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Molecular and cytological characterization of ZTL in *Ipomoea nil*

A. ZIENKIEWICZ[1]*, D.J. SMOLIŃSKI[2], K. ZIENKIEWICZ[2], P. GLAZIŃSKA[1], W. WOJCIECHOWSKI[1]
and J. KOPCEWICZ[1]

Department of Plant Physiology and Molecular Biology of Plants[1], Department of Cell Biology[2], Institute of General and Molecular Biology, Nicolaus Copernicus University, Gagarina 9, PL-87100 Toruń, Poland

Abstract

The ZEITLUPE (ZTL) protein is involved in the control of circadian period, hypocotyl elongation and flowering time in *Arabidopsis thaliana*. The aim of the present work was the identification of the *InZTL* gene and localization of its mRNA in the model short-day plant *Ipomoea nil*. The deduced InZTL protein of 622 amino acid residues contained a LOV domain at the N-terminal part, followed by an F-box domain and six carboxy terminal kelch repeats. Amino acid sequence of InZTL showed 84 % homology with *Mesembryanthemum crystallinum* ZTL (McZTL) and 83 % with *Arabidopsis thaliana* ZTL (AtZTL). Fluorescence *in situ* hybridization (FISH) to *InZTL* mRNA showed its high accumulation in the vascular bundles as well in the guard cells of the cotyledon. Immunolocalization of ZTL protein indicated a similar distribution pattern of ZTL protein as *InZTL* mRNAs.

Additional key words: fluorescence *in situ* hybridization (FISH), short-day plant, circadian clock.

Introduction

Circadian clock controls many different physiological and developmental processes in plants, among others cells elongation, stomatal opening, leaf movement and flowering. The endogenous clock creates about 24-h rhythms (Somers 1999). The clock is set mainly by the alternations in irradiance and temperature at dawn and dusk. The machinery responsible for generating circadian rhythms is composed of three major components: *1*) an input pathway by which environmental cues act to synchronize the clock, *2*) the endogenous oscillator, and *3*) the output pathway as the rhythms of physiological and developmental processes controlled by the clock (Salome and McClung 2004). Irradiance forms the dominant signal in resetting the clock. A close link between the photoreceptors and the clock's components has been demonstrated for different experimental models. In plants both of the photoreceptor molecule families – phytochromes (phy) and cryptochromes (cry) participate in light signal transduction to the oscillator (Fankhauser and Staiger 2002). The ZEITLUPE protein family has also been implicated in light input to the clock (Kim *et al.* 2007, Kiba *et al.* 2007).

Molecular studies of the central oscillator from several different organisms (*e.g.*, *Arabidopsis thaliana, Neurospora crassa, Drosophila melanogaster*) indicate that it works as several interlocking feedback loops consisting of positive and negative elements. Proteins encoded by clock genes act as negative elements that repress their own expression by blocking transcriptional activators which act as positive elements (Dunlap 1999). In *Arabidopsis thaliana* the circadian oscillator is based on the function of following proteins: late elongated hypocotyl (LHY), circadian clock associated1 (CCA1), timing of cab1 expression (TOC1), *A. thaliana* pseudo-response regulators (APRR1) and early flowering4 (ELF4) (Doyle *et al.* 2002, Salome and McClung 2004). In recent years, an increasing number of genes have been identified which function in central oscillator as well input/output pathway, for example: *GI* (*gigantea*), *ELF3* (*early flowering* 3), *LUX/PCL1* (*lux arrhythmo/ phytoclock* 1), *TIC* (*time for coffee*), *TEJ*, *SRR1* (*sensitivity to red light reduced* 1) (Gardner *et al.* 2006).

FKF/LKP/ZTL is a small protein family which consists of three members: LKP1/ZTL, LKP2/FKL and FKF1. Each member contains a LOV, an F-box and kelch repeat domains (Kiyosue and Wada 2000, Somers *et al.*

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Abbreviations: FISH - fluorescence *in situ* hybridization; LD - long day; PCR - polymerase chain reaction; RT-PCR - reverse transcriptase PCR; SD - short day; ZTL - ZEITLUPE.

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* Corresponding author: fax: (+48) 56 6114772, e-mail: agazet@biol.uni.torun.pl

2000). Their LOV domain is a special class of the PAS motif and shows close similarity to the flavin binding region of the *A. thaliana* phototropins (PHOT1 and PHOT2) and the *Neurospora crassa* WC-1 protein. Phototropins and WC1 are blue light photoreceptors in which the LOV domain can bind flavin mononucleotide (FMN) (Briggs and Christie 2002). Downstream of the LOV is a F-box domain involved in the interaction with several ASK proteins (*Arabidopsis* SKP1) which suggests that FKF1/LKP2/ZTL are parts of the SCF (Skp1-Cullin-F-box protein) complex responsible for ubiquitination and proteasome-dependent protein degradation (Yasuhara *et al.* 2004). C-terminal six kelch repeats probably can form a β-propeller structure that is similar to the structure formed by repeats of WD40, found in yeast and mammalian F-box protein. Kelch repeats are involved in protein-protein interactions (Adams *et al.* 2000).

ZTL is the best characterized of the three members of the gene family (*ZTL*, *LKP2*, *FKF1*). The ZTL protein is involved in the control of circadian period, hypocotyl elongation and flowering time regulation in *A. thaliana* (Somers *et al.* 2004). Previous studies have shown interaction between TOC1 and ZTL in the yeast two-hybrid system. The complex SCF^{ZTL} is responsible for degradation of TOC1 via a proteasome dependent pathway and this process is probably responsible for the regulation of the circadian period by the clock (Mas *et al.* 2003). Moreover, Kiba *et al.* (2007) showed that PPR5 levels are negatively regulated by ZTL which in turn regulates the circadian oscillator. Kim *et al.* (2007) reported also that post-translational rhythm on ZTL protein is controlled by ZTL interaction with GI. Additionally, it was shown that ZTL overexpression (OX) delays flowering in *A. thaliana* plants growing under inductive long day conditions. In ZTL OX *A. thaliana* plants a strong reduction of *CO* (*CONSTANS*) and *FT* (*FLOWERING TIME*) transcript levels is found but the mechanism of flowering regulation by the ZTL protein is still not clear (Kim *et al.* 2005).

