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CLONING AND EXPRESSION ANALYSIS OF *LeTIR1* IN TOMATO

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The full-length cDNA of *LeTIR1* gene was isolated from tomato with EST-based in silico cloning followed by RACE amplification. *LeTIR1* contained an open reading frame (ORF) 1872 bp long, encoding 624 amino acid residues. The predicted protein LeTIR1 had one F-box motif and eleven leucine-rich repeats (LRRs), all of which are highly conserved in TIR1 proteins of other plant species. Phylogenetic analysis showed that the LeTIR1 protein shared high similarity with other known TIR1 proteins. Both sequence and phylogenetic analysis suggested that *LeTIR1* is a *TIR1* homologue and encodes an F-box protein in tomato. Semi-quantitative RT-PCR indicated that *LeTIR1* was expressed constitutively in all organs tested, with higher expression in stem than root, leaf, flower and fruit. Its expression level was positively correlated with the auxin distribution in stem or axillary shoot, and was induced by spraying exogenous IAA.

Key words: Tomato, TIR, auxin receptor, IAA, semi-quantitative RT-PCR.

INTRODUCTION

Among the five kinds of endogenous phytohormones, auxin was the one earliest identified. In 1880, Charles Darwin and his son discovered transportable matter which exists at very low concentrations but plays a crucial role in plant phototropism. This matter was ultimately identified as auxin, naturally existing as indole-3-acetic acid (IAA) in plants (Woodward and Bartel, 2005). Auxin is involved in many aspects of plant growth and development (Dharmasiri et al., 2003; Callis, 2005), such as root elongation, adventitious root formation, apical dominance, vascular tissue formation, root geotropism and stem phototropism. It also promotes flowering, embryogenesis and root system formation (Willemsen and Scheres, 2004; Leyser, 2005).

In higher plants, the auxin signal is first received by receptors which then trigger auxin signaling, followed by plant growth and development responses. Recently it has been shown that the F-box protein TIR1 is an auxin receptor which becomes an important component of the SCF^{TIR1} protein complex by combining with SKP1 (Sphase kinase-associated protein 1) and Cullin protein which has ubiquitin ligase E3 activity (Gagne et al., 2002). TIR1 can bind directly to auxin and mediate specific recognition and interaction of the SCF^{TIR1} protein complex with auxin response repressor Aux/IAAs (Gray et al., 2001; Kepinski and Leyser, 2005). Subsequently, Aux/IAAs are ubiquitinated by the ubiquitin ligase E3 activity of the complex (Moon et al., 2004) and degraded through the proteasome pathway (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Finally, repression is extinguished so that the plants grow and develop in response to the auxin signal. In addition to TIR1, three other F-box proteins – AFB1, AFB2 and AFB3 – act as auxin receptors too (Dharmasiri et al., 2005b). Those four F-box proteins work together to regulate plant responses to auxin.

Tomato is one of the world's most widely cultivated fruit vegetables. In tomato, auxin not only regulates plant growth and development but also functions in many processes associated with fruit development such as blossoming, fruit setting and ripening (Wang et al., 2005; Pandolfini et al., 2007), which directly affect fruit production and quality. Little is known about the molecular mechanism of tomato plant responses to the auxin signal. In this study we cloned a homolog of the *TIR1* gene, *LeTIR1*, with EST-based in silico cloning and RACE

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TABLE. 1. Primer sequences for RT-PCR and RACE amplification

Primers	Sequences
LeACTIN-S	5′-CTTCAGTCCACAATCGGTGG-3′
LeACTIN-A	5′-CATTCCGACTTGAGCTGCTG-3′
pLeTIR-S	5′-CATCTTCTTCAACTCTTCCTGG-3′
pLeTIR-A	5′-AGGCCTTCGTCACATACAG-3′
LeTIR3-1	5′-GTATCTGACGATGAAGTGGAC-3′
LeTIR3-2	5′-AGGTCACCTAGTTTGAAGAG-3′
B26	5′-GACTCGAGTCGACATCGATTTTTTTTTTTTTTTT-3′
B25	5′-GACTCGAGTCGACATCGA-3′
LeTIR-S	5′-CATGAGTGGTAGTGACAATCC-3′
LeTIR-A	5′-AATCTCTGTACGGAACCATATAC-3
LeTIRsq-S	5′-CTTCCCATTAATCTAGACCCTC-3
LeTIRsq-A	5′-CAAGGGATGCAGCGTTAC-3

amplification from tomato. Then we profiled its expression patterns with semi-quantitative PCRs. This made it possible for us to further examine the function of LeTIR1 and the responses of the tomato plant to the auxin signal.

