

SPECIAL ISSUE RESEARCH PAPER

Peach [*Prunus persica* (L.) Batsch] *KNOPE1*, a class 1 *KNOX* orthologue to *Arabidopsis* *BREVIPELICELLUS*/*KNAT1*, is misexpressed during hyperplasia of leaf curl disease

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Abstract

Class 1 *KNOTTED*-like (*KNOX*) transcription factors control cell meristematic identity. An investigation was carried out to determine whether they maintain this function in peach plants and might act in leaf curliness caused by the ascomycete *Taphrina deformans*. *KNOPE1* function was assessed by overexpression in *Arabidopsis* and by yeast two-hybrid assays with *Arabidopsis* *BELL* proteins. Subsequently, *KNOPE1* mRNA and zeatin localization was monitored during leaf curl disease. *KNOPE1* and *Arabidopsis* *BREVIPELICELLUS* (*BP*) proteins fell into the same phyletic group and recognized the same *BELL* factors. *35S:KNOPE1 Arabidopsis* lines exhibited altered traits resembling those of *BP*-overexpressing lines. In peach shoot apical meristem, *KNOPE1* was expressed in the peripheral and central zones but not in leaf primordia, identically to the *BP* expression pattern. These results strongly suggest that *KNOPE1* must be down-regulated for leaf initiation and that it can control cell meristem identity equally as well as all class 1 *KNOX* genes. Leaves attacked by *T. deformans* share histological alterations with class 1 *KNOX*-overexpressing leaves, including cell proliferation and loss of cell differentiation. Both *KNOPE1* and a cytokinin synthesis *ISOPENTENYLTRANSFERASE* gene were found to

be up-regulated in infected curled leaves. At early disease stages, *KNOPE1* was uniquely triggered in the palisade cells interacting with subepidermal mycelium, while zeatin vascular localization was unaltered compared with healthy leaves. Subsequently, when mycelium colonization and asci development occurred, both *KNOPE1* and zeatin signals were scattered in sectors of cell disorders. These results suggest that *KNOPE1* misexpression and *de novo* zeatin synthesis of host origin might participate in hyperplasia of leaf curl disease.

Key words: *KNOPE1*, peach, role in cell undetermined fate, response in leaf curl disease, *Taphrina deformans*, transcription factor, zeatin.

Introduction

The plant *KNOTTED1*-like (*KNOX*) genes encode homeo-domain- (HD) containing transcription factors (TFs) and fall into two classes, distinguished by the identity grade of the residues within the HD, intron position, and expression patterns (Reiser *et al.*, 2000; Hake *et al.*, 2004).

Class 1 *KNOX* genes are differentially required for shoot apical meristem (SAM) establishment and function, and constitute a pathway that controls meristem cell fate

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(Hake *et al.*, 2004). In simple leafed species, they are typically expressed in the SAM, but the down-regulation is required both in cell groups (P_0) that will originate a leaf primordium and throughout leaf development (Hay and Tsiantis, 2006).

The *Arabidopsis* SHOOT MERISTEMLESS (*STM*) gene suppresses differentiation throughout the SAM, allowing stem cell amplification before cell recruitment into lateral organs (Scofield and Murray, 2006). *Arabidopsis* *KNAT1*, also named *BREVIPEDICELLUS* (*BP*), *KNAT2*, and *KNAT6* are active in the SAM, but show different spatial patterns of expression compared with that of *STM* (Reiser *et al.*, 2000). Mutants deficient in these genes do not exhibit alterations in SAM development or function (see references in Scofield and Murray, 2006), suggesting that *STM* is critical for SAM development. Genetic studies indicate that each class 1 *KNOX* gene may control different developmental pathways (Hake *et al.*, 2004), although functional redundancy between *STM* and *BP* was assessed (Byrne *et al.*, 2002).

In *Arabidopsis* SAM, *BP* mRNA is excluded from P_0 , and marks the peripheral zone (PZ), rib zone (RZ), and the boundaries at the base of leaf primordia. It localizes in the stem below the SAM and in the cells surrounding stem veins (Lincoln *et al.*, 1994). *BP* is also active in the inflorescence meristem (but not in the floral one), gynoecium, style, and cortical tissues of pedicels (Lincoln *et al.*, 1994; Douglas *et al.*, 2002; Douglas and Riggs, 2005). *BP* loss-of-function mutants (*bp*) exhibit growth reduction of tissues that normally express *BP*, except for the carpel tissues (Douglas *et al.*, 2002; Venglat *et al.*, 2002). The morphological alterations (e.g. shortened internodes and pedicels, enhanced lignin deposition, epinasty of siliques) are due to reduced cell division and impaired differentiation in the affected tissues. Transcript profiling analyses suggest that *BP* also regulates some lignin pathway genes during internode development (Mele *et al.*, 2003). *BP* ectopic expression causes the formation of abnormal leaves with intense lobing at margins and, sporadically, the development of ectopic shoot meristems on the leaf adaxial surface (Chuck *et al.*, 1996; Ori *et al.*, 2000; Frugis *et al.*, 2001). Histological analyses evidence the inhibition of proper leaf cell differentiation (e.g. palisade layer and spongy mesophyll cells are hardly distinguishable), which is proposed not to be caused by direct promotion of cell division by *KNOX* (Scofield and Murray 2006), although cell proliferation was specifically observed during lobe development in *STM*-overexpressing leaves (Lenhard *et al.*, 2002).

Phytohormones and class 1 *KNOX* cooperate to regulate the balance between SAM maintenance and the production of lateral organs (Hay *et al.*, 2004). In the *Arabidopsis* SAM, *STM* inhibits *ASYMMETRIC LEAVES* (*AS*) gene expression, allowing *KNAT1/2/6* activity. Class 1 *KNOX* repress gibberellin synthesis and show reciprocal

up-regulation with cytokinins (CKs). In leaf primordia, the auxin and *AS* cooperate to repress *BP* (Hay *et al.*, 2006). Moreover, *de novo* CK biosynthesis, due to *ISOPENTENYLTRANSFERASES* (*IPT*) up-regulation, was observed in both *Arabidopsis* and rice leaves overexpressing *STM* (Jasinski *et al.*, 2005; Yanai *et al.*, 2005) and *STM/BP* orthologues, respectively (Sakamoto *et al.*, 2006). Conversely, bacterial *IPT* overexpression induced *KNAT1* and *STM* ectopic activity (Rupp *et al.*, 1999).

KNOX and *BELL* proteins form transcriptional complexes, necessary for normal *KNOX* function in the SAM (Bellaoui *et al.*, 2001). The interactions are selective because each *KNOX* recognizes a subset of *BELL*. *BP* specifically binds to *PNY* and *PNF*, encoded by the *PENNYWISE* (*PNY*) and *POUND-FOOLISH* (*PNF*) genes, which contribute to specify inflorescence internode patterning and floral primordia (Smith and Hake, 2003; Smith *et al.*, 2004).

Clearly, *KNOX* have been widely investigated in model species, but much less in commercially important trees. *KNOX* may govern traits of agronomical interest (e.g. for *BP* orthologues: caulis development, tree habitus, etc.) and there is a growing focus on the roles of *KNOX* in stem secondary growth of forest trees (Groover *et al.*, 2006). Studies on fruit tree *KNOX* have been limited to gene characterization (Watillon *et al.*, 1997) and bud expression (Brunel *et al.*, 2002) in apple trees, and to gene function in palm leaf (Jouannic *et al.*, 2007). Here, evidence is provided that peach *KNOPE1* shared equivalence at the expression, functional, and biochemical levels with the class 1 *BP*.

There is a body of evidence that TFs participate in biotic stress responses (Eulgem, 2005), though little is known about the roles of *KNOX*. Transcriptome analyses revealed root *KNAT6* down-regulation after nematode infections (Füller *et al.*, 2007), while *Medicago truncatula* root *KNOX* genes were triggered by rhizobia and nematodes (Koltai *et al.*, 2001). *Taphrina deformans* (Berk.) Tul. is a biotrophic parasitic fungus that causes leaf curl of peach, a disease that seriously affects both crop yield and tree longevity (Rossi *et al.*, 2006, 2007). Histological analyses of expanded leaves infected by *T. deformans* showed that differentiated palisade cells resumed division capacity, generating hyperopic sectors characterized by undistinguishable palisade and spongy mesophyll layers (Syrop, 1975a). This response was accompanied by the increase of CK content (Szirákí *et al.*, 1975). These features strongly resemble the alterations of *BP*-overexpressing leaves (Chuck *et al.*, 1996; Frugis *et al.*, 2001). Hence, experiments were conducted to verify whether *KNOPE1* transcription occurred during leaf hyperplasia, and *KNOPE1* re-activation was observed, together with host zeatin accumulation, at distinct disease stages, suggesting a role for *KNOPE1* in the histological disorders.

