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ORIGINAL PAPER

The peach (*Prunus persica* [L.] Batsch) homeobox gene *KNOPE3*, which encodes a class 2 knotted-like transcription factor, is regulated during leaf development and triggered by sugars

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Abstract Class 1 KNOTTED1-like transcription factors (KNOX) are known to regulate plant development, whereas information on class 2 KNOX has been limited. The peach *KNOPE3* gene was cloned, belonged to a family of few class 2 members and was located at 66 cM in the *Prunus* spp. G1 linkage-group. The mRNA localization was diversified in leaf, stem, flower and drupe, but recurred in all organ sieves, suggesting a role in sap nutrient transport. During leaf development, the mRNA earliest localized to primordia sieves and subsequently to mesophyll cells of growing leaves. Consistently, its abundance augmented with leaf expansion. The transcription was monitored in leaves responding to darkening, supply and transport block of sugars. It peaked at 4 h after darkness and dropped under prolonged obscurity, showing a similar kinetic to that of sucrose content variation. Feeding leaflets via the transpiration stream caused *KNOPE3* up-regulation at 3 h after fructose, glucose and sucrose absorption and at 12 h after sorbitol. In girdling experiments, leaf *KNOPE3* was triggered from 6 h onwards along with sucrose and sorbitol raise. Both the phloem-associated expression and sugar-specific gene modulation suggest that *KNOPE3* may play a role in sugar translocation during the development of agro-relevant organs such as drupe.

Keywords *KNOPE3* · Transcription factor · Peach · Gene regulation by light and sugars

Introduction

Plant *knotted*-like genes (*KNOX*) encode homeodomain (HD) containing transcription factors (TFs) and are pivotal in regulating organ development (reviewed by Hake et al. 2004). They belong, together with BEL-like proteins, to the three amino acid loop extension protein cluster (Burglin 1997). *KNOX* were split into two classes on the basis of identity grade within the HD, intron position and expression patterns (Reiser et al. 2000). The third class, named M, was set after discovering *KNOX* lacking the HD (Kimura et al. 2008; Magnani and Hake 2008).

Class 1 *KNOX* are required for proper development of the shoot apical meristem (SAM) that act to maintain cells in an indeterminate state and are expressed in meristem cells (reviewed by Scofield and Murray 2006). Their functions have been assessed in *A. thaliana* during the diverse processes of vegetative and reproductive development (Hake et al. 2004; Scofield and Murray 2006; Truernit et al. 2006). The class 1 *KNOX* functions are mediated by interactions with cytokinins, auxin and gibberellins

G. Testone and E. Condello have been equally contributed to the work.

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(reviewed by Hay et al. 2004), specific and combinatorial dimerization with BELL (synopsis by Hackbusch et al. 2005), mechanisms of gene hierarchy (Li et al. 2005; Guo et al. 2008).

Class 2 *KNOX* share HD identity ≤55% compared to that of class 1, are ubiquitously expressed, and typically contain an intron in the ELK domain vicinity (Reiser et al. 2000). Referring to dicot species, they have been characterised in *Arabidopsis* (http://arabidopsis.org/), tomato (Janssen et al. 1998), tobacco (Sentoku et al. 1998), barrel medic (Di Giacomo et al. 2008), pea (Hofer et al. 2001) and apple (Watillon et al. 1997). Organ wide-spread expression was reported for all these plants, though tissue-specific transcriptional preferences and alternative splicing events occur in monocots (Kerstetter et al. 1994; Morere-Le Paven et al. 2007; Tamaoki et al. 1995). The *Arabidopsis KNAT3* has been the most deeply investigated, for which expression patterns were profiled in aerial organs (Serikawa et al. 1997) and roots, together with those of class 2 *KNAT4* and *5* (Truernit et al. 2006). Phytohormones, such as cytokinin (Soucek et al. 2007; Di Giacomo et al. 2008; Truernit et al. 2006), abscissic acid and gibberellins (Morere-Le Paven et al. 2007) affect class 2 *KNOX* transcription in distinct organs. Light-mediated regulation of *KNAT3* expression was observed in seedling as its promoter differentially responded to white, red and far-red spectra (Serikawa et al. 1996; Serikawa et al. 1997), whilst *Knox7* was light-triggered in maize embryos (Morere-Le Paven et al. 2007). Class 2 proteins form heterodimers with KNOX and BEL members, for instance: KNAT3 binds to KNAT4 and nine BEL factors, whilst KNAT4 only binds to class 1 BP and BEL6 (Hackbusch et al. 2005). The sub-cellular localization of KNAT3 and 4 is regulated at the post-translational level (Truernit et al. 2006). The tobacco class 2 *NTH201* was shown to localize at plasmodesmata (Yoshii et al. 2008) and is unable to self-traffic between cells, resembling the behaviour of *KNAT3* (Kim et al. 2005).

The diversified expression patterns in many tissues at different times have made it difficult to propose single functions for class 2 *KNOX*. Moreover, role specificity is a result of spatio-temporal regulation of KNOX dimerization with other proteins. The functional redundancy amongst class 2 genes was evoked to explain the absence of phenotypes in silenced mutants (Serikawa et al. 1997); however, plastochron delay, leaf lamina shrink and petiole elongation were described in *Arabidospsis knat3* null mutants (Chandler and Wolfgang 2004). The *KNAT3* knock-out was found to reverse the *eostre* mutant and proposed to underlie both mega-gametogenesis and embryo sac development (Pagnussat et al. 2007), whilst *KNAT7* was alleged to participate in secondary cell wall synthesis (Zhong et al. 2007). The disruption of a *Physcomitrella* class 2 member caused spore aberrancy (Singer and Ashton 2007), whilst that of tobacco *NTH201* hampered both the accumulation of movement protein and the formation of replication complexes of the tobacco mosaic virus (Yoshii et al. 2008).

KNOX are proposed to play crucial roles in the control of tree agronomic traits (Groover et al. 2006; Testone et al. 2008), and to date the knowledge on class 2 members has been quite modest. Consequently, the peach *KNOPE3* gene (phylogenetic ortholog of *KNAT3*) was cloned and mapped genetically on the *Prunus* reference map using cleavage amplified polymorphic sequence (CAPS) markers. Distinct mRNA localization patterns were observed in stem, flower buds, drupes and leaves, though the expression in sieves was common to all the organs, suggesting that *KNOPE3* may participate in transport mechanisms of elaborated sap compounds. Generally, sugars are nutritive and signalling molecules able to control gene expression and plant development (Rolland et al. 2006); source-to-sink translocation of carbohydrates is essential for peach bud break and drupe growth (Maurel et al. 2004; Morandi et al. 2007). Consequently, the *KNOPE3* expression was monitored in experiments causing sugar content variation in leaves. The gene was rapidly induced in sink leaves directly up-taking fructose, glucose and sucrose; it was also triggered in source leaves concomitantly with sucrose and sorbitol increase caused by transport block after stem girdling. Based on these results, a possible role of *KNOPE3* in sugar translocation is proposed and discussed.

Materials and methods

Plant materials and growth conditions

Adult peach plants (*Prunus persica* cultivar 'Chiripa') grown in the IBBA-CNR fields belonged to two clonal lines (S_1 and S_2) obtained by micro-propagation of two seedlings derived from the open pollinated mother plant16 ('Chiripa'). The clone 18 of the S_1 line (named F_0S_{1-18}) was self-pollinated under controlled conditions and seeds were grown to generate F_1S_{1-18} individuals (Testone et al. 2008), which provided most of the materials at distinct developmental stages for this work. Plants were grown in the greenhouse at 22–25°C, 16/8 h of light/dark with a light intensity of 100 μmol m^{-2} s^{-1} of photosynthetically active radiation (PAR). Several clones of *KNOPE3* cDNA and genomic fragments were sequenced and we did not score any nucleotide differences amongst F_1S_{1-18} sister plants, suggesting that *KNOPE3* was tightly conserved (at least in the transcribed regions) and gene regulation mechanisms may be conserved.

Leaf developmental stages

One-year-old F_1S_{1-18} plants were used during (a) vegetative resumption (March) for in situ hybridizations (see following paragraph) and (b) early shoot elongation (April) for gene expression analyses. In April, the shoots prolonging the main axes (trunks) were 28.3 ± 2.2 cm long and bore 8.8 ± 1.2 leaves. To mark leaf growth stages (Table 1), the leaf position on stem (from the apex to the basis), the expansion status and area of laminas and the chlorophyll content were determined. The apical tips (AT) included 1–4 leaflets; the leaves below (mainly the 5th leaf) were folded (F) or fully expanded (E). The same leaf types ($n = 3$) from distinct plants ($n = 3$) were pooled for RNA isolation and RT-PCR experiments, which were repeated for three distinct sampling. Lamina surfaces and chlorophyll content were measured, respectively, by ImageJ software (http://rsb.info.nih.gov/ij) and as described (Porra et al. 1989).

Isolation and sequence analysis of cDNA and genomic clones

The cDNA was synthesized from leaf RNA (see sub-paragraph of RT-PCR) of F_1S_{1-18} plants and PCR-amplified by primers Kn3.1Fw (5′-CCATGGAAGCAGTGATGGC-3′) and Kn3.2Bw (5′-CTTCCTCAGTTGGATATGGCC-3′). These were designed based on the class 2 KNOX conserved amino acidic stretches AMEAVMA (in the KNOX2) and WPYPTEED (in the HD), respectively (see Fig. 1). The 390 base pairs (bp) fragment was cloned, sequenced and confirmed to share high identity with class 2 *KNOX*. The *KNOPE3* full length cDNA was achieved into steps: (a) the 3′ region (PCR product of 1101 bp) was cloned by 3′RACE methodology (INVITROGEN), using the anchor primers provided by the manufacturer and the Kn3.1Fw primer; (b) the 5′ region was isolated using primers retrieved from *KNAP3* of *M. domestica* (acc. n. Z71980), they were Kn3.3Fw (5′-GTATTCATGGCG-TACCACAACC-3′) and Kn3.4Bw (5′-TAGCACCCGTGC CTTCACCGG-3′). PCR amplification produced a single fragment of 880 bp.

Introns were found by amplifying leaf genomic DNA (gDNA) with the following primer couples: Kn3.3Fw/Kn3.10Bw (5′-TTGTGCCTTGCGTTCTGCC-3′); Kn3.5Fw (5′-GCCCAGCTGGCTCAGTCGC-3′)/Kn3.2Bw and Kn3.1Fw/Kn3.6Bw (5′-GCAGCCTTTGAGCATACCTG-3′). The sequences of products from cDNA and gDNA were aligned by ClustalW (http://www.ebi.ac.uk.clustalw) and intron size and locations established.