MATERIALS AND METHODS

PLANT MATERIALS

Tomato cultivar 'Zhongshu NO.4' was used in this study. Different tissues or organs were harvested depending on the experimental requirements. Stock solutions of IAA were prepared by dissolving the required amount of IAA in 100 µl 95% EtOH, increased to the required volume with distilled water, and subsequently diluted to prepare 20 mg/l aqueous solutions of IAA from stock solution. To reduce the endogenous IAA content, shoot apices were removed from the tomato plants. Five days after shoot apex removal, tomato plants with or without shoot apices were used for auxin treatment. Plant material was sampled 0, 15, 30 and 60 min after spraying 20 mg/L IAA solution. Distilled water was sprayed as the control.

RNA EXTRACTION AND cDNA SYNTHESIS

Total RNA was extracted from tomato tissues using TRIzol reagent (Invitrogen, U.S.A.) as described in the manufacturer's instructions. Two milligrams of total RNA were used to synthesize cDNA template with the PrimeScript First Strand cDNA Synthesis Kit (TaKaRa, Dalian, China) according to the manufacturer's instructions.

RACE AMPLIFICATION

The PCR reaction mixture contained 200 ng cDNA, 1 µl B26 (10 mM), 5 µl 10 Taq DNA polymerase buffer containing Mg^{2+}, 4 µl deoxyribonucleotide (dNTP) (2.5 mM), 1 µl primer (10 mM) and 0.5 µl transTaq DNA polymerase (5 U/µl) in a total 50 µl volume. The reactions were initially denatured at 94°C for 5 min followed by 35 cycles of amplification as follows: 30 s at 94°C, 1 min at 55°C, 1 min at 72°C, and a final extension of 10 min at 72°C. The resulting PCR products were used as template for another round of PCR amplification with primers TIR3-2 and B25, with the reaction mixture and PCR cycles as above. The PCR products were electrophoretically separated on 1% agarose gel and purified with the Agarose Gel DNA Purification Kit (TaKaRa, Dalian, China). Then the target fragment was subcloned into cloning vector *pMD18-T* (TaKaRa, Dalian, China). The resulting plasmid was then transferred into *E. coli* strain DH5 α. After screening in selection medium, the positive clones were chosen for sequencing (Sangon, Shanghai, China). Table 1 shows all the primers used.

SEMI-QUANTITATIVE RT-PCR

The expression level of *LeTIR1* was detected with semi-quantitative RT-PCR with specific primers LeTIRsq-S and LeTIRsq-A (Tab. 1). The PCR reaction mixture contained 200 ng cDNA, 1 µl forward primer LeTIRsq-S (10 mM), 1 µl reverse primer LeTIRsq-A (10 mM), 2.5 µl 10 Taq DNA polymerase buffer containing Mg^{2+}, 2 µl deoxyribonucleotide (dNTP) (2.5 mM) and 0.25 µl transTaq DNA polymerase (5 U/µl) in a total 25 µl volume. The reactions were initially denatured at 94°C for 5 min followed by 25 cycles of amplifications as follows: 30 s at 94°C, 30 s at 55°C, 30 s at 72°C, and a final extension of 10 min at 72°C. The PCR products were separated by 1% agarose gel by electrophoresis. *LeACTIN* was used as loading control with primers LeACTIN-S and LeACTIN-A (Tab. 1).