Materials and methods

Plant materials and growth conditions

The IBBA-CNR field-hosted 40 adult peach plants spaced at a distance of 5 m × 5 m and pruned to an open-centre shape (vase). These belonged to two clonal lines (S_1 and S_2) obtained by micropropagation (Giannino *et al.*, 2000, 2003) of two seedlings from the mother plant OP16 (open pollinator 16, *Prunus persica* cultivar 'Chiripa'). The level of homozygosity of 'Chiripa' was not assessed and it was assumed that it was on average 65%, as estimated by microsatellite fingerprinting (Aranzana *et al.*, 2003). To ensure a grade of plant material homogeneity, only the outdoor adult clones within the S_1 line were used for leaf curl disease experiments. S_1 clones showed uniform phenotypical and physiological traits (e.g. blooming time, fruit production); however, they may contain a grade of *in vitro* induced somaclonal variation, which was not assessed.

Plant 18 from the S_1 line (F_0S_{1-18}) was self-pollinated under controlled conditions, by covering flowers with paper bags in the first 2 weeks of March. Fruit were harvested (July) and seeds were sun dried, treated with Caffaro powder (2 g for 1 kg of seeds) containing 16% copper oxichlorure (ISAGRO, Milan, Italy), stored in paper bags at 7 °C, sown after 90 d, and grown in the greenhouse at 22–25 °C, 16/8 h of light/dark with a light intensity of 100 $\mu\text{mol m}^{-2} \text{s}^{-1}$ of photosynthetically active radiation (PAR). The seeds generated F_1S_{1-18} individuals, which provided samples at different developmental stages for gene cloning, Southern blot, tissue-specific expression, *in situ* hybridizations in the SAM, and CK response (more details are given below). The heterozygosity level of the F_0S_{1-18} was not measured; however, F_1S_{1-18} plants share at most two different alleles for each locus, since they are derived from a strict self-fecundation.

Studies of nucleotide polymorphism for genetic marker identification are in progress in our laboratories. Computational analyses of several *KNOPE1* genes have not revealed, so far, any single nucleotide difference between 'Chiripa' and other cultivars (e.g. 'Springcrest'), between the outdoor plants of the S_1 and S_7 lines, and within the sister plants of the F_1S_{1-18} progeny. *KNOPE1* was highly conserved at least in the transcribed and intron regions, and it was assumed that mechanisms of regulation would be conserved throughout the material used.

Plant material and sampling for *KNOPE1* response to *T. deformans* infections

In the open field, 20 adult plants (S_1 and S_2 clones in plot 1) were treated yearly with two sprays of Bordeaux mixture (2%) and Thiram (0.4%) at leaf fall with a 2-week interval, followed by two sprays of Thiram (0.5%) in spring before bud swelling. Two rows of 10 adult clones (S_1 and S_2 clones in plot 2) were set at ~50 m from plot 1, and pesticide was only sprayed during the second of a 3-year interval (2001–2003). These plants exhibited curl disease symptoms typically from April to June, whereas the infection was rare on treated plants. In April–May, expanded leaves (mid-vein length ~8–10 cm) borne on distal positions of growing shoots were collected from healthy and infected trees. The infected samples exhibited red or green curly areas within or at the margins of leaves (see Fig. 5A), so that the pathogen damage was not yet extended. Northern blot, *in situ* localization, and immunolocalization experiments were performed on attacked and healthy leaves ($n=3$), which were sampled from three different S_1 clones of the outdoor treated and untreated plots. Experiments were performed for 3 years (2001–2003).

Isolation and sequence analysis of cDNA and genomic clones

The cDNA was synthesized (see next paragraph) from herbaceous stem RNA of F_1S_{1-18} plants and PCR-amplified by degenerate

primers Kn5Fw [5'-GA(C/T)(C/G)A(A/G)TT(C/T)ATGGA(A/G)(A/G)C(A/G/C/T)TA-3'] and Kn8Bw [5'-AT(A/G)AACCA(A/G)TT(A/G)TT(A/T/G)AT(C/T)TG-3'], which were designed on DQEMEAY (KNOX2) and QINNWF (HD), respectively. A 482 bp fragment was cloned and sequenced, then the full-length *KNOPE1* cDNA was rescued by 5'/3' RACE (rapid amplification of cDNA ends) technology (Invitrogen). Reverse 5' RACE primers for cDNA synthesis were: Kn4Bw (5'-GGAGGAGCATTATTG-TTGCCAAGC-3') and Kn2Bw (5'-CTGCCTTGCCTCAAATC-C-3'), which yielded 916 bp and 734 bp fragments, respectively. 3' RACE primers were: cDNA synthesis oligo(dT) anchor and Kn7Fw (5'-CTCCACTTCGCATCTTCTCACCC-3'). PCR conditions were 500 ng of genomic DNA or 200 ng of cDNA, 1 mM of each primer, 0.5 mM dNTPs, 2.5 U of *Taq* DNA polymerase (TaqQUA, Quiagen), 1/10 of 10× *Taq* buffer, 2.5 mM MgCl_2 , final volume 50 μl . Cycling conditions were an initial cycle at 95 °C for 5 min followed by 35 cycles at 95 °C for 40 s, 57 °C using cDNA or 62 °C using genomic DNA for 30–60 s, and 72 °C for 30–90 s, and a final extension at 72 °C for 5 min.

Introns were found by amplifying genomic DNA of F_1S_{1-18} plant leaves with the following pairs of primers: Kn3Fw (5'-GCCAAGATCATCGCCACCC-3')/Kn4Bw; Kn7Fw/Kn6Bw (5'-ATTCCTCTCCTCTTTCAG-3'); Kn11Fw (5'-AAGGTGGCATTGG-CGGAGT-3')/Kn10Bw (5'-GCAGTTTGCTTTGTCTTTTGG-3'). The PCR product sequences were aligned to set intron size and locations. All PCR fragments were cloned into the pGEM-T easy vector system (Promega).

Yeast two-hybrid assay

KNOPE1 was amplified (cDNA fragment 226–1508) under the conditions reported above except using *Taq* Platinum Pfx (Invitrogen), and enzyme-adapted primers Kn1Fw (5'-GGGATCCGGAT-GGAAGAGTACAACC-3') and Kn10Bw (5'-GCTCGAGGCA-GTTTGTCTTTGTCTTTTGG-3'). The PCR product (50 ng) was ligated in *Bam*HI–*Xho*I sites (underlined nucleotides) in the oriented direction into pENTR1A (Gateway system, Invitrogen), sequenced to check for gene integrity, and cloned into the *TRP1*-marked Gal4 activation domain vector (pDEST22, Invitrogen) through the LR reaction. *Arabidopsis PNF* (Atg27990), *PNY* (NM_120281), *KNAT1/BP* (NM_116884), *STM* (NM_104916), and *BELLI* (NM_123506) were cloned into a *LEU2*-marked Gal4 DNA-binding domain construct (pDEST32) provided by Dr Enrico Magnani. Recombinant pDEST22 and pDEST32 were transferred into *Saccharomyces cerevisiae* (MaV103 and MaV203, respectively) by electroporation (1800 V for one pulse, EasyjectPrima-EQUIBIO) and then plated on selective medium. The recombinant colonies were used in mating-type procedures by plating on -LEU-TRP-deficient medium (YPAD). Plates were overlaid with chloroform, incubated for 5 min, and dried for 10 min under a hood, then X-Gal agarose (20 ml, 1% low-melting agarose, 0.1 M NaH_2PO_4 buffer pH 7.0, 0.25 mg ml^{-1} X-Gal) was poured on the colonies and they were left to harden. Plates were incubated at 30 °C until a blue stain occurred. Inserts were also swapped from prey to bait vectors.

Genetic transformation of *Arabidopsis*

The 1–1508 bp *KNOPE1* cDNA portion was amplified using adapted primers: Kn-5'UTRFw (5'-GTCTAGAGTTTGGCTTCA-CTTCTCGC-3') and Kn-3'UTRBw (5'-GCTCGAGGCA-GTTTGTCTTTTGG-3'), and *Taq* Platinum Pfx (Invitrogen). PCR conditions were the same as above. The amplified fragment was digested with *Xba*I and *Xho*I, and cloned into the binary vector pBA002 (Kost *et al.*, 1998) under the control of the 35S promoter of cauliflower mosaic virus (CaMV). The recombinant plasmid was sequence-checked and transferred to the *Agrobacterium tumefaciens*

strain GV3101 (pMP90) by freeze–thaw (Holstein *et al.*, 1978). The 35S:*KNOPE1* was inserted in *Arabidopsis* Columbia by vacuum infiltration following Bechthold *et al.* (1998). Transformant lines were selected on medium (MS0 and Gambor vitamins) containing phosphinothricin, carbenicillin, and cefotaxime at 10, 500, and 90 ppm, respectively. The *KNOPE1* transcript was detected by RT-PCR using Kn3Fw and Kn4Bw primers.