The final PCR conditions were: 500 ng of gDNA or 200 ng of cDNA, 1 mM of each primer, 0.5 mM dNTPs, *Taq* DNA polymerase (TaqQUIA, Qiagen) 2.5 U, 1/10 of 10× *Taq* Buffer (Qiagen), 2.5 mM $MgCl_2$, in a final volume of 50 µl. Thermocycle conditions included an initial cycle of 95°C for 5 min followed by 35 cycles of 95°C for 40 s, either 55°C (in cDNA based and RACE-PCR experiments) or 60°C (with gDNA) for 30–60 s and 72°C for 30–90 s, final extension at 72°C for 5 min. All PCR fragments were cloned into pGEM-T easy vector system (PROMEGA). The GenBank accession numbers of *KNOPE3* cDNA and genomic sequences are DQ786755 and EU910092.

Alignments and phylogenetic analysis

The alignment of KNOPE3 with other class 2 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 1,000 replicates and the 50% value was accepted as an indicative of a well-supported branch. The class 2 KNOX accession numbers were: PpKNOPE3 DQ786755; MtKNOX3 ABO33480.1; MtKNOX4 ABO33481.1; MtKNOX5 ABO33482.1; MdKNAP3 Z71980; NtH23 BAA25921; NtH201 AB333840; Osh45 BAA08552; LeT12 AAC49918; AtKNAT3 X92392; AtKNAT4 NP196667; AtKNAT5 NP194932; AtKNAT7 AF308451; VvCAO67726; VvCAO63645; VvCAO44300; ZmNP_001105852; PtABK92551; OsBAB55660; NtBAF95776. A few class 1

Table 1 Evolution of lamina area, chlorophyll ($a + b$) content, and relative expression of *RUBCA* and *KNOPE3* genes during leaf development of 1-year-old peach plants after vegetative resumption (April)

Leaf position	Status	Area (cm^2)	Chlorophyll ($a + b$) (µg cm^{-2})	*RUBCA* REL	*KNOPE3* REL
AT	F	1.29 ± 0.85	3.88 ± 2.55	1.00 ± 0.04	1.00 ± 0.08
5	F	7.26 ± 1.76	21.79 ± 5.31	6.54 ± 1.31	5.41 ± 0.13
6	E	11.99 ± 1.98	35.98 ± 5.95	56.69 ± 5.34	15.57 ± 3.03
7	E	17.78 ± 3.42	53.36 ± 10.28	52.71 ± 6.33	17.58 ± 2.23
8	E	19.86 ± 2.25	59.61 ± 6.76	68.59 ± 7.12	19.38 ± 1.22

AT apical tips consisted of a pool from the 1st to 4th leaf, *F* folded lamina, *E* expanded lamina, *REL* relative expression levels, corresponding standard deviations are indicated

Fig. 1 Features of KNOPE3 protein. **a** Alignment of KNOPE3 deduced amino acid sequence (*bolded*) with *Malus domestica* MdKN3 and *Vitis vinifera* KNAT3-like gene (VvCA067726). *Gaps* introduced for better alignment are shown by *dashes*, *asterisks* indicate strictly maintained residues. The typical KNOX1, KNOX2, ELK and the HD are indicated. **b** KNOPE3 (*bolded*) within the phylogenetic tree of class 2 KNOX proteins. Class 1 proteins were used to create an out-group. Bootstraps values (at the branching points) are given for major nodes and based on 1,000 replicates. Orthologous members of each subgroup are bordered and named referring to the respective *Arabidopsis* class 2 KNOX

A

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KNOPE3       MAYHNHLSQQDLPLHHFTDQTQQQHQPFQS-DQHDPNSKPTEPHHPFQPAPNWLNTALLR 59
MdKNAP3      MAYHNHLS-QDLPLHHFTDQTHHQHQQYQS-DQPDPNSKPPEPHHSFQPAPNWLNSALLR 58
VvCA067726   MAFHNQLS-HDMALQHFTDSHLTENTAVLRGILPEQLGQSSSDGAGKPPSHQQLGGGGGG 59
             **:**:** :*:.*:****.    ::         :  .:...      *: : *. .

KNOPE3       NYTNADANNNHNNSPNNNGGGGASNFLNLHVTASDSTTSQASNQWLSQPHRPILHRNHSD 119
MdKNAP3      NFTNTDTNPTNSNNANNNGGG-VSNFLNLHVTASDSAASQASNQWLSQSHRPILHRNHSD 117
VvCA067726   GGPTWLNNAILRQQSQYADGSFLHLQTNSDSTSSPATATTTNQWLSRSMS-----NVGA 114
             . ..     *    :..:  .*.        * . *:* :::: ::*****:.      * .

KNOPE3       VIDDVTAIAGDSMIARTISHDSADLKPDSSLNKTDIVVESGGGGGDGGMINWQNARHKAE 179
MdKNAP3      VNDDVT-VAGDSMIAAALSHDSADLKPDSILNK------NEGGGDGGVMNWQNARHKAE 170
VvCA067726   QNDDVPVSSGSVIAAISADLNGNQEKRNGGNNQNRGDNNGEDMLDCDSGGNWENARYKAD 174
               ***.  :*. : * : . :. : * :.  *:          . .  . ..   **:***:**:

                          KNOX1                                   KNOX2
KNOPE3       ILAHPLYEPLLSAHVACLRIATPVDQLPRIDAQLAQSQNVVAKYSALGHGMVGDDKELDQ 239
MdKNAP3      ILAHPLYEPLLSAHVACLRIATPVDQLPRIDAQLAQSQNVVAKYSALGNGMVGDDKELDQ 230
VvCA067726   ILAHPLYEQLLSAHVSCLRIATPVDQLPRIDAQLAQSQGVVTKYSVLAN-QPLDDKELDQ 233
             ******** ******:**********************.**:***.*.:      *******

                           KNOX2
KNOPE3       FMRHYVLLLCSFKEQLQQHVRVHAMKAVMACWEIEQSLQSLTGVSPGEGTGATMSDDEDD 299
MdKNAP3      FMRNYVLLLCSFKEQLQQHVRVHAMKAVMACWEIEQSLQSLTGVSPGEGTSATMSDDEDD 290
VvCA067726   FMTHYVLLLCSFKEQLQQHVRVHAMKAVMACWELEQSLQSLTGVSPGEGTGATMSDDEDD 293
             ** :******************************:****************.*********

                                                             ELK
KNOPE3       QVDSDANLFDGSMEGHDSMGFGPLIPTESERSLMERVRQELKHELKQGYKEKIVDIREEI 359
MdKNAP3      QVDSDANLFDEGMEGHDSMGFGPLIPTESERSLMERVRQELKHELKQGYKEKIVDIREEI 350
VvCA067726   QADSEINLFDGSLDGPDSMGFGPLVPTETERSLMERVRQELKHELKQGYKEKIVDIREEI 353
             *.**: **** .::* ********:***:******************************

                                         HD
KNOPE3       LRKRRAGKLPGDTTSVLKAWWQSHSKWPYPTEEDKARLVQETGLQLKQINNWFINQRKRN 419
MdKNAP3      LRKRRAGKLPGDTTSVLKAWWQSHSKWPYPTEEDKARLVQETGLQLKQINNWFINQRKRN 410
VvCA067726   LRKRRAGKLPGDTTSLLKAWWQSHSKWPYPTEEDKARLVQETGLHLKQINNWFINQRKRN 413
             ***************:****************************:***************

KNOPE3       WHSNPSTSTVLKSKRKRSNAGENSSDRFG 448
MdKNAP3      WHSNPSTSTVLKSKRKR------------ 427
VvCA067726   WHSNPSSSAVLKTKRKR------------ 430
             ******:*:***:****
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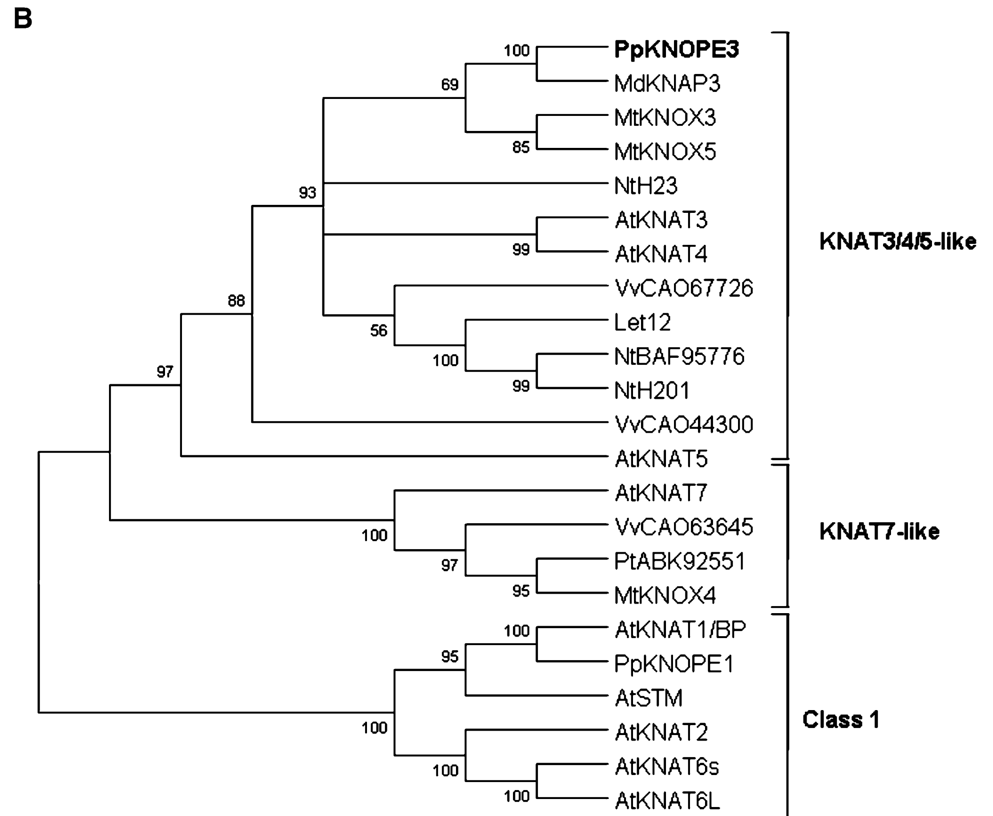
B



KNOX were used to create an out-group; acc. n.: AtKNAT1, U14174; AtSTM, U32344; AtKNAT2, U14175; AtKNAT6L, AB072362; AtKNAT6S, AB072361; PpKN-OPE1 ABD52723.

Southern blot analysis

Extraction of gDNA and Southern blots were performed as previously described (Giannino et al. 2000). In brief, leaf gDNA (30 µg) was digested, fragments were size-fractionated on 0.8% agarose gel and transferred onto nylon membrane (Hybond N+, Amersham) according to Kingston et al. (2001). The probe (25 ng) was radio-labelled with to 5 µl of ^{32}dCTP in 45 µl of TE buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA) and denatured in boiling water according to Rediprime^{TM} II (Amersham) kit instructions. Filters were hybridized at 55°C (cDNA probe 1) and 60°C (genomic probe 2) overnight using a modified Church and Gilbert (1984) buffer (NaPi 0.5 M pH 7.2, SDS 5%, EDTA 10 mM), washed twice (2× and 1× SSC/0.1% SDS) at 55°C (cDNA probe 1) and 60°C (genomic probe 2) for 10 min and exposed to Biomax films (Kodak) for 4–12 h at −80°C. The cDNA probe 1 and the genomic probe 2 included the region between the primers Kn3.7Fw (5′-GTTTCCCCCGGTGAAGGCAC-3′)-Kn3.6Bw and were 735 and 1142 bp long, respectively. *Hin*d III restriction enzyme cut twice (2108–2113 and 2139–2144, acc n. EU910092) in the exons of genomic probe.

