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Ancestry and diversity of *BEL1*-like homeobox genes revealed by gymnosperm (*Gnetum gnemon*) homologs

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Abstract *BEL1*-like homeobox genes encode plant-specific transcription factors, at least some of which are important for ovule development. Here we report *MEL-BEL1–MELBEL4,* the first *BEL1*-like genes from a non-flowering plant, the gymnosperm *Gnetum gnemon.* Our analyses suggest that there was already at least one *BEL1*-like gene present in the most recent common ancestor of extant seed plants about 300 million years ago. Multiple sequence alignments revealed that since this time, not only the DNA-binding homeodomain, but also a protein-protein interaction domain upstream of the homeodomain, termed the BEL domain, has been highly conserved. Sequence comparison of the BEL domain with upstream domains that have been conserved in other TALE homeodomain proteins, i.e. MEIS, KNOX, and PBC, revealed only weak sequence similarity. However, since homology has been shown for MEIS, KNOX, and PBC domains and since KNOX and BEL domains directly interact in vivo, it appears likely that the BEL domain was also derived from an ancestral upstream (MEINOX) domain. **Electronic Supplementary Material** is available if you access this article at http://dx.doi.org/10.1007/s00427-002-0259-7. On that page (frame on the left side), a link takes you directly to the supplementary material.

Keywords *Gnetum gnemon* · Homeobox · *BEL1* · Evolution · TALE homeodomain

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Introduction

Initially, genes containing homeoboxes were found by studying homeotic mutants of the fruit fly *Drosophila*. They encode transcription factors which are essential for many aspects of development (Gehring et al. 1994). Meanwhile, homologs of these genes were also isolated from evolutionarily distant species like plants and fungi (Vollbrecht et al. 1991; Bharathan et al. 1997; Bürglin 1997). The homeobox is 180 bp in length and encodes the homeodomain. It consists of a protein motif which folds into a characteristic structure including three α-helices that constitute the DNA-binding domain (Gehring et al. 1994).

Comparisons of homeodomains and their adjacent sequences reveal the presence of about 20 classes of homeobox genes in plants, fungi and animals (Bürglin 1994; Bharathan et al. 1997; Chan et al. 1998). Plant homeobox genes can be divided into several clearly defined families according to their sequence similarity within the homeodomain and adjacent regions of the corresponding proteins. These proteins are defined as KNOX (*KNOTTED1*-like homeobox), Hd-Zip I and II (homeodomain-leucine zipper), PHD-finger, GLABRA-, and BEL1-(BELL1) like proteins, according to their founding members or structural features (Chan et al. 1998). KNOX as well as BEL1-like proteins belong to the TALE superfamily of homeodomain proteins (Bürglin 1997), which represents a branch distinct from typical homeodomain proteins characterized by an extra three amino acids between homeodomain helix 1 and 2 (TALE: three amino acid loop extension). It has been suggested that the KNOX and BEL1-like genes originated in a gene dupli-

cation that occurred before the separation of the lineages that led to extant plants and animals more than a billion years ago (Bellaoui et al. 2001). Thus one would expect to find *BEL1*-like genes in all land plants. According to an alternative hypothesis (Bürglin 1998), *BEL1*-like and KNOX genes originated by a gene duplication within the plant lineage. As a matter of fact, *BEL1*-like homeobox genes have so far been described only from flowering plants, i.e. *Arabidopsis* (*BEL1*; Reiser et al. 1995), apple (*MDH1*; Dong et al. 2000), and barley (Müller et al. 2001), plant lineages that separated 200 million years ago at most.

Even though the *bel1* mutant in *Arabidopsis* has been studied for a number of years already, much of the biological function of *BEL1* remains enigmatic (Balasubramanian and Schneitz 2000). Mutants of *BEL1* bear ovules that lack the inner and outer integument and exhibit multicellular protuberances which emerge from the central region of the ovule (Modrusan et al. 1994). The mutant ovule morphology correlates to the expression of *BEL1* throughout the entire ovule. The available evidence suggests that *BEL1* mediates the developmental events between ovule initiation and determination of ovule organ identity (Reiser et al. 1995; Gasser 1996). Most likely, *BEL1* controls the entire integument-forming meristematic region by directing it to differentiate into two distinct structures, the inner and outer integument (Gasser 1996). A gene function which plays a central role in ovule development of angiosperms may well have been already present in the most recent common ancestor of all extant seed plants. Here we report sequences of four *BEL1*-like genes from the gymnosperm *Gnetum gnemon*, which exhibits three characteristic envelopes around its ovules of unknown homology to flowering plant organs. The isolation of four *BEL1*-like genes in a gymnosperm indicates that there was already at least one *BEL1*-like gene present in the most recent common ancestor of extant seed plants.