Alignments and phylogenetic analysis

The alignment of *KNOPE1* with other *KNOX* proteins was performed by ClustalW (<http://www.ebi.ac.uk.clustalw>) and optimized by visual inspection (PILEUP program). Phylogenetic trees were constructed by MegaBlast2 (based on the minimum evolution criterion), using bootstrap values performed on 1000 replicates, and the 50% value was accepted as an indication of a well-supported branch. The class 1 *KNOX* accession numbers are: AtKNAT1, U14174; AtKNAT2, U14175; AtKNAT6L, AB072362; AtKNAT6s, AB072361; AtSTM, U32344; GmSBH1, P46608; HaKn1, AY096802; HaKn2, AY096803; HtKn1, CAD58394; InKn1, AB015999; InKn2, AB016000; InKn3, AB016002; LeT6, AF000141; LeTKn1, U32247; LetKn2, U76407; LeTKn3, U76408; LeTKn4, AF533597; MdKN1.1, Z71978; MdKN1.2, Z71979; MtKNOX1, AF308454; NtH1, AY169493; NtH9, AB025713; NtH15, BAA25546; NtH20, AB025714; NtH22, AB025715; NtKn1, AF544052; NtKn2, AF544053; PatARK1, AY755413; PbKn3, AAV49802; Pkn1, BAA31698; PsHOP1, AF063307; PsKn1, AAC32262; PsKN2, AF080105; PtdKn2, AY684937; PtdKn3, AY684938; and PtKN, AAV84585. The class 2 *KNOX* proteins were used to create an outgroup; accession numbers: AtKNAT3, X92392; AtKNAT4, NP_196667; AtKNAT5, NP_194932; and AtKNAT7, AF308451.

Southern blot analysis

The technique was performed as previously described (Giannino *et al.*, 2000). Filters were hybridized at 60 °C, washed twice (2× and 1× SSC/0.1% SDS) at 60 °C for 10 min, and exposed to Biomax films (Kodak) for 4–12 h at –80 °C. Probe 1 spanned the 780–1261 bp of *KNOPE1* cDNA. Probe 2 spanned the 2796–3539 bp of *KNOPE1* genomic sequence, in which *Hind*III cuts at nucleotide 2841.

Northern blot analyses

Total RNA was extracted according to Giannino *et al.* (2000) from 2-year-old F₁S_{1–18} individuals (organ-specific expression) and S₁ clones (response to *T. deformans* infection). RNA (10 µg per lane) was separated on a 1.2% (w/v) agarose–formaldehyde gel and transferred to a Hybond N⁺ membrane (Amersham Biosciences, UK). Hybridization was carried out overnight at 42 °C with Ultrahyb buffer (Ambion) containing formamide, followed by two washes in 2× and 1× SSC/0.1% (w/v) SDS at 60 °C for 10 min and one wash in 0.1× SSC/0.1% (w/v) SDS. Filters were exposed to Kodak BIOMAX films (Amersham Biosciences, UK) for at least 4 h at –80 °C. Probe 3 of *KNOPE1* cDNA spanned the 1303–1681 bp stretch. The *ISOPENTENYLTRANSFERASE 5* (*IPT5*) probe consisted of a 386 bp fragment, which was amplified by *IPT5*Fw (5'-GCGTGCCTGAAATGGATGAG-3') and *IPT5*Bw (5'-AGCGGTCACTGTTCGGCATAG-3'), designed from the apple expressed sequence tag (EST) CN916352, and using leaflet cDNA. The single amplified product was cloned and sequenced, and the gene fragment was named *IPT5* after *Arabidopsis IPT5* because they shared 73% identity. The probes were radiolabelled with [³²P]dCTP using the Ready Primer kit (Amersham Biosciences, UK).

RT-PCR analyses

RNA from 35S:*KNOPE1* and wild-type *Arabidopsis* (Fig. 4A) was isolated by the TRIZOL reagent following the manufacturer's instructions (Invitrogen). For peach RNA, see the paragraph above. RNA was made DNA free (RQ1, Promega) and 3 µg was reverse transcribed (Superscript III, Invitrogen) at 55 °C into a single-stranded cDNA by oligo(dT)₂₀. The *KNOPE1* 311 bp transcript was amplified by Kn3Fw/Kn4Bw primers; 26S rRNA by 26SFw (5'-AGCATTGCGATGGTCCCTGCGG-3')/26SBw (5'-GCCCCGT-CGATTCAGCCAAACTCC-3'), and used to check for equal amounts of cDNA template. A mock PCR was performed using RNA samples to exclude DNA contamination. PCR trials were performed at distinct cycles to assess the variation of transcript abundance before signal saturation, at fixed primer pair and reaction parameters. Amplification products were sequenced to avoid artefacts. The PCR was conducted in a 50 µl total volume containing cDNA (3 µl); the chemical parameters are given above. Cycles were 35 for *KNOPE1* and 20 for 26S rRNA; 15 µl was electrophoresed in a 1% agarose gel. Reverse-transcribed 26S rRNA appeared to be equal in all the tissues tested. In CK response assays (Fig. 7), the optical density (OD) of the signal bands was determined by the ID Image Analysis software (Kodak Digital Science), and the relative OD was graphed as histograms (Microsoft Excel) representing the ratio between the OD of *KNOPE1* and 26S rRNA (checking for equal loading of RNA). RT-PCR experiments were carried out three times with independent RNA extracts. Standard errors were calculated and are indicated on the size bars. All data sets were subjected to Student's *t*-test where *P* < 0.05 is regarded as significant.

Cytokinin response assay

Fully expanded leaves (mid-vein was ~8 cm) including the petioles were excised from the primary axis of 2-year-old F₁S_{1–18} plants (June). They were immersed in sterile tubes filled with 50 ml of 0.1 µM NaOH (control) and 10 µM benzyl aminopurine (BAP)/0.1 µM NaOH. Samples were kept at 22 °C at a light intensity of 100 µmol m⁻² s⁻¹ PAR and removed from solutions after 0.5, 2, and 4 h, and frozen in liquid nitrogen. Non-immersed leaves, which were excised from the plant and rapidly frozen in liquid nitrogen, represented the controls at 0 h. RNA was isolated from a two leaf pool and the assay was repeated three times using leaves borne on three different plants.

In situ hybridization

Excised tissues were fixed, dehydrated, embedded in paraffin, cut into 8 µm sections, and hybridized (55 °C) to a digoxigenin-labelled antisense RNA probe as described by Cañas *et al.* (1994). The cDNA probe 3 was linearized by *Spe*I and *Nco*I. Antisense and sense probes were *in vitro* synthesized by T7 and SP6 polymerases, respectively (Giannino *et al.*, 2000). In experiments performed on the SAM shoot, three apices were excised from 2-year-old F₁S_{1–18} plants (*n*=3) at vegetative resumption (late February). In experiments of the *KNOPE1* response during leaf curl disease, sections of attacked and healthy leaves (*n*=3) were sampled from three different S₁ clones of the outdoor treated and untreated plots.

Cytological–histological analyses and zeatin immunocytolabelling

Both cytohistological procedures and zeatin immunolocalization were carried out using the same material as reported above. Concerning zeatin localization, the leaf portions were pre-embedded, cut with a vibratome (Leica VT1000E, Bensheim, Germany), and incubated with primary antibody against zeatin according to

Dewitte *et al.* (1999). Colloidal gold- (<1 nm) labelled secondary antibodies (1:40, Aurion, Wageningen, The Netherlands) were used. The antibodies can recognize both the conjugated and free CK bases. However, the procedures of fixation (based on paraformaldehyde and glutaraldehyde mixtures) wash away the conjugated forms; hence, the antibodies specifically detected the free zeatin bases, which are biologically active (Dewitte *et al.*, 1999).

Results

KNOPE1 is a class 1 KNOX-like gene

The full-length cDNA of *KNOPE1* is 1681 bp (accession no. DQ358050; for details on cDNA features, see Supplementary Fig. S1 available at *JXB* online) with an open reading frame (ORF) of 1170 bp encoding a deduced polypeptide of 389 amino acids. *KNOPE1* is a 44.41 kDa protein that contains the typical KNOX domains (Fig. 1A)

and shares highest identity with *Malus domestica* KNAP1 (88%), followed by *Populus* spp. KN3 (73%), and *A. thaliana* KNAT1 (59%). The *KNOPE1* HD was 100, 95.2, and 85.7% identical to those of apple KNAP1, poplar KN3, and maize KN1, respectively. The identity grade with the maize KN1 HD is a criterion to assign KNOX proteins to class 1 (Kerstetter *et al.*, 1994). *KNOPE1* fell into the monophyletic KNAT1/BP-like group, in a subclade of arboreal species (Fig. 1B); it was closest to apple KNAP1 (MdKN1). *KNOPE1* and the other arboreal BP-like proteins shared highly conserved stretches at the N-terminus (e.g. RGNFLYAS, FHLQS, and VKTEA shaded in grey in Fig. 1A) not found in herbaceous KNOX proteins. Interestingly, a conserved VKT (position: 64–66) was maintained in six out of nine members of the BP-like group (LeTKN1, NtH20, PtdKN3, *KNOPE1*, MdKN1.1, and MdKN1.2 in Fig. 1B).