Mapping

The F2 interspecific mapping population of the *Prunus* reference map (Joobeur et al. 1998; Dirlewanger et al. 2004) was used in this study. It was obtained by selfing a single F_1 plant from the cross between the almond [*Prunus dulcis* (Mill) D. A. Webb; syn. *P. amygdalus* Batsch] cv 'Texas' and the peach cv. 'Earlygold' (abbreviation T × E). The tree population is maintained at CRA, Centro di Ricerca per la Frutticoltura, Ciampino, Rome. Leaf gDNA was isolated using DNeasy Plant Mini Kit (Qiagen). Primers derived from 'Chiripa' *KNOPE3:* Kn3.5Fw (see above) was in the exonic region, whilst Kn3I2.1Bw (5′-AACTTCGGTGCTCAAGAGCC-3′) fell in intron II. They were used to amplify gDNA from the parents, F1 and F2 progeny of the T × E population. The PCR conditions were: 30 ng of gDNA, 0.4 µM of each primer, 0.5 mM dNTPs, 1.25 U of high fidelity Platinum *Taq* (Invitrogen) for the parental genotypes, sequencing and *Taq* DNA polymerase (Qiagen) for segregation analyses, 1/10 of 10× *Taq* buffer, 2.5 mM $MgCl_2$, in a final volume of 25 µl. Thermocycler conditions included an initial cycle at 95°C for 5 min followed by 35 cycles at 95°C for 30 s, 62°C for 60 s and 72°C for 90 s with a final extension at 72°C for 7 min. The sequences of *KNOPE3* PCR fragments from E and T genotypes are reported in the supplemental data 1. The polymorphic profile generated by *Hin*cII digestion is described in "Results". The PCR products were digested with 5 *Hin*cII units (New England Biolabs) in a final volume of 30 and 15 µl was electrophoresed onto 1.2% agarose gel. *KNOPE3* CAPS was integrated in T × E map (Joobeur et al. 1998; Dirlewanger et al. 2004) using the software MAPMAKER (Lander et al. 1987; Lincoln et al. 1992). Initially, the "group" command assigned the *locus* to linkage groups (LOD threshold >7 and recombination fraction <10). The locus was then placed within the respective T × E linkage group using the "try" and "ripple" commands. After mapping, the "error detection" command was used and no candidate errors were detected for *KNOPE3*. Kosambi's mapping function (Kosambi 1944) was used to convert recombination fractions into centimorgan (cM) map distances.

Semi-quantitative RT-PCR

Total RNA was extracted from various organ tissues (Giannino et al. 2000), DNAse-treated (RQ1, Promega), quantified by the spectrophotometer (NanoDrop technologies) and 3 µg was reverse transcribed at 55°C into a single strand cDNA by oligo-$(dT)_{20}$ by Superscript III reverse transcriptase (Invitrogen), according to the manufacturer's instructions. Table 2 contains details on the genes, primers and PCR conditions used in this work. PCR trials were performed at distinct cycles to assess the variation of transcript abundance before signal saturation, at fixed primer pair and reaction parameters. The Kn3.9fw and Kn3.8bw primers were designed in the 3′untranslated region (3′UTR) and yielded a 424 bp fragment. The expression of peach constitutive gene *ACTIN* 2 (same copy number per cell and stable expression in every cell, An et al. 1996) was monitored to check for equal transcript abundance. The PCR reaction was conducted in 50 µl total volume containing 100 ng of cDNA; the chemical parameters are explained in "Materials and methods"; 15 µl was electrophoresed in a 1% agarose gel.

Real time RT-PCR (qRT-PCR)

All the qRT-PCR experiments included three repetitions of PCR reactions (technical triplicates) and each PCR sample consisted of cDNA retrieved from RNA of a leaf pool ($n = 2$–4 leaves, see details along the text). Total RNA was isolated from leaf pools (100 mg) by RNeasy Plant Mini Kit (Qiagen) and reverse-transcribed as described above, the cDNA (40 ng) was amplified in qRT-PCR reactions in 20 µl total volume containing: PCR reaction buffer 1×; dNTPs 50 µM; $MgCl_2$ 3.5 mM; D-(+)-Trehalose dihydrate (Fluka) 0.6 M; formamide 0.7 M; 0.1 µl of a

Table 2 Genes, primers, PCR conditions and products in expression assays

Gene	Acc. no.	Primers 5′–3′	Ann. temp. (°C)	Base pairs
KNOPE3	DQ786755	Fw: GGATGAATGGAGAAAAGGAAACG	55	424
		Bw: GGAACCATGCTCAGGTCATTG		
RUBCA	DY633832	Fw: GAGGAGAAGCAGACCAAAGAGGAC	55	393
		Bw: CATTGGAACGATTTACCCTGACC		
SDH	AB025969	Fw: TCAAACGCCCAGATTCCCTCG	60	585
		Bw:CACCAACACTCAGAGGCTCGCAC		
S6PHD	AF414988	Fw: TTTTGGACGAGAATAAGGTGTTGG	60	333
		Bw: TCACGCTGGAAATAAGGGTGAGT		
CAT2	AJ496419	Fw: TCATCAGTAAGGCAGGGAAAGTACAC	59	349
		Bw: TGGGCAGGGTTAAACGCAAG		
ACTIN2	DY651796	Fw: CCATTCTTCGTCTGGACCTTGC	55	375
		Bw: GCACAATGTTTCCATATAGGTCTTTCC		

1:100 Sybr Green I dilution in dimethylsulfoxide (Invitrogen); *Taq* DNA polymerase (Qiagen) 0.2 U; 0.3 μM of each primer. Reactions were performed in triplicate at these conditions: 180 s at 95°C, 45 cycles of three-step amplification (30 s at 95°C, 1 min at 58°C and 1 min at 72°C). To test primer specificity, melting curve analysis (from 55 to 95°C with an increasing heat rate of 0.5°C s^{-1}) was performed after amplifications. The threshold cycle (C_t) value of each gene was normalized with that of *ACTIN2* and compared with the C_t of an untreated control using the $2^{-\Delta\Delta C_t}$ method (Livak and Schmittgen 2001). In assays for leaf development, the *KNOPE3* and *RIBULOSE CARBOXYLASE ACTIVASE* (*RUBCA*) expressions were, respectively, calibrated referring to the apical tip values, which were set as 1. The primers used for qRT-PCR are listed in Table 2.

In situ hybridization

One-year-old F_1S_{1-18} plants were used at vegetative resumption (March 2006). Sections were made from growing shoots ($n = 5$) derived from the central axis. Excised tissues were fixed, dehydrated, embedded in paraffin, cut into 8 μM sections and hybridized (53°C) with the digoxygenin-labelled antisense RNA probe as described by Cañas et al. (1994). Probe 3 spanned the 89–536 bp cDNA stretch between primers Kn3.11Fw (5′-TTCAATCCGACCAACACGACC-3′) and Kn3.10Bw, and linearized by SpeI and NcoI. The anti-sense and sense RNA probes were in vitro synthesized by T7 and SP6 polymerases, respectively (Giannino et al. 2000).

Assays of imposed dark

Dark response was carried out in vitro using F_1S_{1-18} seedlings. As for in vitro growth, the woody endocarp was removed mechanically by a nut cracker; seeds were sterilized in 70% ethanol for 1 min, followed by HClO 0.75% for 30 min, six washes in sterile water and a final immersion in sterile water for 12 h, and then dried with blotting paper under laminar flow cabinet. The brown coat was peeled gently by scalpel; seeds were germinated on MS0 (Murashige and Skoog 1962) supplied with 5 g l^{-1} agar (Micro Agar, DUCHEFA), 30 g l^{-1} sucrose to a final pH 5.7 in glass pots under irradiance of 80 μmol s^{-1} cm^{-2} with a photoperiod of 16/8 h light/dark cycle at 22°C. The germination occurred in ca. 4 days and seed vitality was ca. 55%; the seedlings were transferred on fresh agarised-MS0 11 days after sowing (das).

Dark was imposed on 15 das seedlings, which formed primary and secondary roots and bore 4–5 fully expanded leaves. The pots were covered with aluminium foil. The expanded leaves ($n = 4$) were excised in the dark and pooled. A separate plantlet was used for each different sampling time (0.5, 1, 2, 4, 8, 16 h). The dark period lasted 16 h so that treated samples could be compared to controls, which were under the normal range of 16/8 h light/dark. A longer dark time was not approached so as to minimize the interference of circadian effects. The experiment was repeated twice with a new set of seedlings (exceptionally, biological duplicates were performed). The expression of peach *RUBCA* and *CATALASE2* (*CAT2*) genes were assayed as dark/light and stress responsive markers, respectively (Table 2 and supplemental data to Fig. 7). The diagrams of relative mRNA abundance represent actin-normalized values of treated samples with respect to those of untreated controls. Standard deviation for ratios was calculated and represented by bars.

Sugar assays

Sugar feeding was performed via the transpiration stream using the leaflets surrounding the shoot apex (AT, in

Table 1) borne on the central axis of 1-year-old F_1S_{1-18} plants (April 2006). Leaflets ($n = 4$) were excised at the junction between the petiole and subtending stem, and immersed into 2.2 ml tubes containing 1.8 ml of a 50 mM solution (separately: fructose, glucose, sucrose, sorbitol), maintained under irradiance of 50 µmol s^{-1} cm^{-2} to favour transpiration (Krapp et al. 1991), and sampled at 0, 3, 6 and 12 h. To check the occurrence of solution uptake, the overall volume of transpired water by the leaves (V_T) in sorbitol feeding assays was measured by the formula: V_{Tn} $(\%) = (V_{ET} - V_E) \times 100 \times (V_0)^{-1}$; V_{ET}, evapo-transpired volume (µl) from tubes feeding the samples; V_E, evaporated volume from tubes without samples; V_0, starting volume (1,800 µl); n, time of sampling. The V_{Tn} (%) were: 0.00 ± 0.00, 2.37 ± 0.35, 5.23 ± 0.87, 9.27 ± 2.21 at 0, 3, 6 and 12 h post-treatment (hpt), respectively. The V_{Tn} values of pure water and other sugar solutions (50 mM) did not vary significantly over time with respect to those for sorbitol, indicating that solution viscosity did not affect leaf uptake.