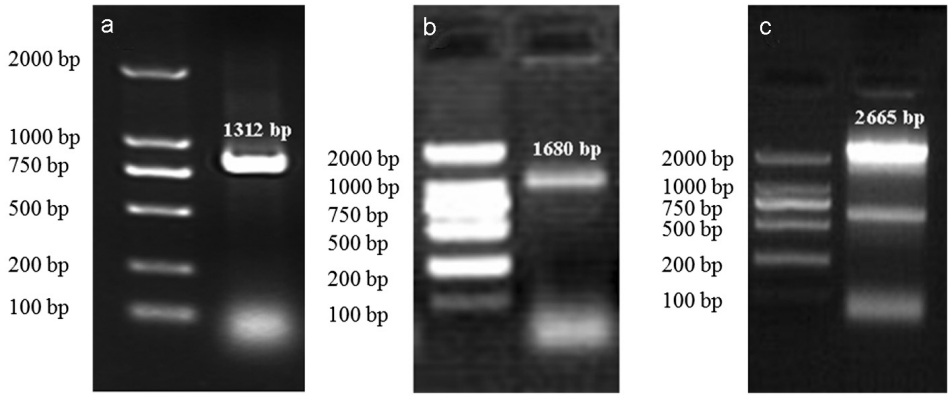

Fig. 1. Molecular cloning of *LeTIR1*. (**a**) RT-PCR product of the cDNA fragment of *LeTIR1*, (**b**) Product of 3'-RACE amplification of *LeTIR1*, (**c**) RT-PCR product of the full length of *LeTIR1*.

RESULTS

EST-BASED IN SILICO CLONING AND RACE AMPLIFICATION OF *LeTIR1* cDNA

The *LeTIR1* EST sequence in GenBank was BLAST-searched with the cDNA sequence of *AtTIR1* (AAF78487), and its homologous EST fragments were obtained. EST BI925157 was the one with the highest similarity to *AtTIR1*. Subsequently, the homologous EST fragments were searched and aligned with DNAMAN® software (Lynnon Biosoft, Vaudreuil, Quebec, Canada) until no more overlapped EST sequences could be prolonged. Consequently, a 1513 bp homologous fragment (*pLeTIR1*) of the *TIR1* gene in tomato was acquired. It contained the start codon and part of the 5'-UTR sequence.

To get the cDNA fragment of *pLeTIR1*, RT-PCR was conducted with primers pLeTIR-S and pLeTIR-A. This yielded a 1312 bp fragment (Fig. 1a). After confirmation by sequencing, *pLeTIR1* was used as core sequence for RACE amplification. Nested RT-PCRs were carried out for 3'-RACE amplification. Primers B26 and TIR3-1 were used for the first round of PCR using tomato cDNA as template. The resulting PCR products and primers TIR3-2 and B25 were used for another round of PCR amplification. A 1680 bp fragment was obtained (Fig. 1b). It was confirmed to be the 3' region of tomato TIR1 by sequencing. Finally, the full-length cDNA sequence of the tomato *TIR1* gene was aligned with DNAman software. Then the specific primers LeTIR-S and LeTIR-A were designed and used for RT-PCR to clone the full-length cDNA. The result showed that the tomato *TIR1* cDNA was 2665 bp in its full length; hereafter it is called *LeTIR1* (GU079663, Fig. 1c). The full-length cDNA of *LeTIR1* contained 3'- and 5'-UTR, 247 bp and 673 bp respectively. Its open reading frame (ORF) was 1872 bp in length and encoded a polypeptide containing 624 amino acid residues with molecular weight 68.6 kD (Fig. 2).

FUNCTIONAL DOMAINS AND GENETIC RELATIONSHIP OF LeTIR1 PROTEIN

We made a homology comparison among TIR1 proteins of different plant species with DNAman software. PpTIR1 (*Prunus persica*) from the dicotyledonous woody plant poplar showed the highest similarity (69.17%) with *LeTIR1*. Other values of similarity with *LeTIR1* were 48% for VvTIR1 (*Vitis vinifera*), 47.16% for AtTIR1 (*Arabidopsis thaliana*), 46.24% for PtTIR1 (poplar), 45.56% for BrTIR1 (*Brassica*), 42.68% for GhTIR1 (*Gossypium hirsutum*) and 43.7% for PsTIR1 (*Lypodiopsida*) (Fig. 3). All those proteins contained the conserved F-box motif and 11 leucine-rich repeats (LRRs) (Fig. 4).

We also used DNAman to create the phylogenetic tree for LeTIR1 and other TIR1-like proteins, shown in Figure 4. LeTIR1 and PpTIR1 grouped into one cluster, indicating that among the TIR1 proteins PpTIR1 had the closest genetic relationship with LeTIR1.

