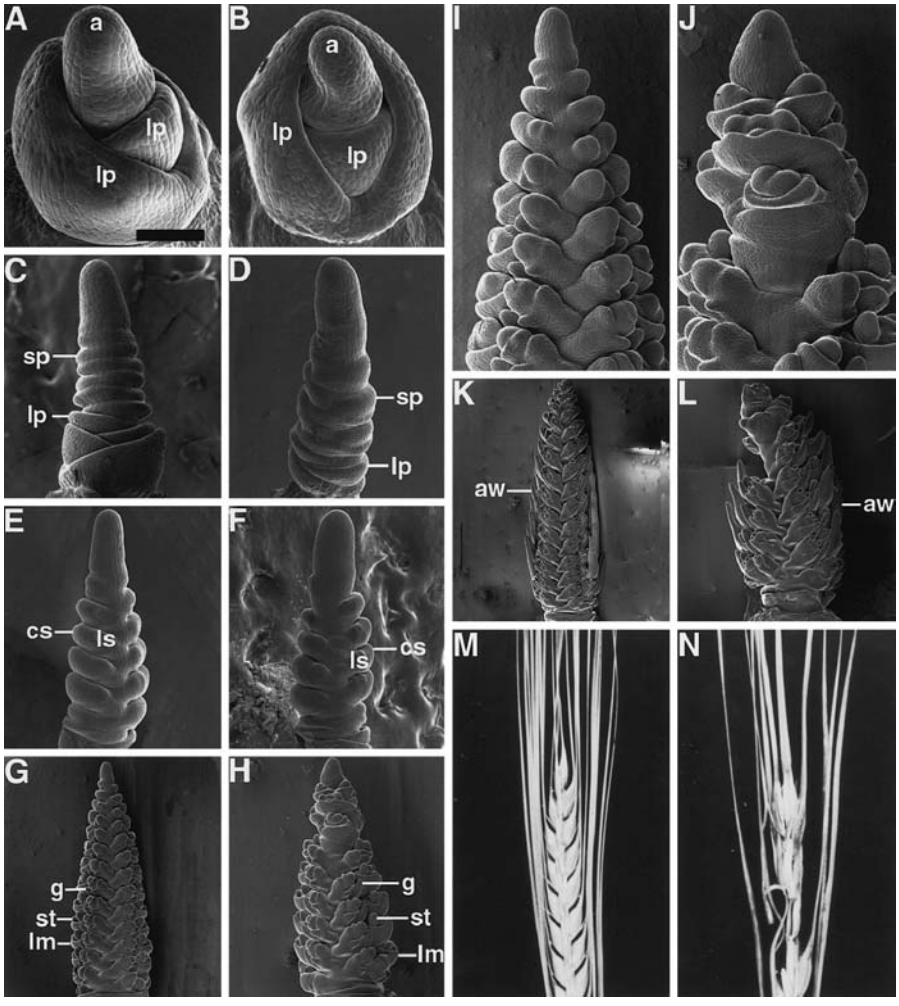

Fig. 4A–N Scanning electron micrographs of *cul2* and wild-type vegetative and inflorescence shoot apices. **A** Wild-type and **B** *cul2* mutant shoot apices at the vegetative stage sampled as 8-day old seedlings. **C** Wild-type and **D** *cul2* shoot apices at the double ridge stage sampled as 13 and 14 day-old seedlings, respectively. **E** Wild-type and **F** *cul2* shoot apices at the triple mound stage sampled as 14- and 17-day old seedlings, respectively. **G, I** Wild-type and **H, J** *cul2* shoot apices at the stamen primordium stage sampled as 19-day old seedlings. **K** Wild-type and **L** *cul2* shoot apices at the awn primordium stage sampled as 23-day old seedlings. **M** Wild-type and **N** *cul2* spikes at physiological maturity. Scale bar in **A** equals: **A** 74 μm; **B** 84 μm; **C** 174 μm; **D** 191 μm; **E** 222 μm; **F** 313 μm; **G** 544 μm; **H** 594 μm; **I** 181 μm; **J** 175 μm; **K** 1.16 mm; **L** 644 μm. *a*, apex; *aw*, awn; *cs*, central spikelet; *g*, glume; *lm*, lemma; *lp*, leaf primordium; *ls*, lateral spikelet; *sp*, spikelet primordium; *st*, stamen primordium