In April, 1-year F_1S_{1-18} plants bore side branches with mature expanded leaves. The stem of the lateral branch closest to the soil was girdled (cortex and sieve removal). The bark ring was performed above and below the leaves laying at the branch basis, so as to hamper the transport towards the trunk and the upper sectors of the side branch. The leaves ($n = 2$) of the lateral branches of girdled and control plants were excised and pooled separately at 0 (10.00 a.m.), 6 (4.00 p.m.), and 30 (4.00 p.m.) hpt. Three repetitions of the experiments using distinct material (biological triplicates) were performed in sugar supply and girdling experiments and the relative gene expression was graphed by dots representing the mean values ± standard error.

Sugar quantification

The assay kits for sorbitol (cat. no. K-SORB) and sucrose, fructose and glucose and (K-SUFRG) were used (MEGAZYME International Ireland, Bray, Ireland), starting from 30 mg of leaf tissues and following the manufacturer's instructions. The type and number of samples were the same as those used for the expression analyses of darkening and girdling assays. Sugar content fold change (Tables 3, 4; Figs. 7, 8) was graphed by dot representing the ratio between mean values from treated samples and those of untreated controls. Standard deviation for ratios was calculated and represented by bars. Student's t test of treated versus control plants was performed and significant values ($P < 0.05$) were indicated by asterisks.

Results

KNOPE3 belongs to the class 2 of *KNOX*

KNOPE3 full-length cDNA was 1894 bp long and encompassed an open reading frame of 1347 bp. The 5′UTR did not contain the typical Kozak and Shine-Dalgarno sequences, whereas the 3′UTR included a poly-adenylation tail (AATAAA, 1852-1857), a U-rich stretch (TTTTCT, 1811-1816) followed by a cleavage site (CA, 1823-1824) and terminal U-rich site (1825-1831), which are frequently located upstream the poly A tail of many plants (Graber et al. 1999).

KNOPE3 deduced protein consisted of 448 amino acids with a calculated weight of 50.05 kD; it contained the typical KNOX domains (Fig. 1a) and the highest overall identity was with *Malus domestica* KNAP3 (91%) followed by *Medicago truncatula* KNOX5 (77%), *Arabidopsis thaliana* KNAT3 and KNAT4 (69 and 70%), *Vitis vinifera* (CAO67726) and *Populus thricocarpa* (ABK92551) class 2 KNOX (64%). Referring to dicotyledonous trees, the identity grade of KNOPE3 C terminus (from the MEINOX domain to the protein end) was 98, 88 and 71%, respectively, compared to those of apple, western balsam poplar and grapevine class 2 KNOX, whilst the N terminus identity fell to 82, 23 and 16%. The KNOPE3 HD was 55% identical to that of class 1 Kn1 of maize and respected one of the three parameters telling class 1 from class 2 proteins (Reiser et al. 2000).

A phylogram (Fig. 1b) was constructed using various class 2 KNOX proteins from dicot and monocot species available in several databases. KNOPE3 was further confirmed to belong to the highly supported monophyletic class 2 proteins and to be closest to apple KNAP3.

Peach class 2 *KNOX* are a small family and *KNOPE3* lays on linkage group 1

KNOPE3 harboured five introns (Fig. 2a, top scheme) maintaining the same positions as those of *Arabidopsis KNAT3* (Fig. 2a, bottom scheme). Intron I and II fell in the KNOX2 sub-domain, intron III lay in the ELK domain, which is a feature that is specific to class 2 KNOX (Kerstetter et al. 1994). Intron IV exists in the HD and intron V was upstream of the stop codon. All the introns were AT rich (65% in average) and possessed GT/AG or GC/AG splice sites (Kitamura-Abe et al. 2004).

Southern blot analysis (Fig. 2b) was first carried out with the *KNOPE3* cDNA probe 1 (Fig. 2a) which encompassed the ELK to 3′UTR stretch and was remotely paralogous (11% nucleotide identity) to that of class 1 *KNOPE1* (acc n. DQ358050). The signal pattern suggested that more than two members of class 2 occurred in the

'Chiripa' genome (Fig. 2b, middle panel). Subsequently, we used the genomic probe 2; spanning the stretch downstream of intron II to the 3′UTR and encompassed two *Hin*d III sites in the exonic regions (H in Fig. 2a), lying 31 bp one from the other. A single band was observed (Fig. 2b, right panel) using EcoRV and XbaI, whilst two signals of ca. 2 kb and 450 bp appeared with *Hin*d III. The ca. 450 bp size was consistent with that of 419 bp predicted by the restriction analysis (Fig. 2a), whilst the 31 bp band was not visible for it fell out of the electrophoretic run. Overall, this hybridization pattern indicated that *KNOPE3* was a single copy per haploid genome.

KNOPE3 was mapped (Fig. 2c) scoring the segregation pattern of a CAPS marker in the *Prunus* T × E population (Joobeur et al. 1998; Dirlewanger et al. 2004). Primer pairs were designed based on the segment of 'Chiripa' *KNOPE3* spanning intron I, and yielded 1113 and 1127 bp PCR products in E and T genotypes, respectively. They were sequenced and the differences consisted of 44 single nucleotide polymorphisms and ten insertion/deletion events (see "Material and methods" and supplemental data 1). The *KNOPE3* alleles from E and T contained two and one *Hin*cII sites, respectively, at distinct positions. *Hin*cII digestion of PCR products generated a three band set of 529, 360, 224 bp in E, and a two band set of 903 and 2224 bp in T (Fig. 2c, top panel). The co-dominant polymorphic profile was confirmed in the F_1, hence the allele segregation was determined in the F_2 T × E population (Fig. 2c, top panel), processed by MAPMAKER EXP 3.0 and *KNOPE3* was positioned at 66 cM of G1 (Fig. 2c, bottom panel), in co-segregation with the AC18 and AC23 markers.

Fig. 2 *KNOPE3* genomic organization. **a** Scheme of peach *KNOPE3* and *Arabidopsis KNAT3* genes. The *black triangles* and *numbers* indicate intron positions and sizes (in base pairs, bp) with respect to the cDNA. Start and stop codons and polyadenylation tails are typed. The cDNA probe 1 spans the ELK-HD domains and a portion of the 3′UTR, whilst the genomic probe 2 includes introns III-V and 3′UTR. The cDNA probe 3 was used for in situ experiments. H, *Hin*d III site. **b** Southern blot analysis. Molecular weights of a DNA marker are in kilo base pairs (kb) (*left panel*). Peach class 2 *KNOX* gene family: gDNA digested with *Eco*RV, *Xho*I, *Xba*I and hybridized with cDNA probe 1 (*middle panel*). Genomic probe 2 was used in combination with *Eco*RV, *Xba*I and *Hin*dIII, the latter cuts the probe twice (*right panel*). **c** *KNOPE3* CAPS in T × E. Polymorphism profile of *KNOPE3* CAPS in the parents (T, E), F_1 and 9T × E F_2 individuals (*top panel*). Map of linkage group 1 (G1) of the *Prunus* reference map and position of *KNOPE3* (in *bold*) (*bottom panel*)

KNOPE3 has a diversified pattern of expression in stem, flowers and fruits

KNOPE3 transcription was assayed in aerial vegetative and reproductive organs by semi-quantitative RT-PCR (Fig. 3) and in situ hybridization (Figs. 4, 5, 6) using the antisense dig-labelled probe 3 (Fig. 2a).

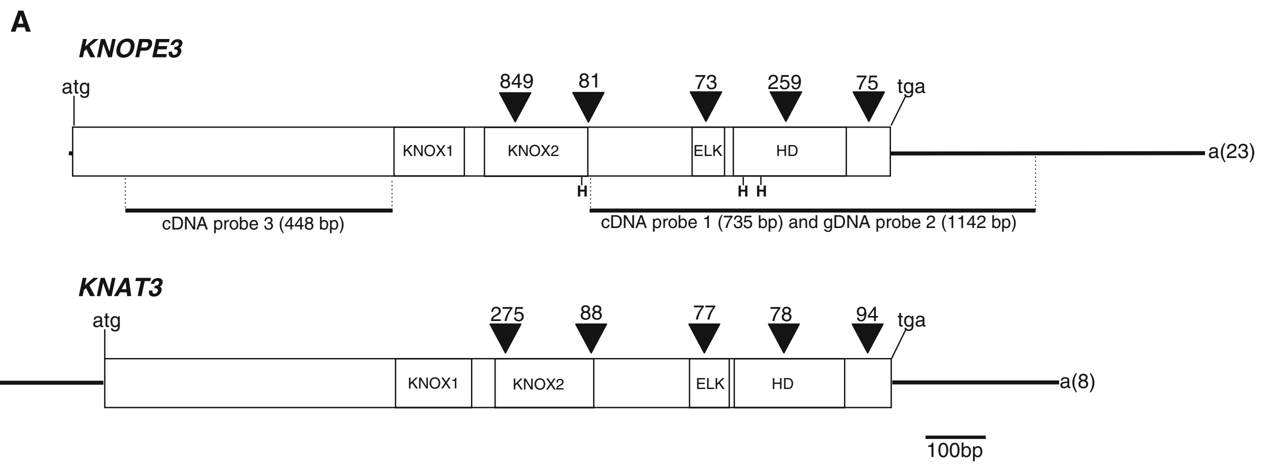
KNOPE3 was expressed in all organ tissues and the lowest transcript level occurred in stems and petals (Fig. 3). Stems of elongating shoots (April) were sectioned at 0.2 mm (Fig. 4a, b) and 5 cm (Fig. 4c–e) underneath the SAM. The transcript signal (blue stain, Fig. 4a) spread evenly in the cortex and was absent in the pith; it also marked the phloem and/or phloem-associated cells (also called hereafter sieve element/companion cell complex, acronym: SE-CCC) of leaf traces (Fig. 4b). At 5 cm, a strong signal featured in both the stem vascular system and leaf traces (Fig. 4c). As for the latter (Fig. 4d), *KNOPE3* mRNA marked: (a) cell groups forming the SE-CCC residing underneath the phloem cap and (b) sporadic cells, likely to be the secondary phloem, which were located upon the cambium layer. As for stem vascular bundles (vsb), the message occurred in secondary phloem cells (Fig. 4e), but was absent in the intra-fascicular cambium, in the phloem cap and xylem cells.

In floral buds before anthesis (February), the gene transcript marked the receptacle, the sepals and the outer layers of anthers (Fig. 5a). More precisely, the signal diffused into the cortical region and peaked within the vsb of the receptacle (Fig. 5a); it occurred in the parenchimatous cells and vsb of sepals, whereas it only featured in vsb of petals (Fig. 5b). With regard to stamens, magnifications showed that the message localized to the endothecium and tapetum cells (Fig. 5c) and to the vsb of filaments connecting the anther lobes (Fig. 5d). *KNOPE3* was not expressed in pollen grains and perithecia (Fig. 5c). As for gynoecium, a faint signal was observed in the outer cells of the stigma orifice, in the style vsb (Fig. 5e) and in a few inner cells of the ovary (Fig. 5f). Fruits of 3 mm diameter (Fig. 5g) were sectioned at early canonical stage S1 (Zanchin et al. 1994) and the transcript marked the vsb of mesocarp and seed funiculus.

