Genetics of Mutations Affecting the Development of a Barley Floral Bract

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Manuscript received February 8, 1999
Accepted for publication November 19, 1999

ABSTRACT

Two groups of mutants that affect the morphology of the lemma, a floral bract of barley, are described. The first comprises phenotypes associated with mutant alleles of *calcaroides* loci. On the lemma of these mutants, a well-organized neomorphic structure is formed, termed the sac. We provide a morphological description of wild-type (WT) and mutant lemmas, based on scanning electron microscopy (SEM), showing that both consist of similar tissues, but that the mutant is characterized by reversed growth polarity. The sac is a unique structure among grasses, and it is remarkable that recessive mutations at five different genetic loci lead to the same organ. The second group of mutants carry recessive alleles of two *leafy lemma* genes, both of which are necessary to cause the transformation of the lemma into a structure having all characteristics of a vegetative leaf, as shown by SEM analysis. The presence of sheath, blade, and ligule in the mutant lemma suggests that wild-type lemma development is interrupted at a leaf-like stage. The genes *cal a*, *b*, *C*, *d*, *23*, *lel1*, and *lel2* have now been mapped at precise positions on linkage groups 2, 7, 7, 3, 7, 5, and 7, respectively. The mutants considered in this article are unaffected in other floral organs. A model for lemma development is suggested.

THE grass leaf develops from a primordium, which grows out from the shoot apical meristem. At later stages of development, the primordium generates files of cells that either extend from the base to the tip of the leaf or produce stem internode tissues (Poethig 1984). The mature leaf consists of the sheath, the blade, and the border between these two domains, the so-called auricle-ligule transition zone. Two leafy organs protect the floret of grasses, the lemma, and the palea, and both are considered to represent reduced vegetative leaves (Arber 1934; Mehlenbacher 1970; Tran 1973; Clifford 1988). The upper part of the lemma forms a long, distal appendage, the awn (see Figure 2).

Several mutations affect the barley lemma. One group of such mutants is characterized by an increase in lemma complexity. The dominant *Hooded* (*K*) mutant (Bossinger *et al.* 1991, 1992) is a member of this group; in this mutant, a flower develops on the lemma in place of the awn. The molecular basis of this phenotype is a mutation in a homeobox gene of the *knox* family (Müller *et al.* 1995). Differences between *Hooded* and wild type (WT) first become apparent as changes in cell size in the adaxial epidermis of the distal part of the lemma, followed by a change in the direction of epidermal cell division (Stebbins and Yagil 1966). Periclinal cell divisions in the subepidermal layer of the *K* awn primordium give rise to an elevated dome from which floral organs differentiate. The orientation of this epiphyllous floret is inverted with respect to the lemma proper (Müller *et al.* 1995). Later, lemma wings grow out at the border between the lemma and the hood. The *calcaroides* (*cal*) mutants of barley (although in several respects phenotypically similar to *K*) display some distinct developmental differences. The name of the mutation derives from the similarity of the mutant lemma to a heel ("*calcar*" in Latin) as observed by Gustaffson (1947) who induced the *calcaroides* phenotype in the Ymer background using γ-rays. A similar mutation was compared previously by Harlan (1931) to *Hooded*. However, this author did not distinguish it clearly from *Hooded* and described the phenotype as subsessile hood. Nötzel (1952) reports the isolation of two X-rays induced mutations that have many similarities with *calcaroides*. Bandlow (1954) divided *cal* mutants more precisely into strongly and weakly affected, the latter being designated *sub-calcaroides*. His genetic results allowed him to propose the existence of two complementation groups. Bandlow also reported the variability in the penetrance of the *calcaroides* phenotype, even among alleles within the two distinct groups.

The mutation *subjacent hood* (*sk*) was isolated by Takahashi *et al.* (1953) and studied by Takahashi and Hayashi (1966). The mutation maps on chromosome 2. Stebbins and Price (1971) claim that *calcaroides* and *subjacent hood* are allelic, and Franckowiack (1995)

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points out that a mutant strain indicated as *cal d* is allelic to *sk*. This gene probably corresponds to the one here described as *calcaroides a1*. The work of Lundqvist and co-workers (Lundqvist 1993) brought order to the genetics of these mutations (see materials and methods). Therefore, the nomenclature used for the collection by Lundqvist and co-workers has been employed in this article.

A description of the *cal* phenotype is provided by Stebbins and Price (1971). The parameters they considered were: mean cell size of the adaxial epidermis at the awn base of WT and *calcaroides* genotypes; the frequency of mitosis *vs.* rate of cell elongation at the base of the awn; the pattern of cell divisions at the base of the awn; and the mitotic index of the dividing cells at the site where the ectopic structure known as sac will develop. They also report the absence of periclinal cell divisions at the base of the mutant awn. Stebbins and Price (1971) postulate an alteration in gibberellin levels during mutant development, a condition that is partly rescued by supplementation of GA_3.

A second type of mutant affecting lemma morphology is represented by the *leafy lemma (lel)* phenotype. This mutant, which illustrates a possible transition step between the vegetative leaf and the lemma proper, was isolated and gross morphology was described for the first time by Bossinger *et al.* (1992).

This article addresses the genetics of lemma development, based on the analyses of *calcaroides* and *leafy lemma* mutants.

MATERIALS AND METHODS

Genetic stocks: *calcaroides* mutants were obtained from U. Lundqvist (Svalöv, Sweden). The mutants were generated by mutagenesis with physical or chemical agents in the genetic backgrounds of the varieties Bonus, Foma, Kristina, and Semira, and (with the exception of *cal 23*) all were assigned to the complementation groups *a*, *b*, *c*, and *d*. The *lel* phenotype was isolated in 1990 at the Istituto Sperimentale per la Cerealicoltura (Fiorenzuola, Italy) in a plot in which the recessive mutant *short awn (lk2*; Kucera *et al.* 1975) was grown. The mutation *lk2* was obtained from the Barley Genetic Cooperative (USDA-ARS, National Small Grain Germplasm Research Facility, Aberdeen, ID). Other genetic strains used were *Hooded* and *Long awn 2* (*Lk2*), obtained from the Barley Genetics Cooperative, and the varieties or breeding strains Havila, Blenheim, Prisma, Carina, Fox, Grit, Aramir, Arda, Panda, Georgie, Gimpel, Angora, FO168A, FO168B, a strain of *Hordeum spontaneum*, Proctor, and Nudinka. All of these genotypes have WT alleles at the *lel* loci. The *K Atlas* strain was made available by G. L. Stebbins (Dept. of Genetics, University of California, Davis, CA).

Complementation tests were carried out by crossing *cal* mutants *inter se* and recording the phenotype of 6–15 F_1 and ~200 F_2 plants. Plants of WT, mutant, and segregating populations were grown in the greenhouse or in the field, either at the Max-Planck-Institute (MPIZ, Köln, Germany) or at the Istituto Sperimentale per la Cerealicoltura (Fiorenzuola, Italy).

Populations used in mutant mapping were generated as described by Castiglioni *et al.* (1998). *cal* and *lel* mutants were crossed to the WT genotypes Proctor and Nudinka, and F_2 populations were generated. F_2 mutant plants were harvested, and DNA from a mixture of 20 seedlings from each selected F_2 plant was used for molecular fingerprinting.