EXPRESSION PATTERN AND AUXIN RESPONSE OF *LeTIR1*

To examine the expression pattern of *LeTIR1* in different tissues and its responses to the auxin signal, a pair of specific primers, LeTIRsq-S and LeTIRsq-A, was designed in 5'-UTR. Semi-quantitative RT-PCRs were run with the specific primers, which showed that *LeTIR1* was constitutively expressed in the different organs tested. Its expression level varied with the organ. It accumulated the highest level of transcripts in stem, and quite a low level in leaves and fruits (Fig. 5a).

TIR1 protein is an auxin receptor in *Arabidopsis*, so we suggest that *LeTIR1* most likely acts as an auxin receptor in tomato, and that it expresses in response to the gradient distribution of endogenous auxin. Because auxin is synthesized mainly in the shoot apex and transported gradually

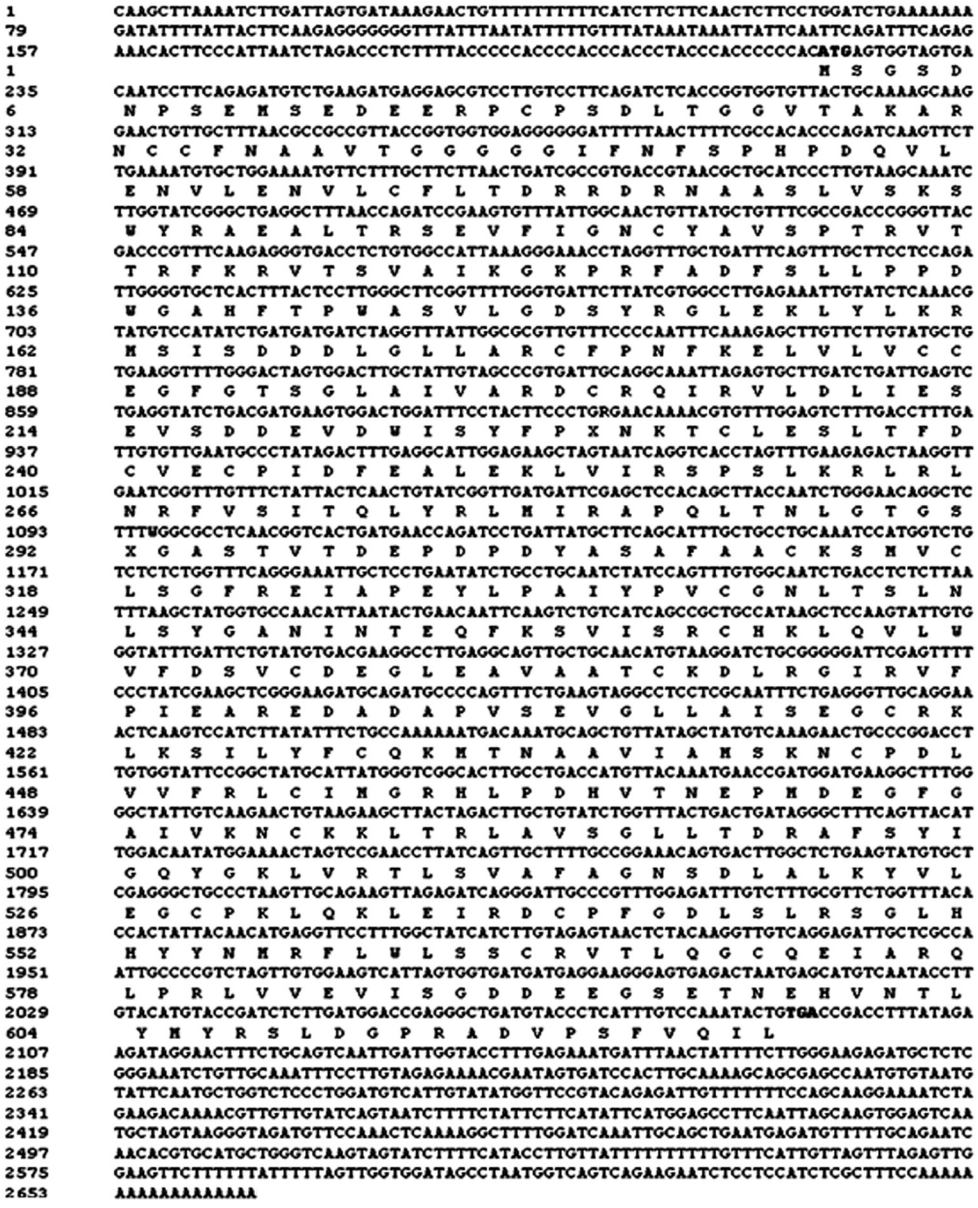

Fig. 2. Nucleotide/deduced amino acid sequences of *LeTIR1*/*LeTIR1*. Sequence of *LeTIR1* cDNA and predicted amino acid sequence of *LeTIR1* protein. The Start and Stop codons are in bold caps.

to the roots, and because the transport activity of IAA is higher in the upper parts than in the lower parts (Kojima et al., 2002), auxin is distributed in a concentration gradient, from high in the shoot apex to low in roots. To examine whether *LeTIR1* expression responds to the gradient distribution of auxin in tomato plants we ran semi-quantitative RT-PCR. We divided the stem into four parts, S1 to S4, and the axillary shoot into three parts, A1 to A3 (Fig. 5b). The cDNA of each part was used as template in a series of semi-quantitative RT-PCRs. The results confirmed that the *LeTIR1* expression