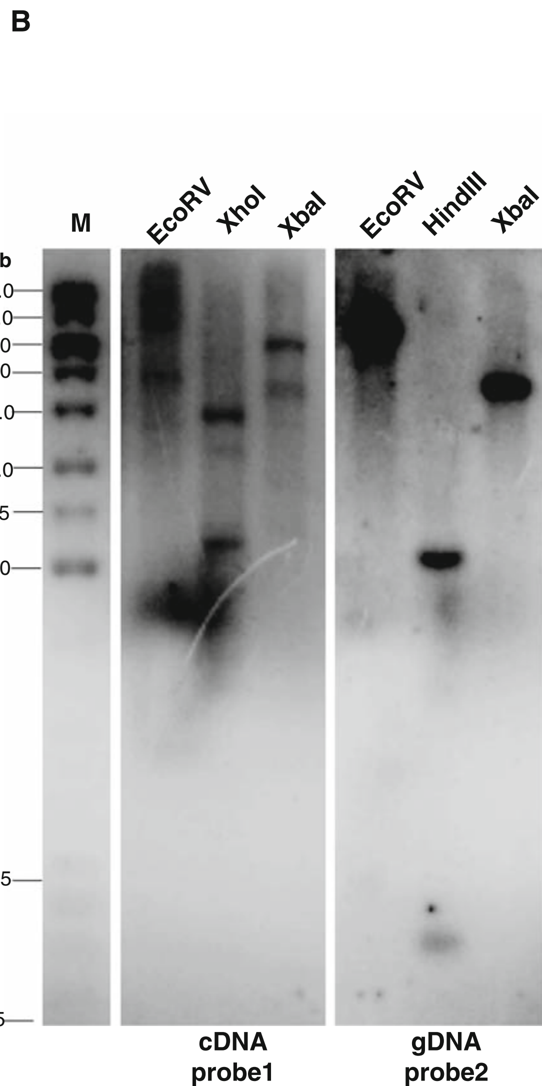
KNOPE3 transcript localizes with different patterns during leaf growth

KNOPE3 expression was monitored in leaves at distinct growth stages borne on the main axis (April), following a top-down direction (Table 1). The lamina area, folding/expansion status, chlorophyll contents and *RUBCA* expression (Watillon et al. 1993) were measured to mark the growth stages and they increased with leaf maturity. The *KNOPE3* transcript levels of fully expanded leaves at the shoot basis were significantly higher than those of upper folded leaves, evidencing that the expression raised in concomitance with growth.

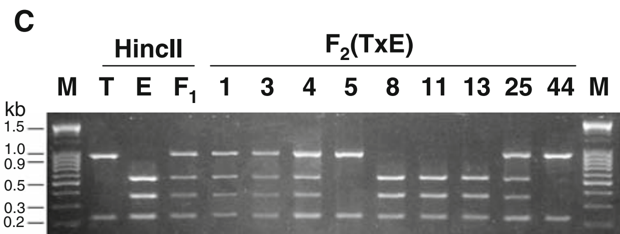
In situ hybridization was performed on the apical meristems and surrounding leaves of shoots at the vegetative burst (late March). In SAM longitudinal sections,



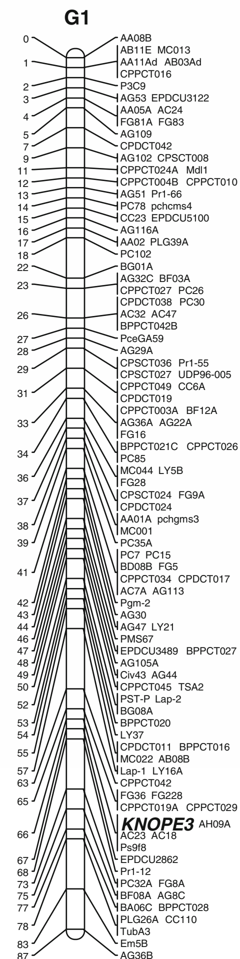
A
KNOPE3
atg
849
81
73
259
75
tga
KNOX1
KNOX2
ELK
HD
a(23)
H
H H
cDNA probe 3 (448 bp)
cDNA probe 1 (735 bp) and gDNA probe 2 (1142 bp)
KNAT3
atg
275
88
77
78
94
tga
KNOX1
KNOX2
ELK
HD
a(8)
100bp



B
M
EcoRV
XhoI
XbaI
EcoRV
HindIII
XbaI
kb
10.0
8.0
6.0
5.0
4.0
3.0
2.5
2.0
0.5
0.25
cDNA probe1
gDNA probe2



C
HincII
F2(TxE)
M T E F1 1 3 4 5 8 11 13 25 44 M
kb
1.5
1.0
0.9
0.5
0.3
0.2



G1
0 AA08B
AB11E MC013
1 AA11Ad AB03Ad
CPPCT016
2 P3C9
3 AG53 EPDCU3122
4 AA05A AC24
FG81A FG83
5 AG109
7 CPDCT042
9 AG102 CPSCT008
11 CPPCT024A Mdl1
12 CPPCT004B CPPCT010
13 AG51 Pr1-66
14 PC78 pchcms4
15 CC23 EPDCU5100
16 AG116A
17 AA02 PLG39A
18 PC102
22 BG01A
23 AG32C BF03A
CPPCT027 PC26
CPDCT038 PC30
26 AC32 AC47
BPPCT042B
27 PceGA59
28 AG29A
29 CPSCT036 Pr1-55
CPSCT027 UDP96-005
31 CPPCT049 CC6A
CPDCT019
33 CPPCT003A BF12A
AG36A AG22A
FG16
34 BPPCT021C CPPCT026
PC85
36 MC044 LY5B
FG28
37 CPSCT024 FG9A
CPDCT024
38 AA01A pchgms3
MC001
39 PC35A
PC7 PC15
41 BD08B FG5
CPPCT034 CPDCT017
AC7A AG113
42 Pgm-2
43 AG30
44 AG47 LY21
46 PMS67
47 EPDCU3489 BPPCT027
48 AG105A
49 Civ43 AG44
50 CPPCT045 TSA2
52 PST-P Lap-2
BG08A
53 BPPCT020
54 LY37
55 CPDCT011 BPPCT016
MC022 AB08B
57 Lap-1 LY16A
63 CPPCT042
65 FG36 FG228
CPPCT019A CPPCT029
KNOPE3 AH09A
66 AC23 AC18
Ps9f8
67 EPDCU2862
68 Pr1-12
73 PC32A FG8A
75 BF08A AG8C
77 BA06C BPPCT028
78 PLG26A CC110
TubA3
83 Em5B
87 AG36B

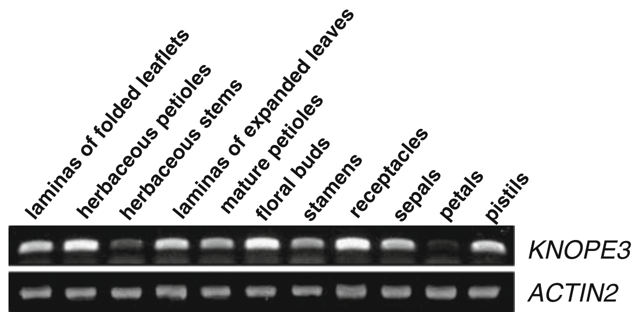


Fig. 3 Detection of *KNOPE3* expression by RT-PCR. Tissues are indicated above the lanes. The *ACTIN2* was amplified to check for cDNA synthesis and equal loading. Transcript molecular sizes are in Table 2

the *KNOPE3* signal was absent in the central and peripheral zones, marked cells of the innermost layer in the rib zone, likely to be the vascular cordon, were intense in the pro-vascular strands at the basis of primordia and developing leaves, and absent in the pith (Fig. 6a). In transversal sections, the overall mRNA pattern showed a signal increase proceeding from the inner to the outer leaves (Fig. 6b). In younger leaves, it mainly localized to the vsb (Fig. 6c), whilst it extended in several group cells of older leaves (Fig. 6d), namely in the vsb and mesophyll cells, but not in sub-epidermal and epidermal layers. In expanded leaves (Fig. 6e), *KNOPE3* message localized to the palisade layer, spongy parenchyma and vsb (Fig. 6f), but was absent in the bundle sheath of main (Fig. 6g) and secondary veins (not shown) and in the epidermis (Fig. 6f). Finally, *KNOPE3* expression occurred in SE-CCC of leaf mid vein (Fig. 6g), which strongly resembled the pattern of stem leaf traces (Fig. 6h). Controls were performed using a sense probe, no signal above background was detected (not shown).

