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The *indeterminate* Gene Encodes a Zinc Finger Protein and Regulates a Leaf-Generated Signal Required for the Transition to Flowering in Maize

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Summary

Flowering in plants is a consequence of the transition of the shoot apex from vegetative to reproductive growth in response to environmental and internal signals. The *indeterminate1* gene (*id1*) controls the transition to flowering in maize. We show by cloning the *id1* gene that it encodes a protein with zinc finger motifs, suggesting that the *id1* gene product functions as a transcriptional regulator of the floral transition. *id1* mRNA expression studies and analyses of transposon-induced chimeric plants indicate that *id1* acts non-cell-autonomously to regulate the production of a transmissible signal in the leaf that elicits the transformation of the shoot apex to reproductive development. These results provide molecular and genetic data consistent with the florigen hypothesis derived from classical plant physiology studies.

Introduction

The shoot apical meristem of higher plants embodies a population of undifferentiated cells that gives rise to both vegetative and reproductive structures. Leaves and axillary meristems emerge from the flanks of the shoot apex during vegetative growth, and inflorescences and flowers are formed during reproductive growth. The point of transition, when the shoot apex begins to produce reproductive structures instead of vegetative structures, is a critical developmental process in flowering plants. In some plants such as maize, the shoot apical meristem undergoes a transformation during the transition marked by a rapid increase in cell division and restructuring of the apex. The end results are the formation of the male inflorescence, or tassel, from which determinate flowers emerge, and the cessation of vegetative growth. After the transition, one or two axillary meristems develop into female inflorescences, or ears, which also bear determinate flowers.

Plants have evolved intricate schemes to coordinate the transition to flowering with optimal environmental conditions and developmental states. Some plants are completely dependent upon environmental signals to evoke flowering, whereas others rely on internal developmental cues perhaps correlated with plant size to signal the time of transition (Lang, 1965; Bernier, 1988; McDaniel et al., 1996). Most plants integrate both environmental and developmental signals to elicit flowering. In *Arabidopsis*, at least 12 late-flowering mutants have been described, showing that many genes function to augment the transition to reproductive growth in this species (Koornneef et al., 1991; Martinez-Zapater et al., 1994), and three of these genes have been cloned and characterized (Lee et al., 1994; Putterill et al., 1995; Macknight et al., 1997). Genetic analysis has supported a model for two pathways of floral induction in *Arabidopsis*; one pathway is constitutive, dependent on developmental signals, and the other pathway relies on environmental signals to induce flowering (Martinez-Zapater et al., 1994; Coupland, 1995). A combination of grafting and genetic experiments with pea have identified genes that act at the shoot apex to make it competent to receive the floral stimulus and other genes that act outside the apex to produce graft-transmissible signals that either induce or inhibit the transition to flowering (reviewed in Weller et al., 1997). In contrast, much less is known about genes controlling this process in monocotyledonous plants, including the agronomically important cereals such as maize.

Maize grown in temperate climates is a vegetatively determinate plant that makes the transition to flowering after initiating a particular number of leaves. *indeterminate* (*id1*) is the only mutation known to specifically and severely alter the ability of maize to undergo the transition to reproductive growth (Singleton, 1946). Homozygous *id1* mutants develop normally at first but eventually produce many more leaves than their wild-type siblings (Figure 1A). As with late flowering mutants from other species, *id1* mutants are unable to undergo a normal transition to flower development and remain in a prolonged state of vegetative growth (Galinat and Naylor, 1951). But in contrast to the well-characterized late-flowering mutants in *Arabidopsis* and pea, severe *id1* mutants produce aberrant inflorescences with vegetative characteristics (Figures 1B and 1C). Determinate flowers that form on the flanks of the *id1* inflorescence revert to vegetative growth and whole plantlets emerge from each spikelet (Figures 1C and 1D). Axillary meristems of *id1* mutants either fail to form a female inflorescence or they are converted into branch-like vegetative structures (not shown). These mutant phenotypes demonstrate that the *id1* gene has an important role in controlling the transition to reproductive development as well as in maintaining the florally determined state.

In this study we describe the molecular characterization of the *id1* gene and analyze its expression pattern. The deduced ID1 protein has two zinc finger motifs that implicate *id1* as a regulator of genes that mediate the transition to flowering. Analysis of plants with transposon excisions that generate somatic chimeras provides evidence that *id1* acts non-cell-autonomously; i.e., *id1* can act outside the shoot apical meristem to mediate the transition. Further, expression studies show that *id1* is expressed in immature leaves and is not detectable

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Figure 1. Comparison of Wild-Type Maize and an *id1-m1*/*id1-m1* Mutant

(A) Plants were grown at the same time in the greenhouse. The wild-type plant on the left (*Id1*/*Id1*) has 13 leaves, a normal tassel, and two ears; its *id1-m1*/*id1-m1* mutant sibling (right) has 20 visible leaves and shows no signs of flowering.

(B) Inflorescence of a severe *id1-m1* mutant that produced 32 leaves and a "tassel" with plantlets emerging from every spikelet.

(C) Comparison of tassel branches from normal and *id1-m1* mutant plants. The normal branch (left) shows anthers emerging from some flowers (florets). The mutant tassel branch (right) has produced florets showing initial stages of proliferation of plantlets from within each spikelet.

(D) Spikelet from an *id1-m1* tassel showing an emerging plantlet. Arrowheads indicate each glume of the spikelet. Scale bar = 1 cm.

