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RESEARCH PAPER

Flower-specific *KNOX* phenotype in the orchid *Dactylorhiza fuchsii*

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GenBank accession numbers: *DfKN1*, JQ229970; *DfKN*, JQ229971; *DfKN3*, JQ229972; *DfKN4*, JQ229973.
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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 *DfKN2* 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
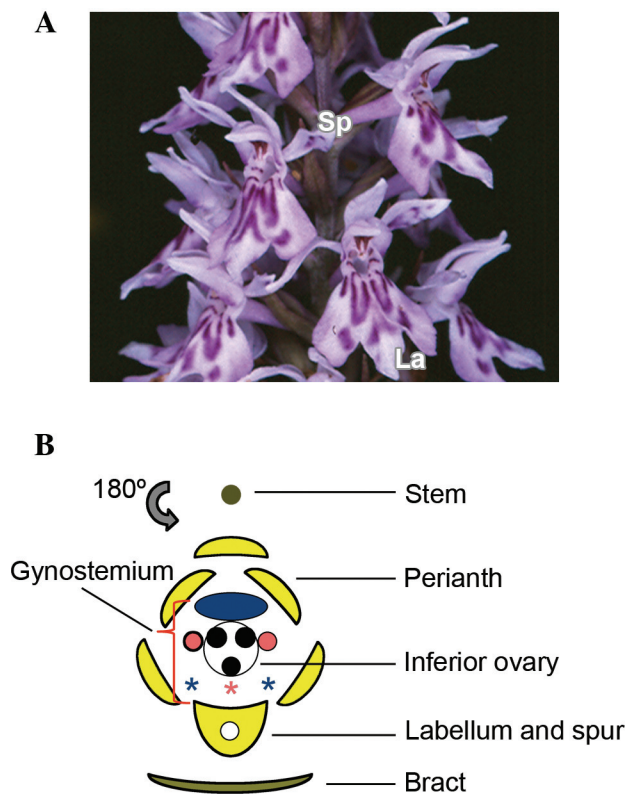
A

B

Fig. 1. The elaborate flowers of *D. fuchsii*. (A) *Dactylorhiza fuchsii* inflorescence, showing the labellum (La) and spur (Sp). (B) Flower diagram. The flower is bilaterally symmetrical and epigynous, that is, the floral parts are inserted on top of a tricarpellate inferior ovary (open circle containing three filled circles), with two perianth whorls (yellow): an outer whorl of sepals and an inner whorl of petals. The enlarged median adaxial petal of the inner whorl, the labellum, is three-lobed with a long spur (open circle). Androecial and gynoecial parts of the *D. fuchsii* flower are fused to form the gynostemium (red brace), consisting of an enlarged fertile anther (blue ellipse) of the outer androecial whorl, and two non-fertile staminodes (pink circles) putatively derived from the inner androecial whorl. The remaining expected stamens (asterisks) never develop. During development, the inferior ovary twists through 180° (a process termed resupination) so that the adaxially formed labellum occupies an abaxial position at anthesis (as pictured).

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 elaborate flowers of the functional diploid orchid *Dactylorhiza fuchsii* (Druce) Soó (Orchidaceae: Orchidinae, 2*n*=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 (*DfKN1–4*) have been isolated that represent the four major Class I KNOX subclades described previously (Sano *et al.*, 2005; Jouannic *et al.*, 2007). *DfKNOX* transcripts are detectable in developing floral organs and constitutive expression of the *DfKN2* 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 'WFIN' motifs of the KNOX and Homeodomain encoding regions. A full set of primer sequences is presented in Supplementary Table S1 at *JXB* online. The 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 *DfKN1*, JQ229970; *DfKN2*, JQ229971; *DfKN3*, JQ229972; *DfKN4*, 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 (Finnzymes, 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 *DfKN2* was cloned directly from cDNA into a modified pGreenII0029 vector between a double CaMV 35S promoter and a single CaMV 35S terminator (Hellens *et al.*, 2000) using gene-specific primers containing the *Eco*RI 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; *DfKN1–4*: 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 *DfKN3* 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 (DfKN1), 327 (DfKN2), and 287 (DfKN4) amino acids, and a partial protein of 287 (DfKN3) 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). DfKN1 occurs in the STM-like subgroup alongside EgKNOX1 and RaSTM, DfKN2 appears in the OSH6-like subgroup with LIGULESS3 and Oskn2, and DfKN3 is confidently placed in the KNAT1/BP-like subgroup in a polytomy outside the main monocot clade. DfKN4 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 DfKN1–4 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 *DfKN1–4* 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 *Dactylorhiza* homologue of *Arabidopsis ACT11* (U27981), was used as an internal control.

