Journal of Experimental Botany, Vol. 54, No. 383, pp. 681–690, February 2003
DOI: 10.1093/jxb/erg077

Journal of Experimental Botany

RESEARCH PAPER

Knotted1-like genes are strongly expressed in differentiated cell types in sunflower[1]

Mariana F. Tioni, Daniel H. Gonzalez and Raquel L. Chan[2]

Cátedra de Biología Celular y Molecular, Facultad de Bioquímica y Ciencias Biológicas, Universidad Nacional del Litoral, CC 242 Paraje El Pozo, 3000 Santa Fe, Argentina

Received 17 June 2002; Accepted 7 October 2002

Abstract

Knotted1-like genes constitute a family of genes whose products are transcription factors involved in several aspects of plant development. In most species, these genes are expressed primarily in meristematic cells and are switched off as leaves develop. In this paper, the expression patterns of three kn1-like genes from sunflower (*Helianthus annuus* L.) are described. Northern blot experiments indicated that these genes are expressed at different levels in several organs of the plant, including flowers, leaves, stems, roots, and embryos. Notably, one of these genes, named *HAKN1*, was also highly expressed in leaves and roots. Using *in situ* hybridization, expression was detected in parenchymatic cells from leaf veins, petiole and lamina, and also in stem and root. Enhanced expression in phloem was also evident in both leaves and stem. Another, *HAKN2*, showed preferential expression in stem, specifically in fascicular and interfascicular cambium and phloem. In flowers, both genes are expressed throughout inflorescence and floral meristems and in developing organ primordia. Strong expression of *HAKN1* in developing involucral bracts was also observed. The results show the existence of some differencies in expression patterns of kn1-like genes in sunflower with respect to other plants. It is proposed that cell- and species-specific factors are involved in determining the developmental responses of plant cells to the expression of kn1-like genes.

Key words: Compositae, gene regulation, homeobox, *Knotted1*, plant architecture, sunflower.

Introduction

Homeobox genes encode a group of transcription factors generally involved in the regulation of developmental processes (Gehring, 1987). These genes contain a region coding for a homeodomain, a 61 amino acid protein motif that interacts specifically with DNA (Qian *et al.*, 1989, 1994; Gehring *et al.*, 1994; Tsao *et al.*, 1995). In plants, the first homeobox was identified in Knotted1 (*kn1*), a maize gene for which dominant mutations affect leaf development (Vollbrecht *et al.*, 1991). The knotted leaf phenotype is due to the aberrant expression in leaves of the *kn1* gene, which is normally active only in meristematic cells (Smith *et al.*, 1992). Additional kn1-like genes have been isolated from maize and other monocot and dicot species (for review, see Chan *et al.*, 1998), indicating that this class of genes constitute a family present throughout the plant kingdom.

The *kn1* family of genes can be subdivided into two classes, I and II, by sequence relatedness and expression patterns (Kerstetter *et al.*, 1994). While class I genes, such as *kn1*, are mainly expressed in apical meristems and embryos, class II genes show a broader range of expression. Based upon expression patterns (Smith *et al.*, 1992; Jackson *et al.*, 1994; Lincoln *et al.*, 1994; Sentoku *et al.*, 1999), analysis of mutants (Müller *et al.*, 1995; Schneeberger *et al.*, 1995; Long *et al.*, 1996; Kerstetter *et al.*, 1997) and overexpression studies (Matsuoka *et al.*, 1993; Sinha *et al.*, 1993; Chuck *et al.*, 1996; Tamaoki *et al.*, 1997) it was proposed that type-I kn1-like genes are involved in the maintenance of meristematic cells in an undifferentiated state. Indeed, overexpression of some class I genes in *Arabidopsis* and tobacco produces the proliferation of meristems on the surface of leaves. The most striking exception to this behaviour has been observed in tomato, where some class I genes are

[1] The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the Accession nos AY096802, AY096803 and AY096804.

[2] To whom correspondence should be addressed. Fax: +54 342 4575219. E-mail: rchan@fbcb.unl.edu.ar

expressed in young leaves, flowers and roots (Hareven *et al*., 1996; Chen *et al*., 1997; Parnis *et al*., 1997; Janssen *et al*., 1998*a*, *b*; Koltai and Bird, 2000). Since overexpression of these genes in tomato produces a phenotype of supercompound leaves, it has been suggested that the deregulation of the expression may be responsible for the characteristic compound leaf architecture of this species (Hareven *et al*., 1996; Chen *et al*., 1997; Parnis *et al*., 1997).

In the present study, three homeobox cDNAs representing class I kn1-like genes from sunflower were isolated and their expression in different organs and cell types was characterized. It was observed that the expression patterns of these sunflower genes resemble the situation in tomato. Sunflower (*Helianthus annuus* L.) is a plant with simple leaves and belongs to the Compositae family, which is characterized by the presence of compound flowers. The results suggest that the effect on plant development of the expression of class I *kn1* genes in defined cell types depends on additional, species-specific, factors.

Materials and methods

Plant material

Sunflower plants (*Helianthus annuus* L. cv. contiflor 15, from Zeneca seeds) were grown in pots filled with soil in a growth chamber at 24–28 °C, 14-h photoperiod and 700 µmol m^{-2} s^{-1} photosynthetic photon flux density. Roots were collected from seedlings grown on trays 72 h after germination. Inflorescences at different developmental stages were harvested and classified as defined by Schneiter and Miller (1981).

Isolation of kn1 cDNA homologues from sunflower

Degenerate oligonucleotides 5′-GGCGGATCCTTNCKYTGRTTD-ATRAACCA-3′ and 5′-GCGGAATTCGAYCARTTYATGGARG-CNTA-3′, corresponding to amino acid regions conserved in class I kn1-like polypeptides, were used as primers to amplify putative sunflower kn1-like cDNA fragments from R1-stage inflorescence RNA reverse transcribed with the first of these oligonucleotides as primer. The amplification product, consisting of one major band of about 500 bp, was purified, digested with *Eco*RI and *Bam*HI and cloned into the pUC119 plasmid treated with the same enzymes. Several clones were sequenced by the dideoxy chain termination method.

The 3′-ends of the transcripts were obtained applying RACE (rapid amplification of cDNA ends) to polyA$^+$ RNA obtained from sunflower R1 inflorescence using specific oligonucleotides (5′-GCGGAATTCRTGGCCATAYCCTTCGGA-3′ for *HAKN1*, 5′-GGCGGATCCATCAGAGAAGGTAGCGT-3′ for *HAKN2*, and 5′-GGAGGATCCCTTGACTGGTGGACTAGA-3′ for *HAKN3*) and primers Qt and Qo according to Frohman (1994). The respective 5′ ends were obtained by PCR on total DNA from cDNA libraries from R1-stage inflorescence (for *HAKN1*) or stems (for *HAKN2*) using one specific oligonucleotide (5′-GAAGTGGAAATAGA-GATAG-3′; or 5′-TCGCATTTGCTTTCATCAG-3′, respectively) and one oligonucleotide that matches vector sequences. PCR products were cloned in pGEM-T Easy (Promega Corp.) and sequenced.