Materials and methods

Total RNA from female cones of *G. gnemon* (Botanical Gardens of Karlsruhe and Bochum, Germany) was isolated using Biomol Total RNA Reagent (Biomol, Germany) according to the manufacturer's instructions. By employing the Dynabeads mRNA Purification Kit (Dynal, Norway), polyA$^+$ RNA was isolated using 100 µg total RNA. Five hundred nanograms polyA$^+$ RNA were reverse transcribed into cDNA using 200 U Superscript II RNase H reverse transcriptase (Gibco BRL, USA) with 25 pmol extension primer (5′-GACTCGAGTCGACAT CTGTTTTTTTTTTTTTT-TTT-3′). This cDNA template was amplified with DNA Taq-polymerase (Roche) using degenerate primers as outlined in the Results and discussion (mb20: 5′-GGYTNTTYGARCACTTTCTTC-3′; mb21: 5′-GGCTCTTTGAACAYTTYYTTC-3′; mb22: 5′-GGCTCTTTGAACACTTTCTTC-3′; mb25: 5′-CATTTCTTCAATYATNGGYTTC-3′; mb26: 5′-CATTTCYTCAATYATTGGYTTC-3′) which were derived from the conserved homeoboxes of *BEL1*-like genes from *Arabidopsis* and barley. Several independent clones containing partial *BEL1*-like homeoboxes from *G. gnemon* were obtained this way. Larger cDNA fragments were amplified by 3′-RACE experiments (Frohman et al. 1988) using the adapter primer (5′-GACTCGAGTCG ACATCTG-3′) and gene specific PCR primers derived from the above mentioned homeobox fragments. Multiple rounds of 5′-RACE with several gene-specific primers (sequences available upon request) were carried out to obtain sequence information upstream of the *MELBEL1* homeobox.

Amplified cDNA fragments were cloned into the pGEM-T vector (Promega, USA). DNA sequences were determined by the MPIZ DNA core facility on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3,700 sequencers using BigDye-terminator chemistry. Premixed reagents were from Applied Biosystems. Oligonucleotides were purchased from GibcoBRL (USA), MWG Biotech (Germany) and Metabion (Germany). Sequence analyses employed MacVector and GCG version 10.0 (Genetics Computer Group, Oxford Molecular Group).

For phylogeny reconstruction, conceptual amino acid sequences of the following *BEL1*-like genes were used: *ATH1* (accession number D43962), *BEL1* (U39944), *BLH1* (AF353094), *BLH2* (AL161590), *BLH3* (AF353093), *BLH4* (AF353092), *JUBEL1* (AF334758), *JUBEL2* (AF334759), *MDH1* (AF053769), *MELBEL1* (AJ318871), and anonymous sequences corresponding to accession nos. AAG12557, At2g16400, At2g27220, At2g27990, T05281, and T48224. Besides *MELBEL1*, all genes had been isolated from *Arabidopsis thaliana,* except *JUBEL1* and *2* which are from *Hordeum vulgare* (barley), and *MDH1* which is from *Malus domestica* (apple). To obtain all *BEL1*-like genes present in the *A. thaliana* genome, the homeodomains of *BEL1, MDH1, JUBEL1*, and *JUBEL2* were used as query sequences in BLAST searches against all *Arabidopsis* protein sequences available at the NCBI database (http://www.ncbi.nlm.nih.gov/) using default parameters.

Phylogeny reconstructions were carried out by first aligning the conserved protein regions 123 amino acids prior to the homeodomain, the homeodomain (as defined by Bürglin 1997), and 9 amino acids downstream of the homeodomain (for alignment, see Fig. 2) with the program PILEUP of the GCG program package (version 10.0) using default parameters. Then the Neighbor-Joining algorithm (Saitou and Nei 1987) within the PHYLIP program package (Protdist version 3.753c, Neighbor-Joining method version 3.5) was employed, using the Dayhoff PAM matrix and calculating 100 bootstrap replicas.