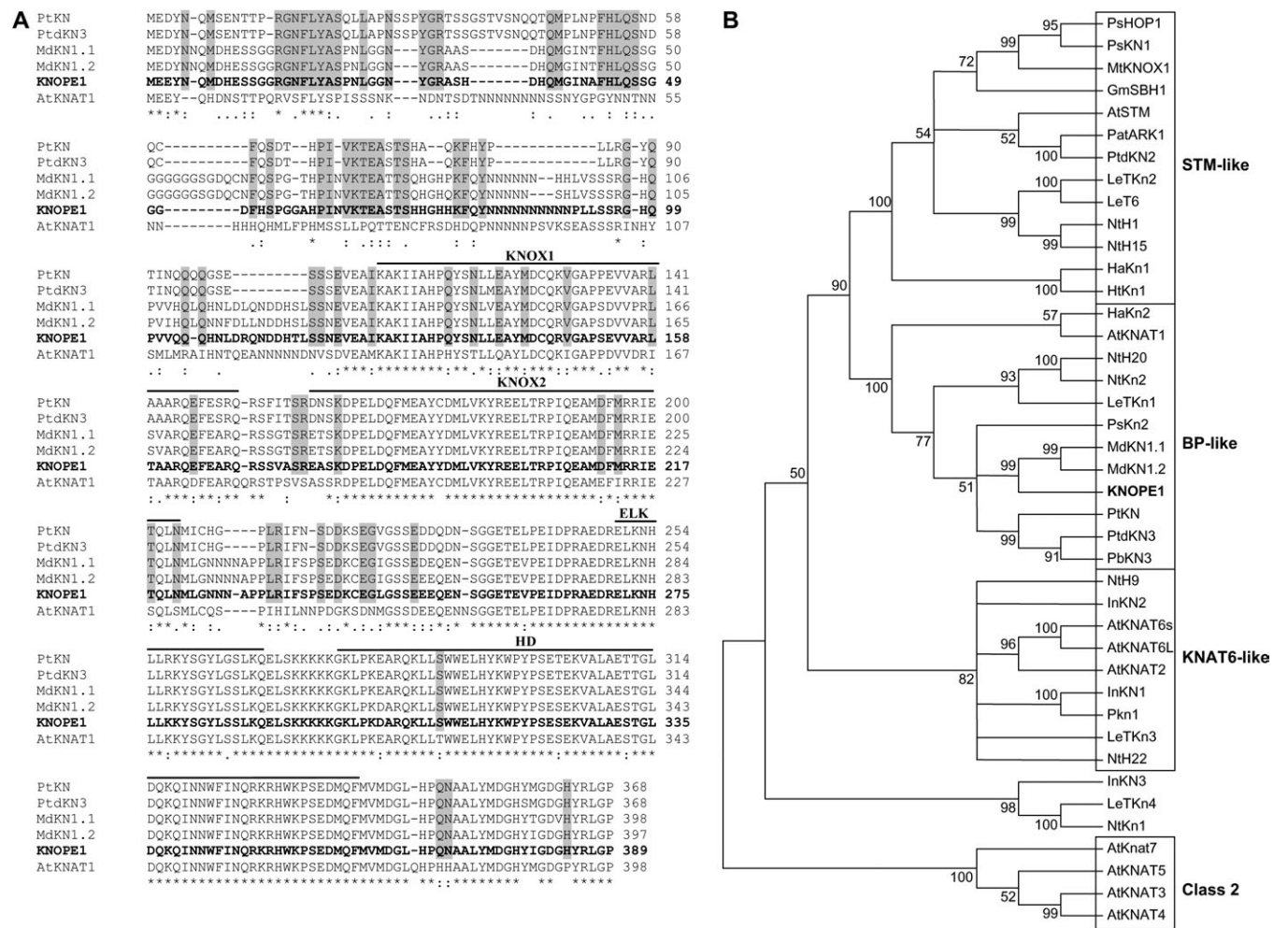


Fig. 1. Features of *KNOPE1* protein. (A) Alignment of the *KNOPE1* deduced amino acid sequence (bold) with *Populus tomentosa* PtKN, *Populus trichocarpa* × *P. deltoides* PtdKN3, *Malus domestica* MdKN1.1 and MdKN1.2, and *Arabidopsis thaliana* KNAT1/BP. Gaps introduced for better alignment are shown by dashes; asterisks indicate strictly conserved residues. Grey shading encompasses amino acids conserved in tree species. The typical KNOX1, KNOX2, ELK domains, and the homeodomain (HD) are indicated. (B) *KNOPE1* (bold) within the phylogenetic tree of class 1 KNOX dicot proteins. Class 2 KNOX proteins were used to create an outgroup. Bootstraps values (at the branching points) are given for major nodes and are based on 1000 replicates. Orthologous members of each subgroup are boxed and are named referring to the respective *Arabidopsis* KNOX.

To estimate the copy number of class 1 members in cultivar 'Chiripa', Southern blot analysis was initially performed with the cDNA probe 1 (Fig. 2A), which spanned the highly conserved KNOX2-HD stretch. The signal pattern (Fig. 2B, left panel) suggested the occurrence of a small gene family. Subsequently, probe 2 based

on the *KNOPE1* genomic sequence, which harboured a cleavage site for *Hind*III (Fig. 2A), was used. A single band was found (Fig. 2B, right panel) when genomic DNA was digested with *Eco*RI and *Eco*RV, whereas two signals were produced when using *Hind*III, and the ~1.8 kb band was consistent with the size predicted by

