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IDENTIFICATION OF A THYMIDINE KINASE (*RUTK1*) HOMOLOG DIFFERENTIALLY EXPRESSED IN BLACKBERRY (*RUBUS* L.) PRICKLES

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Abstract

Thymidine kinase (TK) is a key enzyme in controlling DNA synthesis and plays an important role in cell proliferation. However, our understanding on the *TK* functions in plants is still limited. From an earlier comparative transcriptome analysis of shoot apex of blackberry cv. Boysenberry and its bud mutant cv. Ningzhi 1 with fewer and thinner prickles, we found a unigene homologous to *TK*, *RuTK1* which was differentially expressed between them. In this study, the cDNA and genomic DNA (gDNA) sequences of *RuTK1* were further analyzed. *RuTK1* revealed an open reading frame (ORF) of 660 bp coding for 219 amino acid residues. The gDNA sequence, which contains four exons and three introns, is relatively conserved in most plant *TK* homologs. A phylogenetic analysis revealed that the TK proteins from plants were classified into three groups. In each group, TKs from the same family were relatively concentrated, and *RuTK1* was classified to the dicotyledoneae class and closer to those from Rosaceae. *RuTK1* was highly expressed in prickles at the early stage in Boysenberry compared to in Ningzhi1. In addition, *RuTK1* expression was similarly greater in mature prickles at the late stage in both cultivars, which implies a possible involvement of *RuTK1* in the cell cycle at the early stage of prickle formation. These results provide a novel foundation for the further elucidation of blackberry prickle development mechanism and the functions of TKs in plants.

Key words: Blackberry (*Rubus* L.), Prickle development, Thymidine kinase, Cell cycle, Expression analysis.

Introduction

Thymidine kinase (ATP: thymidine 5'-phosphotransferase, EC 2.7.1.21, TK) has a key function in the synthesis of DNA precursor and subsequently in cell division, which can be found in most living cells (Kit, 1985; Wintersberger, 1997). The reaction of TKs incorporated the thymidine into the DNA followed by the phosphorylation of thymidine and the conversion of deoxyuridylate (dUMP) to thymidylate (dTMP) (Bessman *et al*., 1956). The TKs produce deoxythymidine monophosphate, which can be rapidly catalyzed by monophosphate and diphosphate kinases to 2'-deoxythymidine triphosphate (dTTP) (Fujiwaki *et al*., 2002). High levels of dTTP shift the substrate specifically from pyrimidine to purine nucleotide reduction (Xu *et al*., 2006). Cells must maintain a balanced deoxyribonucleoside triphosphate (dNTP) level throughout the cell cycle that is close to the DNA synthesis rate (Rampazzo *et al*., 2010). TK plays a vital role in determining the dTTP level that is crucial for a cell to keep the balance of four dNTPs as well as to maintain accurate DNA replication (Mutahir *et al*., 2013).

Thymidine kinases in mammalian cells are compartmentalized based on their isoenzyme forms. Thymidine kinase 1 (TK1) is found in the cytoplasm, and thymidine kinase 2 (TK2) is found in mitochondria (Eriksson *et al*., 2002). In mammals, TK1 plays vital roles in DNA replication and DNA repair and is tightly regulated during the cell cycle (Gasparri *et al*., 2009), whereas TK2 is cell cycle-independent (Berk *et al*., 1973). Currently, TK1 tracking and monitoring is widely used in clinical screenings as a proliferation marker in the diagnosis and in the control treatment of malignant tumors (Lamarca *et al*., 2015). In plants, a large proportion of the total activity of thymidine kinase was found in chloroplasts compared to that in the cytosol in plants of the same age (Golaszewski *et al*., 1975). All animals had a tetrameric TK1, whilst plant only exhibited dimeric TK1 (Mutahir *et al*., 2013). Though a certain number of plant *TK* sequences were released in the GenBank database, reports on their specific functions has been limited. In *Oryza sativa*, *TK1* was found to be independent of the cell cycle and expression occurred during all development stages and was higher in non-proliferating tissues (Ullah *et al*., 1999). In *Hevea brasiliensis*, the up-regulation of *TK1* was found to be closely related to resistance to mechanical wounding (Perumal *et al*., 2010). Expression analyses of *TK1a* and *TK1b* in *Arabidopsis* showed that both genes had ubiquitous expression patterns during plant development except a higher transcript level in *TK1a* (Clausen *et al*., 2012). Moreover, it was demonstrated that *TK1a* overexpression provided tolerance to genotoxins that induced double-strand break, and thus contributed to several DNA repair pathways by providing deoxythymidine triphosphate (Pedroza-García *et al*., 2015).