Table 3 Summary of double mutant tillering and inflorescence phenotypes

Double mutant combination	Population size	Chi-square[a]	*P*-value	Tillering phenotype	Inflorescence phenotype
cul2; *als*	184	5.31	0.1502	uniculm	No spikelets on lower half of spike
cul2; *den6*	169	3.09	0.3784	Short uniculm	Short spike with short awns; some lateral spikelets can develop and/or form awns
cul2; *gra-a*	174	6.84	0.0771	usually short uniculm; rarely 2–3 shoots	Short *cul2*-like spike
cul2; *int-b*	191	3.08	0.3801	uniculm	Rachis without spikelets or sterile terminal spikelet at the top of the rachis
cul2; *int-m*	189	1.65	0.6470	uniculm	Long *int-m*-like spike
cul2; *lnt1*	204	4.56	0.2072	uniculm	Range from no spike to production of 1 sterile spikelet to a short *cul2*-like spike
cul2; *mnd1*	192	0.89	0.8281	Short uniculm	No spike; rarely production of a few sterile spikelets
cul2; *cul4*	189	2.22	0.5282	uniculm	Range from no spike to no spikelets on lower half of spike
cul2; *uzu*	191	7.21	0.0655	Short uniculm	Rachis internodes, awns, and spike short like *uzu*; *cul2*-like spikelet arrangement

[a]The chi-square analysis was used to test the fit of the F_2 generation phenotypes observed in each cross to 9:3:3:1

only one-half the height of other double mutants; and no spike emerged. The inflorescence phenotype of the other double mutants observed varied from production of a rachis lacking spikelets to a rachis with a single sterile terminal spikelet as shown in Fig. 5H.

cul2; *mnd1*. The resulting double mutants were short, uniculm plants (Fig. 5B). Most double mutants failed to