Published data concerning the localization of ZTL molecules in plants are few and fragmentary. Kiyosue and Wada (2000) showed a strong LKP1::GUS activity in cotyledons, rosette and cauline leaves of *A. thaliana*. So far, no investigations on the distribution of ZTL in short-day plants have been performed.

The aim of this work was the identification of the *InZTL* gene in *Ipomoea nil* and determination of the distribution of it's transcript and protein in various organs of *I. nil* seedlings. In order to analyze this, RACE-PCR, RT-PCR, fluorescence *in situ* hybridization and immunofluorescence methods were used.

Materials and methods

Plants: Seeds of *Ipomoea nil* (*Pharbitis nil*) Choisy cv. Violet (from *Marutane*, Kyoto, Japan) were soaked in concentrated sulfuric acid for 45 min, rinsed in running tap water for 2 h and imbibed in water overnight. Seedlings were grown for 6 d or 8 weeks in a growth-chamber at 26 °C and irradiance of 130 $\mu mol \cdot m^{-2} \cdot s^{-1}$ (cool white fluorescent tubes, *Polam*, Warsaw, Poland) during 16-h photoperiod (long day, LD) or 8-h photoperiod (short day, SD). Cotyledons, hypocotyls and roots were collected on the 6th day of seedlings cultivation. Flowers were collected on the 26th - 36th day of plant cultivation.

RNA isolation and cDNA synthesis: Frozen samples were ground in liquid nitrogen using a mortar and pestle. Total RNA was isolated from the frozen powder using *TRI REAGENT* (*Sigma-Aldrich*, St Louis, USA) according to the manufacturer's instructions. The final pellet was resuspended in 0.04 cm^3 of deionized water and stored at -80 °C. Total RNA was DNase-treated with deoxyribonuclease I (RNase free) (*Fermentas*, Burlington, Canada). 0.5 μg DNase-treated RNA primed with anchored oligo $(dT)_{19}$ primers were used for first strand synthesis with *RevertAid M-MuLV* reverse transcriptase (*Fermentas*) according to the manufacturer's instructions.

Cloning the full-length cDNA for *InZTL* homolog from *Ipomoea nil* using RACE-PCR: Degenerate primers were used to amplify partial *I. nil* cDNAs homologous to *ZTL* gene: *ZTL* Fp: TCTGGA/TGGA/CTGTGCA/TGATTC, *ZTL* Fp: TGTAAT/CTTT/AGTGCT/CTGTGCA/TGT, *ZTL* Rp: TCG/CTCT/TCG/CTT/CAA/CG/ATCCATA/TGT, *ZTL* Rp: CG/ACTT/CTTAGCA/CAG/AG/ACCA/ CCAAAC. The predicted length of the products is 593 bp. Amplified cDNAs were loaded onto 1.3 % agarose gel and after electrophoresis, purified with *GeneMATRIX* agarose-out DNA purification kit (*EURx*, Gdańsk, Poland) and cloned into *PCR II TOPO* vector (*TOPO TA* cloning kit dual promotor, *Invitrogen*, Carlsbad, USA). Plasmid DNAs of positive clones were isolated with *GeneMATRIX PLASMID MINIPREP* DNA kit (*EURx*) and analyzed by restriction analysis to confirm the presence of the insert and then were sequenced.

The 5' and 3' ends of the *Ipomoea nil ZTL* cDNAs were amplified using *SMART* RACE-PCR kit (*BD Biosciences Clontech*, San Jose, USA) according to the manufacturer's protocols. Amplified cDNAs were cloned and sequenced as described above. The nucleotide sequences reported here is in the Gene Bank with accession number DQ 309278.

DNA sequencing and analysis: All products were sequenced in Institute of Biochemistry and Biophysics (IBB), (Polish Academy of Sciences). A *BLAST* search was performed *via* the NCBI web site. Percentage of amino acid sequence similarity between InZTL and other proteins was performed according to web site (http:// genomatix. de/cgi-bin/dialign/dialign.pl/).

Semi-quantitative RT-PCR: For first-strand cDNA synthesis 0.5 μg the total RNA with oligo $(dT)_{19}$ primer (0.25 μg) and reverse transcriptase (*Fermentas*) were mixed according to the manufacturer's instructions. PCR reactions were performed using 0.002 cm^3 of each reverse transcribed cDNA sample in reaction mixture (0.048 cm^3) containing blue perpetual *Taq* DNA polymerase (*EURx*), buffer B (*EURx*), dNTP set (*EURx*), *InZTL* and *InACT4* primers; *InZTL* Fp: TGAATTCGAGCAAC CCAGAGTGG, *InZTL* Rp: CATGCTACAGGT ATCTCTCGCCACA, *InACT4* Fp; GAATTCCATACT CTGCCTTGGCAATC, *InACT4* Rp; GAATTCGAT ATCCGAAAAGACTTGTATGG. The predicted product sizes for *InZTL* was 450 bp and for *InACT4* was 250 bp. We used 0.001 cm^3 (10 μM) of each forward and reverse primers. PCR reactions were conducted in a programmable thermocycler. The optimal number of PCR cycles for semi-quantitative RT-PCR was 31. PCR cycles were 95 °C for 2 min, 95 °C for 1 min and final extension step of 74 °C for 1 min followed by 74 °C for 1 min. All samples were separated on 1.4 % agarose gel and stained with ethidium bromide. Gels were visualized using *Multi Doc* digital imaging system. An *Ipomoea nil Actin* gene (*InACT4*) was used as a control for the RT-PCR.

Preparation of material for microscopy: Organs of *Ipomoea nil* seedlings were harvested and fixed in 4 % paraformaldehyde and 0.25 % glutaraldehyde in PBS buffer, pH 7.2, overnight at 4 °C. The material was dehydrated in increasing ethanol concentrations, supersatured and then embedded in BMM resin (butyl methacrylate, methyl methacrylate, 0.5 % benzoin ethyl ether, 10 mM dithiothreitol; *Fluka*, Buchs, Switzerland). The embedded material was cut into semithin sections which were placed on microscope slides covered with *Biobond* (*British Biocell International*, Cardiff, UK).

Fluorescence *in situ* hybridization (FISH): The digoxigenin-labelled antisense and sense RNA probes were prepared from the linearized PCR II TOPO vector carrying a *InZTL* cDNA insert using SP6 or T7 RNA polymerase (*F. Hoffmann - La Roche*, Rotkrenz, Switzerland). The sections were hybridized with the probes at 42 °C for 12 h. In order to visualize the probe, primary mouse anti-digoxigenin antibodies (*F. Hoffmann - La Roche*) were added overnight at 4 °C and secondary goat anti-mouse Alexa Fluor 488 (*Molecular Probes*, Eugene, USA) antibodies for 1 h at 37 °C.