Results and discussion

By employing first a PCR with two degenerate homeobox-specific primers (mb21, mb25) and then 3′-RACE and multiple rounds of 5′-RACE, a *MELBEL1* cDNA fragment of 3582 bp was obtained. It comprises the entire homeobox, the subsequent 3′ coding region, the 3′-UTR and also about 1.8 kb of coding sequence upstream of the homeobox (Fig. S1, see Electronic Supplementary Material). Southern hybridization experiments under stringent conditions showed only a single band per lane for all tested restriction enzymes (*Bam*HI, *Eco*RI, *Eco*RV, *Hind*II, *Hind*III, and *Xba*I), suggesting that *MELBEL1* is a single-copy gene (M. Bey, unpublished). *MELBEL2* was first isolated as a homeobox fragment by using primers mb20 and mb25. Then 3′-RACE with a specific primer obtained from the previously isolated *MELBEL2* homeobox was employed. The partial cDNA of *MELBEL2* covers 136 bp of the homeobox, the downstream coding region and the 3′-UTR (Fig. S1, see Electronic Supplementary Material). A partial homeobox of *MELBEL3* was amplified with primers mb22 and mb26; subsequently, 3′-RACE with a gene-specific primer was employed to isolate the 3′ part of *MELBEL3*; it includes 134 bp of the homeobox and the subsequent 3′ coding region (Fig. S1, see Electronic Supplementary Material).

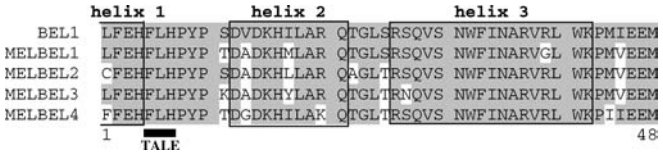

Fig. 1 Multiple amino acid sequence alignment of parts of the homeodomain of MELBEL1 (AJ318871), MELBEL2 (AJ318872), MELBEL3 (AJ318873), MELBEL4 (AJ318774) and BEL1. Positions where a MELBEL sequence deviates from the BEL1 sequence are *highlighted in white*. The three helices of homeodomain proteins are marked by *boxes*, the three-amino-acid-loop-extension (TALE) is indicated by a *black bar*

Of *MELBEL4*, part of the homeobox (148 bp) was isolated with primers mb20 and mb25 (Fig. S1, see Electronic Supplementary Material).

The conceptual amino acid sequences of the homeodomains of all four MELBEL proteins show a high degree of similarity to BEL1 (Fig. 1) and other BEL1-like proteins from angiosperms (Reiser et al. 1995; Dong et al. 2000; Müller et al. 2001). Phylogeny reconstruction employing homeodomain sequences of BEL1-like proteins and of representatives of the other families of homeodomain proteins strongly corroborated the view that all MELBEL proteins are members of the family of BEL1-like proteins (data not shown). All previously characterized *BEL1*-like genes were isolated from flowering plants, thus we report here the first four *BEL1*-like genes from a gymnosperm. Within the BEL1-like proteins from *Gnetum* and angiosperms, 123 amino acids upstream and 9 amino acids downstream of the homeodomain are also conserved well enough for unambiguous multiple sequence alignments (Fig. 2). The conserved upstream sequences comprise an extensive sequence stretch termed the BEL domain (Fig. 2; Bellaoui et al. 2001).

Since sequence similarities have been detected between the KNOX, MEIS (collectively termed MEINOX), and PBC domains (Bürglin 1998), we investigated whether the BEL domain might also share sequence similarities with any of these domains. We attempted to align the BEL domain to the other domains using ClustalX version 1.81 and PSI-blast (Altschul et al. 1997). In addition, we also tried to identify conserved patterns by eye. PSI-blast did not reveal any similarity between any of the domains, nor did we find any convincing matches by eye. In Fig. S2A–C (see Electronic Supplementary Material) we show the best alignments obtained by aligning the BEL domain by eye to the other domains. However, in these alignments, the KNOX and MEIS domains do not line up the same way. Thus, we cannot unambiguously show that the BEL domain was derived from the ancient MEINOX domain, although similarities shown in the alignments in Fig. S2A–C (see Electronic Supplementary Material), in particular of the BEL domain to the PBC-A domain (Fig. S2C, see Electronic Supplementary Material) might indeed be due to a common evolutionary origin. We also used secondary structure prediction employing the PHD program (Rost 1996) to analyze the four domains. The BEL domain is predicted to consist of two long alpha-helices (40 and 30 aa, respectively), while the MEIS, KNOX, and PBC domains all have one long predicted alpha-helix (around 35 aa) only in the second part of each domain (data not shown). Thus possibly one of the helices of the BEL domain might functionally correspond to the helix in the second part of the MEIS, KNOX, and PBC domain.