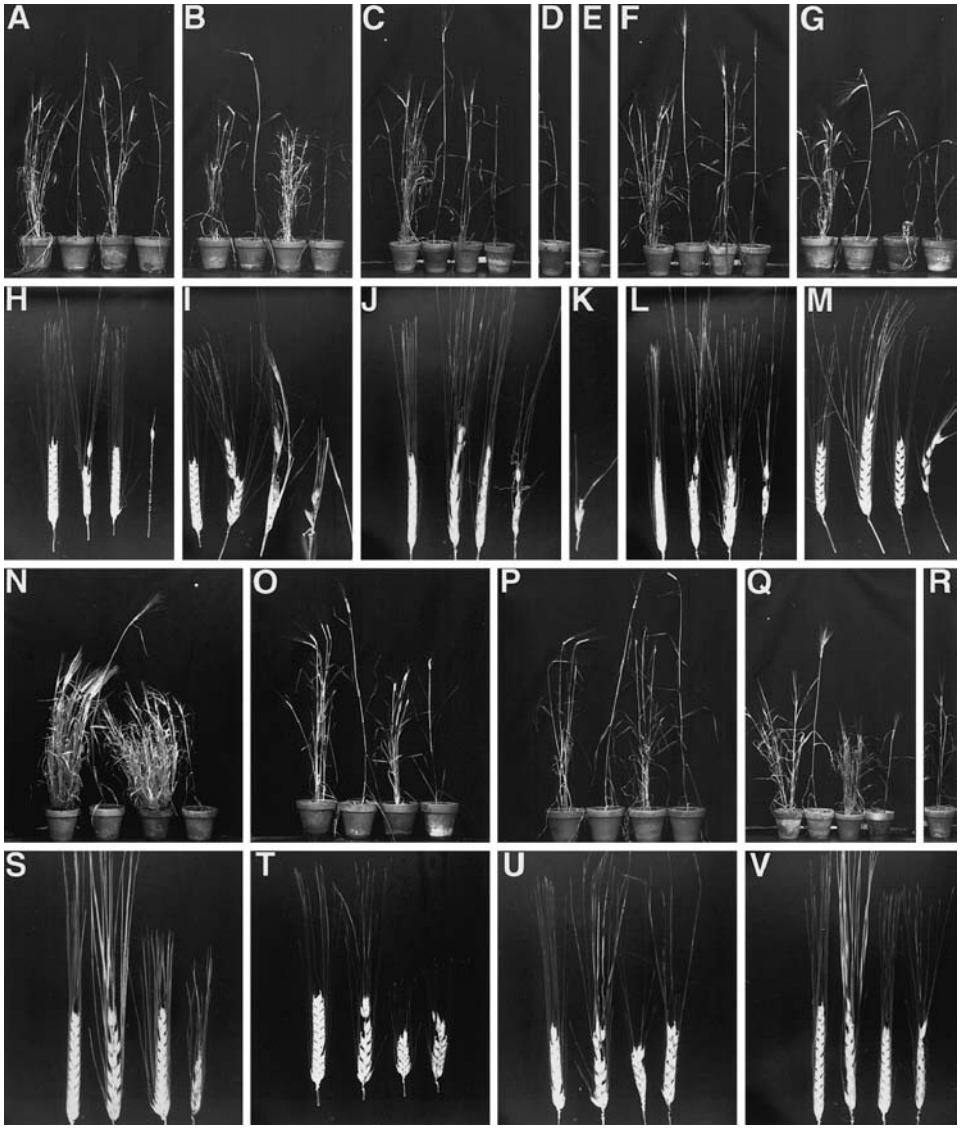

Fig. 5A–V Double-mutant combinations between *cul2* and nine other single gene recessive mutants. In each panel, the order of plants and spikes from left to right is as follows: wild-type (Bowman), *cul2*, the mutant to which *cul2* has been crossed, and the double mutant. **A, H** *cul2*; *int-b*. **B, I** *cul2*; *mnd1*. **C–E, J,K** *cul2*; *lnt1*. **F, L** *cul2*; *als*. **G, M** *cul2*; *cul4*. **N, S** *cul2*; *den6*. **O, T** *cul2*; *uzu*. **P, U** *cul2*; *int-m*. **Q, R, V** *cul2*; *gra-a*

produce a spike; however, occasionally, a few sterile spikelets were generated. One double mutant plant produced the spike shown in Fig. 5I, where the spike branched, forming a few sterile spikelets on one branch and one sterile spikelet on the other.

cul2; *lnt1*. Although all double mutants were uniculm, variation in vegetative phenotypes occurred. The most commonly observed phenotype is shown in Fig. 5C, a uniculm plant with a variable spike phenotype. In some double mutants, growth arrested during internode elongation (Fig. 5D), while in others, the plants died when they reached the two-leaf stage (Fig. 5E). Double mutant inflorescence phenotypes were variable, as well. When spikes were produced, they ranged from the production of one to a few sterile spikelets (Fig. 5K) to a sterile spike missing spikelets near the distal end of the spike similar to *cul2* (Fig. 5J).

cul2; *als*. All resulting double mutants displayed consistent vegetative and inflorescence phenotypes; plants were tall and uniculm (Fig. 5F) and lacked all spikelets on the lower half of the spike and, similar to *cul2*, a few near the distal end of the spike (Fig. 5L). Although *als* inflorescences lack one or two spikelets at the base of the spike as well as a few lower lateral spikelets, the double mutant phenotype was more severe.

cul2; *cul4*. Double mutant plants were uniculm (Fig. 5G). Most double mutant plants formed spikes that resembled those of the *cul2*; *als* double mutants with spikelets missing from the lower half of the inflorescence and a few absent near the distal end of the spike (Fig. 5M).

cul2; *den6*. All double mutant plants were short and uniculm (Fig. 5N) with a short, sterile spike missing a few spikelets near the distal end of the spike (Fig. 5S). The spike often displayed developmental irregularities including the development of some lateral spikelets.