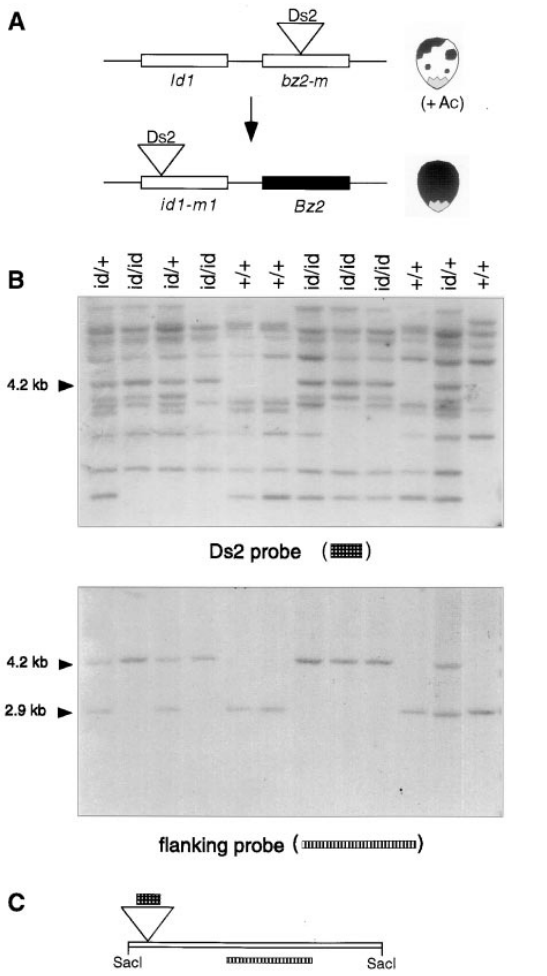


Figure 2. Isolation of an *id1* Genomic Fragment by Transposon Tagging

(A) Illustration of tagging scheme using a *Ds2* transposon inserted within the nearby *bz2-m* allele (indicated by spotted kernel). Selection for germinal excisions (solid kernel) that restored *Bz2* function identified an F2 family that segregated the *id1-m1* allele caused by *Ds2* insertion.

(B) Southern blot of SacI-digested DNA from a family of plants segregating the newly identified *id1* mutation (*id1* = *id1-m1*). The upper blot was hybridized with a *Ds2*-specific probe; the lower panel shows the same blot hybridized with a genomic probe derived from *Ds2*-flanking sequence. The 4.2 kb and 2.9 kb bands are indicated.

(C) Schematic showing relative positions of the probes within the 4.2 kb SacI fragment. Triangle indicates the *Ds2* transposon.

at the shoot apex. Thus, *id1* appears to mediate the transition to reproductive development by regulating the synthesis or transmission of a long-distance signal from the leaves to the shoot apex.

Results

Identification of a Transposon Insertion into the *id1* Gene

The *id1* gene is located on chromosome 1 near the *Bz2* gene that conditions kernel coloration; wild-type *Bz2* kernels are purple, whereas mutant *bz2* kernels are bronze colored. Transpositions of a *Ds2* transposon from the *bz2-m* allele were used for localized mutagenesis of the locus containing the wild-type *Id1* allele (Figure 2A). Out of 600 independent transposition events, one family segregated plants with characteristics of *indeterminate* plants; i.e., they continued to make leaves long after normal siblings had flowered and died. The new mutant was designated *id1-m1* after it was found to be allelic to the first *id1* mutation to be described, *id1-R* (Singleton, 1946), and to another allele, *id1-Compeigne*.

Southern analysis of several hundred plants revealed a 4.2 kb SacI *Ds2*-hybridizing band that consistently cosegregated with the *id1-m1* allele and was absent in plants that did not contain this allele (Figure 2B). The cloned 4.2 kb fragment was found to have a 1.3 kb

Ds2 element inserted 165 bp from one of the SacI sites (Figure 2C). Reprobing the blots with a genomic fragment flanking the insertion showed the expected pattern for *Ds2* insertion; i.e., a 4.2 kb band in *id1-m1* homozygotes, a 2.9 kb band in plants homozygous for the wild-type allele, and both bands in heterozygotes (Figure 2B, lower panel). Southern analysis of other *id1* alleles revealed that *id1-R*/*id1-R* plants had no hybridizing band and that the *id1-Compeigne* allele was associated with an insertion of approximately 3 kb in this region (J. C. and V. S., unpublished data). Therefore, the *id1-R* mutation is caused by a deletion of all or part of the *id1* gene, and the *id1-Compeigne* allele is likely the result of an undetermined transposon insertion.

Determination of the *id1* Coding Region and Gene Structure

Sequence analysis indicated an open reading frame (ORF) near the *Ds2* insertion site with similarities to *Krüppel*-like zinc finger proteins from several species (not shown). Three different cDNA libraries made from 3-leaf seedlings, vegetative apices, and immature ears, respectively, were screened with an ORF probe. However, while cDNAs that encoded zinc finger proteins closely related to *id1* could be isolated, none of them corresponded to the *id1* gene itself (not shown). This suggests that *id1* mRNA may be present only at very low levels or that it is not represented in the libraries that were screened. Subsequent Northern blotting showed *id1* expression only in immature leaf tissue (see below). The complete *id1* cDNA sequence was derived by RT–PCR reconstruction of the *id1* transcribed region using information based on the *id1* genomic sequence and on sequences of cDNA obtained from immature leaves (see Experimental Procedures). The intron/exon structure and deduced amino acid sequence of the *id1* gene are shown in Figures 3A and 3B, respectively.

The *id1* Gene Is a Member of a Zinc Finger Gene Family

The sequence of the *id1* transcript reveals a second zinc finger motif upstream of the first zinc finger motif identified near the *Ds2* insertion site at the 3′ exon/intron junction. Comparison of *id1* to the *id*-like genes isolated from screening cDNA libraries showed a region of extensive similarity that included both zinc finger motifs and the intervening sequence (Figure 3C). Southern analysis with a conserved region probe indicates that there may be as many as ten *id*-like genes in the maize genome (J. C. and V. S., unpublished data).

More recent database searches picked up another gene from potato encoding a protein with striking similarity in the zinc finger region to the *id1* and *id*-like gene products (Figure 3C). This protein, PCP1, is a putative RNA/DNA-binding protein that was isolated by its ability to complement a sucrose transport deficiency in *Saccharomyces cerevisiae* (Kuhn and Frommer, 1995). Searches of *Arabidopsis* expressed sequence tags (EST) and genomic databases revealed an EST (Genbank accession number T04539) and a region of a BAC clone from chromosome IV (R. Martienssen and R. McCombie, personal communication) that encode proteins with a

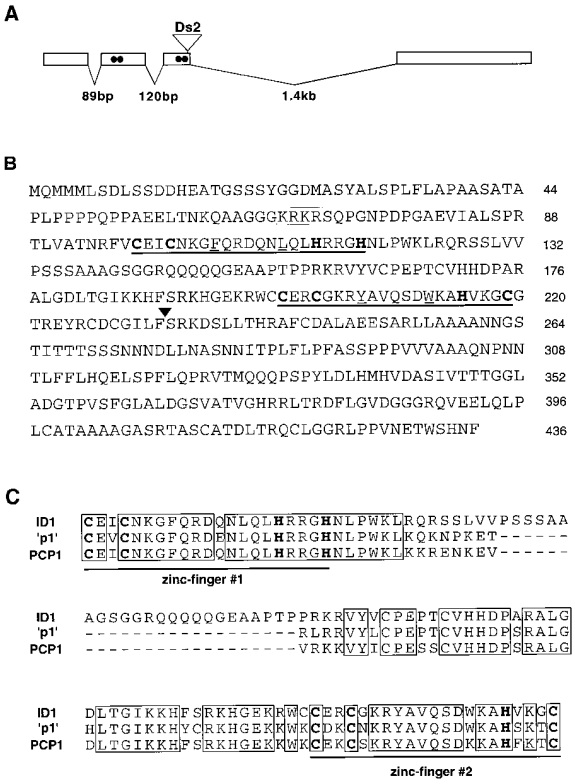


Figure 3. Characterization of the *id1* Gene Structure, Deduced Protein Sequence, and Comparison to *id*-like Genes

(A) Intron/exon structure of the *id1* gene. Open boxes represent exons and thin lines show intron position with size indicated below. Filled double circles show positions of the two zinc finger motifs within the second and third exons. Position of the *Ds2* element near the third exon/intron junction is indicated.

