RESEARCH PAPER

Flower-specific KNOX phenotype in the orchid Dactylorhiza fuchsii

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Abstract

The KNOTTED1-like homeobox (KNOX) genes are best known for maintaining a pluripotent stem-cell population in the shoot apical meristem that underlies indeterminate vegetative growth, allowing plants to adapt their development to suit the prevailing environmental conditions. More recently, the function of the KNOX gene family has been expanded to include additional roles in lateral organ development such as complex leaf morphogenesis, which has come to dominate the KNOX literature. Despite several reports implicating KNOX genes in the development of carpels and floral elaborations such as petal spurs, few authors have investigated the role of KNOX genes in flower development. Evidence is presented here of a flower-specific KNOX function in the development of the elaborate flowers of the orchid Dactylorhiza fuchsii, which have a three-lobed labellum petal with a prominent spur. Using degenerate PCR, four Class I KNOX genes (DfKN1–4) have been isolated, one from each of the four major Class I KNOX subclades and by reverse transcription PCR (RT-PCR), it is demonstrated that DfKNOX transcripts are detectable in developing floral organs such as the spur-bearing labellum and inferior ovary. Although constitutive expression of the DfKNOX2 transcript in tobacco produces a wide range of floral abnormalities, including serrated petal margins, extra petal tissue, and fused organs, none of the vegetative phenotypes typical of constitutive KNOX expression were produced. These data are highly suggestive of a role for KNOX expression in floral development that may be especially important in taxa with elaborate flowers.

Key words: Dactylorhiza fuchsii, evolution, flower development, KNOX genes, labellum, orchids, petal shape, petal spur.

Introduction

The discovery of animal homeobox genes revolutionized our understanding of the molecular basis of animal development and evolution (Gehring, 1998, 2007). Plant homeobox genes, such as KNOTTED1 (KN1) (Vollbrecht et al., 1991), play equally significant roles in evolutionary developmental change. KNOX genes are integral to one of the most defining characteristics of the green plant lineage, their indeterminate vegetative growth, which is dependent on the maintenance of a pluripotent stem-cell niche at the shoot apical meristem (SAM). Expression of Class I KNOX genes is one of the earliest markers for meristematic cell fate (Smith et al., 1992) and prevents the pluripotent cells of the SAM from differentiating (Endrizzi et al., 1996; Long et al., 1996; Kerstetter et al., 1997; Vollbrecht et al., 2000). By contrast, lateral organs derived from the SAM are determinate, often requiring suppression of KNOX expression (Smith et al., 1992; Jackson et al., 1994).

As more KNOX genes have been isolated from an increasingly diverse range of taxa, additional functions in complex leaf morphogenesis have become apparent. When constitutively expressed in simple-leafed species, KNOX genes induce
dramatically lobed and super-compounded leaf phenotypes (Vollbrecht et al., 1991; Sinha et al., 1993; Lincoln et al., 1994; Chuck et al., 1996). These observations are supported by a strong positive correlation between KNOX re-expression in developing leaf primordia and complex leaf morphology in a broad range of angiosperm taxa (Hareven et al., 1996; Bharathan et al., 2002; Hay and Tsiantis, 2006; Piazza et al., 2010; Chatterjee et al., 2011). Work on tomato (Shani et al., 2009, 2010) and Cardamine hirsuta L. (Hay and Tsiantis, 2006; Barkoulas et al., 2008) suggests that re-activation of KNOX expression in leaf primordia facilitates leaflet formation by maintaining a state of prolonged indeterminacy and morphogenetic activity (reviewed extensively by Hay and Tsiantis, 2009, 2010; Canales et al., 2010).

Despite several reports implicating KNOX genes in the development of carpels (Endrizzi et al., 1996; Pautot et al., 2001; Scofield et al., 2007, 2008), few authors have investigated the involvement of KNOX genes in flower development. Recent studies of petal-spur development in snapdragon and Linaria vulgaris provided evidence that KNOX genes may be important in the development of the spur, a floral elaboration that has aided the diversification of several major flowering plant groups (Box et al., 2011).