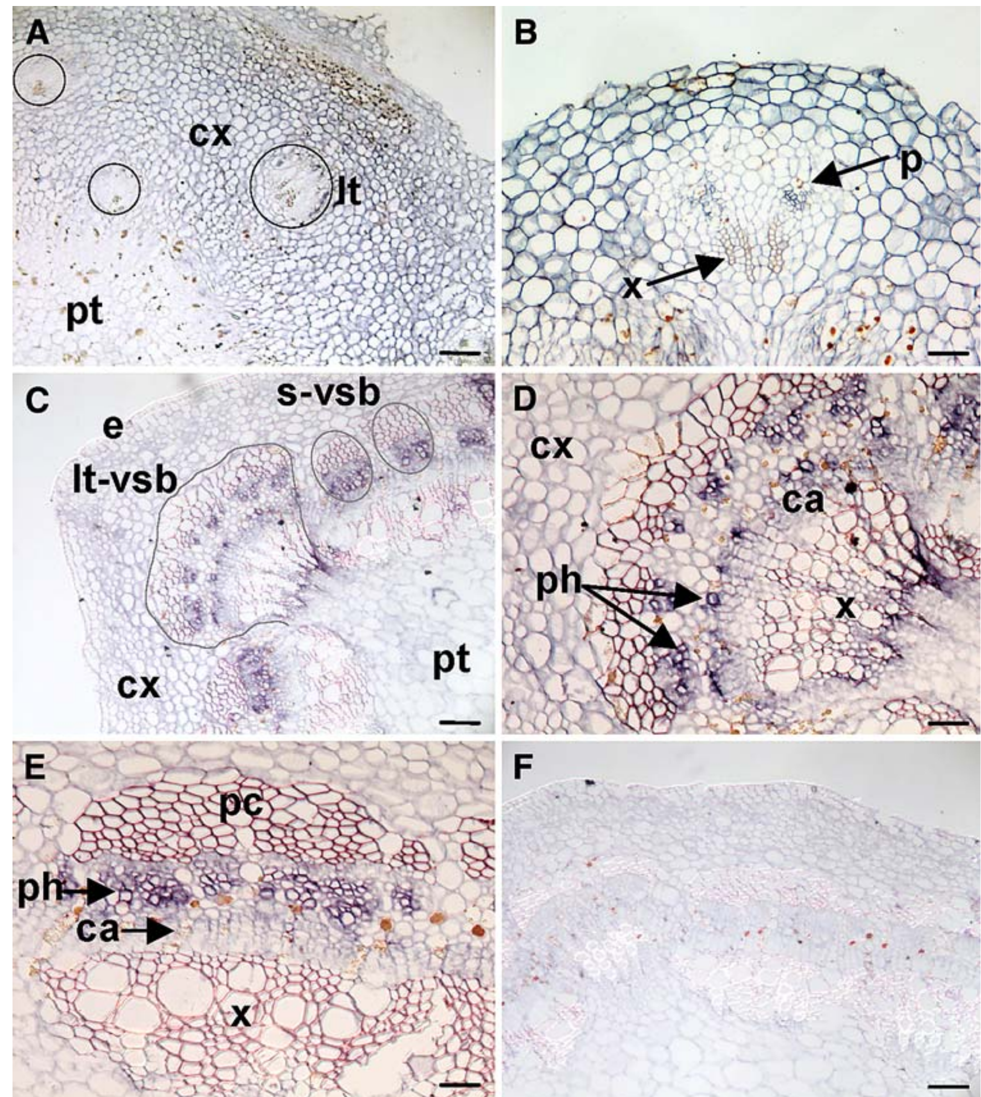


Fig. 4 *KNOPE3* transcript localization in stem The *KNOPE3* mRNA is represented by the intense blue signal. **a** Sections at ca. 0.2 mm below the SAM. *KNOPE3* mRNA was absent in the pith (*p*), spread in the cortex (*cx*) and visible in bundles of leaf traces (*lt*), marked by drawn circles. **b** Leaf trace magnification shows that the message localized to phloem and/or phloem-associated cells (*p*) and not to xylem (*x*). **c** Sections at ca. 5 cm from the apex. Leaf trace (*lt-vsb*) and stem vascular bundles (*s-vsb*) are *circle-marked*. The epidermis is also indicated (*e*). **d** Leaf trace magnification evidenced *KNOPE3* signal in phloematic cells (*ph*) and not in the cambium (*ca*). **e** Close-up of stem vascular system showed an intense signal in phloematic cells, but not in the phloematic cap (*phc*). **f** Control experiment, tissues were hybridized with a sense probe and no signal above background was detected (*size bars* **a** 100 μm, **b** 55 μm, **c** 115 μm, **d**, **e** 30 μm, **f** 80 μm)

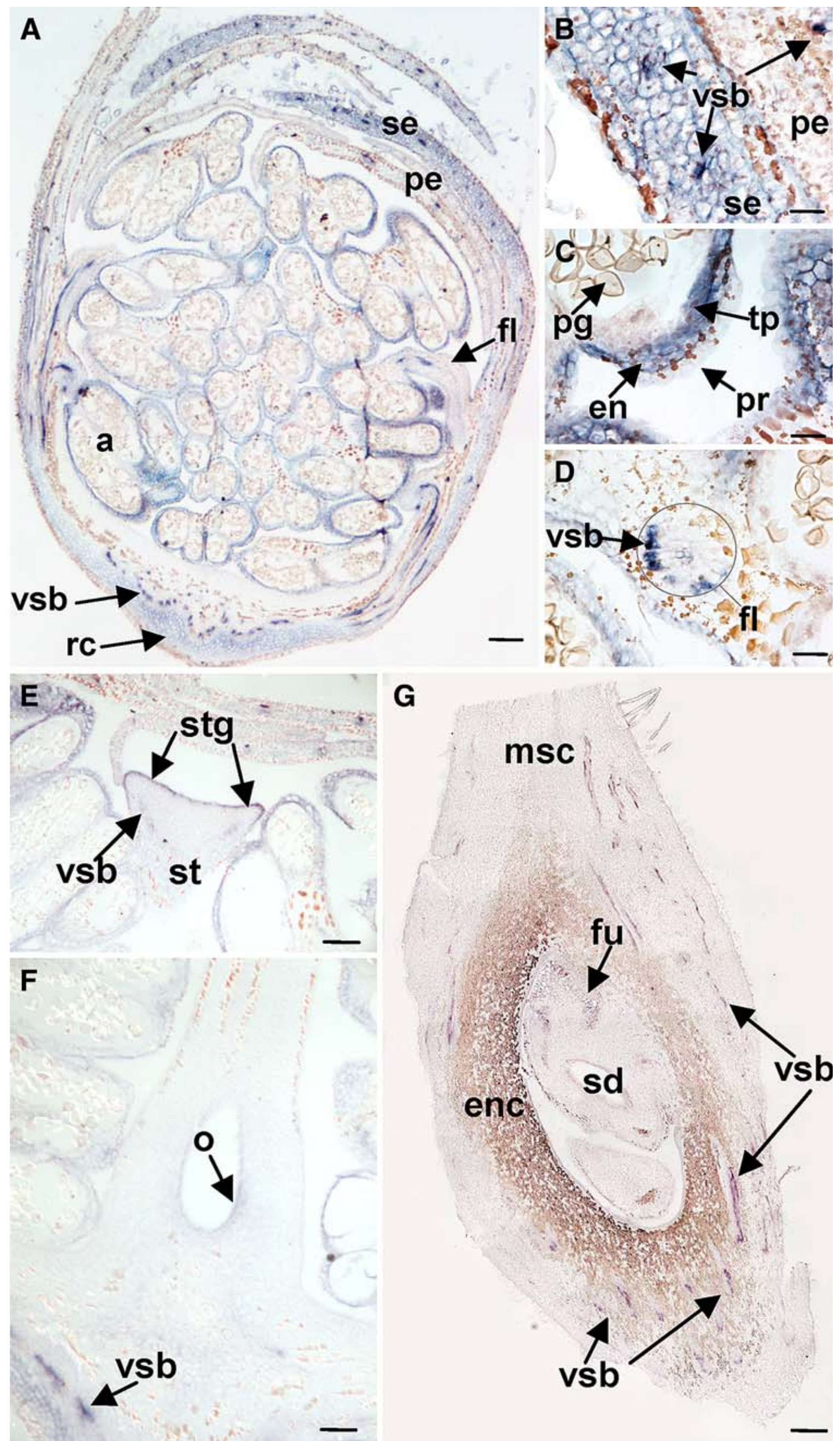


Fig. 5 Transcript localization in flower and fruit. **a–e** Floral buds before anthesis (February) were used for the in situ experiments. **a** The *KNOPE3* message occurred in the parenchymatous cells of receptacle (*rc*), and in the vascular bundles (*vsb*) of several flower components, such as anthers (*a*) filaments (*fl*), petals (*pe*) and sepals (*se*). **b** Magnification of a sepal (*se*) and a petal (*pe*) showed *KNOPE3* mRNA stained sepals more intensely than petals. Intense signal occurred in vsb. **c** The message marked the anther endothecium (*en*) and tapetum region (*tp*), but was absent in perithecium (*pr*) and pollen grain (*pg*). **d** Deep blue signal marked vascular bundles (*vsb*) of anther filament (*fl*). **e**, **f** *KNOPE3* was active in the vascular bundles (*vsb*) of stylus (*st*) and the stigma orifice (*stg*) and faintly in inner cell layers of the ovary (*o*). **g** Fruit at early development stage (S1, 3 mm of diameter). The transcript mainly localized in the vascular bundles (*vsb*) of mesocarp (*msc*); a slight signal was detected in funiculus tissue (*fu*) of seed (*sd*) and endocarp (*enc*) (size bars **a** 200 μm, **b–d** 40 μm, **e**, **f** 135 μm, **g** 500 μm)

