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CLONING THE FULL-LENGTH cDNA OF ACTIN GENE AND ANALYSING ALLIINASE GENE EXPRESSION IN TILLERING ONION

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Abstract

Tillering onion is a herbaceous plant belonging to the Liliaceae family. We cloned the cDNAs of the *actin* gene (*AcACT*, GenBank: MF919598) of tillering onion using rapid amplification of the cDNA ends. The full-length cDNA of *AcACT* was 1,357 bp long with an open reading frame of 1,131 bp encoding 376 amino acids. The amino acid sequence of AcACT shared > 96% similarity with the amino acid sequences of other ACTs and was found (by means of phylogenetic tree analysis) to be closely related to those of *Ananas comosus* and *Papaver somniferum*. *AcACT* expressions showed no significant differences ($p > 0.01$) in two cultivars L-SH and L-SY over three growth periods and under suitable conditions, low temperature, and short-day conditions. In addition, *AcACT* was used as an internal reference gene to analyse the expression of the alliinase gene (*AcALL*). *AcALL* expression trends in the roots, stems and leaves were consistent with those of diallyl disulphide and diallyl trisulphide. Thus, *AcACT* is highly conserved and can be used as a suitable internal reference gene when analysing gene expression in tillering onion.

Keywords: Actin. Alliinase. RACE. Tillering onion.

1. Introduction

Actin is an essential component of the cytoskeleton in higher plants (Bashline et al. 2014). Actin fibres coordinate developmental processes, such as the establishment of cell polarity, guiding the stratum of cell division, cell wall integration and synthesis, placement of organelles, participation in the dynamic tracks of the cytoplasmic cascade, regulation of transport through plasmodesmata, cell-cell communication, responses to pathogen attack, and regulation of cellular turgor pressure and shape changes (Stephan 2017; Duan et al. 2018; Szymanski et al. 2018; Ma et al. 2021). *Actin* genes are highly conserved within species at the nucleotide and protein levels, making them useful indicators when studying the evolution of higher plants (Wang et al. 2016; Wang et al. 2019). In addition, their stable expression during the different developmental stages, under different conditions, and in different tissues implies that *actin* genes can be used as reference genes for normalization (Nakayama et al. 2014; Liu et al. 2015; Kim et al. 2018; Wang et al. 2021). The structure and expression of *actin* genes have been well-characterised in higher plants (Wen et al. 2014). However, only a few *actin* genes from *Allium* species have been cloned and reported.

Tillering onion (*Allium cepa* L. var. *Multiplcans* Bailey syn. var. *Agrogatum* Don) is an annual herbaceous plant of the genus *Allium* and belongs to the Liliaceae family. This cultivar has a greatly

reduced sexual reproduction ability, and reproduces vegetatively by offsetting bulbs. Due to the presence of abundant sulphur and phenolic compounds (Yang et al. 2013; Yang et al. 2018), tillering onion displays strong allelopathy and is often used for rotation, intercropping, or concomitant cultivation in China (Li et al. 2009; Fu et al. 2016; Fu et al. 2019; Li et al. 2020; Fu et al. 2020). Plant allelopathy is a phenomenon of chemical ecology, which is affected by many factors, such as plants, microorganisms, allelochemicals, and environment (Cheng and Cheng 2016). Progress in allelopathy research involving the identification of the molecular mechanisms underlying allelopathy and the functional analysis of allelopathic genes have become important study areas. At present, the research foundation for the molecular biology of tillering onion is weak, which hinders the functional analysis of allelopathic genes needed for the development and utilization of active allelochemicals. As one of the internal reference gene, the *actin* gene could be used to study the spatiotemporal expression characteristics of other allelopathic genes in tillering onion.

In our study, we cloned and characterised the full-length cDNA of the *actin* gene (*AcACT*) and its mRNA distribution in different tillering onion tissues under different conditions. Subsequently, we used *AcACT* as an internal reference gene to evaluate the expression level of the *alliinase* gene (*AcALL*). Alliinase, an enzyme found in many *Allium* species, catalyses the generation of a chemically and therapeutically dynamic compound allicin (diallyl thiosulfate) (Mitrová et al. 2018; Soorni et al. 2021). Allicin is rapidly and spontaneously degraded to stable bioactive phytochemicals, such as diallyl disulphide (DADS), diallyl trisulphide (DATS), methyl allyl trisulphide and diallyl sulphide (Shang et al. 2019; Yoshimoto et al. 2019; Zhao et al. 2021). Cloning the *actin* gene of the tillering onion will promote relevant gene expression studies and genetic engineering research.

2. Material and Methods

Plant cultivars and RNA extraction

The tillering onion cultivars L-SH and L-SY were obtained from Jilin Province, China, and cultured in a plant culture chamber at 21 °C, 65% relative humidity, and 300 µmol photons $m^{-2}s^{-1}$ under 15 h light and 9 h dark conditions. Total RNA was isolated from the leaves of the tillering onion L-SH using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). The integrity and quantification of the isolated samples were investigated using agarose gel electrophoresis and spectrophotometry, respectively.