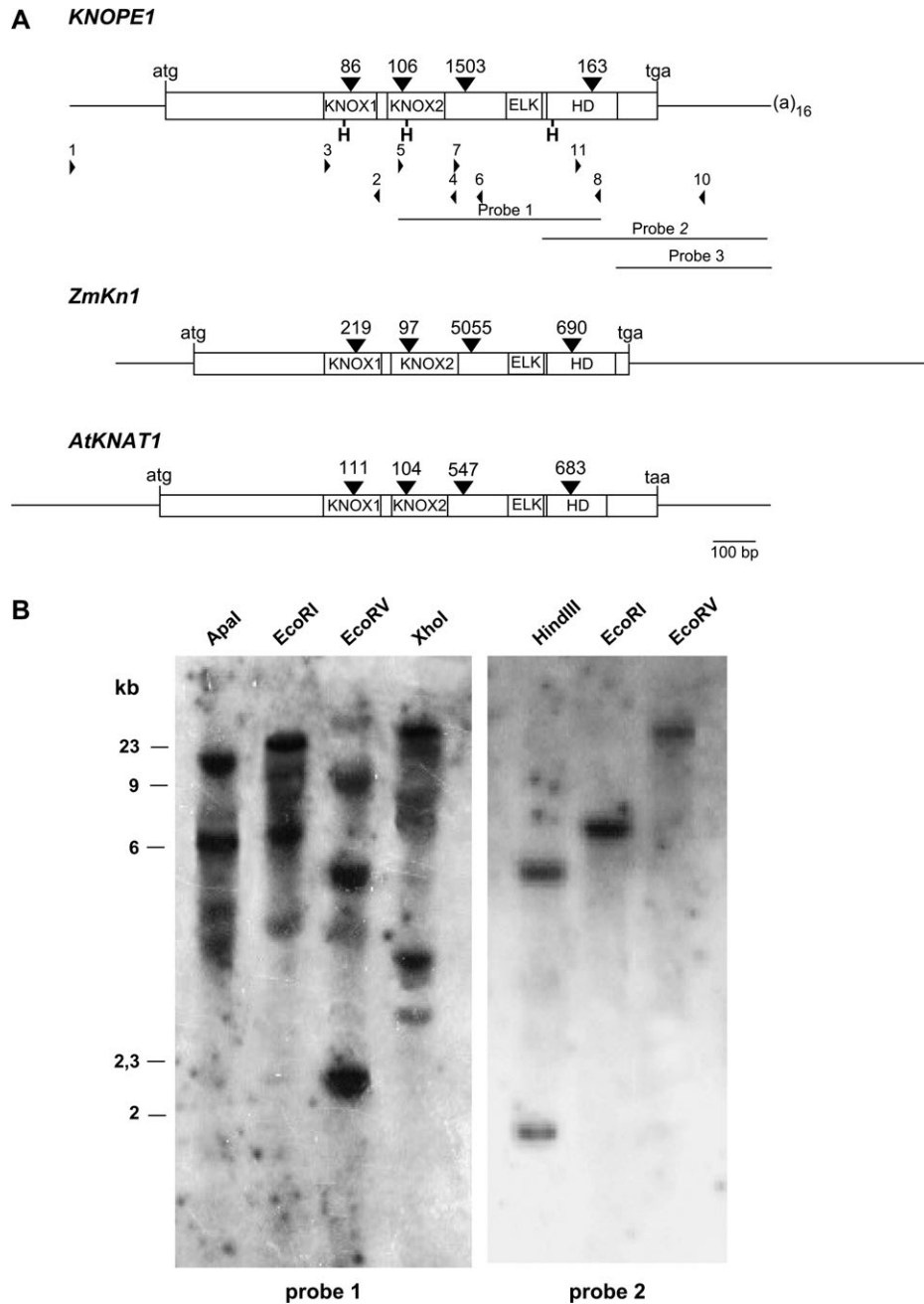


Fig. 2. *KNOPE1* genome organization. (A) Scheme of *KNOPE1*, *Zea mays* (*Zm*) *KN1*, and *Arabidopsis thaliana* (*At*) *KNAT1* genes. The black triangles and numbers indicate intron positions and sizes (in bp). Start and stop codons and the polyadenylation tail are indicated. The cDNA probe 1 spans the KNOX2-HD domains. The genomic probe 2 is *KNOPE1* specific, including intron IV and the 3'-untranslated region. The genomic probe 3 proceeds downstream of the HD and was used for *in situ* experiments. Numbered and oriented arrowheads represent forward and reverse primers used to assemble the gene. H, *Hind*III site. (B) Southern blot analysis. Left panel, *KNOX* gene family: genomic DNA digested with *Apa*I, *Eco*RI, *Eco*RV, and *Xho*I, and hybridized with probe 1. Right panel: genomic probe 2 was used in combination with *Eco*RI, *Eco*RV, and *Hind*III; the latter cuts the probe once. The molecular weights of a co-migrating DNA marker are in kb.

restriction analysis (i.e. 1856 kb). This pattern strongly suggested that *KNOPE1* was a single-copy gene.

KNOPE1 harboured four introns (Fig. 2A), which were flanked by the GT/AG editing motifs and rich in A/T bases. The first two introns lay in the KNOX1 and KNOX2 subdomains, maintaining the same positions as those of *Arabidopsis* *KNAT1/BP* and maize *KN1* (Fig. 2A). The third intron was between KNOX2 and the HD, and the fourth fell within the HD, similar to the intron locations of the above-mentioned *Arabidopsis* and maize proteins. Finally, intron II of *KNOPE1* (accession no. DQ388033) encompassed a stretch (position 907–917) which is conserved in introns II of monocot *KNOX* (Bauer *et al.*, 2004).

KNOPE1 interacts with *BELL1*, *PNF*, and *PNY*, partners of *Arabidopsis* *BP*

To test the hypothesis that *KNOPE1* and *BP* shared biochemical similarities, a yeast two-hybrid assay was carried out (Fig. 3). The interactions were tested using *S. cerevisiae* harbouring the *Arabidopsis* genes *PNF*, *PNY*, *STM*, *BELL1*, and *BP* as prey for the *KNOPE1* bait. Blue signals indicated the binding of *KNOPE1* to *BELL1*, *PNF*, and *PNY*, whereas null staining indicated a lack of interaction of *KNOPE1* with *KNAT1*, *STM*, *KNOPE1* itself, and empty vectors (data not shown). Notably, the *KNOPE1* interaction pattern was essentially the same as that of *BP*. Experiments with swapped prey and bait were performed and the pattern described above did not vary (data not shown).

35S:*KNOPE1* and 35S:*BP* *Arabidopsis* transgenics share common altered traits

The *A. thaliana* ecotype Columbia was transformed with the full-length *KNOPE1* driven by the CaMV 35S promoter. RT-PCR analyses (Fig. 4A) confirmed *KNOPE1* expression in the transgenics. Several independent T₁ lines (an example is shown in Fig. 4C) exhibited alterations in leaf morphology compared with controls (Fig. 4B). The shape of the first two leaves of 35S:*KNOPE1* plants was unaltered, whereas the rosette leaves showed lamina alteration and lobed margins (Fig. 4D) accompanied by sporadic leaflet-like outgrowths (Fig. 4E). There was also pronounced thickening of the central rib and a reduction or loss of dominance of the central vein with secondary ones (Fig. 4F, G). These traits were similar, if not identical, to those observed in the 35S:*BP* *Arabidopsis* (Lincoln *et al.*, 1994).

In peach, *KNOPE1* is down-regulated in leaf primordia flanking the vegetative meristems

Northern analyses and *in situ* hybridization were used to study the strength of spatial expression patterns of *KNOPE1* (Fig. 5). In aerial vegetative organs (Fig. 5A,

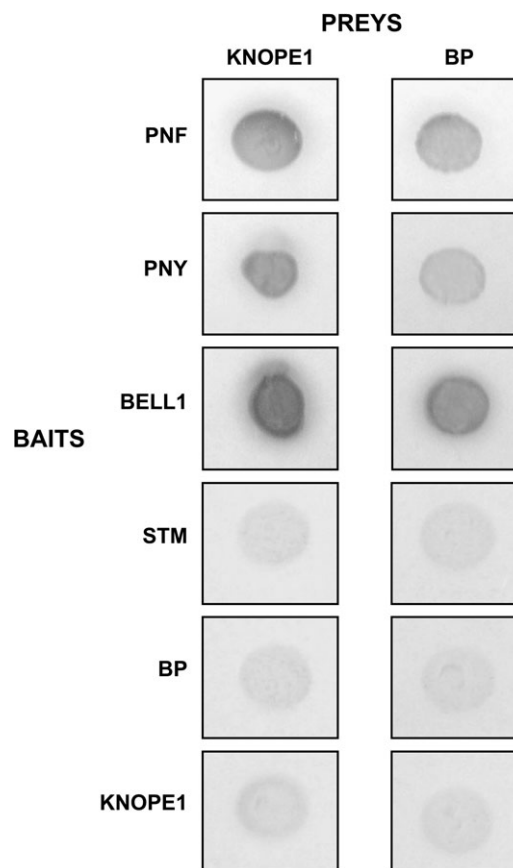


Fig. 3. Yeast two-hybrid assay. Prey proteins *KNOPE1* (left column) and *BREVIPEDICELLUS* (*BP*, right column), and *Arabidopsis* baits *POUNDFOOLISH* (*PNF*), *PENNYWISE* (*PNY*), *BELL1*, *SHOOT MERISTEMLESS* (*STM*), *BP*, and *KNOPE1* itself were expressed in *S. cerevisiae*. The blue staining (here rendered in dark grey) indicates the occurrence of a prey–bait interaction.

left panel, lanes 2–4), the message was most abundant in herbaceous stems of young shoots (May), followed by apical tips (apices and leaflets), and pre-shooting vegetative buds. In leaves (Fig. 5A, central panel), the message occurred in petioles, but was undetected in the laminae. In reproductive organs (Fig. 5A, right panel), the transcript signal was intense in swollen floral buds inclusive of pedicels (February), faint in pistil/ovaries and sepals, and absent in stamens and petals of open flowers (March). Finally, *KNOPE1* expression was also observed in seedling roots (Fig. 5A, left panel, lane 1).

In transverse sections of vegetative SAMs (Fig. 5B), the *KNOPE1* message (purple stain) marked the apical dome (ad), but was absent in developing leaflets (dl). In longitudinal sections (Fig. 5C), it was absent in the tunica, but abundant in the corpus and in the boundaries at the base of leaf primordia. More precisely, the transcript featured in the PZ, but not in the site where the next leaf would form (P₀), in the RZ, and procambial strands (ps). In controls, a sense probe failed to produce a signal (Fig. 5D).