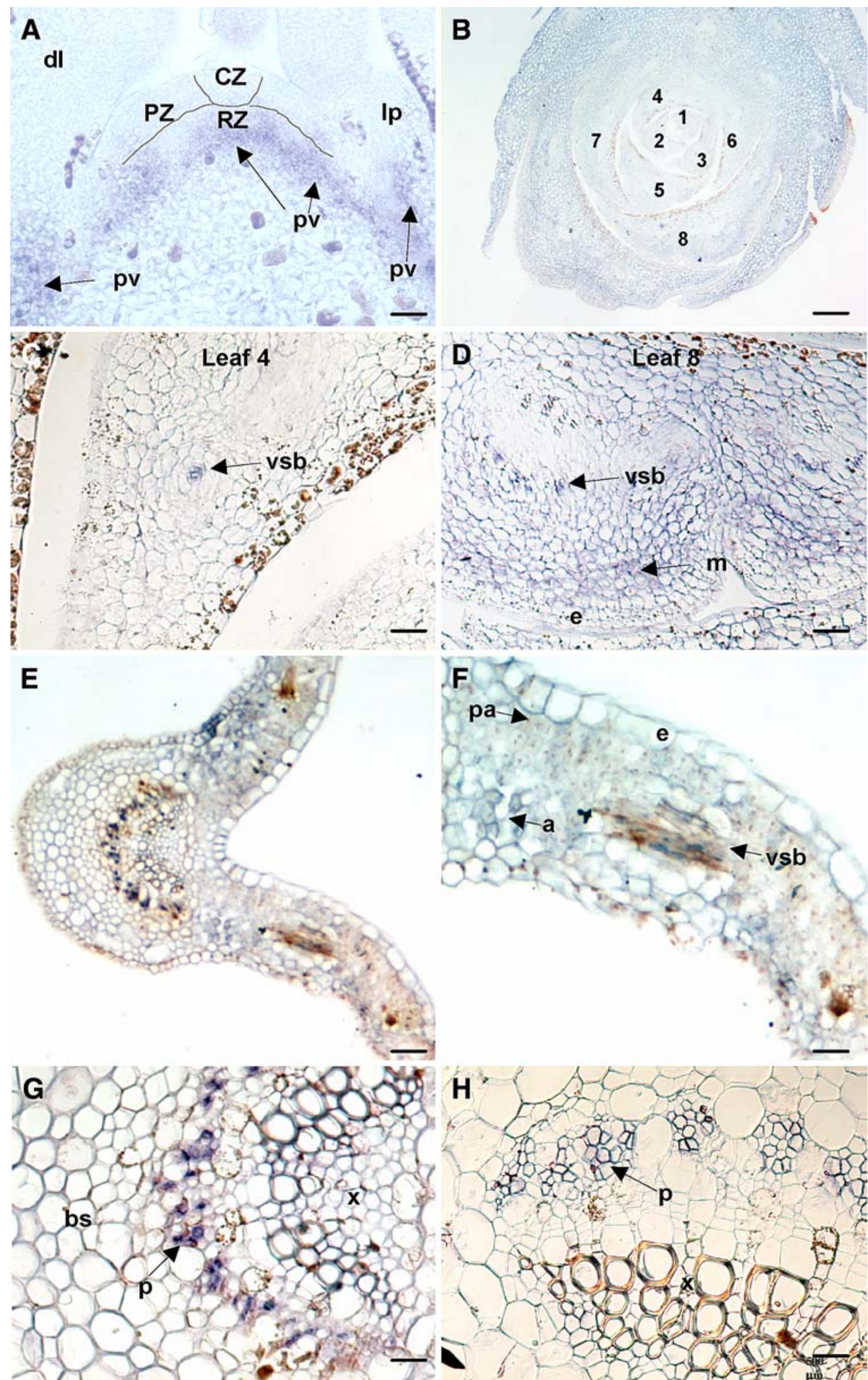


Fig. 6 Transcript localization in vegetative SAM and leaves. **a**–**d** Shoots at the vegetative burst (late March) were analysed. **a** In SAM longitudinal section *KNOPE3* message was absent in the central (*CZ*) and peripheral (*PZ*) zones, confined to the last cells of the rib zone (*RZ*), intense in provascular tissues (*pv*) underlying primordia (*lp*) and growing leaves (*dl*). **b** Shoot transversal section included growing leaves but not the apical meristem and primordia. Leaves were numbered arbitrarily 1–8 following the growth stage. Synoptically, the blue signal spread centripetally from inner (younger) towards outer (older) leaves. **c** Leaf 4 magnification pinpoints the mRNA in vascular bundles (*vsb*). **d** Close-up of leaf 8 shows the mRNA signal spread in the mesophyll (*m*), persisted in bundles (*vsb*), and was absent in the outermost layer of mesophyll and the epidermis (*e*). **e** Fully expanded leaf (main rib length 5 cm, sited 10 cm below the SAM, April). **f** Magnification of lamina showed the *KNOPE3* expression in vascular bundles (*vsb*), palisade layer (*pa*) and aerenchyma (*a*). **g** Mid vein close-up evidenced specific transcript localization in the phloem (*ph*), absence in the xylem (*x*) and bundle sheath (*bs*). **f** In petiole transversal section the mRNA pattern resembled that of the leaf mid vein (*size bars* **a** 25 µm, **b** 280 µm, **c**, **d** 30 µm, **e** 110 µm, **f** 50 µm, **g**, **h** 25 µm)

Leaf *KNOPE3* rapidly responds to light variation and is triggered by sugars

The level of *KNOPE3* transcript was assayed in leaves of darkened seedlings (Fig. 7). Light-exposed in vitro plants were obscured for 16 h and subsequently re-exposed to light. To characterise the physiological status of treated plants, the expression of peach *RUBCA* and *CAT2* genes was monitored to mark dark/light (Watillon et al. 1993; Giannino et al. 2004) and stress events, respectively (supplemental material 2).

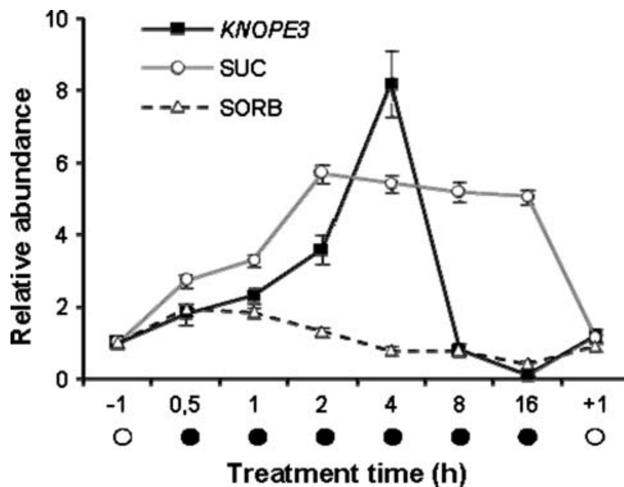


Fig. 7 *KNOPE3* expression pattern and sugar leaf content variation in response to imposed darkening. Time-course of relative *KNOPE3* mRNA levels by qRT-PCR and fold change variation of sorbitol and sucrose leaf contents ±1 h preceding the darkness and subsequent to light restoration, respectively. *White* and *black circles* indicate light-on and darkening, respectively. *Bars* indicate standard errors

The contents of major translocation sugars (Merlo and Passera 1991; Moing et al. 1997) were also determined (Table 3) and the fold variation of sorbitol and sucrose was graphed together with that of *KNOPE3* transcription (Fig. 7). The latter augmented rapidly and climaxed with a ca. eightfold increase at 4 h post-darkness (hpd). An abrupt drop to the control levels occurred at 8 hpd, followed by an intense repression at 16 hpd (Fig. 7). The trend of sucrose content variation was similar to that of *KNOPE3*, suggesting that transport sugar may act on *KNOPE3* expression. The *RUB-CA* transcription was repressed from 0.5 hpd onwards and triggered at 1 h post-light restore. The *CAT2* expression augmented significantly of ca. six times at 16 hpd, suggesting a late stress status (supplemental material 2).

To assess whether *KNOPE3* transcription is modulated by carbohydrates, shoot apical tips were fed with fructose, glucose, sucrose and sorbitol via the transpiration stream and gene expression monitored in a 12 h time lapse under illumination (Fig. 8a). The *KNOPE3* transcription was induced differentially at 3 h post-treatment (hpt) by sucrose, glucose and fructose, maintaining the up-regulation state afterwards, whereas sorbitol feeding led to the transcriptional peak at 12 hpt. A 20- to 30-fold expression raise was observed after sucrose and glucose treatments, a less than tenfold induction was caused by fructose, whilst the sorbitol induction was ca. 15 times higher than controls (samples fed with water).

The sorbitol kinetics was delayed with respect to the other sugars, suggesting that a sorbitol derivate may have mediated the gene triggering rather than the polyol itself. The latter is converted into fructose by the sorbitol dehydrogenase (SDH, EC 1.1.1.14), hence the peach *SDH* transcription was monitored upon sorbitol supply (Fig. 8b). The *SDH* up-regulation was confirmed (Zhou et al. 2006) and found to precede that of *KNOPE3*.

Stem girdling was performed to inhibit sugar transport from mature basal leaves. The *KNOPE3* expression was significantly higher than controls (Fig. 8c) from 6 hpt afterwards, along with the raise of sorbitol and sucrose contents (Table 4) that followed the phloem removal. The down-regulation of the peach *SORBITOL-6-PHOSPHATE DEHYDROGENASE* gene (*S6PDH*), which encodes the enzyme synthesizing sorbitol (EC1.1.1.200), further suggested that sorbitol augment was caused by the translocation block rather than by a de novo synthesis (supplemental material 3).

The wounding effect (petiole cutting and stem cortex removal in sugar supply and girdling experiments, respectively) on *KNOPE3* triggering was negligible, if not

Table 3 Content of major transport sugars in expanded leaves of in vitro seedlings subjected to imposed darkening

Treatment		Sugar content (mg g^{-1}fw)[a]				
Status	Hours	Glucose	Fructose	Sucrose	Sorbitol	Total
Light	−1	6.12 ± 0.79[b]	0.91 ± 0.11	12.22 ± 1.34	36.12 ± 5.05	55.35 ± 5.31[c]
	0.5	13.32 ± 1.59*	1.81 ± 0.21*	37.42 ± 4.86*	61.71 ± 8.02*	114.26 ± 9.56*
	1	7.41 ± 0.66	0.83 ± 0.77	45.13 ± 3.15*	58.96 ± 7.07*	112.33 ± 8.03*
	2	6.24 ± 0.87	0.94 ± 0.13	78.27 ± 9.39*	41.99 ± 4.19	127.44 ± 11.07*
Dark	4	7.63 ± 0.67	1.12 ± 0.09	74.38 ± 7.14*	25.04 ± 2.42*	108.17 ± 11.01*
	8	7.51 ± 0.82	1.03 ± 0.11	71.22 ± 9.25*	24.69 ± 2.96*	104.45 ± 18.73*
	16	7.64 ± 0.72	0.92 ± 0.08	69.32 ± 6.03*	12.94 ± 1.42*	90.82 ± 6.31*
Light	1	5.81 ± 0.63	0.73 ± 0.08	15.21 ± 1.97	28.76 ± 3.16	50.51 ± 3.78

* Significant differences (Student's t-test P<0.05) of darkened versus untreated plants

[a] Mean values were measured from leaf pool ($n = 2$) derived from three distinct experiments

[b] Standard error

[c] Standard deviation for sums

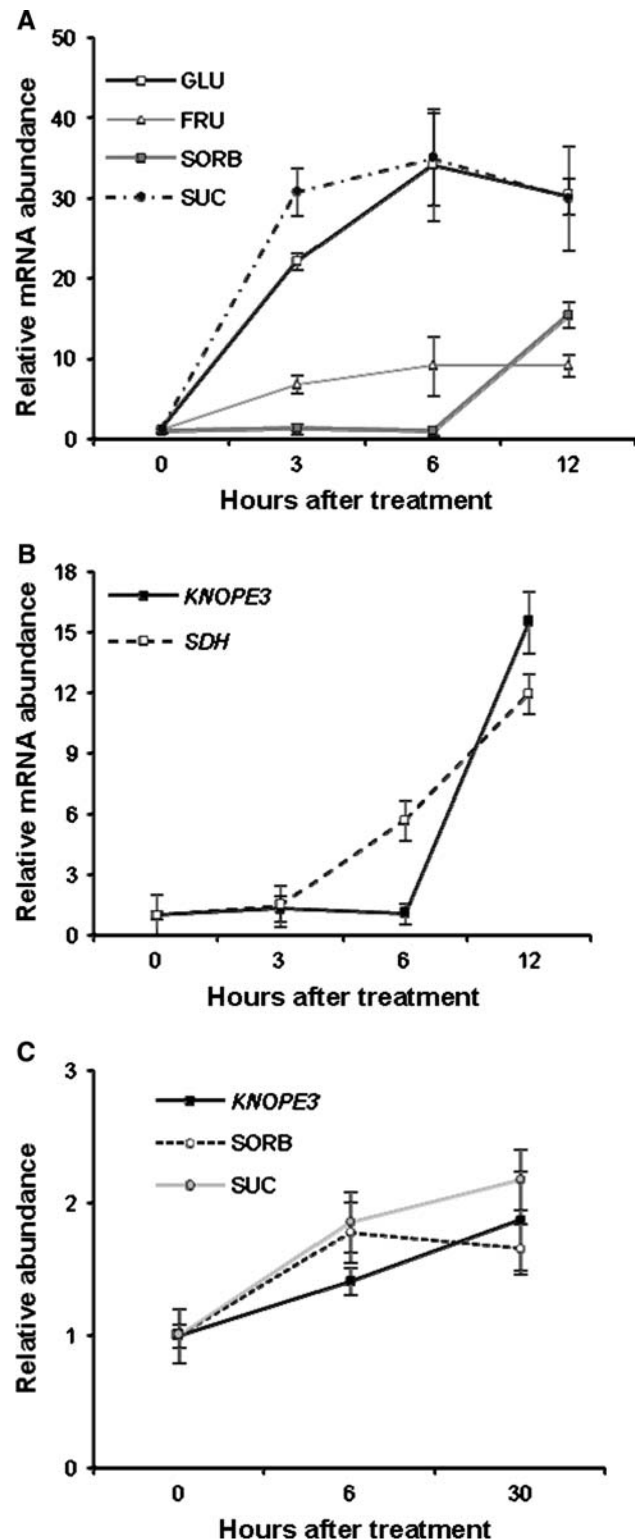


Fig. 8 Time course of *KNOPE3* expression in response to sugar variation. **a** The relative mRNA abundance of *KNOPE3* determined by qRT-PCR in young (sink) leaves fed with 50 mM glucose (GLU, *open square*), fructose (FRU, *open triangle*), sorbitol (SORB, *filled square*) and sucrose (SUC, *filled circle*). **b** The *KNOPE3* expression (*filled square*) was compared with that of *SORBITOL DEHYDROGENASE* (*SDH*, *open square*) in sorbitol uptake assay. **c** Stems bearing mature (source) leaves were girdled and the leaf *KNOPE3* expression kinetics (*filled square*) was graphed together with sorbitol (SORB, *open circle*) and sucrose (SUC, *filled circle*) content variation

absent. This is viewable in the supporting material to Fig. 8a and c (named supplemental material 5), explicating *KNOPE3* expression in control samples. To further check whether *KNOPE3* was triggered by mechanical damages, gene transcription was measured in leaves after their blades were tweezer-injured (Supplemental material 4, Response to leaf injury). No significant variation of the message levels occurred along 6 h after wounding, whilst the expression of *CATALASE* gene stress markers (*CAT1* and *CAT2*) increased from 3 hpt onwards. Hence, the *KNOPE3* up-regulation appeared to be specifically and rapidly caused by glucose, fructose and sucrose.