Phylogenetic analysis

Sequences encoding kn1 homologues were recovered by homology search in nucleotide sequence databanks using the program BLASTP (Altschul *et al*., 1990) with sunflower hakn1, hakn2 and hakn3 protein sequences. Phylogenetic analysis was performed using programs from the PHYLIP group on an alignment of protein sequences generated by ClustalW (Thompson *et al*., 1994). Only full-length amino acid sequences were used for the analysis.

RNA isolation and analysis

Total RNA was prepared as described (Almoguera and Jordano, 1992). For RNA blot analyses, 20 µg of total RNA were denatured with formamide and formaldehyde, separated in a 1.5% (w/v) agarose/6% formaldehyde gel, and blotted onto nylon membranes (Hybond N, Amersham-Pharmacia) essentially as described by Sambrook *et al*. (1989).

Hybridization was performed overnight at 65 °C in 6× SSC (1× SSC is 0.15 M NaCl, 0.015 M Na_3-citrate, pH 7.0), 0.1% (w/v) polyvinylpirrolidone, 0.1% (w/v) BSA, 0.1% (w/v) Ficoll, and 0.5% (w/v) SDS. Fragments corresponding to the 3′-non coding region of the three cDNAs were labelled with [^{32}P]dATP (1× 10^8 dpm µg^{-1}) by random priming (Sambrook *et al*., 1989) and used as probes. Filters were autoradiographed using Bio-Max films and transcreen (Kodak) overnight. To check the amount of total RNA loaded in each lane, filters were then re-probed with a 25S rDNA from *Vicia faba* under similar conditions as those described above, except that hybridization was performed at 62 °C. To check the amount of total mRNA present in each sample, similar filters were hybridized with a polydT (20-mer) probe labelled with [^{32}P]dATP (1× 10^8 dpm µg^{-1}) with terminal transferase as described in Sambrook *et al*. (1989). For this probe, hybridization and washing were performed at 42 °C.

In situ *hybridization*

Tissue preparation and *in situ* hybridization were carried out essentially as described by Burgess (1995). Plant material was fixed overnight in 3.7% formaldehyde, 5% acetic acid, 47.5% ethanol at room temperature, dehydrated through an ethanol series, and embedded in Histoplast (Biopack). Sections (8–10 µm thick depending on the material) were mounted on slides coated with 50 µg ml^{-1} poly-D-lysine (Sigma Chemical Co., St Louis, MO) in 10 mM Tris-HCl, pH 8.0, and dried overnight at 37 °C. After removing the paraffin with xylene, sections were rehydrated by an ethanol series and treated with 1 µg ml^{-1} proteinase K for 30 min at 37 °C and then with acetic anhydride in 100 mM triethanolamine, pH 8.0, for 10 min at room temperature. After a brief wash with H_2O, sections were used for hybridization.

Digoxigenin-labelled RNA sense, antisense and control probes were synthesized using the DIG-RNA labelling mix (Boehringer Mannheim) and T3 or T7 RNA polymerase, according to the manufacturer's instructions. Labelled RNA was precipitated with LiCl and ethanol and its concentration and integrity was checked in agarose gels. Clones containing the 3′-non-coding regions of *HAKN1*, *HAKN2* and *HAKN3* in pBluescript SK$^-$ were linearized with appropriate restriction enzymes and used as templates. In some experiments, sections hybridized with sense probes showed patterns of label similar to those observed with the corresponding antisense probe (although with significantly lower intensity). This may be due to contamination of the sense probe with antisense RNA originated from unspecific initiation by RNA polymerase, as previously reported by Müller *et al*. (2001). In those cases, an antisense probe for a rat steroid hormone receptor was also used as a negative control to exclude signals arising from unspecific hybridization. PolydT was labelled with digoxigenin-UTP with terminal transferase (Promega).

Tissue sections were prehybridized in a moist chamber for 30 min at 44 °C in 300 µl per slide of a solution containing 50% formamide,

4× SSC, 5% (w/v) dextran sulphate, 0.02% (w/v) polyvinylpirrolidone, 0.02% (w/v) BSA, 0.02% (w/v) Ficoll, and 0.25 mg ml^{-1} yeast tRNA. Hybridization was carried out overnight under similar conditions with 1 µg ml^{-1} probe. After they were hybridized, sections were washed twice in 2× SSC at 44 °C for 15 min, and once in 500 mM NaCl, 10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA at 37 °C for the same time. Single-stranded RNAs were removed from the sections with a 30 min incubation in 10 µg ml^{-1} RNase A in the same buffer, followed by washes (15 min each) in 2× SSC, 1× SSC, 0.5× SSC (at 37 °C), and 0.2× SSC (at room temperature). For probe detection, slides were washed twice with 20 mM Tris-HCl, pH 7.5, 0.48 M NaCl, blocked for 30 min in 1% (w/v) Blocking Reagent (Boehringer Mannheim), incubated for 2 h at room temperature with alkaline phosphatase labelled anti-digoxigenin (Boehringer Mannheim) diluted 1:500 in the same buffer, and developed after two new washes using the BCIP/NBT colorimetric system. Development was carried out until satisfactory signals were obtained (usually overnight). After stopping the reaction with 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, sections were mounted with 15% (w/v) gelatine, 50% glycerol in water with traces of phenol. Digital images were generated using a Coolpix 995 digital camera (Nikon) mounted on an optical microscope, and processed with Adobe Photoshop 5.0. When polydT was used as probe, *in situ* hybridization experiments were carried out as described by Mine *et al*. (2001).

Results

Isolation of three sunflower kn1-like cDNAs

In order to characterize kn1-like homeobox containing sequences from sunflower, several partial clones were isolated using an RT-PCR strategy on mRNA isolated from immature inflorescences. Sequencing of the different clones revealed three different cDNAs that represent members of the kn1 family that were named *HAKN1, HAKN2* and *HAKN3*. Sequences corresponding to the 5′ and 3′ ends of *HAKN1* and *HAKN2* were obtained by PCR as described in the Materials and methods. For *HAKN2*, two sequences that differed by the presence of 21 extra base pairs within the protein coding region were found. This results in the inclusion of seven extra amino acids in a highly variable region of the MEINOX domain (Bürglin, 1997). The two cDNAs may represent allelic forms of the same gene, since a hybrid sunflower cultivar was used as a source of RNA. Alternatively, these forms may originate from alternative splicing of a single gene; the additional sequence begins with the dinucleotide 5′-GT-3′, known to be present at the 5′ end of introns. For *HAKN3*, efforts to isolate clones encoding the 5′ region of the transcript by either RACE or library screening were unsuccessful, perhaps due to its low abundance.