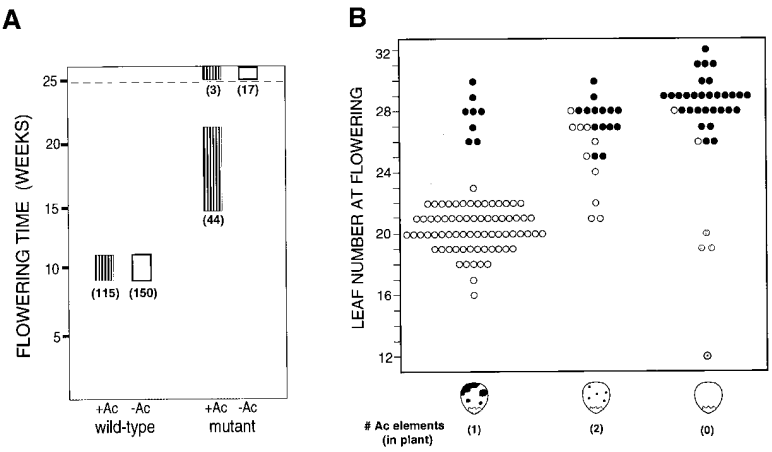
(B) Deduced amino acid sequence of the ID1 protein. The two zinc finger sequences are underlined; conserved cysteine (C) and histidine (H) residues are in bold and conserved hydrophobic residues are double underlined. Position of a potential SV40-like nuclear localization signal is boxed (Raikhel, 1992). A filled triangle shows the relative position of the *Ds2* element within the *id1* coding sequence.

(C) Comparison of the *id1* zinc fingers and intervening region with that of an *id*-like gene from maize, *"p1"*, and to PCP1 from potato (Kuhn and Frommer, 1995).

similar level of homology to the zinc finger motifs above (data not shown). The similarity between the products of *id1*, the *id*-like genes from maize, and the genes from potato and *Arabidopsis* extends beyond the zinc finger region by approximately 30 amino acids on either side of the sequence shown in Figure 3C. The overall identity of these genes in this 160 amino acid region is approximately 70% (data not shown). No sequence could be classed as being more similar to any other in the conserved region, although the *id1* gene is unique in that the region between the zinc fingers has an additional 24 amino acids (Figure 3C).

Effect of *Ac* on the *id1-m1* Phenotype

The *Ds2* transposon in the *id1* gene is expected to excise in the presence of an *Ac* element, giving rise to revertant tissues in the plant as well as derivative alleles that are transmitted through the germ line. Differences in the



C

Allele	(# isolates)	Footprint	Phenotype
Id1		ATCCTCTTCTCCAGGTACATCAT	++
id1-m1(-Ac)	(1)	ATCCTCTTCTCCAGG Ds2 TCTCCAGGTACATCAT	--
id1-X7	(4)	ATCCTCTTCTCCAG (+7) **A**CTCCAGGTACATCAT	--
id1-X71	(1)	ATCCTCTTCTCCAG (+7) **G**CTCCAGGTACATCAT	--
id1-X5	(2)	ATCCTCTTCTCC (+5) **A**CTCCAGGTACATCAT	--
id1-X10	(1)	ATCCTCTTCTC (-10) AT	--
id1-X18	(2)	AT (-18) AT	--
id1-X3	(2)	ATCCTCTTCTCC (+3) TCCAGGTACATCAT	-
id1-X31	(1)	ATCCTCTTCTCCAG (+3) CAGGTACATCAT	-
Id1-Rev	(1)	ATCCTCTTCTCCAGG (+0) TACATCAT	++

Figure 4. Effects of *Ac* on Flowering of *id1-m1* Mutants and Generation of Derivative Alleles

(A) Flowering time of wild-type (left) and *id1-m1*/*id1-m1* mutant plants (right) with *Ac* (hatched boxes) and with no *Ac* (open boxes). Each box indicates the range of flowering time denoted as time of initial pollen shedding. The number of plants in each group is shown in parentheses. The dashed line designates the end of the growing season. Plants that did not flower during the growing season are shown above the dashed line.

(B) Effect of *Ac* dosage on flowering time of *id1-m1* mutants. All plants were grown in a greenhouse from kernels generated by crosses between *id1-m1* homozygous mutants with one *Ac* element. Segregation of *Ac* results in kernels with three distinct spotting patterns that are diagrammed at the bottom of the graph; the number of *Ac* elements in the corresponding plant grown from each type of kernel is shown in parentheses. Plants that made normal flowers are shown by open circles, and plants that made proliferous tassels (with plantlets emerging from spikelets) are shown by filled circles. Shaded circles represent three plants in which *Ds2* had excised and left 3 bp footprints (see below). The revertant allele *Id1-Rev* is indicated by the circle with a dot.