Scanning electron microscopy: Plants were grown in the greenhouse at 18° with 16 hr of light per day. Floral development was monitored starting when the inflorescence was 2–3 mm long, through the lemma primordium stage (7–12 leaves emerged), up to the stage when the spikelet was fully developed. Samples were processed for scanning electron microscopy (SEM) analysis by standard protocols or by the replica technique (Williams *et al.* 1987). The resin-filled replicas were polymerized at 70° for 24 hr, mounted on SEM stubs with epoxy, and sputter-coated with a 25-nm layer of gold (Sputter Coater Balzers SCD 004) in an argon atmosphere at 0.007 mbar and a current of 20 mA. Alternatively, the tissue was fixed in 3% glutaraldehyde, 50% ethanol, and 10% acetic acid at 4° (*cal* analyses) or in 5% formaldehyde, 50% ethanol, and 5% acetic acid (*lel* analyses). Fixed tissue was dehydrated in dimethoxymethane (DMM) for 24 hr and was critical point-dried in a CPD Balzers 030. Samples were coated with gold and were examined with a DSM-940 Zeiss SEM, operated at an accelerating voltage of 10–15 kV. An average of 15 specimens for each *cal* locus have been analyzed at different developmental stages: the triple mound, stamen primordium, awn primordium, and white anther stages, and at the time when the tip of the ear begins to degenerate.

Amplified fragment length and restriction fragment length polymorphisms and sequence-tagged site markers: The procedure and primer nomenclatures adopted for amplified fragment length polymorphism (AFLP) are described in Castiglioni *et al.* (1998). Restriction fragment length polymorphism (RFLP) analysis was done according to Pecchioni *et al.* (1993). RFLP probes were provided by S. Tanksley (Cornell University, Ithaca, NY) and A. Graner (Institut für Resistenzgenetik, Grünbach, Germany); the clones pB11 and pcP387 were obtained from P. Shewry (University of Bristol, Bristol, UK); P27-46 was from D. Bartels (MPIZ, Köln, Germany); and CDR29 was from M. Grossi (Istituto Sperimentale per la Cerealicoltura; Fiorenzuola, Italy). One of 10 sequence-tagged site (STS) markers tested (Tragoonrung *et al.* 1992) revealed a polymorphism between the mutant *lel* and Nudinka, and segregated in linkage with the *lel* phenotype. The primer sequences used to uncover this polymorphism were 5′ ATCCAGTTCTTGTGCACCTG 3′ and 5′ AGCTACGTGGATCACACCAC 3′. PCR reactions were carried out as in Tragoonrung *et al.* (1992), and the amplification products were digested with *Hae*III-*Rsa*I to reveal polymorphisms.

Linkage analysis: Linkage analyses were based on DNA data from F_2 recessive plants as in Castiglioni *et al.* (1998). Where not otherwise specified, the F_2 mutant plants were from crosses with Nudinka. The sizes of the populations homozygous for recessive alleles used in AFLP mapping were 49, 25, 20, 46, 20, 25, and 34 F_2 individuals, respectively, for the alleles *cal a1*, *cal b19*, *cal C15*, *cal d4*, *cal 23* (in crosses with Proctor), *cal 23* (with Nudinka), and *lel*. RFLP studies of *lel* loci were carried out with 38 plants. The use of only mutant plants for linkage mapping minimizes the number of DNA extractions required and maximizes the number of meiotic events screened, even though, in relatively small populations, close linkage between marker and mutant frequently precludes the detection of recombinants. In AFLP analysis, when linkage between a band and a given mutant phenotype was established, the map position of the polymorphism was assigned based on the map of Castiglioni *et al.* (1998); see also the internet site http://www.mpiz-koeln.mpg.de/salamini/. Segregation data for AFLP, RFLP, and STS markers were analyzed with the MAPMAKER program (UNIX version/EXP3; Lander *et al.* 1987)

with LOD score value 3 and maximum distance 50 cM. A virtual marker, showing 100% linkage to the mutant phenotype in F_2 plants, represented the locus of interest.

Markers linked to the *lel* phenotype, which mapped to a specific linkage group, were analyzed independently from markers that were also linked to *lel* but mapped to a different linkage group. The chromosomal assignment of the polymorphic locus revealed by the probe P27-46 was based on bread wheat (cv. Chinese Spring) lines carrying ditelochromosomes of barley cv. Betzes (obtained from A. Islam, University of Adelaide, Adelaide, Australia). The polymorphism revealed by the probe CDR29 was mapped to barley linkage group 5 using dihaploid lines and computerized RFLP data made available by M. Heun (Heun *et al.* 1991).

RESULTS

Genetics: *calcaroides:* Available *cal* mutants were previously assigned to the loci (Lundqvist 1993) *cal a* with alleles *a1, a3, a5, a6, a7, a8, a16, a17, a20*; *cal b* with alleles *b2* and *b19*; *cal c* with allele *C15*; *cal d* with alleles *d4, d14*, and *d22*. *cal 23* was not assigned to a specific locus. The genetics of the 16 *cal* mutants was examined again by monitoring the phenotype of F_1 and F_2 populations. Dominance/recessiveness of *cal* alleles was assessed based on crosses between mutants and the WT varieties Proctor and Nudinka. With the exception of *C15*, all *calcaroides* mutations were recessive to the WT. In a further experiment, the alleles *cal a1, a3, b2, b19, C15, d4, d14*, and *23* were crossed to each other, to WT, and to *K*, and populations of ~150–200 F_2 plants were grown and classified in each case. The results were as follows: (i) Crosses between the alleles *cal a* and *b*, *b* and *d*, and *a* and *d* resulted in WT F_1's; the corresponding F_2 populations segregated with a 9:7 WT to mutant ratio, and almost always with a 3:1 ratio in crosses with Proctor and Nudinka. This result confirms the previous assignment of *cal a*, *cal b*, and *cal d* to separate loci. (ii) F_1 plants from crosses between *cal C15* and alleles of the groups *cal a* and *d* had a mutant phenotype. F_2 plants of the same crosses segregated WT and mutant plants. (iii) In the crosses of *cal C15* to *b2* and *b19*, F_2 WT phenotypes were absent. *cal C15*, *cal b2*, and *cal b19* should thus be considered as alleles of the same genetic locus or as two very tightly linked loci. (iv) All tests involving the mutant *cal 23* indicated that it defines a genetic locus different from *a*, *b*, *c*, and *d*. (v) In the crosses between *K* and *cal a1, a3, b2, b19, C15, d4, d14*, and *23*, F_2 WT plants were detected. This indicates that *Hooded* is not allelic to any of the *calcaroides* loci, a finding

TABLE 1

Dimension of populations, χ^2 value, and their significance in *cal* crosses[a]

	F_1		F_2		
Cross	*cal*	WT	*cal*	WT	χ^2
cal a × WT	0	8	42	148	0.85[b] ($P > 0.3$)
cal b × WT	0	5	58	120	5.46[b] ($P < 0.05$)
cal C × WT	10	0	41	145	0.86[b] ($P > 0.3$)
cal d × WT	0	11	48	158	0.31[b] ($P > 0.5$)
cal 23 × WT	0	6	30	125	2.60[b] ($P > 0.1$)
cal a × *cal b*	0	7	69	75	1.01[c] ($P > 0.3$)
cal a × *cal C*	15	0	116	31	0.52[c] ($P > 0.4$)
cal a × *cal d*	0	10	72	81	0.68[c] ($P > 0.4$)
cal a × *cal 23*	0	15	67	88	0.01[c] ($P > 0.9$)
cal b × *cal C*	14	0	>300	0	∞[c] ($P < 0.01$)
cal b × *cal d*	0	9	75	110	0.77[c] ($P > 0.3$)
cal b × *cal 23*	0	14	118	133	1.08[c] ($P > 0.2$)
cal C × *cal d*	17	0	144	26	1.33[d] ($P > 0.2$)
cal C × *cal 23*	13	0	123	33	0.59[d] ($P > 0.4$)
cal d × *cal 23*	0	15	60	98	1.94[d] ($P > 0.1$)
	F_2 plants		F_2 WT plants		
cal a × *K*[e]	99		>15		
cal b × *K*[e]	96		>15		
cal C × *K*[e]	91		7		
cal d × *K*[e]	79		>15		
cal 23 × *K*[e]	80		>15		

[a] The data refer to crosses for alleles *cal a1*, *cal b19*, *cal C15*, and *cal 23*; the WT and *K* genotypes used in the crosses indicated are Nudinka and *K Atlas*, respectively.