Evidence is presented here suggesting a flower-specific role for KNOX genes in the development of the elaborated flowers of the functional diploid orchid Dactylorhiza fuchsii (Druce) Soó (Orchidaceae: Orchidinae, 2n=40; Hagerup, 1944; Paun et al., 2011), which resembles the majority of European orchids in possessing a three-lobed labellum petal with a prominent spur (Fig. 1; see Box et al., 2008, for a detailed description of D. fuchsii floral morphology and Rudall and Bateman, 2002, for orchids more broadly). Four Class I KNOX genes (DfKNO1–4) have been isolated that represent the four major Class I KNOX subclades described previously (Sano et al., 2005; Jouanne et al., 2007). DfKNO transcripts are detectable in developing floral organs and constitute expression of the DfKNO2 transcript in tobacco produces a wide range of floral abnormalities but none of the vegetative phenotypes typical of constitutive KNOX expression. These data are highly suggestive of a role for KNOX expression in floral development that may be especially important in taxa with elaborate flowers.

Materials and methods

Plant materials

Wild specimens of Dactylorhiza fuchsii were collected with permission from Southeast England and Nockberge National Park, Austria (permits SP3-NS-865/2007 002/2007; SP3-NS-865/2007 004/2008).

Isolation of DfKNOX genes

DfKNOX transcripts were isolated by degenerate RT-PCR with primers designed using the CODEHOP algorithm (Rose et al., 1998, 2003) to anneal to the highly conserved ‘DQFM’ and ‘WFIM’ motifs of the KNOX and Homeodomain encoding regions. A full set of primer sequences is presented in Supplementary Table S1 online. Full-length coding sequences were obtained by 5’ and 3’ RACE using the GeneRacer kit (Invitrogen, Life Technologies, UK), re-sequenced and deposited in GenBank (GenBank ID DfKNO1, JQ229970; DfKNO2, JQ229971; DfKNO3, JQ229972; DfKNO4, JQ229973).

RNA extraction and RT-PCR

RNA was extracted using a phenol/chloroform-LiCl method (Sambrook and MacCallum, 2001) and cDNA was prepared from 1 µg of DNase-treated RNA using Superscript III (Invitrogen, Life Technologies, UK). Gene-specific primers (see Supplementary Table S1 at JXB online) were designed for tissue-specific expression analysis by semi-quantitative RT-PCR. To avoid amplification from contaminating genomic DNA, primers were designed to span predicted introns (Czechowski et al., 2005). RT-PCR was performed on three independent sets of biological samples using 4 µl of 1:100 diluted cDNA template in a 32-cycle PCR reaction with Phusion DNA polymerase (Finzymes, Finland), performed according to the manufacturer’s instructions. OrACT, the Dactylorhiza homologue of Arabidopsis ACT11 (U27981), was used as an internal control.
Constitutive expression in tobacco

The full-length coding sequence of DfKNO2 was cloned directly from cDNA into a modified pGreenII0029 vector between a double CaMV 35S promoter and a single CaMV 35S terminator (Helling et al., 2000) using gene-specific primers containing the EcoRI restriction site (see Supplementary Table S1 at JXB online). The direction of the insert was confirmed by PCR and the construct was transferred to Agrobacterium tumefaciens strain GV3101 by electroporation (Mattanovich et al., 1989) and used to transform leaf segments of tobacco (Nicotiana tabacum cv. Samsun) (Horsch et al., 1985). Successful transformants were selected with kanamycin and the presence of the transfer DNA (T-DNA) was confirmed by gene-specific PCR using a genomic DNA template. In each case, expression of the T-DNA was assayed by semi-quantitative RT-PCR.

Phylogenetic analysis

Protein alignment was performed using MAFFT server (Katoh and Toh, 2008) with default settings. Phylogenetic analyses were conducted in MEGA5 (Tamura et al., 2011) using the Neighbour–Joining method (Saitou and Nei, 1987). Evolutionary distances were computed using the JTT matrix-based method (Jones et al., 1992) with a gamma shape parameter of 0.9. The resulting tree was subject to 1000 bootstrap replicates (Felsenstein, 1985). GenBank accession numbers for all the sequences used in this analysis are listed in Supplementary Table S2 at JXB online.