Immunofluorescence labeling: A rabbit antibody, raised against *Arabidopsis thaliana* ZTL, that was a generous gift from Prof. D. Sommers, was used in immunolocalization studies. ZTL protein was detected by incubating with a primary anti-ZTL antibody in 1 % bovine serum albumin (BSA) in PBS (1:200), pH 7.2, for 2 h at room temperature, and the secondary goat anti-rabbit antibody Alexa Fluor 488 (*Molecular Probes*) in 1 % BSA in PBS (1:500) for 1 h at 37 °C. Control reactions were performed without the primary antibody. DNA was stained with 4',6-diamidino-2-phenylindole (DAPI) (*Fluka*).

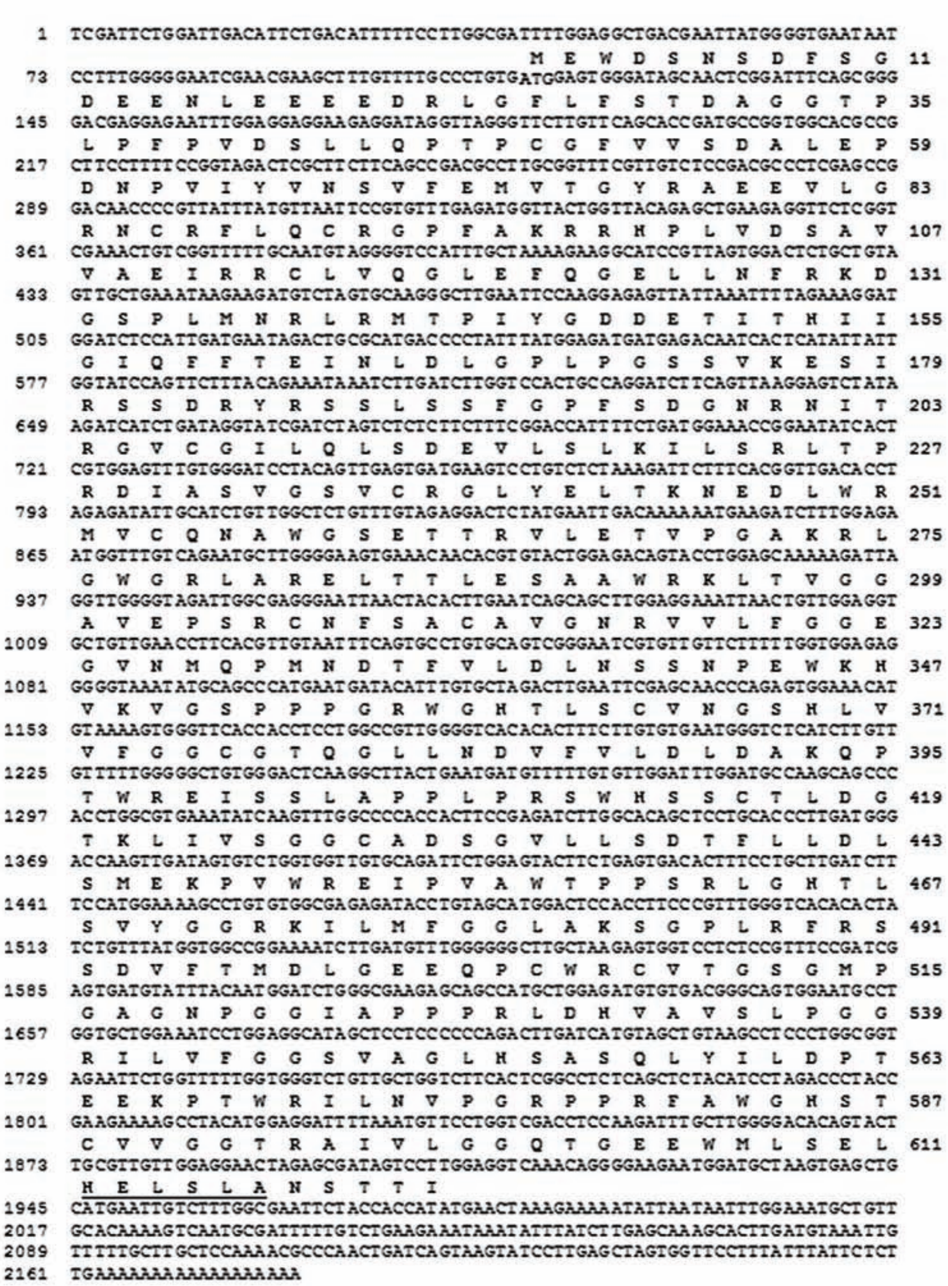
Microscopy: Samples were analyzed with a *Nikon Eclipse 80i* fluorescence microscope. The *CPI Plan Fluor 100* (numerical aperture, 1.3) *DIC H/N2* oil immersion lens and narrow band filters (UV-2EC, B-2EC, G-2EC) were used. The results were registered with a *Nikon DS-5Mc* color cooled digital camera and *Nikon NIS* elements software (*Nikon Europe*, Langen, Germany).