[b] χ^2 values are given for the expected ratio of 3:1 (WT:*cal*).

[c] χ^2 values are given for the expected ratio of 9:7 (WT:*cal*).

[d] χ^2 values are given for the expected ratio of 13:3 (WT:*cal*).

[e] Presence of WT plants in F_2 supported the absence of allelism between *cal* and *K* mutants.

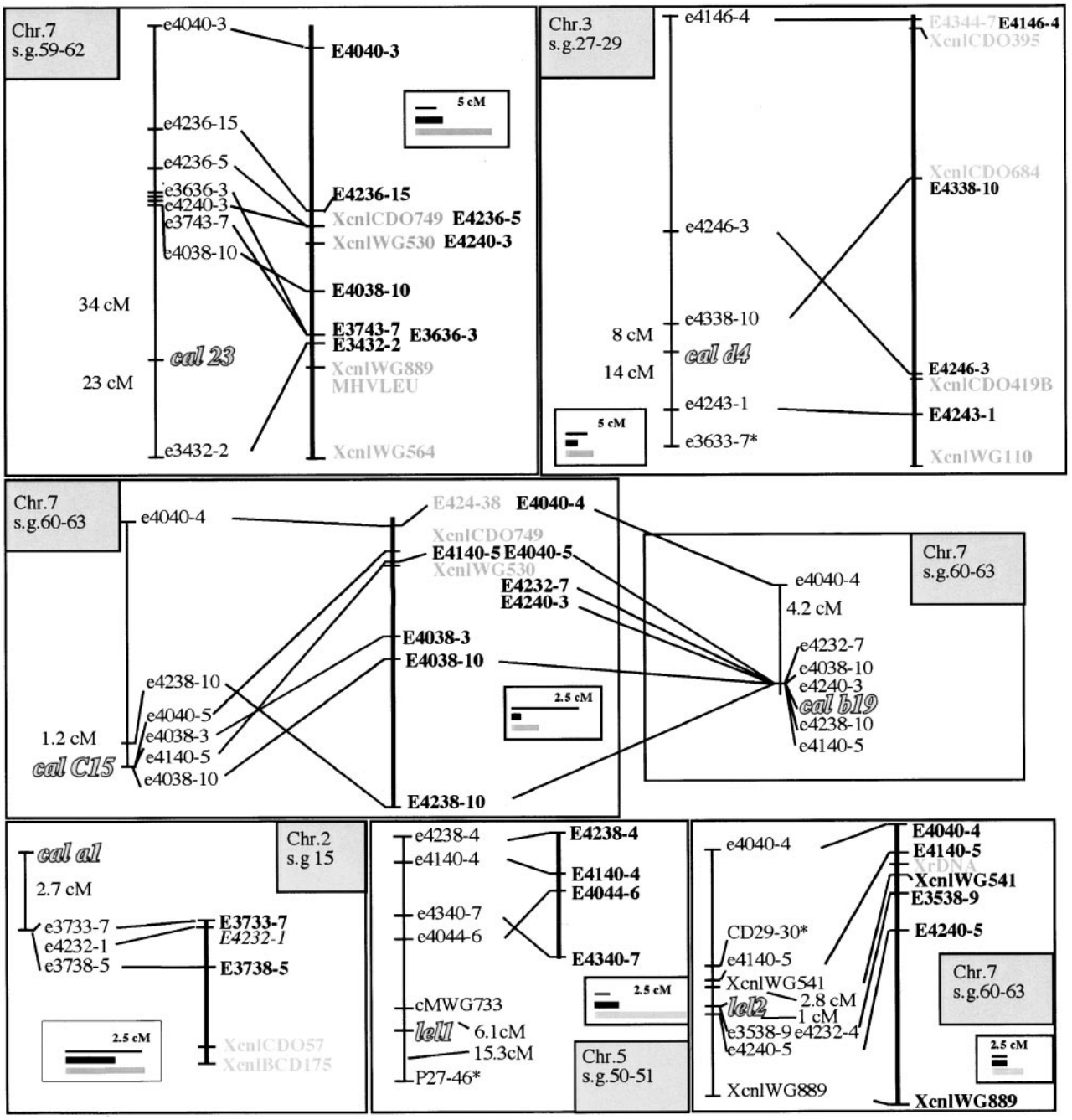

Figure 1.—Map positions of *cal* and *lel* loci based on marker segregation in the crosses analyzed here (thin vertical lines), compared to sublinkage (s.g.) and linkage groups as defined in Castiglioni *et al.* (1998; thick vertical bars on the right). The horizontal bars in boxes are distance scales, for the *cal* or *lel* × WT map (thin line); Proctor × Nudinka (P × N) map (thick line); P × N map calculated with the MAPMAKER Error Detection option (gray line). Shaded markers are AFLPs or RFLPs for which linkage to *cal* loci has not been tested. Positions of the markers in the "*cal* map" and in the Castiglioni *et al.* (1998) map are connected. The asterisks indicate markers not mapped in the P × N map.

consistent with the mapping data presented later in this article. Examples of crosses with *cal a1*, *cal b19*, *cal C15*, and *cal 23* are reported in Table 1.

Leafy lemma: When *lel* plants were crossed to four WT genotypes, the F_1 plants were phenotypically WT, and the corresponding F_2 populations segregated 2409 WT and 179 *lel* plants. The $\chi^2_{15:1}$ value for the segregation was equal to 2.9 ($P > 0.10$). It was concluded that the *lel* phenotype in the cross analyzed was caused by two independently segregating mutations. The genetic loci controlling the *lel* phenotype are thus referred to as *lel1* and *lel2*. A further experiment clarified why it was possible to isolate the *lel* mutant in the *short awned* mutant strain *lk2*. The F_2 population of the *lel* × *lk2* cross

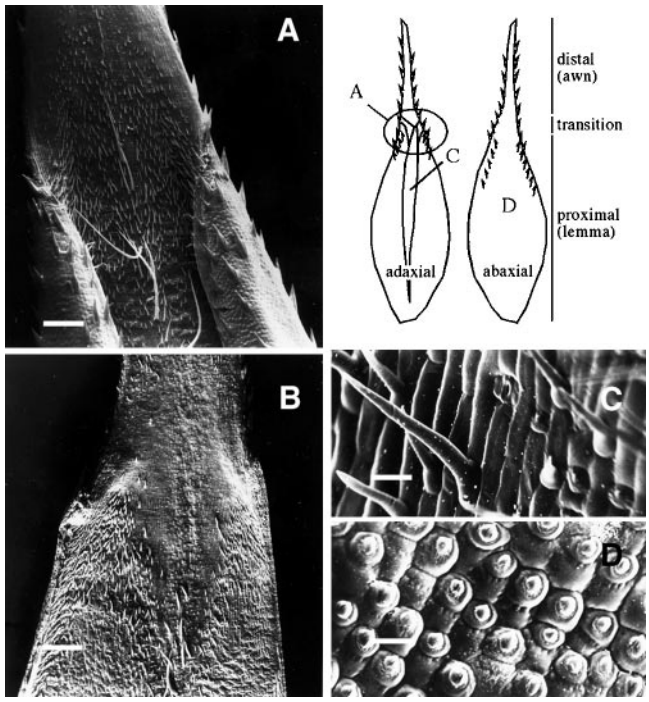

Figure 2.—WT lemma. The letters in the drawing refer to the positions of SEM micrographs. (A) Transition region between lemma and awn, where long hairs grow along the lemma rib. (B) Same region in the *cal a1* mutant. The row of hairs is split into two, as is evident near the initiation point of the dome. (C and D) Adaxial and abaxial surfaces, respectively, of the WT lemma. Stomata are visible between long hairs in A. Bars: A, 290 μm; B, 246 μm; C, 22 μm; D, 26 μm.

