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Evolutionary and polymorphic organization of the *knotted1* homeobox in cereals

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Abstract Homeobox genes are the master control genes harbouring the homeobox which is crucial for developmental associated functions. One homeobox gene, *knotted1*, which has a role in leaf development, is conserved in plants and might have arisen from a single ancestral gene. Using PCR, we identified multiple *kn1* homeoboxes in diverse cereals and showed a cereal/species-specific organization correlating them to evolutionary changes. We postulate the insertion of a large intron preceded by duplication of the *kn1* homeobox in the lineage leading to rice.

Key words Homeobox · Intron · Cereals · Phylogeny · Ancestry

Introduction

The homeobox was first identified in *Drosophila* (McGinnis et al. 1984) and later in several genes that are involved in development in diverse groups of animals and plants (Gehring, 1987). Different homeodomains recognize diverse DNA binding sites and act as transcription factors (Scott et al. 1989). The third helix (recognition helix) and the amino terminal region of the homeodomain are very important in determining the specificity of DNA binding (Triesman et al. 1989; Affolter, 1990). The first family of homeobox genes reported in plant species was the *knotted1* (*kn1*) in maize which is involved in leaf development (Hake et al. 1989; Vollbrecht et al. 1991). *Kn1*-like homeobox genes have recently been reported in other monocots, such as rice (Matsuoka et al. 1993) and barley (Muller et al. 1995), and several dicot species such as Arabidopsis (Lincoln et al. 1994), tomato (Hareven et al. 1996) and soybean (Ma et al. 1994). The *knotted1* genes may also have a role in flower development as shown by their expression in the flowers of rice (Tamaoki et al. 1996). Intron duplication in the *kn1* homeobox is associated with the conversion of the awned phenotype to the hooded phenotype of seed in barley (Muller et al. 1995).

Sequence similarity of the homeobox in *kn1*-like genes across monocot and dicot plant species suggests a functional significance that is conserved over a vast evolutionary time scale (Vollbrecht et al. 1993). Genetic studies have shown that many of these genes are organized into regulatory gene families that are related to each other by descent from a common ancestral gene. Homeobox genes should, therefore, prove to be useful in studying evolutionary changes in plants.

In the study presented here, we show the differential organization of the *kn1* homeobox in diverse cereals, revealing the presence of multiple *kn1* homologues, their crop/species-specific organization and variations in intronic lengths, and we correlate them to cereal evolution.

Materials and methods

Plant material

Seeds of oat, barley, wheat and rye and their respective wild relatives were obtained from USDA-ARS, National Small Grains Collection, Aberdeen, USA, and those of barley were obtained from the Swedish Agricultural University, Sweden. Seeds of the remaining cereals were obtained from various agricultural research stations in India.

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Polymerase chain reaction (PCR) amplification and analysis

Total genomic DNA was extracted from the leaves by the CTAB method as described by Ramakrishna et al. (1994). Primers from the basic region (5′ AAAGGGAAGCTCCCCAAGGA 3′) and helix 3 region (5′ GGCTTCCAGTGCCGCTTCCG 3′) were synthesized and used for PCR amplification in a volume of 10 µl containing 50 ng of DNA, 0.15 µ*M* of each primer at 200 µ*M* of dGTP and dTTP, 25 µ*M* of dCTP and dATP, 4.625×10^4 bq α-[^{32}P] dATP and α-[^{32}P] dCTP, 0.24 U *Taq* DNA polymerase, 50 m*M* KCl, 10 m*M* TRIS-HCl (pH 8.0) and 1.5 m*M* $MgCl_2$. DNA amplifications were performed in a Perkin-Elmer Cetus thermal cycler with the following profile: (1) 94°C for 4 min for 1 cycle, (2) 94°C for 1 min, 55°C for 1 min and 72°C for 2 min, for 30 cycles and (3) 72°C for 5 min for 1 cycle, as described by Wu and Tanksley (1993). Amplification products were separated on a 6% polyacrylamide denaturing gel containing 7 *M* urea and 0.5 × TBE buffer (pH 8.0) and electrophoresed at 60 W constant power. A similar PCR amplification using each dNTP at a concentration of 200 µ*M* without radioactivity was performed in a 50-µl reaction. Amplification products were loaded on a 1.2% agarose gel and separated by electrophoresis in TAE buffer.

