ORIGINAL PAPER

Impact of *InMIR319* and light on the expression of *InTCP4* gene involved in the development of *Ipomoea nil* plants

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Abstract MicroRNAs regulate gene expression by guiding the cleavage or attenuating the translation of target mRNAs. In *Arabidopsis thaliana*, the subset of class II *TCP* genes (plant-specific group of transcription factors) contains an miR319-binding site. One of them, *AtTCP4*, regulates negatively leaf growth and positively leaf senescence. In addition, miR319 targeting of *TCP4* is critical for petal and stamen development and affects flowering time. The aim of this work was to identify the cDNA of *InTCP4* gene and In-miR319 precursor in *Ipomoea nil* (*Pharbitis nil*). The cDNA sequence of *InTCP4* shows a significant similarity to the cDNA members of the *TCP* family of other plant species and contains nucleotides complementary to miR319. The identified sequence In-pre-miR319 creates a long hairpin structure and mature miRNA sequence is located in a similar place as in precursors found in other plant species. Accumulation of *InTCP4* mRNA and In-pre-miR319 was examined in various organs of *I. nil* plants. We found that the *InTCP4* is strongly expressed in cotyledons of *I. nil* seedlings while the In-pre-miR319 accumulates mainly in the hypocotyls of seedlings. Moreover, we investigate the role of *InTCP4* in the flowering induction, flower development and cotyledon senescence in *I. nil*. We indicate that the *InTCP4* expression is controlled by both light/clock and miR319. Both *InTCP4* and *InMIR319* probably participate in the regulation of such processes as do their homologues in other plant species, the development of cotyledons, leaves and flower elements. The main function of *InMIR319* seems to be the regulation of *InTCP4* organ localization.

Keywords *InTCP4* · *Ipomoea nil* · microRNA · miR319 · In-pre-miR319

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Introduction

MicroRNAs about 19–21 nt in length are involved in silencing target genes, by inactivation of their expression in plants usually at the transcriptional level. MiRNAs are obtained as a result of *MIR* gene expression encoding precursor RNA, which is sliced to the mature miRNA molecule. The miRNA nucleotide sequence length of 19–21 nt is sufficiently long to bind complementary-specific mRNA encoding some protein, and lead to its degradation or inhibit the process of translation. What is more, the miRNA sequence and the complementary sequence in the target mRNA are highly conserved within kingdoms (Reinhart et al. 2002; Zhang et al. 2007; Voinnet 2009).

On the basis of research conducted on *Arabidopsis thaliana*, a number of miRNAs involved in the regulation of various developmental processes were identified (Bonnet et al. 2006; Jones-Rhoades et al. 2006; Rubio-Somoza and Weigel 2011). Among others, it has been shown that the miRNA and its target genes regulate different stages of development of leaves, from the initiation of their formation to senescence (Kidner 2010; Pulido and Laufs 2010).

Leaf development is a comprehensive process that involves the transition from a small group of undifferentiated cells to a structure of a particular size and shape, highly organized, composed of different cell types with specialized functions. During this developmental sequence of events, growth and differentiation must be coordinated by a complex regulatory network in which small RNAs, mainly microRNA, but also ta-siRNAs (trans-acting small interfering RNA), begin to play an important role as a new class of gene expression regulators (Kidner 2010; Pulido and Laufs 2010). The main function of leaves is to carry out the process of photosynthesis and transpiration; they are the sites of intensive gas exchange between the plant and the environment as well. The leaves and cotyledons are also places of photoperiodic signal perception in the photoperiodic flowering induction, where the formed flowering signal is transported to the apical meristem, which leads to the transformation of vegetative shoot apical meristem into generative (Thomas and Vince-Prue 1997; Izawa et al. 2002; Hayama and Coupland 2004; Imaizumi and Kay 2006).

In the mechanism of formation of the leaves, the miR160 target genes are *ARF10* (*AUXIN RESPONSE FACTOR*), *ARF16* and *ARF17* (Mallory et al. 2005; Wang et al. 2005; Liu et al. 2007). Next, miR164 regulates the size of the border between the organs by modulating expression of *CUC1* (*CUP-SHAPED COTYLEDONI1*) and *CUC2* (Laufs et al. 2004; Mallory et al. 2004; Baker et al. 2005; Sieber et al. 2007). Two miRNAs, miR156 and miR172, are involved in the phase transition from juvenile to mature plants of *A. thaliana* and *Zea mays* (Aukerman and Sakai 2003; Lauter et al. 2005; Wu et al. 2009). In the process of leaf senescence in *A. thaliana* two miRNAs were described: miR164 and miR319, for which the target genes are *ORE1* and *TCP2, -3, -4, -10, -24*, respectively (Woo et al. 2001; Schommer et al. 2012). All targets of miR319 are closely related members of the class II subclass of *TCP* genes (Palatnik et al. 2003). In plants, the genes encoding TCP-specific transcription factors containing a conserved DNA-binding domain named TCP. This domain does not show, at the level of sequence, any homology to other known DNA-binding domains, but forms a structure similar to the bHLH domain (basic Helix–Loop–Helix). Such a domain protein is described in TB1 (TEOSINTE BRANCHED1) from maize, CYC (CYCLOIDEA) of *Antirrhinum* and PCF1 and 2 (PCNA PROMOTER BINDING FACTOR) of rice, hence the name of this domain (Cubas et al. 1999; Martin-Trillo and Cubas 2010).

Ipomoea nil (called also *Pharbitis nil*) is one of the most commonly used model short day plants (SDP) to study the process of photoperiodic flower induction. Treatment of *I. nil* seedlings at an early stage of development with a single cycle of induction (16 h of darkness) is sufficient for the plants to produce generative buds (Vince-Prue and Gressel 1985; Hayama et al. 2007). The place of perception of the photoperiodic signal may be in cotyledons of seedlings as young as 5 days old. This is the time when these organs have the highest sensitivity to the photoperiodic night. This is probably related to the achievement by the cotyledons of the appropriate stage of development (Ogawa and King 1990; Hayama et al. 2007).

In our previous paper, we described the first miR172 target gene *InAP2-like* identified in *I. nil* which is involved in the regulation of photoperiodic flower induction in *I. nil* (Glazińska et al. 2009). Studies indicate that in *A. thaliana* and tomato, miR319 and its target genes from the *TCP* family are involved in the regulation of both morphogenesis and leaf senescence. This probably occurs through control of the expression of JA biosynthesis genes, such as *LOX2* (*LIPOXYGENASE 2*) (Schommer et al. 2008). More than that, the *rTCP* mutants of *A. thaliana* with an altered miRNA-binding site, and thus "resistant" to miR319 show, in addition to changes in leaf morphology, also delayed flowering. miR319a targeting of *TCP4* is also critical for petal and stamen development in *Arabidopsis* (Schommer et al. 2012).

Numerous studies have shown that JA is involved in the regulation of many processes such as flower development, senescence and organ abscission (Ueda and Kato 1980; Turner et al. 2002; Yoshida 2003; Acosta and Farmer 2010). JA is an inhibitor of photosynthetic enzymes, inhibits the biosynthesis and accelerates the degradation of carboxylase ribulose-1,5-bisphosphate, and the rate of breathing and the proteolytic activity (Parthier 1990). JA can thus affect the "biological" age of the cotyledons, and thus adjust the time sensitivity of plants to the inductive photoperiod. The *I. nil* study demonstrated that disruption of the endogenous level of JA during night inhibits the induction of flowering (Maciejewska et al. 2004; Kęsy et al. 2012); therefore we identify *InTCP4* cDNA and In-pre-miR319 from *I. nil* cotyledons, observe its expression in various organs of plants and investigate the role of *In-TCP4* in flowering induction. We also examine their participation in flower development and cotyledon senescence of *I. nil*.

Materials and methods

Plant material

Seeds of *Ipomoea nil* Chois cv. *Violet* (Marutane Seed Co., Kyoto, Japan) were treated according to Glazińska et al. (2009).

Organ expression

Plants were grown under LD conditions for 5 days and then were exposed to a 16-h-long inductive night. The apexes, cotyledons, hypocotyls and roots were picked after a short night in 6 days of growth and immediately frozen in liquid nitrogen and stored at −80 °C. In addition, pistils, stamens, sepals and petals were harvested from fully open flowers. Leaves were harvested from 3-week-old plants (Fig. 4).

Each experiment was repeated at least three times. All data are presented as mean ± standard error (SE).

Expression analysis during pistil and stamen development

For development analysis of pistils and stamens, flower buds were collected at various stages of growth (Fig. 4. Stage 1–5), from the appearance of flower buds with visible stamens and pistils to fully open flowers. Flower elements, pistils and stamens, were collected from all stages of growth. Thereafter, material was frozen in liquid nitrogen and stored at −80 °C for RNA isolation.

Expression analysis under photoperiodic conditions

The expression analysis of *InTCP4* was performed in four photoperiodic variants. In all variants, the seedlings of *I. nil* were grown under LD conditions for 5 days and subsequently divided into two portions. The first one was left under LD conditions (8 h of darkness—negative flowering control) (variant I), the second was transferred to SD conditions (16-h night) (variant II–IV). In variant II, the seedlings were irradiated in hour 8 of the darkness with a 15-min-long pulse of red light (R, 1.5 µmol m^{-2} s^{-1}, fluorescent tubes TLD 15 Red/18 W, Philips, Holland). In variant III, IAA applications on cotyledons were performed once before starting the 16-h inductive night. IAA (0.5 mM) applications to both sides of the cotyledons were performed with the use of a small soft brush (about 50 µL/plant). Variant IV was control plants without any treatment (positive flowering control).

Cotyledons (without petioles) were harvested every 3 h after transfer to the darkness (variant I and IV). In experiments with the influence of IAA and a 15-min-long pulse of red light (variant II, III) for *InTCP4* expression analysis, cotyledons were picked every 3 h after hormone or light application. Subsequently, the cotyledons were frozen in liquid nitrogen and stored at −80 °C. All manipulations during the dark period were done under dim green safe light.

Expression analysis during cotyledon senescence

The 6-day-old plants were divided into four variants. Three subjected to ageing, which was induced by placing a dark (variant I) or exogenous application of phytohormones, accelerating the ageing process, such as jasmonic acid methyl ester (JAMe) at a concentration of 100 µM (variant II), and abscisic acid (ABA) with a concentration of 0.5 mM (variant III). Some plants were left at LD for further growth as a control (variant IV). Samples were collected in the next 6 days of growth after the phytohormone application or inserted into the darkness. Before starting the experiment, and at each harvest, to monitor the ongoing process of ageing, the amount of chlorophyll in the cotyledons was investigated. The material collected was placed immediately in liquid nitrogen and stored at −80 °C.

