Copyright © 2004 by the Genetics Society of America

Genetics of Barley *Hooded* Suppression

Cristina Roig,*[,1] Carlo Pozzi,*[,1,2] Luca Santi,* Judith Müller,* Yamei Wang,* Maria Rosaria Stile,* Laura Rossini,† Michele Stanca‡ and Francesco Salamini*[,3]

**Max-Planck-Institut für Züchtungsforschung, 50829 Cologne, Germany,* †*University of Milan, 20133 Milan, Italy and* ‡*Istituto Sperimentale per la Cerealicoltura, 29017 Fiorenzuola d'Arda, Italy*

Manuscript received August 4, 2003
Accepted for publication January 9, 2004

ABSTRACT

The molecular basis of the barley dominant *Hooded* (*K*) mutant is a duplication of 305 bp in intron IV of the homeobox gene *Bkn3*. A chemical mutagenesis screen was carried out to identify genetical factors that participate in *Bkn3* intron-mediated gene regulation. Plants from recurrently mutagenized *KK* seeds were examined for the suppression of the hooded awn phenotype induced by the *K* allele and, in total, 41 *suK* (*su*ppressor of *K*) recessive mutants were identified. Complementation tests established the existence of five *suK* loci, and alleles *suKB-4*, *suKC-33*, *suKD-25*, *suKE-74*, and *suKF-76* were studied in detail. All *K*-suppressed mutants showed a short-awn phenotype. The *suK* loci have been mapped by bulked segregant analysis nested in a standard mapping procedure based on AFLP markers. *K* suppressor loci *suKB*, *B*, *E*, and *F* all map in a short interval of chromosome 7H, while the locus *suKD* is assigned to chromosome 5H. A complementation test between the four *suK* mutants mapping on chromosome 7H and the short-awn mutant *lks2*, located nearby, excluded the allelism between *suK* loci and *lks2*. The last experiment made clear that the short-awn phenotype of *suK* mutants is due to a specific dominant function of the *K* allele, a function that is independent from the control on hood formation. The *suK* loci are discussed as candidate participants in the regulation of *Bkn3* expression.

THE floret of grasses is protected by two leafy organs, the lemma and the palea, both representing reduced vegetative leaves (Arber 1934; Dahlgren *et al.* 1985; Clifford 1988; Pozzi *et al.* 2000). In several species of the family, the upper part of the lemma develops into the awn, a long distal appendage. In barley, several mutants are known in which the development of the lemma is affected (discussed in Pozzi *et al.* 1999). Among these are the recessives *calcaroides* and *leafy lemma* (Pozzi *et al.* 2000) and the dominant mutant *Hooded* (Stebbins and Yagil 1966). In *Hooded* plants, an extra flower develops at the site of transition between lemma and awn. In this region, the mutant differs from the wild type (WT) in the size of the cells of the adaxial epidermis and in the direction of cell division (Stebbins and Yagil 1966). In the subepidermal layer of the *K* awn primordium, periclinal divisions generate a dome, from which ectopic floral organs differentiate in an inverted orientation with respect to the lemma proper (Müller *et al.* 1995). The molecular basis of this phenotype is a mutation in the homeobox gene *Bkn3*, which is a member of the *knox* plant homeodomain family (Müller *et al.* 1995).

In plants, *knox* genes play an important role in the establishment and development of leaf primordia (Bharathan and Sinha 2001). Some of them, referred to as class I (Kerstetter *et al.* 1994), drastically alter the meristematic activity and the shape and compoundness of leaves when expressed in transgenic plants (Müller *et al.* 1995; Chuck *et al.* 1996; Parnis *et al.* 1997; Lin and Müller 2002). A particular feature of the barley *Bkn3* gene (Müller *et al.* 1995) and of its maize ortholog *Kn1* (Vollbrecht *et al.* 1991) is that dominant, homozygous viable mutations are associated with DNA insertions in the large intron (IV in barley, III in maize). The importance of this noncoding region as the putative regulatory region was recently put forward (Inada *et al.* 2003; Santi *et al.* 2003). This noncoding region makes it possible to screen for second-site mutations to identify loci coding for factors that putatively participate in intron-mediated regulation of *Bkn3*. This article describes such a suppressor screen, together with the mapping to linkage groups of the concerned loci.

MATERIALS AND METHODS

Plant material and generation of *K*-suppressed lines: The barley line *K*BGS152 [Barley Genetics Stock Center (BGSC), Fort Collins, CO) is homozygous for the dominant allele *K* of the *Hooded* locus and for the recessive allele *vrs1* of the *Two/six row spike* locus. The line was propagated at the Max-Planck-Institut für Züchtungsforschung (MPIZ, Köln, Germany) and was used for the suppressor screen.

[1]These authors contributed equally to this work.

[2]*Present address:* Fondazione Parco Tecnologico Padano, via Haussmann 7, 26900 Lodi, Italy.

[3]*Corresponding author:* Max-Planck-Institut für Züchtungsforshung, Carl-von-Linnè-Weg 10, D-50829 Cologne, Germany.
E-mail: salamini@mpiz-koeln.mpg.de

Three consecutive mutagenic treatments were carried out. In the first one, 500 g of seeds were soaked for 18 hr at 5° in H_2O and treated for 2 hr with 0.001 M sodium azide (NaN_3) in 0.1 M phosphate buffer (pH 3.0) at room temperature. Seeds were washed in running tap water for 30 min and sown in the field. Single ears were harvested from each M_0 plant, and 2697 M_1 families of 10 plants each were grown at the Istituto Sperimentale per la Cerealicoltura, Fiorenzuola, Italy. Families segregating plants in which the *K* phenotype was suppressed were identified and single non-*Hooded* plants were harvested, while remaining lines were harvested in bulk. A seed sample of this bulk harvest was again mutagenized. The second treatment, involving $\sim 7 \times 10^4$ seeds, was carried out at the MPIZ using NaN_3 under the conditions described. The M_2 families that did not segregate for the WT-reverted phenotypes were mutagenized a third time, using approximately the same number of seeds. About 4.5×10^4 plants were harvested in bulk after the second and third mutagenic treatments and phenotypes other than *vrs1* and *K* were discarded during field selection. Field mutagenesis was carried out without ear bagging.

Two lots of 8×10^4 seeds, obtained from the third mutagenic treatment, were germinated in micropots (96-well plates of 50×33 cm with wells 2 cm in diameter and 2 cm deep) under greenhouse conditions. Each plant produced from one to three fertile seeds and ear morphology was sufficiently clear to allow selection of *K*-suppressed lines.

The *K*Atlas strain was made available by G. L. Stebbins (Department of Genetics, University of California, Davis, CA). The wild type (WT) lines Nudinka (ears with two rows), Vogelsanger Gold (six rows), and Atlas (six rows), used in genetic studies and phenotypic comparisons, were from the MPIZ collection. The *lks2* mutant was from the BGSC.

