Indian Journal of Agricultural Sciences **85** (11): 1508–12, November 2015/Article

Prokaryotic and tissue-specific expression of *RglKAT* gene from *Rehmannia glutinosa*

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Recevied: 20 October 2013; Accepted: 28 August 2015

ABSTRACT

A 1640-bp full-length cDNA of the 3-ketoacyl-CoA thiolase gene was cloned from *Rehmannia glutinosa* L. leaf using RT-PCR and RACE. Bioinformatics analyses indicated that it contained one 1395-bp open reading frameencoding a thiolase of 464 amino acids with the predicted molecular weight of 49 kDa, and had a similarity of 73-82% to other species at the nucleotide level and 63-88% to other species at the amino acid level, respectively. The recombinant *RglKAT* was expressed in a bacterial system in an active form, analyzed by PAGE-CBB staining and purified using Ni-agarose chromatography. Real Time quantitative PCR analysis showed that relative expression levels of the *RglKAT* gene differed between *R.glutinosa* tissues, with the highest levels in the petals and the lowest in the early senescing stems. These results revealed that *RglKAT* gene could be cloned and heterogeneously expressed, and related to *R. glutinosa* growth and development.

Key words: Cloning, Expression, 3-Ketoacyl-CoA thiolase (KAT), *Rehmannia glutinosa*, Real Time quantitative PCR

Rehmannia glutinosa L. is a perennial herbaceous species belonging to the Scrophulariaceae family. Its economic importance results from the medicinal activity present in extracts of its tuberous roots. This species is widely distributed in China, Korea, Japan, Vietnam and others. It is extensively used in traditional Chinese clinical practice in fresh, dried and steamed forms in terms of the processing methods for the treatment of fever, strengthening liver function, nervous breakdown, diabetes, hypertension, hematopoietic, immunoenhancing, and tonic purposes (Kim *et al.* 2012, Sun *et al.* 2010). Up to date, *R. glutinosa* gene cloning and expression have been reported as follows: internal reference *genes,* root development-related genes, expansin genes, terpenoid biosynthetic genes and their tissue-specific expression (Sun *et al*. 2008, Hou *et al.* 2011, Sun *et al.* 2009, Sun *et al.* 2010, Sun *et al.* 2012, Zang *et al.* 2012), while we also studied transposase gene (Zhou *et al*. 2012) and vacuolar H+-pyrophosphatase gene (Zhou *et al*. 2013). However, now there is no report on cloning and expression of *3-ketoacyl-CoA thiolase* (KAT) gene for *Rehmannia glutinosa*.

In the present study, one *KAT* gene from *R. glutinosa* leaf was cloned, expressed in *E.coli* and different *R. glutinosa* tissues for us to better understand its functions.

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MATERIALS AND METHODS

Rehmannia glutinosa variety Wen85-5 plants were grown in the experimental field of College of Life Sciences, Henan Normal University, Xinxiang, Henan, China. Their fresh leaves, stems, roots, petals, receptacles, stamens and pistils were harvested in their corresponding growth phases such as vegetative growth phase, full-blooming phase and early senescence phase, frozen immediately in liquid nitrogen, and stored at -80 ºC for total RNA isolation using RNA Extraction Kit (Lifefeng Biotechnology Co., Shanghai, China). The RNA concentration and integrity were determined as reported by Dyer *et al.* (2009). First strand cDNA was synthesised using 5 µg of the total RNA with RNase H-reverse transcriptase AMV and oligo $d(T)_{20}$ according to the manufacturer's instructions.

The sequence of the conserved domains of five known auxin-regulated genes involved in organ size, including *Zea mays* (HQ875338.1), *Oryza sativa* (DQ641272.1), *Arabidopsis thaliana* (NM-115853.4), *Brassica rapa pekinensis* (FJ171724.1) and *Vitis vinifera* (FJ171725.1), was chosen as the template for designing the following degenerate primers: P1: 5′AAYTAYTTYAGYTTRGAGTC3′, P2: 5′ AATBGGRACYAAYARCARCATA3′; P3: 5′ GGT CTAACRGCRTCWYTGTT3′. After each cDNA was

