tion of genes expressed in diploid cells to pollen tube guidance.

The *pop2, pop3* pollen tube guidance defect results from alteration of both pollen and pistil components. In pairwise reciprocal crosses with homozygous single mutants (*pop2/pop2* or *pop3/pop3*), the double mutant (*pop2/pop2, pop3/±*), or wild type (Table 2), all combinations produced seeds except a cross between pollen from a *pop2, pop3* plant and *pop2, pop3* pistils. These observations indicate that *POP2* and *POP3* function in both the pollen and pistil, consistent with a model in which these genes encode molecules present on the surface of pollen tubes and pistil cells, much like the agglutinins required for adhesion of mating yeast cells (*17*).

In many plant species, self-fertilization is inhibited at a late stage, sometimes through self-incompatibility mechanisms (*12*). Self-sterility in these systems, as in the *Arabidopsis pop2, pop3* mutant, may involve defects in pollen tube guidance and redundant signaling pathways active in male and female tissues.

REFERENCES AND NOTES

1. R. E. Pruitt and M. Hülskamp, *Arabidopsis*, E. M. Meyerowitz and C. Somerville, Eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1994), pp. 467–483; M. Hülskamp, K. Schneitz, R. E. Pruitt, *Plant Cell* **7**, 57 (1995).
2. J. P. Hill and E. M. Lord, *Am. J. Bot.* **74**, 988 (1987).
3. C. S. Goodman, *Cell* **78**, 353 (1994).
4. D. Preuss, B. Lemieux, G. Yen, R. W. Davis, *Genes Dev.* **7**, 974 (1993).
5. H. C. C. Foote *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **91**, 2265 (1994); J. B. Nasrallah, J. C. Stein, M. K. Kandasamy, M. E. Nasrallah, *Science* **266**, 1505 (1994); S. J. Hiscock, J. Doughty, A. C. Willis, H. G. Dickinson, *Planta* **196**, 367 (1995); A. Y. Cheung, H. Wang, H. Wu, *Cell* **82**, 383 (1995); P. N. Dodds, A. E. Clarke, E. Newbigin, *ibid.* **85**, 141 (1996).
6. M. Kandasamy, J. B. Nasrallah, M. E. Nasrallah, *Development* **120**, 3405 (1994).
7. M. Hülskamp, K. Schneitz, R. E. Pruitt, *Plant Cell* **7**, 57 (1995).
8. Seed stocks (Landsberg ecotype) were mutagenized and screened for sterility, pollen germination, and pollen tube growth as described in Preuss *et al.* (*4*).
9. E. M. Lord and L. C. Sanders, *Dev. Biol.* **153**, 16 (1992).
10. Of crosses between emasculated mutant pistils and wild-type pollen, 116/133 were successful, and of crosses between emasculated wild-type pistils and mutant pollen, 76/91 were successful, with average seed sets of 41 ± 8 (n = 11) and 31 ± 15 (n = 37), respectively; wild-type *Arabidopsis* pistils contain about 50 ovules. Similar pollinations with mutant pollen and mutant pistils yielded no seed (n = 52).
11. L. P. Taylor and R. Jorgensen, *J. Heredity* **83**, 11 (1992); Y. Mo, C. Nagel, L. P. Taylor, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 7213 (1992).
12. S. R. Seavey and K. S. Bawa, *Botan. Rev.* **52**, 195 (1986).
13. Genetic mapping was performed as described [A. Konieczny and F. M. Ausubel, *Plant J.* **4**, 403 (1993); C. J. Bell and J. R. Ecker, *Genomics* **19**, 137 (1994)]. The sterile mutant (Landsberg ecotype) was crossed to a wild-type Columbia strain, and mutant F_2 plants were scored. Recombination break-point analysis was used to map *POP2* to chromosome 3 at 37.8 ± 1.3 cM (within a 4.8-cM interval between m560 and *DMC1*) and *POP3* to a 6-cM interval between nga106 and *PI* (genetic distances correspond to those reported in September 1996 by C. Lister and C. Dean, available at http://genome-www.stanford.edu/Arabidopsis/ww/Vol3ii/home.html). No loci, other than those indicated in Fig. 2, showed significant linkage to the sterile phenotype.
14. K. K. Niyogi, R. L. Last, G. R. Fink, B. Keith, *Plant Cell* **5**, 1011 (1993).
15. Markers flanking *POP2* (nga162, g4711) and *POP3* (nga139, nga151) defining an ~30-cM interval were used to determine the genotype of 68 fertile strains (*13*). From 64 of these, ~25 F_3 seeds were planted and ~95% germinated, indicating that all combinations of *pop2* and *pop3* are viable. The ratio of sterile to fertile F_3 plants was determined; 40 plants predicted to lack either *pop2* or *pop3* (or both) produced only fertile F_3 progeny. Of 24 fertile plants predicted to contain both mutant alleles (*pop2/POP2*$^+$, *pop3/POP3*$^+$ or *pop2/POP2*$^+$, *pop3/pop3*), 21 produced sterile progeny. The three exceptions (3/64 or ~5%) most likely were due to double crossovers, which are expected to occur at a frequency of $(0.3)^2$ or ~9%.
16. J. P. Mascarenhas, *Plant Cell* **5**, 1303 (1993).
17. P. N. Lipke and J. Kurjan, *Microbiol. Rev.* **56**, 180 (1992).