Blackberry (*Rubus* L.) belongs to the *Eubatus* subgenus within the *Rubus* genus and is an excellently nonwood forest tree in the Rosaceae family. Blackberry fruit is an important source of cellulose, vitamin E, natural pigment and phenolic compounds (Beattie *et al*., 2005). Large differences exist between different blackberry genotypes, which have made the progress in genetics and molecular biology analyses within this genus relatively slow. RNA sequencing (RNA-Seq) has just emerged in recent years as a novel transcriptome analysis technology using deep sequencing. RNA-Seq provides more accurate digital signals and detects greater flux and a wider range of overall transcriptional activity without probes designed based on known sequences (Wang *et al*., 2009). At present, transcriptome sequencing has been widely used in the tree species in gene mining and for transcription-level analyses (Shi *et al*., 2011). Using RNA-Seq, traits that controlled mature fruits of the thornless blackberry cv. Lochness have been recently revealed (Garcia-Seco *et al*., 2015).

Boysenberry (*ursinus* × *idaeus* lineage Rubus), a *Rubus* L. hybrid, has good performance in production with early maturity, large fruit with strong growth potential. However, the presence of dense thorns on canes and leaves makes the field operation of boysenberry rather spiny. Ningzhi 1 is a new variety derived from Boysenberry by natural mutation in the root neck (Zhang *et al*., 2013). This variety appears with a thin-thorn phenotype and contains far less thorns on its stems. Previous observations using paraffin sectioning and scanning electron microscopy on Ningzhi 1 indicated that the number of cells inside the stem prickles was greatly reduced (Zhang *et al*., 2013). Through an earlier RNA-Seq analysis of annual shoot apex of Boysenberry and Ningzhi 1 (unpublished data), we found that the expression level of a thymidine kinase gene (named *RuTK1*) in Ningzhi 1 was obviously down-regulated (1.5-fold) compared to that in Boysenberry. Our assumption is this difference in expression may be related to a reduced level of dTTP available for DNA synthesis and thereby cell division in prickle cells of Ningzhi 1.

In this study, we investigated the function of *RuTK1* by evaluating its expression pattern during the prickle cell development. The functional predictions of TKs may provide new evidence for investigating the functions of *TK* genes in plants. The results provided new insights into the mechanisms of prickle development in blackberry plants, and the regulation of the cell cycle in its initiation and morphogenesis.

Materials and Methods

Plant material and cultivation: Different tissue samples, including young stems and stem tips, young leaves and different stem bark sections containing prickles were collected from annual plants of Boysenberry cv. and the thinner- and fewer-thorn mutant cv. Ningzhi 1. Plants were field-grown conventionally in the nursery garden at the Institute of Botany, Jiangsu Province and Chinese Academy of Sciences. After sampling, tissues were quickly frozen in liquid nitrogen and stored at -80°C until use.

Cloning of cDNA and gDNA sequences of *RuTK1*: Total RNA was extracted from young leaves of Boysenberry using an RNA Extraction Kit from Bioteke Co., Ltd. (Beijing, China) according to the manufacturer's instruction. First strand cDNA synthesis was performed using 2 μg RNA and Oligo $(dT)_{18}$.

A pair of specific primers for full-length cDNA and gDNA cloning of *RuTK1* was designed as follows: TK1-F: 5'- ATC ATG GCT TCC TTC AAA C-3' and TK1-R: 5'-AAC TGG AGC TGC TTC TGA A-3'. The reaction assay of 20 μL contained 1× PCR buffer, 0.4 μL 10 mM dNTPs, 2 μL sense/antisense primers (10 μM), 1 U *Taq* polymerase and 1 μL cDNA template (1 μg/μL). The PCR reaction proceeded as follows: a pre-denaturation step at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing for 30 s at 60°C and extension at 72°C for 60 s and a final 10 min extension at 72°C. Amplification products were separated by electrophoresis on a 1% agarose gel. After recovery, the PCR products were ligated into the pUM19-T vector. This construct was used to transform competent *E. coli* DH5α cells, which were then placed in LB medium (containing 100 mg/L ampicillin) to screen for recombinants. Positive clones were confirmed by PCR amplification and submitted for sequencing at Invitrogen. Co. (Shanghai, China).