Discussion

The peach *KNOPE3* full length cDNA was isolated and assigned to the class 2 KNOX (Reiser et al. 2000). The gene consisted of six exons and five introns, maintaining a highly similar organization to that of *Arabidopsis KNAT3*. It was a single copy per haploid genome in the cultivar 'Chiripa' and belonged to a small family class 2 KNOX genes. In our lab, five class 1 *KNOPE* members were assessed in 'Chiripa', whilst the class 2 *KNOPE4*, distinct from *KNOPE3,* was retrieved by cDNA sequencing (unpublished). Hence, at least six *KNOPE* genes occur in the peach genome. *KNOPE3* was positioned at 66 cM on G1 of the *Prunus* 'Texas' × 'Earlygold' genetic map and fell into a region where a QTL for leafing date was localized in almond (Sanchez Perez et al. 2007). The deduced protein KNOPE3 maintained the typical KNOX organization, including the N-terminal MEINOX, the ELK and the HD domains. The ELK domain of class 1 KNOX was proposed to be a nuclear localization signal (NLS, Reiser et al. 2000) and BEL dimerization was required for proper import to the nucleus (Cole et al. 2006). Class 2 KNOX are regulated at the post-translational level to reach the nucleus (Truernit et al. 2006), though the precise amino acidic signals are unknown. Interestingly, the C terminus of KNOPE3 contained (http://cubic.bioc.columbia.edu/predictNLS/) a predicted NLS (RKRNWHSNPSTSTVLKSKRKR, pos. 416–436, Fig. 1). Additionally, KNOPE3 harboured the same binding motif (WFIN pos. 411–414, Fig. 1) as that of class 1 KNOX, which recognize the TGACAGG/CT DNA stretch (Hake et al. 2004).

The *KNOPE3* expression was widespread in several organs (Fig. 3), though the mRNA abundance varied in distinct tissues, in accordance with the behaviours of other class 2 *KNOX* (Reiser et al. 2000). In peach SAM, *KNOPE3* was confined to pro-cambial strands underneath

Table 4 Leaf sugar contents in girdling experiments

Sugar content (mg g^{-1}fw)[a]	Treatment time (h)		
	0	6	30
Sorbitol			
Untreated	19.12 ± 1.84[b]	17.94 ± 2.46	22.33 ± 2.58
Girdled	–	31.99 ± 6.31*	36.98 ± 2.11*
Sucrose			
Untreated	9.23 ± 0.87	8.34 ± 1.22	11.09 ± 1.52
Girdled	–	12.53 ± 0.78*	13.85 ± 1.77*

* Significant differences (Student's *t* test $P < 0.05$) of girdled versus untreated plants

[a] Mean values were based on leaf pool ($n = 2$) derived from three distinct experiments

[b] Standard error

primordia and developing leaves (Fig. 4a). In the stem below the apex, the transcript featured in the cortex and vascular bundles, whereas it was confined to SE-CCC in stem distal sectors, where the secondary growth had started. Overall, the gene was repressed in the tunica of apical meristems and in cambia, pith, xylem and phloem cap of stems. The mRNA localization profile in undisclosed flower buds evidenced *KNOPE3* action in the vsb of stamen threads, pistils, sepals, petals, receptacle and in tapetum/endothecium of anthers. The patterns were consistent with those reported for tomato *Let12* in flower buds (Janssen et al. 1998). The *KNOPE3* mRNA sequence is retrievable from EST databanks of drupe at S1–S4 stages (http://www.itb.cnr.it/estree/), proofing that the gene works during the whole fruit development. In situ hybridizations showed that the message localized to the vasculature innervating the mesocarp and the seed funiculus, suggesting that *KNOPE3* maybe play a role in fruit nourishment.

With regard to leaf development, *KNOPE3* mRNA abundance increased with growth, being lower in apical curled than basal expanded leaves (Table 1). The *KNOPE3* mRNA was first seen in the vsb of younger leaves and then in SE-CCC and mesophyll of older leaves. In expanded leaves, it persisted in SE-CCC, palisade and spongy layers, but was absent in the epidermis, bundle sheaths and collenchyma. The message of *Arabidopsis KNAT3* localized to vsb of leaf primordia, petioles of older leaves and hypocotyls of seedlings. Additionally, GUS assays showed that gene promoter activity declined from young to older leaves, being intense in veins and blade of the former, whilst strong in vascular net and diffuse in lamina of the latter. Hence, authors suggested that *KNAT3* repression occurred with leaf development (Serikawa et al. 1997). The *KNOPE3* mRNA loc patterns were partially consistent with those of *Arabidopsis KNAT3*; however, qRT-PCR experiments showed that *KNOPE3* transcript increased with leaf growth. This was supported by in situ experiments showing that *KNOPE3* domains expanded from vsb to mesophyll in parallel with leaf maturity. Hence, *KNOPE3* and *KNAT3* may not share the same functions in leaf development despite sharing high identity.

The *KNOPE3* message occurs in pro-vascular strands and is associated to bundles of all aerial organs, suggesting that it may play a role throughout the functioning of vascular system. The intense localization signal inside the SE-CCC suggests that *KNOPE3* mRNA may be either transported in the sap, similarly to class 1 messages (Kim et al. 2005), or produced directly by the nucleated companion cells of phloem. The class 1 KNOX protein localize to vasculature associated cells of leaves (Harrison et al. 2005; Hay and Tsiantis 2006) and are able to self-move, contrary to the class 2 KNAT3 (Kim et al. 2005) and NTH201, which localizes to plasmodesmata (Yoshii et al. 2008). Supposing that KNOPE3 maintains these skills, it might act at the level of connections between companion cells and adjacent sieve elements.

The *KNOPE3* expression in green and photosynthetically active tissues, such as leaf mesophyll and sepal parenchyma (Vemmos and Goldwin 1994), suggests that gene activity is light regulated. In fact, obscuring the leaves caused a fast and intense expression increase, followed by a drastic down-regulation; the light restore triggered the gene immediately. In prolonged darkness, the *KNOPE3* repression agrees with that of *Arabidopsis KNAT3* in 6-day etiolated seedlings (Serikawa et al. 1996, 1997). However, in the latter case, multiple factors in addition to light absence can account for *KNAT3* repression, whilst our experiments indicate a *KNOPE3* fast sensing to both dark and light. Interestingly, the "high and low" transcription trend of *KNOPE3* overlapped the "rise and fall" area of sucrose content. This is not unexpected since microarray analyses show that many genes are co-regulated by both light and sugar (Thum et al. 2008). In plants exposed to darkening, starch breakdown occurs and carbohydrate metabolism genes are reported to respond positively (reviewed by Smith et al. 2003; Rolland et al. 2006); moreover, senescence and stress defense mechanisms are also induced (Weaver and Amasino 2001; Lin and Wu 2004). The results from imposed obscurity suggest that the rapid increase of leaf *KNOPE3* transcription may be caused by the quick variation of sugar content (mainly sucrose) together with the light turn-off. The role of light and carbohydrates is further supported by computational analyses (http://www.dna.affrc.go.jp/htdocs/PLACE) performed on *KNOPE3* orthologues, namely *Arabidopsis KNAT3* and grapevine CAO67726, which both hosted several light and sugar responsive motifs in the promoters (not shown).

Experiments of sugar uptake from sink leaflets showed that glucose, fructose and sucrose specifically triggered *KNOPE3* transcription and more rapidly than sorbitol. The delayed sorbitol action and the clear difference between fructose and sucrose/glucose kinetic patterns (Fig. 8a)

sustained a sugar-specific effect. The insignificant effect of wounding further corroborated the sugar peculiarity. The quick *KNOPE3* up-regulation was consistent with that of several *Arabidopsis* homeobox TFs, including *KNAT3*, occurring at 3 h after glucose treatment of seedlings. Moreover, de novo protein synthesis was proposed to mediate the glucose induction (Price et al. 2004). In this context, the production of KNOPE3 antibodies would be auspicial to assess whether mRNA up-regulation associates to protein accumulation (as well as to confirm the mRNA and protein co-localization), considering that transcript and protein levels may not correlate as a result of multiple regulatory mechanisms (Bassett 2007; Schutze et al. 2008). *KNOX* alternative splicing is known to occur (reported in "Introduction"), but no specific data on post-translational modification have been available to our knowledge so far. The *KNOPE3* late response to sorbitol suggests that sorbitol metabolites (and not the polyol itself) ignite the *KNOPE3* up-regulation. The *SDH* induction preceded that of *KNOPE3* after sorbitol supply, hence the fructose released by SDH might be a candidate of *KNOPE3* triggering. Another candidate may be the glucose produced by the sorbitol oxidase (SOX, Yamaki 1980). SOX plays a negligible role in peach and apple sink leaflets (Lo Bianco et al. 1999; Zhou et al. 2006) and its expression was not tested because there has not been any sequence available in any plant database so far. Hence, it is not excluded that SOX-derived glucose may also mediate *KNOPE3* response to sorbitol. Finally, leaf *KNOPE3* up-regulation was observed to proceed along with the endogenous increase of transport sugars due to the block of source-to-sink trafficking. Class 2 KNOX may have a role in carbohydrate translocation by acting on genes encoding sugar carriers and/or enzymes of sugar metabolism. For instance, computational analyses show that the *S6PDH* genes of poplar and apple (acc n. P28475 and AF057134) harbour several KNOX binding motifs. This prompts our future investigation towards the elucidation of class 2 KNOX role in nutrient transport, which is pivotal for organ differentiation, growth and development.

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