18. We thank W. E. Browne, D. Charlesworth, R. W. Davis, C. Gasser, B. Hauser, A. Shapiro, V. Walbot, and O. Rossanese for helpful discussions; G. Copenhaver, B. Keith, S. Kron, J. Mayfield, and L. Mets for critical reading of the manuscript; and L. DuPree and R. Choi for technical assistance. The *Arabidopsis* Biological Resource Center provided *ms1* seeds. Supported by an NIH Genetics and Regulation Training Grant (L.K.W.) and by grants from the American Cancer Society, U.S. Department of Energy, and NSF (D.P.). This project was initially supported by R. W. Davis (NSF grant DMB-9106011).

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Diversification of C-Function Activity in Maize Flower Development

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The *Arabidopsis* gene *AGAMOUS* is required for male and female reproductive organ development and for floral determinacy. Reverse genetics allowed the isolation of a transposon-induced mutation in *ZAG1*, the maize homolog of *AGAMOUS*. *ZAG1* mutants exhibited a loss of determinacy, but the identity of reproductive organs was largely unaffected. This suggested a redundancy in maize sex organ specification that led to the identification and cloning of a second *AGAMOUS* homolog, *ZMM2*, that has a pattern of expression distinct from that of *ZAG1*. C-function organ identity in maize (as defined by the A, B, C model of floral organ development) may therefore be orchestrated by two closely related genes, *ZAG1* and *ZMM2*, with overlapping but nonidentical activities.

The discovery of conserved genetic mechanisms of flower morphogenesis in dicots such as *Arabidopsis thaliana* and *Antirrhinum majus* (*1–3*) suggests that an ancestral floral developmental plan has been preserved during angiosperm evolution (*4*). Monocots diverged from dicots about 180 million years ago and have evolved flowers that are distinct from those of dicots. Among the monocots, flowers in the grasses are the most highly derived. We previously reported (*5*) the isolation of ZAG1, the maize homolog of AGAMOUS (AG) from *Arabidopsis* and PLENA (*PLE*) from *Antirrhinum*, both of which are required for reproductive organ development (*6–8*). Although the onset of ZAG1 expression in the cells that give rise to stamens and carpels is analogous to that observed for AG and *PLE*, ZAG1 expression (unlike AG and *PLE* expression) diminishes during stamen development (*5*). Here, we identified a transposon-induced mutant allele of ZAG1 and used it to examine the functional homology between these monocot and dicot genes.