Results

Sequence homology of DfKNOX genes

Four DfKNOX transcripts (see Supplementary Fig. S1A at JXB online; DfKNO1–4; GenBank ID JQ229970–JQ229973) were isolated from a mixed cDNA pool derived from floral and vegetative tissues at a range of developmental stages using a combination of degenerate RT-PCR and RACE. Although most of the DfKNO3 coding sequence and 5′-untranslated region (UTR) were obtained in this way, it was not possible to identify the remaining 3′-sequence beyond that encoding the ‘WFIN’ motif of the homeodomain. Translating each coding sequence generated full-length proteins of 298 (DfKNO1), 327 (DfKNO2), and 287 (DfKNO4) amino acids, and a partial protein of 287 (DfKNO3) amino acids. All of the translated proteins included the MEINOX (KNOX1 and KNOX2), GSE box, ELK, and homeodomain motifs typical of other published KNOX transcription factors (see Supplementary Fig. S1B at JXB online).

Phylogenetic analysis of previously described KNOX protein sequences obtained from a broad range of species firmly places the four DfKNOX proteins alongside other monocot KNOX sequences in the four well-characterized Class 1 KNOX subgroups (Fig. 2; for branch lengths, see Supplementary Fig. S2 at JXB online). DfKNO1 occurs in the STM-like subgroup alongside EgKNOX1 and RaSTM, DfKNO2 appears in the OSH6-like subgroup with LIGULESS3 and OsKNO2, and DfKNO3 is confidently placed in the KNAT1/BP-like subgroup in a polytomy outside the main monocot clade. DfKNO4 is resolved to the KNAT2/6-like subgroup in a well-supported monocot clade that includes DOH1 and DnSTM from Dendrobium. A list of the most similar sequences to the DfKNO1–4 proteins is presented in Supplementary Table S3 at JXB online.

Expression patterns of DfKNOX genes

Gene-specific semi-quantitative RT-PCR was used to determine the accumulation of DfKNO1–4 transcripts in vegetative and floral tissues from mature and developing floral buds. Due to the limited number of plants available to us, it was not possible to evaluate transcript accumulation in the SAM, as the SAM becomes exhausted at the inflorescence apex and sampling during the rosette stage resulted in the death of the plant. The results presented here are representative of a minimum of three biological replicates. In each case, ORACT, the Dactyloctentia homologue of Arabidopsis ACTII (U27981), was used as an internal control.

DfKNO1, 2, and 4 transcripts are detectable in mature and developing floral buds, whereas only DfKNO2 and 4 transcripts can be detected in the leaves (Fig. 3A). In the first phase of analysis, DfKNO3 transcripts were undetectable by RT-PCR in either leaves or floral buds. In each case, DfKNO1, 2, and 4 are expressed to a high level in floral buds in which the labellum and spur are developing.

Developing floral buds at the earliest stages of labellum elaboration and spur expansion were dissected into several parts to determine the precise floral localization of DfKNO1–4 transcripts (Fig. 3B). Floral dissections divided the developing floral bud into the floral bract, the elaborate labellum (which bears the spur), the five remaining perianth parts (three sepals and two lateral petals), the gynostemium (fused androecium and non-ovary gynoecial elements) and the inferior ovary, which contains numerous ovules (Fig. 1). From this analysis, it is clear that floral expression of DfKNO1 is predominantly due to transcript accumulation in the ovary. DfKNO2 expression is more ubiquitous, being detectable in the bract, the non-spur-bearing perianth organs, the spur-bearing labellum, gynostemium, and ovary (Fig. 3C). Despite the apparent absence of DfKNO3 expression in whole floral buds (Fig. 3B), DfKNO3 transcripts are readily detectable in the bract, non-spur-bearing perianth organs, and ovary, whereas DfKNO4 transcripts are barely detectable in the bract and ovary.

Constitutive expression of the DfKNO2 transcript in tobacco

RT-PCR results strongly indicate that DfKNO2 may play an important role in floral development. To understand this potential function, the DfKNO2 transcript was constitutively expressed in tobacco under the cauliflower mosaic virus (CaMV) 35S promoter. To ensure that any phenotype observed resulted from the T-DNA, three independent transgenic tobacco lines were generated carrying the empty CaMV 35S, as confirmed by PCR using genomic DNA (see Supplementary Fig. S3 at JXB online). Visual inspection of the empty CaMV 35S lines found them to resemble wild-type tobacco closely (Fig. 4A). These empty vector lines were used as controls for phenotypic comparison. In total, 11 independent transgenic tobacco lines were generated carrying the DfKNO2 transcript under the CaMV 35S promoter. Despite confirming the presence of the T-DNA in all 11 independent lines by PCR using genomic DNA (data not shown), only four of these lines were shown to carry the T-DNA and express the DfKNO2 transcript to a readily detectable level when assayed by RT-PCR (see Supplementary Fig. S3 at JXB online). Vegetative and floral phenotypes were subsequently recorded from these four independent 35S::DfKNO2 transgenic tobacco lines by visual inspection.