cul2; *uzu*. The vegetative double mutant phenotype was a short, uniculm plant (Fig. 5O). Displaying aspects of both *cul2* and *uzu*, the short double mutant spike had reduced awns and rachis internodes like *uzu*, while the

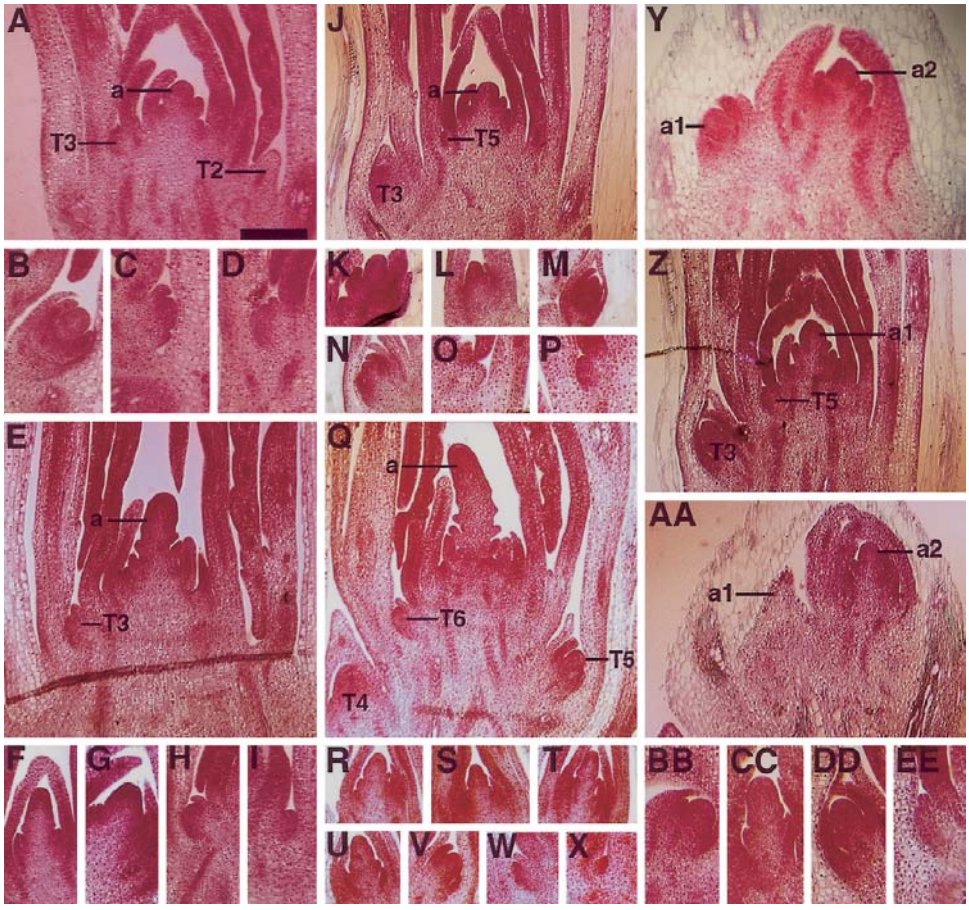

Fig. 6A–Z, AA–EE Shoot apex and axillary meristem structure in developing *gra-a* seedlings. Developing wild-type seedlings at **A** 1-week and **E** 2-weeks. Wild-type tiller buds developing in the axils of the **B** coleoptile, **C** second and **D** third leaves at 1-week. Wild-type tiller buds developing in the axils of the **F** first, **G** second, **H** third and **I** fourth leaves at 2-weeks. **J** *gra-a* with one apical meristem and tiller buds developing in the axils of the **K** coleoptile, **L** first, **M** second, **N** third, **O** fourth and **P** fifth leaves at 1-week. **Q** *gra-a* with one apical meristem and tiller buds developing in the axils of the **R** first, **S** second, **T** third, **U** fourth, **V** fifth, **W** sixth and **X** seventh leaves at 2-weeks. **Y** *gra-a* with two apical meristems at 1-week. **Z** first and **AA** second *gra-a* apical meristems at 2 weeks with developing tiller buds in the axils of the **BB** coleoptile, **CC** first, **DD** third and **EE** fifth leaves. Scale bar in **A** equals: **A** 235 mu m; **B** 228 μm; **C** 199 μm; **D** 161 μm; **E** 250 μm; **F** 251; **G** 192 μm; **H** 240 μm; **I** 172 μm; **J** 247 μm; **K** 256 μm; **L** 245 μm; **M** 290 μm; **N** 327 μm; **O** 176 μm; **P** 157 μm; **Q** 230 μm; **R** 277 μm; **S** 357 μm; **T** 313 μm; **U** 281 μm; **V** 286 μm; **W** 245 μm; **X** 157 μm; **Y** 121 μm; **Z** 300 μm; **AA** 284 μm; **BB** 213 μm; **CC** 177 μm; **DD** 176 μm; **EE** 125 μm. *a*, apex; *a1*, first apex in *gra-a*; *a2*, second apex in *gra-a*; *T2*, *T3*, *T4*, *T5* and *T6*, developing tillers under the second, third, fourth, fifth and sixth leaves, respectively

arrangement of the spikelets resembled that of *cul2* (Fig. 5T).

cul2; *int-m*. All double mutants were tall and uniculm (Fig. 5P) with a long spike that exhibited characteristics of *int-m* spikes; fused or fasciated spikelets at the distal end of the spike (Fig. 5U).

cul2; *gra-a*. Most double mutants were short, uniculm plants (Fig. 5Q) with a shortened *cul2*-like spike (Fig. 5V). Occasionally, however, double-mutant plants produced two or, even more rarely, three shoots (Fig. 5R).