We used reverse genetic technology to screen for transposon insertions in the ZAG1 gene (*9*). A ZAG1 mutant allele, designated *zag1-mum1*, was isolated that carried a Robertson's *Mutator* (*Mu*) transposable element inserted adjacent to the region encoding for the K domain (Fig. 1, A and B), a motif thought to be involved in protein-protein interactions (*10*). No ZAG1 transcript was detected in RNA samples from *zag1-mum1* homozygotes upon hybridization with a probe 3′ of the *Mu* insertion site, and only reduced amounts of transcript were detectable with a ZAG1 probe 5′ of the insertion site (Fig. 1C). These results suggest that in the *zag1-mum1* allele, transcription proceeds into but not through the *Mu* element, and that the chimeric message generated is unstable. Any possible translation product of the chimeric transcript (Fig. 1B) would lack 77 amino acids of the normal protein, including the COOH-terminus of the K box. Mutations

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that generate similarly truncated protein products for the *Arabidopsis AP1* MADS-box gene generate a severe *ap1* mutant phenotype (*11*). Taken together, these observations suggest that *zag1-mum1* is a null allele for ZAG1 function.

A recessive floral mutant phenotype cosegregated with plants homozygous for the *zag1-mum1* allele (Fig. 2). Mutant female flowers showed a transformed pistil in which two silks developed, one in an abaxial position (like the single silk of a wild-type pistil) and the other in an adaxial position. In most mutant florets, abaxial and adaxial carpels did not fuse along their margins and the resulting pistil structure remained open. Enclosed within these organs, some mutant flowers additionally developed a variable number of extra silks. Most mutant flowers produced silks that failed to emerge through the husk leaves, consequently yielding seedless ears. When husk leaves were peeled back to uncover the silks, manual pollination resulted in a number of viable seeds. This indicates that at least some florets developed a functional ovary and egg. In contrast, the tassels of mutant plants were phenotypically normal and produced fertile pollen.

To determine more precisely the point during female inflorescence development when mutant florets departed from the normal course of development, we performed scanning electron microscope (SEM) analysis (*12, 13*) (Fig. 3). Early ear development proceeded normally in mutant plants through those stages involving the establishment of spikelet and flower primordia (*14*). Immediately after stamen initiation, wild-type and mutant floral meristems exhibited a distinguishable morphology; the mutant meristem appeared substantially enlarged on its adaxial flank, from which a second silk primordium arose to produce the double set of silks. At all times, stamen development in the mutant paralleled that observed in the wild type, and only carpel development was altered.

AG and *PLE* specify stamen and carpel identity as well as prevent the indeterminate growth of the floral meristem (*6–8, 15, 16*). These roles constitute the C-function activity as defined by the A, B, C model of floral organ development (*4, 17*). Our analysis of the *zag1-mum1* mutant suggests that ZAG1 is necessary for only a portion of these activities in maize. Analysis of the *zag1-mum1* mutant phenotype shows that the absence of ZAG1 activity results in a change in the fate of the cells that are destined to become carpel primordia. This group of cells now acquires the ability to proliferate and reiterate a program of organ initiation, typical of an indeterminate pattern of growth. However, in contrast to loss-of-function *ag* or *ple* mutants, *zag1* mutant plants have normal pollen-producing stamens and carpelloid features in the innermost sets of organs. These data indicate that the *zag1-mum1* mutation affects determinacy and that any role of ZAG1 in organ identity is obscured by another gene with partially redundant activity.

We isolated a gene, *ucsd78b* (*18*), that is more similar to AG and ZAG1 than to any other gene in the EMBL and GenBank databases. In addition, ZAG1 and *ucsd78b* are on chromosomal segments 6L-080 and 8L-056, respectively (*19, 20*), which are known to carry synonymous genes that were introduced into maize by alloploidization (*21–23*). Recently (*20*), a partial sequence of the *ucsd78b* gene was reported and termed ZMM2, and we adopt this designation here. Through the MADS domain, ZMM2 (the complete sequence of ZMM2 cDNA can be found at GenBank accession number L81162) is more similar to AG than is ZAG1 (Fig. 4A). However, over the portion of the proteins spanning the MADS box through the K box, ZAG1 has higher

