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Research article

Cloning and molecular characterization of rutin degrading enzyme from tartary buckwheat (*Fagopyrum tataricum* Gaertn.)

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ABSTRACT

Rutin and quercetin, abundant in tartary buckwheat, have physiological and pharmacological functions and play roles in abiotic stress tolerance in plant. Rutin degrading enzymes (RDE) are the key enzymes for rutin metabolism. However, the RDE coding sequence information has not been available. In this study, a 1515-bp coding sequence of RDE was cloned from tartary buckwheat (named *FtRDE*) using 5′ and 3′ RACE, based on the FtRDE protein sequence. The recombinant RDE (rRDE) expressed in *P.pastoris* with glycosylation modification degraded rutin into quercetin and the Glu171 and Glu382 were indispensable residues for catalytic activity. *FtRDE* was highly expressed in seed filling stage and response to ABA and MeJA, confirmed by qRT-PCR and *FtRDE* promoter activity analysis in mesophyll protoplast. This study provided a new approach for the large-scale preparation of RDE by heterologous expression and production of quercetin by hydrolyzing rutin, and could be helpful for understanding the *FtRDE* function under stress conditions.

1. Introduction

Tartary buckwheat (*Fagopyrum tartaricum* (L.) Gaertn) is a typical crop used as both medicines and food, due to its well-balanced amino acid composition and bioactive flavonoids in the seeds (Pomeranz and Robbins, 1972). Rutin, one of the most important bioactive flavonoids in buckwheat seeds, accounts for approximately 80% of total buckwheat flavonoid content (Couch et al., 1946; Jiang et al., 2007). Rutin has been used in pharmaceutical drugs because of its antibacterial (Lee and Lee, 2010), antihyperglycaemic (Calzada et al., 2017) and antioxidative properties (Yang et al., 2008). Quercetin is the metabolic precursor of rutin, which is more efficient in oxidant removal (de Araújo et al., 2013) and cell protection (Chen et al., 2006) than rutin. Quercetin also contribute the main source of bitter taste of buckwheat seeds limited its use in food products (Suzuki and Morishita, 2016). Rutin degrading enzymes (RDEs), abundant in tartary buckwheat seeds, can efficiently convert rutin to quercetin, potentially meeting the demands of quercetin production in the industry (Chen and Gu, 2011).

Yasuda and Nakagawa first reported the existence of RDEs in tartary buckwheat seeds and purified two distinct RDEs from tartary buckwheat seeds with the same Km for rutin, through a series of chromatographic procedures (Yasuda and Nakagawa, 1994). Cui and Wang, 2012 purified a rutin-hydrolyzing enzyme (RHE) with rutin-degrading activity. In the previous work of our laboratory, Chen and Gu established an isoabsorptive spectrophotometric method (ISM) for rapidly monitoring rutin-degrading enzyme (RDE) activity (Chen and Gu, 2011). Zhang et al. established a rapid purification method based on the characteristics of organic solvent tolerance of RDE, and obtained the partial protein sequence by mass spectrometry (Zhang et al., 2017). However, there have been no reports of *FtRDE* gene sequence information and recombinant expression, limiting the in-depth study of its catalytic mechanism and biological function.

Gene transcriptional regulation studies could help to further understand the function of RDEs in tartary buckwheat. Promoter not only controls the transcription initiation of the downstream gene through the interaction between cis-acting elements and trans-acting factors, but also mediates the expression of certain genes in specific tissues, specific environments, and special developmental stages (Gu et al., 2013; Guan et al., 2016). Functional identification of the cis-acting elements of a promoter plays an important part in the investigation of gene regulatory mechanisms. Flavonoids and flavonoid metabolic enzyme genes were often involved in plant stress tolerance processes

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(Ning and Wang, 2018; Tu et al., 2016), and were regulated by exogenous hormone treatment, such as ABA and MeJA (Fernando et al., 2010; Sandhu et al., 2011). Therefore, isolation of *FtRDE* promoter and detection of its expression pattern and response to different hormone treatments are important for revealing the biological function of *FtRDE*.

For those reasons, the aim of this study was to isolate the RDE-coding gene for heterologous expression and explore the active sites and catalytic mechanism, as well as to detect the transcriptional regulation of *FtRDE*. Heterologous expression of *FtRDE* can be used to develop an affordable technique for quercetin preparation. Determination of active sites provides the ideal target sites for the development of RDE-knockout germplasm with good taste. Promoter activity and expression pattern analysis help to further study the biological function of *FtRDE* in tartary buckwheat.