gra-a apical and axillary development

To explore the *cul2*; *gra-a* genetic interaction further, histological sections were made from the apical meristem region of *gra-a* and wild-type seedlings (Fig. 6). Wild-type plants sampled at 1 week had formed three tiller buds (Fig. 6A–D). At 2 weeks, the shoot apical meristem of wild-type plants was beginning the transition from vegetative to reproductive, indicated by the accumulation of primordia on the apex (Fig. 6E). Additionally, most wild-type plants had formed four tiller buds by 2 weeks (Fig. 6F–I).

Two different phenotypes were observed in the histological sections made from developing *gra-a* seedlings. The most commonly observed *gra-a* phenotype is displayed in Fig. 6J–X. Similar to wild-type, the *gra-a* shoot apical meristem was vegetative at 1 week (Fig. 6J) and transitioning from vegetative to reproductive at 2 weeks (Fig. 6Q). However, by the same developmental stage, *gra-a* had produced more tiller buds than wild-type plants. At 1 week, six of seven *gra-a* plants had produced four to five tiller buds (Fig. 6K–P), while, at 2 weeks, 11

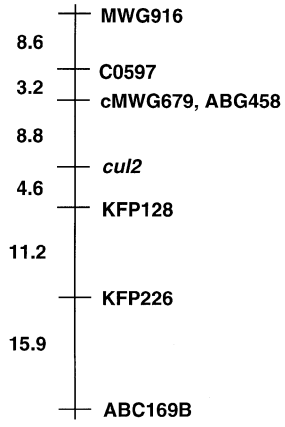

Fig. 7 Molecular map location of *cul2* on barley chromosome 6H. RFLP markers are shown on the right. All distances are expressed in centimorgans on the left

of 14 *gra-a* plants had produced six to seven tiller buds (Fig. 6R–X).

A second, less common, phenotype was also observed in developing *gra-a* seedlings. At 1 week, one plant appeared to have produced a second shoot apical meristem; no tiller buds had formed (Fig. 6Y). Three of the 2-week-old *gra-a* plants sampled also produced two shoot apical meristems. Fig. 6Z shows the first shoot apical meristem observed in one of these plants. The second shoot apical meristem (Fig. 6AA) was located behind the first and was not revealed until several serial sections later. Anywhere from three to six tiller buds developed in these three *gra-a* plants (Fig. 6CC–FF). Therefore, these data show that *gra-a* can occasionally produce two shoot apical meristems, which may result from the development of multiple apical meristems or bifurcation of the shoot apical meristem. In addition, *gra-a* plants with one apical meristem appear to develop more axillary tiller buds than *gra-a* plants with two apical meristems.