Primer design and synthesis

The primers were designed according to the partial sequences of the *actin* gene from *Allium cepa* L. (Accession: GU570135.2) using Primer 5.0 software. All the primers were synthesised by Sangon Biotech (Shanghai, China) to clone the conserved regions of *AcACT*, 5′ and 3′ RACE, and ORF. Primers were also used for analysing gene expression (Table 1).

Cloning the full-length cDNA for the target gene *AcACT*

The total RNA that was isolated was reverse transcribed using a PrimeScript™ II High Fidelity real time reverse transcription-polymerase chain reaction (RT-PCR) kit (TaKaRa, Otsu, Shiga, Japan) to obtain first-strand cDNA, which was then used as a template for PCR. After detection and purification, the target gene PCR product was cloned into the pMD18T vector (TaKaRa, Otsu, Shiga, Japan). Following sequencing, a BLAST search confirmed that the target fragment had a high sequence identity with homologous genes. The core fragment was used to design gene-specific primers (GSP) that could elongate the 5′ upstream and 3′ downstream regions by RACE. The 3′ RACE-ready and 5′ RACE-ready cDNAs were synthesized using 3′-Full RACE Core Set with PrimeScript™ RTase and 5′-Full RACE kit with TAP (TaKaRa, Otsu, Shiga, Japan), respectively.

For 3′ RACE of *AcACT*, two 3′-GSP (AcACT3-1 and AcACT3-2) and two universal primers (3′ outer primer and 3′ inner primer) were used to perform nested PCR amplification. In the initial step, AcACT3-1 and the 3′ outer primer were used along with the 3′ RACE-ready cDNA as templates, whereas for the

subsequent step, AcACT3-2 and the 3′ RACE inner primer were used along with the products of the PCR reaction as templates. Likewise, for 5′ RACE, two 5′-GSP (AcACT5-1 and AcACT5-2) and two universal primers (5′ outer primer and 5′ inner primer) were used as templates. Nested PCR reaction systems and procedures were performed according to the instructions of the kits. Both the ends of the target gene were collected and sequenced. ContigExpress (Vector NTI Advance11.5) was used to align and assemble the sequences of the 5′-uncovered region (5′ RACE), core fragment, and 3′-uncovered region (3′ RACE). The ORF of the target gene was subsequently amplified using the primer pair AcACT-F/R by RT-PCR. PCR amplification was performed for 5 min at 94 °C, followed by 35 amplification cycles (30 s at 94 °C, 30 s at 55 °C, and 90 s at 72 °C) and for 10 min at 72 °C. After purification, the amplified product was subcloned into pMD18T, transformed into competent DH5a cells of *Escherichia coli*, and sequenced. The target gene was independently amplified by PCR and was sequenced three times to confirm the experimental results.

Table 1. List of primers information.

Function	Primers	Sequence (5′→3′)
Conserved region	ACT-F	ACACGGCCTGGATAGCAACAT
clone	ACT-R	AGAGCAGTATTCCCAAGCATT
3′ RACE	3′ Adapter	TACCGTCGTTCCACTAGTGATTTCACTATAGGTTTTTTTTTTTTTTTTTVN
	3′ Outer Primer	TACCGTCGTTCCACTAGTGATTT
	3′ Inner Primer	CGCGGATCCTCCACTAGTGATTTCACTATAGG
	AcACT3-1	GAAAGCCAGTCTCTCCAC
	AcACT3-2	CTCAGTCCAAAAGGGGTA
5′ RACE	5′ Outer Primer	CATGGCTACATGCTGACAGCCTA
	5′ Inner Primer	CGCGGATCCACAGCCTACTGATGATCAGTCGATG
	AcACT5-1	CTCTCTGTTTGCCTTGGGGTTC
	AcACT5-2	CACAGGATGTTCTTGAGGAGCA
ORF clone	AcACT-F	ATGGCTGAAGAGGATATCCA
	AcACT-R	TCAGAAGCACTTTCTGTGCA
qRT-PCR	AcACTB-qRT-F	TGACCTCACCGACTCCCTAAT
	AcACTB-qRT-R	ACTTCTGGGCACCTGAACCTC
	AcALL-qRT-F	TTTTCAACCCAGTTAGCA
	AcALL-qRT-R	CAAGGAGTGGCAGTCATA

Sequence alignment

The obtained nucleotide and deduced amino acid sequences were analysed by bioinformatic tools in the NCBI website (www.ncbi.nlm.nih.gov) and multiple sequence alignments were analysed by the DNAMAN software. The phylogenetic tree for AcACT was constructed by the neighbour-joining method in MEGA 7.0 software with 1,000 replicates (Rusinko and Mcpartlon 2017; Kumar et al. 2018).