Molecular cloning of *InTCP4* cDNA

The tissue of *I. nil* (1.0–1.5 g) was homogenized in a sterile chilled mortar with a pestle. Total RNA was isolated from cotyledons with the GeneMATRIX Universal RNA Purification Kit (EurX Gdansk, Poland) according to the manufacturer's instructions. First-strand cDNA was synthesized with 1 µg of total RNA and 0.5 µg anchored oligo (dT)$_{19}$ primer according to the protocol Transcriptor High Fidelity cDNA Synthesis Kit (ROCHE Diagnostics GmbH, Germany). PCR, using degenerated primers 5′-WGTY-GARGTRCAAGGYGGTCACAT-3′ (forward) and 5′-AGGWGTGTWACTGGACTGAAGGGGW-3′ (reverse), was performed in the T3 Thermocycler (Biometra, Göttingen, Germany). Primers were designed from sequences conserved between *TCP4* from *A. thaliana*, *Solanum lycopersicum* and *Malus domestica*. All primers used were synthesized by Genomed S.A. Warsaw, Poland. Mixtures and conditions of PCR reaction were prepared according to Glazińska et al. (2009). An amplified cDNA fragment was isolated from an agarose gel with the GeneMATRIX Agarose Out DNA Purification Kit (EurX, Gdansk, Poland), cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California) and sequenced by Genomed S.A. Warsaw, Poland. A full-length cDNA coding for *InTCP4* was isolated using the BD SMART RACE cDNA Amplification Kit (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). mRNA used in 5′- and 3′-RACE-PCR was isolated with Dynabeads Oligo (dT)$_{25}$ (Dynal Biotech, Oslo, Sweden). Gene-specific primers for 5′-RACE (5′-AGCAGCTTCAGCAACAGGAGGATAC TTG-3′ outer primer, 5′-GGCACGGTGGTGGTGGTGG AGACG-3′ inner primer) and 3′-RACE (5′-CGTAGCC GAGCCGATCCTGGACATC-3′ outer primer, 5′-GGGTT AACGGTACTGGGTTTCCAGGCG-3′ inner primer) were picked from a cDNA sequence of the identified clone using FastPCR 5.4.30 software. PCR reactions were performed using the Advantage 2 PCR Enzyme System (Clontech-Takara Bio Europe, Saint-Germain-en-Laye, France). The

RACE products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, California) and sequenced by Genomed S.A. Warsaw, Poland.

The identified cDNAs of the *InTCP4* gene were analysed using ClustalW (http://www.ebi.ac.uk/clustalw), BLAST 2.2.25 (http://www.ncbi.nlm.nih.gov/BLAST) ExPASY (http://www.expasy.org) and Search miR-Base::Sequences (http://microrna.sanger.ac.uk/sequences/). The phylogenetic analysis was made using the Phylogeny.fr web service (http://www.phylogeny.fr/version2_cgi/simple_phylogeny.cgi). Sequence alignments were carried out using the MUSCLE software and then the Gblocks program was used to eliminate poorly aligned positions and divergent regions. The phylogenetic analysis was made using the PhyML program. The phylogenetic tree was constructed using the TreeDyn program.

Identification of In-pre-miRNA319 sequence

Basic Local Alignment Search Tool (BLAST) (Altschul et al. 1990) was used to search for the fragment pri-miR319 including the pre-miRNA sequence in the EST sequences database of *I. nil*. The MiR319 sequence was obtained from the miRBase database (ftp://mirbase.org/pub/mirbase/12.0/) (Griffiths-Jones et al. 2008). Because sequences of miR319 show conservation across different kingdoms (Axtell and Bartel 2005; Zhang et al. 2006), we use the miRNAs from *A. thaliana*. EST no: CJ744218 that potentially contain miRNA sequences were obtained after the BLAST search. The RNAstructure software (http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html) was used to compute their secondary structure.

The presence of the identified sequence in the plant was confirmed by the RT-PCR reaction with specific primers 5′-ACTGAGTGAATGATGCGGGAGACAA-3′ (forward) and 5′-CCAATGAAAAACCCTTTCTCCAGC-3′ (reverse) with GoTaq Hot Start Colorless Master Mix (Promega Corporation, USA) according to the manufacturer's instructions. An amplified DNA fragment was isolated from an agarose gel with the GeneMATRIX Agarose Out DNA Purification Kit (EurX, Gdansk, Poland), cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and sequenced by Genomed S.A. Warsaw, Poland. The identified sequences were analysed using Search miRBase web software.

Quantitative RT-PCR analysis of *InTCP4* gene and In-pre-miR319 expression

The gene expression analysis was performed by real-time PCR (qPCR) with a LightCycler 2.0 Carousel-Based System (ROCHE Diagnostics GmbH, Germany) and

Fig. 1 a Nucleotide and deduced amino acid sequence of InTCP4. ▶ Amino acid sequences marked in *grey* represent TCP domain. *Underlined* nucleotide sequences correspond to target site for miR319. **b** The phylogenetic relationship of *InTCP4* compared with the *TCP* genes family from various plant species. GeneBank Accession Nos. from *top* to *bottom* HM210875.1, AF175965.1, HM210876.1, EF091574.1, AY205603.1, AB531021.1, NM_180258.2, GQ505958.1. **c** Alignment of part of nucleotide sequences of *InTCP4* with homologous genes from other plant species and mature miR319a, -b, and -c from *A. thaliana*. *Stars* denote identical nucleotides

LightCycler TaqMan Master Kit (ROCHE Diagnostics GmbH, Germany). The templates for qRT-PCR were prepared the same way as for molecular cloning of *InTCP4* cDNA.

Gene-specific primers and a UPL probe were designed using Universal ProbeLibrary Assay Design Center (http://www.roche-applied-science.com/sis/rtpcr/upl). The qPCR reactions were made for the following genes: *InTCP4*, *In-CAB2* (*CHLOROPHYLL A/B BINDING PROTEIN2*) (GenBank: D78207), *InLOX2* (identified EST sequences in the NCBI database of *I. nil*: CJ761975), and *InACT4* (GenBank: HM802138.2). Actin was used as reference endogenous control for normalization purposes. This technique was also used to analyse the accumulation of In-pre-miR319.

The qPCR conditions included an initial denaturation step 95 °C for 10 min followed by 45 cycles of 95 °C for 10 s, 58 °C for 30 s, 72 °C for 1 s followed by 1 cycle of cooling at 40 °C for 30 s.

qPCR analyses were performed with the gene-specific primers: *InTCP4* (61 bp) 5′-GTTAACCCGAATTTCGACCA-3′ (forward) and 5′-CATCTCCGGGGGTCTCTT-3′ (reverse) with hydrolysis probe UPL 105, In-pre-miR319 (76 bp) 5′-TTCCCTTTTCCACACTAATCG-3′ (forward) and 5′-AACTGGCAAACACCAACAAA-3′ (reverse) with hydrolysis probe UPL20, *InLOX2* (70 bp) 5′-CACGCGGACAAGAAGGAT-3′ forward) and 5′-TCAGGATTCCAATCAGGTCA-3′ (reverse) with hydrolysis probe UPL 64, *InCAB2* (78 bp) 5′-CGGAAAGGCTGTGAAGGTC-3′ (forward) and 5′-CCAGCTGTCTTCCTCATGGTA-3′ (reverse) with hydrolysis probe UPL 97, *InACT4* (65 bp) with the gene-specific primers 5′-GGAAATACAGTGTCTGGATTGGA-3′ (forward) and 5′-CCACATCTGTTGGAATGTGC-3′ (reverse) with hydrolysis probe UPL 139 (ROCHE Diagnostics GmbH, Germany). qPCR mixtures were made as follows: 1 µL of 1st strand cDNA, 0.4 µL of gene-specific primer solution (10 µM), 0.2 µL of gene-specific hydrolysis probe and 4 µL of Master Mix (containing reaction buffer, dNTP mix and DNA Polymerase) from LightCycler TaqMan Master (ROCHE Diagnostics GmbH, Germany). The

(a)