Two hypotheses have been proposed to explain how the PBC, KNOX, MEIS, and *BEL1* gene families could have evolved from a single TALE homeobox gene:

1. The separation of the lineages that gave rise to extant plants and animals more than a billion years ago generated a MEIS/PBC ancestor in animals and a KNOX/BEL1 ancestor in plants. Later, independent gene duplications took place in both lineages which resulted in the relatively recent paralogous pairs of MEIS and PBC genes in animals and KNOX and *BEL1*-like genes in plants (Bürglin 1998). However, this hypothesis was formulated prior to the identification of the BEL domain.
2. Alternatively, a duplication event of the ancestral TALE homeobox gene, which gave rise to KNOX/MEIS and BEL/PBC precursor genes, had already occurred in a common ancestor of animals and plants (Bellaoui et al. 2001). Under this hypothesis, KNOX and *BEL1*-like genes from plants and MEIS and PBC genes from animals would be ancient rather than recent paralogs. The *BEL1*-like and PBC genes would be orthologs, and the same relationship would apply to KNOX and MEIS genes.

Our sequence analysis of the BEL, KNOX, MEIS, and PBC domains cannot distinguish between the two hypotheses. The same is true for phylogeny reconstructions using the TALE homeodomain, which only very weakly revealed a KNOX/BEL clade and did not reveal a PBC/MEIS clade (Bürglin 1997).

Interestingly, however, KNOX proteins interact with BEL1-like proteins via the KNOX domain (Bellaoui et al. 2001). Two BEL1-like proteins from barley, JUBEL1 and 2, interact with the barley KNOX proteins BKN1 and BKN3 via a part of the BEL domain (as shown in Fig. 2; Müller et al. 2001). This may be taken as circumstantial evidence that the BEL domain and the KNOX domain share a common evolutionary origin, implying that the BEL domain was also derived from the ancestral MEINOX domain in some way, although the similarity of the BEL domain to either MEIS, KNOX, or PBC domains is only weak (Fig. S2A–C, see Electronic Supplementary Material). Therefore, interaction of KNOX and BEL1-like proteins is mediated by possibly homologous domains which originated more than 1 billion years ago (under hypothesis 2) or at least 300 million years ago [since both, *BEL1*-like and KNOX homeobox genes have been identified in both, gymnosperms and angio-