segregated 144 *Lel* and 51 *lel* plants (3:1 ratio; $\chi^2_{3:1} = 0.15$, $P > 0.75$), supporting the conclusion that the *lk2* strain was homozygous for a recessive mutation at one of the two *lel* loci (named *lel1*). In the cross between *lel* and *Lk2*, F_2 segregation pattern of 181 *Lel* and 5 *lel* plants was observed. In this population, the number of plants with the *lel* phenotype was even less than that expected for a 15:1 segregation ratio, but the $\chi^2_{15:1}$ value was just within the bounds of significance. These results suggested that *lel1* may be a recessive allele of the *lk2* locus. The allelic state at the two *lel* loci was tested in several barley varieties and breeding strains, and all F_2 populations analyzed segregated in agreement with a 15:1 ratio for *Lel* to *lel*. This result is also compatible with the assumption that *lk2* is allelic to *lel1*. This hypothesis, which must still be proven definitively, explains why the *lel* mutant was isolated only in the *lk2* strain, and why it was not reported previously in lists of available barley mutants (Søgaard and von Wettstein-Knowles 1987).

Mapping: To map *cal* and *lel* loci, RFLP, STS, and AFLP markers were used. F_2s were produced from crosses between lines bearing alleles of *cal* and *lel* loci and the WT genotypes Nudinka and Proctor. Mutant F_2 plants were selected and were used in linkage studies. In the case of the dominant *cal C15* mutant, WT F_2 plants were analyzed. Linkage data are summarized for all mutant loci in Figure 1. In this figure, the positions of markers derived from the map of Castiglioni *et al.* (1998) that are linked to the mutant loci are also included. Marker orders in the two maps are consistent, with few exceptions. These discrepancies were expected considering the relatively low number of F_2 plants used in this study, as well as the local ambiguity of marker order in very dense linkage maps (discussed in Castiglioni *et al.* 1998).

cal a1 mapped on linkage group 2, 2.7 cM distal to markers e3733-7, e3738-5, and e4232-1. The three markers did not recombine in our cross, and are located in a 2-cM interval on the map of Castiglioni *et al.* (1998).

cal b19 mapped on chromosome 7, 4.2 cM from marker e4040-4 (two recombinants among the plants studied, coupling configuration), and showed no recombination with markers e4238-10, e4140-5, or e4038-10 (repulsion), or with e4232-7 and e4240-3 (coupling). The interval defined by these markers spanned ~6 cM. The region between markers e4040-4 and e4238-10 in the map of Castiglioni *et al.* (1998) encompassed 25 cM when calculated with the MAPMAKER Error Detection command.

cal C15 was linked to markers e4040-4 (five recombinants, repulsion configuration), e4238-10 (one recombinant, repulsion), e4140-5, e4038-10, e4040-5, and e4038-3 (all with no recombinants, repulsion). Markers e4040-4 and the cluster of five markers cited above were separated by 8 cM. Mapping results thus support the conclusion that the recessive *cal b* alleles and *cal C15* map to two very tightly linked loci or represent alleles of the same genetic locus.

cal 23 mapped on chromosome 7, linked to e3432-2 (7 recombinants, coupling), e3743-7 (8 recombinants, coupling), e4038-10, e3636-3, and e4236-5 (11 recombinants, coupling), e4236-15 and e4240-3 (8 recombinants, coupling), and marker e4040-3 (12 recombinants, coupling). The region was delimited by markers e3432-2 and e4040-3. Based on complementation tests, *cal 23* and *cal b* alleles defined two distinct genetic loci. To test the extent of linkage between *cal 23* and *cal b*, the *cal 23* × *cal b19* F_2 population was analyzed. In the case of absence of recombination between the two loci, a 1:1 segregation ratio was expected. The ratio actually observed, in a total of 251 plants, was not statistically different from 9:7, suggesting the absence of close linkage between the two loci (Table 1).

cal d4 mapped on chromosome 3 in the region defined by the markers e4146-4 and e3633-7. The locus was linked to markers e4146-4 (21 recombinants, coupling configuration), e4246-3 (19 recombinants, coupling), e3633-7 (2 recombinants, repulsion), e4338-10 (7 recombinants, coupling), and e4243-1 (10 recombinants, coupling).

We attempted to localize the two *lel* genes using an RFLP approach on 38 F_2 *lel* plants derived from the

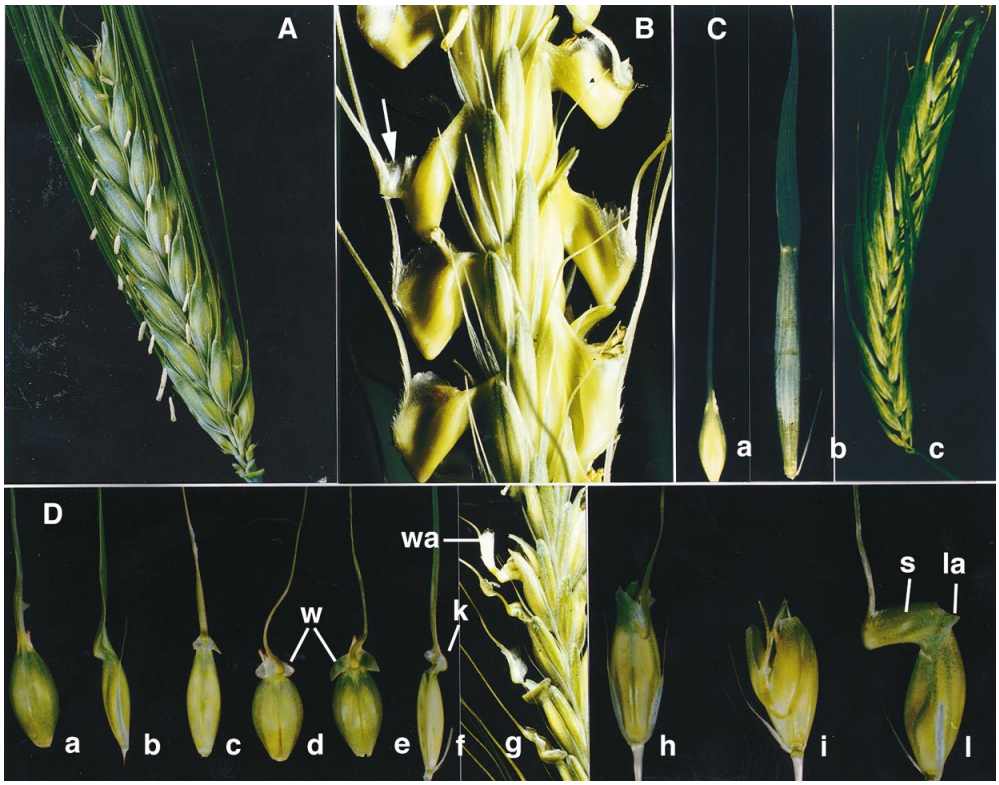

Figure 3.—Gross morphology of the *calcaroides* and *leafy lemma* inflorescence and floret phenotypes, as compared to the WT illustrating the phenotypes associated with different *cal* alleles (details in the text). (A) WT spike. (B) *cal 23* spike. The arrow points to a sac appendage, a fringe of tissue developing at the base of the sac awn. (C, a) WT flower; (C, b) *leafy lemma* floret, in which the transition and distal zones are similar to a leaf blade. (C, c) *leafy lemma* spike. (D) Different degrees of penetrance of the *calcaroides* phenotype: wings, wings on the awn, lateral appendages, and the sac are indicated. s, sac; w, wing; la, lateral appendages; k, knee; wa, wings on awn.