generated by RT-PCR and sequence was verified, the conserved sequence was used as a template for designing primers for 3′ - RACE (outer primer GCCAAGTGAGTGATGGTGCTGGAGCT and inner primer CATGAAGA GAAGTGTTGCCATGC) and 5′ - RACE (outer primer GTAATACCCATTGGAAGGAGAC and inner primer CACAGTTCTAACAGGTACAGTTTCAGGG). Finally, one full length cDNA was obtained by aligning the conserved cDNA fragment, 3′ -RACE product and 5′ -RACE product using DNAMAN 4.0 software, whose open reading frame (ORF) was predicted by NCBI online and amplified PCR with a pair of primers (forward primer 5′ CGCGGATCCATGGAGAAAG CAACTG3[1] and reverse primer 5′ CCGCTCGAGCTTGCAAAGTGGACAT3′). The ORF was cloned into the pMD19-T vector and sequenced by Beijing Hua Gene Company Limited. Alignment of its nucleotide sequence and that of orthologous genes in NCBI was performed using the online tool ClustalW2. Its coding sequence was predicted by similarity with those of other KATs in the database using the multiple sequence alignment capabilities of BLAST (http://www.ncbi.nlm.nih.gov/blast). ExPASy Proteomics Server (http://www.expasy.ch/tools/protparam.html) was used to calculate physical and chemical parameters for the deduced amino acid sequence, including the molecular weight, theoretical isoelectric point (pI) and amino acid composition. Expert Protein Analysis System proteomics server (http://www.expasy.org) and Predict Protein server (http: // www.Predictprotein.org/) were used to predict the structure and function of the encoded protein.

Prokaryotic expression of *RglKAT* was performed as stated by Dyer *et al.* (2009) with some modifications. After the ORF of *RglKAT*, flanked by *Bam*HI and *Xho*I sites, and the bigger fragment of vector pET-32a were recombined to construct the prokaryotic expression vector pET-32a-*RglKAT,* it was transformed into *E. coli* BL21 to obtain transformed BL21 harbouring pET-32a-*RglKAT,* detected by enzyme digestion, PCR and sequencing. RglKAT expression was induced by IPTG, analysed by PAGE-CBB staining and purified using His-tagged protein purification according to the manufacturer's instructions.

Tissue-specific expression was analysed as stated by Kumar *et al.* (2013), with some modifications. RT-PCR was performed using an AMV one step RT-PCR kit. Specific primers for the *RglKAT* gene were used for RT-PCR and *R. glutinosa TIP41* primers, forward primer 5′ TGGCTCAGAGTTGATGGAGTGCT3[1] and reverse primer, 5′ CTCTCCAGCAGCTTTCT CGGAGA 3′, (Hou *et al.* 2011) were used as a control. Real time qPCR analysis was performed using the ABI 7500 Real Time PCR instrument. The experiments were repeated three times with similar results. The thermal cycler conditions were as follows: 10 min at 95°, followed by 40 cycles at 95° for 15 s, 60° for 1 min and 72° for 5 min. The fluorescent product was detected in the last step of each cycle. Amplification, detection and data analysis were conducted using a Rotor-Gene 6000 real-time rotary analyser. Amplicons were diluted 16-fold and used to generate a calibration curve for determination of the PCR amplification efficiency (Eff) for each gene. The relative mRNA expression (Relative quantity) for target genes was determined with the formula as stated by Liu *et al.* (2012) after obtaining the *Ct* values of *RglKAT* and *TIP41*, respectively. Data were analysed with the 7500 system SDS software, version 1.4. The $2^{-\Delta\Delta Ct}$ method was used to analyse the expression levels of *RglKAT* (Livak and Schmittgen 2001).

For the relative expression ratio of *RglKAT* gene in the *R. glutinosa*, results were expressed as the mean ± standard error of mean (SEM). Differences in the expression of *RglKAT* gene were analysed by one way ANOVA followed by Tukey's multiple comparison test. All statistical analyses were computed using SPSS v17.0.

RESULTS AND DISCUSSION

A 1640 bp cDNA was cloned from *R.glutinosa* young leaf in vegetative phase using RT-PCR and RACE,composed of a 52 UTR of 68 bp, a 32 UTR of 177 bp and an open reading frame (ORF) encoding a protein of 464 amino acids (Fig 1). The resulting protein was composed of 20 basic amino acids,whose theoretical isoelectric point (pI) and molecular weight were found to be 7.98 and 49 kD,