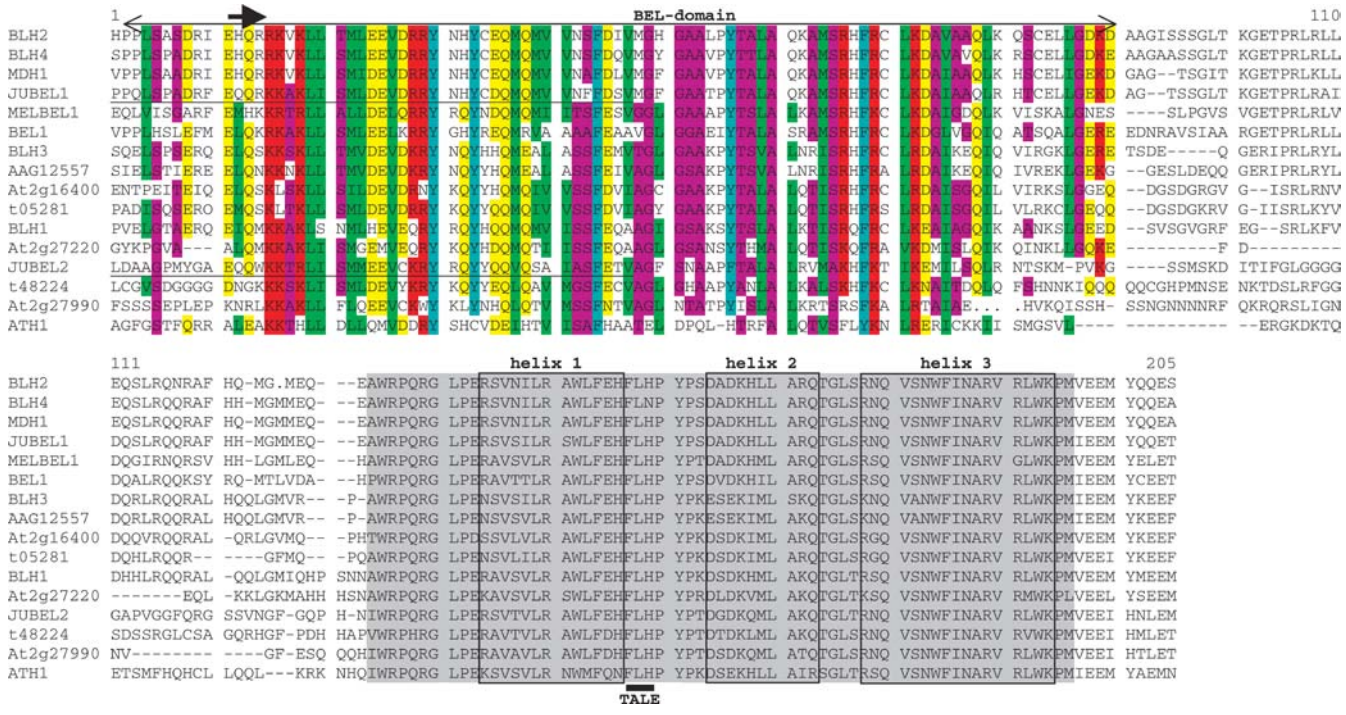

Fig. 2 Multiple sequence alignment of the conserved amino acids of all available BEL1-like proteins. The BEL domain (according to Bellaoui et al. 2001) contains conserved amino acid positions which are labeled in *color*. The amino acids are marked, if ≥50% of the proteins show the same amino acid or conserved residue substitutions (according to Bordo and Argos 1991) at a given position (*red* KR, *yellow* NDEQ, *pink* GSTA, *green* ILMV, *turquoise* PWY). *Underlined* parts of the sequences of JUBEL1 and JUBEL2 mark predicted coiled-coil domains which interact with barley KNOX proteins (Müller et al. 2001). The homeodomain is shaded in *gray*, the characteristic three DNA-binding helices are indicated by *boxes*, and the three-amino-acid-loop-extension (TALE) is labeled below the sequences by a *black bar*. This amino acid alignment starting at the position marked by a *black arrow* was used for constructing the phylogenetic tree in Fig. 3

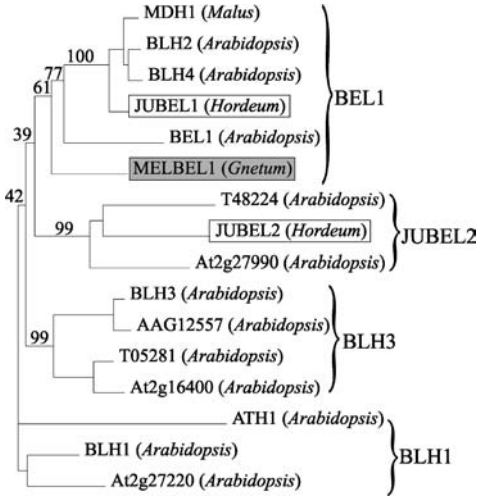

Fig. 3 Phylogenetic tree of all available BEL1-like proteins utilizing the multiple alignment of Fig. 2 and employing the Neighbor-Joining algorithm. Genus names of species from which the respective genes were isolated are given in *parentheses* after the protein names. The *Gnetum* protein is indicated by a *gray box*, proteins from monocots are marked with *open boxes*. Proteins that are not boxed have been derived from eudicots. The *numbers* next to some nodes that are of special relevance here give bootstrap percentages. Subfamilies are labeled by *brackets* at the right margin

sperms (this work; Chan et al. 1998; Sundas-Larsson et al. 1998)]. Thus, even though the protein sequences strongly diverged, they may have maintained their function. The weak sequence similarity between the BEL domain and the KNOX, MEIS, and PBC domains (in contrast to the high sequence similarity within the BEL1-like proteins) would be compatible with a quite ancient origin of the *BEL1*-like genes. Perhaps more sequence information from other plants will help to resolve the issue in the future.

Outside the conserved BEL, SKY (data not shown; Bellaoui et al. 2001), and homeodomains, BEL1-like proteins are highly divergent, and only one short common sequence motif can be found (Fig. S3, see Electronic Supplementary Material). This motif is present at various positions upstream of the homeodomain of the different proteins and its function is so far unknown. Different degrees of conservation of this motif (Fig. S3, see Electronic Supplementary Material) correspond to different subfamilies of BEL1-like proteins (see below).

The sequences which could be aligned unambiguously (Fig. 2) were used for phylogeny reconstruction in order to clarify the relationships between MELBEL1 and all the other BEL1-like proteins known. Our analysis comprises probably all BEL1-like sequences from the completely sequenced genome of the flowering plant *A. thaliana* (The Arabidopsis Genome Initiative 2000), retrieved from the NCBI server (http://www.ncbi.nlm.nih.gov/). Previous

phylogeny reconstructions of homeodomain proteins involved only three BEL1-like proteins at most (Bürglin 1997), so we provide the first extensive analysis here.