PCR product cloning, sequencing and Southern hybridization

PCR products from different species were separated on agarose gels, eluted and cloned into either pGEM T-vector (Promega) or pMOS T-vector (Amersham). Sequencing was performed using the Sequenase version 2.0 DNA sequencing kit (US Biochemical Corp). The cloned plasmids were labelled by random priming and used for hybridization. Simultaneously, DNA amplifications were performed as described above, and the amplified products were run on 1% agarose gel and blotted onto Hybond N + membrane (Amersham). Hybridization was performed at 60°C in 5 × SSC, 5 × Denhardt's reagent, 1% SDS and 0.1 × BLOTTO. The washing stringency was 1 × SSC, 1% SDS twice at room temperature for 10 min each and once at 60°C for 15 min. Autoradiography was performed by exposing the blot to X-ray film.

Results

Arrangement and organization of the *kn1* homeobox in cereals as analysed on polyacrylamide gels

To study the phylogenetic conservation of the *knotted1* homeobox in diverse cereals, we used primers based on reported maize and rice *kn1* homeobox sequences to amplify this region, which also harbours an intron. The primers were designed to specifically amplify the *knotted1* homeobox region. All the cereals tested, maize, rice, barley, wheat, rye, oat, sorghum, sugarcane and pearl millet, gave strong amplifications, indicating a high sequence conservation of the *kn1* homeobox in these cereals.

Figure 1 shows the polymorphic arrangement of amplified products of the *kn1* homeobox in cereals as analysed on a sequencing gel. Maize, its relative teosinte and different species of oat show significant variation in intronic lengths (Fig. 1 A, lanes 1–5, 7, 8). No common bands were found in the two maize genotypes (lanes 2, 3) and its relative teosinte (lane 1), suggesting the divergence of intronic lengths within the homeobox. A band of 380 bp is common in three oat species, *Avena fatua*, *A. vaviloviana* and *A. sativa* (Fig. 1 A, lanes 4, 7 and 8, respectively), whereas *A. fatua* and *A. sativa* have identical patterns (lanes 4, 8). In lane 8, although the bands are not clearly visible, they were distinct on the original X-ray film. A wild wheat species, *Aegilops longissima* (lane 6), was included to compare its *kn1* homeobox organization to that of other cereals. In the case of barley (lanes 9–12), different species show a similar profile as was also observed in rye (lanes 13–15). However, the profile of *Hordeum marinum* (lane 11) is slightly divergent by a small variation in the migration of the 260 bp band. *Hordeum marinum* (lane 11) and *H. bogdani* (lane 12) are also characterized by a faint higher molecular-weight band. Similar differences with reference to rye were observed where *Secale cereale* ssp. *ancestrale* (lane 14) shows a slightly divergent band pattern by the absence of the 260 bp band which is present in other rye species. Sugarcane (lane 16) and pearl millet (lane 17), each represented by a single species, show distinct single bands which are unique to them.

Figure 1B depicts the *kn1* homeobox organization in different rye (lanes 1–3) species as compared to different wheat species (lanes 4–8). Among these, *Triticum timopheevi* (lane 7) has a different pattern, especially with reference to the 255-bp band, which is seen even in the case of *Aegilops tauschii* (lane 8) where some other minor variations are observed. However, the overall organization of the *kn1* homeobox in wheat is strikingly similar to that of rye. This is well-supported by the classification of wheat and rye in subtribe triticineae in Gramineae.

Figure 1 C shows the *kn1* homeobox organization in different rice species (lanes 1–7). Ten different rice cultivars representing the indica and japonica subspecies of rice in which we have previously shown polymorphisms with micro- and minisatellites (Ramakrishna et al. 1994, 1995; Gupta et al. 1994) show identical patterns with reference to the *kn1* homeobox (data not shown). Wild rice ancestors having the A genome, represented by *Oryza nivara* and *O. longistaminata* (lanes 1, 2) show a similar *kn1* homeobox organization to that of the cultivars (not shown in figure). The wild rice species belonging to other genomes show extensive variations (lanes 3–7); for example, *Oryza alta*, a wild rice species, shows several bands (lane 4) indicating the presence of multiple *kn1* homeoboxes with varying intronic lengths. Lengths of *kn1* homeoboxes are strikingly different in each of the cereals analysed, except in the case of wheat and rye where they are organized in a similar manner.