***AcACT* mRNA distribution**

Total RNA was isolated from roots, stems and leaves of the tillering onion cultivars L-SH and L-SY at three growth period points (20, 40 and 60 d) under suitable conditions (21 °C, 65% relative humidity, 15 h light and 9 h dark), low temperature (15 °C, 65% relative humidity, 15 h light and 9 h dark), and short-day conditions (21 °C, 65% relative humidity, 9 h light and 15 h dark) for *AcACT* expression analysis .

Plasmid pMD18T-*AcACT* was extracted using a MiniBEST Plasmid Purification kit Ver. 4.0 (TaKaRa, Otsu, Shiga, Japan). The standards were quantified using the NanoDrop2000 system (Thermo, Wilmington, DE, USA). The number of standard copies per µL was calculated using the following equation: Copies per µL = (Ac x Pc x 10^{-9}) / (n × mw), where Ac is the Avogadro constant (6.02 × 10^{23} molecules per mole), Pc is the plasmid concentration (ng per µL), n is the length of the double-stranded DNA, and mw is the molecular weight (average mw of 660 Da for one base pair) (Malandraki et al. 2017). Serial dilutions of 10^1 to 10^8 plasmids (20 ng µL^{-}) were prepared in Milli-Q water and quantitative PCR (qRT-PCR) was used to measure the Ct value of each of the concentration standard. A standard curve was drawn with the Ct value as the ordinate and the logarithm of the number of standard copies per µL as the abscissa.

The qRT-PCR reaction system contained 25 µL of 2×KAPA SYBR®FAST qPCR Master Mix ABI Prism™ (KAPA Biosystems, Cape Town, South Africa), 1 µL of diluted 10× cDNA, 2.5 µL of sense primer, 2.5 µL of anti-sense primer (10 µM), and 22 µL of Milli-Q water. The amplified PCR procedure was as follows: 95 °C for 3 min, 40 cycles of 95 °C for 15 s and 60 °C for 60 s, followed by gradual heating from 65 °C to 95 °C in order to generate the melting curves. Four repetitions were done per sample. After the PCR amplification, the results were analysed using ABI 7500 software (v. 2.3) (Thermo Fisher, Waltham, MA, USA). The Ct method was used to analyse gene expression.

AcALL expression and high-performance liquid chromatography analysis (HPLC) of allicin

The roots, stems, and leaves of 3-week-old plants of cultivars L-SH and L-SY were collected to analyse *AcALL* expression by qRT-PCR and allicin concentrations by HPLC. The reaction system and qRT-PCR procedure were performed as described above. The $2^{-\Delta\Delta CT}$ technique was used to analyse the gene expression of *AcALL* (Livak et al. 2001).

Tillering onion plants were ground thoroughly in liquid nitrogen. Pure methanol (5.0 mL) was added to each fresh tissue sample (5.0 g). The mixture was vortexed and sonicated for 5 min, stored at 4 °C for 36 h, and centrifuged at 16,000×g for 10 min to separate the crude extract from the tissue debris. The allicin concentrations in the crude extract were determined by a Waters 1525 HPLC system (Waters Co., Milford, MA, USA) equipped with a 1525 binary pump, a 2489 UV detector, and a 2707 autosampler. The samples were fractionated at 25 °C using a C_{18} column (250 mm × 4.6 mm I.D, 5 µm; Waters Co., Milford, MA, USA) at a flow rate of 1.0 mL min^{-1} with a mobile phase comprising 70% acetonitrile and 30% water. The run time was 25 min and the eluents were monitored at 215 nm (Xu et al. 2016; Zhang et al. 2013). Allicin (purity >90%), which degraded to DADS and DATS, was purchased from Toronto Research Chemicals Inc., Toronto, Canada.

Data analysis

SPSS version 22.0 software (IBM Cororation, Armonk, NY, USA) was used to analyse the data. Fisher's least significant difference test was performed for significance analysis with $p < 0.01$ or $p < 0.05$.

3. Results

Quality assessment of the extracted total RNA

The RNA samples showed clear bands corresponding to 28S, 18S, and 5S rRNA following 1% agarose gel electrophoresis (Figure 1A), indicating that the samples were intact. The sample OD_{260}/OD_{280} ratios were equal to or above 2.0, thus indicating that the purity of the RNA met the quality requirements for reverse transcription.

5' and 3' RACE

The ACT-F/ACT-R primer pairs were used to obtain and sequence cDNA fragments of *ACT* (309 bp) (Figure 1B). The 5'-uncovered regions were acquired by synthesising the primers AcACT5-1 and AcACT5-2 using their corresponding cloned fragments. These specific primers were used along with the 5' Outer/inner primers for nested PCR amplification during 5' RACE. This yielded a band at a position that corresponded well with the expected size of 430 bp (Figure 1C).

To clone the 3'-uncovered regions, specific primers AcACT3-1 and AcACT3-2 were designed based on the cloned core fragments. These specific primers were used along with the 3' Outer/inner primers for nested PCR amplification during 3' RACE. This yielded a band that corresponded well with the expected size of approximately 1,102 bp (Figure 1D).