cross *lel* × Nudinka. The assumption was that two groups of markers would deviate from random segregation, those linked to *lel1* and *lel2*. RFLP and STS markers distributed across barley linkage groups in the Proctor × Nudinka map of Heun *et al.* (1991) and in the Igri × Franka map of Graner *et al.* (1991) were first tested for their capacity to reveal polymorphism between the parents of the mapping populations. Among the markers that revealed polymorphism, STS marker Pstl-337, the RFLP markers XcnlWG541, XcnlWG889, and cMWG733, and the cDNA marker P27-46 defined genetic loci tightly linked to the *lel* phenotype. Pst1-337, XcnlWG541, and XcnlWG889 were known to be associated with chromosome 7 (Heun *et al.* 1991; Tragoonrung *et al.* 1992). They define a chromosomal region in which one of the two putative *lel* genes was located: to this gene the symbol *lel2* was assigned based on the results of crosses between RFLP-fingerprinted F_2 *lel* plants (from the cross with Nudinka) and the *lk2* strain. The designation *lel* was already assigned to the recessive allele in this strain (see above). The AFLP analysis of 34 F_2 *lel* plants derived from the cross *lel* × Nudinka confirmed the location of *lel2*: this locus maps on chromosome 7 in the region defined by AFLP markers e4240-5 and e4140-5. *lel2* is linked to markers e4240-5 and e4140-5 (no recombinants, repulsion configuration), e3538-9 and e4232-4 (no recombinants, coupling).

The marker cMWG733, also linked to the *lel* phenotype, is mapped by Graner *et al.* (1991) to linkage group 5. This chromosome was thus considered as the putative site of the *lel1* gene. Two cDNA markers, P27-46 and CDR29, were also found to be linked to the *lel* phenotype. Since these markers had not yet been mapped, further experiments were carried out. The polymorphism revealed by the marker P27-46 was initially assigned to chromosome 5 based on the 12 barley ditelosomic addition lines of wheat (Islam *et al.* 1981). The wheat addition lines bearing the barley chromosomes 1, 2, 3, 4, 6, and 7 had the characteristic RFLP pattern of Chinese Spring. We inferred that marker P27-46 was located on linkage group 5 (the wheat addition lines for the barley chromosome 5 were not available; Islam *et al.* 1981). The marker CDR29 revealed polymorphism between Nudinka and *lel*, as well as between the WT varieties Nudinka and Proctor. A total of 75 dihaploid lines from the cross Nudinka × Proctor (Heun *et al.* 1991) were tested with the marker CDR29. The locus defined by this probe was assigned to linkage group 5, a location confirmed by a further experiment: two hordein loci defined by the probes pB11 and pcP387, both located on chromosome 5, were also found to be linked to CDR29. Based on AFLP analysis, *lel1* mapped on chromosome 5 and was linked to the AFLP markers e4140-4 (five recombinants, repulsion), e4340-7 (three recombinants, repulsion), e4044-6 (two recombinants, repulsion), and e4238-4 (five recombinants, coupling).

Morphology: *The WT lemma:* The lemma comprises three distinct domains: a basal part, a transition zone between this and the awn, and the awn itself. The lemma

TABLE 2

Description of *cal* alleles and WT[a]

Locus	Allele	Recessive (r) *vs.* dominant (d)	Sac	Wings	Sac appendages	Awn wings	Ectopic structures on leaves	Leaf knots	Leaf curling
cal a	*cal a1*	r	++ (apical)	++	−	−	−	+	+
	cal a3	r	−	++	−	−	−	−	−
	cal a5	r	+++	+ (basal)	−	−	−	−	−
	cal a6	r	+	+++ (apical)	−	−	−	+	−
	cal a7	r	−	+++	−	−	−	−	−
	cal a8	r	+++	++ (basal)	−	−	−	−	−
	cal a16	r	+++[c]	−	−	−	−	+	++
	cal a17	r	−	+	−	−	−	+	−
	cal a20	r	+++[c]	−	−	−	−	+	++
cal b	*cal b2*	r	−	−[b]	−	−	−	++	++
	cal b19	r	−	−[b]	−	−	−	++	++
cal C15	*cal C15*	d	+	++	−	++	−	++	−
cal d	*cal d4*	r	+++	++ (basal)	−	−	−	−	+
	cal d14	r	+++ (apical)	++	−	+	+	−	++
	cal d22	r	+++	−	−	−	−	−	+
cal 23	*cal 23*	r	+++	−	++	++	−	−	−
WT	*Nudinka*	−	−	−	−	−	−	−	−

[a] For each genotype, 10–20 plants were analyzed at the developmental stages mentioned in materials and methods.
[b] Absence of wings but presence of the knee (see text).
[c] In some florets, the sac is absent, leaving only the knee.
+, Presence of the ectopic structure indicated; −, absence; +, ++, and +++, increasing degree of expression of the ectopic structure; basal, restricted to the first three to five basal spikelets of the ear; apical, confined to the apical part of the ear.

is inserted on the rachilla (the spikelet axis) and, together with the palea, encloses the floret (Figure 2). SEM analysis indicated that the adaxial, "internal," surface of a differentiated lemma is made up of different cell types and bears several types of hairs. The abaxial part was smooth with few hairs. On the adaxial surface of the transition zone, long and short hairs were present. As seen in Figure 2, A and C, long hairs were concentrated along the midrib, extending to the basal part of the awn; short hairs flanked long hairs. Distally, on the adaxial lemma, patches of short hairs formed two rows internal to the lemma folds (Figure 2A). Stomata were present between short and long hairs (Figure 2C), but not where hairs were absent. The central and proximal regions of the midrib were characterized by long hairs and files of stomata. The abaxial lemma surface was made up of compact cells with hair primordia (Figure 2D), and files of short hairs were present along the keels and on the proximal margin of the folds (Figure 2, diagram).

The calcaroides lemma: At the tip of the lemma proper, in a position corresponding to the transition between lemma and awn in WT, *calcaroides* mutants bear a well-organized ectopic structure, the sac (Figure 3B; Figure 3A shows the WT ear). In contrast to the *Hooded* phenotype (Stebbins and Yagil 1966), in *cal* plants the sac does not develop into an epiphyllous flower. Moreover, in *cal* mutants it bears a distal awn.

The first morphological difference that arises between the lemma primordia of awned and *calcaroides* genotypes is marked by a change in the overall length of the organ. According to Stebbins and Price (1971), at this stage the cells located at the base of the awn are observed to divide transversely more frequently than in the WT (*i.e.*, the spindle is oriented transversely to the long axis of the awn), while periclinal divisions are almost absent. This leads to alterations in cell distribution and organization: the files of cells produced by transversal cell divisions and arranged perpendicularly to those present on the lemma proper will result in the formation of the sac. When analyzed by SEM, this ectopic structure showed several remarkable features: it was quite constant in position on different ears of a given mutant, and it consisted of tissues common to other organs of the barley inflorescence.