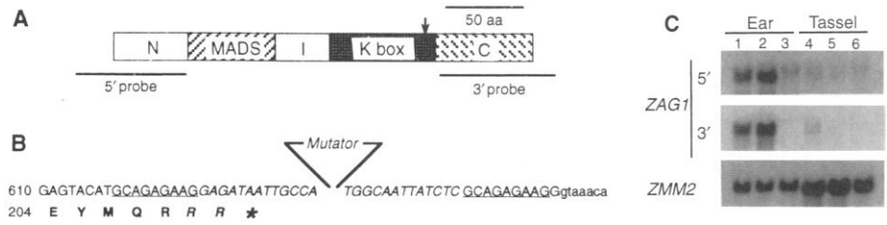

Fig. 1. Isolation and molecular characterization of the *zag1-mum1* allele. (**A**) Schematic representation of domains in the predicted ZAG1 protein. The functions of the MADS box, K box, and intervening region (I) have been recently discussed (*10, 24*). *ZAG1*, like a subfamily of MADS-box genes (including *AG* and *PLE*), encodes a stretch of amino acids NH_2-terminal to the MADS domain (N) as well as COOH-terminal to the K box (C) (aa, amino acids). The arrow shows the location of the *Mu* element in *zag1-mum1*. The positions of the probes used in (C) are indicated. (**B**) Insertion site of the *Mu* element in *zag1-mum1*. *ZAG1* exon and intron sequences are represented by capital and lowercase letters, respectively. *Mu* sequences are in italics. The characteristic 9-bp host sequence duplication associated with *Mu* insertions is underlined. A possible chimeric translation product, terminating within the *Mu* element (asterisk), is shown beginning with amino acid 204 (*25*). (**C**) *ZAG1* mRNA accumulation in *zag1-mum1* plants. Total RNA was isolated from developing ears and tassels of segregating individuals identified as homozygous for the *ZAG1* wild-type allele (lanes 1 and 4), heterozygous (lanes 2 and 5), or homozygous for the *zag1-mum1* allele (lanes 3 and 6). Blots were hybridized with *ZAG1*-specific probes hybridizing either 5′ or 3′ to the location of the *Mu* element in *zag1-mum1*. The presence of *ZMM2* transcript (see Fig. 4) serves as a control for RNA quality.

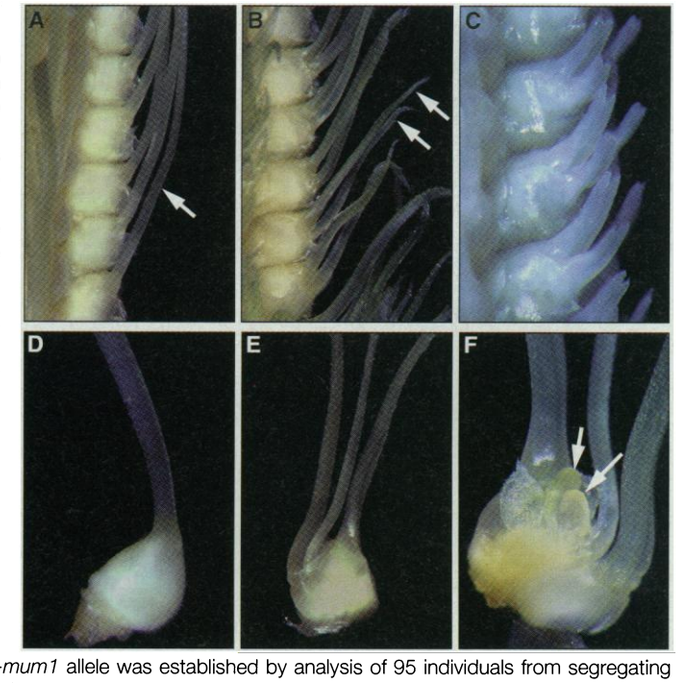

Fig. 2. Phenotype of wild-type and *zag1-mum1* ear florets. (**A**) Wild-type ear florets with single silks (arrow). (**B**) Ear of *zag1-mum1* mutant showing two silks per flower (arrows). (**C**) An earlier stage of a mutant ear. (**D**) Wild-type pistil. (**E**) Pistil structure of *zag1* mutant showing a third silk emerging from inside the carpel. (**F**) The pistil in (E) was opened mechanically to show the enclosed developing primordia (arrows). Note the presence in the center of an undifferentiated mass of tissue and a pair of organs bearing trichomes and resembling the sterile accessory organs of the flower. Cosegregation of this mutant phenotype with homozygosity for the *zag1-mum1* allele was established by analysis of 95 individuals from segregating families of either F_2 or backcross populations.

sequence identity with AG than does ZMM2 (87% versus 75%, respectively).