Isolation of the *AcACT* cDNA and sequence alignment

The *AcACT* gene was successfully cloned by RT-PCR with the primer pair AcACT-F/R and submitted to GenBank (Accession: MF919598.1). The full-length cDNA of *AcACT* was 1,357 bp long with a 5' untranslated region (UTR) of 79 bp, ORF of 1,131 bp, and 3' UTR of 147 bp (Figure 1E). The region encoded a polypeptide containing 376 amino acids (Figure 2) with a predicted molecular weight of 41.62 kDa and an isoelectric point of 5.31.

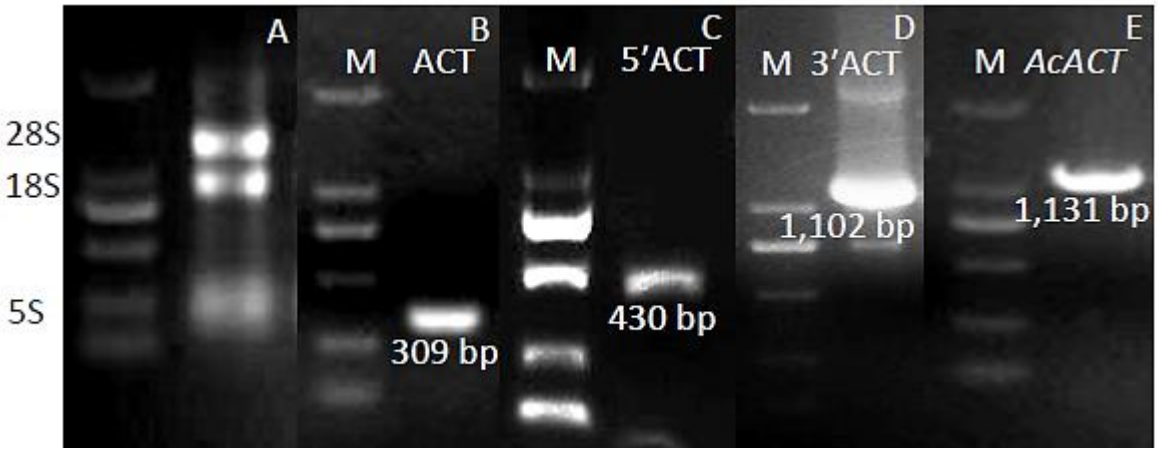


Figure 1. Gel electrophoresis analysis. A - Total RNA bands; B, C and D - RACE PCR product bands and E - PCR products produced by the full-length *AcACT* cDNA from tillering onion. M: DL2000 Marker.

The deduced amino acid sequences of AcACT shared over 96% similarity with homologous proteins found in the NCBI. The highest sequence similarity (98.93%) was with that of actin from *Asparagus officinalis* (Accession: XP_020277053.1). The alignment of the amino acid sequences of AcACT from tillering onion with those from five other plant species is shown in Figure 3A.

A phylogenetic tree was constructed to investigate the evolutionary relationship between plant ACT proteins. The results showed that AcACT had the highest sequence identity with ACT from *Ananas comosus* (Accession: XP_020102227.1) (Figure 3B).

Analysis of *AcACT* expression

A linear correlation (R^2 = 0.9993) was observed between Ct and the logarithm of the DNA copy number (Figure 4A). In the qRT-PCR analysis, only one melt peak was observed, which suggested that the primers had high specificity and that there was one unique PCR product (Figure 4B). The expression levels of *AcACT* were not significantly different among the three growth periods and under suitable conditions, low temperature, and short-day conditions for the two tillering onion cultivars ($p > 0.01$) (Figure 4C).

Analysis of *AcALL* expression

AcALL gene expression was examined in the roots, stems and leaves of tillering onion using *AcACT* as the reference gene by qRT-PCR. As shown in Figure 5A, *AcALL* expression was the highest in roots and the lowest in leaves for the two cultivars L-SH and L-SY ($p < 0.05$).

Measurement of allicin concentrations in different plant tissues using HPLC

HPLC was used to measure the allicin concentrations in the roots, stems and leaves of the cultivars L-SH and L-SY. The concentrations of the stable allicin derivatives DADS and DATS were measured in our study (Figure 5B). As shown in Figure 5C, the concentrations of DADS and DATS were the highest in the roots and lowest in the leaves of both the cultivars ($p < 0.05$).