Identification of second-site *K* suppressors: The crossing program was conducted by manual cross-pollination: two ears were emasculated for each cross and fertilized using anthers of the appropriate genotype. A total of 6–18 F_1 seeds were harvested from each cross and F_1 plants were grown for phenotypic assessment. When necessary, F_2 seeds were harvested from single F_1 plants for further genetic analysis. Molecular fingerprinting and testcrosses were carried out to eliminate contaminants due to accidental outcrossing and to distinguish between intragenic and second-site mutations, respectively.

Amplified fragment length polymorphism (AFLP) molecular profiles diverging even marginally from those of the mutagenized *K*BGS152 line were assumed to originate from contaminants. Contaminating lines were found to have awn length comparable to that of WT lines. Moreover, contaminants derived from *Vrs1* (two-rowed ear) pollen were also easily detected, because the phenotype conferred by this allele is dominant.

Testcrosses were conducted by crossing *K*-suppressed lines to the WT line Nudinka (genotype *kk*). The symbol *suK* was assigned to extragenic suppressor, the lowercase initials indicating the recessive nature of the suppressing alleles. The genotype of these suppressed lines is indicated as *suK suK*, *K K*, the WT condition being *SuK SuK*, *k k*. Dominance was assessed through crosses of *K*-suppressed lines with *K*BGS152 and with *K*Atlas.

Complementation tests and phenotypic comparisons of *suK* lines: For *suK* lines, complementation tests were carried out using various crossing schemes. Each cross was repeated up to six times, and each repetition generated from 4 to 28 F_1 seeds. All F_1 plants were grown to maturity and their lemma phenotypes were recorded. A further validation of the complementation test was carried out on the basis of the analysis of F_2 populations. This concerned a specific set of crosses among *suK* mutants mapped to chromosome 7H, sublinkage groups 5–7. Their F_2 generations were grown and the presence of *K* and *suK* phenotypes controlled, as well as their segregation ratio.

F_2 plants or F_3 segregating families from the cross *suK* × *K*Atlas were used to compare plant and ear traits of *suK* and *K* phenotypes. Plants were grown in 20-well plastic plates of 50×33 cm in the greenhouse or in the field in rows of plants spaced 10 cm apart with 20 cm between rows. In the same F_2 populations, grown in either the greenhouse or the field, segregation ratios were also recorded.

The allelism test between *suK* mutants and the *lks2* line was carried out by analyzing the F_1 and F_2 generations, as described for the suppressor lines. In this case, the phenotypic description included the measurements of the length of the awn considering those of the more distal spikelets of the ear. For awn length, three classes were considered (Table 4): A, long awn; B, short awn (awn length 2–8 cm; control genotype was *lks2*, with an average awn length of 7.5 cm, evaluated in 19 plants; *suKB-4* was 5.1 cm in 12 plants; *suKC-33* was 5.9 cm in 15 plants; *suKD-25* was 5.1 cm in 15 plants; *suKE-74* was 4.3 cm in 13 plants; *suKF-76* was 6.5 cm in 15 plants); C, WT or almost awnless (9–15 cm; control genotype was the variety Proctor, awn of 12.9 cm evaluated in 15 plants, and the variety Nudinka, 13.7 cm in 15 plants).

Amplified fragment length polymorphism analysis: Populations used in mapping experiments were generated from crosses between *suK* mutants and *K*Atlas and between *K*Atlas or *K*BGS152 and the WT line Nudinka. F_2 plants were harvested, DNAs were extracted (DNeasy plant mini kit, QIAGEN, Hilden, Germany) and pooled if necessary or used as such for molecular fingerprinting. The procedure and primer combinations used for AFLPs were as described in Castiglioni *et al.* (1998).

Mapping of *suK* loci: The mapping procedure described by Castiglioni *et al.* (1998) was used and integrated as follows. Mutants representative of each of the different complementation groups were crossed with *K*Atlas. Homozygous *suK suK*, *K K* (awned lemma) or *SuK SuK*, *K K* (*Hooded* lemma) F_3 lines were harvested. AFLP analysis of 15 bulked DNAs for both types of lines allowed the identification of amplified fragments in linkage with the *suK* loci. In the next step, the *suK*-linked AFLP fragments were amplified from single F_3 lines. Four to seven AFLP loci were sufficient to define a map around each *suK* locus; loci were not considered with genetic distances among each other if exceeding 10 cM.

To position the *suK*-linked AFLP loci into the map of Castiglioni *et al.* (1998), crosses were performed between the *K* genotypes *K*BGS152 and *K*Atlas and the WT line Nudinka. In these crosses, linkage of at least two *suK*-linked AFLP loci with AFLP bands already mapped by Castiglioni *et al.* (1998) was established for each *SuK* locus.

The segregation data were analyzed with the MAPMAKER program (UNIX version/EXP3; Lander *et al.* 1987) with a LOD score value of 3 and a maximum distance of 50 cM. A virtual marker, showing 100% linkage to the mutant phenotype in F_2 plants, represented the locus of interest.

RESULTS

Isolation and complementation analysis of extragenic *suK* mutants: The large dimension of the barley genome made it necessary to design a recurrent mutagenesis experiment during which only mutants in the M_2 generation were selected. A total of three recurrent mutagenic treatments, conducted on $\sim 1.6 \times 10^5$ *KK* seeds, yielded 41 second-site suppressed mutants, exhibiting an awn