Nucleotide and amino acid sequence analysis: Calculation of the molecular weight and isoelectric point of the RuTK1 was performed using ExPASy tools (http://www.expasy.org). Conserved functional domain analysis was predicted using the SMART program (http://smart.embl-heidelberg.de/). By the The Basic Local Alignment Search Tool (BLAST), plant TKs were found and used to perform multiple sequence alignments. A phylogenetic tree of TKs from different species was constructed using MEGA 4.0 software. Genome structure was displayed using the Gene Structure Display Server 2.0 (Hu *et al*., 2015).

Real-time quantitative PCR analysis: Total RNA from three tissue samples (young leaves, young stems and stem tips) and different parts (bottom, medium and top) of epidermis tissue containing prickles was isolated from annual vegetative stems of Boysenberry and Ningzhi 1. Random primers were used to transcribe cDNA. A SYBR GREEN dye assay and a fluorescence quantitative PCR instrument (Roche) were used to analyze gene expression by relative quantitative real-time fluorescent PCR. Blackberry (*Rubus spp*.) *actin* gene transcript (GenBank accession No. HQ439556) was used as an internal reference.

PCR reactions were performed in 20 μL volumes and contained 10 μL 2 ×qPCR Master Mix, 0.4 μL sense/antisense primers (10 μM), 2 μL cDNA template (1 μg/μL) and 7.2 μL ddH_2O. The amplification conditions for real-time fluorescence quantitative PCR were as follows. An amplification curve was performed at 94°C for 30s, followed by 45 cycles of 94°C for 5 s and 53°C for 30 s with a single point detection signal at 53°C. Dissolution conditions were 95°C for 0 s, 60°C for 15 s and 95°C for 0 s with a continuous detection signal. Primers for *RuTK1* were designed as follows: TK2-F: 5'-CGA ATC CCT TTC CTC GCT CT-3' and TK2-R: 5'-GGA CCC ATG ATG ACG TGG AC-3' generated a product with a theoretical size of 91 bp, act-F: 5'- AGG CCG TTC TTT CGC TGT-3' and act-R: 5'- GCC AGG TCC AAA CGA AGG A-3' generated a product with a theoretical size of 133 bp.

The analysis of the fluorescent value variation curve and the melting curve was performed after the PCR reaction was complete. Relative quantitative gene expression levels were compared by $2^{-\Delta\Delta Ct}$ (Livak & Schmittgen, 2001). For these calculations, $\Delta\Delta Ct$= (Ct Target-Ct Tubulin)X- (Ct Target-Ct Tubulin) Y, X and Y represented different samples.

Results

Cloning of *RuTK1* from blackberry: Using the specific primers, the full-length sequences of *RuTK1* cDNA and gDNA were obtained by RT-PCR and PCR amplifications, which were then deposited in GenBank under accession Nos. KP322701 and KP322702, respectively. The cDNA sequence of *RuTK1* was 99.9% identical to the previous

transcriptome data, which contained an ORF of 660 bp coding for 219 amino acid residues. This peptide had a predicted protein molecular weight of 23.87 kDa and a predicted isoelectric point of 7.42. At the both nucleotide and amino acid level, RuTK1 had 93% and 95% homology to those sequences in *Fragaria vesca*, respectively. As determined by a blastp search, RuTK1 contained a Walker A motif (p-loop). This motif is commonly present in all ATP binding proteins, including kinases such as TK and other ATPases such as ABC- transporters and the ABC_FeS_Assembly transporter family.

Comparing with the cDNA sequence, the gDNA of *RuTK1* covered a length of 1,152 bp, which was found to contain four exons (119 bp, 145 bp, 122 bp and 271 bp in length) and three introns (95 bp, 257 bp and 123 bp in length). The introns all had typical GT-AG cleavage borders. By a GenBank sequence search, six plant annotated genomic *TK* sequences were identified (Fig. 1). A comparison of the sequence structures of the plant *TKs* showed that most contained four exons and three introns, similar to *RuTK1*. MnTK (*Morus notabilis*) contained five exons and four introns. All TK genomic sequences had second and third exons of identical lengths; the lengths of the fourth exons were similar.