Comparison of the proteins encoded by these genes with other members of the knotted family indicated that they belong to class I, sharing between 75% and 100% identical amino acids within the homeodomain with other members of this subfamily. *HAKN1* and *HAKN3* are closely related in sequence within the coding region, but diverge in 3′-non-coding sequences. To analyse the correspondence of the sunflower genes with knotted genes from other species,

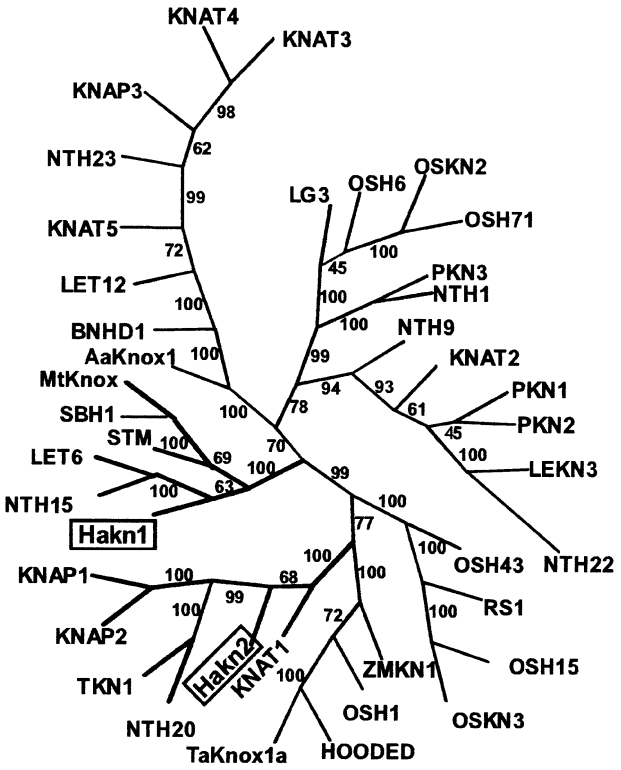

Fig. 1. Phylogenetic analysis of members of the plant knotted family. Protein sequences encoding full-length kn1-like polypeptides were identified in databanks by homology search and aligned using ClustalW. A phylogenetic tree derived from this alignment, using the PHYLIP group of programs, is shown. The tree is a Neighbor–Joining consensus one generated by Consensus after bootstrap analysis of 100 trees performed with Protdist (with Dayhoff's PAM matrix) followed by Neighbor. Numbers indicate bootstrap values for each of the groups. The sequences used for the analysis and their corresponding accession nos are: *Oryza sativa* OSH1 (JQ2379), OSH5 (BAA31688), OSKN2 (AAK11580), OSKN3 (AAK11581), OSH43 (BAA79225), OSH6 (BAA79224) and OSH71 (BAA79226), *Glycine max* SBH1 (P46608), *Arabidopsis thaliana* KNAT1 (P46639), KNAT2 (P46640), KNAT3 (P48000), KNAT4 (P48001), KNAT5 (P48002) and STM (Q38874), *Nicotiana tabacum* NTH1 (BAA76750), NTH9 (BAA76903), NTH22 (BAA79225), NTH20 (BAA76904), NTH15 (T01735) and NTH23 (T02220), *Zea mays* LG3 (P56669), RS1 (T03946) and ZMKN1 (CAA43065), *Lycopersicon esculentum* LET6 (T04317), LET12 (T04318), LEKN3 (AAD00252) and TKN1 (Q41330), *Ipomoea nil* PKN1 (BAA31698), PKN2 (BAA31699) and PKN3 (BAA31698), *Hordeum vulgare* HOODED (S58871), *Triticum aestivum* TaKNOX1a (AAF32398), *Acetabularia acetabulum* AaKNOX1 (AAD51632), *Medicago truncatula* MtKNOX (AA627464), *Malus×domestica* KNAP1 (O04134), KNAP2 (O04135) and KNAP3 (O04136), and *Brassica napus* BNHD1 (P46606).

a phylogenetic tree employing protein sequences from several monocots and dicots together with the sequence deduced from the *knox1* gene from *Acetabularia* was constructed (Fig. 1). The phylogenetic tree shows that hakn1 maps in a different clade than hakn2. The hakn1 clade contains STM, Let6 and NTH15, among others, while hakn2 maps together with KNAT1, NTH20, TKn1, KNAP1, and KNAP2. It is likely that the proteins within each group have acquired specialized functions during evolution from a common ancestor. For instance, muta-

tions in *Arabidopsis STM* produce plants defective in meristem maintenance (Long *et al*., 1996), while deletion of *KNAT1* affects mainly floral pedicel development (Douglas *et al*., 2002; Venglat *et al*., 2002). The tree also indicates that divergence of these groups has occurred before the separation of monocot and dicot plants. Notably, there is no monocot representative in the hakn1 clade, suggesting that the corresponding ancestor has been lost in monocots. If a similar analysis is made with homeodomain sequences only, a similar tree is obtained (not shown), suggesting that the different protein domains have evolved together from a common ancestor.

Expression studies of sunflower kn1-like genes

In order to characterize the expression patterns of the sunflower kn1-like genes during flower development, RNA from different stages (R1 to R5 according to the classification developed by Schneiter and Miller, 1981) was isolated and analysed by RNA blots (Fig. 2A). Expression of *HAKN1* was readily detected at all stages, with a slight decrease in transcript levels upon flower development. The signals obtained with *HAKN2*, and specially with *HAKN3*, were less intense, suggesting that these genes are expressed at lower levels. This agrees with the number of clones representing each gene that were identified after the RT-PCR isolation procedure. In the case of *HAKN2*, similar expression levels were observed at different stages of flower development, while *HAKN3* was almost undetectable (Fig. 2A). Expression was also monitored after pollination. During embryo development, *HAKN1* transcripts were detected at low levels that increased with maturation (Fig. 2B), while the expression of the other two genes was significantly lower.