Analysis of the *kn1* homeobox of a greater size in diverse cereals on agarose gels

To detect the presence of *kn1* homeobox regions of a greater size in diverse cereals which could not be

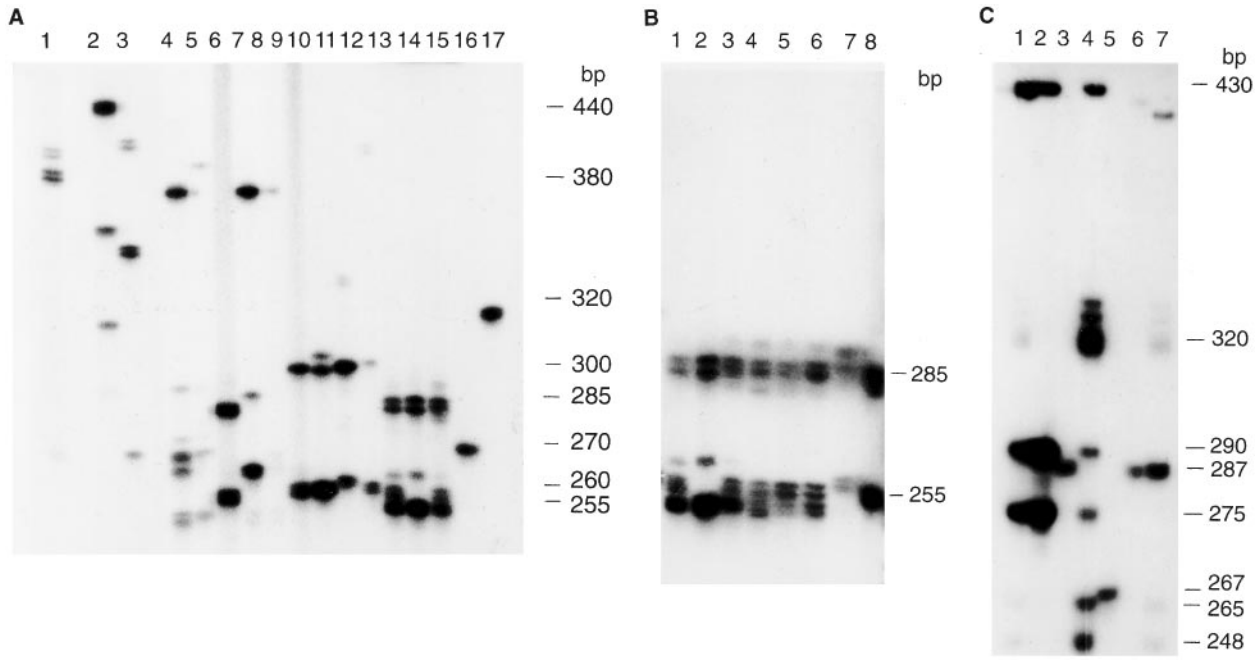

Fig. 1A–C Radioactive PCR amplification of the *knotted1* homeobox from different cereals. Genomic DNA of the following cereals was amplified and loaded on 6% denaturing polyacrylamide gel. **A** *Lanes 1 Zea diploperennis* (Teosinte), *2 Z. mays* var. 'CHIO3 DK 93' (maize), *3 Z. mays* var. 'DK 94 6714' (maize), *4 Avena fatua* (oat), *5 A. eriantha* (oat), *6 Aegilops longissima* (wheat), *7 Avena vaviloviana* (oat), *8 A. sativa* (oat), *9 H. vulgare* ssp *vulgare* (barley), *10 H. lechleri* (barley), *11 H. marinum* (barley), *12 H. bogdani* (barley), *13 S. strictum* (rye), *14 S. cereale* ssp *ancestrale* (rye), *15 S. cereale* ssp *cereale* (rye), *16 Sacharum officinarum* (sugarcane), *17 Pennisetum americanum* (pearl millet). **B** *Lanes 1 Secale strictum* (rye), *2 S. cereale* ssp *ancestrale* (rye), *3 S. cereale* ssp *cereale* (rye), *4 Triticum. diccoccoides* (wheat), *5 T. aestivum* cv. 'Chinese Spring' (wheat), *6 T. aestivum* landrace, Narsinghad yellow (wheat), *7 T. timopheevi* (wheat), *8 Aegilops tauschii* (wheat). **C** Rice species: *Lanes 1 Oryza nivara*, *2 O. longistaminata*, *3 O. officinalis*, *4 O. alta*, *5 O. granulata*, *6 O. malampuzhansis*, *7 O. minuta*