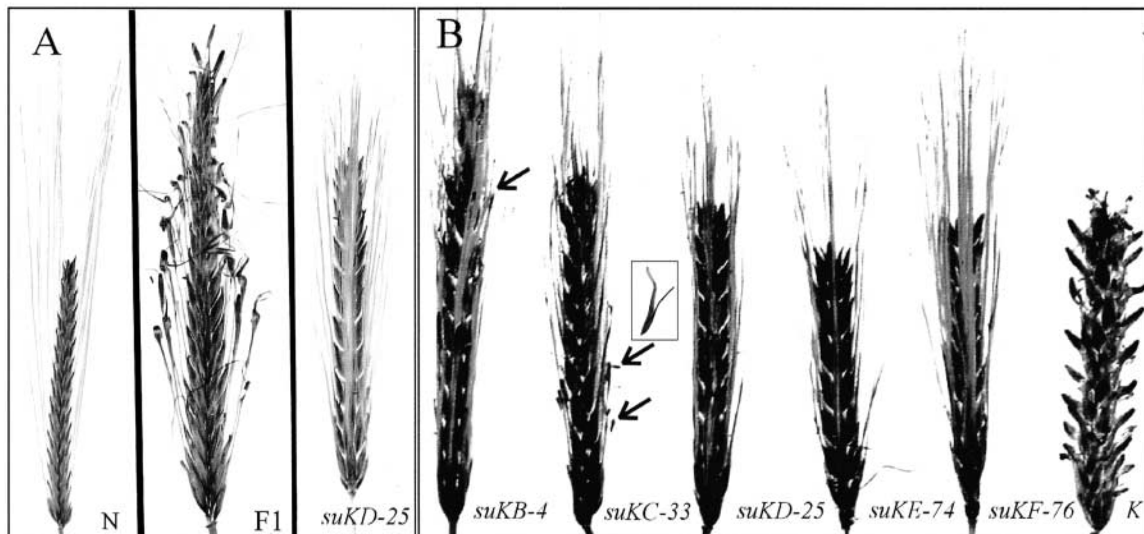

FIGURE 1.—(A) Phenotypic analysis supporting the nonintragenic nature of the *suK* mutant with respect to the *K* locus. The appearance of the *Hooded* phenotype in F_1 supports the recessivity for the *suK* locus. Thus, the genotype assigned to the mutant line is *suKD-25 suKD-25*, *K K* and to the WT variety, Nudinka *SuKD-25 SuKD-25*, *k k*. N, WT variety Nudinka. (B) Short-awn phenotype of *suK suK*, *K K* mutant compared to *Hooded K*BGS152 (*K*). Arrows, instability of the *suK* phenotype in mutants *suKB-4* and *suKC-33*.

in place of the extra floret present on the lemma of *Hooded* plants. AFLP fingerprinting of all the isolated WT revertants confirmed that they represent *suK* mutations as opposed to pollen contaminations. Testcrosses were conducted to distinguish among intragenic and extragenic suppressors of the *K* phenotype. Contaminants and lines bearing *Bkn3* intragenic mutations were expected to generate *kk* F_1 plants with the non-*Hooded* phenotype, while extragenic suppressor lines were expected to generate F_1's with the *K* phenotype (Figure 1A). Testcross analysis allowed the identification of the mutants *suK4*, *suK9* to *suK35*; *74* to *78*; and *81* to *86*, *88*, and *89* as representing extragenic suppressors of the *K* phenotype.

Diallelic F_1 populations: Sets of diallelic crosses were allowed to conclude allelism for 37 mutants (*suK9* to *32*, *34*, *35*, *77* to *79*, *81* to *86*, *88*, and *89*). The result was achieved in two steps: at first, six mutants were found allelic upon phenotypic inspection of the F_1 generations of all their possible pairwise crosses. A second diallelic cross, involving one of the previous six mutants and an additional four, again revealed complete allelism, suggesting the predominance among the isolated mutants of alleles belonging to one locus. Eventually, *suK* 25 and 28, representative of this locus, were crossed to all remaining mutants: 37 of 41 *suK* resulted in being allelic and the locus was designated *suKD*. The four mutants that complemented *suKD* were tentatively assigned the symbols *suKB-4*, *suKC-33*, *suKE-74*, and *suKF-76*. The assumption that they represented alleles of four different complementation groups was verified by diallelic crosses among the mutant lines *suKB-4*, *suKC-33*, *suKD-25*, *suKE-74*, and *suKF-76*. All the resulting F_1 plants were *Hooded*, supporting the conclusion that the 41 *suK* mutants represented five different complementation groups. For the loci *suKB*, *C*, *E*, and *F*, only one allele was recovered.

Allelism tests in F_2 populations: Allelism of those *suK* mutants mapping to a short region of chromosome 7H was controlled also in the F_2 generation. Short-awn, non-*Hooded* F_2 populations were expected in the case of allelism among *suK* mutants. Because of the tight linkage existing between the genetic loci involved, a shift in the segregation ratio from 9 *K* and 7 *suK* to 1 *K* and 1 *suK* was expected. F_2 data were available for the crosses *suKB-4* × *suKC-33* [the result of the segregation was 34 *K* and 45 *suK*; $\chi^2_{1:1} = 1.53$, statistically not significant (NS)], *suKB-4* × *suKE-74* (66 *K* and 56 *suK*; $\chi^2_{1:1} = 0.82$, NS), *suKB-4* × *suKF*-76 (88 *K* and 85 *suK*; $\chi^2_{1:1} = 0.05$, NS), and *suKC-33* × *suKF-76* (94 *K* and 84 *suK*; $\chi^2_{1:1} = 0.56$, NS). It was concluded also that F_2 generation analysis indicated that the *suK* mutants mapping to chromosome 7H represented four different genetic loci.

Phenotypic and genetic analyses of *suK* mutants and of their F_1 hybrids: Crossing of representative mutants belonging to the different complementation groups with the line *K*Atlas (Table 1) supported the conclusion that each locus behaved according to Mendelian rules, with suppression occurring only when the recessive alleles were homozygous. In the F_2's, the homozygous recessive class was slightly underrepresented, particularly for *suKB-4* and *E-74*. In all mutants, the length of the awn was reduced by ~50% compared to WT. The suppressed phenotype was unstable in that few lemmas,

TABLE 1

Segregation ratios recorded in F_2 populations derived from crosses between *suK* mutants (genotype *suK suK, K K*) and *K*Atlas (*SuK SuK, K K*)

suK parent	No. of plants *K*	No. of plants *suK*	$\chi^2_{3:1}$
suKB-4	2311[a]	674	9.33**
suKC-33	282[b]	92	0.03***
suKD-25	177	54	0.32***
suKD-25	257[b]	84	0.02***
suKE-74	148[c]	33	4.42*
suKE-74	309[b]	69	9.17**
suKF-76	38[c]	12	0.03***
suKF-76	255[b]	98	1.44***

*, **, significant (respectively, $P < 0.05$ and $P < 0.01$); ***, not significant ($P = 0.05$).
[a] Field data, 1999.
[b] Greenhouse data, 2000.
[c] Greenhouse data, 1999.

from otherwise fully suppressed ears, developed a rudimentary extra floret (arrows in Figure 1B). This phenotype was analyzed in detail for mutants *suKB-4*, *C-33*, *D-25*, *E-74*, and *F-76*. Only *suKB-4* and *suKC-33* showed an intraplant phenotypic penetrance <100%, at least when plants were grown under field conditions. The incomplete penetrance of *suKC-33* was more obvious in ears produced from lateral tillers (data not shown). When *suK* mutants were crossed to the WT two-rowed Nudinka line, the resulting *suK SuK, k K* F_1 plants had lemmas carrying extra florets in a more elevated position as compared to *K*BGS152 (Figure 1A). The *Hooded* phenotype recorded in complementing F_1's involving *suKB-4* showed a small percentage of lemmas with a rudimentary awn, suggesting that this allele is not completely recessive. All other complementing F_1's were indistinguishable from *K*BGS152. Homozygous *suK* plants from *suK* × *K*Atlas crosses were smaller when compared to *K* plants (Table 2) and characterized by reduced spike length and number of fertile nodes. However, only *suKE-74* was significantly different from *K* in plant and ear characters.

Mapping *suK* loci to barley linkage groups: Data derived from *suK* × *K*Atlas F_2 populations allowed the construction of an AFLP map around each *suK* locus. The segregation of the same AFLP *suK*-linked markers in the crosses between *K* and WT (Nudinka) genotypes made it possible to precisely position these AFLP loci ("bridge markers") in the Nudinka × Proctor map of Castiglioni *et al.* (1998).