4. Discussion

We successfully cloned the full-length cDNA of *AcACT* from tillering onion using the RACE technique. The transcripts contained 1,357 bp. It was found that the deduced amino acid sequence of AcACT shared more than 96% similarity with the homologs from other plant species, which was unsurprising since the actin genes are known to be highly conserved. The phylogenetic tree analysis indicated that AcACT was most closely related to ACT in *Ananas comosus*. It also indicated that few *actin* genes have been cloned from other *Allium* species.

qRT-PCR is often considered the gold standard for the quantitative analysis of gene expression (Huggett et al. 2005; Kozera et al. 2013). However, the accuracy of qRT-PCR is highly dependent on reliable internal reference genes (Cheng et al. 2011). These genes must be constantly and stably expressed under different conditions, such as growth periods or tissue types, and also remain unaffected by experimental parameters (Schmittgen and Zakrajsek 2000). There was no statistical difference ($p > 0.01$) found in the expression of *AcACT* among the different tillering onion samples (Figure 4C). To determine the suitability of the cloned *AcACT* to be used as an internal reference gene, we evaluated the expression of *AcALL* in the roots, stems, and leaves of tillering onion plants from two different cultivars. In addition, we used HPLC to examine the products produced after the enzymatic degradation of allicin in these tissues. Using *AcACT* as the internal reference gene, changes in *AcALL* expression between the different tissues from the two different cultivars were found to be consistent with the HPLC data on allicin accumulation. Therefore, we concluded that *AcACT* can be used as an internal reference gene for the study of gene expression in tillering onion. Overall, the *AcACT* gene was successfully cloned and may provide a reference for further research on gene expression in tillering onion and other *Allium* species.

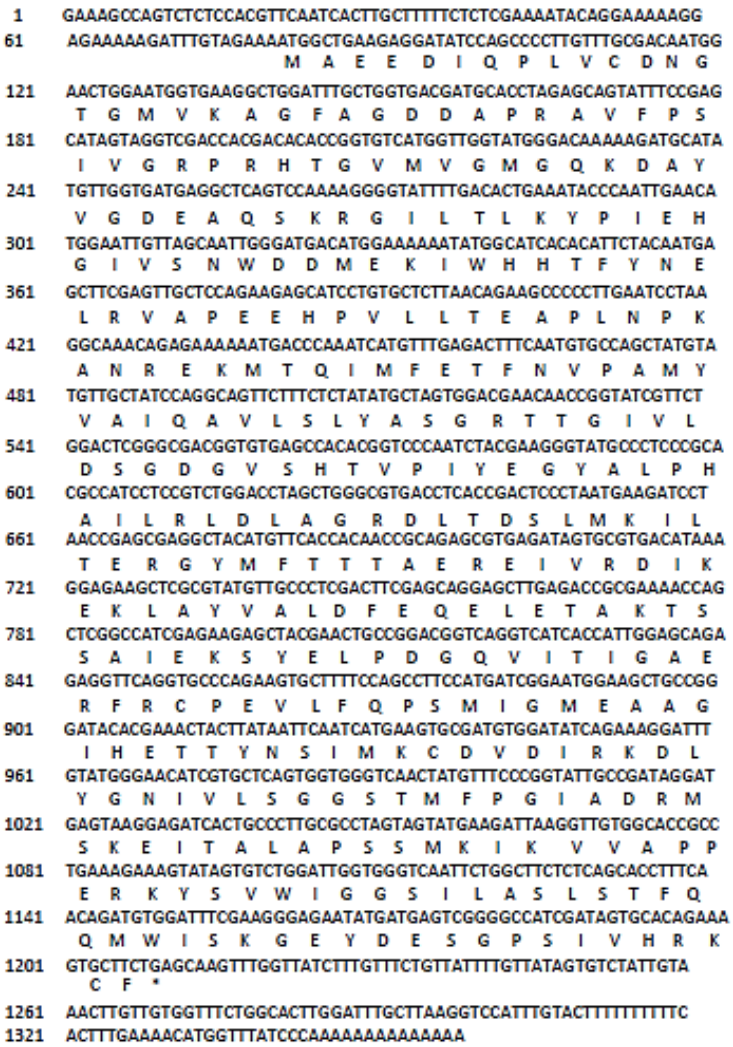
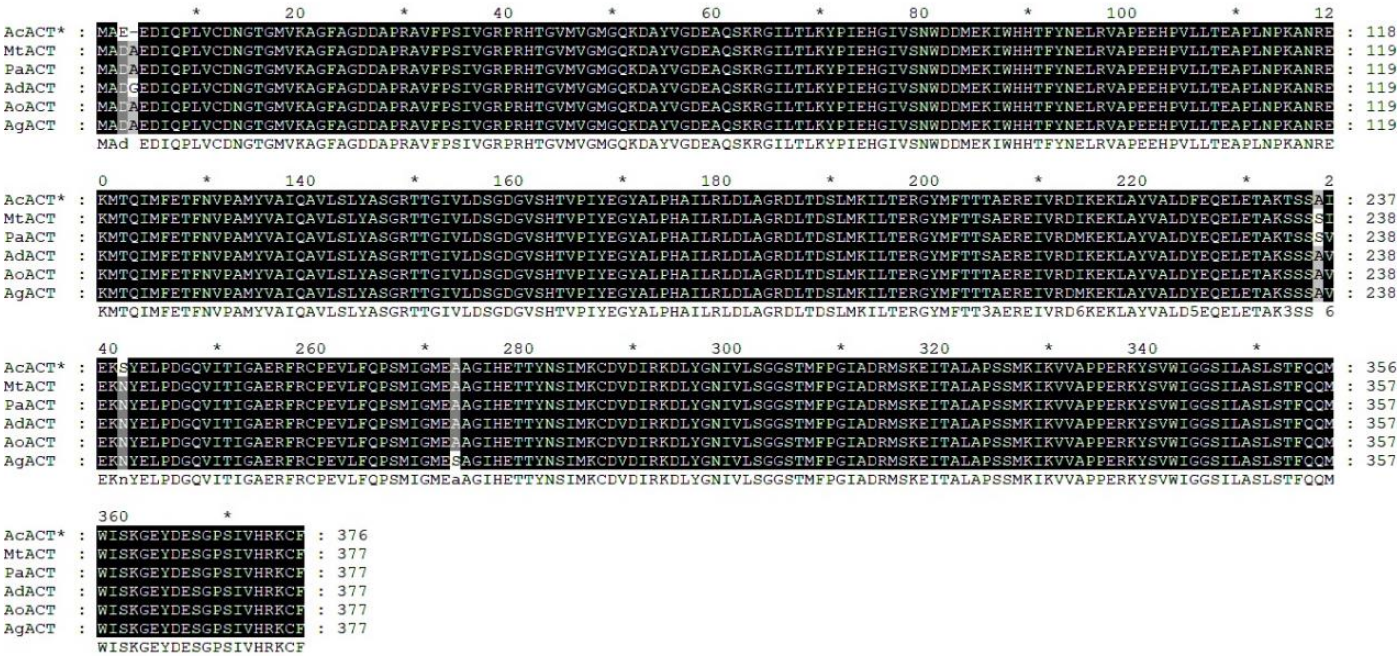