DfKN1, *2*, and *4* transcripts are detectable in mature and developing floral buds, whereas only *DfKN2* and *4* transcripts can be detected in the leaves (Fig. 3A). In the first phase of analysis, *DfKN3* transcripts were undetectable by RT-PCR in either leaves or floral buds. In each case, *DfKN1*, *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 *DfKN1–4* 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 *DfKN1* is predominantly due to transcript accumulation in the ovary. *DfKN2* 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 *DfKN3* expression in whole floral buds (Fig. 3B), *DfKN3* transcripts are readily detectable in the bract, non-spur-bearing perianth organs, and ovary, whereas *DfKN4* transcripts are barely detectable in the bract and ovary.

Constitutive expression of the DfKN2 *transcript in tobacco*

RT-PCR results strongly indicate that *DfKN2* may play an important role in floral development. To understand this potential function, the *DfKN2* 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 *DfKN2* 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 *DfKN2* 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::*DfKN2* transgenic tobacco lines by visual inspection.

Tobacco transformants constitutively expressing the *DfKN2* transcript did not differ significantly from wild-type and empty
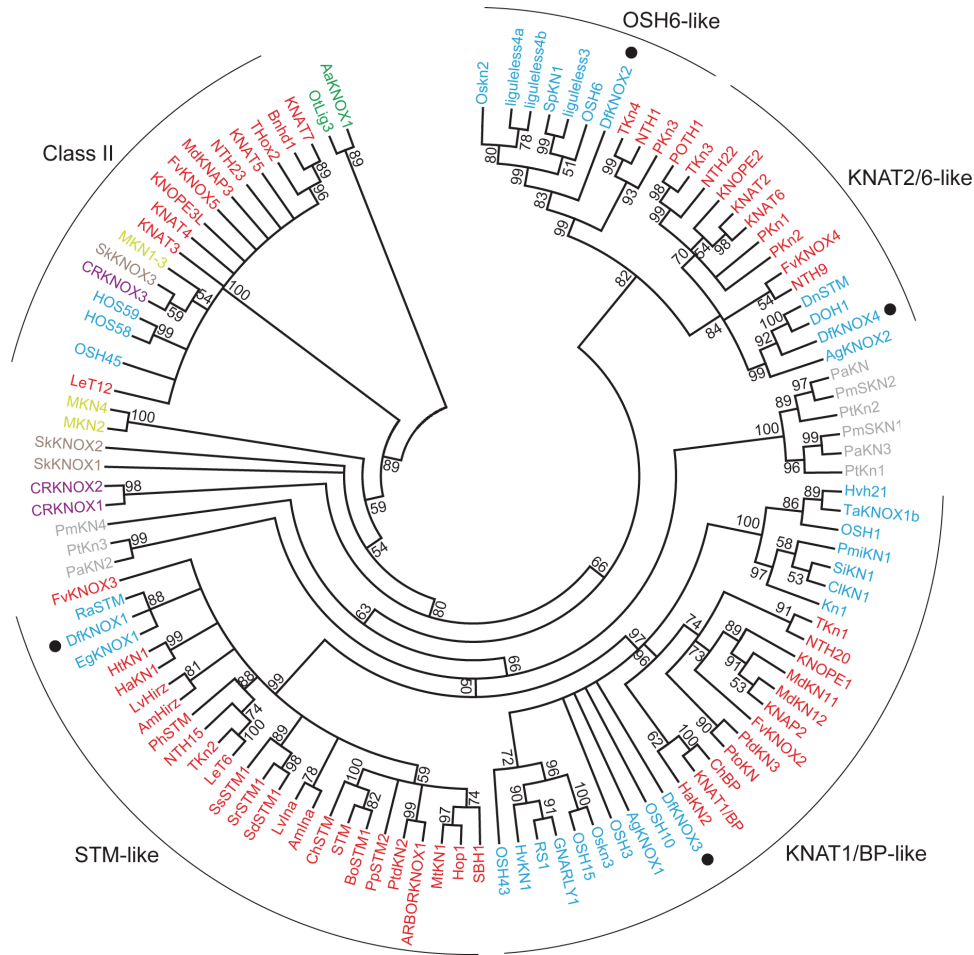
Fig. 2. Phylogenetic analysis of DfKNOX proteins. Neighbour–Joining (NJ) tree of DfKNOX proteins and their relatives in the Class 1 KNOX group. Bootstrap values are indicated above the nodes; those with less than 50% support were collapsed. DfKNOX proteins are indicated by closed circles. The colour of the protein names indicate higher classifications as follows: algae, green; bryophytes, yellow; lycophytes, brown; monilophytes, purple; gymnosperms, silver; eudicots, red; monocots, blue. Braces indicate the Class II and major Class I KNOX subgroups. For clarity, the tree is represented as a circular cladogram (for a circular phylogram, showing proportional branch lengths, see Supplementary Fig. S2 at *JXB* online). A list of species names, and the accession numbers of the protein sequences used, are detailed in Supplementary Table S2 at *JXB* online.