The suppressor loci *suKB*, *C*, *E*, and *F* all mapped to barley chromosome 7H in linkage subgroups 5, 6, and 7 (Figure 2A). The locus *suKD* was assigned to linkage group 5H between linkage subgroups 66 and 67 (Figure 2B). A summary of the data on bridge AFLP markers, providing their distances in centimorgans from *suK* loci, is in Table 3.

The unexpected localization of all four nonallelic *suK* mutants, *B-4*, *C-33*, *E-74*, and *F-76*, to a short linkage region of chromosome 7H was confirmed by AFLP analysis in the *K*Atlas × Nudinka cross (Figure 2A). Loci *suKF-76* and *suKE-74* were separable by crossing over as indicated by their distal and proximal, respectively, recombination with the AFLP marker E41M46-5. A similar observation was made for *suKE-74* and *suKC-33* and the AFLP marker E42M43-2. On the basis of recombination data, the possibility cannot be ruled out that *suKC-33* and *suKB-4*, although complementing, are alleles of the same genetic locus.

The short awns of *suK* mutants suggest a test of allelism to the short-awn mutant *lks2*: On the basis of mapping information, the short-awn mutant *lks2* was a candidate to correspond to one of the four *suK* loci mapping on chromosome 7H, sublinkage groups 5–7. In fact, the marker loci E35M40-1 and E34M40-4, which are tightly linked to the *lks2* locus (Pozzi *et al.* 2003), also mapped very near to *suKF-76* and *suKC-33* (Figure 2A).

A complementation assay based on F_1 and F_2 generations of *lk2* × *suK* crosses was designed to test the allelic state of the two types of mutants (Table 4 and Figure 3). The phenotypes recorded in the F_1 (genotype *Lks2 lks2, SuK suK, K k*) were unexpected in that the *lks2*

TABLE 2

Plant and ear characters (average values ±SD) measured in populations segregating *suK* and *K* phenotypes from crosses between *suK* mutants and the line *K*Atlas

Mutant	No. of plants per phenotypic group	Plant height (cm) *suK*	Plant height (cm) *K*	Spike length (cm) *suK*	Spike length (cm) *K*	No. of fertile nodes per ear row *suK*	No. of fertile nodes per ear row *K*
suKB-4	80	56.7 ± 9.4	63.2 ± 10.2	5.8 ± 1.4	5.9 ± 1.2	5.9 ± 1.6	5.9 ± 1.6
suKC-33	81	52.3 ± 10.5	55.0 ± 12.5	4.8 ± 1.4	4.8 ± 1.5	5.0 ± 1.9	5.3 ± 2.1
suKD-25	49	53.7 ± 10.2	54.9 ± 13.4	3.9 ± 1.4	4.6 ± 2.2	4.9 ± 2.2	5.1 ± 2.1
suK E-74	49	54.8 ± 11.8	67.8 ± 13.6	3.9 ± 1.4	5.7 ± 1.1	3.9 ± 1.8	6.3 ± 2.1
suKF-76	63	48.2 ± 10.2	57.8 ± 12.7	3.8 ± 1.2	4.9 ± 1.8	3.3 ± 1.6	5.4 ± 2.3

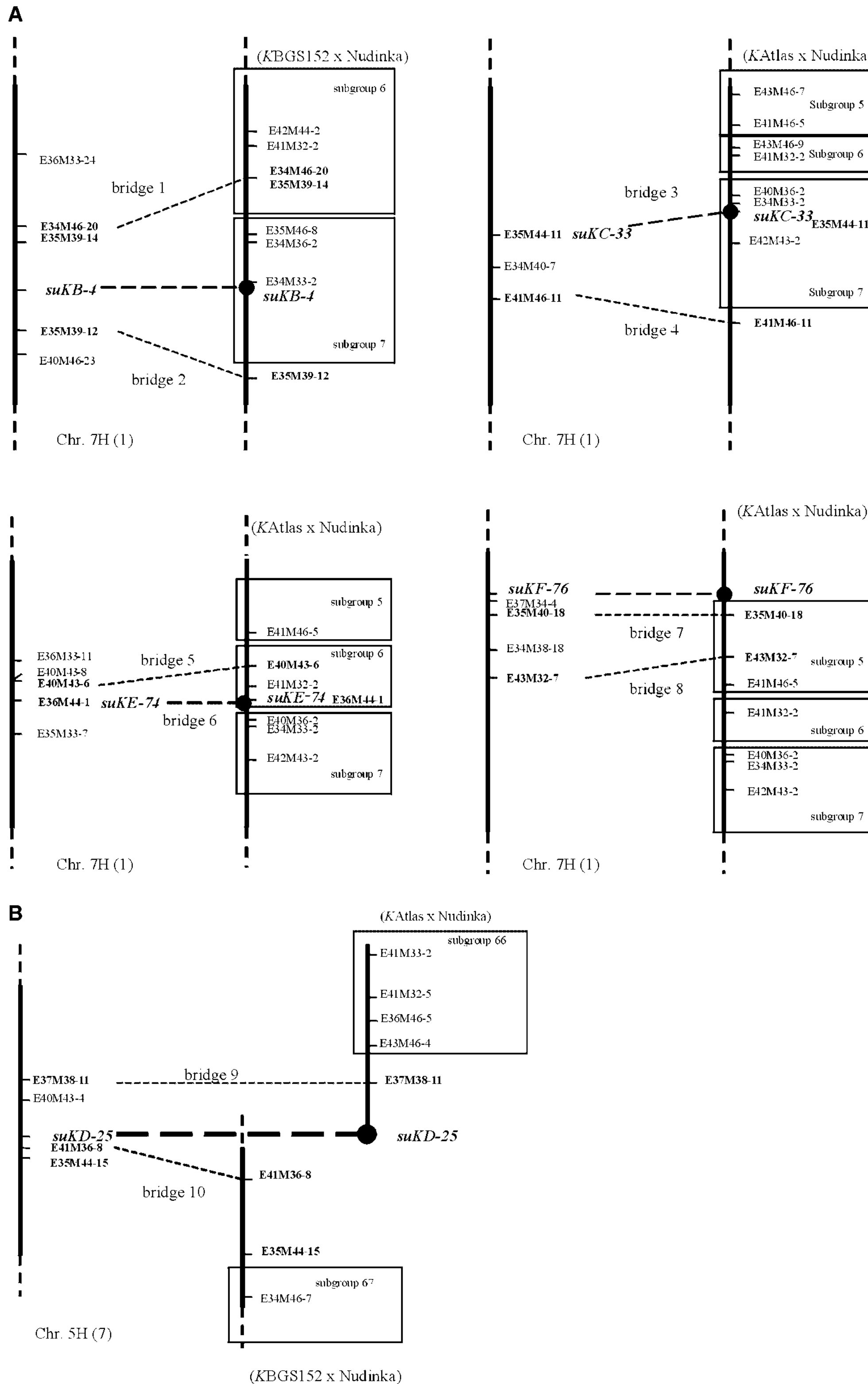

Figure 2.—Assignment of *suK* loci to linkage groups and subgroups. (A) Mapping of *suKB*, *C*, *E*, and *F* loci by AFLP markers to a short region of chromosome 7H. Bridge markers are joined by a transversal dotted line and are mapped in the crosses *suK* × *K*Atlas (left) and *K*Atlas × Nudinka or *K*BGS152 × Nudinka (right). AFLP markers not in boldface type and included in the "subgroups" boxes are also mapped in the Proctor × Nudinka population (Castiglioni *et al.* 1998) and can be used as links to the restriction fragment length polymorphism integrated map of Castiglioni *et al.* (1998). (B) Mapping of the *suKD* locus. Bridge AFLP markers have been mapped via *K*Atlas × Nudinka and *K*BGS152 × Nudinka crosses.