During the experiments performed to analyse transcript levels in flowers, samples containing total RNA from roots and leaves were occasionally introduced. Surprisingly,

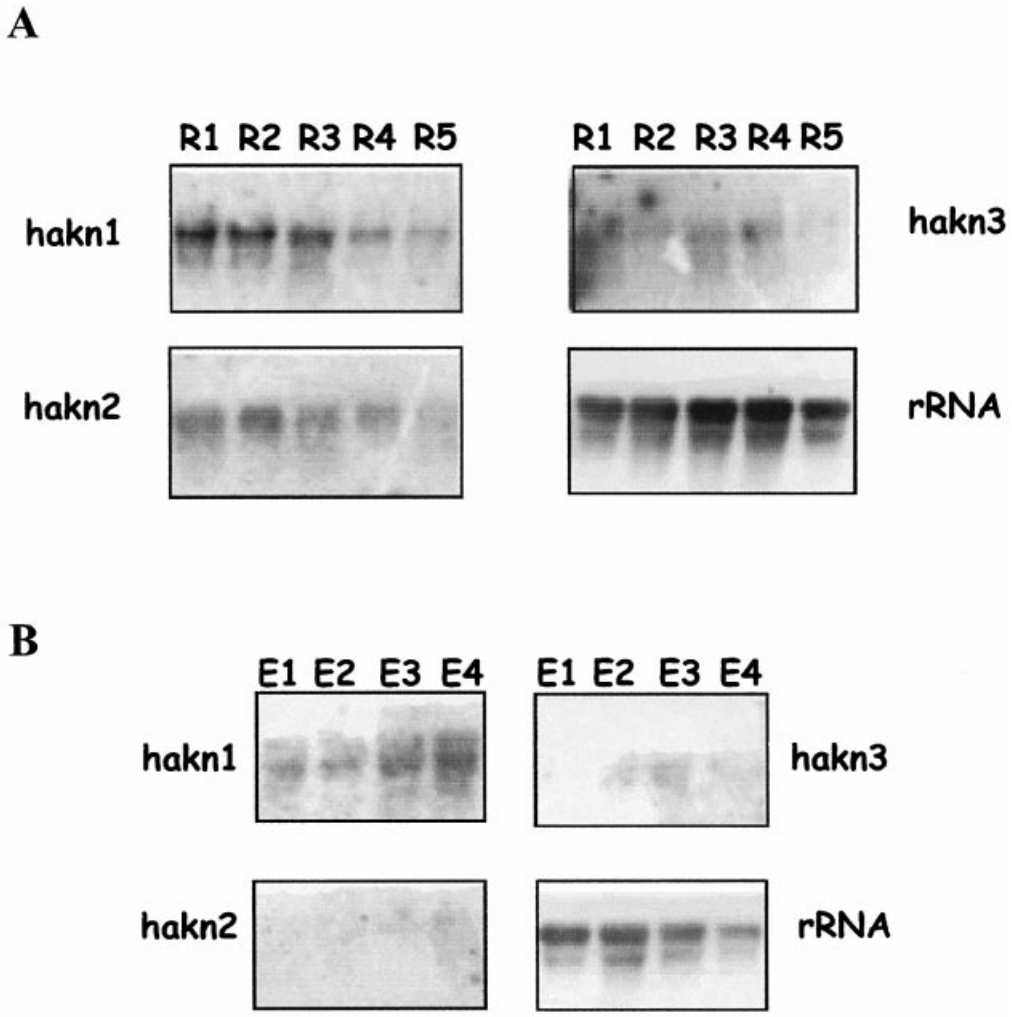

Fig. 2. Northern analysis of *HAKN1*, *HAKN2* and *HAKN3* expression during flower and embryo development. Total RNA (20 μg) extracted from sunflower inflorescence in stages R1 to R5 (panel A) or embryos harvested 1–4 weeks after pollination (E1 to E4, panel B) was hybridized with ^{32}P-labelled specific probes prepared from the 3′-non-coding regions of *HAKN1*, *HAKN2* and *HAKN3*. After autoradiography, the blots were rehybridized with a 25S rDNA probe.

strong signals were detected in these samples, mainly for *HAKN1*. A complete experiment using total RNA from several parts of the plant is shown in Fig. 3. It can be observed that transcript levels for *HAKN1* are considerably higher in leaves (both petiole and blade) and roots than in mature (R5 stage) flowers. *HAKN2* is also expressed in these parts of the plant, but shows preferential expression in stems (specially in internodal regions). Experiments with RNA from leaves and stems at different developmental stages indicated that transcript levels decrease during development. Thus, class I *kn1* genes are expressed at relatively high levels in differentiated organs of sunflower.

To determine the specific cell types that express these *kn1* genes, *in situ* hybridization studies were performed. During early stages of inflorescence development (R1 stage), *HAKN1* is expressed in the apical portion of the capitulum, a structure that bears the inflorescence meristem at the centre with floral meristems progressively developing towards the periphery (Fig. 4A). A detailed view of developing flowers indicates that *HAKN1* expression occurs throughout the floral meristem (Fig. 4B, C). This expression pattern closely matches that of total mRNA as indicated by sections probed with polydT (Fig. 4G–I). In more developed flowers, expression in petals is reduced and the label is mainly observed in stamen and carpel primordia (not shown). In addition to flowers, intense signals were also obtained in young involucral bracts (Fig. 4A–C). These signals also decline upon organ development.

Hybridization of transverse sections of leaf lamina detected *HAKN1* transcripts in parenchymatic and epidermal cells (Fig. 5A). Strong signals were obtained in parenchymatic cells in transverse sections of leaf veins (Fig. 5B, C). Detailed analysis also revealed expression in phloem both in the lamina (Fig. 5C) and petiole (not shown). Control experiments using polydT as probe indicated that *HAKN1* transcript levels do not match those of total mRNA. In fact, stronger signals were obtained in the lamina than in leaf veins with polydT (Fig. 5H). Young roots showed *HAKN1* expression in procambium and parenchymatic tissue (Fig. 6A–C). In stem cross-sections, enhanced expression of *HAKN1* was also observed in phloem cells (Fig. 6D), while pith parenchymatic cells were also labelled (Fig. 6D, E).

Expression of *HAKN2* was detected in floral meristems and developing floral organ primordia at different developmental stages (Fig. 7A, B). Strong expression was also observed in interfloral bracts, with decreasing intensity towards the border of the capitulum. *In situ* hybridization of stem internodal sections detected *HAKN2* transcripts in a ring of cells constituting the cambial cylinder and derivative phloem cells (Fig. 7C), suggesting the existence of cell-specific up-regulation of this gene in stems.