The obtained phylogenetic tree revealed that the family of BEL1-like proteins is separable into at least three defined subfamilies, all including *Arabidopsis* proteins, which have been termed the BEL1-like, JUBEL2-like and BLH3-like subfamily, respectively (Fig. 3). Our tree shows a fourth group of proteins, BLH1-like proteins (comprising ATH1, BLH1 and At2g27220, all from *Arabidopsis*), whose relationships are uncertain, however, due to low bootstrap support.

Since our tree is unrooted, it is not clear for every subfamily whether they represent monophyletic protein/gene clades. TGIF (accession no. NP_033398), a TALE homeodomain protein from mouse (Bürglin 1997), was used as outgroup in the otherwise unchanged dataset. Including this sequence in the phylogenetic tree supports the view that BEL1-, JUBEL1-, and BLH3-like proteins represent clades, but suggests that BLH1-like proteins are paraphyletic (rather than monophyletic). Rooting the tree with TGIF also did not change the position of MELBEL1 at the base of the subfamily of BEL1-like proteins (data not shown).

The BEL1-like subfamily includes BEL1 (*Arabidopsis*), MDH1 (apple), JUBEL1 (barley), MELBEL1 (*Gnetum*), and other *Arabidopsis* BEL1-like proteins. MELBEL1 is placed at the base of the BEL1-like subfamily, suggesting that it is an ortholog of BEL1 (for a definition of orthology, see Theissen 2002). However, the low bootstrap support of this placement and the limited sampling of *BEL1*-like genes from *Gnetum* imply that this hypothesis needs further critical assessment.

As already stated above, the presence of *BEL1*-like genes in both a gymnosperm and several angiosperms strongly suggests that the most recent common ancestor of extant gymnosperms and angiosperms 300 million years ago contained at least one *BEL1*-like gene. The presence of both *BEL1*-like and *JUBEL2*-like subfamily members in a eudicot (*A. thaliana*) as well as in a monocot (barley) indicates that the most recent common ancestor of monocots and eudicots, assumed to have existed 150–200 million years ago, already contained at least two *BEL1*-like genes. The topology of the tree in Fig. 3 would even be compatible with the view that there were already at least three different *BEL1*-like genes in the most recent common ancestor of angiosperms and gymnosperms. This seems reasonable since *MELBEL1* is probably a member of the *BEL1*-like subfamily, which makes it conceivable that the *BEL1*-, *JUBEL2*- and *BLH3*-like subfamilies originated before the gymnosperm/angiosperm split. However, it could also be that MELBEL1 occupies the basal position in the family tree of BEL1-like sequences, and that in angiosperms the gene family diversified only after the gymnosperm/angiosperm split. Unfortunately, a suitable outgroup sequence from lower plants to root the *BEL1* subfamily tree is not available. This may change if *BEL1*-like genes can be isolated from ferns or mosses. Answering the question as to whether there are *BLH1-, BLH3-* or *JUBEL2*-like genes in gymnosperms may also help to clarify the issue.

Northern hybridization with a *MELBEL1*-specific probe showed transcript accumulation in leaves as well as male and female reproductive cones of *G. gnemon* (Fig. S4, see Electronic Supplementary Material). In leaves, the signal appears stronger than in reproductive organs. In parallel to our findings, expression of the apple homolog *MDH1* was also found in leaves and reproductive organs (Dong et al. 2000), and *BEL1* is expressed in *Arabidopsis* leaves and flowers, too, with similar intensity (Reiser et al. 1995). Thus, the almost ubiquitous expression patterns of these three *BEL1*-like genes may point to a general conservation of gene expression at least within the *BEL1*-like subfamily.

Although we have no information about the function of the *MELBEL* genes, their similarity to *BEL1*, which controls ovule formation in *Arabidopsis*, suggests a function for the *MELBEL* genes also in the development of the reproductive structures of *G. gnemon*. Detailed studies on the expression of the *MELBEL* genes in male and female reproductive units by in situ hybridization may help to determine the function of these genes. Such studies also promise insights into the evolution of the ovule, which is still enigmatic (Bateman and DiMichele 1994). For example, homology between the integuments of angiosperm ovules and the envelopes of *Gnetum* ovules is controversial (Winter et al. 1999), but the *MELBEL* genes may be informative markers.

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