ZAG1 and *ZMM2* RNAs accumulate in spatially overlapping but nonidentical patterns. *ZMM2*, like *ZAG1*, is a floral-specific gene that is expressed in tassels as well as in ears (Fig. 4, B and C). In addition, *ZMM2* is expressed in the *zag1-mum1* mutant inflorescences (Fig. 1C). However, the relative abundance of *ZAG1* and *ZMM2* transcripts in developing male and female inflorescences is different. *ZAG1* RNA accumulates more in developing ears than in tassels (Figs. 1C and 4C) (5). In contrast, the *ZMM2* transcript is more abundant in the tassel. Like *ZAG1*, *ZMM2* is expressed in carpels and stamens, though in stamens the expression is much stronger (Fig. 4C). The expression profile alone suggests that *ZMM2* itself may participate in regulating the development of stamens and carpels, and this is supported by the phenotype observed in the *zag1* mutant. Because of the extensive sequence similarity between *ZAG1* and *ZMM2*, it is likely that they share some redundancy in function. If this is the case, the preferential accumulation of *ZAG1* RNA in carpels and *ZMM2* RNA in stamens could explain the phenotype in *zag1-mum1* mutant plants, in which only carpel development is disturbed.

These results suggest that, unlike *Arabidopsis* and *Antirrhinum*, C-function activity in maize is orchestrated by two closely related MADS-box genes that have evolved distinct yet partially redundant roles in flower development. Isolation of *zmm2* mutants by the same reverse genetic approach and the construction of *zag1 zmm2* double mutants will help to determine the specific contribution of these two genes to male and female reproductive organ development in maize.

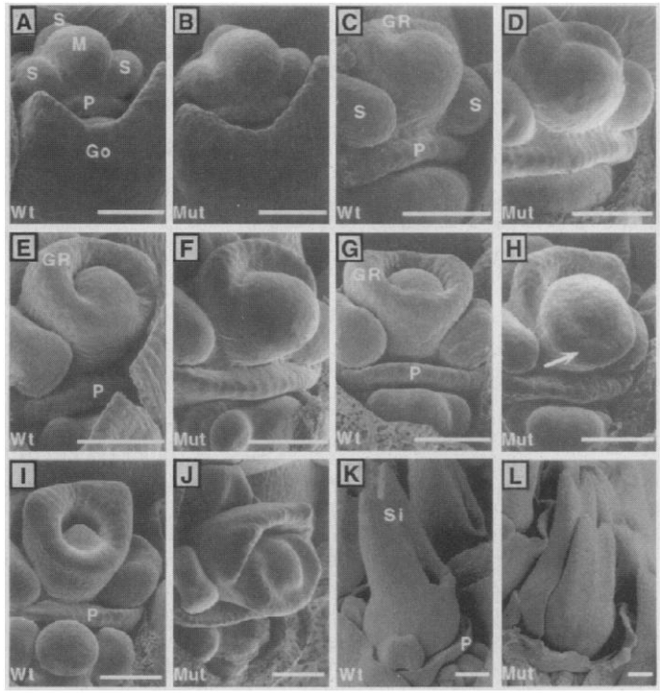

Fig. 3. SEM micrographs of floral development in the wild type (Wt) and in *zag1-mum1* mutants (Mut). The abaxial side of flowers is to the back. (**A** and **B**) Initiation of stamen (S) and palea (P) primordia on the flanks of the meristem (M) in the upper flower. Note the prominent adaxial side of the meristem in the mutant. The outer glume (Go) was partially removed in the subsequent images. (**C** to **G**) Gynoecial ridge (GR) initiation. Initiation of the two abaxial carpels (*26*) is recognized by the simultaneous initiation of the GR. Shortly after, development of the single adaxial carpel becomes noticeable (E), giving rise together to a complete GR that grows to enclose the meristem (G). Mutant flowers also initiate a GR (D) but, in contrast to wild-type development, the GR does not extend to the adaxial region (F), reflecting a failure of the third carpel primordium to initiate. During these stages, the meristem in the mutant continues proliferating and remains only partially encircled by the GR. (**H**) Initiation of a second, adaxial set of carpel primordia (arrow) in the mutant. (**I** to **L**) Early silk development. As the wild-type pistil continues to develop, the single silk differentiates from two growth centers, each emerging from one of the abaxial carpels. This process is accompanied by the differentiation of the ovule and finally the formation (K) of the immature silk (Si). Two growth centers are observed on each set of carpels forming the mutant pistils (J). Note that development of the adaxial silk lags behind that of the abaxial silk. In most mutant flowers the meristem does not produce a normal ovule primordium, but instead it continues to produce a large mass of tissue that finally extrudes from the surrounding carpels (*14*). Scale bars, 100 μm.