Discussion

Sunflower belongs to the Compositae family, with a terminal inflorescence (head or capitulum) composed of

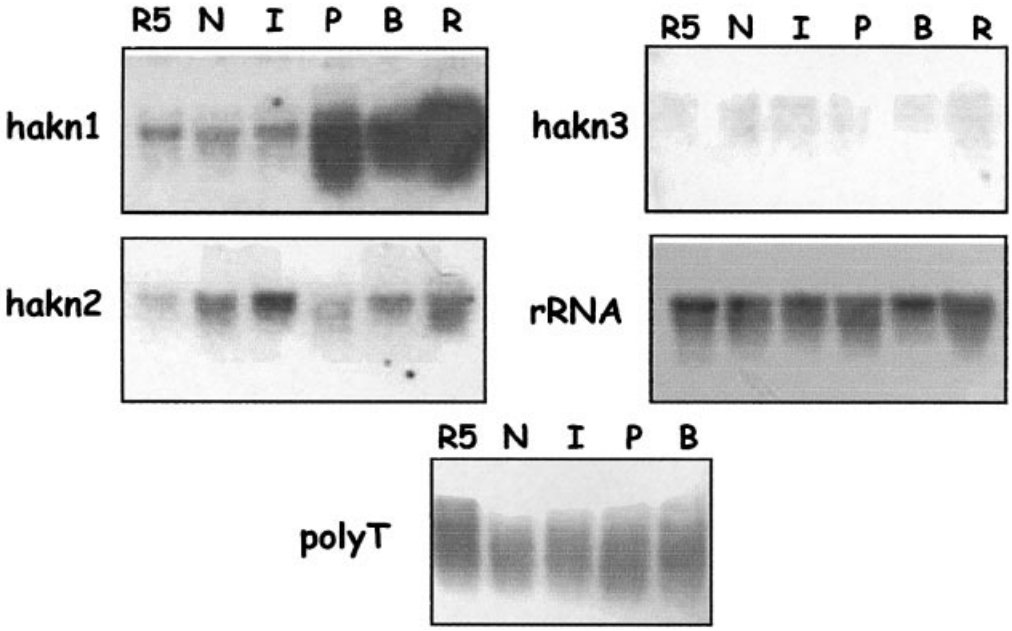

Fig. 3. Northern blot analysis of *HAKN1*, *HAKN2* and *HAKN3* transcript levels in different sunflower organs. RNA was extracted from flowers in developmental stage R5, stem nodes (N) and internodes (I), leaf petioles (P) and blades (B), and roots (R). Total RNA (20 μg) was probed with ^{32}P-labelled specific probes for each of the genes under study. After autoradiography, the blot was rehybridized with a 25S rDNA probe. In the lower panel a similar blot hybridized with a polydT probe is shown.

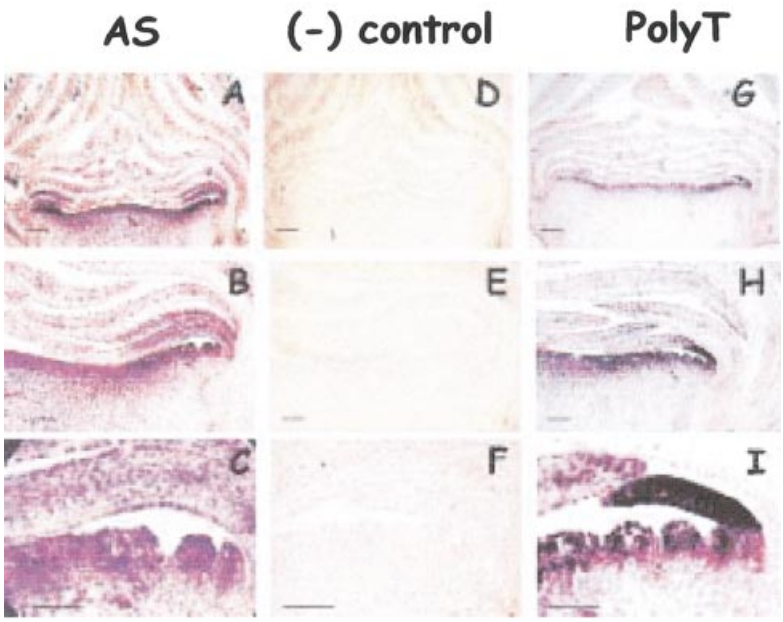

Fig. 4. *In situ* hybridization of *HAKN1* transcripts in sunflower inflorescence. Longitudinal sections of a sunflower inflorescence in the developmental stage R1 were hybridized with antisense *HAKN1* (A–C), sense *HAKN1* (D–F) or polydT (G–I) probes. (A), (D) and (G) show a complete inflorescence including the inflorescence meristem at the centre and developing floral meristems towards the borders. Label is also observed in the innermost involucral bracts in (A) and (G). (B, C) Details of the same capitulum. Scale bars: (A, D, G) 500 µm; (B, E, H) 200 µm; (C, F, I) 100 µm.

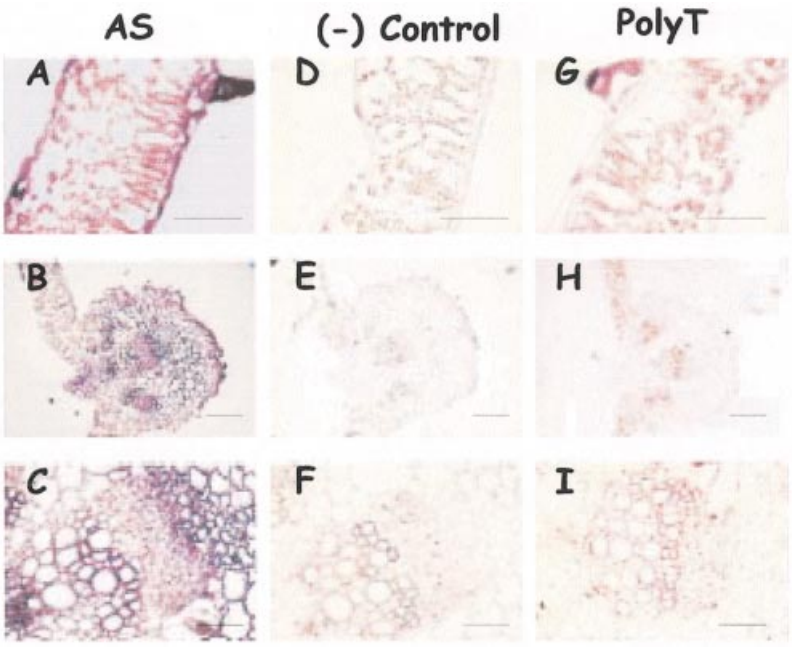

Fig. 5. *In situ* hybridization of *HAKN1* transcripts in leaves. Cross-sections of sunflower leaves showing *HAKN1* expression in lamina (A, D, G) and leaf vein (B, C, E, F, H, I). A–C, D–F and G–I were hybridized with antisense *HAKN1*, negative control and polydT probes, respectively. (C), (F) and (I) are details of (B), (E) and (H). Scale bars: (A, D, G, C, F, I) 100 µm; (B, E, H) 200 µm.