levels were positively correlated with the auxin distribution in both stem and axillary shoot, that is to say, it decreased gradually in response to the decline of auxin concentration from shoot apex to base, demonstrating a positive response of *LeTIR1* expression to the endogenous auxin level (Fig. 5c,d).

In addition to its response to endogenous auxin, the response of *LeTIR1* expression to exogenous IAA was also monitored with semi-quantitative RT-PCR. The result indicated that the expression level of

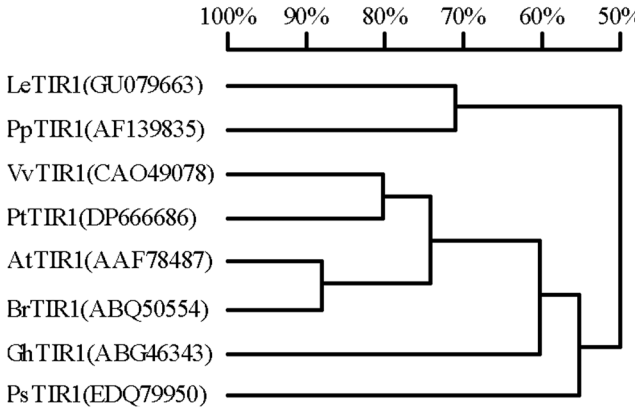

Fig. 3. Phylogenetic tree analyses of TIR-like proteins. LeTIR1(GU079663) from *Solanum lycopersicum*; PpTIR1(AF139835) from *Prunus persica*; VvTIR1 (CAO49078) from *Vitis vinifera*; PtTIR(DP666686) from *Populus*; AtTIR(AAF78487) from *Arabidopsis thaliana*; BRTIR1(ABQ50554) from *Brassica*; GhTIR1(ABG46343) from *Gossypium hirsutum*; PsTIR1(EDQ79950) from *Lypodiopsida*.

LeTIR1 gradually increased at 15, 30 and 60 minutes after IAA spraying (Fig. 5e). However, endogenous IAA may induce the expression of genes related to its signaling pathway. To further confirm that *LeTIR1* was induced by exogenous IAA, the shoot apex was removed from the tomato plant to reduce endogenous IAA content. Then the tomato plants without the shoot apex were sprayed with 20 mg/L IAA solution. Semi-quantitative RT-PCR was performed to analyze the expression of *LeTIR1* in response to IAA treatment, and showed that the expression level of *LeTIR1* was positively induced by IAA but not by distilled water (Fig. 6a,b). This is further confirmation that *LeTIR1* expression positively responded to the auxin level.