Results

Isolation and sequence analysis of *InZTL*: To obtain the *ZTL* homologue from *Ipomoea nil* we amplified partial *I.* cDNA homologous to *ZTL* genes using PCR primers. Primers were designed for conserved nucleotide sequences of *ZTL* genes from available plant species. 595 bp PCR product was obtained. Sequence analysis of the PCR product showed that the clone has homology of 79 % with *Mesembryanthemum crystallinum ZTL* and 73 % with *Arabidopsis thaliana ZTL.* In the next step of our investigations we amplified the 5' ends of the *I. nil ZTL* cDNA using the *SMART* RACE-PCR kit. The 3' sequences of *InZTL* were obtained by completing the sequence of existing partial cDNAs. The 5' end was amplified from cDNA synthesized from mRNA corresponding to the time of the transcript peak as determined with semi-quantitative RT-PCR analysis. The RACE-PCR products were cloned into PCR II *TOPO* vector and sequenced. The *InZTL* was 2180 bp long and contained 183 bp of the 3'-untranslated region, and a poly(A) tail. DNA sequence analysis revealed that *InZTL* has a full length putative open reading frame (Fig. 1). The nucleotide sequence shown here is in the GenBank (NCBI) with accession number DQ309278. The deduced InZTL protein had 622 amino acid residues with a molecular weight of 67,730 kDa. InZTL protein showed 84 % homology to *M. crystallinum* ZTL (McZTL) and 83 % to *A. thaliana* ZTL (AtZTL). The InZTL protein had a LOV domain at the N-terminal part, followed by an F-box domain and six carboxy terminal kelch repeats. The LOV domain of InZTL showed 86 % similarities to the LOV domain of McZTL and AtZTL and 39 and 38 % to *A. thaliana* NPH1 LOV1 and LOV2, respectively. The F-box of InZTL showed sequence homology to the F-box domain of *A. thaliana* TIR1 (TRANSPORT INHIBITOR RESPONSE1) (22 % similarities), UFO (UNUSUAL FLORAL ORGANS) (17 % similarities), McZTL and AtZTL (70 - 60 %). InZTL sequence analysis showed the presence of conserved amino acid motifs HELSLA, specific for ZTL proteins from *A. thaliana* and *M. crystallinum* (Fig. 1). These results led us to assume that the obtained sequence represents the ZTL protein and not FKF1 or LKP2.

Organ-specific expression of *InZTL*: We also investigated the organ specific expression of *InZTL* mRNA in *I. nil* seedlings. Total RNA was isolated from cotyledons, hypocotyls and roots of seedlings and from flower parts (petals, sepals, stamens, pistils). The *InZTL* mRNA was present in all organs. The highest level of *InZTL* transcripts was observed in cotyledons and sepals and the lowest in roots, petals and stamens (Fig. 2).

***InZTL* mRNA localization:** Fluorescence *in situ* hybridization (FISH) was used for tissue localization of *InZTL* mRNA in cotyledons of *I. nil* seedlings. The anatomy of the *I. nil* cotyledon was observed using methylene blue staining (Fig. 3). *InZTL* transcripts were detected using 1200 bp long RNA probe which is complementary to the