Tobacco transformants constitutively expressing the DfKNO2 transcript did not differ significantly from wild-type and empty
35S transgenic control plants with respect to vegetative morphology (Fig. 4B). Each of the four 35S::DfKN2 lines produced normal leaves and grew to an equivalent height to wild-type and control plants. In terms of floral morphology, representatives from each of the four independent 35S::DfKN2 lines differed significantly from the flowers of wild-type and empty 35S transgenic control plants. Although the severity of floral phenotypes varied both within and between independent transgenic lines, many of the floral phenotypes can be regarded as relatively mild. All transgenic lines produced flowers that developed to anthesis and were able to self-fertilize, producing large quantities of seed. The flowering time of transformants was similar to that of wild-type tobacco plants. Flowers from plants exhibiting the mildest phenotypes were borne on inflorescences that were wild type in terms of floral density and branching pattern. The flowers themselves were almost wild type in appearance, with a five-lobed, pale pink corolla. In many cases, the corolla lobes were recurved and the margins moderately serrated (Fig. 4B, C).

Floral traits characteristic of more severe phenotypes were also observable in some lines, predominantly affecting the corolla lobes and margins. The corolla lobes at the proximal region of the corolla tube were more dissected than in wild-type plants, often with a reduced amount of lobe tissue resulting in flowers with a minimal corolla (Fig. 4C, D). Although the numbers of floral organs were not affected in any of the tobacco transformants, many flowers had a reduced corolla tube, short stamen filaments, and a long style (Fig. 4B, red arrow), and produced excessive petal tissue at the base of the corolla tube (Fig. 4B, white arrow). In more extreme cases, neighbouring buds appeared to be fused. Within such buds, floral organs failed to develop to maturity such that the corolla tube did not form (Fig. 4D), although this extreme phenotype was rare.
DfKNOX genes are expressed in developing flowers

RT-PCR demonstrates that DfKNI–4 transcripts are detectable in a broad range of tissues, including mature and developing flowers. In developing flowers, transcripts are detectable in the non-spur-bearing perianth organs (DfKNI, 3), the spur-bearing labellum (DfKNI2), gynostemium (DfKNI2), and ovary (DfKNI–4). Although KNOX genes have been implicated in carpel (Endrizzi et al., 1996; Pautot et al., 2001; Scofield et al., 2007, 2008) and spur (Golz et al., 2002; Box et al., 2011) development, roles in floral development have been largely overlooked in broader syntheses of KNOX gene function.

DfKNOX gene expression supports a function in carpel development

Constitutive expression and/or silencing of Arabidopsis STM and KNAT2 results in severely disrupted carpel formation (Endrizzi et al., 1996; Pautot et al., 2001; Scofield et al., 2007, 2008). Similar experiments in strawberry (Chatterjee et al., 2011) have also demonstrated that KNOX genes affect fruit development. RT-PCR clearly shows that all DfKNOX transcripts are readily detectable in the developing carpels, so KNOX genes could also play a role in orchid carpel development.

In Arabidopsis, carpels are formed from a residual population of stem cells located at the centre of the floral meristem (FM) (Blázquez et al., 2006); explaining early KNOX gene expression in developing ovaries. However, expression of DfKNI–4 transcripts is detectable long after initiation of the gynostemium, suggesting that KNOX genes are also involved in the patterning of later-forming tissues. Arabidopsis STM can also directly promote the development of carpels and the associated meso- and meso- tentic placental tissues of the ovary, independently of LEAFY and AGAMOUS (Pautot et al., 2001; Scofield et al., 2007) which are normally required to terminate stem-cell maintenance and permit the development of reproductive tissues (Busch et al., 1999; Lenhard et al., 2001; Lohmann et al., 2001). Although a direct role for KNOX proteins in carpel development cannot be excluded, it is more likely that they influence the normal patterning of carpel tissues through interactions with other proteins.