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1    gaaaatagagtgttaatttttcggtataatatacaaatttgtaatttagagagagaaagaaatagagg
69   ATGGAGAAAGCAACTGAGAGGCAGAGAGTTTTGCTGCAGCATCTCCGTCCGTCCTTCACTTCGTCGTCTCTA
1    M  E  K  A  T  E  R  Q  R  V  L  L  Q  H  L  R  P  S  F  T  S  S  S  L
141  GAAGATATTGAATCTTCCGTTTCCGCATCTATATGTTCAGCAGGGGACAGTGCAGCATACCATCGTTCTTCT
25   E  D  I  E  S  S  V  S  A  S  I  C  S  A  G  D  S  A  A  Y  H  R  S  S
213  GTCTTTGGTGACGATGTGGTTATAGTGGCTGCCTATCGAACTGCACTGTGCAAGTCTAAGAGAGGTGGCTTC
49   V  F  G  D  D  V  V  I  V  A  A  Y  R  T  A  L  C  K  S  K  R  G  G  F
285  AAAGATACCTATCCTGATGATTTACTAGCACCTGTTTTGAGGGCAGTGGTAGAAAAAACAAATGTAAATCCA
73   K  D  T  Y  P  D  D  L  L  A  P  V  L  R  A  V  V  E  K  T  N  V  N  P
357  AATGAAGTTGGGGACATTGTTGTGGGCACGGTGTTGGCACCGGGCTCCCAAAGAGCAAGTGAATGCAGGATG
97   N  E  V  G  D  I  V  V  G  T  V  L  A  P  G  S  Q  R  A  S  E  C  R  M
429  GCTGCATTTTATGCTGGTTTCCCTGAAACTGTACCTGTTAGAACTGTGAACAGGCAATGTTCATCTGGCCTT
121  A  A  F  Y  A  G  F  P  E  T  V  P  V  R  T  V  N  R  Q  C  S  S  G  L
501  CAAGCTGTAGCTGATGTAGCTGCAGCTATCAAAGCTGGATTTTATGACATTGGGATTGGTGCTGGGTTGGAG
145  Q  A  V  A  D  V  A  A  A  I  K  A  G  F  Y  D  I  G  I  G  A  G  L  E
573  TCGATGACCGTTAATCCTATGGCTTGGGAAGGATCAGTCAATCCAAGAGTAAAATCGATGGCACAAGCACAA
169  S  M  T  V  N  P  M  A  W  E  G  S  V  N  P  R  V  K  S  M  A  Q  A  Q
645  GATTGTCTCCTTCCAATGGGTATTACTTCAGAAAATGTCGCCCATCGTTTTGGAGTGACAAGGCAGGAGCAA
193  D  C  L  L  P  M  G  I  T  S  E  N  V  A  H  R  F  G  V  T  R  Q  E  Q
717  GATCAGGCTGCAGTTGATTCGCACAGAAAGGCTGCTGCTGCCACTGCATCAGGCAAATTTAAAGATGAGATA
217  D  Q  A  A  V  D  S  H  R  K  A  A  A  A  I  A  S  G  K  F  K  D  E  I
789  ATACCAGTGAAAACCAAGATTGTGGACCCTAAATCTGGGGATGAGAAACCGGTTACGATATCTGTTGATGAT
241  I  P  V  K  T  K  I  V  D  P  K  S  G  D  E  K  P  V  T  I  S  V  D  D
861  GGCATACGACCAAATACCTCAGTGGCAGACCTGGGCAAGCTGAAACCTGTTTTCAAGAAAGATGGCTCAACC
265  G  I  R  P  N  T  S  V  A  D  L  G  K  L  K  P  V  F  K  K  D  G  S  T
933  ACTGCTGGGAATTCTAGCCAAGTGAGTGATGGTGCTGGAGCTGTGCTCCTCATGAAGAGAAGTGTTGCCATG
289  T  A  G  N  S  S  Q  V  S  D  G  A  G  A  V  L  L  M  K  R  S  V  A  M
1005 CAGAAAGGGCTTCCCATCCTTGGTGTATTCAGGAGCTTTGCTGCAGTTGGTGTAGATCCTGCGATCATGGGT
313  Q  K  G  L  P  I  L  G  V  F  R  S  F  A  A  V  G  V  D  P  A  I  M  G
1077 GTTGGTCCAGCC GTTGCAATTCCTGCAGCAGTCAAATCTGCTGGTCTTGAACTTGGGGACATCGATCTTTTC
337  V  G  P  A  V  A  I  P  A  A  V  K  S  A  G  L  E  L  G  D  I  D  L  F
1149 GAGATAAATGAGGCATTTGCTTCGCAATTCGTGTACTGCCGTAAGAAGCTTGAACTTGATCCTGAGAAGATC
361  E  I  N  E  A  F  A  S  Q  F  V  Y  C  R  K  K  L  E  L  D  P  E  K  I
1221 AATGTGAATGGAGGTGCCATGGCAATTGGGCATCCTTTGGGTGTTACAGGAGCTCGCTGTGTTGTTACCTTA
385  N  V  N  G  G  A  M  A  I  G  H  P  L  G  V  T  G  A  R  C  V  V  T  L
1293 TTGCATGAAATGAAGCGTCGTGGCAAAGATTGCCGCTTTGGCGTGGTGTCAATGTGTATAGGCACTGGGATG
409  L  H  E  M  K  R  R  G  K  D  C  R  F  G  V  V  S  M  C  I  G  T  G  M
1365 GGCGCTGCTGCTGTTTTTGAAAGGGGTGACTCCTGTGACGAGCTTTCCAATACACGAACAGTCGGAACACAC
433  G  A  A  A  V  F  E  R  G  D  S  C  D  E  L  S  N  T  R  T  V  G  T  H
1437 AATTTTCTGTCCAAGGATGCTCGATAAactccaatgtccacttgcaagttccaataaattttaaatctgca
457  N  F  L  S  K  D  A  R  *
1509 ttcagaagtcaataatatactgcaataaattacttactgatttatcacaacagctgtggtgcagtttaat
1579 gatctctgtatttttctgtactgatgtattattgtctgtactttggaatcaaaaaaaaaaaa
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Fig 1 Nucleotide and deduced amino acid sequences of the *RglKAT* from *Rehmannia glutinosa* leaf. 3', 5'-untranslated regions are shown as small letters, coding region sequence is shown as capital letters; the upper sequence indicates the nucleotide and the lower shows the amino acid; asterisk represents stop codon; the start codon and the stop codon are boxed; Poly A tail is shaded in bold.

