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

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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 differences 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

Homebox 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
expressed in young leaves, flowers and roots (Hareven et al., 1996; Chen et al., 1997; Parnis et al., 1997; Janssen et al., 1998a, b; Koltai and Bird, 2000). Since over-expression 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 these sunflower genes resemble the situation in tomato. Sunflower genes characterized by the presence of compound flowers. The expression patterns of these sunflower genes resemble the situation in tomato.

**Materials and methods**

**Plant material**

Sunflower plants (Helianthus annuus L. cv. conti°or 15, from Zeneca seeds) were grown in pots ®lled 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 classi®ed as de®ned by Schneiter and Miller (1981).

**Isolation of kn1 cDNA homologues from sunflower**

Degenerate oligonucleotides 5′-GGCGGATCCCTNCKYTGRRTTDATRAAcCTT3′ and 5′-GGCGGATCTGYACTTGAAGTT3′ 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 ®nese reverse transcribed with the ®rst of these oligonucleotides as primer. The ampli®cation product, consisting of one major band of about 500 bp, was puri®ed, digested with EcoRI and BamHI and cloned into the pUC119 plasmid treated with the same enzymes.

**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 hkn1, hkn2 and hkn3 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 Na2-citrate, pH 7.0), 1% (w/v) polyvinylpirrolidione, 0.1% (w/v) BSA, 0.1% (w/v) Ficol, and 0.5% (w/v) SDS. Fragments corresponding to the 3′-non-coding region of the three cDNAs were labelled with [32P]dATP (1 × 106 dpm μg−1) by random priming (Sambrook et al., 1989) and used as probes. Filters were autoradiographed using Bio-Max films and transcribe (Kodak) overnight. To check the amount of total RNA loaded in each lane, filters were then re-probed with a 25S RNA 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 polyT (20-mer) probe labelled with [35S]dATP (1 × 106 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 ®xed overnight in 3.7% formaldehyde, 5% acetic acid, 47.5% ethanol at room temperature, dehydrated through an ethanol series, and embedded in Histoplant (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 rehybridized 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 H2O, sections were used for hybridization.

Digoxygenin-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 signi®cantly lower intensity). This may be due to contamination of the sense probe with antisense RNA originated from unspeci®c 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 unspeci®c 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) polyvinylpyrolidone, 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.

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 result is 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, 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 hkn1 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 especially 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,....

![Figure 2](http://jxb.oxfordjournals.org)
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 knl genes are expressed at relatively high levels in differentiated organs of sunflower.

To determine the specific cell types that express these knl 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
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...
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. 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.
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 knl-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., 1998a, b; Koltaï 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 knl-like gene Pskn1 was not detected in pea compound leaves (Hofer et al., 2001). Expression of knl-like genes in flowers, leaves and roots was also observed in the monocot barley (Müller et al., 2001). Notably, the ectopic expression of the knl 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 knl 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 knl-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 knl-like proteins through direct interactions or post-translational modifications.

It has been postulated that knl-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 knl 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.

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