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     M A E N R Q Q Q Q G S S S R L G L R N A G G E I V E V Q G G
  1  ATGGCGGAGAATCGTCAGCAGCAACAGGGGAGTTCGTCGAGATTGGGCCTAAGGAACGCCGGCGGCGAGATAGTGGAAGTCCAAGGCGGC
     H I V R S T G R K D R H S K V C T A K G P R D R R V R L A A
 91  CACATTGTGAGGTCCACGGGGCGGAAAGACCGCCACAGCAAGGTCTGCACAGCCAAAGGCCCCAGGGACCGCCGGGTCCGCCTAGCCGCC
     H T A I Q F Y D V Q D R L G Y D R P S K A V D W L I N K A K
181  CACACTGCTATTCAGTTCTACGATGTCCAGGATCGGCTCGGCTACGACCGCCCCAGCAAGGCCGTCGATTGGCTCATCAATAAGGCCAAA
     P A I D E L A E L P A W K P S T V N P N F D Q E D A Q K Q H
271  CCCGCCATTGACGAGCTTGCTGAGCTACCCGCCTGGAAACCCAGTACCGTTAACCCGAATTTCGACCAAGAAGATGCCCAGAAACAGCAC
     Q E T P G D A N L L D N V A G P S S K R S M I M Q E S E N E
361  CAAGAGACCCCCGGAGATGCCAATTTGTTGGATAATGTCGCTGGGCCTTCTAGTAAGAGAAGCATGATAATGCAAGAGAGTGAAAATGAA
     S S F L P P S L N S D S I A D T I K S F F P M G A S E Q P I
451  TCGAGCTTTTTGCCTCCTTCTTTGAATTCTGACTCCATTGCTGATACTATTAAGTCCTTCTTCCCCATGGGAGCTTCAGAGCAGCCTATT
     P N S S S M Q F Q S F A Q H S D L L S S Q N Q D L R L S L Q
541  CCTAATTCTTCCTCTATGCAGTTCCAGAGCTTTGCCCAGCACTCCGATTTACTGTCTTCCCAGAACCAAGATTTGAGGCTTTCTTTACAG
     S F Q D P T I L L H H Q N Q Q A A Q H H Q N P A S H R A I L
631  TCATTTCAAGATCCTACTATCCTTCTCCACCACCAAAACCAGCAAGCAGCTCAGCACCACCAGAACCCGGCTAGCCATAGAGCCATTTTA
     T G F D A S G W S E H N Q H R F P G W H G G G G D A A A A
721  ACCGGGTTCGATGCTTCAGGCTGGTCTGAGCACAATCAGCATAGGTTTCCGGGTTGGCACGGTGGTGGTGGTGGAGACGCCGCTGCTGCC
     A S C S A S G S G G A A A S A T G G Y L F N S P Q Q P L F Q
811  GCCTCCTGCTCCGCTAGCGGCAGTGGTGGCGCAGCAGCTTCAGCAACAGGAGGATACTTGTTCAATTCCCCACAGCAGCCACTGTTCCAG
     Q L F G G Q N Q F F P S Q R G P L Q S S Y S P S V R A W I D
901  CAGTTGTTTGGTGGGCAGAACCAGTTTTTTCCTTCTCAGAGGGGACCCCTTCAGTCCAGTTACTCGCCTTCGGTTCGTGCTTGGATCGAC
     P S P A I A I A S V D P N Q Q H Y Q L P I Y P S S L S G I G
991  CCATCACCCGCGATAGCCATTGCCAGTGTTGATCCAACCAGCAGCACTACCAGCTACCCATTTACCCATCATCACTATCGGGTATAGGA
     F S T G V G E F S G F R I P A R I Q G E E E E H D G I S D K
1081 TTCTCCACGGGAGTGGGGGAATTCTCTGGGTTTCGCATTCCTGCACGAATCCAAGGTGAAGAAGAGGAGCACGATGGCATTTCCGACAAG
     P S S A S S D S R R
1171 CCATCCTCTGCTTCCTCTGACTCCCGTCGTTGAATTAGCTCCCAATAATCTCTCGTTTCTTCTCTCCAAGGATATGATGAATTAGTTATG
1261 CAAGAGATCTTGGAGGCAGAGAAGGAAAGGGGAGTTTGCCAGGTCTAAACCAATGGCGGTCACCCGAGGTAGAAGACTTGTTGGCATAGT
1351 TTCAGGTTTTTCTATCCCGGGCATTATTGTTATCTGTATTAGTTCATGTCGATTAATATCAAGTTTTACATTTGTGTATGATTGAGTGGG
1441 CTTCCCGAGCTCAACTTTGTCTAGTTTGAGGATTATGTTGTGTTTCTATTGCCATCCTAATCAGGGAGCTTACTCCTTGCTTG
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(b)
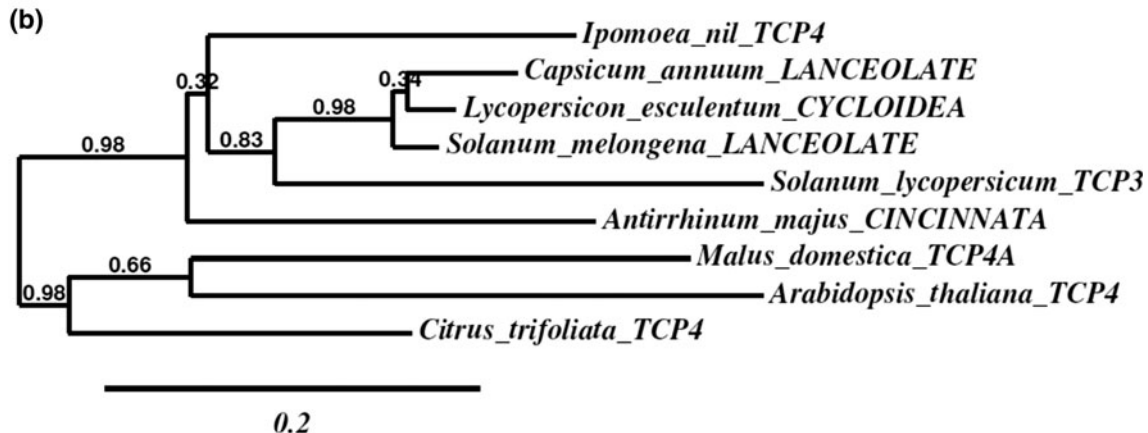
(c)

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                         ** *  ************
Ath-miR319a    (1)    -----AGGGAGCTCCCTTCAGTCCAA--------
Ath-miR319b    (1)    -----AGGGAGCTCCCTTCAGTCCAA--------
Ath-miR319c    (1)    -----AAGGAGCTCCCTTCAGTCCAA--------
    InTCP4   (934)    TCTCAGAGGGGACCCCTTCAGTCCAGTTACTCGC
    AtTCP4  (1277)    TCTCAGAGGGGTCCCCTTCAGTCCAGTTACAGTC
      CaLa  (1318)    ACACAGAGGGGACCCCTTCAGTCCAGTAACACAT
    CtTCP4  (1334)    ACACAGAGGGGTCCCCTTCAGTCCAGTAACACGC
     LeCYC  (1473)    TCTCAGAGGGGACCCCTTCAGTCCAGTAACACAT
   MdTCP4A  (1033)    TTTCAGAGGGGACCCCTTCAGTCCAGTAACTCGC
    SlTCP3  (1364)    TCTCAGAGGGGACCCCTTCAGTCCAGTAACACAT
      SmLA   (901)    TCTCAGAGGGGACCCCTTCAGTCCAGTTACTCAC
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computer application used for the analysis was LCS4.0 (ROCHE Diagnostics GmbH, Germany) and for the calculations and graphs, MS Office Excel (Microsoft).

qPCR reactions were performed in triplicate for each RNA sample. All data are presented as mean ± standard error (SE).
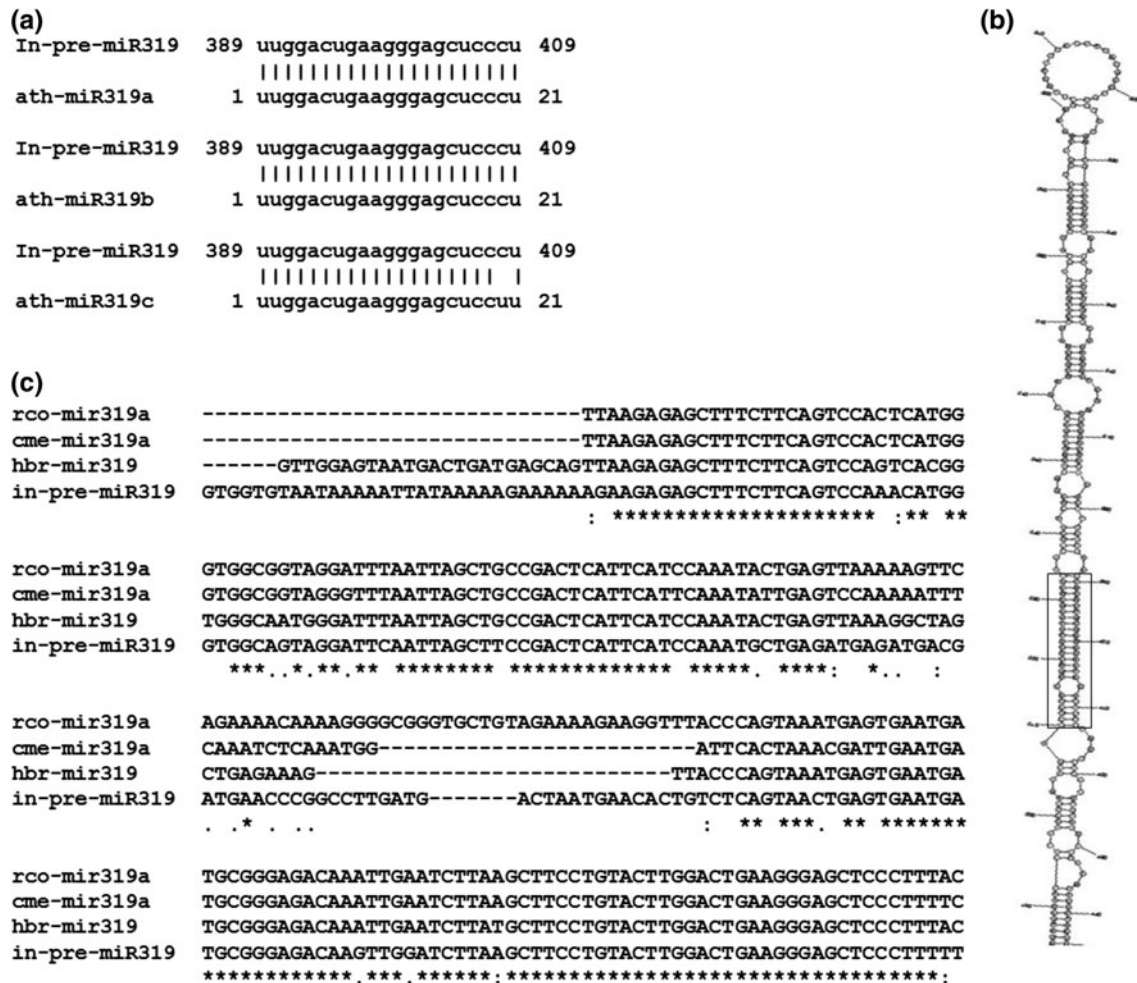
(a)

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In-pre-miR319    389 uuggacugaagggagcucccu 409
                     |||||||||||||||||||||
ath-miR319a        1 uuggacugaagggagcucccu 21

In-pre-miR319    389 uuggacugaagggagcucccu 409
                     |||||||||||||||||||||
ath-miR319b        1 uuggacugaagggagcucccu 21

In-pre-miR319    389 uuggacugaagggagcucccu 409
                     |||||||||||||||||| |
ath-miR319c        1 uuggacugaagggagcuccuu 21
```

(b)

(c)