(C) Footprint sequence for eight types of germinal derivative alleles generated by excision of *Ds2* from the *id1-m1* locus. The 8 bp target site for *Ds2* insertion into the wild-type gene is underlined. The inverted "V" indicates the exon/intron splice junction. Nucleotides changed by *Ds2* excision are shown in bold. The phenotype of each mutant is shown on the right. Normal plants (double plus) made 12–14 leaves and wild-type inflorescences; severe *id1* mutants (double minus) made more than 25 leaves and often produced proliferous tassels. Moderate *id1* mutant phenotypes (minus) were observed with the derivative alleles *id1-X3* and *id1-X31*, which made 19 or 20 leaves and normal tassels.

id1-m1 phenotype related to the absence or presence of *Ac* could give clues about how the *id1* gene functions to mediate the transition to reproductive growth. For example, it may be possible to determine whether or not *id1* acts cell-autonomously from analysis of sectored plants generated by *Ds2* excisions. In order to monitor *Ac* activity in sectored plants, we constructed a recombinant in which the *id1-m1* allele was linked to the *bz2-m* marker allele. In *id1-m1 bz2-m* recombinants, *Ac* activity is indicated by the mutability of *bz2-m* as visible sectors of purple pigmentation in the kernel. In early stages of growth, mutant plants with *Ac*, i.e., *id1-m1*(+*Ac*), were indistinguishable from *id1* mutants with no *Ac*. Both classes of mutant plants continued to produce leaves 11 weeks after planting, whereas normal siblings, regardless of the presence of *Ac*, made between 11 and 13 visible leaves before producing a tassel inflorescence at this stage (Figure 4A). At 15 weeks after planting, some of the *id1-m1*(+*Ac*) plants produced normal tassels, and a few initiated ears with vegetative characteristics. When the season ended 25 weeks after planting, *id1-m1*(−*Ac*) plants had produced over 25 leaves and showed no signs of flowering. At this time, all but three of the *id1-m1*(+*Ac*) plants had made tassels that shed pollen (Figure 4A). Some of these plants produced ears that could be crossed with pollen from other *id1-m1* (+*Ac*) mutants. Most of the ears, however, exhibited extensive vegetative characteristics that precluded crossing.

We conclude that *id1-m1* plants (with three exceptions, see below) had a significantly attenuated phenotype in response to the presence of *Ac*. This partial restoration of wild-type characteristics is consistent with excisions of *Ds2* in response to *Ac*, resulting in reversion of *id1-m1* to a functional *Id1* gene. However, we never observed plants that displayed identifiable revertant sectors from *id1* mutant phenotype to wild type. Rather, attenuation of the *id1* mutation by *Ac* resulted in an overall reduction in the severity of the mutant phenotype. No significant differences were observed between the expression of the mutant phenotype in the

tassel and in the ear from any one *id1-m1*(+*Ac*) plant, even though the two inflorescences arise from different populations of cells in the shoot meristem (McDaniel and Poethig, 1988).

As described above, several *id1-m1*(+*Ac*) mutants flowered early enough and made flowers that were sufficiently normal, so a few mutant siblings could be crossed to each other. These plants produced ears with viable kernels that segregated spotted and bronze kernels 3:1, indicating that each parent had a single *Ac* element. The *Ac-Ds* transposon family exhibits a negative dosage phenomenon that is unique to maize; i.e., transposons excise later in development with increasing copies of *Ac*, resulting in smaller somatic sectors (McClintock, 1949). The spotting pattern on *bz2-m* kernels was used as an indicator of the number of *Ac* elements present in the progeny of each kernel. Kernels with large purple sectors have a single *Ac* element, whereas kernels with very few, tiny spots have two or three *Ac* elements present in the endosperm and generally two elements in the embryo. The flowering times of mutant plants with different doses of *Ac* are compared in the experiment shown in Figure 4B. Mutants with a single *Ac* produced significantly fewer leaves and flowered earlier than mutants with no *Ac*. More importantly, *id1-m1* mutants with two *Ac* elements made almost as many leaves as plants with no *Ac* and many exhibited reversion to vegetative growth. Plants with no *Ac* had the most severe mutant phenotype, producing more leaves than siblings with one or two *Ac* elements and also exhibiting floral reversion in nearly all tassels (Figure 4B). This suggests that *Ac*-mediated attenuation of the *id1-m1* mutant phenotype exhibits a negative dosage effect with respect to *Ac* copy number.

Derivative Alleles of *id1-m1* Have Altered Flowering Times

In the previous experiment, three plants did not show attenuation of the *id1-m1* phenotype by *Ac* (Figure 4A). These three plants were siblings that came from spotted kernels, indicating the presence of an *Ac* element, but they resembled plants with no *Ac* since they did not flower. Sequencing revealed that these plants were new derivative alleles caused by *Ds2* excision from the *id1-m1* locus, which left behind a 5 bp duplication resulting in a potential frame shift in the third exon of *id1* (Figure 4C). Such a *Ds2* footprint is expected to generate a stable null allele, which would explain the nonresponsiveness to the effects of *Ac*.

Other alleles derived by *Ds2* excision from *id1-m1* were found by examining progeny of *id1-m1* plants in which the *Ac* had been segregated away. Absence of *Ac* was important in this experiment to ensure that the observed phenotypes were due to germinal excision events and not large somatic revertant sectors. The flowering time for some of these plants is shown in Figure 4B. Several derivatives had either 5 bp or 7 bp insertions that would result in a frameshift in the *Id1* ORF (Figure 4C). The derivative *id1-X10* had a 10 bp deletion that removed the splice donor site, which probably causes a disruption of the normal transcript and results in a severe phenotype. Three independent excision alleles with 3 bp insertions were isolated; two had identical footprints (*id1-X3*) and the other had a different sequence (*id1-X31*), but the overall result was the addition of a single serine residue to the putative coding sequence. All three of these plants made fewer leaves than the most severe *id1-m1* mutants, and they did not show reversion to vegetative growth in the tassels (shaded circles in Figure 4B). Loss of function due to an extra serine residue 12 amino acids downstream of the second zinc finger motif suggests that even minor changes in this region of the ID1 protein have substantial effects on gene function.

Finally, while all the derivatives exhibit mutant phenotypes, one derivative allele was completely normal; i.e., it made 12 leaves and produced fully normal flowers. Southern blots showed that this plant was heterozygous for the *id1-m1* locus. Sequence analysis revealed that *Ds2* had excised precisely, completely restoring the functional *Id1* gene structure (Figure 4C). This plant is a true germinal revertant and not a contaminant, because its parents had been crossed after all normal plants had completely shed their pollen. The possibility that the revertant phenotype is due to large somatic revertant sectors can be eliminated because an *Ac* element was not present. Although excision of *Ds* from a locus more often causes a change in the DNA sequence, precise excisions of *Ds* elements from various genes have been reported previously (Scott et al., 1996; Goodrich et al., 1997).

id1 Is Expressed Early in Development in Immature Leaf Tissue

The expression pattern of *id1* in different parts of the plant and at different times of development was examined by Northern hybridization with an *id1*-specific probe. PolyA$^+$ RNA was prepared from plants early in development (3 days postgermination) to late in development (after flower formation) and from various parts of the plant. Temporally, *id1* mRNA was detected in vegetative shoots as early as 3 days after germination (Figure 5A, longer exposure), and its expression increased as plants approached the floral transition stage. Expression of *id1* was also detected later in development in plants that had undergone the floral transition as well as in plants that had formed flowers (Figure 5C, lanes I and LL). Therefore, *id1* mRNA is present throughout the postembryonic life of the sporophytic plant.