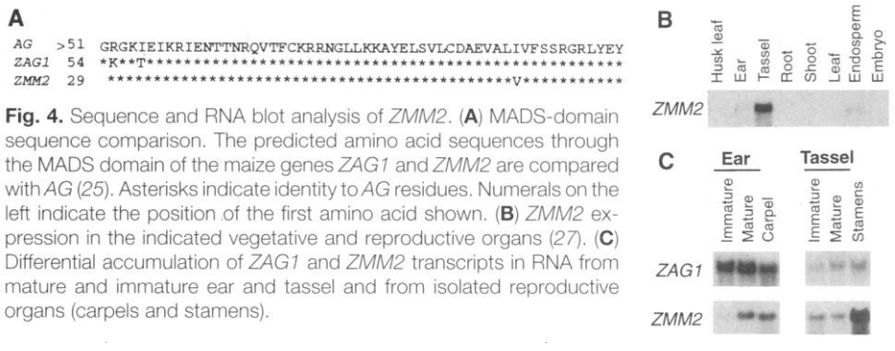

Fig. 4. Sequence and RNA blot analysis of *ZMM2*. (**A**) MADS-domain sequence comparison. The predicted amino acid sequences through the MADS domain of the maize genes *ZAG1* and *ZMM2* are compared with *AG* (*25*). Asterisks indicate identity to *AG* residues. Numerals on the left indicate the position of the first amino acid shown. (**B**) *ZMM2* expression in the indicated vegetative and reproductive organs (*27*). (**C**) Differential accumulation of *ZAG1* and *ZMM2* transcripts in RNA from mature and immature ear and tassel and from isolated reproductive organs (carpels and stamens).

REFERENCES AND NOTES

1. D. Weigel and E. M. Meyerowitz, *Cell* **78**, 203 (1994).
2. H. Ma, *Genes Dev.* **8**, 745 (1994).
3. M. F. Yanofsky, *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **46**, 167 (1995).
4. E. S. Coen and E. M. Meyerowitz, *Nature* **353**, 31 (1991).
5. R. J. Schmidt *et al., Plant Cell* **5**, 729 (1993).
6. J. L. Bowman, *ibid.* **1**, 37 (1989).
7. M. F. Yanofsky *et al., Nature* **346**, 35 (1990).
8. D. Bradley, R. Carpenter, H. Sommer, N. Hartley, E. Coen, *Cell* **72**, 85 (1993).
9. The Pioneer Hi-Bred collection of 24,000 F_1 maize plants mutagenized by means of a Robertson's *Mutator* element was screened for *Mu*-containing alleles of the *ZAG1* gene by a reverse genetics–based technology [R. J. Bensen *et al., Plant Cell* **7**, 75 (1995)]. Polymerase chain reactions (PCRs) were initially performed on pooled and subsequently on individual DNA samples using a *Mu*-specific primer [5′-CCCTGAGCTCTTCGTC(CT)ATAATGGCAATTATCTC-3′] to the conserved region of the *Mu* terminal inverted repeat in combination with four different primers specific for *ZAG1* exon sequences (*5*). Cloning and sequencing of the corresponding amplified PCR products confirmed that one family (1482A2) carried a *Mu* element inserted in the *ZAG1* gene. RNA isolation, blotting, and hybridization procedures were performed as described (*5*). *ZAG1*-5′ encompasses nucleotides 1 to 236; *ZAG1*-3′ spans nucleotides 716 to 936 immediately 3′ of the *Mu* insertion.
10. B. Davies and Zs. Schwarz-Sommer, *Results Probl. Cell Differ.* **20**, 235 (1994).
11. A. Mandel and M. Yanofsky, unpublished data.
12. Developing ears were dissected and fixed in FAA (50% ethanol, 5% acetic acid, 3.7% formaldehyde) for 24 hours at 4°C. Specimens were dehydrated through a graded ethanol series and then critical point–dried with CO_2. Coating was performed with gold and palladium. The specimens were examined with a SEM at an accelerating voltage of 10 kV.
13. P. C. Cheng, R. I. Greyson, D. B. Walden, *Am. J. Bot.* **70**, 450 (1983).
14. M. Mena, B. Ambrose, R. B. Meeley, S. P. Briggs, M. F. Yanofsky, R. J. Schmidt, data not shown.
15. L. E. Sieburth, M. P. Running, E. M. Meyerowitz, *Plant Cell* **7**, 1249 (1995).
16. Y. Mizukami and H. Ma, *Plant Mol. Biol.* **28**, 767 (1995).
17. J. L. Bowman, D. R. Smyth, E. M. Meyerowitz, *Development* **112**, 1 (1991).
18. For isolation of *ZMM2* (*ucsd78b*) cDNA, PCR was performed on a size-selected fraction of a Hind III digest of inbred T232 containing the restriction fragment length polymorphism (RFLP) that maps to *ucsd78b* (*19*). Use of primers MADS6.1 [5′-CGGAATTC(AG)TN(CG)A(AG)ATNAA(AG)(AC)GNATNGANAA-3′] and *ZAG1*-MADS.3 (5′-TTGTTGGCGTACTCGTAGAGG-3′) yielded a product with high sequence similarity to the *ZAG1* MADS region.