allele demonstrated that it could suppress, to a different degree, the formation of the hood in *K k* plants in the presence of a WT *SuK* allele. For the same F_1, a short-awn phenotype, even in the presence of the long awn dominant allele *Lks2*, was noted and interpreted as due to a dominant effect of the *K* mutation (see below and discussion). Under this assumption, in addition to the control of hood formation, *K* should provide a second specific function related to the reduction of awn length.

In detail, F_1 plants of the crosses between *lks2* and *suKB-4* and *lks2* and *suKC-33* resulted in nonhooded with short-awn lemmas (Figure 3B). F_1 plants from *lks2*

TABLE 3

Size of the populations used in *suK* mapping, bridge AFLP markers, and assignment of *suK* loci to linkage and sublinkage groups

Locus	Size of F_2 population: *suK* × *K*atlas	*K*Atlas × Nudinka	*K*BGS15 2 × Nudinka	Bridge AFLP markers[a]: No.	Symbol		Linkage-sublinkage group[b]	Distances (cM): To AFLP bridge marker[c]	Between AFLP bridge markers
suKB	85		85	1	E34M46	20 N	7H-6	16.9	45[e]
				2	E35M39	12 S	7H-7[f]	9.9	
suKC	30	69		3	E35M44	11 C	7H-7	0.0	26[d]
				4	E41M46	11 S	7H-7[f]	7.8	
suKE	33	69		5	E40M43	6 N	7H-6	5.6	8.5[d]
				6	E36M44	1 C	7H-6	0.0	
suKF	32	69		7	E35M40	18 S	7H-5	5.3	17.8[d]
				8	E43M32	7 S	7H-5	22.4	
suKD	32	69	85	9	E37M38	4 N	5H-66 or 67[f]	15.9	36.1[d,e]
				10	E41M36	8 S	5H-66 or 67[f]	1.1	

[a] N, distal (north in the map of Castiglioni *et al.* 1998) with respect to the *suK* locus; S, proximal (south); C, cosegregating marker. The numbering of AFLP markers is as in Figure 2.
[b] Linkage group numbering is as in Pozzi *et al.* (2003).
[c] Data from the cross *suK* × *K*Atlas.
[d] Data from the cross *K*Atlas × Nudinka.
[e] Data from the cross *K*BGS152 × Nudinka.
[f] Marker positioned at the border of a sublinkage group.

crosses with *suKE-74* and *suKF-76* were less sensitive to *lks2* repression of hood formation, showing a very elevated hood phenotype (Figure 3C). The presence of short-awn characters in at least two different F_1's did not allow the establishment of the complementation state between *lks2* and the mutants *suKB-4*, *suKC-33*, *suKE-74*, and *suKF-76*.

F_2 populations were generated. The goal was to search, in a cross of a specific *suk* mutant, for a discriminatory large fraction of *kk* (WT, non-*Hooded*) plants with a long awn phenotype, concomitantly with *Hooded* plants. This would have been considered an indication of the nonallelic state of *lks2* with the *suK* allele. Putative interactions between *suK* mutants and *lks2* are well illustrated by the results of the cross *lks2* × *suKD-25*, which, involving loci mapping to different chromosomes, represents a control case for the absence of allelism, because in F_2 the cross allows the combination of all alleles

TABLE 4

Results of crosses between *lks2* and *suK* mutants

Cross	F_1 plants: No.	Phenotype	F_2 WT plants: Total no.	Awn length[c]: A	B	C	D	F_2 *K* plants[b]: Phenotype	$\chi^2_{9(A):43(B+C):12(D)}$
lks2 × *suKD-25*[a,b]	10	Short awn	65	17	36	12	13	*Hooded*	1.13 (NS)
lks2 × *suKB-4*[b]	10	Short awn	66	21	45	0	23	*Hooded* (8 plants elevated hood)	
lks2 × *suKC-33*[b]	10	Short awn	51	20	31	0	18	Different degree of penetrance of *Hooded*	
lks2 × *suKE-74*[b]	9	Very elevated hood	51	23	28	0	35	*Hooded* and elevated hood	
lks2 × *suKF-76*[b]	5	Very elevated hood	51	26	25	0	34	*Hooded* and elevated hood	

NS, not significant.
[a] For this cross, the predicted phenotype segregation ratio is 9 Hooded, 43 short awn (including awnless), and 12 long awn; see additional material at http://www.diprove.unimi.it/.
[b] See the F_2 phenotype in Figure 3.
[c] A, long awn; B, short awn; C, almost awnless. For details, see materials and methods.

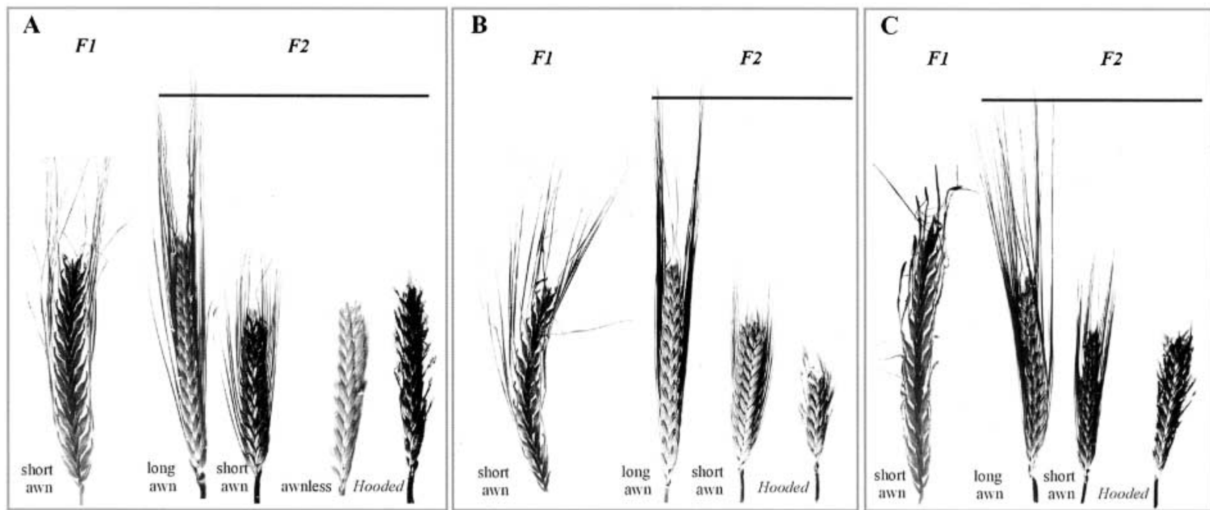

Figure 3.—Phenotypes recorded in the F_1 and F_2 generations of the crosses *lks2* × *suK*. (A) *lks2* × *suKD-25*; (B) *lks2* × *suKC-33*; (C) *lks2* × *suKE-74*. Note in C that the short-awn phenotype of the F_1 is also characterized by a very elevated hood.