respectively. Prediction of domain structure and three-dimensional structure showed that the protein possessed the characteristic sequence of thiolases and basic secondary structure composed of four parts, including α-helixes (38.58%), random coils (35.78%), β-sheets (15.3%) and β-turns (10.34%) . After sequencing and assembling the cDNA fragments, the total coding sequence of *RglKAT* was obtained and submitted to GenBank and GenBank accession No. JX983188 was given. Comparison of RglKAT with the KATs of other species showed a high similarity of 73-82% at the nucleotide level and 63-88% at the amino acid level (Table 1).

In vitro RglKAT expression in *E.coli* BL21 harbouring pET-32a-*RglKAT* was induced with 1.0 mM/ml IPTG. The recombinant His-tagged fusion protein was purified using His-tag affinity columns. The purity of RglKAT enzymes was examined by SDS–PAGE, on which one single band corresponding to approximately 64 kDa proteins was observed (Fig 2). The resultant proteins had the identical molecular mass as predicted based on the deduced amino acid sequence.

Real-time quantitative PCR analysis revealed that mRNA transcripts can be detected in the 13 tissues mentioned above, but expression levels varied to different degrees (Fig 3, Table 2 and Table 3). Differential expression between

Table 1 Homology of KAT between *Rehmannia glutinosa* and other plant species

Species	Accession no.	Nucleotide (%)	Amino acid (%)
Petunia x hybrida	ACV70033.1	—[a]	88
Vitis vinifera	XP_002285653.1	—[a]	88
Vitis vinifera	XM_002285617.1	82	—[b]
Mangifera indica	CAA53078.1	—[a]	88
Arabidopsis thaliana	NP_180873.1	—[a]	87
Arabidopsis thaliana	NM_100351.4	79	—[b]
Cucumis sativus	CAA47926.1	—[a]	86
precursor – cucurbit	S72532	—[a]	86
Brassica napus	CAA63598.1	—[a]	86
Helianthus annuus	AAQ77242.1	—[a]	86
Ricinus communis	XP_002518136.1	—[a]	85
Glycine max	XP_003555712.1	—[a]	83
Lotus japonicus	BT134810.1	81	—[b]
Populus trichocarpay	XM_002299248.1	81	—[b]
Elaeis guineensis	AEQ94132.1	—[a]	81
Brachypodium distachyon	XP_003570403.1	—[a]	79
Zea mays	NP_001241698.1	—[a]	79
Hordeum vulgare subsp. Vulgare	AK356359.1	74	—[b]
Triticum aestivum	BAI66423.1	—[a]	78
Triticum aestivum	AB539589.1	73	—[b]
Medicago truncatula	XP_003604976.1	—[a]	78
Chlamydomonas reinhardtii	XP_001697225.1	—[a]	66
Coccomyxa subellipsoidea C-169	EIE21619.1	—[a]	63