A new primer, 5′-ACACGACGAACCGGCAGGTG-3′, was derived from this sequence and used in combination with a λ ZAP–specific primer (Stratagene) to amplify a portion of the *ZMM2* (*ucsd78b*) transcript from our ear and tassel cDNA libraries (*19*). A full-length cDNA was subsequently isolated from the tassel library by screening at high stringency with a probe from the 3′ end of the partial clone. RFLP analysis on recombinant inbreds confirmed that the correct locus had been obtained.

19. M. Mena, M. A. Mandel, D. R. Lerner, M. F. Yanofsky, R. J. Schmidt, *Plant J.* **8**, 845 (1995).
20. G. Theissen, T. Strater, A. Fischer, H. Saedler, *Gene* **156**, 155 (1995).
21. M. M. Goodman, C. W. Stuber, K. Newton, H. H. Weissinger, *Genetics* **96**, 697 (1980).
22. J. F. Wendel, C. W. Stuber, M. D. Edwards, M. M. Goodman, *Theor. Appl. Genet.* **72**, 178 (1986).
23. T. Helentjaris, D. Weber, S. Wright, *Genetics* **118**, 6442 (1989).
24. J. L. Riechmann, B. A. Krizek, E. M. Meyerowitz, *Proc. Natl. Acad. Sci. U.S.A.* **93**, 4793 (1996).
25. Abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
26. T. A. Kiesselbach, *The Structure and Reproduction of Corn* [*Univ. Nebr. Agric. Exp. Stn. Res. Bull. 161* (1949)].
27. Analysis of RNA expression was performed as described in Fig. 1. A 500–base pair (bp) Hpa I–Nde I fragment was used as the *ZAG1* probe in Fig. 4B. The *ZMM2*-specific probe (see below) was a 400-bp Xho I fragment isolated from the 3′ end of the cDNA. Both probes were labeled to comparable specific activities. The faint *ZMM2* hybridization detected in endosperm is likely a result of nucellar tissue, which often contaminates endosperm tissue obtained from early developmental stages. RNA from immature ears and tassels was isolated from developing inflorescences that were <3 cm in length. Hybridization with a tubulin probe produced signal in every lane.
28. We thank T. Fox and M. Albertson for use of their tassel cDNA library. Supported in part by postdoctoral fellowships from the North Atlantic Treaty Organization and Ministerio de Educación y Ciencia (to M.M.) and from the Pioneer Discovery Research Program (to R.B.M.) and by grants from the U.S. Department of Agriculture and the University of California Biotechnology and Research Foundation (to R.J.S. and M.F.Y.).