The development of the sac varied in different *cal* mutants, as revealed by repeated comparative observations. When the phenotype associated with all 16 alleles of *cal* loci was considered, a phenotypic series could be constructed. For example, in *cal 23* an almost completely developed sac was present on every floret (Figure 3B), while in *cal b2* frequently only an enlargement of the basal third of the awn was evident (Figure 3D, a and b). All other *cal* phenotypes fall between these two extremes. In *cal a3, a6, a7,* and *a17,* only a few florets of the ear carried malformations. These alleles, like those of the *cal b* and *d* loci, are associated with the formation of pronounced wings (Figure 3D, c–f). The sac, more-

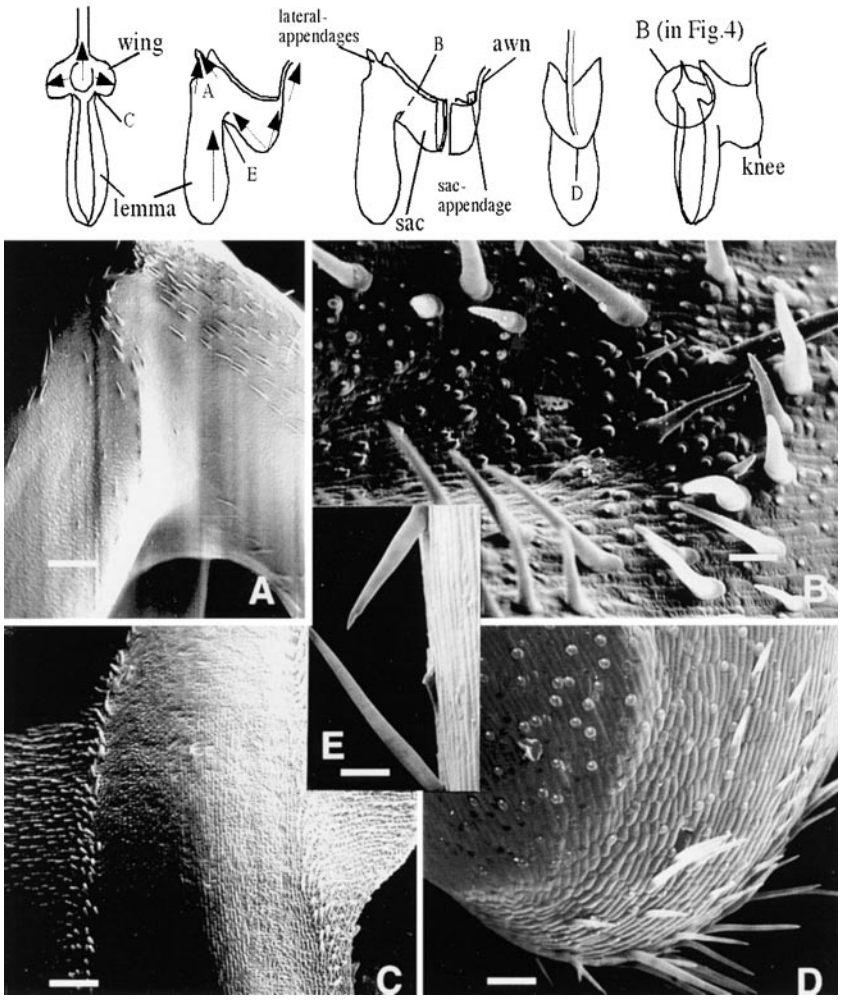

Figure 4.—SEM view of the major morphological consequences of the inversion of polarity in the *cal* phenotype. The letters in the scheme refer to the positions of micrographs. The arrows in the drawing indicate the orientation of hairs. (A) Abaxial lateral view of the lemma (left) and sac (right). The orientation of the hairs indicates the polarity of growth. (B) Adaxial view of the region where the inversion of growth polarity occurs between the lemma (left) and the sac (right) tissue. (C) Abaxial view of the wings, marked by the presence of hairs growing at right angles to those of the awn tissue (central part of the picture). (D) Abaxial view of the knee. The hairs are oriented acropetally, while in the proximal part of the sac they grow basipetally, *i.e.*, oriented toward the lemma-sac interface (see drawing for details). (E) Detail of the proximal region of the sac, at the interface with the lemma. The tissue of the sac (lower hair) is opposite to the tissue of the lemma (upper hair). Bars: A, 200 μm; B, 44 μm; C, 127 μm; D, 75 μm; E, 38 μm.

over, in some cases was found together with the other ectopic structures listed in Table 2: wings, two protruding structures located at the base of the awn (Figure 3D, b–f); sac appendages (arrow in Figure 3B); wings on awns as in *cal C15* (one example is shown in Figure 3D, g); ectopic tissue formation on vegetative leaves; leaf knots and leaf curling. At maturity, in some instances the sac was almost as large as the lemma, and two lateral appendages developed at the border between the lemma and the sac (Figure 3D, h–l). In some flowers, wing-like structures formed at the interface between the sac and the awn.

SEM analysis revealed that the abaxial surface of the *cal* lemma and sac consisted of cells typical of the WT lemma, *i.e.*, compressed in shape and with short hair primordia. The salient observation concerning the mutant lemmas was that on the proximal part of the sac the hairs were oriented in a direction opposite to that seen on the lemma proper (Figure 4, drawing and A). In the same position, this change in growth polarity was also seen on the adaxial lemma surface (Figure 4B). The abaxial surface of the wings bears hairs that are directed acropetally to the awn / wing interface and then become oriented towards the tip of the wing (Figure 4, drawing and C). The orientation of cell growth along the abaxial surface of the lemma-sac complex was as follows: (i) on the lemma: acropetal; (ii) on the distal part of the sac: acropetal, pointing towards the awn (Figure 4D); (iii) on the proximal part of the sac: basipetal, pointing towards the lemma (Figure 4E); and (iv) on the awn: acropetal. Thus, two inversions of the direction of cell differentiation were evident: the first occurs at the proximal border of the sac, and the second occurs at the knee, the tip of the sac. Further details on the orientation of sac tissues are presented in the legend to Figure 4. The adaxial surface of the sac was characterized by several cell types and tissue patterns. In early phases of development of the ectopic structure, the midrib-associated row of long hairs on the lemma bifurcated at the sac border, while short hair primordia characterized the tissue between lemma and sac (Figure 2B).

In mutant florets with ectopic wings only, the cells of the wings were growing in a direction perpendicular to that of awn cells (Figure 5A). In florets in which a complete sac develops, the inversion of differentiation polarity was also evident on the adaxial surface, in the region where the lemma tissue, marked by compressed cells and hair primordia, impinged on the sac tissue,

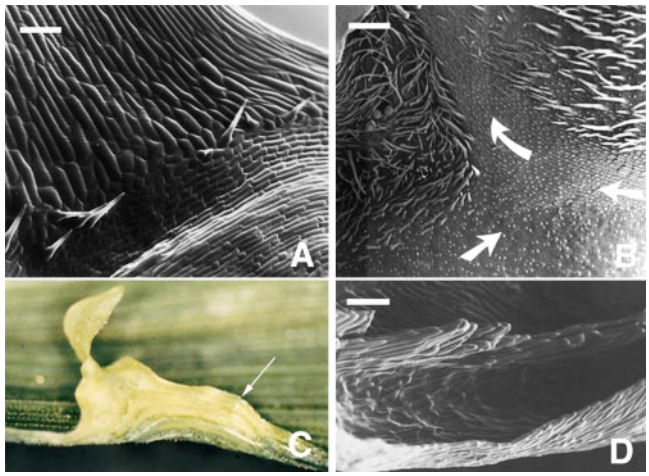

Figure 5.—Adaxial view of wing and lateral appendage tissues. (A) Wing tissue, with elongated cells oriented perpendicular to the awn tissue (lower part of the picture). Hairs are present at the interface between awn and wing. (B) Lateral appendage, as diagrammed in the scheme in Figure 3. Three tissues are evident: compact cells of the lemma (bottom part, indicated by the leftmost arrow) that impinge on the sac tissue (rightmost arrow); more compact and hairy cells of the sac, directed toward the tip of the appendage (upward arrow); hairy tissues, with opposite polarity, of the lemma and the sac (left and right, respectively). (C) Prong of thin tissue on the barley leaf of mutant *cal d14*. The picture was taken on the blade, ~5 cm from the ligule/auricle. The arrow indicates the position where micrograph D was taken. (D) SEM view of the ectopic fringe on the blade, as in C. Bars: A, 97 μm; B, 263 μm; D, 200 μm.

which has long hairs and larger cells (Figure 4B). The sac wings consisted of two alternating tissues: one hairy with stomata and the other made up of compact cells (Figure 5B). Long hairs and stomata on the midrib, as seen on the internal surface of the sac, were also characteristic of the lemma surface. In both tissues, the cells were elongated and lacked crenulations (not shown). An awn develops at the distal end of the sac (Figure 3D, l).