KNOPE1 is triggered and its mRNA differentially localized at distinct stages of curl disease

Northern analyses indicated that *KNOPE1* and *IPT5* were up-regulated in leaves naturally infected by the fungus and exhibiting distortions (Fig. 6A). To monitor *KNOPE1* mRNA localization during the disease, reference was made to (i) the model of Syrop (1975a, b), who defined the *T. deformans* developmental stages (S1–S5) associated with cytohistological modifications of almond leaf, and (ii) histological data from peach leaf curl disease (Bassi

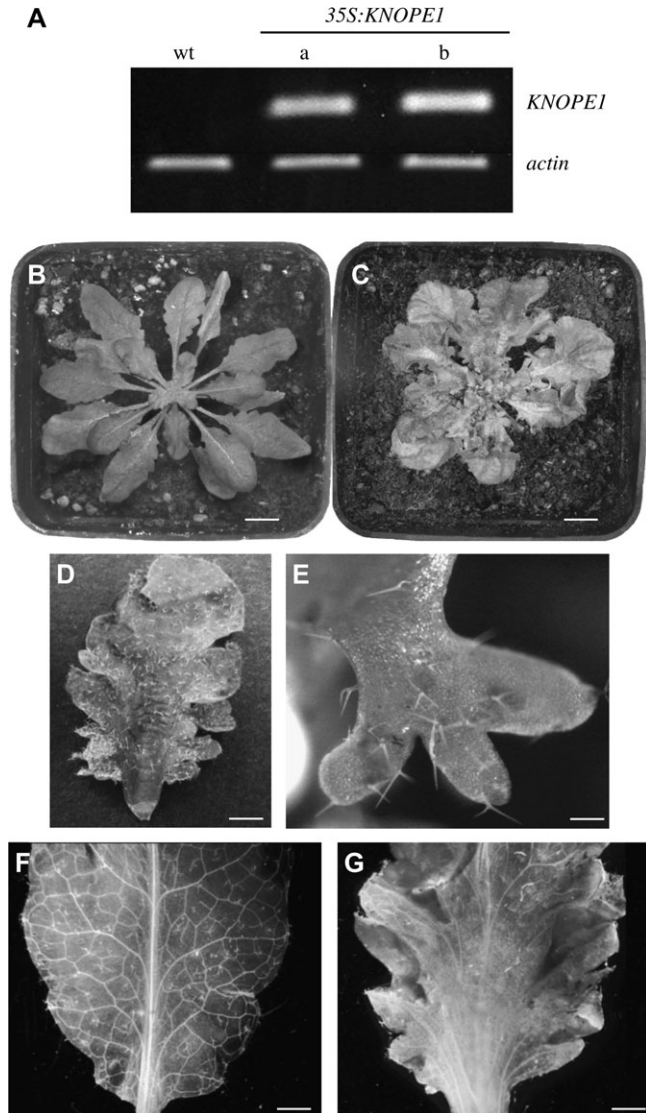


Fig. 4. Phenotypes of *35S:KNOPE1 Arabidopsis*. (A) RT-PCR analysis. a and b, two distinct *35S:KNOPE1* T₁ individuals; wt, wild type; *actin 8* was used as a control for cDNA synthesis and loading. (B) Wild-type Columbia and (C) *35S:KNOPE1 Arabidopsis*. (D) *35S:KNOPE1* leaf exhibiting lamina alteration and lobed margins and (E) leaflet-like outgrowths. (F) Wild-type and (G) *35S:KNOPE1* leaves devoid of chlorophyll highlight vascular modifications. Size bars: 0.4 cm in (B) and (C); 0.2 cm in (D); 0.2 mm in (E); 0.15 cm in (F) and (G).

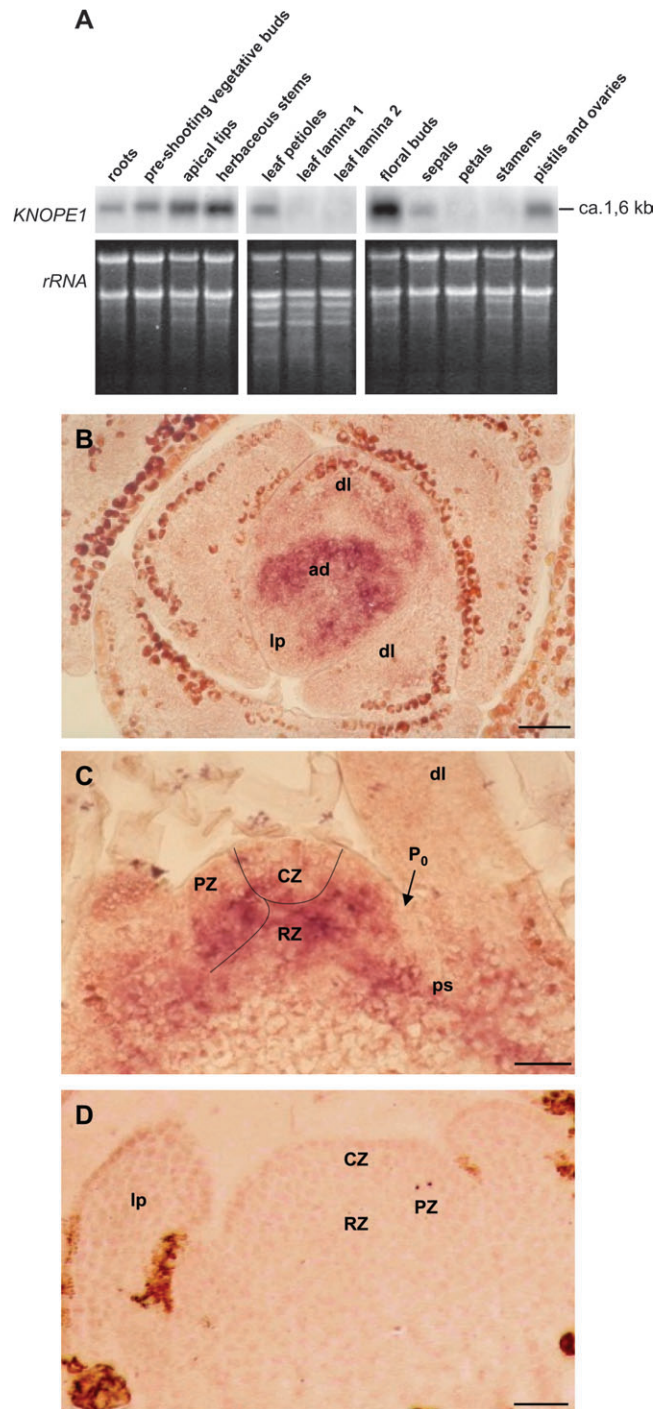


Fig. 5. The *KNOPE1* pattern of expression. (A) Organ-specific expression by northern analysis. Organs examined are indicated above the lanes. Lamina 1 and 2 refer to expanded leaves bearing a major vein of ~8 cm and 12 cm, respectively. The *KNOPE1* transcript size was ~1.6 kb as estimated by comparison with a co-migrating RNA marker. r-RNA, ethidium bromide-stained rRNA was photographed to check for equal loading. (B–D) *In situ* hybridization in vegetative meristems. The antisense probe 3 (Fig. 2A) was used and the purple stain indicates *KNOPE1* mRNA. (B) Transverse and (C) longitudinal sections of the SAM at vegetative resumption. (D) Control reaction with a sense probe. ad, apical dome; dl, developing leaf; lp, leaf primordium CZ, PZ, RZ, central, peripheral, and rib zones; P₀, site where the next leaf primordium will be formed; ps, procambial strands. Size bars: 35 μm in (B), (C), and (D).