Although *id1* expression is not specific to a particular time of development, we found that *id1* mRNA is present only in particular structures (specifically, immature leaves [Figures 5A and 5B]). Analysis of different parts of 3-leaf and 5-leaf seedlings (i.e., before the floral transition) showed that *id1* is not expressed in leaf blades but is expressed within shoots that contain a whorl of immature leaves and the shoot apical meristem (Figure 5A). We determined by dissection of standard B73 inbred seedlings that the shoot apical meristem of plants with eight visible leaves is at, or near, a stage of transition to flowering. The stems of 8-leaf plants were dissected into five equal 2 cm pieces from which mRNA was extracted and expression of *id1* analyzed (Figure 5B). Northern analysis showed that *id1* is expressed at relatively high levels in the whorl of unexpanded leaves

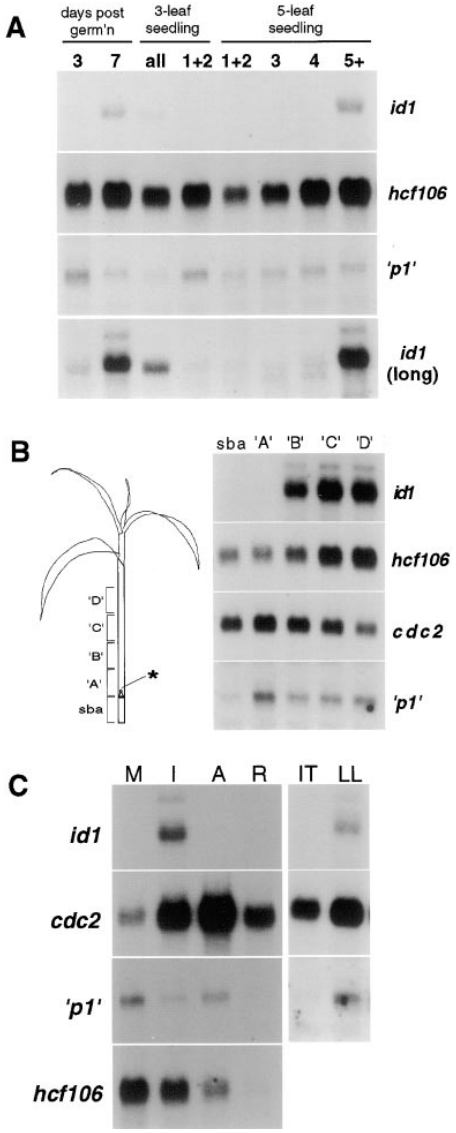


Figure 5. Northern Analysis of *id1* Gene Expression

(A) Expression of *id1* in shoots of 3- and 7-day-old plants and in entire 3-leaf seedling shoots (all) or from leaves 1 and 2 only (1 + 2). Plants with five visible leaves were dissected into leaves 1 + 2, leaf 3, leaf 4, and leaf 5 + shoot (including apex). Blots were reprobed sequentially with the maize *cdc2ZmA* gene and then with *hcf106*, a gene required for chloroplast biogenesis (Martienssen et al., 1989), and a cDNA specific for the maize *id*-like gene *"p1"*.

(B) Expression of *id1* in different portions of a B73 inbred seedling at the transition to flowering stage (eight visible leaves). The first four leaves, including sheath, were removed and the remaining shoot was partitioned into five equal 2 cm portions as illustrated (left). Segment A contains the shoot apical meristem (asterisk); sba refers to the stem below the apex, which contains no leaf tissue.

(C) Expression of *id1* in a plant that has initiated all of its leaves and has begun floral development (ten visible leaves) is shown in the left four lanes. The two right lanes are mRNA samples from a mature plant with a fully formed tassel that has not yet emerged. M, mature leaf derived from fully expanded leaves; I, immature leaf; A, apex transformed into tassel primordium; R, root; IT, immature tassel; LL, the last leaf enclosing the immature tassel. The *id1*-specific probe hybridized to a 1.6 kb band, the approximate size predicted from the cDNA. A minor band of 1.9 kb that consistently hybridizes with the *id1* probe may be an unspliced precursor RNA.

above the shoot apex, but it is not detectable in the part of the shoot that contained the apex nor is it expressed in the internode stem below the apex. Expression of *id1* mRNA therefore is confined to immature leaves and not the shoot apex.

Northern analysis with posttransition plants (ten or more visible leaves) reveals that *id1* continues to be expressed in immature leaves and remains detectable, although at lower levels, in the last immature leaf that surrounds the tassel before it emerges from the whorl of leaves (Figure 5C, lane LL). No *id1* mRNA was detected in mature leaves or in roots or floral tissues at any stage of plant development. Conversely, the *id*-like gene *"p1"* is expressed in both mature and immature leaves as well as in the apical region, but it is absent in root and floral tissues (Figures 5B and 5C).

In situ hybridization experiments provided further evidence that *id1* is expressed in immature leaf tissue and not at the apex. Transverse sections through the stems of B73 inbred seedlings with eight visible leaves showed *id1*-specific mRNA in the inner, immature leaf of the whorl and not in the outer leaf (Figures 6A and 6B). Expression was not detectable at the shoot apical meristem and the bases of surrounding leaves (Figure 6C), although expression of the *Knotted* (*Kn*) gene was confined to the shoot apical meristem (Figure 6D) as reported previously (Jackson et al., 1994). Longitudinal sections through the shoot apical meristem similarly showed no *id1* expression (unpublished data). As expected, *id1* mRNA was not detected by in situ hybridization in any tissue sections of *id1-R* mutant plants (data not shown). Although *id1* expression appears to be greater in the outer epidermal layers of the inner leaves, there is also detectable staining in the leaf mesophyll cells, which might be less intense because of large vacuoles (Figure 6B).

In contrast, expression analysis of the *Arabidopsis LD* gene by Northern hybridization (Lee et al., 1994) and *CO* by RT–PCR analysis (Putterill et al., 1995) showed that these genes are expressed in all aerial parts of the plant, and in situ hybridization experiments showed that *CO* expression is localized to the vegetative shoot apical meristem and several leaf primordia (Simon et al., 1996). *FCA* transcripts were detected in roots as well as shoots (Macknight et al., 1997). Therefore, the expression pattern of *id1* differs from previously characterized flowering time genes in that *id1* expression is restricted to immature leaves and is absent from the shoot apex.