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1     TCGATTCTGGATTGACATTCTGACATTTTTCCTTGGCGATTTTGGAGGCTGACGAATTATGGGGTGAATAAT
                                              M  E  W  D  S  N  S  D  F  S  G   11
73    CCTTTGGGGGAATCGAACGAAGCTTTGTTTTGCCCTGTGATGGAGTGGGATAGCAACTCGGATTTCAGCGGG
       D  E  E  N  L  E  E  E  E  D  R  L  G  F  L  F  S  T  D  A  G  G  T  P   35
145   GACGAGGAGAATTTGGAGGAGGAAGAGGATAGGTTAGGGTTCTTGTTCAGCACCGATGCCGGTGGCACGCCG
       L  P  F  P  V  D  S  L  L  Q  P  T  P  C  G  F  V  V  S  D  A  L  E  P   59
217   CTTCCTTTTCCGGTAGACTCGCTTCTTCAGCCGACGCCTTGCGGTTTCGTTGTCTCCGACGCCCTCGAGCCG
       D  N  P  V  I  Y  V  N  S  V  F  E  M  V  T  G  Y  R  A  E  E  V  L  G   83
289   GACAACCCCGTTATTTATGTTAATTCCGTGTTTGAGATGGTTACTGGTTACAGAGCTGAAGAGGTTCTCGGT
       R  N  C  R  F  L  Q  C  R  G  P  F  A  K  R  R  H  P  L  V  D  S  A  V  107
361   CGAAACTGTCGGTTTTTGCAATGTAGGGGTCCATTTGCTAAAAGAAGGCATCCGTTAGTGGACTCTGCTGTA
       V  A  E  I  R  R  C  L  V  Q  G  L  E  F  Q  G  E  L  L  N  F  R  K  D  131
433   GTTGCTGAAATAAGAAGATGTCTAGTGCAAGGGCTTGAATTCCAAGGAGAGTTATTAAATTTTAGAAAGGAT
       G  S  P  L  M  N  R  L  R  M  T  P  I  Y  G  D  D  E  T  I  T  H  I  I  155
505   GGATCTCCATTGATGAATAGACTGCGCATGACCCCTATTTATGGAGATGATGAGACAATCACTCATATTATT
       G  I  Q  F  F  T  E  I  N  L  D  L  G  P  L  P  G  S  S  V  K  E  S  I  179
577   GGTATCCAGTTCTTTACAGAAATAAATCTTGATCTTGGTCCACTGCCAGGATCTTCAGTTAAGGAGTCTATA
       R  S  S  D  R  Y  R  S  S  L  S  S  F  G  P  F  S  D  G  N  R  N  I  T  203
649   AGATCATCTGATAGGTATCGATCTAGTCTCTCTTCTTTCGGACCATTTTCTGATGGAAACCGGAATATCACT
       R  G  V  C  G  I  L  Q  L  S  D  E  V  L  S  L  K  I  L  S  R  L  T  P  227
721   CGTGGAGTTTGTGGGATCCTACAGTTGAGTGATGAAGTCCTGTCTCTAAAGATTCTTTCACGGTTGACACCT
       R  D  I  A  S  V  G  S  V  C  R  G  L  Y  E  L  T  K  N  E  D  L  W  R  251
793   AGAGATATTGCATCTGTTGGCTCTGTTTGTAGAGGACTCTATGAATTGACAAAAAATGAAGATCTTTGGAGA
       M  V  C  Q  N  A  W  G  S  E  T  T  R  V  L  E  T  V  P  G  A  K  R  L  275
865   ATGGTTTGTCAGAATGCTTGGGGAAGTGAAACAACACGTGTACTGGAGACAGTACCTGGAGCAAAAAGATTA
       G  W  G  R  L  A  R  E  L  T  T  L  E  S  A  A  W  R  K  L  T  V  G  G  299
937   GGTTGGGGTAGATTGGCGAGGGAATTAACTACACTTGAATCAGCAGCTTGGAGGAAATTAACTGTTGGAGGT
       A  V  E  P  S  R  C  N  F  S  A  C  A  V  G  N  R  V  V  L  F  G  G  E  323
1009  GCTGTTGAACCTTCACGTTGTAATTTCAGTGCCTGTGCAGTCGGGAATCGTGTTGTTCTTTTTGGTGGAGAG
       G  V  N  M  Q  P  M  N  D  T  F  V  L  D  L  N  S  S  N  P  E  W  K  H  347
1081  GGGGTAAATATGCAGCCCATGAATGATACATTTGTGCTAGACTTGAATTCGAGCAACCCAGAGTGGAAACAT
       V  K  V  G  S  P  P  P  G  R  W  G  H  T  L  S  C  V  N  G  S  H  L  V  371
1153  GTAAAAGTGGGTTCACCACCTCCTGGCCGTTGGGGTCACACACTTTCTTGTGTGAATGGGTCTCATCTTGTT
       V  F  G  G  C  G  T  Q  G  L  L  N  D  V  F  V  L  D  L  D  A  K  Q  P  395
1225  GTTTTTGGGGGCTGTGGGACTCAAGGCTTACTGAATGATGTTTTTGTGTTGGATTTGGATGCCAAGCAGCCC
       T  W  R  E  I  S  S  L  A  P  P  L  P  R  S  W  H  S  S  C  T  L  D  G  419
1297  ACCTGGCGTGAAATATCAAGTTTGGCCCCACCACTTCCGAGATCTTGGCACAGCTCCTGCACCCTTGATGGG
       T  K  L  I  V  S  G  G  C  A  D  S  G  V  L  L  S  D  T  F  L  L  D  L  443
1369  ACCAAGTTGATAGTGTCTGGTGGTTGTGCAGATTCTGGAGTACTTCTGAGTGACACTTTCCTGCTTGATCTT
       S  M  E  K  P  V  W  R  E  I  P  V  A  W  T  P  P  S  R  L  G  H  T  L  467
1441  TCCATGGAAAAGCCTGTGTGGCGAGAGATACCTGTAGCATGGACTCCACCTTCCCGTTTGGGTCACACACTA
       S  V  Y  G  G  R  K  I  L  M  F  G  G  L  A  K  S  G  P  L  R  F  R  S  491
1513  TCTGTTTATGGTGGCCGGAAAATCTTGATGTTTGGGGGGCTTGCTAAGAGTGGTCCTCTCCGTTTCCGATCG
       S  D  V  F  T  M  D  L  G  E  E  Q  P  C  W  R  C  V  T  G  S  G  M  P  515
1585  AGTGATGTATTTACAATGGATCTGGGCGAAGAGCAGCCATGCTGGAGATGTGTGACGGGCAGTGGAATGCCT
       G  A  G  N  P  G  G  I  A  P  P  P  R  L  D  H  V  A  V  S  L  P  G  G  539
1657  GGTGCTGGAAATCCTGGAGGCATAGCTCCTCCCCCCAGACTTGATCATGTAGCTGTAAGCCTCCCTGGCGGT
       R  I  L  V  F  G  G  S  V  A  G  L  H  S  A  S  Q  L  Y  I  L  D  P  T  563
1729  AGAATTCTGGTTTTTGGTGGGTCTGTTGCTGGTCTTCACTCGGCCTCTCAGCTCTACATCCTAGACCCTACC
       E  E  K  P  T  W  R  I  L  N  V  P  G  R  P  P  R  F  A  W  G  H  S  T  587
1801  GAAGAAAAGCCTACATGGAGGATTTTAAATGTTCCTGGTCGACCTCCAAGATTTGCTTGGGGACACAGTACT
       C  V  V  G  G  T  R  A  I  V  L  G  G  Q  T  G  E  E  W  M  L  S  E  L  611
1873  TGCGTTGTTGGAGGAACTAGAGCGATAGTCCTTGGAGGTCAAACAGGGGAAGAATGGATGCTAAGTGAGCTG
       H  E  L  S  L  A  N  S  T  T  I
1945  CATGAATTGTCTTTGGCGAATTCTACCACCATATGAACTAAAGAAAAATATTAATAATTTGGAAATGCTGTT
2017  GCACAAAAGTCAATGCGATTTTTGTCTGAAGAAATAAATATTTATCTTGAGCAAAGCACTTGATGTAAATTG
2089  TTTTTGCTTGCTCCAAAACGCCCAACTGATCAGTAAGTATCCTTGAGCTAGTGGTTCCTTTATTTATTCTCT
2161  TGAAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequence of the *InZTL*. The position of the nucleotide is shown on the left and the position of amino acids on the right.

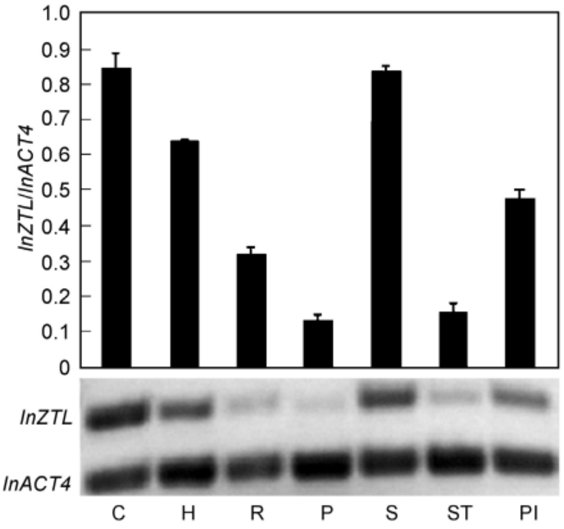


Fig. 2. Tissue specific expression of *InZTL*. Total RNA was isolated from different tissues of *Ipomoea nil* seedlings. Semi-quantitative RT-PCR was performed on the total RNA and the resulting band intensities were normalized to the *InACT4*. Analyses were performed on three replicates (each replicate from a different plant). Values are means ± SE of three independent experiments. C - cotyledons; H - hypocotyls; R - roots, P - petals, S - sepals, ST - stamens, PI - pistils.

fragment of *InZTL* mRNA. *InZTL* transcripts were present in all examined tissues (Fig. 7). The highest level of fluorescence indicating the *InZTL* mRNA was observed in vascular bundle area (Figs. 5, 7). Strong labeling was present in xylem mesophyll cells and weaker in phloem cells (Fig. 5). Relatively high signal was also observed in the palisade and spongy mesophyll cells (Fig. 6). The lowest fluorescence level was present in epidermal cells (Fig. 7), excluding guard cells, where the level of the signal was significant (Fig. 6). Analysis of the cellular InZTL mRNA distribution showed its presence both in the cell nucleus and cytoplasm in all examined tissues, however the cytoplasmic level was much more higher than in the cell nucleus (Fig. 5). No fluorescence was observed in the control (Fig. 4).