Sequence alignments of RuTK1 and other plant TK peptides: Deduced amino acid sequences of *RuTK1* and the other six plant *TK* sequences in Figure 1 were further compared (Fig. 2). All of the TK sequences contained fairly conserved thymidine kinase domains, as predicted for members of the ABC_ATPase superfamily. This domain was also similar to that of thymidine kinases from bacteria and viruses (Black & Hruby, 1991). Lengths of the amino acid sequences deduced from the second to the third exons were strictly conserved in these sequences. This conservation was found in plant *TKs* first.

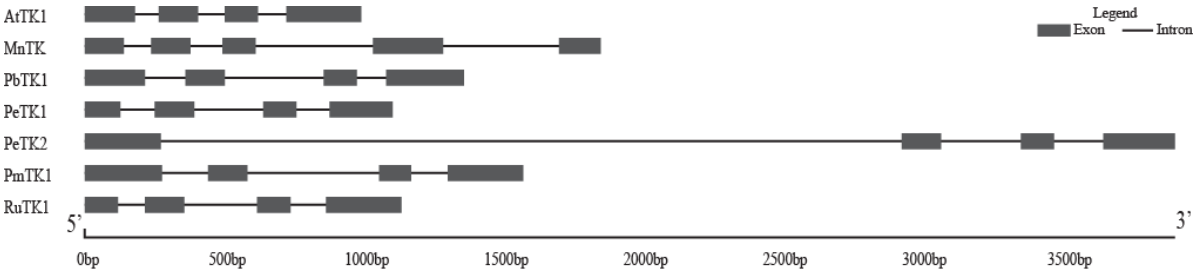

Fig. 1. Genomic diagram of *RuTK1* and other plant *TK* genes. Sequence names and GenBank accession numbers were as follows: *AtTK1* (*Arabidopsis thaliana*, NC_003074), *MnTK* (*Morus notabilis*, NW_010368056), *PbTK1* (*Pyrus bretschneideri*, NW_008988159), *PeTK1* (*Populus euphratica*, NW_011499929), *PeTK2* (*Populus euphratica*, NW_011499936), *PmTK1* (*Prunus mume*, NC_024129) and *RuTK1* (*Rubus* hybrid, KP322702).