Discussion

The deduced amino acid sequence of the ID1 protein shows two zinc finger motifs with the pattern HH-CC and HH-HC, respectively. Since zinc finger proteins are associated with DNA/RNA binding activity and the particular type of zinc finger in ID1 has some similarity to a class of animal transcription factors, we suggest that ID1 could act as a regulatory protein that controls the transcription of genes required for the transition to flowering. The existence of related genes in maize with extensive similarities in the zinc finger and surrounding regions suggests the possibility of some functional redundancy with *id1*. However, the one *id*-like transcript

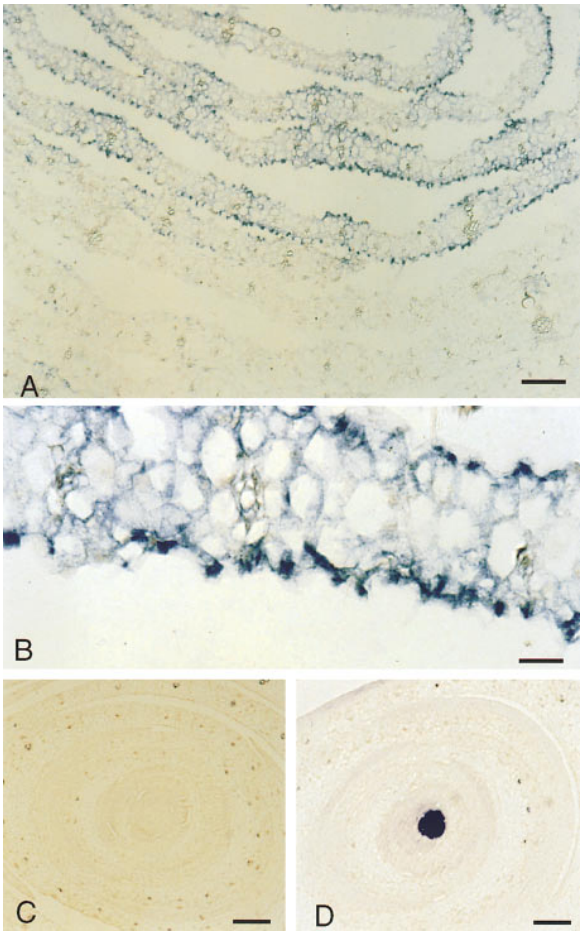


Figure 6. In Situ Hybridization in Seedlings with Eight Visible Leaves

(A) Transverse section through the stem 5 cm above the apex with *id1* anti-sense probe. Two concentric leaves are shown wrapped within the whorl; *id1* staining is evident in the inner leaf whorl. Scale bar = 300 μm.

(B) Same section as (A). Scale bar = 100 μm.

(C) Transverse section through the shoot apical meristem hybridized with *id1* anti-sense probe. Scale bar = 300 μm.

(D) Transverse section through the apex of same plant as (C) hybridized with a *Kn*-specific anti-sense probe showing staining in the shoot meristem (Jackson et al., 1994). Scale bar = 300 μm.

that we characterized in detail (*"p1"*) had a markedly different expression pattern from that of *id1* (Figure 5), so it is likely that some of the *id*-like genes of maize regulate developmental processes other than flowering.

Arabidopsis is the only other plant species in which genes that specifically regulate the transition to flowering have been isolated. The *LUMINIDEPENDENS* (*LD*) gene encodes a possible homeodomain protein (Lee et al., 1994; Aukerman and Amasino, 1996), and the CONSTANS (CO) gene product contains GATA factor–like zinc finger motifs (Putterill et al., 1995). More recently the FCA gene product was reported to have similarities to RNA-binding proteins, suggesting a potential role in controlling gene expression at the level of RNA processing (Macknight et al., 1997). All three of these genes have a postulated role in controlling the transition to flowering. Although *id1* is similar in that it likely has a regulatory function, the *id1* gene product has no significant similarity to these three *Arabidopsis* proteins. *id1* is the only flowering transition gene isolated from a monocot, and there is presently no evidence for or against the existence of orthologs of this gene in dicots.

The *id1* mutation causes other defects in reproductive development besides delayed flowering, i.e., floral aberrations caused by reversion to vegetative growth that have not been reported for flowering time mutants described in dicots. We note that a floral reversion phenotype is associated with mutation of floral meristem identity genes such as *floricaula* of *Antirrhinum* and *LEAFY* of *Arabidopsis* (Coen et al., 1990; Weigel et al., 1992). In these cases, loss of gene function causes a transformation of flowers into inflorescence shoots. In addition, under conditions of noninductive short-day photoperiods, plants heterozygous for a *leafy* null mutation show a high rate of floral reversion, with shoots emerging from within flowers (Okamuro et al., 1996). In some species such as *Arabidopsis*, meristem identity genes such as *LEAFY* may play an important role in establishing as well as maintaining floral meristem identity (Blázquez et al., 1997). Therefore, there likely are differences between monocots and dicots in the genetic regulatory networks for the induction of flowering and for the maintenance of the florally differentiated state.

Evidence for Non-Cell-Autonomous Action of the *id1* Gene

The overall attenuation of the *id1-m1* phenotype by *Ac* together with the absence of visible phenotypically revertant sectors is characteristic of non-cell-autonomous gene action. An alternative explanation would be that the presence of *Ac* partially restores *Id1* function in every cell carrying the *id1-m1* mutation. The phenotypes of mutations generated by transposon insertions can depend upon the presence of regulatory transposons (McClintock, 1955). In the *Ac-Ds* system, such an effect was shown to occur with the *Kn* gene of maize (Hake et al., 1989). However, we believe that this explanation is not applicable to the effects of *Ac* on *id1-m1* for the following two reasons. First, attenuation of the *id1-m1* mutation exhibited a negative dosage effect with the *Ac* copy number. Plants with one *Ac* had a less severe *id1* phenotype than plants with two *Ac* elements. Such a negative dosage effect is typical of excision events in the *Ac-Ds* system; i.e., *Ds2* transposes from the mutant locus later with more *Ac* elements, resulting in smaller revertant sectors. By contrast, the *Ds2*-induced gain-of-function *Kn* mutation exhibits a positive dosage effect with *Ac* (Hake et al., 1989). Second, for all suppressible transposon-induced mutations that have been molecularly characterized to date, the transposon is inserted in a noncoding region of the gene, so that transcripts encoding a functional protein can be generated without transposon excision. In *id1-m1*, the *Ds2* element is inserted into a coding exon of the *id1* gene near the zinc finger domain at a location where even minor alterations of the amino acid sequence severely affect function. Therefore, it is likely that attenuation of the *id1-m1* phenotype is due to excisions of *Ds2* rather than suppression by *Ac*.