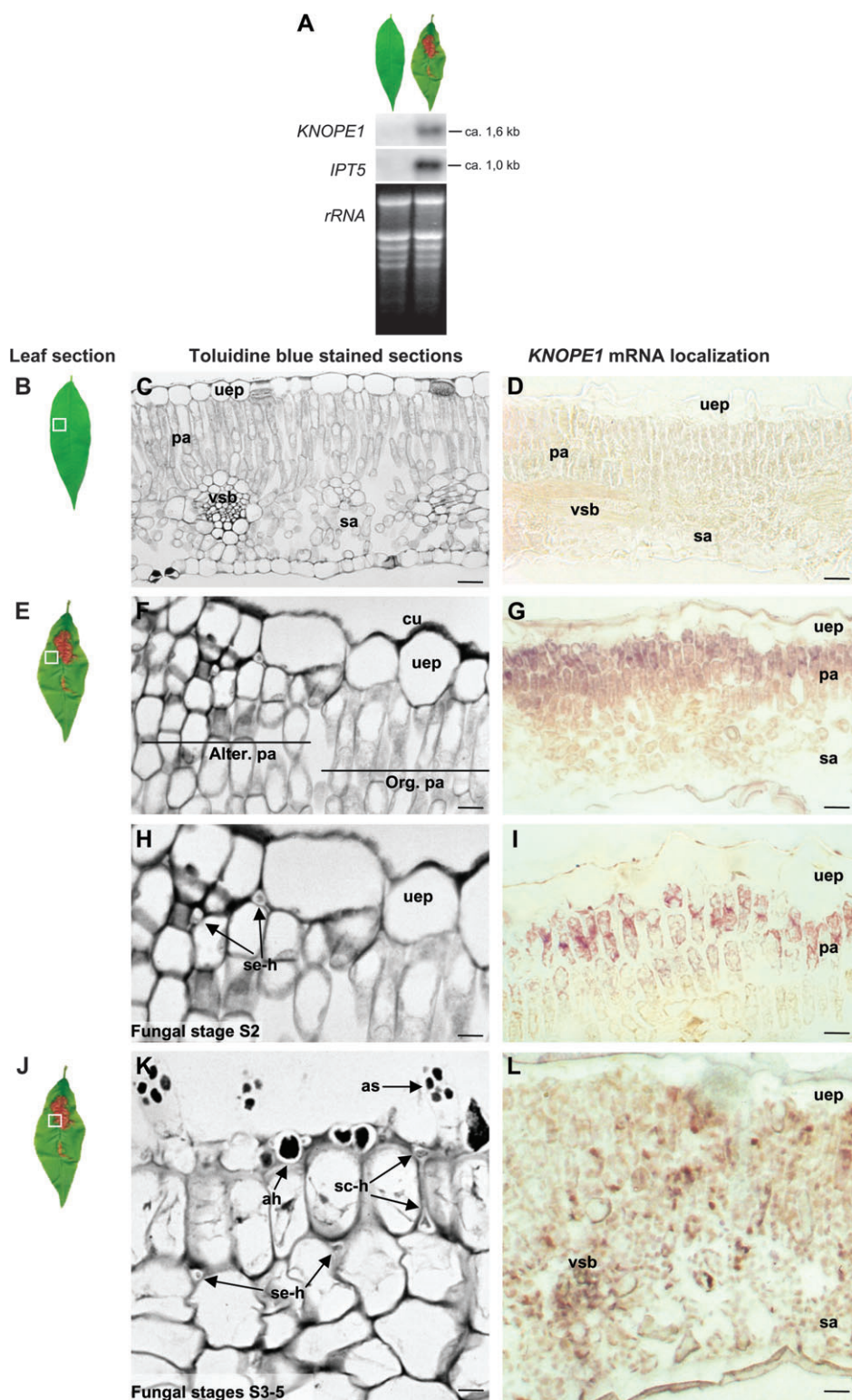


Fig. 6. *KNOPE1* response in leaves affected by curl disease. (A) Healthy (left) and infected (right) leaves are photographed (major vein of ~8 cm). In northern blots, the RNA was derived from a three-leaf pool, and the pictured results are typical of three distinct experiments. The *KNOPE1* and *IPT5* transcript sizes are indicated in kb. r-RNA, ethidium-bromide-stained rRNA. (B), (E), (J) Leaf type and position of sectioned areas (white box) for the *in situ* experiments. Healthy (B) and infected (E, J) leaves. (C), (F), (H), (K) Toluidine blue-stained sections of healthy (C) and infected leaves showing fungal stages S2 (F and the magnification H) and S3–S5 (K). (D), (G), (I), (L) *KNOPE1* mRNA (visible as purple stain) in healthy (D) and infected leaves at S2 (G and the magnification I), and at S3–S5 (L). uep, upper epidermis; pa, palisade layer; vsb, vascular bundle; sa, spongy aerenchyma; cu, cuticle; alter. and org. pa, altered palisade with isodiametric cells and organised palisade with elongated cells, respectively; se-h, subepidermal hyphae; sc-h, subcuticular hyphae; ah, ascogenous hyphae; as, ascospores. Size bars: 15 μ m in (H); 20 μ m in (F) and (K); 25 μ m in (I); 50 μ m in (C), (D), (G), and (L).

et al., 1984). Table 1 shows a synopsis of *T. deformans* developmental stages, and symptoms and histological features of infected leaves, and correlates them to the respective panels in Fig. 6. Tissues used for *in situ* hybridizations were: sectors of healthy leaves (Fig. 6B) at S1 (Fig. 6C), healthy sectors without distortions of infected leaves (Fig. 6E) sited at the border of curly areas, at S2 (Fig. 6F–H), and curly sectors of infected leaves (Fig. 6J) at S3–S5 (Fig. 6K). In the lamina of healthy expanded leaves, the *KNOPE1* transcript was undetected in all cell types (Fig. 6D). At S2, the *KNOPE1* message (Fig. 6G–I) localized uniquely to the cells of the palisade layer (pa), whereas it spread in a scattered manner (Fig. 6L) at S3–S5, including the vascular bundles (vsb) and the spongy parenchyma (sa).

Zeatin accumulation is a host response associated with histological disorders of leaf curl disease

The triggering of *IPT5* (Fig. 6A) and the histological alteration of curly areas suggested the occurrence of changes in CK homeostasis. Hence, zeatin immunolocalization was performed on tissue sectors (Fig. 7A, C, E) consistent with those where *KNOPE1 in situ* hybridization was carried out (Fig. 6D, G, L). Zeatin mainly localized in the vascular bundles (vsb) and in sporadic mesophyll cells of both uninfected leaves (Fig. 7B) and green sectors of affected leaves (Fig. 7D). In contrast, an intense signal spread diffusely in curly hyperplastic sectors (Fig. 7F) and, at high magnifications, the zeatin signal was observed inside the host cells (rc) interacting with subepidermal mycelium (Fig. 7G, H). Notably, the anti-zeatin antibodies cross-hybridized with the subcuticular hyphae (sc-h) and ascogenous cells (not shown), but not with subepidermal hyphae (se-h), suggesting that *T. deformans* was able to produce CK-like compounds at late developmental stages (Johnston and Trione, 1974). As for

controls, tissues were hybridized with a sense probe in the *in situ* experiments, whereas immunoreaction was performed without primary anti-zeatin antibody in immunolocalization experiments. In both cases, signal above background was not detected (see Supplementary Fig. S2 at *JXB* online).

Leaf KNOPE1 transcription is prompted by cytokinin treatment.

To ascertain *KNOPE1* responsiveness to CK, expanded leaves were immersed in 10 μ M BAP and transcription was monitored in a time lapse of 0.5, 2, and 4 h (Fig. 8A). A mock PCR and water control reactions were performed and no signal occurred (not shown). A very faint signal of *KNOPE1* mRNA was observed in non-immersed control leaves (Fig. 8A, lane 0 h), very probably due to gene activity in petioles (see Fig. 5A), which were included in the sampling of the experiments. In leaves soaked in BAP-free solution, *KNOPE1* expression increased after 0.5 h, but fell below the level of non-immersed leaves within 4 h (Fig. 8B, white histograms), suggesting that a response to stress effects (e.g. anoxia) may have occurred. The message abundance of BAP-treated leaves was higher than that of non-immersed leaves, and the transcriptional increment was maintained from 0.5 h onwards (Fig. 8B, grey histograms). Consequently, *KNOPE1* mRNA was significantly more abundant in leaves treated with BAP than in those soaked in BAP-free solution during the whole time lapse, suggesting a specific response to the CK treatment.

Discussion

The peach *KNOPE1* full-length cDNA was isolated, and its deduced protein belonged to class 1 KNOX, falling in a clade of the BP-like group. The gene is likely to be

Table 1. Synopsis of *Taphrina deformans* developmental stages, and symptoms and histological features of infected leaves

Stage name	<i>Taphrina deformans</i> ontogeny ^a	Fig. 6 panels	Leaf visible traits	Leaf histological features	Fig. 6 panels
S1	Absent	C, D	None (healthy)	Normal	C, D
S2	Hyphae—subepidermal	F, H	Red sectors (infected)	Bundle sheath cells rich in red pigments Start of subepidermal cell division Palisade and spongy cells start to be indistinguishable	F, H F, H
S3	Hyphae subepidermal subcuticular	K L	Red sectors Curliness Thickening	Cells rich in red pigments Cell division in upper layers Aerenchyma still visible	K L
S4	Ascogenous layer	K	Red sectors Severe curliness Thickening	Cells rich in red pigments Cell division and enlargement	K
S5	Mature asci Ascospores	K K	Tissue distortions	Palisade and spongy mesophyll layers are indistinguishable	K, L

^a Adapted from Syrop (1975a) and Bassi et al. (1984).

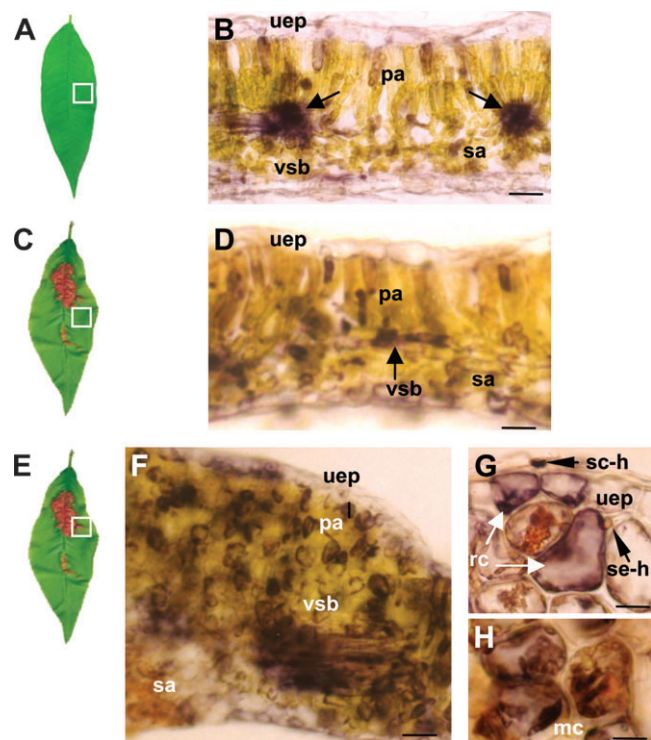


Fig. 7. Zeatin immunolocalization in leaves affected by curl disease. (A), (C), (E) Leaf type and position of sectioned areas (white box) for the zeatin immunolocalization experiments. Healthy (A) and infected (C, E) leaves. (B), (D), (F), (G), and (H) Zeatin (arrowed deep purple stain) in healthy (B) and affected leaves at S2 (D) and S3–S5 (F–H). (G) and (H) Magnifications of leaves at S3–S5 show zeatin accumulation in the epidermal and subepidermal (G) and mesophyll (H) host cells. uep, upper epidermis; pa, palisade layer; vsb, vascular bundle; sa, spongy aerenchima; se-h, subepidermal hyphae; sc-h, subcuticular hyphae; rc, reactive epidermal and subepidermal cells; mc, mesophyll cells. Size bars: 100 μm in (F); 75 μm in (B) and (D); 15 μm in (G) and (H).