Ectopic tissue also formed on vegetative leaves of some *calcaroides* mutants (Table 2), and consisted of a prong of thin tissue displaying a pale yellow color. The position of this tissue on the leaf blade was variable and could be present close to the auricles (just increasing the auricle surface) or in the middle of the leaf laminae (Figure 5, C and D), and it could be present on both sides of the blade or on one side only.

Leafy lemma: Wild-type and *lel* phenotypes of ear and lemma are shown in Figure 3, A and C (a–c). The WT lemma terminates with the awn. The *lel* lemma has a basal zone that is wider and more elongated, a transition zone, and a distal domain similar in shape to a leaf blade (Figure 3C, b). The overall shape of the *lel* lemma recalls that of the typical grass leaf: sheath and blade are separated by the auricle-ligule zone.

In all crosses analyzed, F_2 *lel* plants always had awnless lemmas. In *lel* plants, the caryopsis was longer than normal and was partially naked; there was a tendency for the rachillas to bear more than one floret, and ear internodes were longer. SEM analysis of the base of the *lel* lemma showed that this region is almost identical to that of the wild type (Figure 6, b). The transition zone of the *lel* lemma is marked by a fringe similar to a rudimentary ligule (Figure 6, t). At the tip of this structure, the cells were separated from each other and were elongated in shape, a morphology typical of mature ligules of vegetative leaves (Figure 6, li). Distal to the *lel* fringe, columns of cells within a transversal area protruded from the adaxial lemma surface (Figure 6, arrow). These cells characterize the upper zone of the *lel* lemma, which had all the features of a leaf lamina (Figure 6, u and bl): elongated cells and hairs not restricted to the longitudinal rib and different from those of the awn. The basal part of the WT and *lel* lemma resembled the epidermis of the sheath of the vegetative leaf (Figure 6, b and sh), while the lemma transition zone of *lel* is similar to the ligule-auricle region. The vegetative WT leaf lamina was almost identical in tissue pattern to the distal part of the *lel* lemma.

DISCUSSION

The results presented in this article (i) correct the previous genetic analysis of *cal* (*cal C* is reported to segregate WT plants in the F_2s with *cal b*; Lundqvist 1993); demonstrate (ii) the existence of the new complementation group *cal 23*; (iii) the very tight linkage between *cal b* and *cal C* mutations; (iv) the dependence of the *leafy lemma* phenotype on two genes; (v) the precise mapping of all *cal* and *lel* loci to linkage groups; and provide (vi) the SEM analysis of *cal* and *lel* phenotypes. These data clarify the genetics of these two groups of lemma mutations, and they offer a basis for a developmental interpretation of the barley lemma. Discrepancies with previously published data concerning the linkage between *cal b* and *cal C* loci (Lundqvist 1993) are attributable to difficulty in correctly assigning to F_2 plants the *cal* phenotype. In fact, *cal C15* often displays a low level of penetrance. This is the reason why we have taken particular care to carefully classify in several repeated crosses the phenotypes in F_2. Our genetic conclusions on segregation data of *cal b* × *cal C* crosses are consistent with the mapping data of the two mutations.

In grasses, leaf and lemma are considered to be homologous organs (Clifford 1988). In barley mutants like *leafless (lfl)*, for example, the leaf undergoes a lemma-like modification (Tsuchiya 1969). The foliar origin of the lemma is also apparent in the *lel* phenotype: lemma and awn are transformed into a vegetative leaf, reduced in size but with sheath, blade, and ligule. The notion that the awn represents at least in part a modified leaf blade (Dahlgren *et al.* 1985) and the lemma proper a modified leaf sheath (Clifford 1988) can thus be

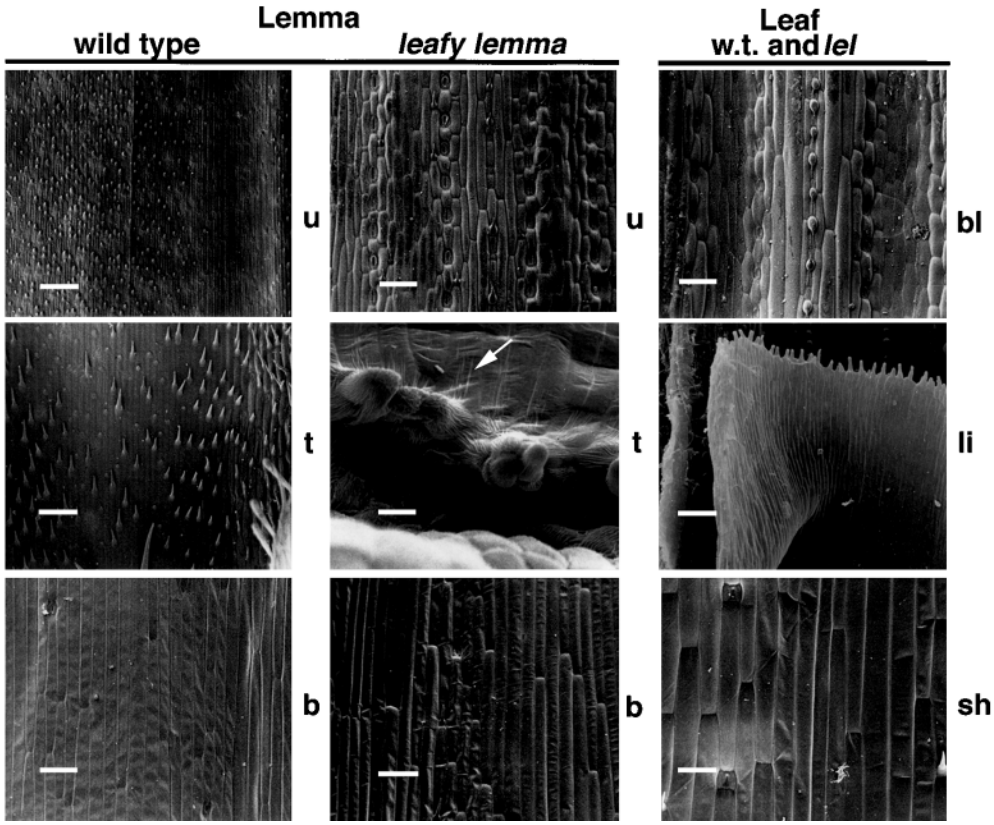

Figure 6.—SEM views of *leafy lemma* and WT lemma, compared to leaf and ligule. Lemma and leaf are described separately, with the two columns on the left side depicting the lemma. (b) Basal region of the lemma in WT and *leafy lemma*. (t) Transition zone between lemma and awn in WT and *leafy lemma*. A fringe of tissue indicates an incipient ligule in *leafy lemma*. The arrow points to cylindrical cells above the ectopic ligule. (u) Upper, distal region of the lemma. The *leafy lemma* has a leaf-like character, as is evident by comparison with WT leaf. (sh) WT and *leafy lemma* leaf sheath. (li) Developing ligule at the border between leaf sheath and blade in WT and *leafy lemma*. (bl) WT and *leafy lemma* leaf blade. Bars: all pictures, except *leafy lemma* (t) and leaf (li), 100 μm; t, 20 μm; li, 200 μm.

considered correct. Our data indicated that mutations in only two genetic loci are sufficient to control the evolutionary step that transforms a photosynthetic organ into a protective one. This case illustrates how second-site mutations contribute to unravelling developmental pathways, even when complex gene networks and gene redundancy are involved. The existence of the *lel* phenotype establishes that one alternative to lemma development is its regression to a leaf-like state.