resolved on polyacrylamide gel, PCR-amplified products were analysed on an agarose gel. Figure 2 A shows the presence of 1.2-kb band in wheat species *Triticum timopheevi* (lane 3), of a 1.3-kb band in rice cultivar 'Basmati-370' (lane 14) and of a 1.1-kb band in the wild rice species, *O. officinalis* (lane 15), in addition to the bands visible on the polyacrylamide gel. The presence of a high-molecular-weight band in both cultivated and wild rice species suggests the possibility of the insertion of a large intron in rice. To examine this possibility, we analysed several rice cultivars and wild species, as shown in Fig. 2B. All of the rice cultivars (lanes 2–6) and wild rice species, *O. nivara* and *O. rufipogon*, belonging to the A genome (lanes 7, 8), considered to be the ancestors of cultivated rice, show a 1.3-kb band. Wild rice species, *O. minuta*, *O. punctata*, *O. officinalis* and *O. latifolia* show a 1.1-kb band (lanes 9–11, 13). In addition, *O. punctata* shows a band at 0.8-kb (lane 10). Interestingly, *O. alta* a non-A-genome rice shows a 1.3-kb band (lane 12) observed in A-genome rices. *O. granulata*, a wild rice species recently classified as G genome (Agarwal et al. 1997) gave a unique band at 0.9 kb (lane 14).

Homology of the *kn1* homeoboxes of cereals based on Southern hybridizations

In order to analyse the homology among different cereal *kn1* homeoboxes harbouring the introns, we cloned and sequenced these regions from wheat, KNAL1 (263 bp), oat, KNAV1 (288 bp) and rice, KNOFF1 (1 kb), and Southern blot hybridization of the same gel as shown in Fig. 2A was performed successively with the above probes (Fig. 3). As seen in Fig. 3A, the *kn1* homeobox of wheat (*Aegilops longissima*) gave strong signals with the *kn1* homeobox of other wheat genotypes, namely *Triticum timopheevi* (lane 4), 'Chinese Spring' (lane 6) and its own (lane 9), while moderate hybridization was observed with *Aegilops tauschii* (lane 5). Other cereals also showed weak cross hybridization. Interestingly, none of the high-molecular-weight bands observed in *T. timopheevi*, 'Basmati-370' and *O. officinalis* (lanes 3, 14, 15) hybridized to the wheat *kn1* homeobox, probably because of the presence of a divergent large intron. When the blot was hybridized to the *kn1* homeobox region of oat (*Avena vaviloviana*), as shown in Fig. 3B, signals were seen in oat (lane 2) (faint because of weak amplification), wheat (lanes 3–5, 9), barley (lanes 6, 7), maize (lane 8) and rye (lane 12). When the blot was hybridized to the *kn1* homeobox region of rice (*O. officinalis*), as shown in Fig. 3C, hybridization was observed with the 1.1-kb fragment of *O. officinalis* (lane 15) and also with