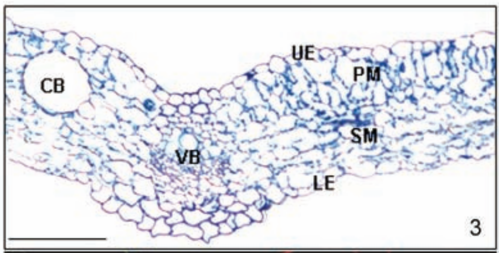
Immunolocalization of ZTL protein: Immunolocalization of InZTL protein in cotyledons of *I. nil* seedlings showed its presence in all tissues (Fig. 8). The highest level of fluorescence, indicating the presence of ZTL protein, was observed in vascular bundle area, mainly in the xylem cells (Fig. 11). Relatively high signal was located in guard and subsidiary cells (Fig. 9) as well as in the palisade and spongy mesophyll cells (Figs. 8, 10). Weak fluorescence was observed also in the epidermal cells and no signal was observed in the cotyledon bodies (Fig. 8). In the majority of cotyledon cells the signal was detected both in the cytoplasm and in the cell nucleus (Figs. 9, 10). There was no fluorescence in the control (Fig. 12). Similar to cotyledon high level of ZTL protein was also observed in the root vascular bundle (Fig. 13). Fluorescence was localized both in the cyto-plasm and nucleus (Fig. 13) of the mesophyll cells. Weaker signal occurred in the root cortex cells (Fig. 14). In cotyledon strong fluorescence was present in the upper part as well around vascular tissues (Fig. 11). In turn, in the root of the seedling clear decrease of fluorescence level was observed from vascular bundle to epidermis (Fig. 14).

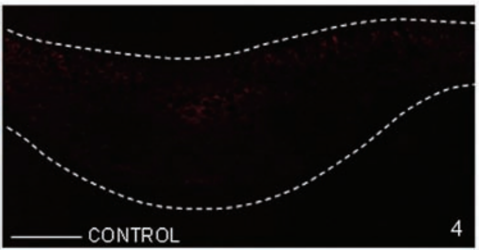
Discussion

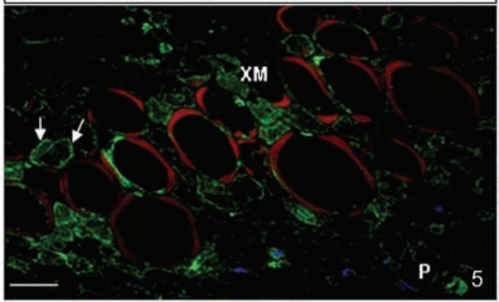
The *InZTL* gene is predicted to encode a 622 amino acid polypeptide with three distinct motifs: LOV and F-box domains and six kelch repeats (Fig. 1). All these domains were also identified in proteins which belong to ZEITLUPE family (ZTL, FKF1 and LKP2) in *A. thaliana* (Somers *et al*. 2000). Moreover, Boxall *et al*. (2005) found all three of these motifs in McZTL and McFKF1 proteins from *M. crystallinum*. Our results indicate that LOV, F-box and kelch repeats motifs found in InZTL protein are highly conserved between ZEITLUPE proteins from different species. This suggests the importance of these domains for ZTL function in plants. The *A. thaliana* ZTL protein is involved in the control of circadian period, hypocotyl elongation and flowering time regulation (Somers *et al*. 2004). High sequence homology of InZTL to *A. thaliana* ZTL (83 %) may suggests that this protein plays a similar function in *I. nil*.

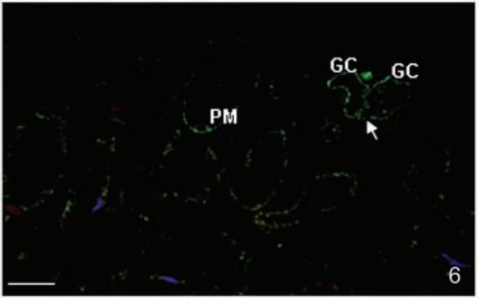
Organ-specific accumulation of *InZTL* mRNAs was observed in all examined organs of *I. nil* seedlings. The highest level of *InZTL* mRNA was observed in cotyledons (Fig. 2). In *A. thaliana* containing the ZTL::GUS construct, the highest GUS activity was also detected in cotyledons, as well in rosette and cauline leaves (Kiyosue and Wada 2000). High level of *InZTL* transcript was also detected in hypocotyl of *I. nil* seedlings (Fig 2.). On the contrary low level of the *InZTL* mRNA was present in roots. Transcripts of all *ZEITLUPE* mRNAs, as well of several circadian clock molecules (*e.g*. PHYA and PHYB), were detected in roots of *A. thaliana*, but information about circadian clock machinery in root cells is quite limited and its organization and role remains unclear (Hall *et al*. 2001, Schultz *et al*. 2001). In *I. nil* flowers, significantly high levels of the *InZTL* mRNA were observed in sepals and pistils and the lowest in petals and stamens. Kiyosue and Wada (2000) using RT-PCR showed that in *A. thaliana* the *AtZTL* mRNA level is higher in flowers than in roots and stem, but lower than in leaves. In *A. thaliana* plants transformed with *ZTL::GUS* construct, the level of its expression was significantly higher in petals than in sepals (Kiyosue and Wada 2000). Although the fact that the *AtZTL* gene is specifically expressed in some flower parts, the authors don't speculate about its role in generative development of *A. thaliana*.