In *cal* mutants, the sac reveals a further developmental possibility. This organ, which is unusual in the grass family, is organized quite precisely. It is remarkable (i) that the tissues that contribute to the sac have attributes typical of those of floral bracts; and (ii) that mutations at five different loci give rise to the same ectopic structure. This supports the conclusion that the genetic program leading to sac formation represents a developmental alternative for the barley lemma.

The *cal* sac structure shows similarity to the hood of the *K* mutant, which bears epiphyllous flowers originating from an ectopic meristem organized at the transition zone of the lemma. The homeobox gene *knox-3*, encoded at the *K* locus, has the capacity to induce epiphyllous structures on the leaves of transgenic tobacco (Müller *et al.* 1995). Moreover, the *K* ortholog *Kn1* from maize reproduces the hood when expressed in barley (Williams-Carrier *et al.* 1997). Additional cases of epiphylly are provided by the *Hsfl-0* (Bertrand-Garcia and Freeling 1991; Schichnes and Freeling 1998) and *Lxm1-0* mutants in maize. In the latter, a whole ectopic leaf flap arises symmetrically around a lateral vein, without any evidence for a meristem or a primordium. In several respects the *cal* sac can be considered a lemma-like appendage of the lemma proper, organized in the absence of a visibly active meristem; it can still be considered a case of leaf epiphylly, albeit a very special instance. An alternative interpretation would consider the new organ to be the result of a cryptic morphogenetic pathway that is revealed only when the normal developmental pathway of the lemma is disrupted. This hypothesis would consider *calcaroides* as negative regulators of *knox* genes. A similar role has been proposed for the *rough sheath2* gene of maize (Tsiantis *et al.* 1999). The observation that recessive mutations at several loci generate the same neomorphic organ is new. In plants, neomorphic mutations affecting characters of the leaf or of leaf-homologous organs are mainly dominant. This is the case for maize mutations that cause ectopic expression of a homeodomain protein (Freeling 1992; Smith *et al.* 1993; Schneeberger *et al.* 1995; Fowler and Freeling 1996) or the formation of the spur in Aquilegia petals, which depends on mutation(s) in one or two genes (Prazmo 1965). In this latter case, although the gene involved has alleles with reverted phenotypical effects (*i.e.*, only the dominant alleles support the presence of the spur), the spur is a structure quite similar to the sac of *calcaroides* mutants. In *Antirrhinum majus*, the semidominant mutation *Hir-*

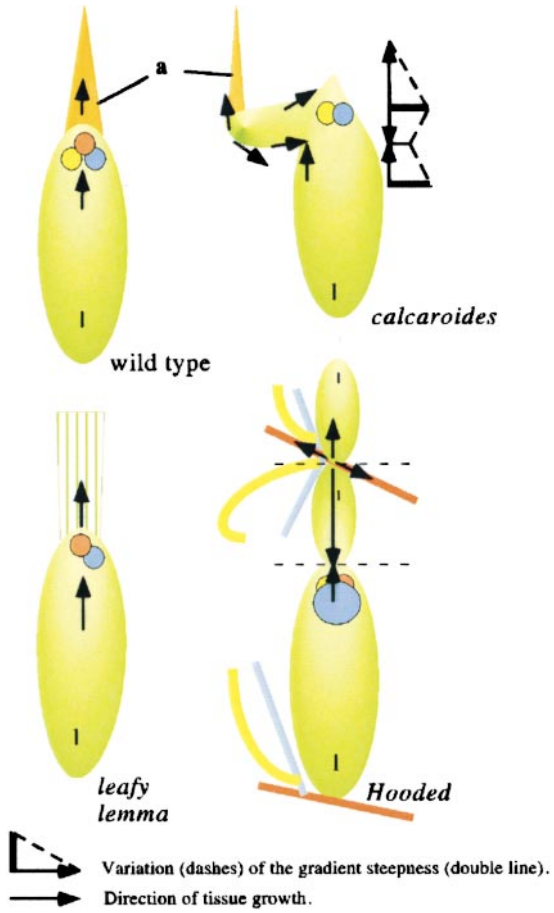

Figure 7.—Summary of the possible alternative states of development of the barley lemma, depending on the local inhibitory action of *lel* and *cal* genes, the expression of *Knox* genes, and putative gradients of morphogens. Red line: rachilla; yellow: palea; blue: floret axis; green to yellow gradient: putative morphogen gradient; red circle: local inhibitory action of *cal* gene products; yellow circle: local inhibitory action of *lel* gene products; blue circles: site and increasing levels of local expression of *Knox* gene(s) in the lemma primordium; a, awn; l, lemma.

zina also shows the neomorphic formation of a spur (Stubbe 1966). The sac, although dependent on recessive alleles, is an attribute of the lemma that has not yet been reported for wild-type grass species (Arber 1934; Dahlgren *et al.* 1985).

The *cal* sac and the lemma proper consist of very similar tissues. But the two organs show (in *cal* mutants as well as in *K*) opposite directions of growth; *i.e.*, a change in tissue polarity is involved. A cell is polarized when some of its physiological or developmental processes are biased along one preferred direction. The same term also refers to preferential expression of features along a specialized axis of a multicellular structure (Sachs 1991). Plant patterning, in many instances, has been attributed to the early establishment of prepatterns of morphogens (discussed in Meinhardt 1996). In the transition domain of the *cal* lemma, modification of a gradient triggered by the downregulation of patterning genes may generate new gradients, resulting in switches in polarity and reorganization of developmental patterns.

In summary, it is concluded that the lemma / awn transition region has a special capacity to resume cell division, leading to neomorphic patterning of the organ. The corresponding transition region of a normal leaf (the petiole to lamina in dicots or the sheath to lamina domains in monocots) has similar local attributes, as revealed by phenomena like epiphylly or activity of "groove meristems" (Bell 1991), as well as by ectopic effects induced precisely in this region by homeobox genes expressed in transgenic plants (Sinha *et al.* 1993; Müller *et al.* 1995; Chuck *et al.* 1996). In *lel* and *cal* alleles, as well as in the *leafless* mutant (Tsuchiya 1969), the identity of floral organs is not changed; this may indicate that the grass lemma may not belong to the floret perianth.

The model we provide in Figure 7 summarizes alternative developmental states of the barley lemma. The model considers the generation of tissues with inverted polarity as being the result of the interaction between growth and gradients of morphogens (discussed in Lawrence 1992; Bohn 1974). Auxin, due to its synthesis localized in meristems and subsequent polar transport (Sachs 1991), and levels of expression of homeobox genes active in meristem origin and maintenance may play a role in this process (Jackson *et al.* 1994).

We thank N. Pecchioni for his assistance in the analysis of the *lel* mapping data, and S. Effgen and D. Pagani for their excellent technical assistance. We also thank the Istituto di Genetica Vegetale, Universitá Cattolica del S. Cuore Piacenza, for providing SEM facilities for *lel* analysis. We are grateful to U. Lundqvist for her collaboration. C.P. received a European Community Grant (contract no. BIO4CT965023); K.J.M. was sponsored by the German Research Ministry (BMBF); and P.F., V.T., and A.M.S. were in part supported by MiPA, Special Project "Biotecnologie Vegetali." Part of this work was sponsored by the European Gramineae Mapping Project.

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Communicating editor: V. L. Chandler