of considered loci (Figure 3A). In F_2, awn length was under a complex control: 11 of 65 non-*Hooded* plants were awnless while 17 had normal awns (the latter are *kk* genotypes in which the *K* function on awn length is absent; Figure 3A). A fraction of *Hooded* plants was also observed. The hypothesis that in the F_2 population the *Lks2-*, *suKD-25 suKD-25*, *kk* genotypes occur within the long awn phenotypic class is accepted in Table 4 at the 5% confidence interval ($\chi^2_{[2]} = 1.13$). The segregation data of this cross (Table 4) fit, statistically, the phenotypic ratio expected under the following assumptions: (i) *lks2* dominantly suppresses the hood formation in *Kk* plants (allowing the appearance of short-awn phenotypes); (ii) *lks2* recessively conditions the short-awn phenotype in *kk* (WT) plants; and (iii) in *K* plants, homozygous recessive for the *suKD-25* allele, the hood is suppressed.

The following data, summarized in Table 4, consider the crosses between *lsk2* and the four *suK* mutants mapping on chromosome 7H. The presence in all F_2 populations of a substantial fraction of long-awn plants supported the conclusion that *lks2* was not allelic to any of the four *suK* mutants tested. The numerical analysis of the F_2 phenotypic segregation ratios of the four F_2 populations was not attempted because of the existence of tight linkages among the concerned loci and of the different degree of dominance of *lks2* on hood suppression, resulting in F_1's either with short-awn phenotypes (as in *suKB-4* and *suKC-33*) or in lemmas with a very elevated hood (as in *suKE-74* and *suKF-76*).

F_2 data, nevertheless, supported the concept, already evident from the consideration of F_1 results, that the short-awn phenotype of all *suK* mutants is a character under control of the *K* allele and not of the *suK* recessive alleles.

DISCUSSION

Plant homeobox genes have a role in the formation of leaf primordia (Poethig 1997; reviewed in Brutnell and Langdale 1998; Pozzi *et al.* 1999; Sinha 1999), as evident from the effect of mutations in class I *Knox* genes (Vollbrecht *et al.* 1991; Kerstetter *et al.* 1994), which result in dominant mutant phenotypes (Chen *et al.* 1997; Parnis *et al.* 1997; Chan *et al.* 1998). Overexpression of the same genes has morphogenetic effects on leaf shape (Kano-Murakami *et al.* 1993; Matsuoka *et al.* 1993; Sinha *et al.* 1993; Lincoln *et al.* 1994; Müller *et al.* 1995; Chuck *et al.* 1996; Sato *et al.* 1996; Tamaoki *et al.* 1997; Williams-Carrier *et al.* 1997), while loss-of-function mutants (with some exceptions; Long *et al.* 1996) have no obvious phenotypes.

Models considering homeobox gene regulation should take into account the finding that *knox* genes show partial functional redundancy (Chen *et al.* 1997; Martienssen and Dolan 1998; Bharathan *et al.* 1999; Pozzi *et al.* 1999). Furthermore, classes I and II of *knox* genes interact with each other and with members of the *Bell* homeobox family (Bellaoui *et al.* 2001; Müller *et al.* 2001), supporting the possibility that the loss of single components of the system may have relatively minor effects on phenotype. In this context, the *Hooded* mutation represents a special case. In the *Hooded* mutant, the barley *knox* gene *Bkn3* is overexpressed due to a 305-bp duplication in intron IV (Müller *et al.* 1995). The regulatory role of this intron is consistent with the existence of similar dominant insertional mutants in the corresponding intron of the maize ortholog *Knotted1* (reviewed in Santi *et al.* 2003). To shed light on the molecular network involving *Bkn3*, a number of experimental strategies have been adopted. The 305-bp duplicated element in the intron of *Bkn3* has been used as

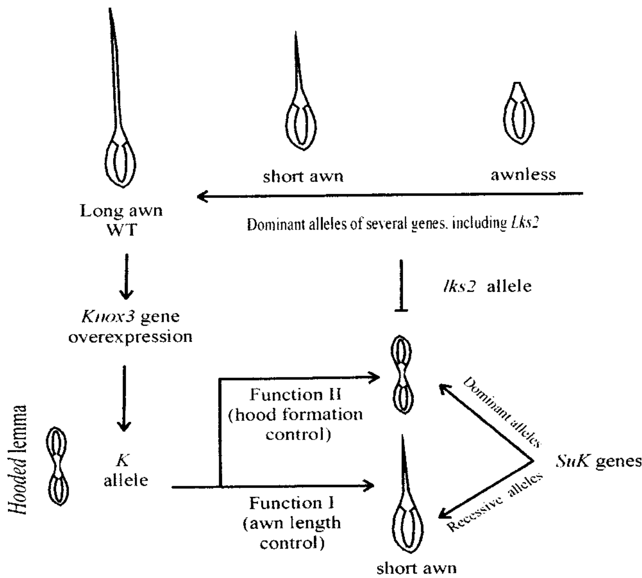

Figure 4.—Schematic of phenotypes, genotypes, and genes affecting lemma organization, including the role played by the *Bkn3* homeobox gene.

a bait in a yeast one-hybrid screen. This approach led to the isolation of four barley cDNA clones encoding for putative binding proteins, one of which one is extensively characterized (Santi *et al.* 2003). The screen for second-site suppressors of *Hooded*, described in the present article, represents a complementary genetic support to the molecular investigation of intron-mediated homeobox gene regulation (Santi *et al.* 2003). Our results indicate that five genetic loci exist that, when mutated, suppress *in trans* the *Hooded* syndrome, producing the replacement of the ectopic *K* flower with awns much shorter than those in the WT. Genetic tests designed to clarify whether *suK* mutations mapped to loci controlling awn length (Søgaard and von Wettstein-Knowles 1987; Franckowiak 1997; Franckowiak *et al.* 1997; Lundqvist *et al.* 1997; Pozzi *et al.* 2003) allowed us to understand that the *K* allele present in *suK* lines—and not the *suK* alleles *per se*—induced the appearance of the short awn. The finding that the gene *Lks2* (whose recessive allele causes the formation of short awns) mapped in the vicinity of the *suK* cluster on chromosome 7H (Pozzi *et al.* 2003) prompted us to test its allelism with *suKB-4*, *suKC-33*, *suKE-74*, and *suKF-76*. The allelic state was not assessed, but, interestingly, plants carrying the *lks2* allele together with *K* were characterized by the absence of hood formation and reduction in awn length. This led to the assignment of two distinct functions of the dominant *K* allele: the reduction of awn length and the formation of the hood. Only the latter can be suppressed by both *suK* and *lks2* alleles. In Figure 4, phenotypic interactions and genotypes supporting this conclusion are presented. The reported findings imply that short-awn and awnless loci should be considered as candidates in participating in the regulatory network controlling homeobox gene expression during barley lemma formation.