DISCUSSION

Great progress has been made in research on auxin signal transduction in plants in the last few years. In 2005, two articles in the same issue of *Nature* stat-

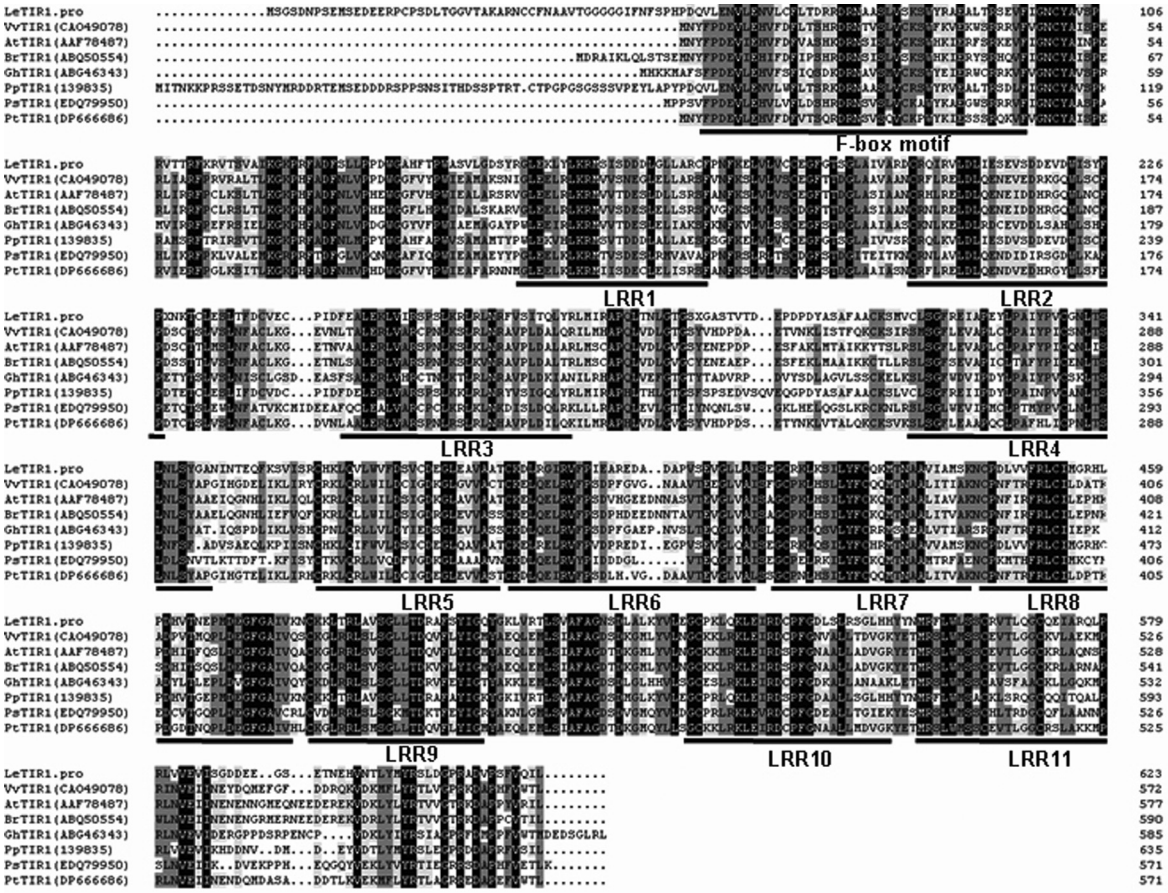

Fig. 4. Alignment of selected TIR1-like proteins. LeTIR1(GU079663) from *Solanum lycopersicum*; PpTIR1(AF139835) from *Prunus persica*; VvTIR1(CAO49078) from *Vitis vinifera*; PtTIR(DP666686) from *Populus*; AtTIR(AAF78487) from *Arabidopsis thaliana*; BRTIR1(ABQ50554) from *Brassica*; GhTIR1(ABG46343) from *Gossypium hirsutum*; PsTIR1(EDQ79950) from *Lypodiopsida*. The underlined sequences were conserved domains as named.