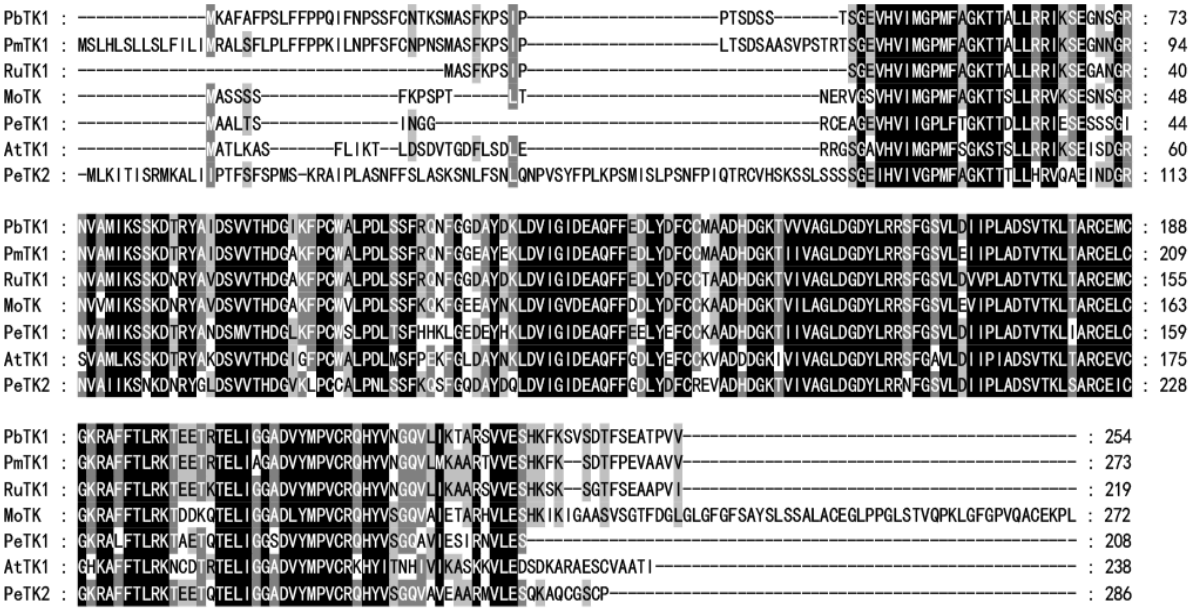

Fig. 2. Multiple alignments of deduced amino acid sequences of *RuTK1* with six other *TK* genes as in Fig. 1. Sequence names and GenBank accession numbers were as follows: AtTK1 (*Arabidopsis thaliana*, NM_111659), MnTK(*Morus notabilis*, XM_010114497), PbTK1(*Pyrus bretschneideri*, XM_009366920), PeTK1(*Populus euphratica*, XM_011041037), PeTK2(*Populus euphratica*, XM_011041898), PmTK1(*Prunus mume*, XM_008231651) and RuTK1(*Rubus* hybrid, KP322701). The sequences between the two vertical dashed lines corresponded to conserved thydimine kinase domains. The three conserved exon-intron borders in plant *TK* genes were marked with white triangles. The sequences between the dashed lines close to the N-terminus and the seven short black underlines showed unique sequence characteristics of each member of the plant ABC_ATPase superfamily.

Phylogenetic analysis of plant TK proteins: Amino acid sequences of 62 plant TKs deposited in GenBank, including blackberry RuTK1, were used in the phylogenetic analysis (Fig. 3). Partial sequence of another thymidine kinase gene (named *RuTK2*) was also found by sequence annotation after RNA-Seq. This sequence was added to the phylogenetic analysis of plant TK proteins for comparison. All sequences were classified into three groups. RuTK1 and RuTK2 which were closer to other Rosaceae plants had been classified into group I and group II, respectively. A comparison of members from groups I and group II revealed that sequences in group I contained approximately 230 amino acid residues; sequences in group II all contained approximately 280 amino acid residues.

A combination and comparison of groups I and II revealed that one to five TKs from each plant were currently deposited in GenBank. The earliest report on plant TK protein functions was from *Arabidopsis* (Clausen *et al*., 2012). *Arabidopsis* contained two members, one each in groups I and II. Several larger plant families, such as subgroup A1 (Rosaceae), B1 (Leguminosae) and C1 (Cucurbitaceae) in group I, had members of the same kind in the subgroups A2 (Rosaceae), B2 (Leguminosae) and C2 (Cucurbitaceae) in group II. The members of Solanaceae were mainly distributed in subgroup D in group I; sequences were rarely found in group II. Other plants contained sequences distributed only in group I or II; this may due to the limited number of current members, or to distinct, genus-dependent traits.

The genera in groups I and II were dicotyledonous plants; group III contained monocotyledons. *Elaeis guineensis* was the sole monocotyledonous woody plant in group III; others were all gramineous plants.

Relative expression analysis of *RuTK1* in different tissues: Expression patterns of *RuTK1* in different tissues of Boysenberry and Ningzhi 1 were further identified (Fig. 4). Expression was relatively higher in the leaves of Boysenberry, followed by expression in stem tips. In contrast, *RuTK1* expression was relatively lower in the stems and stem tips of Boysenberry; expression was higher in Ningzhi 1.

Relative expression analysis of *RuTK1* in different stem bark sections with prickles: The primary aim of this study was to identify the expression pattern of *RuTK1* in prickles. Thus, different stem bark sections with prickles from Boysenberry and Ningzhi 1 were separated in bottom, medium and top sections. This was in contrast to divisions by prickle development, such as old, middle and young stages. In these sections (Fig. 5), *RuTK1* expression was higher in the bottom and top sections of Boysenberry; a relatively significantly lower level of expression was observed in those of Ningzhi1. In Ningzhi 1, expression was also higher in the bottom sections; expression was relatively lower in the top section and similar with that in the medium section of Boysenberry.