suK mutants, particularly *suKE-74*, are somewhat weaker than *K* (Table 1). This is also evident in the F_2 segregation as a deficiency in the homozygous recessive class (Table 2), findings consistent with some of the pleiotropic effects noted when plant *knox* genes are expressed in model species (Chan *et al.* 1998; discussed in Müller *et al.* 2001). Also, the phenotypic instability of some *suK* mutants is in agreement with the observations of Yagil and Stebbins (1968). These authors reported that variations in light and temperature could cause metabolic changes in the distal end of the lemma, resulting in variable penetrance of the *K* phenotype. Recent data support the hypothesis that hormones can mediate homeobox gene expression and their possible interactions with the environment (Tsiantis *et al.* 1999).

The experiments reported in this article were performed under the hypothesis that the products encoded by *suK* loci may be related to the intron-mediated regulation of *Bkn3* (Müller *et al.* 1995). Evidence has been provided that a 305-bp fragment of the *Bkn3* intron IV controls *Bkn3* gene expression in specific developmental domains *in planta*. This is mediated by a $(GA/TC)_8$ sequence binding site of the nuclear-targeted *BBR* protein (Santi *et al.* 2003). The *BBR* gene is one of four cDNAs isolated on the basis of a yeast one-hybrid screen and comaps with the *lks5* locus on chromosome 4H, sublinkage group 38 (Santi *et al.* 2003). The expression of *BBR* in tobacco leads to a pronounced leaf shape modification, as expected if *BBR* interacts with leaf primordia-related homeobox gene expression. The isolation of the *BBR* gene supports the assumption that distinct genetic loci affect the *Bkn3*-mediated phenotype. A further cDNA isolated during the one-hybrid screen, indicated with the symbol *Beil*, maps to the barley sublinkage group 6 on chromosome 7H (Wang 2001), and it is a candidate to represent one of the *suK* loci mapping to the same chromosomal region.

The genetic control of awn length in barley is well characterized: *lks* loci affecting the trait have been described (Tsuchiya 1973; Franckowiak 1997; Franckowiak and Lundqvist 2002), and one of these loci hosts the *lks2* mutant (Takahashi *et al.* 1953; the symbol has been modified in *lks2*: Franckowiak and Lundqvist 2002). The position of the locus has been recently integrated into a barley molecular linkage map (Pozzi *et al.* 2003) and assigned to chromosome 7H, sublinkage group 6. Also, the loci *suKB*, *C*, *E*, and *F* map close together on chromosome 7H. This observation is striking in light of the fact that for each of the four tightly linked loci only one allele was recovered. The possibility was considered that the four linked mutants on chromosome 7H might represent mutually complementing alleles of the same genetic locus. However, recombination occurring between their loci and linked AFLP markers

and the analysis of F_1 and F_2 generations of *suK* crosses exclude the one-locus hypothesis. Correspondence of the four *suK* loci to *lks2* was evaluated in consideration of the hypotheses that the *suK* short-awn phenotype could have originated from (i) the recessive state at a short-awn locus of the original (unknown) WT variety, which generated the dominant mutation *Hooded*, or (ii) the hood-suppressive effect of *suK* recessive alleles, which also controls awn length. Both hypotheses turned out to be incorrect, also on the basis of *lks2* × *suK* cross results, which showed that the short-awn phenotype of *suK* mutants is contributed by the *K* allele itself. Moreover, due to the dominance of the *K* allele in F_1 crosses, the short-awn phenotype was also found to be dominant over the awn length control carried out by the WT allele *Lks2*.

We thank Donata Pagani for careful technical assistance.

LITERATURE CITED

Arber, A., 1934 *The Gramineae: A Study of Cereals, Bamboos and Grasses.* Cambridge University Press, Cambridge, UK.

Bellaoui, M., M. S. Pidkowich, A. Samach, K. Kushalappa, S. E. Kohalmi *et al.*, 2001 The Arabidopsis *BELL* 1 and *KNOX* TALE homeodomain proteins interact through a domain conserved between plants and animals. Plant Cell **13:** 2455–2470.

Bharathan, G., and N. R. Sinha, 2001 The regulation of compound leaf development. Plant Physiol. **127:** 1533–1538.

Bharathan, G., B. J. Jannsen, E. A. Kellog and N. Sinha, 1999 Phylogenetic relationships and evolution of the *KNOTTED* class of plant homeodomain proteins. Mol. Biol. Evol. **16:** 553–563.

Brutnell, T. P., and J. A. Langdale, 1998 Signals in leaf development. Adv. Bot. Res. **28:** 36–42.

Castiglioni, P., C. Pozzi, M. Heun, K. J. Müller, V. Terzi *et al.*, 1998 An AFLP-based procedure for the efficient mapping of mutants and DNA probes in barley. Genetics **149:** 2039–2056.

Chan, R. L., G. M. Gago, C. M. Palena and D. H. Gonzalez, 1998 Homeoboxes in plant development. Biochim. Biophys. Acta **1442:** 1–19.

Chen, J. J., B. J. Janssen, A. Williams and N. Sinha, 1997 A gene fusion at a homeobox locus: alterations in leaf shape and implications for morphological evolution. Plant Cell **9:** 1289–1304.

Chuck, G., C. Lincoln and S. Hake, 1996 *KNAT1* induces lobed leaves with ectopic meristems when overexpressed in Arabidopsis. Plant Cell **8:** 1277–1289.

Clifford, H. T., 1988 Spikelet and floral morphology, pp. 21–30 in *Grass Systematics and Evolution*, edited by T. R. Soderstrom, K. W. Hilu, S. C. Campbell and M. E. Barkworth. Smithsonian Institution Press, Washington, DC/London.

Dahlgren, R. H. T., H. T. Clifford and P. F. Yeo, 1985 *The Families of the Monocotyledons: Structure, Evolution and Taxonomy.* Springer-Verlag, New York/Berlin/Heidelberg, Germany.

Franckowiak, J., 1997 Revised linkage maps for morphological markers in barley, *Hordeum vulgare*. Barley Genet. Newsl. **26:** 9–21.