hundreds of flowers of two different types: ray (sterile) flowers in the periphery, and rings of disk (fertile) flowers in the centre (actually formed by radiating arcs from the centre of the head) (Seiler, 1997). Fertile flowers develop sequentially from the periphery to the centre of the head (Hernández and Green, 1993). This constitutes an interesting system to observe changes in expression patterns dependent on flower development, since an inflorescence

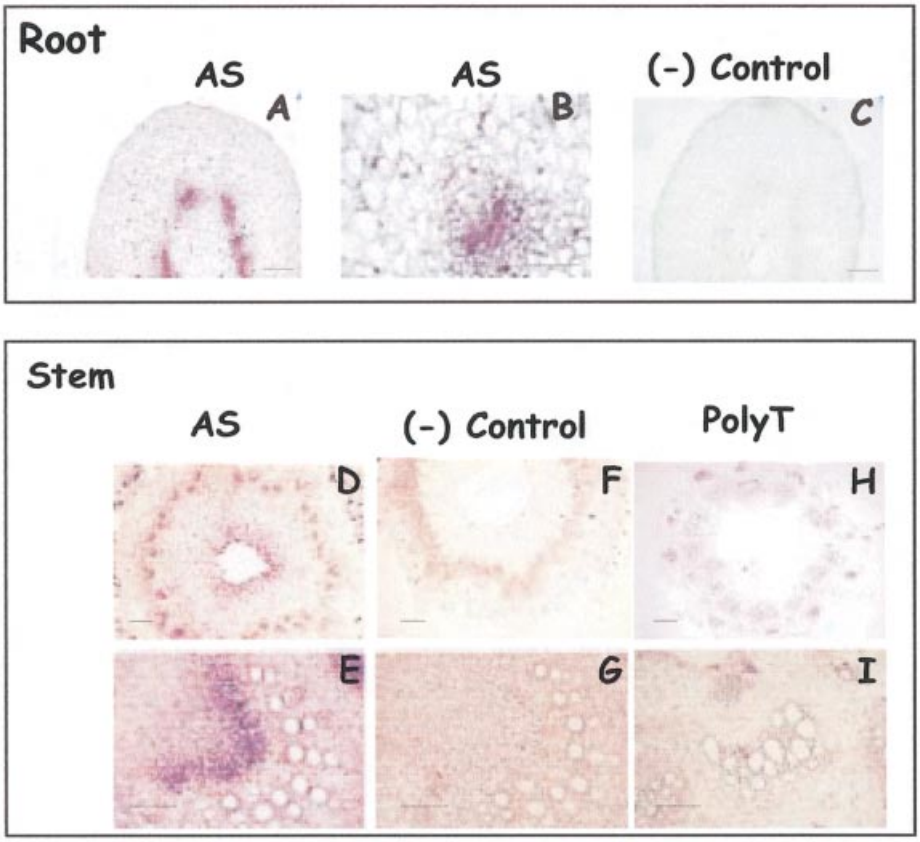

Fig. 6. *In situ* hybridization of *HAKN1* transcripts in root and stem. (Upper panel) cross-section through a sunflower root hybridized with an *HAKN1* antisense probe (A, B) or a negative control probe (C). (B) is a magnification of (A) showing expression in a cambial pole. (Lower panel) cross-section through a sunflower stem hybridized with an antisense *HAKN1* probe (D, E), a negative control probe (F, G) or a polydT probe (H, I). Note preferential expression of *HAKN1* in phloem. Scale bars: (A, C) 200 µm; (D, F, H) 500 µm; (B, E, G, I) 100 µm.

in a given stage contains flowers at different developmental stages. Since kn1-like genes function in meristem maintenance, and their expression patterns reflect branching and lateral organ formation, the expression of this class of genes during capitulum development in sunflower was characterized. The results clearly show that in this species class I *kn1* genes are expressed in differentiated cell types in several plant organs, including flowers, leaves, stems, and roots. This expression pattern differs from those observed in other monocots and dicots. In maize, *kn1* and related class I genes are down-regulated as leaves and floral organs are initiated, and were not detected in roots (Smith *et al*., 1992; Jackson *et al*., 1994; Kerstetter *et al*., 1994), with the sole exception of *rs1* (Schneeberger *et al*., 1995). In *Arabidopsis*, the kn1-like gene *STM* is expressed with a similar pattern, although expression in stems and developing flowers was also detected (Long *et al*., 1996). In sunflower, expression of kn1-like genes was detected in all cells of the apical part of the capitulum, including inflorescence and floral meristems, and developing floral organs. Down-regulation of expression is then retarded with respect to maize and *Arabidopsis*. In addition, strong *HAKN1* expression was observed in parenchymatic cells of leaf petiole and lamina. These results may indicate that the regulation of the expression of these genes differs in sunflower from that observed in other plants. The fact that transcript levels for *HAKN1* and *HAKN2* vary differently according to organ type suggests the existence of specific regulation for each of these genes. Specific expression of *HAKN2* was observed in cambial cells of the stem. This expression pattern has not been reported previously for any kn1-like gene, although expression in stems (outside the apical meristem) has been reported for some of them (Lincoln *et al*., 1994; Long *et al*., 1996; Parnis *et al*., 1997; Tamaoki *et al*., 1997; Watillon *et al*., 1997). Expression of *HAKN2* in cambial cells is in accordance with the orthodox view that kn1-like genes are involved in meristem maintenance, since this cambial cells serve as stem cells responsible for the radial growth of the stem. Expression of *HAKN1* and *HAKN2* was also detected in phloem cells of the stem. Expression in phloem has been reported previously for *NTH15* in tobacco (Tamaoki *et al*., 1997).

Down-regulation of kn1-like genes seems to be a prerequisite for organ differentiation, since the ectopic

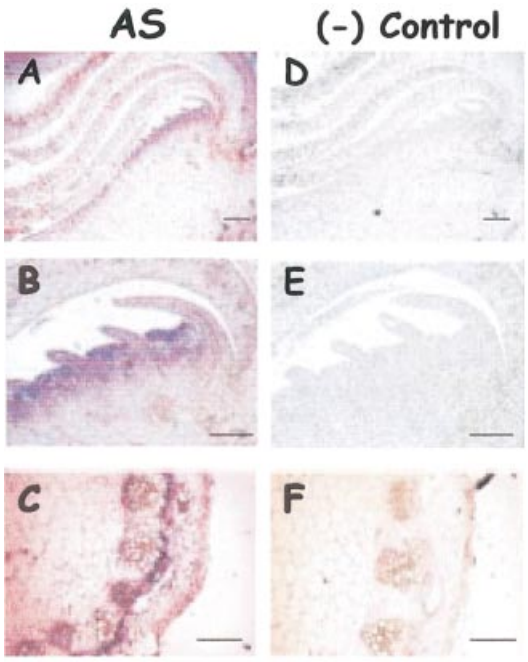

Fig. 7. *In situ* hybridization of *HAKN2* transcripts in different sunflower organs. Transversal sections of a sunflower capitulum were hybridized with an *HAKN2* antisense probe (A, B) or a negative control probe (D, E). (B) and (E) show a magnification of the external part of the capitulum shown in (A) and (D), respectively. Label is observed across the floral meristem and in interfloral bracts. Petal primordia are visible in the outermost flower. (C, F) Transversal sections of a stem internode hybridized with an antisense (C) or sense (F) *HAKN2* probe. Label is observed in a ring of cambial cells and also in derivative phloem cells. Scale bars: (A, D) 200 µm; (B, E) 100 µm; (C, F) 500 µm.