```
rco-mir319a    ------------------------------TTAAGAGAGCTTTCTTCAGTCCACTCATGG
cme-mir319a    ------------------------------TTAAGAGAGCTTTCTTCAGTCCACTCATGG
hbr-mir319     ------GTTGGAGTAATGACTGATGAGCAGTTAAGAGAGCTTTCTTCAGTCCAGTCACGG
in-pre-miR319  GTGGTGTAATAAAAATTATAAAAAGAAAAAAGAAGAGAGCTTTCTTCAGTCCAAACATGG
                                            : ******************** :** **

rco-mir319a    GTGGCGGTAGGATTTAATTAGCTGCCGACTCATTCATCCAAATACTGAGTTAAAAAGTTC
cme-mir319a    GTGGCGGTAGGGTTTAATTAGCTGCCGACTCATTCATTCAAATATTGAGTCCAAAAATTT
hbr-mir319     TGGGCAATGGGATTTAATTAGCTGCCGACTCATTCATCCAAATACTGAGTTAAAGGCTAG
in-pre-miR319  GTGGCAGTAGGATTCAATTAGCTTCCGACTCATTCATCCAAATGCTGAGATGAGATGACG
                ***..*.**.** ******* ************ *****. ****:  *..  :

rco-mir319a    AGAAAACAAAAGGGGCGGGTGCTGTAGAAAAGAAGGTTTACCCAGTAAATGAGTGAATGA
cme-mir319a    CAAATCTCAAATGG-----------------------ATTCACTAAACGATTGAATGA
hbr-mir319     CTGAGAAAG-------------------------TTACCCAGTAAATGAGTGAATGA
in-pre-miR319  ATGAACCCGGCCTTGATG-------ACTAATGAACACTGTCTCAGTAACTGAGTGAATGA
                .  .*  ...            :  ** ***. ** *******

rco-mir319a    TGCGGGAGACAAATTGAATCTTAAGCTTCCTGTACTTGGACTGAAGGGAGCTCCCTTTAC
cme-mir319a    TGCGGGAGACAAATTGAATCTTAAGCTTCCTGTACTTGGACTGAAGGGAGCTCCCTTTTC
hbr-mir319     TGCGGGAGACAAATTGAATCTTATGCTTCCTGTACTTGGACTGAAGGGAGCTCCCTTTAC
in-pre-miR319  TGCGGGAGACAAGTTGGATCTTAAGCTTCCTGTACTTGGACTGAAGGGAGCTCCCTTTTT
                ***********.***.******:*****************************:
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Fig. 2 Alignment of the nucleotide sequences of In-pre-miR319 with **a** mature miR319a, -b, and -c from *A. thaliana*, **b** precursors of miR319 of other plant species indicate the highest homology. *Stars* denote identical nucleotides. miRBase Accession Nos. from *top* to *bottom* MI0013406, MI0023233, MI0022056. **c** Secondary stem-loop structures of the In-pre-miR319. Localization of mature miR319 is indicated on the stem of the precursor sequence

Measurement of the relative amounts of chlorophyll in cotyledons

The amount of chlorophyll was determined by the method of CCl (chlorophyll content index) with the device Opti-Sciences CCM-200 (Opti-Sciences, Inc., USA). Each measurement of relative chlorophyll content was performed on at least three different plants of the experimental variant. Each measurement was performed three times. The data obtained were then subjected to statistical analysis.

Mapping of the *InTCP4* mRNA internal cleavage sites

To determine the internal cleavage site in *InTCP4* mRNA, RNA ligase-mediated rapid amplification of cDNA ends (5′ RACE) was performed with the First Choice RLM-RACE Kit (Ambion). The procedure was based on Glazińska et al. (2009). A unique gene-specific DNA fragment was first amplified with the 5′ RACE outer primer (Ambion) and gene-specific primer (5′-TACCTCGGGTGACCGCCAT TGG-3′) and then with the 5′ RACE inner primer (Ambion) and nested gene-specific primer (5′-AGCAGAGG ATGGCTTGTCGGA-3′). The RACE products were cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA, USA) and sequenced by Genomed S.A. Warsaw, Poland.

Results

Isolation of cDNA for the *InTCP4* gene

The cDNA sequence of the gene *TCP4* homologue was identified with RT-PCR with degenerate primers. These primers were designed to conserve sequences encoding the TCP domain, and a sequence complementary to miR319

based on cDNA sequence comparisons of the *TCP* gene family present in different plant species. After the RT-PCR reaction, a single product, with a molecular weight of 800 bp, was obtained (data not shown). The obtained reaction products were isolated, cloned and sequenced. Comparison of the sequenced cDNA fragment with the sequences chosen by BlastN showed its highest homology to cDNAs of the *TCP* gene family from other plant species. To isolate the full cDNA sequence of the gene *InTCP4*, the 5′ and 3′ RACE method was used. The obtained reaction
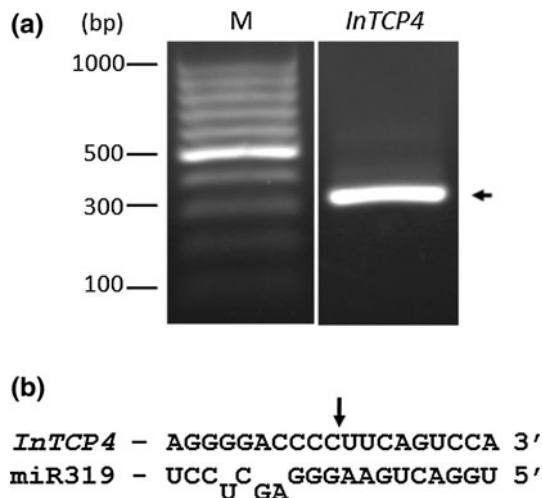
Fig. 3 5′- RACE to determine the cleavage site. **a** Nested PCR product (line *InTCP4*), spanning between the primer and cleavage sites, was used for cloning into a vector for DNA sequencing. Lane *M* represents 100 bp DNA ladders. **b** The *InTCP4* mRNA cleavage site is designated at the 11th base from the 5′ end, indicated by the *arrow*

products were isolated and cloned in the same way as for the reaction with degenerate primers. After connection all identified fragments formed a full-length cDNA gene *In-TCP4*, 1,523 bp in length (Fig. 1a). Then, the sequence was deposited in the NCBI database (National Center for Biotechnology Information) as *I. nil TCP* family transcription factor *TCP4* mRNA, complete cds no: HM357790.

The deduced InTCP4 protein had 400 aa residues and possesses TCP domains, characteristic for transcriptional factors from the TCP family (Fig. 1a).

An analysis of phylogenetic relationships between the identified cDNA of the *InTCP4* sequence and the cDNAs of the *TCP* gene family revealed that isolated cDNA was very closely related to *CYCLOIDA* (*CYC*) from *Lycopersicon esculentum*, and *LANCEOLATE* (*LA*) from *Capsinum annum* and *Solanum melongena*. A high degree of similarity of the sequence was also found for cDNA *CINCINNATA* (*CIN*) *Antirrhinum majus*, *TCP4A Malus domestica*, and *TCP4 A. thaliana* (Fig. 1b). Analysis of *InTCP4* cDNA using the software available on http://microrna.sanger.ac.uk/sequences/ also revealed the sequence complementary to miR319. This sequence is located at the end of the protein coding sequence, as in the other *TCP* genes identified in other plant species (Fig. 1c).

Identification of In-pre-miRNA319 sequence

To identify the precursor of miR319, the *I. nil* EST sequence database was searched with the miR319 sequence derived from *A. thaliana*. The BlastN program indicated that SEQ ID CJ744218 showed high homology to the
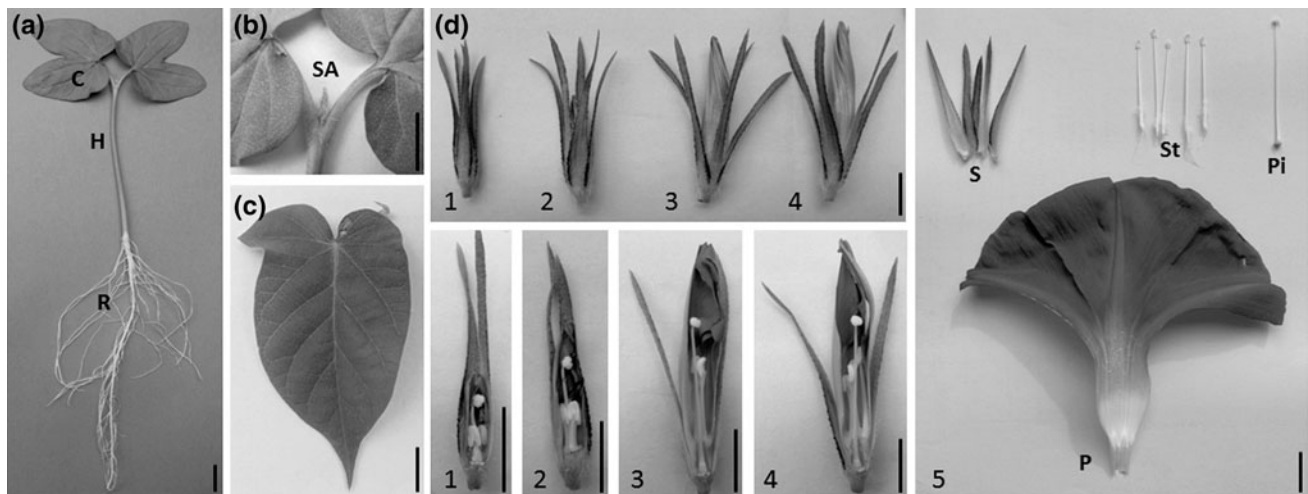