Discussion

Blackberry and red raspberry are the most commercially important brambles worldwide. Their prickles have been revealed as an unappealing trait in a variety of commercial crops (Clark *et al*., 2007). Prickles are simply outgrowths of epidermal and, in some cases, cortical tissues that lack vasculature (Coyner *et al*., 2005). Currently, the molecular events of plant prickle development and structure data are still limited. A comparison of raspberry and rose prickles showed that *Rubus* prickles were modified glandular trichomes that continued to grow and this structure eventually hardens into their final prickle morphology as an outgrowth of epidermal tissue (Kellogg *et al*., 2011). Raspberry prickles consist entirely of epidermal tissue, while blackberry prickles are composed of both epidermal and underlying cortical tissues (Kellogg, 2009). The correlation between the presence of glandular trichomes and prickles in blackberry suggests that glandular trichomes are indeed involved in blackberry prickle development (Kellogg *et al*., 2011). Our previous studies comparing stem prickles of Boysenberry cv. and the thin-prickle bud mutant cv. Ningzhi 1 indicated that prickles of both originated from the stem epidermis. Prickles on Boysenberry stems contained a large number of cortical cells, while in Ningzhi 1, a relatively small number of cells form the prickle (Zhang *et al*., 2013). This finding of differences in prickle morphology and cell quantity in prickles offers a clue for continuing seeking the prickle formation from trichomes.

Trichomes are appendages originating from the epidermal cells of leaves, stems and floral organs in plants and exist in diverse forms (Werker, 2000). They serve a variety of functions, such as protecting against insect and pathogen attack, reducing water loss, and increasing tolerance to abiotic stress conditions (Levin, 1973). For all types of trichomes, the cells that are destined to become trichomes must undergo a similar transition from the mitotic cell cycle to an endoreduplication cycle (Ishida *et al*., 2008). Researches have also shown that cell cycle control plays an important role in trichome initiation (Schnittger & Hülskamp, 2002; Yang & Ye, 2013). Whether some cell cycle-related genes function as identical activators for trichomes is still an interesting question.

In blackberry, the prickle developmental signals sent by the glandular trichomes are not only received in the epidermis but also penetrate to the underlying cortical cells, which results in the proliferation of cortical tissues forming the base of the prickle (Kellogg *et al*., 2011). Transcriptome study facilitates revealing new regulators of trichome formation in *Rubus*. *RuTK1* was a putative down-regulated candidate differentially expressed in the thin-prickle mutant cv. Ningzhi 1 from Boysenberry cv. detected by RNA-seq. In this study, we first cloned and analyzed the *RuTK1* sequence in blackberry. The nucleotide sequence of *RuTK1* was largely different from the corresponding sequence in virus (Xuan *et al*., 1996). We also compared the genomic structures of TKs from different plants according to the annotated data in GenBank (Fig. 1). The genomic structures in plants were relatively conserved, providing a fascinating clue towards cloning and elucidating their functions.