—[a], No nucleotide sequence shown in GenBank; —[b], No amino acid sequence shown in GenBank

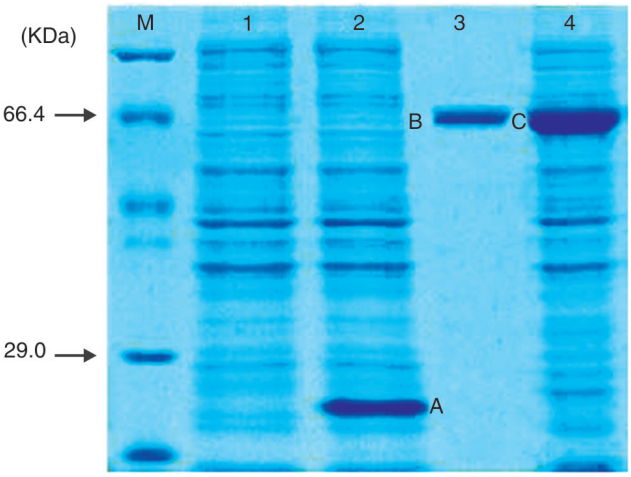

Fig 2 Coomassie brilliant blue-stained SDS-polyacrylamide gel electophoresis of total proteins from *E. coli* BL21 (DE3) harbouring pET-32a encoding bacterial protein or pET-32a-RglKAT encoding recombinant protein (bacterial protein and RglKAT), respectively, and the purified recombinant protein. LaneM, molecular mass standards [Premixed Protein Marker (Low), takara Code No.: 3595A]; before (lane 1) and after (lane 2 and lane 4) induction with isopropyl-β-D- thiogalactopyranoside; lane3, the purified *RglKAT* protein; A, bacterial protein; B and C, recombinant protein

stem and root; between stem and leaf at young seedling phase; between petals and stamens; between petals and pistils, between petals and receptacle at flowering phase; and between stems in young seedling and ones in mature phase reached extremely significant levels ($P<0.01$). Moreover, the mRNA expression of the *RglKAT* gene was the highest in the petals and lowest in the early senescing stems among them.

The middle fragment of *RglKAT* gene was amplified from *R. glutinosa* RNA by RT-PCR with a pair of degenerate primer designed according to the conserved areas of five known plant *ARGOS* genes as mentioned above. Based on its sequence, 3′ -end and 5′ -end fragments were generated

Table 2 Analyses of significant differences for relative expression level (REL) of *R glutinosa RglKAT* gene in the same tissue at three different developmental phase (α=0.01)

Developmental phase	REL in root	REL in stem	REL in leaf
Full flowering phase	1.498BC	1.201BC	1.023BC
Young seedling phase	0.573C	1.562AB	0.274C
Mature phase	0.899BC	0.009C	1.424BC

Data are expressed as mean ± SEM (n = 3).

Table 3 Analyses of significant differences for relative expression level (REL) of *R glutinosa RglKAT* gene in different tissue at flowering phase (α=0.01)

Tissues	Petal	Receptacle	Stamen	Pistil
REL	4.798A	2.164BC	1.582BC	0.345C

Values are expressed as mean ± SEM (n = 3).

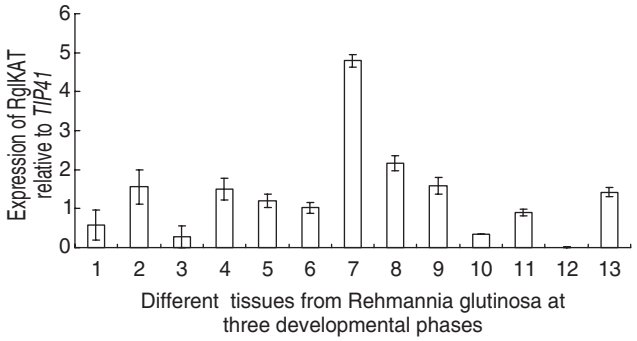

Fig 3 Relative expression levels of *RglKAT* gene to *TIP41* gene in the different tissues from *Rehmannia glutinosa* plants at three different phases during growth and development by RT-qPCR. Relative expression profile of *RglKAT* mRNA to *TIP41* as an internal control for cDNA. Values are means ± SEM (n = 3) indicating significant difference ($p<0.01$). 1-3: roots, stems and leaves of young plants at vegetative phase; 4-10: roots, stems, leaves, petals, receptacles, stamens and pistils of full blooming plants at generative phase; 11-13: roots, stems and leaves of plants at early senesence phase.