Fig. 4 Development of *I. nil* seedlings and flowers. **a** 5- day-old seedling. **b** Shoot apices of 5-day-old seedling. **c** Leaf of mature plant. **d** Development of flowers. *Upper panel*—whole flower buds, *below*—buds with flower elements shown. *On the right*—flower elements of a fully open flower. *Numbers (1–5)* represent the stages of flower growth from the first flower bud with easily visible stamens and pistils to fully open flowers. The symbols are: *C* cotyledons, *H* hypocotyls, *R* roots, *SA* shoot apices, *L* leaf, *ST* stamen, *PI* pistil, *P* petal, *S* sepals. *Black line*—bar 1 cm
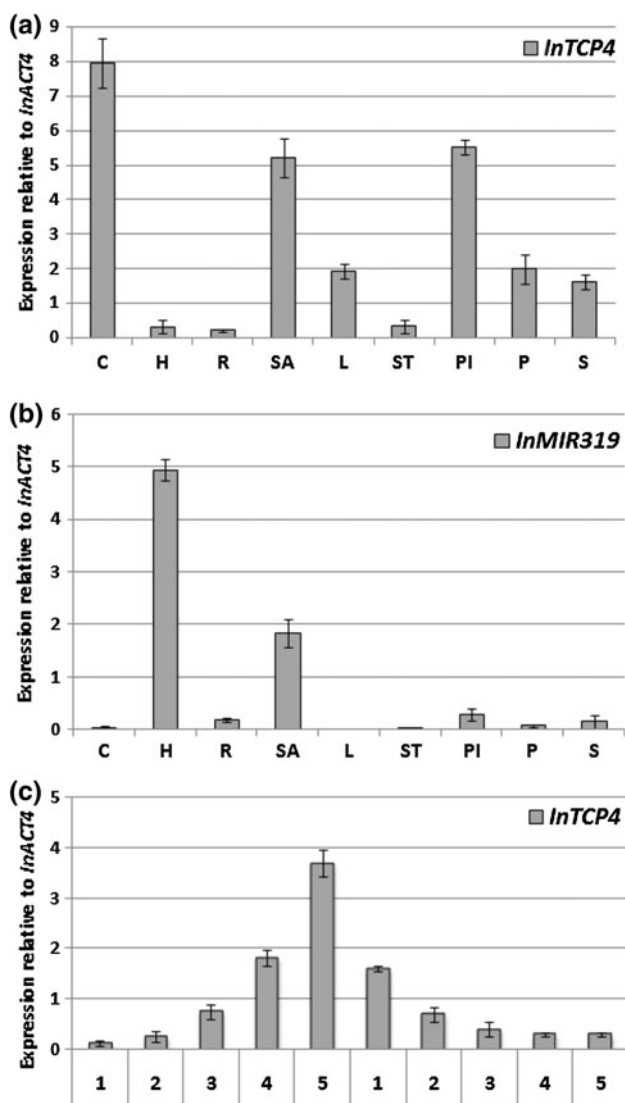
Fig. 5 The level (related to *InACT4*) in various plant tissues of *I. nil*, **a** *InTCP4* transcript and **b** In-pre-miR319 accumulation. The symbols are: *C* cotyledons, *H* hypocotyls, *R* roots, *SA* shoot apices, *L* leaf, *ST* stamen, *PI* pistil, *P* petal, *S* sepals. **c** The transcript level of *InTCP4* (related to *InACT4*) during stamen and pistil development of *I. nil*. *Numbers* (*1–5*) represent the stages of flower growth from the first flower bud with easily visible stamens and pistils to fully open flowers

similarity to the miR319 precursor sequence of a number of plant species, the highest to *Cucumis melo*, *Hevea brasiliensis* and *Ricinus communis* (Fig. 2c).

MiR319 mediates cleavage of *InTCP4* mRNA

The specific cleavage site of the *InTCP4* mRNA was determined by 5′ RACE, which takes advantage of the monophosphate present at the 5′ terminus of the 3′ cleavage fragment (Kasschau et al. 2003). The distinct PCR band of the expected size was observed for the *InTCP4* target (Fig. 3). Cloning and DNA sequencing of this amplified product mapped the 5′ end of the cleavage products to the designated position at the middle of the region complementary to miR319.

Expression patterns of the *InTCP4* and *MIR319* genes in various organs of *I. nil*

Accumulation of *InTCP4* and *MIR319* mRNA was examined in the vegetative and generative tissues of seedlings and mature *I. nil* plants (Fig. 4). The highest levels of *InTCP4* transcripts were indicated in cotyledons and next in shoot apices of 6-day-old seedlings. The high level of expression was also observed in pistils. A much lower level of mRNA *InTCP4* was observed in petals, sepals and leaves (Fig. 5a).

The results of RT-qPCR showed that In-pre-miR319 accumulates mainly in the hypocotyls of seedlings. In other organs, except shoot apexes of seedlings, the level of In-pre-miR319 was very low or almost absent (Fig. 5b).

Analysis of transcript level of *InTCP4* during pistil and stamen development

For analysis of pistil and stamen development, flower elements were isolated from generative buds collected at various stages of growth (Fig. 4), from the appearance of flower buds with easily visible stamens and pistils (stages 1–4) to fully open flowers (stage 5).

The transcript level of *InTCP4* increases during pistil growth (Fig. 5c). During the growth of stamens, *InTCP4* expression pattern is opposite and the lowest level occurs in the stamens of open flowers (Fig. 5c).

InTCP4 expression under LD and SD conditions

To investigate the possible participation of *InTCP4* in the mechanism of flower induction in *I. nil*, gene expression was examined in cotyledons harvested during LD (8-h darkness/16-h light) and SD conditions (16-h darkness/8-h light) (Fig. 6).
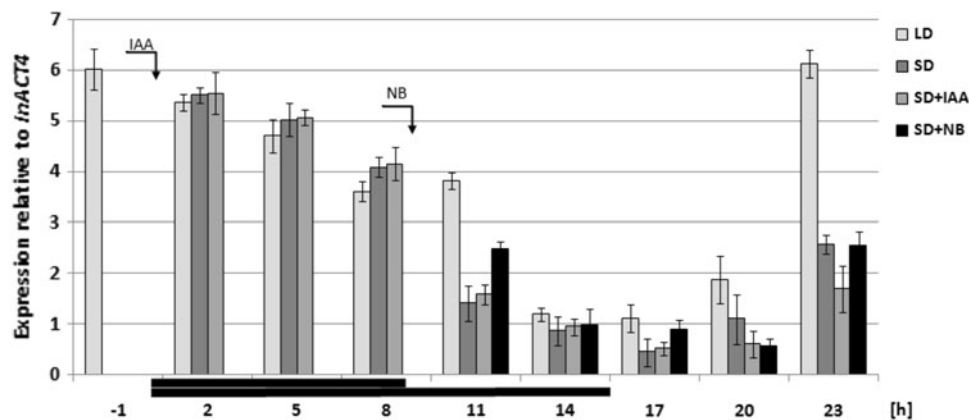
mature and precursor molecule of miR319 of other plant species (data not shown). We confirmed the presence of the identified sequence in plants using the RT-PCR reaction. An amplified DNA fragment was sequenced and analysed for the presence of sequences of mature miR319 (Fig. 2a) and secondary structure (Fig. 2b) with Search miR-Base::Sequences and RNAstructure software, respectively. A putative sequence of the mature molecule 21 nt miR319a/b occurs between 389 and 409 bp and is located in the stem forming a hairpin structure (Fig. 2a, b). The identified sequence was compared with the stem-loop pre-miR319 sequences of other plant species and shows great

Fig. 6 The level (related to *InACT4*) of *InTCP4* transcript (three replicates) in the cotyledons of plants cultivated under different light conditions and treated with IAA. The symbols are: *LD* long day; *SD* short day; *SD + IAA*—SD and IAA treatment before transfer to darkness; *SD + NB*—16-h darkness broken (night break) with 15 min red light. *Black lines*—dark period; *arrows* indicate IAA application to cotyledons and 15 min of red light

We observed that the level of *InTCP4* transcript successively decreased during the night. In light, a slight temporary increase in transcriptional activity occurred followed by a decrease and then gradual increase in expression during the day. For LD conditions, the amount of mRNA went back to the level before the night (Fig. 6). We also investigated the effect of factors that repress the flowering induction of *I. nil* on expression of *InTCP4*. As inhibitory factors, we used the IAA treatment before transfer of seedlings to inductive darkness and a night break in the 8th h with 15 min of red light in the 16-h inductive night. The obtained results indicated that auxin does not affect the expression of the gene. A night break causes a short-term increase in *InTCP4* expression and then a decrease in a similar way as in SD conditions (Fig. 6).

The investigation of the possible role of *InTCP4* in cotyledon senescence of *I. nil*

To investigate the possible role of *InTCP4* in cotyledon senescence of *I. nil*, we analysed the expression of *InTCP4* and jasmonic acid biosynthesis gene (*InLOX2*) during the various stages of ageing (Fig. 7c, d). As ageing-inducing factors, continuous darkness, jasmonic acid methyl ester (JAMe) and abscisic acid (ABA) were used. Changes in the amount of chlorophylls and *InCAB2* gene expression were used in the experiment as indicators of the ongoing process of senescence (Fig. 7a, b).

All the factors that induce ageing caused a decrease in the relative content of chlorophyll, and the expression of *InCAB2*. The strongest decrease was observed under the influence of darkness. In plants grown under control conditions (LD), a slight decrease was observed on the tested parameters due to the presence of the natural ageing process of the cotyledons, as a result of the appearance of the

leaves on the 8th day of growth of seedlings. *InLOX2* gene transcription activity in plants grown in the light and in the dark remained at a low level. The application of ABA and JAMe promotes the expression of *InLOX2*; however, the stimulatory effect of phytohormones is getting weaker in the days of the experiment (Fig. 7c). In cotyledons of the plants treated with ABA and JAMe compared to those grown in the light (control) we observed a slight increase in the level of transcript *InTCP4*, while darkness causes a significant decrease in the expression of a gene (Fig. 7d).

Discussion

Representatives of the class of *TCP* genes are important regulators of plant growth and development. They participate in the creation of the proper symmetry of flowers and petals, plant architecture, leaf morphogenesis, senescence, and regulate processes such as embryonic growth and circadian rhythm (Navaud et al. 2007; Giraud et al. 2010; Martin-Trillo and Cubas 2010). The activity of the part of the *TCP* gene family in *A. thaliana* is controlled by the family of *MIR* genes encoding regulatory micro RNA molecules—miR319 (Schommer et al. 2012).