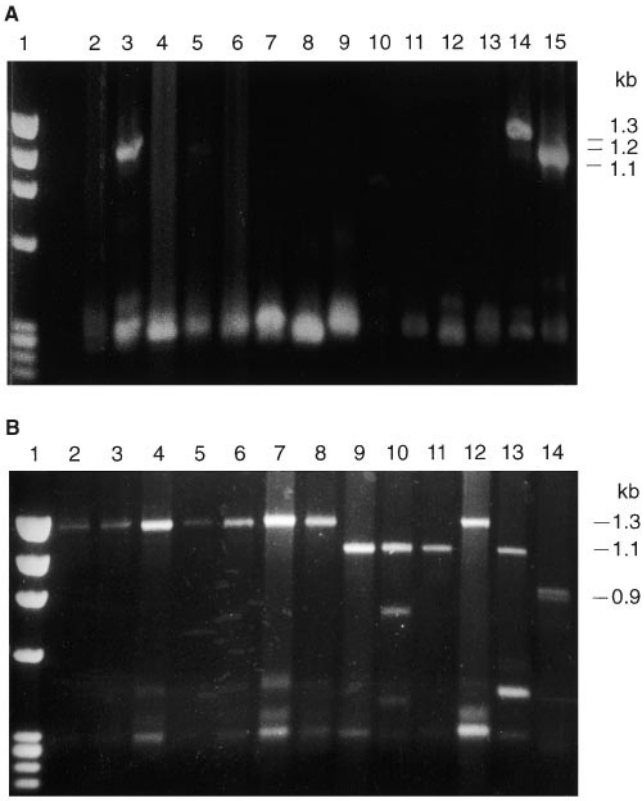

Fig. 2A, B PCR-amplified products from diverse cereals run on 1% agarose gel to detect the presence of high-molecular-weight *kn1* homeobox regions. **A** *Lanes 1* phi × *Hae*III (molecular-weight marker), *2 Avena sativa* (oat), *3 Triticum timopheevi* (wheat), *4 Aegilops tauschii* (wheat), *5 Triticum aestivum* cv 'Chinese Spring' (wheat), *6 Hordeum vulgare* (barley), *7 H. marinum* (barley), *8*, *Zea mays* var 'CHIO3 DK93' (maize), *9 Aegilops longissima* (wheat), *10 Sorghum bicolor* var 'CSV4(G)' (sorghum), *11 S. bicolor* var 'TAM 428(B)' (sorghum), *12 Secale cereale* ssp *ancestrale* (rye), *13 S. cereale* ssp *cereale* (rye), *14 Oryza sativa* cv 'Basmati-370' (rice), *15 O. officinalis* (rice). **B** *Lanes 1* phi × *Hae*III (molecular-weight marker), *2 Oryza sativa* cv 'Indrayani', *3 O. sativa* cv 'Ambemohr', *4 O. sativa* cv 'Taichung 65', *5 O. sativa* cv 'Norin-49', *6 O. sativa* cv 'Fujisaka', *7 O. nivara*, *8 O. rufipogon*, *9 O. minuta*, *10 O. punctata*, *11 O. officinalis*, *12 O. alta*, *13 O. latifolia*, *14 O. granulata*

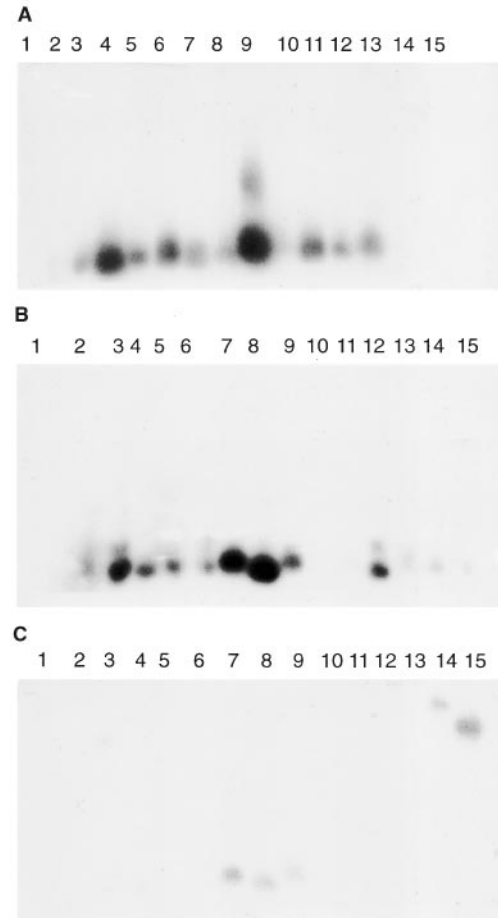

Fig. 3A–C Southern hybridization of PCR-amplified *kn1* homeobox from diverse cereals with cloned *kn1* homeobox of **A** wheat (*Aegilops longissima*), **B** oat (*Avena vaviloviana*) and **C** rice (*Oryza officinalis*). The gel described in Fig. 2A was blotted and hydridized successively to the above probes. *Lanes 1–15* are as described in Fig. 2A

the 1.3-kb band of 'Basmati-370' (lane 14). The lower molecular-weight fragments did not show hybridization except in few cases (lanes 7–9) which may be due to very strong amplification, as is evident from Fig. 2A (lanes 7–9), and less homology.