Franckowiak, J., and U. Lundqvist, 2002 Descriptions of barley genetic stocks for 2001. Barley Genet. Newsl. **32:** 49–137.

Franckowiak, J., U. Lundqvist and T. Konishi, 1997 New revised names for barley genes. Barley Genet. Newsl. **26:** 4–8.

Kano-Murakami, Y., T. Yanai, A. Tagiri and M. Matsuoka, 1993 A rice homeotic gene, *OSH1*, causes unusual phenotypes in transgenic tobacco. FEBS Lett. **334:** 365–368.

Kerstetter, R., E. Vollbrecht, B. Lowe, B. Veit, J. Yamaguchi *et al.*, 1994 Sequence analysis and expression patterns divide the maize *knotted1*-like homeobox genes into two classes. Plant Cell **6:** 1877–1887.

Inada, D. C., A. Bashir, C. Lee, B. C. Thomas, C. Ko *et al.*, 2003 Conserved noncoding sequences in the grasses. Genet. Res. **13:** 2030–2041.

Lander, E. S., P. Green, J. Abrahamson, A. Barlow, M. J. Daly *et al.*, 1987 MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics **1:** 174–181.

Lin, J., and K. J. Müller, 2002 Structure and development of epiphylly in *knox*-transgenic tobacco. Planta **214:** 521–525.

Lincoln, C., J. Long, J. Yamaguchi, K. Serikawa and S. Hake, 1994 A *knotted-1*-like homeobox gene in Arabidopsis is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. Plant Cell **6:** 1859–1876.

Long, J. A., E. J. Moan, J. I. Medford and M. K. Barton, 1996 A member of the *knotted* class of homeodomain proteins encoded by the *STM* gene of *Arabidopsis.* Nature **379:** 66–69.

Lundqvist, U., J. D. Franckowiak and T. Konishi, 1997 New and revised description of barley genes. Barley Genet. Newsl. **26:** 22–43.

Martienssen, R., and L. Dolan, 1998 Patterns in vegetative development, pp. 262–297 in *Arabidopsis. Annual Plant Reviews*, Vol. I, edited by M. Anderson and J. Roberts. Sheffield Academic Press, Sheffield, UK.

Matsuoka, M., H. Ichikawa, A. Saito, Y. Tada, T. Fujimura *et al.*, 1993 Expression of a rice homeobox gene causes altered morphology of transgenic plants. Plant Cell **5:** 1039–1048.

Müller, K. J., N. Romano, O. Gerstner, F. Garcia-Maroto, C. Pozzi *et al.*, 1995 The barley *Hooded* mutation caused by a duplication in a homeobox gene intron. Nature **374:** 727–730.

Müller, J., Y. Wang, R. Franzen, L. Santi, F. Salamini *et al.*, 2001 *In vitro* interactions between barley TALE homeodomain proteins suggest a role for protein-protein associations in the regulation of *knox* gene function. Plant J. **26:** 1–13.

Parnis, A., O. Cohen, T. Gutfinger, D. Hareven, D. Zamir *et al.*, 1997 The dominant developmental mutants of tomato, *Mouse-ear* and *Curl*, are associated with distinct modes of abnormal transcriptional regulation of knotted gene. Plant Cell **9:** 2143–2158.

Poethig, R. S., 1997 Leaf morphogenesis in flowering plants. Plant Cell **9:** 1077–1087.

Pozzi, C., K. J. Müller, W. Rohde and F. Salamini, 1999 Leaf development, pp. 145–165 in *Development: Genetics, Epigenetics and Environmental Regulation*, edited by V. E. A. Russo, D. J. Cove, R. Jaenish and F. Salamini. Springer-Verlag, Berlin/Heidelberg, Germany.

Pozzi, C., P. Faccioli, V. Terzi, A. M. Stanca, S. Cerioli *et al.*, 2000 Genetics of mutations affecting the development of a barley floral bract. Genetics **154:** 1335–1346.

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

Santi, L., Y. Wang, M. R. Stile, K. Berendzen, D. Wanke *et al.*, 2003 The GA octodinucleotide repeat binding factor BBR participates in the transcriptional regulation of the homeobox gene Bkn3. Plant J. **34:** 813–826.

Sato, Y., M. Tamaoki, T. Murakami, N. Yamamoto, Y. Kano-Murakami *et al.*, 1996 Abnormal cell divisions in leaf primordia caused by the expression of the rice homeobox gene *OSH1* lead to altered morphology of leaves in transgenic tobacco. Mol. Gen. Genet. **251:** 13–22.

Sinha, N., 1999 Leaf development in angiosperms. Annu. Rev. Plant Physiol. Plant Mol. Biol. **50:** 419–446.

Sinha, N. R., R. E. Williams and S. Hake, 1993 Overexpression of the maize homeobox gene *KNOTTED-1* causes a switch from determinate cell fates. Genes Dev. **7:** 787–795.

Søgaard, B., and P. von Wettstein-Knowles, 1987 Barley: genes and chromosomes. Carlsberg Res. Commun. **52:** 123–196.

Stebbins, G. L., and E. Yagil, 1966 The morphogenetic effects of the *Hooded* gene in barley. I. The course of development in *Hooded* and awned genotypes. Genetics **54:** 727–741.

Takahashi, R., J. Yamamoto, S. Yasuda and Y. Itano, 1953 Inheritance and linkage studies in barley. Ber Ohara Inst. Landwirtsch. Biol. Okayama Univ. **10:** 29–52.

Tamaoki, M., S. Kusaba, Y. Kano-Murakami and M. Matsuoka, 1997 Ectopic expression of a tobacco homeobox gene, *NTH15*, dramatically alters leaf morphology and hormone levels in transgenic tobacco. Plant Cell Physiol. **38:** 917–927.

Tsiantis, M., M. I. N. Brown, G. Sibinski and J. A. Langdale, 1999 Disruption of auxin transport is associated with aberrant leaf development in maize. Plant Physiol. **121:** 1163–1168.

Tsuchiya, T., 1973 New linkage maps of barley. Barley Genet. Newsl. **3:** 99–103.

Vollbrecht, E., R. Kerstetter, B. Lowe, B. Veit and S. Hake, 1991 The developmental gene *knotted-1* is a member of a maize homeobox gene family. Nature **350:** 241–243.

Wang, Y., 2001 Expression analyses of barley *Knox* homeobox genes and characterization of putative upstream regulators of *Bkn3* (Barley *Knox3*), the *Hooded* gene. Ph.D. Thesis, University of Köln, Köln, Germany.

Williams-Carrier, R. E., Y. S. Lie, S. Hake and P. G. Lemaux, 1997 Ectopic expression of the maize *Kn1* phenocopies the *Hooded* mutant in barley. Development **124:** 3737–3745.

Yagil, E., and G. L. Stebbins, 1968 The morphogentic effects of the *Hooded* gene in barley. II. Cytological and environmental factors affecting gene expression. Genetics **62:** 307–319.

Communicating editor: V. Sundaresan