To identify the *InTCP4* cDNA in *I. nil* plants, the RT-PCR technique and degenerated primers were used. Primers were designed for the sequence encoding DBD domain, as well as for the sequence containing nucleotides complementary to miR319. Using a 3′ and 5′ RACE-PCR reaction, we obtained the full sequence of *InTCP4* cDNA. The complete cDNA (GenBank accession number EF216864) consists of 1,523 bp and encodes 400 amino acids (Fig. 1a). An analysis of phylogenetic relationships between the identified cDNA of the *InTCP4* sequence and the cDNAs of the *TCP* gene family revealed that isolated
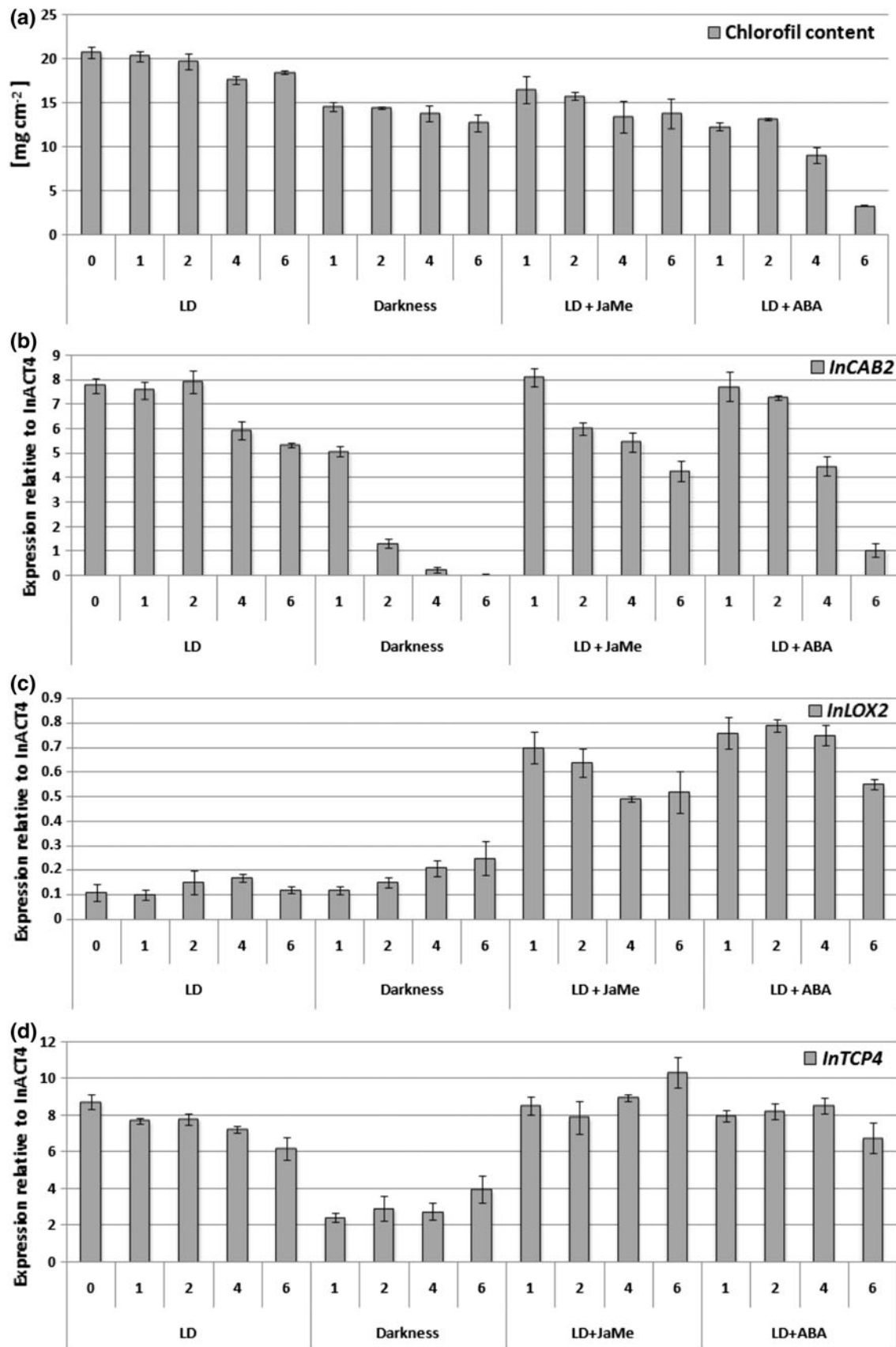
cDNA was very closely related to *CYC* from *L. esculentum*, *LA* from *C. annum* and *S. melongena*. A relatively high degree of homology was also indicated for *CIN A. majus*, *TCP4A M. domestica*, and *TCP4 A. thaliana* (Fig. 1b). Comparison between *InTCP4* mRNA and miR319a, -b, -c, -d from *A. thaliana* indicates that the isolated cDNA sequence contains miR319-complementary sequence and the strongest complementary sequence occurs to miR319c (Fig. 1c). The miRNA target site is located outside the TCP domain, near the 3′ part of the coding region, as in the other *TCP* genes identified in other plant species (Fig. 1c). The interaction between the *InTCP4* and miR319, as in other plant species (Schommer et al. 2012), has up to six mismatches, which is higher than in other known plant miRNA target pairs (Fig. 1c) but still, the predicted free energy is good enough for efficient interaction and guiding cleavage of the target mRNA (Palatnik et al. 2003). A direct role for miR319 in reducing *InTCP4* mRNA levels was demonstrated by the identification of *InTCP4* cDNA fragments whose 5′ termini are consistent with the products expected from miR319-directed mRNA cleavage (Fig. 3).

Members of the TCP family share an approximately 60-residue-long TCP domain that binds to DNA. Secondary structure prediction revealed that this domain forms a basic region followed by two helices separated by a loop (Cubas et al. 1999; Kosugi and Ohashi 2002; Aggarwal et al. 2010), even though it shares little sequence similarity with the classical bHLH domain. TCP domain class II starts from an N-terminal aminoacid Lys (K), which is conserved in 93 % of the members of the TCP protein family and contains three–residue insertion in the basic domain that is absent from class I (Aggarwal et al. 2010). Analysis of the putative amino acid InTCP4 sequence showed the presence of Lys and all other amino acids required for creating a functional TCP domain class II (Fig. 1a). This TCP domain is most similar to the domain present in CIN *A. majus* (data not shown), which indicates that InTCP4 may take part in the regulation of similar processes. The *CIN* gene is responsible for regulating the growth of the leaf blade and influencing the morphogenesis of flower petals (Crawford et al. 2004). Similar functions are also played by another gene with high similarity to the nucleotide sequence *InTCP4* of *I. nil*, namely *LA* from *S. melongena*. The *LA* gene regulates development of the leaves, and is also a target gene for *MIR319* (Ori et al. 2007).

To compare the expression of *InTCP4* and *MIR319*, we decided to identify the sequence of miR319 precursor. Because the sequence of miR319 showed conservation across different plant species (Zhang et al. 2006) we use the *A. thaliana* miR319 sequence from miRBase to identify pre-miRNA in the *I. nil* EST database. Based on comparison of the BlastN indicated sequence EST no. CJ744218 with mature and precursor sequences of miR319 from miRBase, we found the existence of *I. nil* miR319 precursor. We confirmed the presence of the identified sequence in *I. nil* using the RT-PCR reaction. An amplified DNA fragment was sequenced and analysed for the presence of sequences of mature miR319 (Fig. 2a) and secondary structure (Fig. 2b). The miR319 miRNAs are widely distributed in plants, including mosses (Arazi et al. 2005; Axtell and Bartel 2005; Axtell et al. 2007), and their precursors, which have long fold-backs with an extended upper stem above the miRNA, are also highly conserved (Palatnik et al. 2003; Li et al. 2005; Warthmann et al. 2008). The identified sequence In-pre-miR319 also creates a long hairpin structure and putative mature miRNA sequence is located in a similar place (Fig. 2b) as in precursors found in other plant species. It can therefore be concluded that the identified sequence is a precursor miR319 and encodes a mature, functional miRNA molecule.

Identification of In-pre-miR319 allowed the authors to examine the expression of *InTCP4* and *InMIR319* using the qRT-PCR technique. Accumulation of *InTCP4* and *MIR319* mRNA was examined in the vegetative and generative organs of *I. nil* plants (Fig. 4). High levels of *InTCP4* mRNA in cotyledons accompanied lack of accumulation of In-pre-miR319; the opposite situation occurs in hypocotyls: high expression of *InMIR319* causes mRNA of *InTCP4* to be almost absent (Fig. 5). This finding confirms that the amount of *InTCP4* transcripts is regulated by miR319, at the level of degradation of the mRNA. However, this relationship does not occur in all organs. In apexes, there are transcripts of both genes, which is probably associated with their participation in the regulation forming the leaves, as described in tomato (Ori et al. 2007). In *A. thaliana*, there are three genes encoding miR319 (*MIR319a*, -*b*, -*c*), *MIR319a* and *MIR319b* encoding identical mature miRNA and the expression patterns for the three *MIR319* genes are distinct, suggesting that these genes may have largely unique developmental functions (Nag et al. 2009; Schommer et al. 2012). It can therefore be assumed that other unidentified *MIR319* genes also exist in *I. nil* that may influence the accumulation of mRNA *InTCP4* in other tissues. Identified In-pre-miR319 contains a sequence of mature miRNA319 identical to miR319a/b *A. thaliana* (Fig. 2a) and *InMIR319* indicates a similar tissue expression pattern to *MIR319a* and *MIR319b*

in *A. thaliana* (Nag et al. 2009). The results of Nag et al. (2009) suggest that miR319a/b might not play an important role in leaf development, but in petal and stamen development. In *A. thaliana* *jaw-D* mutants (overexpression of *MIR319* gene) have slightly shorter hypocotyls than wild-type plants, whereas increased *TCP* activity leads to longer hypocotyls (Palatnik et al. 2003; Schommer et al. 2008; Sarvepalli and Nath 2011). In our study, we observed in hypocotyls of *I. nil*, high accumulation of In-pre-miR319 (Fig. 5b). These data confirm that the regulation of *TCP4* mRNA level by miRNA is important for proper hypocotyl growth in various plant species.

InTCP4 is expressed also in the generative organs of mature *I. nil* (Fig. 5a). In addition, the transcript level of *InTCP4* increases or decreases during pistil and stamen growth, respectively (Fig. 5c). The high level of *InTCP4* transcript in pistils seems to be a particularly interesting fact because of the lack of reports of involvement of homologues of *TCP4* in the development of this organ. *TCP4* and miR319a, *A. thaliana* regulate the normal development of stamens and petals (Schommer et al. 2008; Nag et al. 2009). Analysis of *cin* mutants in *Antirrhinum* showed that the miRNA-regulated *TCP* gene, *CIN*, affects petal lobe development by controlling epidermal cell differentiation and growth (Crawford et al. 2004).

Apart from affecting the organ development, the TCPs have also been shown to be involved in the processes of phase change. The *TCP4* mutant and *jaw-D* display a moderate late-flowering phenotype with an increase in leaf number compared to the wild type (Palatnik et al. 2003; Schommer et al. 2008; Sarvepalli and Nath 2011). Plants with the hyper-active form of TCP4 flower earlier (Sarvepalli and Nath 2011). The places of perception of the signal in the path of photoperiodic flowering induction are cotyledons and/or leaves (Vince-Prue and Gressel 1985; Ogawa and King 1990), so high expression of *InTCP4* in cotyledons of *I. nil* (Fig. 5a) suggests that it may play an important function in flower induction or another process.