Discussion

In this study, we showed the presence of many novel *kn1* homologues in cereals such as wheat, rye, oat, sorghum, pearl millet and sugarcane. Previous reports indicate *knotted1* homeobox genes to be members of a large class of transcription factors involved in various cellular processes present throughout the plant and animal kingdom. The *knotted1* homeodomain (HD) is similar to yeast and human homeodomains, which is consistent with their evolution from a common ancestral sequence before the divergence of the plant, animal and fungal kingdoms 10^9 million years (myr) ago (Grueneberg et al. 1992). The increase in the homeobox gene family by gene duplications is assumed to have provided an increasing developmental capacity for the formation of complex body plans as seen in vertebrate evolution (Pendleton et al. 1993). An analogous diversity of evolutionary mechanism might be found in the case of the *knotted1* genes. *Kn1* like HDs may have been components of an ancient regulatory mechanism involved in divergent developmental programmes. The evolutionary conservation of *kn1*-like HDs in diverse cereals can be explained by the presence of ancient *kn1*-like HD proteins which underwent evolutionary changes for assorted developmental functions. The *kn1* class appears to be absent in *Drosophila* and all animals (Vollbrecht et al. 1993). However, their conservation and organization in plants suggest that they might have evolved from a common ancestor.

Dicots and monocots have diverged over millions of years on an evolutionary time scale. The presence of the *knotted1* homeobox region has been reported previously in dicots like *Arabidopsis*, tomato and soybean; in diverse cereals like rice, maize and barley; and in the present study in wheat, oat, rye, sorghum, pearl millet and sugarcane. Such a widespread occurrence indicates that these homeobox genes have a functional significance that has been conserved over a vast evolutionary distance. This is similar to the observation made in the case of the unusual floral organs (*UFO*) and the *FIMBRIATA* (*FIM*) genes of *Arabidopsis* and *Antirrhinum*, respectively. Both these genes share molecular structure and expression patterns, but there are a few differences in their functions and genetic interactions suggesting that these differences may reflect changes in important functional pathways that could have occurred during the evolutionary divergence between *Antirrhinum* and *Arabidopsis* (Ingram et al. 1995).

The presence of a large number of bands in most of the cereals in our study suggests that the homeobox in *kn1* represents one member of a fairly large gene family with variable intronic lengths. This is well-supported by the identification of multiple *kn1* homologues in maize (Kerstetter et al. 1994). The size of the homeobox is invariant as has been reported from studies of dicots and monocots. Hence, all of the changes in the length are due to the intronic region in *kn1* homeobox of the cereals. MADS box gene functions have also evolved by a change in the length of introns (Ma et al. 1991).

Rice, maize and wheat have been isolated for more than 60 myr. The *kn1* homeobox (with the intron) seems to have diverged in the process of evolution of cereals as shown by our data. Speciation of ancestral wheats and rice occurred in the same period. The large intron present in rice and the 'Timopheevi' wheat might have arisen by duplication followed by insertion of the large intron, which is divergent in nature. Since the intron was present in all the rice species examined in the present study, it could mean that this intron might have been inserted prior to the speciation of rice. The presence of a large intron in the *kn1* homeobox was observed only in 'Timopheevi' wheat. As reported earlier, plant transposable elements such as the *Stowaway* and *Tourist* elements located in the introns of genes are present in *O. sativa* but absent in non-A-genome types, *O. punctata* and *O. eichengeri*, which suggests insertion around the divergence date of the A genome (14–17 myr). In contrast, this element was inserted recently in maize as it was present in an inbred line and absent in another inbred line and also in teosinte – *Z. mays* ssp. *mexicana* (Bureau and Wessler 1994a, b).

Another interesting observation is that the amplification pattern is similar between the wild species and cultivars of rye, barley and wheat, suggesting that not many alterations took place in the evolutionary history of these cereals. The length of the *kn1* homeoboxes of wheat and rye are organized in a similar manner. This is well-supported by the classification of wheat and rye in subtribe triticineae in Gramineae. The closeness of wheat and rye is also evident from the man-made crop, triticale, which is derived from a cross between wheat species *Triticum turgidum* and rye, *Secale cereale*. In the case of rice, however, all rice cultivars show a similar pattern, whereas its wild relatives appear more diverse, which suggests that rice has undergone rearrangements during domestication. It is also known that the process of domestication leads to a reduction in genetic variability, which supports the monomorphic organization in the case of rice cultivars. The lack of variability in the *kn1* homeobox can be explained by the time elapsed since the initiation of domestication, which might have been too short on an evolutionary time scale for any differences to occur. This theory also holds true for other cereals.

The isolation of many of the *kn1* genes from evolutionarily distant and morphologically distinct taxa allows us to study the conservation of developmental functions and analyse the genetic factors responsible for morphological diversification. It is possible that *kn1* homologues have additional functions in diverse cereals as suggested by the expression of *OSH1* in the flowers of rice.

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