expression of some of these genes, either in mutants or transgenic plants, produces altered leaves and/or flowers (Smith *et al*., 1992; Matsuoka *et al*., 1993; Sinha *et al*., 1993; Müller *et al*., 1995; Schneeberger *et al*., 1995; Chuck *et al*., 1996; Tamaoki *et al*., 1997). The expression patterns obtained in sunflower are difficult to reconcile with this model. One explanation would be that expression of the corresponding proteins is post-transcriptionally down-regulated as organs develop. This would mean that regulation operates at a different level in sunflower than in other plants. If this is not the case, one must assume that sunflower organs show a different response to the expression of these genes. A similar situation occurs in tomato where expression of kn1-like genes has been detected in flowers, leaves and roots (Hareven *et al*., 1996; Chen *et al*., 1997; Parnis *et al*., 1997; Janssen *et al*., 1998*a*, *b*; Koltai and Bird, 2000). Although expression in developing leaves has been related to the compound leaf phenotype of tomato, no alterations have been detected in flowers. In addition, expression of the kn1-like gene *Pskn1* was not detected in pea compound leaves (Hofer *et al*., 2001). Expression of kn1-like genes in flowers, leaves and roots was also observed in the monocot barley (Müller *et al*., 2001). Notably, the ectopic expression of the *kn1* gene *knox3* originated from the *hooded* mutation alters flower, but not leaf development in this plant (Müller *et al*., 1995), and the ectopic expression of maize *kn1* in barley phenocopies the *hooded* mutation (Williams-Carrier *et al*., 1997), while the same gene alters leaf development in maize. These observations indicate that the response of cells to the expression of kn1-like genes depends on specific factors that seem to be cell-type- and species-specific. These factors may operate on the stability and/or activity of kn1-like proteins through direct interactions or post-translational modifications. It is noteworthy that kn1-like proteins interact with other homeodomain proteins from the same family (both class I and II) and from the Bell family (Bellaoui *et al*., 2001; Müller *et al*., 2001), and that the overexpression of a barley Bell protein in tobacco produces developmental alterations in leaves similar to those observed with kn1-like proteins (Müller *et al*., 2001).

It has been postulated that kn1-like genes are major determinants of plant architecture. According to this view, changes in expression patterns of these genes may have contributed to the evolution of plant form. Whether the particular expression patterns observed in sunflower are related to the characteristic architecture of the inflorescence in this plant is a matter that deserves further investigation. In this regard, loss-of-function mutations in the *kn1* and *STM* genes produce plants with a reduced number of flowers (Clark *et al*., 1996; Endrizzi *et al*., 1996; Kerstetter *et al*., 1997) and deletion of the *KNAT1* gene affects pedicel development (Douglas *et al*., 2002; Venglat *et al*., 2002), suggesting a connection between the activity of these genes and inflorescence architecture.

Acknowledgements

We gratefully acknowledge Ruth Heinz and Esteban Hopp (INTA, Castelar, Argentina) for help in the construction of the sunflower inflorescence cDNA library, André Steinmetz and colleagues (IBMP-CNRS, Strasbourg, France) for the kind gift of the stem cDNA library, and Karina Ribichich for help in the initial stages of the *in situ* hybridization studies. We also thank Kimitaka Yakura, Kanazawa University, Japan, for sending us a *Vicia faba* rDNA clone and Richard Amasino, University of Wisconsin-Madison, for helpful suggestions on the manuscript. This work was supported by grants from CONICET, ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica), Universidad Nacional del Litoral and Fundación Antorchas (Argentina). RLC and DHG are members of CONICET; MFT is a fellow of the same Institution.

References

Almoguera C, Jordano J. 1992. Developmental end environmental concurrent expression of sunflower dry-seed-stored low-molecular-weight heat-shock protein and Lea mRNAs. *Plant Molecular Biology* **19,** 781–792.

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. 1990. Basic local alignment search tool. *Journal of Molecular Biology* **215,** 403–410.

Bellaoui M, Pidkowich MS, Samach A, Kushalappa K, Kohalmi SE, Modrusan Z, Crosby WL, Haughn GW. 2001. The *Arabidopsis* BELL1 and KNOX TALE homeodomain proteins interact through a domain conserved between plants and animals. *The Plant Cell* **13,** 2455–2470.

Burgess SR. 1995. *In situ* hybridization to plant tissue sections. In: Jones H, ed. *Methods in molecular biology,* Vol. 49. *Plant gene transfer and expression protocols.* Totowa, NJ: Humana Press Inc**.** 301–316.

Bürglin TR. 1997. Analysis of TALE superclass homeobox genes (MEIS, PBC, KNOX, Iroquois, TGIF) reveals a novel domain conserved between plants and animals. *Nucleic Acids Research* **25,** 4173–4180.

Chan RL, Gago GM, Palena CM, Gonzalez DH. 1998. Homeoboxes in plant development. *Biochimica et Biophysica Acta* **1442,** 1–19.

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

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

Clark SE, Jacobsen SE, Levin JZ, Meyerowitz EM. 1996. The *CLAVATA* and *SHOOT MERISTEMLESS* loci competitively regulate meristem activity in *Arabidopsis*. *Development* **122,** 1567–1575.

Douglas SJ, Chuck G, Dengler RE, Pelecanda L, Riggs D. 2002. *KNAT1* and *ERECTA* regulate inflorescence architecture in *Arabidopsis*. *The Plant Cell* **14,** 547–558.

Endrizzi K, Moussian B, Haecker A, Levin JZ, Laux T. 1996. The *SHOOT MERISTEMLESS* gene is required for maintenance of undifferentiated cells in *Arabidopsis* shoot and floral meristems and acts at a different regulatory level than meristem genes *WUSCHEL* and *ZWILLE*. *The Plant Journal* **10,** 967–979.

Frohman MA. 1994. Cloning PCR products. In: Mullis KB, Ferré F, Gibbs RA, eds. *The polymerase chain reaction.* Boston: Birkhauser, 14–37.

Gehring WJ. 1987. Homeoboxes in the study of development. *Science* **236,** 1245–1252.