Another relevant observation is that even though the flowers of many *Ac*-containing *id1-m1* plants appeared normal, presumably due to reversion by *Ds2* excision, the gametophytes within these flowers always carried the mutant allele. Generation of a revertant sector that completely restores normal tassel formation would require an early excision event that covers the whole shoot apical meristem including both epidermal (L1) and subepidermal (L2) layers of the developing inflorescence (Dawe and Freeling, 1990). Therefore, pollen derived from a revertant tassel would necessarily be revertant as well, resulting in wild-type progeny in the next generation. Such was not the case; with one exception, the progeny of *id1-m1*(+*Ac*) mutants were all mutants. The exception was a germinal revertant that resulted from a precise excision of the *Ds2* element. Since the siblings of this revertant were still mutant, we assume that reversion occurred late in gametophyte development and that it did not affect tassel or ear initiation. These results are most simply explained by proposing that the mode of action of the *id1* gene product is non-cell-autonomous and that excision events outside the apex can restore normal tassel formation.

The earlier flowering times observed with one *Ac* element as opposed to two *Ac* elements can now be interpreted as follows. We anticipate that more functional ID1 would be generated by the frequent excisions expected with one *Ac* element than by the infrequent excisions with two *Ac* elements. If a critical level of ID1 is required for signaling the transition of the apex, this level will be reached earlier in *id1-m1* plants that have frequent excisions of the *Ds2* element. Similarly, the *CO* gene of *Arabidopsis* displays a dose-dependent induction of flowering, since extra copies of *CO* or ectopic expression promotes earlier flowering (Putterill et al., 1995; Simon et al., 1996). It should be noted that whereas *CO* is semidominant, *id1* is completely recessive, and there appears to be no difference in flowering time between plants with one or two functional *id1* genes (Singleton, 1946), perhaps due to dosage compensation.

Since plants with no *Ac* do ultimately flower, although imperfectly, the *id1* gene may be partially redundant with a second gene or floral-induction pathway. It is possible that one of the *id*-like genes is partially able to substitute for *id1* function. We note that mutations other than *id1* that severely affect the flowering transition in maize have not been isolated, suggesting that redundant genes may be detectable only in a loss-of-function *id1* mutant background.

Model of *id1* Gene Action: Support for the Florigen Model

Numerous studies of the last century have provided insights into the physiology of the floral transition. One of the most significant conclusions from these studies is that the flowering transition is triggered by a signal that originates in the leaves (Bernier, 1988; O'Neill, 1992). This led to the proposal that a diffusible substance, sometimes referred to as "florigen," is made in leaves in response to environmental and/or developmental cues and is diffused or transported to the apex where it triggers the transition to reproductive development. Our analysis of the *id1* gene provides evidence that is consistent with the florigen model. We find that *id1* is expressed in specific vegetative structures such as immature leaves, even though its effect is manifested at the shoot apex. In addition, genetic evidence based on transposon-mediated effects on the mutant phenotype suggest that the action of *id1* is non-cell-autonomous. We propose that revertant somatic sectors in immature leaves act as sites of ID1 production. Functional ID1 protein in the leaf then mediates the synthesis of a transmissible substance(s) that migrates to the apex to signal the transition to flowering. It is also possible that the *id1* gene product itself migrates from its site of synthesis in the leaves to the apex. Intercellular migration was reported for the KNOTTED homeobox protein (Lucas et al., 1995). However, we consider this possibility to be unlikely, since KNOTTED migration is limited to a few cell layers and cannot account for the type of long-distance signaling proposed for *id1*.

Our results suggest a model for the regulation of flowering time by *id1*. Maize plants generally flower after making a fixed number of leaves, indicating that flowering is initiated by an endogenous signal that is dependent upon leaf number. Detection of *id1* mRNA in 3-day-old seedlings suggests that ID1 protein is expressed in leaf tissue throughout shoot development. In addition, studies with *id1-m1* plants with different *Ac* doses indicate that flowering of *id1-m1* plants is accelerated by increasing levels of functional *id1* gene product. We suggest that the amount of ID1 in vegetative tissues must increase to a critical level to signal the transition and that this critical level is reached after a particular number of leaves are generated. If *id1* regulates the synthesis of a diffusible factor, as we propose, then the level of ID1 in leaves would reflect the concentration of diffusible factor that is received by the apex, and a threshold level for *id1* product may reflect the requirement for a critical concentration of this factor for the initiation of reproductive growth. The idea of a critical or threshold level for *id1* is consistent with observations from studies with excised maize shoot apices showing that the meristem relies on signals from other parts of the plant to determine the extent of vegetative growth (Irish and Nelson, 1988, 1991; Irish and Jegla, 1997). Similar experiments in tobacco demonstrated that a critical leaf number is required for shoot apices to become determined for reproductive development (Singer and McDaniel, 1986).

The observation that *id1* continues to be expressed late in development after flowers are formed suggests an additional role in maintaining the florally determined state. Consistent with this observation, even severe *id1* mutants eventually undergo a transition in which the shoot apex is converted to an inflorescence-like structure, but the floral meristems that form are capable of further vegetative growth as evidenced by the emergence of plantlets containing shoots and roots from within the spikelets. In some plants such as *Impatiens* and wheat, an inflorescence or flower will revert to vegetative development when the floral stimulus is removed (Fisher, 1972; Battey and Lyndon, 1990; Pouteau et al., 1997).

Conclusions

We have reported the isolation and characterization of the *id1* gene from maize. *id1* is the first gene isolated from a monocot that has an important role in signaling the transition from vegetative to reproductive growth, and it has some distinctive features as compared to genes isolated from dicots that regulate the same process. The action of *id1* is non-cell-autonomous, and a critical level of *id1* may be required for the floral transition. *id1* could regulate the floral transition either by acting at the shoot apex to make it competent to receive the floral stimulus or outside the apex to regulate the production or transmission of a floral signal that is sent to the apex. Since *id1* is expressed in immature leaves but not at the shoot apex, we suggest that *id1* acts through the second mechanism. Together with genetic evidence that suggests *id1* acts in a non-cell-autonomous manner, we propose that *id1* directly or indirectly regulates the synthesis of a florigenic substance or substances that is transmitted to the apex, consistent with physiological studies of the last century.