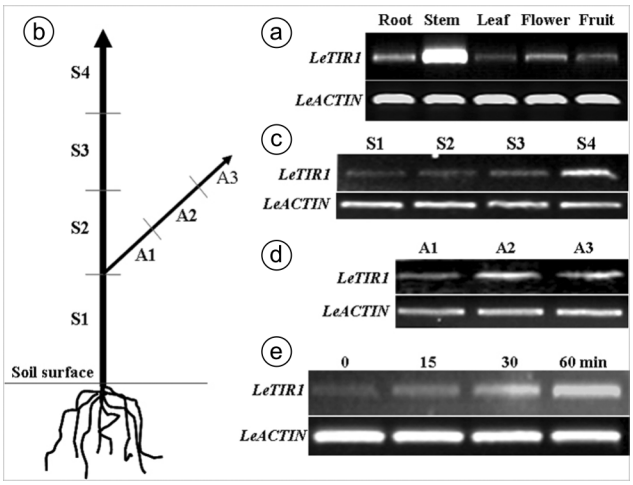

Fig. 5. Semi-quantitative RT-PCR analysis of *LeTIR1* expression. (**a**) Expression levels of *LeTIR1* gene in different organs, (**b**) Sketch of the different parts for stem (S1–S4) and axillary shoot (A1–A3), (**c**) Expression levels of *LeTIR1* in part S1 to S4 of plant stem, (**d**) Expression levels of *LeTIR1* in part A1 to A3 of axillary shoot, (**e**) Expression levels of *LeTIR1* in response to IAA spraying treatments. Transcriptional levels were normalized to expression of a tomato *LeACTIN* gene.

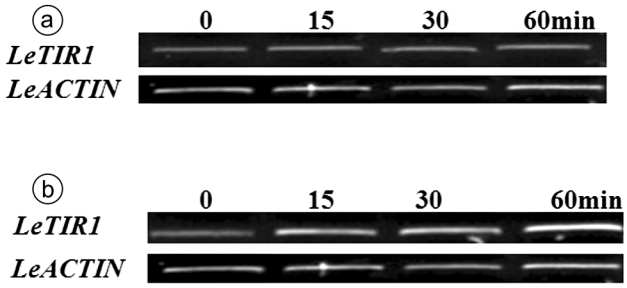

Fig. 6. *LeTIR1* expression in response to exogenous IAA spraying in decapitated tomato plants. (**a**) Expression levels of *LeTIR1* in response to distilled water spraying in decapitated tomato plant, (**b**) Expression levels of *LeTIR1* in response to IAA spraying treatments in decapitated tomato plant. Transcriptional levels were normalized to expression of a tomato *LeACTIN* gene.

ed unequivocally that the F-box protein TIR1 is an auxin receptor (Dharmasiri et al., 2005b; Kepinski and Leyser, 2005), but auxin receptor genes were poorly understood in tomato. In this study we isolated the full-length cDNA of the tomato *LeTIR1* gene with EST-based in silico cloning followed by RACE amplification. The predicated protein encoded by *LeTIR1* contained a conserved F-box motif which is likely involved in the degradation of AUX/IAA protein via ubiquitination and the proteasome pathway (Ruegger et al., 1997). The LeTIR1 protein contained 11 leucine-rich repeats conserved in other TIR1 proteins (LRRs, LxxLxLxxN/CxL; Gagne et al., 2004). They were involved in protein interaction at the C-terminal, which is necessary in substrate recognition (Gagne et al., 2004). In addition, the F-box motif and LRRs were indispensable for TIR1 protein to form the SCF^{TIR1} complex. Thereafter the complex recognizes and interacts with the Aux/IAA protein, followed by ubiquitination and degradation of the Aux/IAA protein. Finally, repression of the auxin response is extinguished in the plant (Dharmasiri et al., 2005a; Kepinski and Leyser, 2005). Homology analysis and the phylogenetic tree showed a conserved functional domain both in the LeTIR protein and in other TIR1-like proteins from different plant species. This indicates that *LeTIR1* likely encodes an auxin receptor protein in tomato.