Localization of *InZTL* mRNA and ZTL protein in cotyledon of *I. nil* seedlings indicated their highest levels in the vascular bundle area, especially in xylem mesophyll cells. Lower signal was present in phloem cells and palisade and spongy mesophyll cells. Many molecules involved in flowering were localized in vascular bundles of cotyledons or leaves in *A. thaliana.* However, major proteins controlling the flowering, like CO and FT, were found to be expressed specifically in vascular bundles of leaves (Takada and Goto 2003). In contrast, photoreceptors are expressed in almost all tissues of *A. thaliana* leaves. PhyB and cry2, for example, were localized in the epidermis, mesophyll and vascular bundles in cotyledons. Interestingly, Endo *et al.* (2005) showed that phyB-GFP expressed in mesophyll cells affects the flowering by suppressing FT expression in vascular bundles, whereas phyB-GFP expressed in vascular bundles does not. In turn Endo *et al.* (2007) indicated that the vascular bundles are the major functional site of cry2-mediated regulation of flowering. Moreover, flowering was unaffected by cry2-GFP expression in mesophyll or epidermis. Thus, the obtained localization pattern of *InZTL* mRNA and ZTL protein also may indicate the functional site of these molecules in *Ipomoea nil.*









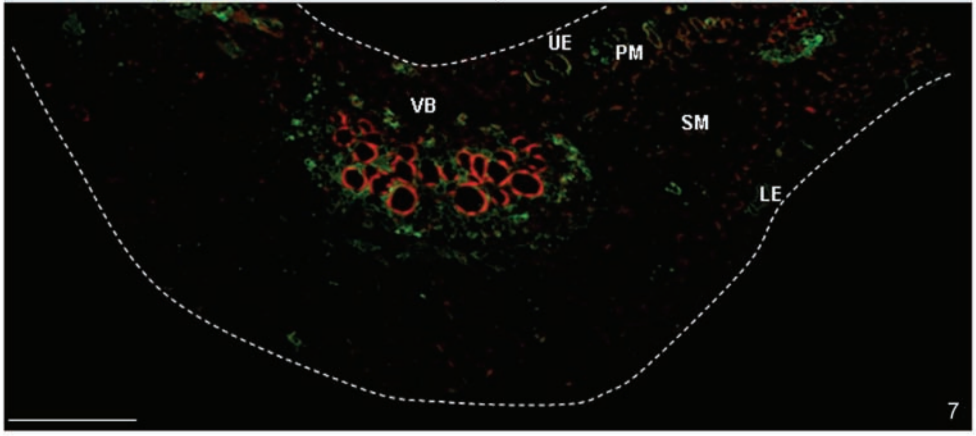


Fig. 3. Cotyledon of 5-d-old *Ipomoea nil* seedling after methylene blue staining (*bar* = 250 µm; CB - cotyledon body, LE - lower epidermis, PM - palisade mesophyll, SM - spongy mesophyll, VB - vascular bundle, UE - upper epidermis).

Figs. 4 - 7. Localization of *InZTL* mRNA in the cotyledon of *Ipomoea nil* seedling. Hybridization signal is visible in green and autofluorescence is visible in red. Blue color representing DNA (GC - guard cell, LE - lower epidermis, P - phloem, PM - palisade mesophyll, SM - spongy mesophyll, VB - vascular bundle, UE - upper epidermis, XM - xylem).

Fig. 4. Control reaction performed with sense probe. No labeling of the cotyledon can be seen (*bar* = 250 µm).

Fig. 5. Vascular bundle area. High level of the signal is present in xylem mesophyll cells (*arrows*). Weak fluorescence is visible in phloem cells cytoplasm (*bar* - 25 µm).

Fig. 6. Epidermal and mesophyll cells. Low signal can be seen in the upper epidermis, excluding guard cells (*arrow*) where the strong fluorescence is visible. Labeling is also present in the cytoplasm of spongy and palisade mesophyll cells (*bar* = 25 µm).

Fig. 7. Cotyledon cross section. The highest signal is visible in the vascular bundle area, mainly in the xylem elements. Weaker fluorescence is present also in spongy and palisade mesophyll area as well as in epidermal cells (*bar* = 250 µm).

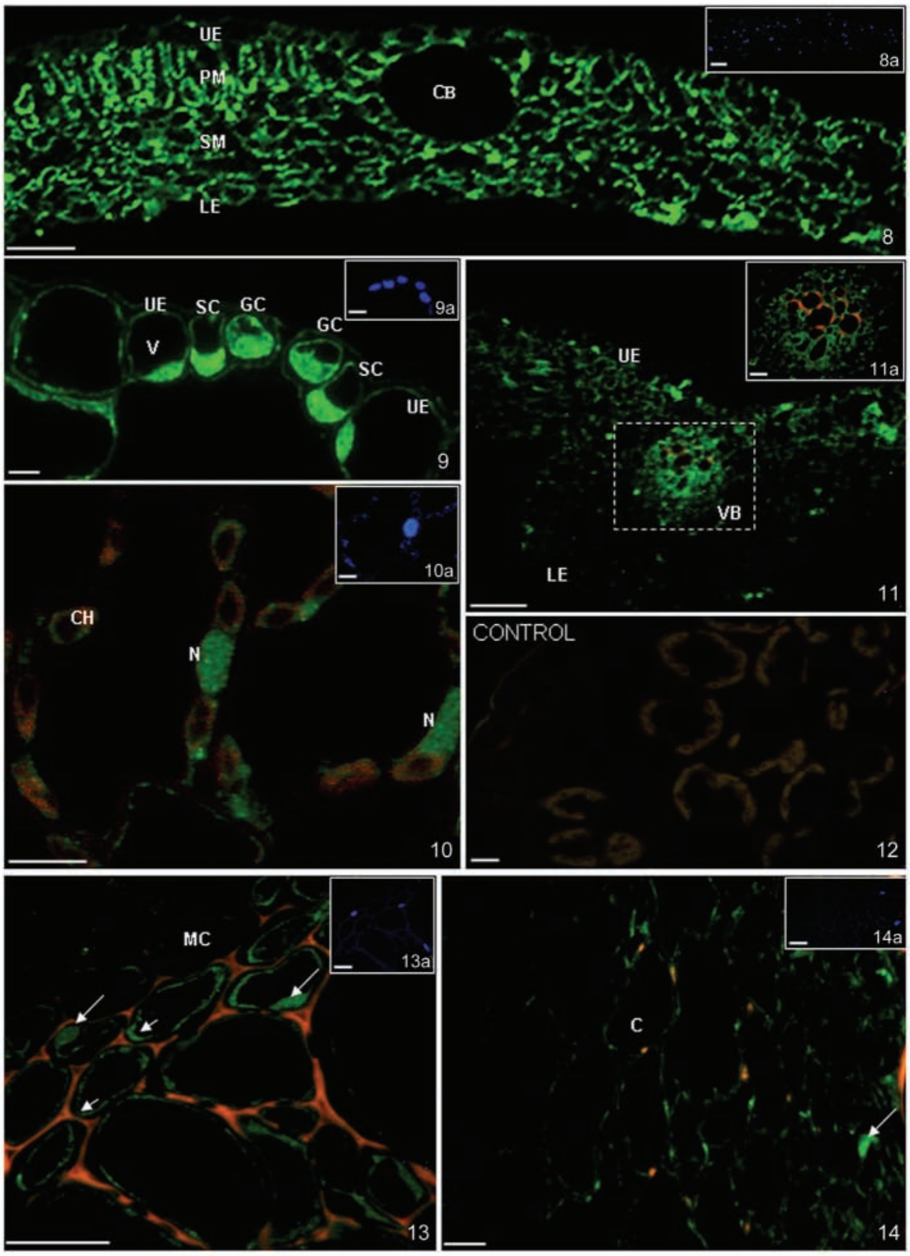


Fig. 8, 8a. The signal is visible in epidermal and mesophyll cells. There is a lack of green fluorescence in cotyledon body (*bar* = 250 µm).