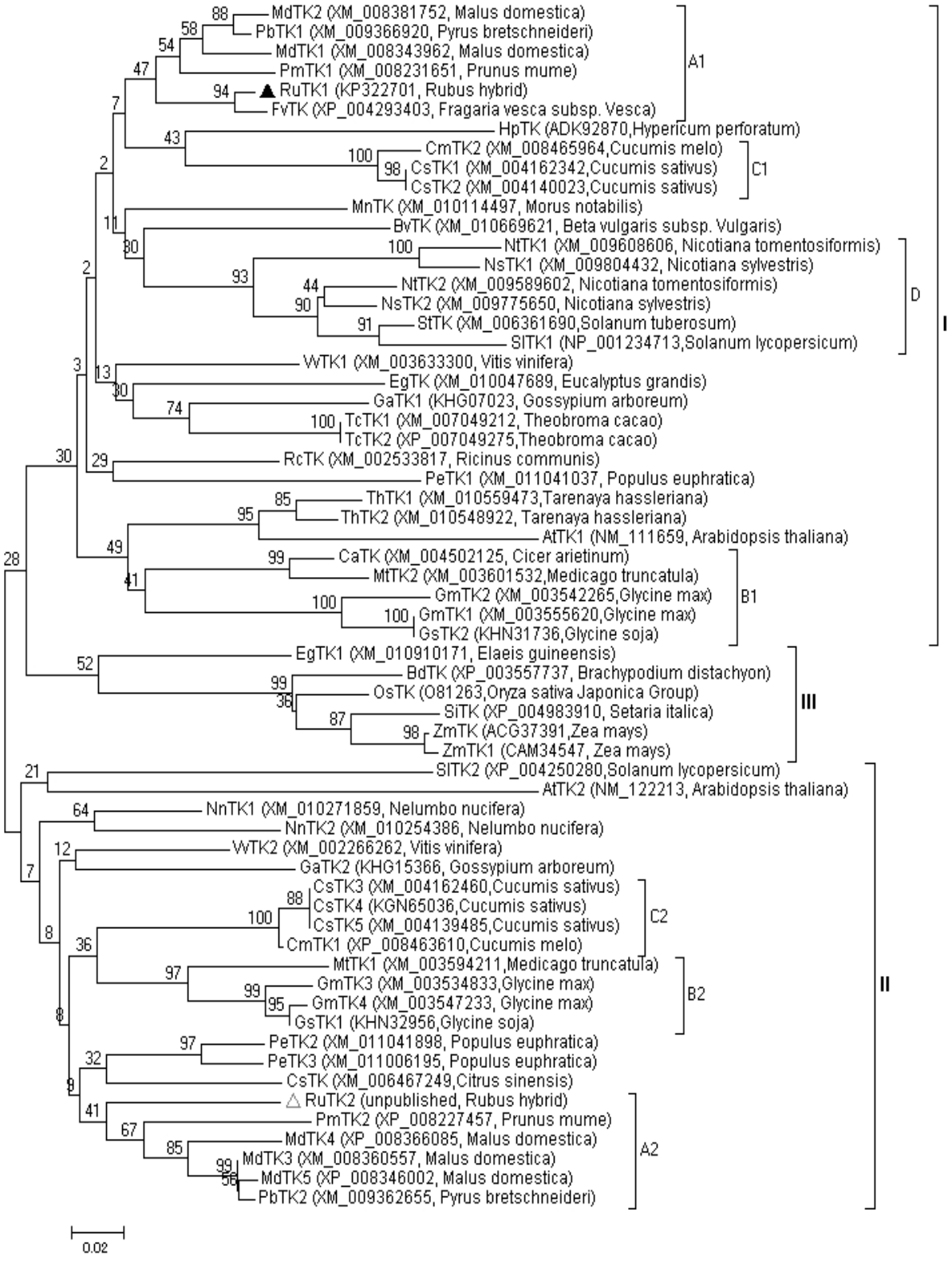

Fig. 3. Phylogenetic analysis of RuTK1 (marked with a black triangle) and other plant TK proteins. Sequence alignment was performed using Clustal X (version 1.8). The phylogenetic tree was constructed using the neighbor-joining (NJ) method (with 1,000 replicates for the bootstrap values and complete deletion data) with MEGA software (version 4.0). The scale bar (0.02) indicated the average number of amino acid substitutions per site. GenBank accession numbers and taxon names were also shown on the tree. An additional blackberry RuTK2 peptide (unpublished, partial sequence) was also shown and marked with gray triangle.

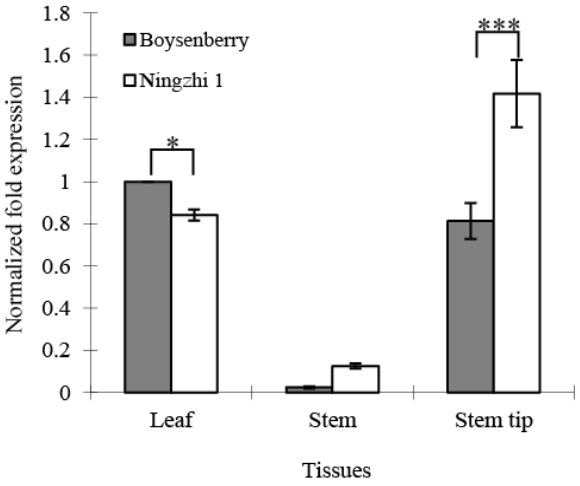

Fig. 4. Expression profiles of *RuTK1* in different tissues of Boysenberry and Ningzhi 1 as measured by real-time quantitative RT-PCR. Relative *RuTK1* expression levels were standardized to the constitutive level of expression of the *actin* gene. Error bars were based on three replicates. Data were compared by Student's *t* test. *P* values of 0.05 or less were considered to be statistically significant.