It has been documented that the *AtTIR1* gene is constitutively expressed in rosette leaf, stem, inflorescence and siliques of *Arabidopsis thaliana*, and that the AtTIR1 protein accumulates with the increase of auxin (Dharmasiri et al., 2005a). Similarly, in our work, *LeTIR1* was constitutively expressed in different organs tested, with the highest level of transcripts accumulated in stem. In this study, the *LeTIR1* transcripts followed a gradient distribution, decreasing from the shoot apex to the base in both stem and axillary shoots (Fig. 5b-d); this distribution of *LeTIR1* transcripts is similar to the endogenous auxin distribution along stem and axillary shoots from apex to base (Leyser, 2005). Thus, the expression level of *LeTIR1* follows the distribution and concentration of endogenous auxin in tomato. The expression level of *LeTIR1* increased with exogenous IAA spraying, demonstrating that *LeTIR1* expression was induced by IAA, as in other plants (Dharmasiri et al., 2005b).

CONCLUSION

LeTIR1 exhibits some typical characteristics of an auxin receptor, as shown by sequence analysis, the expression pattern and auxin response. We suggest that *LeTIR1* likely encodes an auxin receptor in tomato. Further work should identify its functions in tomato.

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REFERENCES

CALLIS J. 2005. Auxin action. *Nature* 435: 436–437.

DHARMASIRI N DHARMASIRI S, JONES AM, and ESTELLE M. 2003. Auxin action in a cell free system. *Current Biology* 13: 1418–1422.

DHARMASIRI N, DHARMASIRI S, ESTELLE M. 2005a. The F-box protein TIR1 is an auxin receptor. *Nature* 435: 436–437.

DHARMASIRI N, DHARMASIRI S, WEIJERS D, LECHNER E, YAMADA M, HOBBIE L, EHRISMANN JS, JÜRGENS G, and ESTELLE M. 2005b. Plant development is regulated by a family of auxin: receptor F-box proteins. *Developmental Cell* 9: 109–119.

GAGNE JM, DOWNES BP, SHIU SH, DURSKI AM, and VIERSTRA RD. 2002. The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis. Proceedings of the National Academy of Sciences, USA* 99: 11519–11524.

GAGNE JM, SMALLE J, GINGERICH DJ, WALKER JM, YOO SD, YANAGISAWA S, and VIESTRA R. 2004. *Arabidopsis* EIN3-binding F-box 1 and 2 form ubiquitin-protein ligases that repress ethylene action and promote growth by directing EIN3 degradation. *Proceedings of the National Academy of Sciences, USA* 101: 6803–6808.

GRAY WM, KEPINSKI S, ROUSE D, LEYSER O, and ESTELLE M. 2001. Auxin regulates SCF^{TIR1}-dependent degradation of Aux/IAA proteins. *Nature* 414:271–276.

KEPINSKI S, and LEYSER O. 2005. The *Arabidopsis* F-box protein TIR is an auxin receptor. *Nature* 435: 446–451.

KOJIMA K, OHTAKE E, and YU Z. 2002. Distribution and transport of IAA in tomato plants. *Plant Growth Regulation* 37: 249–254.

LEYSER O. 2005. Auxin distribution and plant pattern formation: how many angels can dance on the point of PIN? *Cell* 121: 819–822.

MOON J, PARRY G, and ESTELLE M. 2004. The ubiquitin-proteasome pathway and plant development. *The Plant Cell* 16: 3181–3195.

PANDOLFINI T, MOLESINI B, and SPENA A. 2007. Molecular dissection of the role of auxin in fruit initiation. *Trends in Plant Science* 12: 327–329.

RUEGGER M, DEWEY E, HOBBIE L, BROWN D, BERNASCONIP TJ, MUDAY G, and ESTELLE M. 1997. Reduced naphthylphthalamic acid binding in the *tir3* mutant of *Arabidopsis* is associated with a reduction in polar auxin transport and diverse morphological defects. *The Plant Cell* 9: 745–757.

WANG H, JONESA B, LI Z, FRASSE P, DELALANDE C, REGAD F, CHAABOUNI S, LATCHÉ A, PECH JC, and BOUZAYEN M. 2005. The tomato Aux/IAA transcription factor IAA9 is involved in fruit development and leaf morphogenesis. *The Plant Cell* 17: 2676–2692.

WILLEMSEN V, and SCHERES B. 2004. Mechanisms of pattern formation in plant embryo genesis. *Annual Review of Genetics* 38: 587–614.

WOODWARD AW, and BARTEL B. 2005. Auxin: regulation, action, and interaction. *Annals of Botany* 95: 707–735.