Fig. 9, 9a. Labeling is present both in nuclei and cytoplasm of guard and epidermal cells. No fluorescence can be seen in the vacuoles (*bar* = 40 µm).

Fig. 10, 10a. Spongy mesophyll cell. The highest signal occurs in the cell nucleus in the form of clusters with different size and shape. A low fluorescence can be seen also in the cytoplasm (*bar* = 50 µm).

Fig. 11. The highest signal is present in the vascular bundle area (*bar* = 250 µm). Fig. 11a. Magnification of the area marked with the dashed line in Fig. 11 (*bar* = 50 µm).

Fig. 12. Control reaction conducted by omitting the incubation with the antibody against the ZTL protein. No labeling of the cotyledon tissues can be seen (*bar* = 40 µm).

Figs. 13, 14. Localization of the ZTL protein in the root of *Ipomoea nil* seedling. Insets 13a and 14a show DAPI staining. The signal is visible in green and autofluorescence is visible in red. Blue color representing DNA. (C - cortex, MC - mesophyll cell).

Fig. 13. An increased fluorescence is visible in the area of vascular elements. The signal is present both, in the nuclei (*larger arrows*) and the cytoplasm (*minor arrows*) of xylem mesophyll cells (*bar* = 50 µm).

Fig. 14. A weak fluorescence can be seen in the nucleus and cytoplasm of the cortex mesophyll cells (*arrow*) (bar = 40 µm).

The lowest level of *InZTL* transcripts and InZTL protein were observed in epidermal cell, excluding guard cells, where the signals were much higher. It is well known that the stomatal movements show the circadian rhythms regulated by the environmental and physiological conditions as well by the biological clock. Gorton *et al.* (1989) analyzed the circadian stomatal movements in *Vicia faba*. They isolated the guard cells from plants grown under 12-h photoperiod. It was shown that circadian stomatal movements persist also after their exposure to continuous light. Moreover, the authors found that even in guard cells isolated from epidermis, circadian stomatal movements still occurred, even after they were separated from each other. These results suggests that every guard cells has its own circadian clock, which acts independently from other cells (Gorton *et al.* 1989). This hypothesis was supported by several different experiments. Somers *et al.* (1998) found that in *toc1 A. thaliana* mutants the circadian period of stomatal rhythms in low irradiance was shorter than in wild type plants. Thus, it seems that each guard cell has its own oscillator composed of LHY, CCA and TOC1 proteins, however, coordination of processes is still unknown as well as the role of irradiance. Several reports suggest the potential role of blue light photoreceptors like phototropins and zeaxanthin (Kinoshita and Shimazaki 2002, Zeiger *et al.* 2002). Thus, we can not exclude that the ZTL protein, which also acts as blue light photoreceptor, may be involved in the mechanism of circadian stomatal movements.

Additionally we showed relatively high content of ZTL protein in roots of *I. nil* seedlings. It is known that expression of many root-specific genes is under circadian clock control, however, the molecular basis still remains unknown. Circadian expression was shown for genes encoding nitrate reductase, H1 histone and chalcone synthase (Abd-el Baki *et al.* 2000, Corlett *et al.* 1998). Moreover, the circadian expression of *XSP30* (*XYLEM SAP PROTEIN 30 kDa*) was observed only in root tissues and its mRNA was not detectable in upper parts of *Cucumis sativus* seedlings (Oda *et al.* 2003). The *XSP30::GUS* construct was detected in root tip, secondary roots, xylem mesophyll and pericycle. The content of XSP30 protein also showed circadian oscillations. In turn, the highest levels of *CHS::GUS* construct in *A. thaliana* roots were observed in meristematic region as well in the secondary roots. Strong expression of *PHYA* gene coding phytochrome A, was also shown in the cells of root tip in *A. thaliana* (Hall *et al.* 2001). These results strongly suggest that root cells also posses the circadian machinery and the ZTL protein localization in *I. nil* roots seems to support this hypothesis.

Cellular localization of *InZTL* mRNA in *I. nil* seedling cotyledons showed its presence mainly in the cytoplasm while in other examined tissues the ZTL protein was detected both in the cytoplasm and the nucleus. Similarly, in *A. thaliana* the AtZTL protein as well other ZEITLUPE proteins were localized both in the cytoplasm and the nucleus (Kiyosue and Wada 2000). Kiyosue and Wada (2000) identified the putative nuclear localization sequence (NLS) in AtZTL. We identified a similar sequence also in ZTL which is consistent with our immunolocalization results. Nuclear localization of ZTL protein most probably reflects its interactions with other components in nucleus. Indeed, it was shown that in *A. thaliana* the ZTL protein binds to LKP2 and ASK1 proteins (Fukamatsu *et al.* 2005). It seems possible that nuclear distribution reflects similar interactions of ZTL in *I. nil*. Analysis of the ZTL nuclear localization pattern indicated that it accumulates in numerous spots present in the nucleus of the mesophyll cells (Fig. 10). Similar distribution pattern was also observed for TOC1 protein in *A. thaliana*. Moreover, specific colocalization of AtLKP2 protein with CO protein in distinct nuclear bodies was observed (Fukamatsu *et al.* 2005).

Our results indicated that *InZTL* expression level is different in various tissues of *I. nil* seedlings and may reflect tissue-specific function of these molecules. Undoubtedly, a lot of further experiments will be needed to fully understand the role of ZTL in *I. nil*, however, our report provides many valuable data concerning this problems.

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