Genetic map location of *cul2*

To determine the location of *cul2* on the molecular marker linkage map, we mapped *cul2* with RFLP markers. The *cul2* gene has been previously mapped onto chromosome 6(6H) of the barley morphological map (Franckowiak 1996). Furthermore, *rob1*, a gene tightly linked to *cul2*, has been mapped onto chromosome 6(6H) of the barley morphological map (Franckowiak 1996) as well as the barley molecular map (Kleinhofs 1996). Using this information, RFLP markers mapping within 10 to 20 centimorgans (Kleinhofs and Graner 1999) of the *rob1* mutation were chosen to screen the Bowman and *cul2* parents of an F_2 population segregating for *cul2*. Seven RFLP probes were found to be polymorphic for the Bowman-*cul2* and Bowman parents. DNA was extracted from 169 individual F_2 plants and, using DNA gel-blot analysis, hybridized to the seven polymorphic RFLP probes. Linkage analysis positioned *cul2* between ABG458/cMWG679 and KFP128 (Fig. 7). Placement of the *cul2* locus on the RFLP map will facilitate future positional cloning efforts.

Discussion

CUL2 is required for outgrowth of vegetative axillary meristems

Histological sections show that axillary meristems are initiated in *cul2* mutant plants, but these meristems fail to grow out and develop into tillers (Fig. 3). In addition, *cul2* was epistatic to all other genes influencing tillering examined in this research (Fig. 5 and Table 3). Even mutations causing excessive tiller production, like *den6*, *gra-a*, *int-m* and *mnd1*, were unable to stimulate outgrowth of axillary meristems in a double mutant combination with *cul2*. These results show that the CUL2 gene product plays a role in promoting tiller development.

The double mutant combination between *cul2* and *gra-a*, however, occasionally produced two and, more rarely, three shoots (Fig. 5 and Table 3). Further examination of *gra-a* plants, shows that *gra-a* infrequently generates two shoot apical meristems (Fig. 6). These two apical meristems may be the result of the development of multiple apical meristems or bifurcation of the shoot apical meristem. The two or three shoots occasionally formed by *cul2*; *gra-a* double mutant plants are most likely the result of shoot development from multiple *gra-a* apical meristems rather than the development of tillers from axillary meristems. Therefore, *cul2* does not repress the growth of apical meristems.

Other research results further suggest that the wild-type CUL2 gene product plays a major role in the outgrowth of axillary meristems. Woodward and Marshall (1989) observed no stimulation of tiller outgrowth in uniculm barley following the application of plant growth regulators known to cause increased tiller production in cereals. Furthermore, decapitation of the shoot apical meristem in *cul2* plants also fails to release axillary buds (Kirby 1973; Muehlbauer, unpublished results).

CUL2 is required for normal barley inflorescence development

The *cul2* mutation affects the phyllotactic arrangement of spikelets and spikelet development of the inflorescence in both 2-row and 6-row barley. Scanning electron micrographs of developing reproductive *cul2* shoot apical meristems show abnormal spike development most commonly in the form of a clump of central and lateral spikelets in the upper portion of the spike and lack of spikelet development near the distal end of the spike (Fig. 4). Furthermore, development of the *cul2* inflorescence during the double ridge and triple mound stages lags behind wild-type. Similar observations of abnormal

cul2 inflorescence development have been observed previously. *cul2* plants were reported to reach the double ridge stage later than conventional tillering cultivars and produce absent, sterile, and/or distorted spikelets during inflorescence development (Badra and Klinck 1981; Dofing and Karlsson 1993; Dofing 1996). Badra and Klinck (1981) also reported the occurrence of branched rachises, which were not observed in this study.