by 3′- and 5′-RACE, respectively. However, it was not any *ARGOS* orthologous gene but one fragment of KAT gene predicted by bioinformatics methods. This could indicate that these pairs of degenerate primers were not specific to *ARGOS* genes but hybridised to other genes in the *Rehmannia* genome possibly due to its complexity (tetraploid) or no *ARGOS* gene in the *R.glutinosa* tested. These results revealed that some genes for *R. glutinosa* could be cloned using degenerate primers, consistent with our previous findings (Zhou *et al.* 2012, Zhou *et al.* 2013).

KAT is typically involved in the degradative process of fatty acid β-oxidation and is widely distributed in fungi, animals and plants (Carrie *et al.* 2007). Within the plant kingdom, KATs have been cloned from several species to date (Table 1). There are five loci in the *Arabidopsis* genome with sequence similarity to genes encoding known thiolase proteins (Germain *et al.* 2011), divided into Type I and Type II classes. Based on chromosome position, three loci (*KAT 1, 2* and *5*) are typically involved in acetyl-CoA formation in fatty acid β-oxidation, of which *KAT2* expression level was several-fold higher than that of its two paralogues in most tissues (Footitt *et al.* 2007). In this study, Blast search analysis indicated that *RglKAT* gene was a member of the Type I KAT class gene family with 73-82% identity with other species at the nucleotide level and 63-88% identity at the amino acid level, respectively (Table 1) and had the characteristic sequence of thiolases. Moreover, *RglKAT* protein which *RglKAT* expressed in *E. coli* (Fig 2) is similar to AtKAT proteins, KAT2 and KAT5, 48 kDa (Carrie *et al.* 2007). This findings indicated that this gene could be one KAT gene. To my knowledge, this is the first report on *KAT* gene cloning from *R. glutinosa*.

In the past, KAT gene expressions in higher plant tissues were not detected by RT-qPCR but Northern blot and classical Western blot (Footitt *et al.* 2007). Northern blot and Western blot analyses for *Arabidopsis* 3-ketoacyl-CoA thiolase (*KAT2)* protein expression in wild-type organs showed that KAT2 mRNA and protein were detected in some vegetative and reproductive organs tested, indicating that KAT2 protein plays a role in *Arabidopsis* plant growth and reproduction (Footitt *et al.* 2007) . However, detection of mRNA on a Northern blot does not allow precise quantification, while classical Western blots are nonquantitative. RT-qPCR can relatively quantify mRNA. Relative quantification is based on internal reference genes to determine fold-differences in expression of the target gene. The quantification is expressed as the change in expression levels of mRNA interpreted as cDNA. Relative quantification is easier to carry out as it does not require a calibration curve as the amount of the studied gene is compared to the amount of a control housekeeping gene. In this study, RT-qPCR study of *RglKAT* expression revealed that *RglKAT* mRNA could be efficiently detected in all the 13 R. glutinosa tissues tested with the highest expression level in the petals of the plants at full-blooming stage and the lowest in the stems of plants at vegetative growth phase.

RglKAT gene was successfully cloned from *R. glutinosa* leaves by RT-PCR and RACE techniques, and recombinant *RglKAT* protein encoded by the gene was successfully obtained. Spatial and temporal expression pattern of this *RglKAT* gene in *R. glutinosa* showed its close association with *R. glutinosa* growth and development.

ACKNOWLEDGMENT

The present work was supported by National Natural Science Funds for Distinguished Young Scholoar (No. 31500262), Key Research Program of Science and Technology of Education department of Henan Province (No.14B180028), Basic and frontier technology research program of Henan Province (No.092300410009), Natural science foundation of the education department, Henan Province (No. 2011A180022) and Program for Innovative Research Team (in Science and Technology) in University of Henan Province (13IRTSTHN009).

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