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CRNF, a Molluscan Neurotrophic Factor That Interacts with the p75 Neurotrophin Receptor

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A 13.1-kilodalton protein, cysteine-rich neurotrophic factor (CRNF), was purified from the mollusk *Lymnaea stagnalis* by use of a binding assay on the p75 neurotrophin receptor. CRNF bound to p75 with nanomolar affinity but was not similar in sequence to neurotrophins or any other known gene product. CRNF messenger RNA expression was highest in adult foot subepithelial cells; in the central nervous system, expression was regulated by lesion. The factor evoked neurite outgrowth and modulated calcium currents in pedal motor neurons. Thus, CRNF may be involved in target-derived trophic support for motor neurons and could represent the prototype of another family of p75 ligands.

The survival, differentiation, and plasticity of vertebrate neurons are influenced by neurotrophic factors, the best characterized of which are the neurotrophins (*1*). Invertebrate neuronal systems have provided useful models for development and regeneration of the nervous system; however, efforts to clone neurotrophin or neurotrophin receptor–like sequences in invertebrates have so far been unsuccessful (*2*). Indeed, it has been argued that neurotrophic factors may be a comparatively late addition to the arsenal of mechanisms controlling nervous system development and regeneration, and that such factors may be required only in long-lived organisms with complex nervous systems (*2*). To examine the existence of neurotrophin-related molecules in an invertebrate, we focused on the snail *Lymnaea stagnalis*, which is used as a model in cellular and molecular neuroscience (*3*, *4*). *Lymnaea* hemolymph or central nervous system (CNS)–conditioned medium (CM) evoke neurite outgrowth from snail motor neurons. This activity can be mimicked by murine nerve growth factor (NGF), which furthermore has acute effects on calcium currents in *Lymnaea* neurons (*4*).

Initial attempts to clone a *Lymnaea* neurotrophin homolog by polymerase chain reaction (PCR) on the basis of conserved regions of vertebrate neurotrophins (*5*) were not successful. Therefore, we adopted a functional approach to target neurotrophin family members. All neurotrophins interact with two receptor types—ligand-specific receptor tyrosine kinases of the Trk family (*6*) and a shared receptor termed p75 (*7*). Starting from the premise that a putative molluscan neurotrophin homolog might also bind to the p75 receptor, we assayed *Lymnaea* CM and hemolymph for inhibition of binding of ^{125}I-labeled NGF to A875 human melanoma cells, which express high levels of p75 but no Trk receptors. Hemolymph and CM-derived fractions inhibited NGF binding to p75 in a dose-dependent manner (Fig. 1A). Further fractionation revealed that NGF-displacing fractions from both CM and hemolymph had identical chromatographic elution properties and contained an almost identical protein mass of 13.1 kD.

The higher amounts of the 13.1-kD protein in hemolymph enabled its purification to homogeneity from a pool of 7 liters of snail hemolymph, using matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) to monitor the purification (*8*). The final purified fraction (Fig. 1B) contained a major component of 13.1 kD and a minor component of 13.97 kD, as analyzed by MALDI-MS (Fig. 1C). Internal and NH_2-terminal peptide sequences of both proteins were identical and novel. These sequences served to design primers for PCR and eventual isolation of a cDNA clone (*9*), which encoded an open reading frame of 121 amino acids, including an 18-residue putative signal sequence followed by a mature product of 103 amino acids containing all the peptide sequences previously obtained (Fig. 1E). The sequence was not significantly similar to any known protein or DNA sequence in the public databases. The protein molecular mass predicted from the cDNA sequence is 12.5 kD, which is close to the measured masses of the purified CRNF isoforms and suggests that the latter may arise from different extents of glycosylation on the single consensus *N*-glycosylation site (Fig. 1E). The sequence contained a high number of cysteine residues, comprising over 10% of the protein, hence the name CRNF (cysteine-rich neurotrophic factor).

An anti-peptide antiserum was raised (*9*) against a synthetic peptide based on the COOH-terminal region of the CRNF se-

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