To investigate the possible participation of *InTCP4* in the mechanism of flower induction in *I. nil* we investigated the effect of LD and SD conditions and factors that repress the flowering induction of *I. nil* on expression of *InTCP4* (Fig. 6). We observed that the level of *InTCP4* transcript successively decreased during both long and short night and we revealed that light positively influences the level of *InTCP4* transcript accumulation. In addition, *InTCP4* expression showing a diurnal cycle in the cotyledons of seedlings cultivated under both the SD and LD conditions seems to be the consequence of an endogenous rhythm. Oscillations in the level of *InTCP4* mRNA are also observed in the case of IAA-treated plants (Fig. 6). The application of IAA stimulates the biosynthesis of JA, JAMe and ethylene, thus inhibiting flower induction of *I. nil*

(Maciejewska et al. 2004; Kęsy et al. 2008, 2010, 2012; Frankowski et al. 2009). No response to exogenous IAA application, and consequently to an increase in the level of jasmonic acid, indicates that the expression of *InTCP4* is not influenced by these phytohormones. This was confirmed by the results of our experiment with the induction of senescence in the cotyledon of *I. nil* by application of JAMe (see below). Based on the results obtained, it can be concluded that *InTCP4* is not directly involved in photoperiodic flower induction of *I. nil*. Research in *A. thaliana* showed that the *TCP4* gene shows daily oscillations of expression, with similar patterns as in our work (Giraud et al. 2010). Giraud et al. (2010) suggest that TCP transcription factors appear to be the central regulators in circadian clock input sites, the clock oscillator itself, and a myriad of clock output sites, directing and regulating the expression of genes.

Examination of leaf senescence revealed that *jaw-D* leaves had a delay in senescence, while high levels of *TCP4* caused a premature onset of this process (Schommer et al. 2008). The mechanistic pathway that leads to the activation of senescence by the TCPs is currently unknown, although there might be interactions with the regulation of JA biosynthesis, as this phytohormone has been proposed to be a critical factor in senescence (Buchanan-Wollaston et al. 2003; van der Graaff et al. 2006; Schommer et al. 2008). Therefore, it was decided to examine changes in the expression of *InTCP4* and *InLOX2* during cotyledon senescence. Due to the very low expression of *InMIR319* in cotyledon, its activity has not been investigated. Senescence was induced by darkness, JAMe, and ABA (Weaver et al. 1998; Gan 2004). The following stages of the cotyledons' senescence process were controlled by analysis of photosynthetic parameters, such as chlorophyll content, which is typical of an ageing marker (Oh et al. 1996; Woo et al. 2001; Gan 2004; Lim et al. 2007). Furthermore, we analysed the expression of the gene associated with the function of the photosynthetic apparatus, *InCAB2* encoding the chlorophyll-binding protein (Lim et al. 2007).Van der Graaff et al. (2006) showed that the *CAB* gene is strongly and continuously negatively regulated during the developmental and darkness-induced ageing of leaves. Based on the analysis of the level of chlorophylls and *InCAB2* gene expression, we can conclude that all used factors caused ageing of cotyledons of *I. nil* (Fig. 7a, b). A slight decrease in the parameters in plants cultivated under control conditions (LD) (Fig. 7a, b) shows the natural cotyledons ageing due to the appearance of leaves in the 8-day-old seedlings (the second day of the experiment). In plants without access to light compared to those growing in LD we observed a strong reduction in the level of *InTCP4* mRNA (Fig. 7d). The decrease in *InTCP4* expression in the dark confirms the results of our experiments in LD and SD

conditions and indicates the important influence of light on transcriptional activity of the gene. In the cotyledons of plants grown in LD (Fig. 6d) the expression of *InTCP4* shows a similar pattern as *InCAB2* (Fig. 7b), which may indicate a relationship of *InTCP4* with ontogenetic senescence of cotyledons in *I. nil*. *InTCP4* expression does not correlate with accumulation of *InLOX2* mRNA, indicating a lack of direct linkage of these two genes in *I. nil*. *InLOX2* gene transcription activity increases in the 2nd and 4th days of the experiment in plants grown in the light, and in the darkness remains at a low level, while the application of ABA and JAMe strongly promotes *InLOX2* expression (Fig. 7c). Van der Graaff et al. (2006) showed a coordinated increase in *AtLOX2* gene expression only during ontogenetic ageing of the leaves, and not during ageing induced by darkness. This suggests that the jasmonic pathway in leaves is specifically activated by development cues. Furthermore, the *A. thaliana LOX2* responds positively to wounding and JAMe (Bell et al. 1995; Jensen et al. 2002). Our results confirm the data by van der Graaff et al. (2006) that activation of the *LOX2* gene is associated with developmental senescence rather than induced by darkness. In addition, the results confirm the presence of a positive feedback loop, namely, that the JAMe stimulates the expression of jasmonic biosynthesis genes (Jensen et al. 2002; Acosta and Farmer 2010).

To conclude, the results of this study do not provide a clear answer whether *InTCP4* is involved in ageing cotyledons of *I. nil*, but it did not exclude the possibility. Perhaps it is involved in the regulation of this process, not by induction of expression of genes involved in jasmonic acid biosynthesis, but by other genes which were not studied. Similar results were obtained by Sarvepalli and Nath (2011). They observed that the expression level of *AtLOX2* was significantly reduced in *tcp4-1* mutant plants, but it was not up-regulated in *TCP4:VP16-C* plants (hyper-active TCP4). These data raise the possibility that *TCP4* mediates senescence by an LOX2-independent pathway (Sarvepalli and Nath 2011).

Based on the results obtained in our research, it can be concluded that both light (clock) and miR319 control the level of *InTCP4* transcript. The main function of *InMIR319* seems to be the regulation of *InTCP4* organ localization. Our research suggests that *InTCP4* and *InMIR319* participate in the control of such processes as do their homologues in other plant species, i.e., the development of cotyledons, leaves and flower elements.

Author contribution Paulina Glazińska designed and carried out the experiments, analysed the data and wrote the manuscript. Emilia Wilmowicz and Waldemar Wojciechowski carried out the experiments. Kamil Frankowski was responsible for the preparation of figures. Jan Kopcewicz helped in preparing the manuscript.

References

Acosta IF, Farmer EE (2010) Jasmonates. In: Arabidopsis Book 8:e0129. doi:10.1199/tab.0129

Aggarwal P, Das Gupta M, Joseph AP, Chatterjee N, Srinivasan N, Nath U (2010) Identification of specific DNA binding residues in the TCP family of transcription factors in *Arabidopsis*. Plant Cell 22:1174–1189. doi:10.1105/tpc.109.066647

Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. J Mol Biol 215:403–410

Arazi T, Talmor-Neiman M, Stav R, Riese M, Huijser P, Baulcombe DC (2005) Cloning and characterization of micro-RNAs from moss. Plant J 43:837–848. doi:10.1111/j.1365-313X.2005.02499.x

Aukerman MJ, Sakai H (2003) Regulation of flowering time and floral organ identity by a microRNA and its *APETALA2-Like* target genes. Plant Cell 15:2730–2741. doi:10.1105/tpc.016238

Axtell MJ, Bartel DP (2005) Antiquity of microRNAs and their targets in land plants. Plant Cell 17:1658–1673. doi:10.1105/tpc.105.032185

Axtell MJ, Snyder JA, Bartel DP (2007) Common functions for diverse small RNAs of land plants. Plant Cell 19:1750–1769. doi:10.1105/tpc.107.051706

Baker CC, Sieber P, Wellmer F, Meyerowitz EM (2005) The early extra petals1 mutant uncovers a role for microRNA miR164c in regulating petal number in *Arabidopsis*. Curr Biol 15:303–315. doi:10.1016/j.cub.2005.02.017

Bell E, Creelman RA, Mullet JE (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. Proc Natl Acad Sci USA 92:8675–8679

Bonnet E, Van de Peer Y, Rouzé P (2006) The small RNA world of plants. New Phytol 171:451–468. doi:10.1111/j.1469-8137.2006.01806.x

Buchanan-Wollaston V, Earl S, Harrison E, Mathas E, Navabpour S, Page T, Pink D (2003) The molecular analysis of leaf senescence—a genomics approach. Plant Biotechnol J 1:3–22

Crawford BC, Nath U, Carpenter R, Coen ES (2004) *CINCINNATA* controls both cell differentiation and growth in petal lobes and leaves of *Antirrhinum*. Plant Physiol 135:244–253. doi:10.1104/pp.103.036368

Cubas P, Lauter N, Doebley J, Coen E (1999) The TCP domain: a motif found in proteins regulating plant growth and development. Plant J 18:215–222. doi:10.1046/j.1365-313X.1999.00444.x

Frankowski K, Kesy J, Wojciechowski W, Kopcewicz J (2009) Light- and IAA-regulated ACC synthase gene (PnACS) from *Pharbitis nil* and its possible role in IAA-mediated flower inhibition. J Plant Physiol 166:192–202. doi:10.1016/j.jplph.2008.02.013

Gan S (2004) The hormonal regulation of senescence. In: Davies PJ (ed) Plant hormones: biosynthesis, signal transduction and action!. Kluwer Academic Publishers, Dordrecht, pp 561–581

Giraud E, Ng S, Carrie C, Duncan O, Low J, Lee CP, Aken OV, Millar AH, Murcha M, Whelan J (2010) TCP transcription factors link the regulation of genes encoding mitochondrial proteins with the circadian clock in *Arabidopsis thaliana*. Plant Cell 22:3921–3934. doi:10.1105/tpc.110.074518

Glazińska P, Zienkiewicz A, Wojciechowski W, Kopcewicz J (2009) The putative miR172 target gene *InAPETALA2-like* is involved in the photoperiodic flower induction of *Ipomoea nil*. J Plant Physiol 166:1801–1813. doi:10.1016/j.jplph.2009.05.011

Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ (2008) miRBase: tools for microRNA genomics. Nucleic Acids Res 36:D154–D158. doi:10.1093/nar/gkm952

Hayama R, Coupland G (2004) The molecular basis of diversity in the photoperiodic flowering responses of *Arabidopsis* and Rice. Plant Physiol 135:677–684. doi:10.1105/tpc.107.052480

Hayama R, Agashe B, Luley E, King R, Coupland G (2007) A circadian rhythm set by dusk determines the expression of *FT* homologs and the short-day photoperiodic flowering response in *Pharbitis*. Plant Cell 19:2988–3000. doi:10.1105/tpc.107.052480

Imaizumi T, Kay SA (2006) Photoperiodic control of flowering: not only by coincidence. Trends Plant Sci 11:550–558. doi:0.1016/j.tplants.2006.09.004

Izawa T, Oikawa T, Sugiyama N, Tanisaka T, Yano M, Shimamoto K (2002) Phytochrome mediates the external light signal to repress *FT* orthologs in photoperiodic flowering of rice. Genes Dev 16:2006–2020. doi:10.1101/gad.999202