Experimental Procedures

Maize Stocks and Genetic Analysis

Maize *bz2-m* stocks were a gift from Kelly Dawe (University of Georgia). Other maize stocks were obtained from the Maize Genetics Stock Center (Urbana, IL) and Virginia Walbot (Stanford University). *id1-R* and *id1-Compeigne* alleles were obtained from Benjamin Burr (Brookhaven National Laboratory, Upton, NY). Inbred B73 seed was a gift of Zuo-Yu Zhao (Pioneer Hi-Bred International). The strategy used to obtain a transposon-tagged allele of *id1* is reported in detail elsewhere (Colasanti and Sundaresan, 1992). The effect of *Ac* dosage on the *id1-m1* phenotype was observed by recording the flowering time of *id1* mutants with zero, one, or two copies of *Ac* based on the kernel spotting pattern. Kernels derived from crosses of two *id1-m1* homozygous plants were classified as having one *Ac* element (large spots), two *Ac* elements (tiny spots), or no *Ac* elements (no spots). These were grown in greenhouses so that a complete life cycle could be observed. The number of leaves made by wild-type and *id1* mutant plants was determined by counting from the first node visible above the ground. Plants were grown outdoors and in greenhouses at Uplands Farm Agricultural Field Station, Cold Spring Harbor, New York.

Southern Blot Analysis and Isolation of Genomic Subclones

DNA was extracted from maize leaves as described by Chen and Dellaporta (1994), digested with restriction enzymes, and transferred to nitrocellulose membranes according to standard protocols (Sambrook et al., 1989). Probes were ^{32}P-labeled using a random priming DNA labeling kit (Boehringer-Mannheim), and hybridization was performed using standard 50% formamide hybridization buffer with 10% dextran sulfate. A 109 bp *Ds2*-specific probe (from Sarah Hake Plant Gene Expression Center, Albany, CA) was used to identify cosegregating *Ds2*-hybridizing bands. A 4.2 kb SacI fragment containing a portion of *id1* gene was isolated from a subgenomic library constructed from 100 μg of SacI-digested genomic DNA from an *id1-m1* mutant. Purified DNA was ligated with SacI-digested pLITMUS29 (New England Biolabs) and transformed into DH10B cells by electroporation. Approximately 60,000 transformants were plated on nitrocellulose membranes and screened with a labeled *Ds2* probe. One recombinant plasmid containing the 4.2 kb SacI fragment was isolated (Figure 2C). The *id1* genomic region was sequenced by primer walking; i.e., oligonucleotides complementary to every 200–300 bp of genomic DNA were synthesized and used in sequencing reactions.

Screening cDNA Libraries for *id1* and *id*-like Sequences

A 165 bp *Ds2*-flanking genomic DNA fragment with a putative open reading frame (ORF) was labeled and used to screen three B73 maize cDNA libraries. Approximately 2×10^6 plaques were screened from a 3-leaf seedling library (made by Alice Barkan, University of Oregon, Eugene, OR), and 1×10^6 plaques each were screened from an immature ear library and a library made with mRNA from 3- to 4-week-old vegetative apices (from Bruce Veit, Plant Gene Expression Center, Albany, CA). Phage clones that hybridized to the probe were isolated and plasmids with inserts were excised by an in vivo protocol recommended by the manufacturer (Stratagene).

Isolation of a Genomic Clone and Determination of the *id1* Transcribed Region

A genomic clone containing the entire *id1* coding region was isolated from a B73 DNA library (from David Jackson, Plant Gene Expression Center, Albany, CA) by screening with a 1.0 kb probe derived from the 1.4 kb intron of *id1* (see Figure 3). One recombinant phage, λ1a, was isolated from approximately 1×10^6 plaques screened. Two overlapping fragments from the genomic insert of λ1a were subcloned into pLITMUS29. Plasmid pZY12 contained a 4.0 kb BamHI fragment that included most of the *id1* coding region, and pZY15 contained a 1.3 kb SacI fragment that overlapped by 110 bp with the 5′ end of the BamHI fragment. RT–PCR identified the *id1* transcribed region. Total RNA was isolated from immature leaves of 3-week-old plants by grinding in liquid nitrogen and extraction with Trizol reagent (Bethesda Research Laboratories). PolyA$^+$ RNA was isolated by Oligotex spin elution (Qiagen). Reverse transcriptase (SuperscriptII) was used to synthesize cDNA from 1 μg of purified mRNA using the manufacturer's protocol (Bethesda Research Laboratories). Oligonucleotides spanning the genomic region were used as primers in PCR reactions with cDNA as template. Amplification products that consistently reamplified with nested primers and that were smaller than products amplified from genomic DNA templates were cloned into pLITMUS29 and sequenced. Comparison of amplified sequences to genomic sequences revealed the intron positions.

Northern Blot Analysis

PolyA$^+$ RNA was isolated from tissues using the method described above, and 1 μg of each sample was electrophoresed on 1.1% formaldehyde agarose gels and transferred to nylon membranes (Genescreen). A 650 bp fragment of genomic DNA specific to the *id1* transcribed region was used to probe Northern blots using standard conditions (Sambrook et al., 1989). A 900 bp DNA fragment unique to the *id*-like gene *"p1"* was amplified from a cDNA clone and used as probe. The *cdc2ZmA* probe was prepared as described previously (Colasanti et al., 1991), and the *hcf106* probe was made from a 1.1 kb fragment of cDNA clone (provided by Mark Settles, Cold Spring Harbor Laboratory).

In Situ Hybridization

Plant tissues were prepared from maize B73 or *id1* mutant seedlings with eight visible leaves and fixed and hybridized by the method of Jackson (1991) except that FAA (3.5% formaldehyde, 10% acetic acid, 50% ethanol) was used as fixative. Fixed tissue was dehydrated, embedded in Paraplast, sectioned at 10 μm thickness, and attached to ProbeOnPlus slides (Fisher Biotech). Two overlapping regions of *id1*-specific sequence were cloned into transcription vector pSPT18 (Boehringer-Mannheim) to create plasmids p390 and p400 (with 390 bp and 400 bp inserts, respectively). Riboprobes were prepared following directions provided by the manufacturer (Boehringer-Mannheim). Anti-sense *id1* riboprobe was prepared from p390 by linearizing with HindIII and in vitro transcription with T7 RNA polymerase in the presence of 11-digoxigenin-dUTP; anti-sense riboprobe from p400 was prepared by linearizing with BamHI and transcription with SP6 RNA polymerase. Riboprobes were mixed and used at a total concentration of 2 ng/μl/kb for each slide. A *Kn* anti-sense riboprobe was provided by the Hake Laboratory. Immunological digoxigen–nucleic acid detection followed recommended protocols (Boehringer Mannheim) except that antibody conjugate was diluted 1:1250.

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