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.
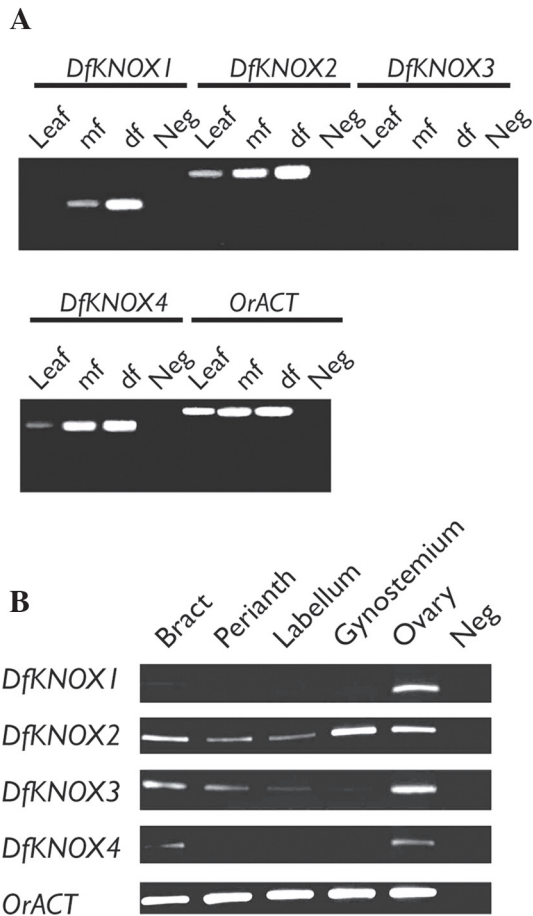
A

B

Fig. 3. Accumulation of *DfKNOX* transcripts in various organs. (A) Vegetative versus floral gene-specific RT-PCR of *DfKN1–4* using *OrACT* as an internal control (mf, mature flower, df, developing flower). (B) Floral tissue-specific RT-PCR of *DfKN1–4*.

Discussion

DfKN4 *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 I *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 DfKN4 and *Dendrobium* DOH1 proteins, DfKN4 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.

DfKNOX *genes are expressed in developing flowers*

RT-PCR demonstrates that *DfKN1–4* 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 (*DfKN2*, *3*), the spur-bearing labellum (*DfKN2*), gynostemium (*DfKN2*), and ovary (*DfKN1–4*). 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 *DfKN1–4* 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 meristematic 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.