Figure 2. The ORF and deduced amino acid sequences of *AcACT* from tillering onion.

A



B

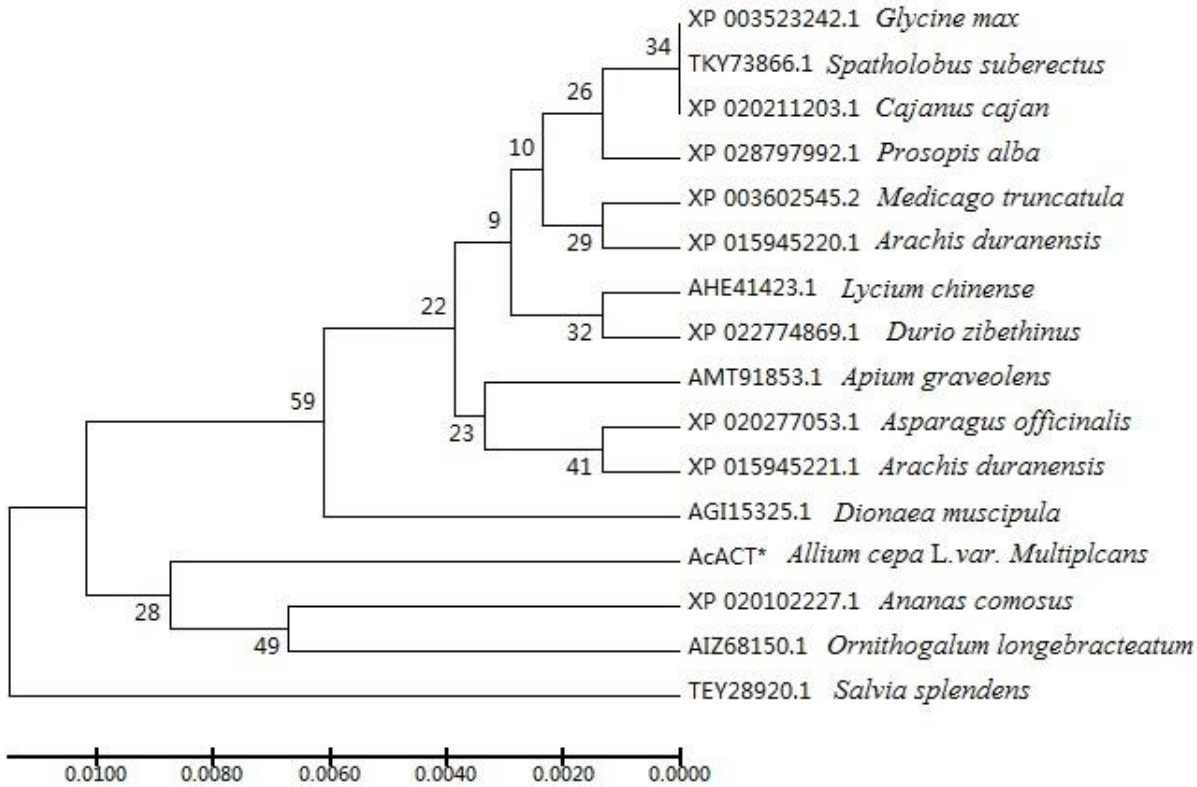


Figure 3. A - Alignment of the deduced amino acid sequence of AcACT from tillering onion and its homologs from different plants species. MtACT: *Medicago truncatula*, Accession: XP_003602545.2; PaACT: *Prosopis alba*, Accession: XP_028797992.1; AdACT: *Arachis duranensis*, Accession: XP_015945221.1; AoACT: *Asparagus officinalis*, Accession: XP_020277053.1 and AgACT: *Apium graveolens*, Accession: AMT91853.1. B - Phylogenetic tree analysis of plant ACT (actin) proteins. The bootstrap values are shown on the branches, the genetic distance scale (substitutions per amino acid) is shown at the bottom of the tree, and the query protein AcACT is indicated by an asterisk (*).