The variety of novel double-mutant inflorescence phenotypes identified in crosses between *cul2* and some of the other genes influencing barley axillary development provide further evidence of the importance of the wild-type *Cul2* gene during normal barley inflorescence development (Fig. 5 and Table 3). Synergistic interactions during inflorescence development were observed between *cul2* and *als*, *den6*, *int-b*, *lnt1*, *mnd1* and *cul4*. The double-mutant inflorescence phenotypes with *cul2* in combination with mutants which produce significantly fewer tillers (*als*, *int-b*, *lnt1* and *cul4)* and significantly more tillers (*den6* and *mnd1*) than wild-type plants, exhibited very few or no spikelets on the inflorescence. These data suggest that the CUL2 gene product functions in combination with the ALS, INT-B, LNT1, CUL4, DEN6 and MND1 gene products to direct proper axillary development in the inflorescence.

Genetic control of vegetative and inflorescence axillary development in barley

Observations of vegetative and inflorescence phenotypes of the single mutants in this study suggest that the processes of vegetative and inflorescence axillary development in barley are under separate, but not distinct, genetic control. *cul2* mutant plants initiate axillary meristems during vegetative development, but these axillary meristems fail to continue to differentiate into tillers (Fig. 3). During *cul2* inflorescence development, though, most initiated axillary meristems do continue to develop and produce fertile spikelets (Fig. 4). This trend is further observed in the tiller and inflorescence phenotypes of the other mutants in this study. Mutants displaying less vegetative axillary development by producing fewer tillers than wild-type (*als*, *int-b*, *lnt1*, *cul4* and *uzu*) exhibit axillary development in the inflorescence (Figs. 1 and 2). However, variations in spikelet morphology and arrangement in the inflorescences of these mutants, except *cul4*, are present (Table 2 and Fig. 2). Mutants that produce more tillers than wild-type (*den6*, *gra-a*, *int-m* and *mnd1*) tend to show less inflorescence axillary development (Figs. 1 and 2). The *gra-a* mutant is dwarfed, producing a short wild-type-like inflorescence. Both *den6* and *int-m* spikes are shortened and appear to produce fewer kernels in comparison with wild-type spikes. *mnd1*, which can branch in the inflorescence, produces even smaller spikes with far fewer fertile spikelets.

Examination of other genes from grasses that influence branching shows that vegetative branching and inflorescence development are regulated by similar genetic pathways. The maize *tb1* mutation results in an increase in tiller number, and branches from upper nodes are converted from female ears (wildtype) to male tassels (*tb1* mutant). These results indicate that the *Tb1* gene regulates tiller number and also inflorescence development. In addition, Jensen et al. (2001) isolated the perennial ryegrass *TERMINAL FLOWER1*-like gene (*LpTFL*). Overexpression of the *LpTFL* gene in transgenic Arabidopsis resulted in delayed flowering and excessive lateral branching (Jensen et al. 2001). Furthermore, analysis of the *LpTFL* promoter driving the *uidA* gene encoding ß-glucuronidase (GUS) in Arabidopsis showed axillary meristem but not apical meristem GUS expression. These authors suggest that the *LpTFL* gene is a repressor of flowering and a regulator of axillary meristem identity in ryegrass.

Our double mutant analysis showed different genetic interactions in vegetative versus inflorescence development from the same double-mutant combination (Fig. 5 and Table 3). *cul2* was epistatic to all mutants examined for tillering; with the exception of the occasional plant from the *cul2*; *gra-a* double-mutant combination. However, the *uzu* mutant displayed an additive interaction with *cul2* for inflorescence development. In addition, *cul2* was epistatic to *gra-a*, and *int-m* was epistatic to *cul2* for inflorescence development. Synergistic interactions were observed with *cul2* in combination with *als*, *den6*, *int-b*, *lnt1*, *mnd1* and *cul4*. These data show that the same double mutant can exhibit differing genetic interactions during vegetative compared to inflorescence development. Taken together, our morphological and genetic data indicate that there are separate but not distinct genetic pathways for axillary vegetative and inflorescence development.

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