a single copy within a family of four or five class 1 members in the ‘Chiripa’ genome. Just like the maize and *Arabidopsis* orthologues, it is organized into five exons and four introns, and did not harbour any intron inside the ELK, another typical feature of class 1 *KNOX* (Reiser *et al.*, 2000). Intron II included a stretch found in introns II of maize and rice class 1 genes (Bauer *et al.*, 2004). This motif might have been selected for a common function throughout monocot and dicot *KNOX*.

Protein–protein interaction assays indicated that *KNOPE1* bound to a set of *Arabidopsis* BELL1-like proteins, which also heterodimerized with BP (Smith *et al.*, 2003). *KNOPE1* overexpression in *Arabidopsis* caused mutant phenotypes, which were highly similar to *35S:BP Arabidopsis* lines. *KNOPE1* transcript localization in peach SAM strongly resembled that of BP in *Arabidopsis* (Lincoln *et al.*, 1994). *KNOPE1* mRNA was undetected in SAM P₀ cells, and leaf primordia (Fig. 5C) and lamina of expanded leaves (Figs 5A, 6D), consistent with the idea that class 1 *KNOX* down-regulation is a key step in leaf initiation and organogenesis and that the

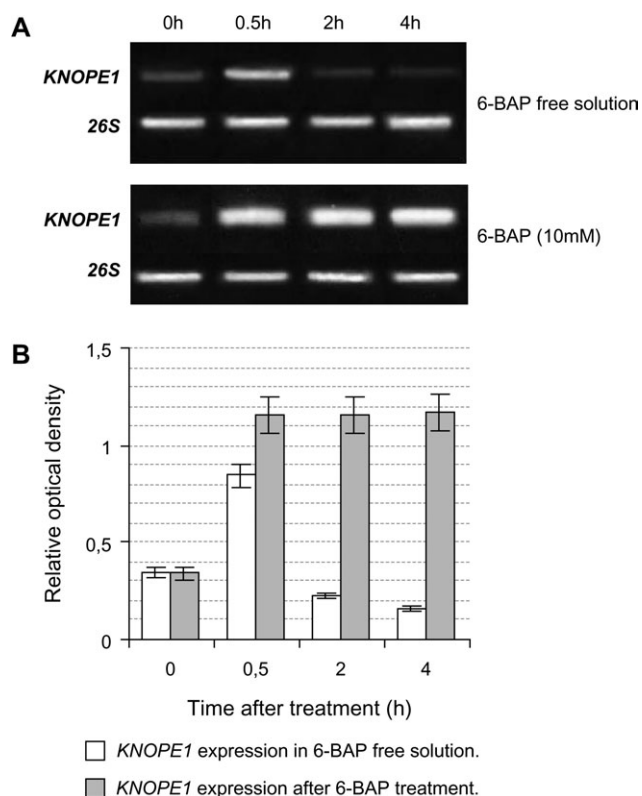


Fig. 8. *KNOPE1* response in cytokinin-treated leaves. (A) RT-PCR for *KNOPE1* in leaves immersed in a 6-benzyl aminopurine (6-BAP) solution and sampled after 0.5, 2, and 4 h. Non-immersed leaves (0 h) and leaves soaked in 6-BAP free solution were used as controls. The 26S was used as control for the amplification. (B) Relative optical density to estimate *KNOPE1* message abundance with respect to 26S. Analyses were performed three times with independent RNA extracts (each deriving from two leaf pools). Bars indicate standard errors and the gel bands represent one of the experiments.

exclusion of *KNOX* proteins from leaves results in a simple leaf shape (Hay and Tsiantis, 2006).

Organs containing tissues with meristematic activity show *KNOPE1* expression (Fig. 5A) and most of them (including roots, see Truernit *et al.*, 2006) coincide with those of *Arabidopsis* in which BP is active (Lincoln *et al.*, 1994; Venglat *et al.*, 2002). Intriguingly, *KNOPE1* was detected in pedicels of expanded leaves (Fig. 5A). BP signal is absent in the petioles of *Arabidopsis* leaves (Hay and Tsiantis, 2006), though found at the petiole base in other works (Ha *et al.*, 2003), and constantly present as a ring encircling the stem leaf traces (Douglas and Riggs, 2005). Petioles of peach leaves harbour a wide vascular bundle, characterized by an active cambium, and undergo lignification. In our laboratories, *KNOPE1* transcript was detected in both the intrafascicular cambium and phloem-associated cells of growing petioles (unpublished results), suggesting that *KNOPE1* may participate in maintaining the meristem identity of cambium cells.

Interestingly, *KNOPE1* expression was triggered in expanded leaves attacked by *T. deformans*, and the

message occurred specifically in the palisade cells invaded by the fungus at early stages of development (S2; Syrop, 1975a). At S2, host cell de-differentiation and division are not observed, though several modifications rapidly occur at the subcellular level (Syrop, 1975b). The *KNOPE1* message localization to the palisade—but not to the epidermal cells—suggests that a programme of indeterminate cell fate is established in specific cell types. At later fungal stages (S3–S5), numerous dividing isodiametric cells (hypertrophy) replace the leaf palisade layer, and few other cells undergo hyperplasia, both leading to tissue distortions (Bassi *et al.*, 1984). The chaotic misexpression of *KNOPE1* may be responsible for the maintenance of cell indefinite identity, thus preventing further differentiation. In this context, *KNOPE1* behaviour is consistent with that of class 1 *KNOX* when these are overexpressed in leaves (Scofield and Murray, 2006).

KNOX up-regulation can induce CK synthesis *de novo* by triggering plant *IPT* genes (Jasinski *et al.*, 2005; Sakamoto *et al.*, 2006), which act to increase zeatin production (Sakakibara, 2006). Hence, it is salient that the peach *IPT5* was up-regulated in infected leaves. The present experiments showed a higher number of zeatin-immunoreactive mesophyll cells in curly sectors than those from both uncurled areas of attacked leaves and healthy leaves. Interestingly, zeatin was localized inside the host cells. Such an event, together with the *IPT* triggering, indicated that zeatin accumulation was a plant response. The role of CKs in comparable plant–biotroph interactions is complex. A host CK increase may promote (Walters and Mc Roberts, 2006) nutrient sink and senescence delay, but favour suppression of rapid host cell death (hypersensitive response). However, a high CK concentration is also reported to induce cell necrosis (Carimi *et al.*, 2003). A mutual interaction underlies class 1 *KNOX* genes and CK (Rupp *et al.*, 1999; Sakamoto *et al.*, 2006), and *KNOPE1* was triggered in leaves treated with BAP, probably due to a specific response from petiole cambium cells. Consequently, at later stages of fungus development, *KNOPE1* and zeatin may share reciprocal (up) regulations to induce and maintain meristematic identity, thus preventing cell differentiation.

Taphrina deformans is an obligate biotrophic fungus, and *in vitro*-based infections have been unsuccessful (see Supplementary Fig. S3 at *JXB* online). Although the occurrence of other pathogens (bacteria, viruses, and mycoplasmas) could not be absolutely excluded, histological analyses allowed monitoring and the fungus movement/stages could be strictly related to the host responses. On this basis, it is proposed that *KNOPE1* expression in the palisade may belong to an early response process occurring when leaf cells interact with subepidermal hyphae. *KNOPE1* activation may mark the start of indefinite cell fate programmes and cause the *IPT* up-regulation that leads to zeatin overproduction (Jasinski

et al., 2005). After the onset of hyperplasia, *KNOPE1* and zeatin may establish a mutual interaction that maintains the abnormal leaf development. This model requires further experiments, dissecting the overlapping pathways in which *KNOPE1* is involved during the disease course.

Supplementary data

Figure S1. Features of *KNOPE1* cDNA.

Figure S2. Control experiments of Fig. 6.

Figure S3. Information on *Taphrina deformans* infection.

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