DfKNI2 is expressed during early phases of spur development

Aside from carpel development, the most widely predicted floral role for KNOX genes is in the development of floral elaborations such as spurs. Antirrhinum mutants ectopically expressing the KNOX genes HIRZINA4 and INVAGINATA produce ectopic petal-tubes that resemble the spurs of closely-related taxa such as Linaria (Golz et al., 2002). The L. vulgaris orthologues of HIRZINA and INVAGINATA also exhibit floral expression and induce sac-like outgrowths on flowers when constitutively expressed in tobacco (Box et al., 2011). Of the four DfKNOX genes identified in this work, DfKNI2 transcripts are readily detectable in the developing labellum which bears the spur primordium (Box et al., 2008). The broad expression pattern of DfKNI2 resembles that of HIRZINA and INVAGINATA in

Discussion

DfKNI4 may function to maintain the SAM

The fundamental function of Class 1 KNOX genes in SAM maintenance (Endrizzi et al., 1996; Long et al., 1996; Kerstetter et al., 1997; Vollbrecht et al., 2000) has been demonstrated in a phylogenetically broad range of angiosperm taxa. In this work, a representative sample of KNOX genes has been isolated from each of the four major subgroups of the Class 1 KNOX family (Bharathan et al., 1999; Reiser et al., 2000; Jouannic et al., 2007). Using similar degenerate PCR strategies, an identical profile of Class 1 KNOX sequences have been identified in other closely related diploid orchid taxa, such as Gymnadenia sp. (MS Box et al., unpublished data). We are therefore confident that one or more of the DfKNOX genes presented here performs the key KNOX gene function of maintaining the D. fuchsii SAM. Given the significant homology of the DfKNI4 and Dendrobium DOH1 proteins, DfKNI4 is the most likely candidate to perform this role. However, as many KNOX genes have been shown to act redundantly in maintaining the SAM (Byrne et al., 2002; Belles-Boix et al., 2006), several of the identified DfKNOX genes could be involved in SAM maintenance.

Fig. 3. Accumulation of DfKNOX transcripts in various organs. (A) Vegetative versus floral gene-specific RT-PCR of DfKNI–4 using OrACT as an internal control (mf, mature flower, df, developing flower). (B) Floral tissue-specific RT-PCR of DfKNI–4.
Fig. 4. Constitutive expression of the DfKN2 transcript in tobacco. (A) Empty-vector tobacco control (boxed). (B) Despite obvious floral aberrations, constitutive expression of the DfKN2 coding sequence did not produce vegetative phenotypes typical of constitutive KNOX expression. Floral abnormalities can vary significantly within the same inflorescence. Many flowers have a reduced corolla tube and stamens with a long stigma extending beyond the end of the corolla tube (red arrow). The production of additional petal tissue generates flowers in which the corolla tube elongates perpendicular to the ovary (white arrow, enlarged). (C) A range of floral abnormalities can be observed in the corolla lobes, which are commonly recurved, serrated or dramatically reduced (arrow). (D) More severely affected flowers are fused to neighbouring buds. Dissection of the minor bud demonstrates the presence of a full complement of floral organs that fail to complete development (arrow).
snapdragon mutants (Golz et al., 2002) and wild-type L. vulgaris (Box et al., 2011). Expression of DfKNOX in the developing label- 

um, carrying the spur primordium, suggests that KNOX genes 

may be involved in the morphogenesis of floral elaborations in 

petaloid monocots such as orchids, as well as in eudicots.

**Constitutive expression of the DfKNOX transcript in 

tobacco has an unusual flower-specific phenotype**

The function of DfKNOX was investigated by constitutively 

expressing its coding sequence in tobacco. Although no sac-like 

outgrowths on the petals or ectopic spurs were observed, a range 

of floral phenotypes was observed in 35S::DfKNOX plants that 

are similar to those previously described when other Class I KNOX 

genes were constitutively expressed in Arabidopsis and tobacco 

(reviewed by Hake et al., 2004; Hay et al., 2004; Shani et al., 

2006). Floral phenotypes included wrinkling of the corolla tube, 

increased corolla dissection and reduced corolla tube/Spacer length (Kano-Murakami et al., 1993; Sinha et al., 1993). Reduction 

in the length of the corolla tube and Spacers may be the result of 

reduced cell division, as previously suggested for the small 

leaves observed in tobacco plants constitutively expressing KN1 

from maize (Sinha et al., 1993). Dissection of the corolla tube 

has also been observed previously in 35S::KNOX tobacco plants 

and may provide further evidence for KNOX in defining organ 

boundaries. Disruption of cotyledon separation is observed in 

Arabidopsis stm mutants (Chuck et al., 1996), and STM and 

KNAT6 have been shown to redundantly define the boundaries 

between the SAM and the cotyledons in combination with CUP-

SHAPE COTYLEDONS (Aida et al., 1997, 1999; Belles-Boix 

et al., 2006). Some of the more unusual 35S::DfKNOX floral 

phenotypes have also been reported in the Arabidopsis KNOX 

literature; instances where neighbouring buds are fused together 

or hang downwards have been observed in mutants of PENNY-

WISE (PNW) (Byrne et al., 2003).