Gehring WJ, Affolter M, Bürglin T. 1994. Homeodomain proteins. *Annual Review of Biochemistry* **63,** 487–526.

Hareven D, Gutfinger T, Parnis A, Eshed Y, Lifschitz E. 1996. The making of a compound leaf: genetic manipulation of leaf architecture in tomato. *Cell* **84,** 735–744.

Hernández LF, Green PB. 1993. Transductions for the expression of structural pattern: analysis in sunflower. *The Plant Cell* **5,** 1725–1738.

Hofer J, Gourlay C, Michael A, Ellis THN. 2001. Expression of a class-1 knotted1-like homeobox gene is down-regulated in pea compound leaf primordia. *Plant Molecular Biology* **45,** 387–398.

Jackson D, Veit B, Hake S. 1994. Expression of maize *KNOTTED1* related homeobox genes in the shoot apical meristem predicts patterns of morphogenesis in the vegetative shoot. *Development* **120,** 405–413.

Janssen B-J, Lund L, Sinha N. 1998*a*. Overexpression of a homeobox gene, Let6, reveals indeterminate features in the tomato compound leaf. *Plant Physiology* **117,** 771–786.

Janssen B-J, Williams A, Chen J-J, Matern J, Hake S, Sinha N. 1998*b*. Isolation and characterization of two knotted-like homeobox genes from tomato. *Plant Molecular Biology* **36,** 417–425.

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

Kerstetter RA, Laudencia-Chingcuanco D, Smith LG, Hake S. 1997. Loss-of-function mutations in the maize homeobox gene, *knotted1*, are defective in shoot meristem maintenance. *Development* **124,** 3045–3054.

Koltai H, Bird DM. 2000. Epistatic repression of *PHANTASTICA* and class 1 *KNOTTED* genes is uncoupled in tomato. *The Plant Journal* **22,** 455–459.

Lincoln C, Long J, Yamaguchi J, Serikawa K, Hake S. 1994. A *knotted1*-like homeobox gene in *Arabidopsis* is expressed in the vegetative meristem and dramatically alters leaf morphology when overexpressed in transgenic plants. *The Plant Cell* **6,** 1859–1876.

Long JA, Moan EI, Medford JI, Barton MK. 1996. A member of the knotted class of homeodomain proteins encoded by the STM gene of *Arabidopsis*. *Nature* **378,** 66–69.

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

Mine I, Okuda K, Menzel D. 2001. Poly(A)$^+$ RNA during vegetative development of *Acetabularia peniculus*. *Protoplasma* **216,** 56–65.

Müller KJ, Romano N, Gerstner O, Garcia-Maroto F, Pozzi C, Salamini F, Rohde W. 1995. The barley *Hooded* mutation caused by a duplication in a homeobox gene intron. *Nature* **374,** 727–730.

Müller J, Wang Y, Franzen R, Santi L, Salamini F, Rohde W. 2001. *In vitro* interactions between barley TALE homeodomain proteins suggest a role for protein–protein associations in the regulation of *Knox* gene function. *The Plant Journal* **27,** 13–23.

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

Qian YQ, Billeter M, Otting G, Müller M, Gehring WJ, Wüthrich K. 1989. The structure of the *Antennapedia* homeodomain determined by MNR spectroscopy in solution: comparison with prokaryotic repressors. *Cell* **59,** 573–580.

Qian YQ, Furukubo-Tokunaga K, Müller M, Resendez-Perez D, Gehring WJ, Wüthrich K. 1994. Nuclear magnetic resonance solution structure of the fushi tarazu homeodomain from *Drosophila* and comparison with the *Antennapedia* homeodomain. *Journal of Molecular Biology* **238,** 333–345.

Sambrook J, Fritsch EF, Maniatis T. 1989. *Molecular cloning: a laboratory manual,* 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Schneeberger RG, Becraft PW, Hake S, Freeling M. 1995. Ectopic expression of the *knox* homeo box gene *rough sheath1* alters cell fate in the maize leaf. *Genes and Development* **9,** 2292–2301.

Schneiter AA, Miller JF. 1981. Description of sunflower growth stages. *Crop Science* **21,** 901–903.

Seiler GJ. 1997. Anatomy and morphology of sunflower. In: Schneiter AA, ed. *Sunflower technology and production,* Agronomy monograph no. 35. Madison, WI: American Society of Agronomy, 67–111.

Sentoku N, Sato Y, Kurata N, Ito Y, Kitano H, Matsuoka M. 1999. Regional expression of the rice *KN1*-type homeobox gene family during embryo, shoot, and flower development. *The Plant Cell* **11,** 1651–1663.

Sinha NR, Williams Jr EE, Hake S. 1993. Overexpression of the maize homeo box gene, *KNOTTED-1*, causes a switch from determinate to indeterminate cell fates. *Genes and Development* **7,** 787–795.

Smith LG, Greene B, Veit B, Hake S. 1992. A dominant mutation in the maize homeobox gene, *Knotted-1*, causes its ectopic

expression in leaf cells with altered fates. *Development* **116,** 21–30.

Tamaoki M, Kusaba S, Kano-Murakami Y, Matsuoka M. 1997. Ectopic expression of a tobacco homeobox gene, *NTH*15, dramatically alters leaf morphology and hormone levels in transgenic tobacco. *Plant and Cell Physiology* **38,** 917–927.

Thompson JD, Higgins DG, Gibson TJ. 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. *Nucleic Acids Research* **22,** 4673–4680.

Tsao DHH, Gruschus JM, Wang L, Nirenberg M, Ferretti JA. 1995. The three dimensional solution structure of the NK-2 homeodomain from *Drosophila*. *Journal of Molecular Biology* **251,** 297–307.

Venglat SP, Dumonceaux T, Rozwadowsky K, Parnell L, Babic V, Keller W, Martienssen R, Selvaraj G, Datla R. 2002. The homeobox gene *BREVIPEDICELLUS* is a key regulator of inflorescence architecture in *Arabidopsis*. *Proceedings of the National Academy of Sciences, USA* **99,** 4730–4735.

Vollbrecht E, Veit B, Sinha N, Hake S. 1991. The developmental gene *Knotted-1* is a member of a maize homeobox gene family. *Nature* **350,** 241–243.

Watillon B, Kettmmann R, Boxus P, Burny A. 1997. *Knotted*1-like homeobox genes are expressed during apple tree (*Malus domestica* [L.] Borkh) growth and development. *Plant Molecular Biology* **33,** 757–763.

Williams-Carrier RE, Lie YS, Hake S, Lemaux PG. 1997. Ectopic expression of the maize *kn1* gene phenocopies the *Hooded* mutant of barley. *Development* **124,** 3737–3745.