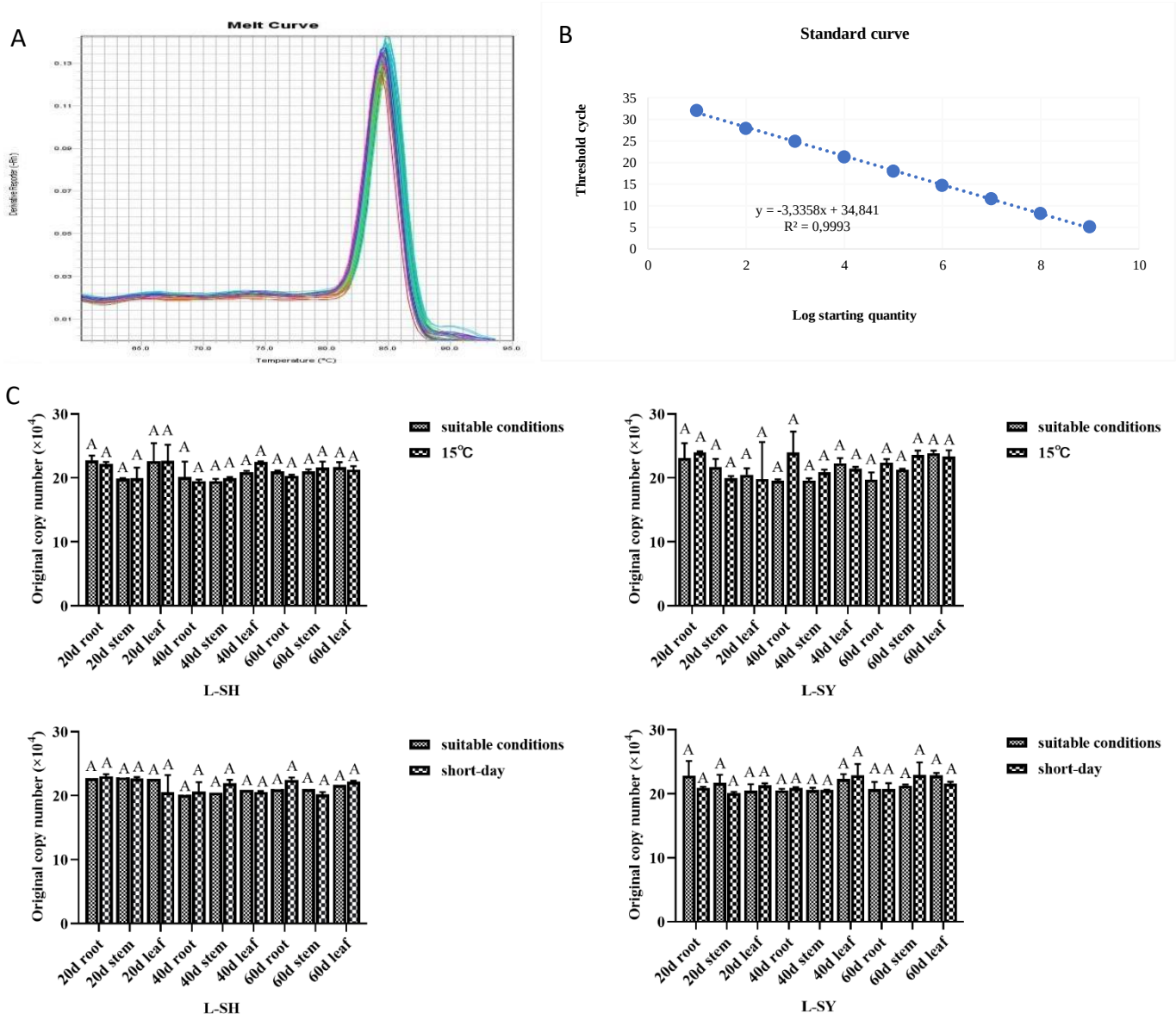


Figure 4. Expression assays of *AcACT* at different phases of tillering onion growth. A - Melt peak of the *AcACT* gene, B - standard curve, and C - absolute quantitative assays of expression level: $p > 0.01$. The same letter on a bar implies no significant difference.