Most remarkably, although 35S::DfKNOX plants have relatively 

severe floral aberrations, they are devoid of vegetative phenotypes.

Typically, transgenic tobacco plants constitutively expressing 

KNOX are dwarfed, with shortened internodes and thickened 

leaves that are often reduced in size, with more or less severe 

wrinkling of the lamina and disrupted leaf symmetry (Kano-

Murakami et al., 1993; Matsuoka et al., 1993; Nishimura et al., 

2000). However, the phenotypes observed in 35S::DfKNOX 

tobacco plants are exclusively floral. The only orchid KNOX 

gene that has been previously characterized is DOH1. Constitu-

tive expression of DOH1 in Dendrobium grex Madame Thong-In 

(Yu et al., 2000) completely suppressed shoot organization and 

development, and showed the expected defects in leaf morphol-

ogy. Similarly, typical KNOX constitutive expression phenotypes 

were expected to be observed in 35S::DfKNOX tobacco plants.

RT-PCR demonstrated that lack of DfKNOX expression was not 

responsible for the absence of the expected leaf phenotypes. The 

CaMV 35S promoter has been shown to drive the expression of 

transgenes in all tissues from an early developmental stage 

(Harpster et al., 1988; Benfey et al., 1989). The timing and level 

of KNOX expression can affect the severity of the phenotype 

(Shani et al., 2009), so it is possible (although unlikely) that the 

DfKNOX transgene was more highly expressed in floral tissues.

A further possibility is that the DfKNOX protein did not 

accumulate in the leaves. DfKNOX protein levels were not mea-

sured and so this possibility cannot be conclusively excluded but 

there is no previous report of such a phenomenon in a 35S::KNOX 

plant. It is also possible that the evolutionary distance separating 

D. fuchsii and Nicotiana has resulted in a chance interaction of 

the DfKNOX protein with a flori ally expressed KNOX-interacting 

protein in tobacco. Similarly, the DfKNOX protein may have dis-

rupted an interaction that normally occurs in tobacco leaves.

Tobacco has been commonly used as a heterologous host for a 

variety of KNOX genes derived from equally distantly related species (e.g. maize) yet no floral-specific phenotype has previ-

ously been documented.

**Conclusions**

Four KNOX genes have been isolated from the orchid D. fuchsii 

and it has been shown that they are predominantly expressed 

in developing floral organs such as the spur-bearing label lum 

(DfKNOX) and the inferior ovary (DfKNOX1–4). A growing body of 

evidence supports a role for KNOX genes in the development of 

elaborate flowers and the angiosperm carpel. We believe that 

the tendency for DfKNOX to specifically alter floral morphology 

when constitutively expressed in a heterologous host may reflect 

a predominantly floral role for this gene in D. fuchsii develop-

ment. Such a floral-specific function for a KNOX gene has not 

previously been described and suggests that KNOX genes have a 

role in flower development that merits further study.

**Supplementary data**

Supplementary data can be found at JXB online.

- Supplementary Table S1. Primers used for the isolation and analysis of DfKNOX genes.
- Supplementary Table S2. KNOX protein sequences used for phylogenetic analyses.
- Supplementary Table S3. Most similar sequences to Dactylorhiza KNOX amino acid sequences.
- Supplementary Fig. S1. Structural organization of DfKNOX genes.
- Supplementary Fig. S2. Circular phylogram of the NJ tree in Fig. 2 comparing DfKNOX proteins and their relatives in the Class 1 KNOX group showing proportional branch lengths.
- Supplementary Fig. S3. In four independent 35S::DfKNOX tobacco lines the presence of the T-DNA was confirmed by PCR using genomic DNA template (A) and constitutive expression assayed by RT-PCR (B).

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