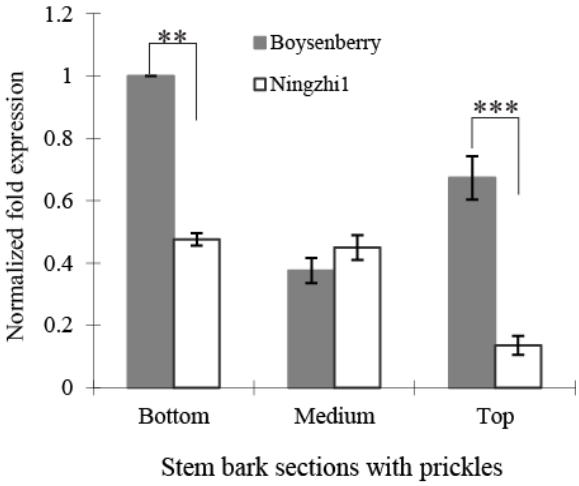

Fig. 5. Expression of *RuTK1* in different blackberry stem bark sections containing prickles from Boysenberry and Ningzhi 1. Expression was measured by real-time quantitative RT-PCR. Relative levels of *RuTK1* expression were standardized to the constitutive level of expression of the *actin* gene. Error bars were based on three replicates. Data were compared by Student's *t* test. *P* values of 0.05 or less were considered to be statistically significant.

Several studies have shown that TK plays certain important roles in the cell cycle. In mammals, TK proteins have been classed into cytoplasmic isoforms and mitochondrial isoforms. The former were shown to be related to cell division and proliferation, and the latter had no relation to cell cycle functions (Eriksson *et al*., 2002). TK was initially found to be related to the cell cycle by Hotta & Stern (1963). In synchronously dividing tissue cultures of *Jerusalem artichoke*, an increase in TK enzyme activity was coincident with the onset of DNA replication. This finding suggested that the TK was closely linked with the preparation for the cell division (Harland *et al*., 1973). In recent study, a phylogenetic analysis of TK1 from different organisms showed that vertebrate sequences were clustered into a single group, and plant and bacterial TK1 sequences were clustered into a separate group (Mutahir *et al*., 2013). The phylogenetic analysis in this study summarized the classifications of the currently annotated TK proteins in plants, which were seemingly classified by cotyledon number (Fig. 3). Sequences from dicotyledonous plants were further classified into group I and II, and monocotyledon plants formed group III. According to the aligned sequences, the large primary difference between plant TKs in group I and group II was that the peptide lengths were approximately 230 and 280 amino acid residues, respectively. This finding was not supported by experimental evidence; however, these classifications somewhat followed the distribution of thymidine kinase activities in the cytosol and chloroplast (Golaszewski *et al*., 1975).

Thymidine monophosphate, the product of the reaction catalyzed by thymidine kinase, is in turn phosphorylated to thymidine diphosphate by the enzyme thymidylate kinase and further to thymidine triphosphate which is included in a DNA molecule (Fujiwaki *et al*., 2002). The activity of the cytosolic TK1 enzyme is high in proliferating cells and peaks during the S-phase of the cell cycle, while is very low in resting cells (McAllister *et al*., 2014). After cell division is completed, TK1 is downregulated and degraded intracellularly, and thus called such a salvage enzyme for supplying thymidine monophosphate for DNA repair (McAllister *et al*., 2014). The thymidine kinase level in serum therefore serves as a measure of malignant proliferation, indirectly as a measure of the aggressivity of the tumor (Lamarca *et al*., 2015). Recently, the thymidine kinase was found to be present in plants and the study on the molecular function is still onset (Clausen *et al*., 2012; Mutahir *et al*., 2013; Pedroza-García *et al*., 2015). In the present experiment, from the expression patterns in different stem sections corresponding to the different stages of prickle development, *RuTK1* was highly expressed in early prickles in Boysenberry compared with that in Ningzhi 1. Similarly higher expression was found in prickles at the late stages in both Boysenberry and Ningzhi 1, which implied that *RuTK1* may appear in the cells during cell division and prickle formation at an early stage. This result was consistent with other publications that the TK1 levels in fetal tissues during development were higher than those of the corresponding tissues later in human (Herzfeld & Greengard, 1980) and rat (Machovich & Greengard, 1972). Therefore, the relative down-regulation of the *RuTK1* in Ningzhi1 prickles may cause the reduction of cell numbers and thus the thin and weak prickles compared with its parent cultivar Boysenberry. The research on *RuTK1* in this species and functional predictions of TKs provides new evidence for understanding the prickle formation mechanisms in blackberry and investigating the functions of *TK* genes in plants.

Acknowledgments

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