Jensen AB, Raventos D, Mundy J (2002) Fusion genetic analysis of jasmonate-signalling mutants in *Arabidopsis*. Plant J 29:595–606. doi:10.1046/j.0960-7412.2001.01241.x

Jones- Rhoades MW, Bartel DP, Bartel B (2006) MicroRNAs and their regulatory roles in plants. Annu Rev Plant Biol 57:49–53. doi:10.1146/annurev.arplant.57.032905.105218

Kasschau KD, Xie Z, Allen E, Llave C, Chapman EJ, Krizan KA, Carrington JC (2003) P1/HC-Pro, a viral suppressor of RNA silencing, interferes with *Arabidopsis* development and miRNA function. Dev Cell 4:205–217. doi:10.1016/j.devcel.2011.02.003

Kęsy J, Maciejewska B, Sowa M, Szumiak M, Kawałowski K, Borzuchowska M, Kopcewicz J (2008) Ethylene and IAA interactions in the inhibition of photoperiodic flower induction of *Pharbitis nil*. Plant Growth Regul 55:43–50. doi:10.1007/s10725-008-9256-9

Kęsy J, Frankowski K, Wilmowicz E, Glazińska P, Wojciechowski W, Kopcewicz J (2010) The possible role of *PnACS2* in IAA-mediated flower inhibition in *Pharbitis nil*. Plant Growth Regul 61:1–10. doi:10.1007/s10725-010-9443-3

Kęsy J, Wilmowicz E, Maciejewska B, Frankowski K, Glazińska P, Kopcewicz J (2012) Independent effects of jasmonates and ethylene on inhibition of *Pharbitis nil* flowering. Acta Physiol Plantarum 33:1211–1216. doi:10.1007/s11738-010-0649-9

Kidner CA (2010) The many roles of small RNAs in leaf development. J Genet Genomics 37:13–21. doi:10.1016/S1673-8527(09)60021-7

Kosugi S, Ohashi Y (2002) DNA binding and dimerization specificity and potential targets for the TCP protein family. Plant J 30:337–348. doi:10.1046/j.1365-313X.2002.01294.x

Laufs P, Peaucelle A, Morin H, Traas J (2004) MicroRNA regulation of the *CUC* genes is required for boundary size control in *Arabidopsis* meristems. Development 131:4311–4322. doi:10.1242/dev.01320

Lauter N, Kampani A, Carlson S, Goebel M, Moose SP (2005) microRNA172 down-regulates *glossy15* to promote vegetative chase change in maize. Proc Natl Acad Sci USA 102:9412–9417. doi:10.1073/pnas.0503927102

Li Y, Li W, Jin YX (2005) Computational identification of novel family members of microRNA genes in *Arabidopsis thaliana* and *Oryza sativa*. Acta Biochim Biophys Sin (Shanghai) 37:75–87. doi:10.1093/abbs/37.2.75

Lim PO, Kim HJ, Nam HG (2007) Leaf senescence. Annu Rev Plant Biol 58:115–136. doi:10.1146/annurev.arplant.57.032905.105316

Liu PP, Montgomery TA, Fahlgren N, Kasschau KD, Nonogaki H, Carrington JC (2007) Repression of AUXIN RESPONSE FACTOR10 by microRNA160 is critical for seed germination and post-germination stages. Plant J 52:133–146. doi:10.1111/j.1365-313X.2007.03218.x

Maciejewska BD, Kęsy J, Zielinska M, Kopcewicz J (2004) Jasmonates inhibit flowering in short-day plant *Pharbitis nil*. Plant Growth Regul 43:1–8. doi:10.1023/B:GROW.0000038241.00771.bd

Mallory AC, Dugas DV, Bartel DP, Bartel B (2004) MicroRNA regulation of NAC-domain targets is required for proper formation and separation of adjacent embryonic, vegetative, and floral organs. Curr Biol 14:1035–1046. doi:10.1016/j.cub.2004.06.022

Mallory AC, Bartel DP, Bartel B (2005) MicroRNA- directed regulation of *Arabidopsis* AUXIN RESPONSE FACTOR17 is essential for proper development and modulates expression of early auxin response genes. Plant Cell 17:1360–1375. doi:10.1105/tpc.105.031716

Martin-Trillo M, Cubas P (2010) TCP genes: a family snapshot ten years later. Trends Plant Sci 15:31–39. doi:10.1016/j.tplants.2009.11.003

Nag A, King S, Jack T (2009) miR319a targeting of TCP4 is critical for petal growth and development in *Arabidopsis*. Proc Natl Acad Sci USA 106:22534–22539. doi:10.1073/pnas.0908718106

Navaud O, Dabos P, Carnus E, Tremousaygue D, Hervé C (2007) TCP transcription factors predate the emergence of land plants. J Mol Evolution 65:23–33. doi:10.1007/s00239-006-0174-z

Ogawa Y, King RW (1990) The inhibition of flowering by non-induced cotyledons of *Pharbitis nil*. Plant Cell Physiol 31:129–135

Oh SA, Lee SY, Chung IK, Lee CH, Nam HG (1996) A senescence-associated gene of *Arabidopsis thaliana* is distinctively regulated during natural and artificially induced leaf senescence. Plant Mol Biol 30:739–754

Ori N, Cohen AR, Etzioni A, Brand A, Yanai O, Shleizer S, Menda N, Amsellem Z, Efroni I, Pekker I, Alvarez JP, Blum E, Zamir D, Eshed Y (2007) Regulation of *LANCEOLATE* by miR319 is required for compound-leaf development in tomato. Nat Genet 39:787–791. doi:10.1038/ng2036

Palatnik JF, Allen E, Wu X, Schommer C, Schwab R, Carrington JC, Weigel D (2003) Control of leaf morphogenesis by microRNAs. Nature 425:257–263. doi:10.1038/nature01958

Parthier B (1990) Jasmonates: hormonal regulators of stress factors in leaf senescence. J Plant Growth Regul 9:445–454. doi:10.1007/BF02041942

Pulido A, Laufs P (2010) Co-ordination of developmental processes by small RNAs during leaf development. J Exp Bot 61:1277–1291. doi:10.1093/jxb/erp397

Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP (2002) MicroRNAs in plants. Genes Dev 16:1616–1626. doi:10.1101/gad.1004402

Rubio-Somoza I, Weigel D (2011) MicroRNA networks and developmental plasticity in plants. Trends Plant Sci 16:258–264. doi:10.1016/j.tplants.2011.03.001

Sarvepalli K, Nath U (2011) Hyper-activation of the TCP4 transcription factor in *Arabidopsis thaliana* accelerates multiple aspects of plant maturation. Plant J 67:595–607. doi:10.1111/j.1365-313X.2011.04616.x

Schommer C, Palatnik JF, Aggarwal P, Chetelat A, Cubas P, Farmer EE, Nath U, Weigel D (2008) Control of jasmonate biosynthesis and senescence by miR319 targets. PLoS Biol 6:1991–2001. doi:10.1371/journal.pbio.0060230

Schommer C, Bresso EG, Spinelli SV, Palatnik JF (2012) Role of microRNA miR319 in plant development. In: Sunkar R (ed) MicroRNAs in plant development and stress responses. Signaling and communication in plants, vol 15. Springer, Heidelberg, pp 29–47

Sieber P, Wellmer F, Gheyselinck J, Riechmann JL, Meyerowitz EM (2007) Redundancy and specialization among plant microRNAs: role of the *MIR164* family in developmental robustness. Development 134:1051–1060. doi:10.1242/dev.02817

Thomas B, Vince-Prue D (1997) Photoperiodism in plants, 2nd edn. Academic Press Inc, San Diego, p 428

Turner JG, Ellis C, Devoto A (2002) The jasmonate signal pathway. Plant Cell (Suppl.): S153–S164. doi:10.1105/tpc.000679

Ueda J, Kato J (1980) Identification of a senescence-promoting substance from wormwood (*Artemisia absinthum* L). Plant Physiol 66:246–249

van der Graaff E, Schwacke R, Schneider A, Desimone M, Flugge UI, Kunze R (2006) Transcription analysis of arabidopsis membrane transporters and hormone pathways during developmental and induced leaf senescence. Plant Physiol 141:776–792. doi:10.1104/pp.106.079293

Vince-Prue D, Gressel J (1985) Pharbitis nil. In: Halevy AH (ed) Handbook of flowering IV. CRC Press Inc, Boca Raton, pp 47–81

Voinnet O (2009) Origin, biogenesis, and activity of plant microRNAs. Cell 136:669–687. doi:10.1016/j.cell.2009.01.046

Wang JW, Wang LJ, Mao YB, Cai WJ, Xue HW, Chen XY (2005) Control of root cap formation by microRNA-targeted auxin response factors in *Arabidopsis*. Plant Cell 17:2204–2216. doi:10.1105/tpc.105.033076

Warthmann N, Das S, Lanz C, Weigel D (2008) Comparative analysis of the *MIR319a* microRNA locus in *Arabidopsis* and related *Brassicaceae*. Mol Biol Evol 25:892–902. doi:10.1093/molbev/msn029

Weaver LM, Gan S, Quirino B, Amasino RM (1998) A comparison of the expression patterns of several senescence-associated genes in response to stress and hormone treatment. Plant Mol Biol 37:455–469. doi:10.1023/A:1005934428906

Woo HR, Chung KM, Park JH, Oh SA, Ahn T, Hong SH, Jang SK, Nam HG (2001) ORE9, an F-box protein that regulates leaf senescence in *Arabidopsis*. Plant Cell 13:1779–1790. doi:10.1105/TPC.010061

Wu G, Park MY, Conway SR, Wang JW, Weigel D, Poethig RS (2009) The sequential action of miR156 and miR172 regulates developmental timing in *Arabidopsis*. Cell 138:750–759. doi:10.1016/j.cell.2009.06.031

Yoshida S (2003) Molecular regulation of leaf senescence. Curr Opin Plant Biol 6:79–84. doi:10.1016/S1369-5266(02)00009-2

Zhang B, Pan X, Cannon CH, Cobb GP, Anderson TA (2006) Conservation and divergence of plant microRNA genes. Plant J 46:243–259. doi:10.1111/j.1365-313X.2006.02697.x

Zhang B, Wang Q, Pan X (2007) MicroRNAs and their regulatory roles in animals and plants. J Cell Physiol 210:279–289. doi:10.1002/jcp.20869