DfKN2 *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 *HIRZINA* 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, *DfKN2* transcripts are readily detectable in the developing labellum which bears the spur primordium (Box *et al.*, 2008). The broad expression pattern of *DfKN2* resembles that of *HIRZINA* and *INVAGINATA* in
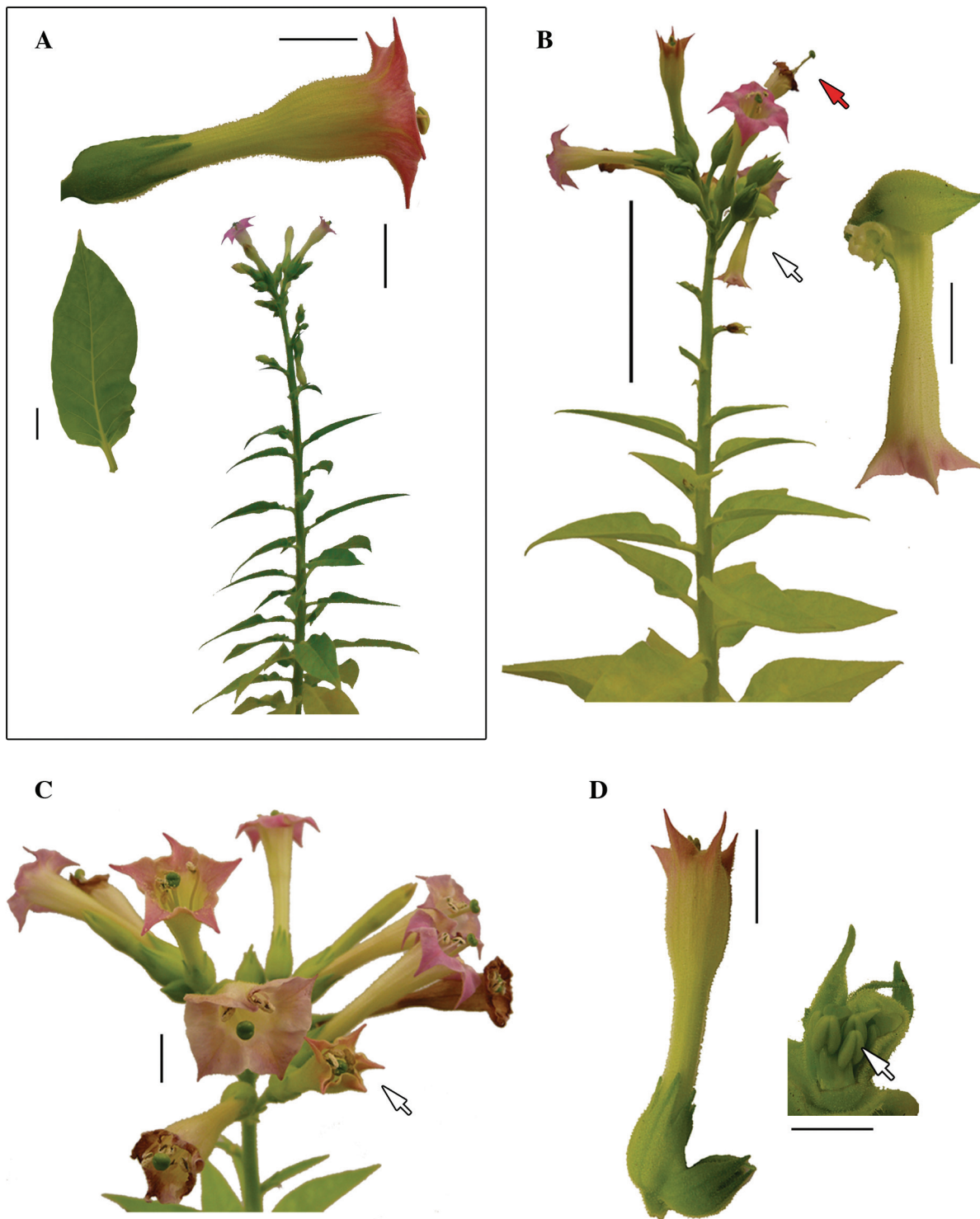
Fig. 4. Constitutive expression of the *DfKN2* transcript in tobacco. (A) Empty-vector tobacco control (boxed). (B) Despite obvious floral aberrations, constitutive expression of the *DfKN2* coding sequence did not produce vegetative phenotypes typical of constitutive *KNOX* expression. Floral abnormalities 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 *DfKN2* in the developing labellum, 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 DfKN2 *transcript in tobacco has an unusual flower-specific phenotype*

The function of *DfKN2* 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::*DfKN2* 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/anther length (Kano-Murakami *et al.*, 1993; Sinha *et al.*, 1993). Reduction in the length of the corolla tube and anthers 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-SHAPED COTYLEDONS* (Aida *et al.*, 1997, 1999; Belles-Boix *et al.*, 2006). Some of the more unusual 35S::*DfKN2* 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* (*PNY)* (Byrne *et al.*, 2003).

Most remarkably, although 35S::*DfKN2* 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::*DfKN2* transgenic tobacco plants are exclusively floral. The only orchid *KNOX* gene that has been previously characterized is *DOH1*. Constitutive 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 morphology. Similarly, typical *KNOX* constitutive expression phenotypes were expected to be observed in 35S::*DfKN2* tobacco plants.

RT-PCR demonstrated that lack of *DfKN2* 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 *DfKN2* transgene was more highly expressed in floral tissues.

A further possibility is that the DfKN2 protein did not accumulate in the leaves. DfKN2 protein levels were not measured 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 DfKN2 protein with a florally expressed KNOX-interacting protein in tobacco. Similarly, the DfKN2 protein may have disrupted 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 previously 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 labellum (*DfKN2*) and the inferior ovary (*DfKN1–4*). 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 *DfKN2* to specifically alter floral morphology when constitutively expressed in a heterologous host may reflect a predominantly floral role for this gene in *D. fuchsii* development. 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.

Supplemtary 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::*DfKN2* 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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