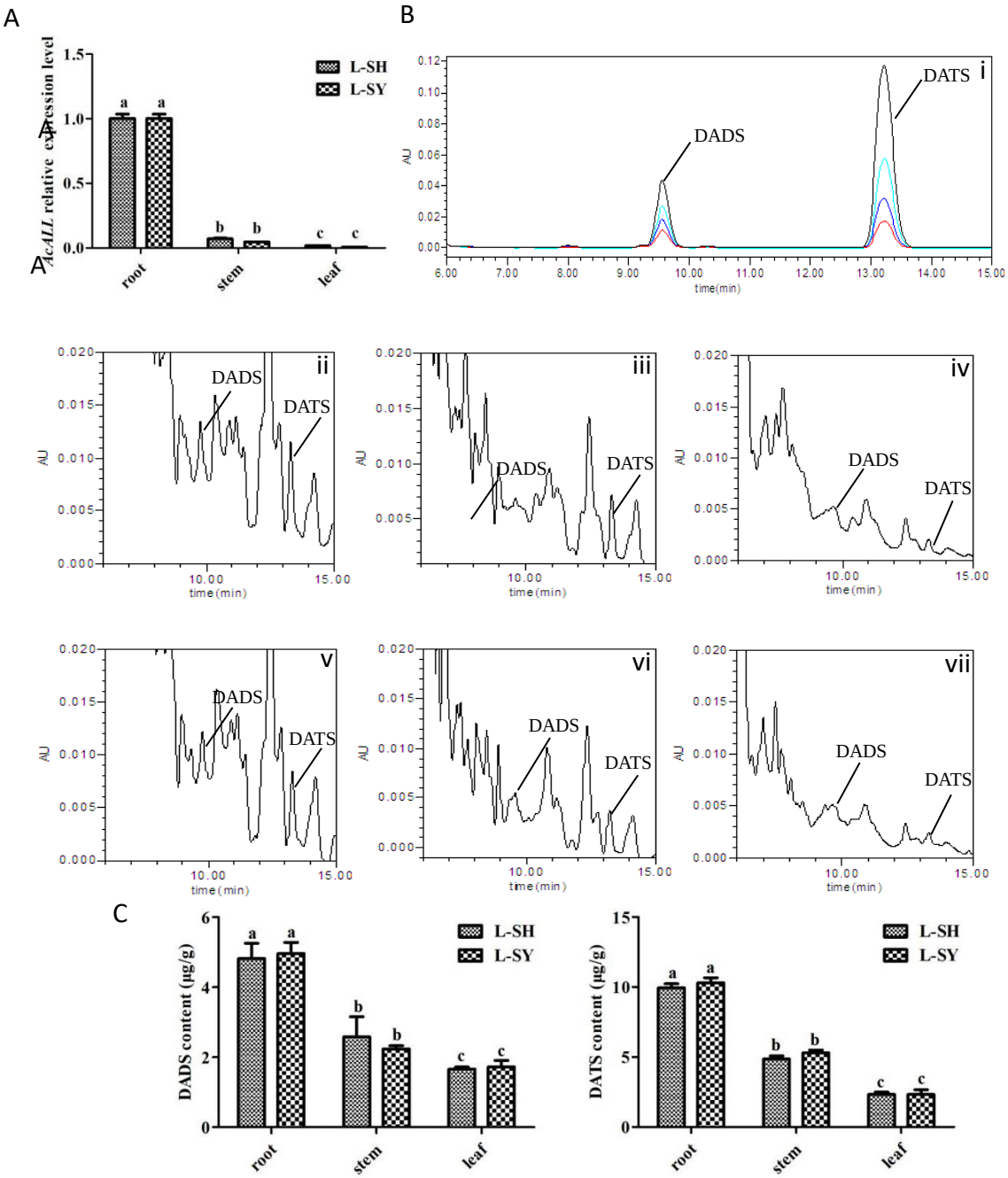


Figure 5. A - AcALL expression levels in tillering onion analysed by fluorescence-based qRT-PCR. Values represent the means ± SD from four replicates. B - HPLC analysis of allicin accumulation in different tissues of tillering onion: (i) Allicin standards: diallyl disulphide (DADS) and diallyl trisulphide (DATS); (ii) L-SH root; (iii) L-SH stem; (iv) L-SH leaf; (v) L-SY root; (vi) L-SY stem and (vii) L-SY leaf. C - Allicin concentrations in different tillering onion tissues according to the HPLC analysis.

5. Conclusions

In summary, the full-length cDNA of *Actin* was successfully cloned. *AcACT* was found to be highly conserved and showed suitability for use as a reference gene for the analysis of gene expression in tillering onion.

Authors' Contributions: YANG, Y.: conception and design, acquisition of data, analysis and interpretation of data, critical review of important intellectual content, drafting the article; FU, Y.S.: acquisition of data, analysis and interpretation of data, drafting the article; YANG, Q.F.: acquisition of data; LI, C.K.: analysis and interpretation of data. All authors have read and approved the final version of the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

Ethics Approval: Not applicable.

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