2. Materials and methods

2.1. Materials and treatment

Tartary buckwheat (*Fagopyrum tartaricum*) 'Yu 6–21' seeds purchased from the Yulin Academy of Agricultural Sciences (Yulin, China) were grown in the horticultural field at the Northwest A&F University under natural conditions. Approximately 4 weeks later, the fully expanded leaves from four-leaf seedling were harvested for the protoplast isolation and DNA extraction. Different tissues were collected from filling stage seedlings for *FtRDE* gene tissue-specific expression analysis and gene cloning. To determine the influence of abscisic acid (ABA) and methyl jasmonate (MeJA) on the expression levels of *FtRDE*, the buckwheat seedlings were grown in Hoagland nutrient solutions under greenhouse conditions with a 16/8 h light/dark cycle at 25 °C. When the two cotyledons of seedlings fully expanded, 100 μM ABA and MeJA were used to treat the seedlings, and then the young leaves were collected after treatment for 0 h, 3 h, 6 h, 12 h, 24 h, and 48 h respectively for RNA extraction. Total RNA Kit and DNA Kit were from OMEGA (USA). All synthetic oligonucleotide primers used in this study were obtained from GENEWIZ (China). Phusion® High-Fidelity PCR Master Mix with HF Buffer and NEBuilder® HiFi DNA Assembly Master Mix were from NEB (USA). SMARTer RACE cDNA Amplification Kit was from Clontech (USA). DEAE Sephadex A-50 was from GE Healthcare (USA). Eclipse XDB-C18 column (250 mm × 4.6 mm i.d., 5 μm particle size) was from Agilent (USA). Cellulase R-10 and macerozyme R-10 were purchased form Yakult (Japan).

2.2. Cloning of FtRDE full-length cDNA

For *FtRDE* full-length cDNA cloning, total RNA was isolated from filling period seeds and poly(A)$^+$-RNA was isolated using Oligotex-dT$_{30}$ super resins (Roche, Switzerland). First strand cDNA was synthesized with M-MLV reverse transcriptase (NEB, USA). Degenerate primers for RACE were designed according to the internal peptide sequences determined previously (Zhang et al., 2017). For 3′ RACE PCR, *FtRDE* gene-specific primers (GSP) were designed according to peptide sequences 'GDVADDFYHR' (5′ - GGNGACGTNGCNGATGATTTYTAYCATCG -3′) and 'TDNHATTSFFK' (5′ - ACTGATAAYCATGCWACNACNTCNTTYTTYAA -3′, where N = A/C/G/T, Y = C/T, W = A/T.) As for 5′ RACE, GSPs were designed according to the 3′ RACE result). All the procedures were according to the 5′/3′ RACE kit. The PCR products were cloned into a pBlunt vector (CloneSmart, China) for sequencing. The signal peptide was predicted with the SignalP 4.1 Server (http://www.cbs.dtu.dk/services/SignalP/). The molecular weight and theoretical isoelectric point (pI) of the mature RDE were calculated using ExPASy (http://www.expasy.org/).

2.3. Expression, purification and enzymatic properties analysis of the recombinant RDE (rRDE) in Pichia pastoris

The RDE coding sequence without the predicted 26-residue signal peptide was amplified and then cloned into the yeast pPICZ(α)A (*EcoR* I and *Sal* I restriction. Recombinant plasmid was linearized with *Sac*I and transformed into *P. pastoris* SMD1168H. The operations for *P. pastoris* transformation and cultivation were performed according to the EasySelect™ Pichia Expression Kit (Invitrogen, USA). After induction for 72 h with daily addition of methanol (0.5% v/v), the culture supernatants were subjected to SDS-PAGE to test for expression of rRDE. For rRDE purification, the culture medium supernatant was collected after centrifugation at 10,000 × *g* for 20 min. The supernatant was filtered through a 0.2 μm filter membrane and then was dialyzed overnight against 20 mmol/L Tris-HCl (pH 8.0). The sample was then applied to a DEAE-Sepharose and the rRDE protein was eluted with a linear gradient of NaCl (20–500 mmol/L). Elution was monitored by the absorbance at 280 nm. 1 mL fractions were collected for SDS-PAGE analysis of the presence of rRDE, and then fractions containing the target protein were pooled. Native RDE (nRDE) was also purified from buckwheat seeds according to the reported procedure (Zhang et al., 2017).

The effect of pH on nRDE and rRDE were evaluated in a range of different buffers from pH 3–8 (50 mM HAc-NaAc, pH 3–6; 50 mM Tris-HCl, pH 7–8). The effect of temperature on nRDE and rRDE activity was determined between 20 °C and 70 °C. The enzymatic activity of rRDE was measured at different substrate rutin concentrations (1, 2, 3, 4, 5, 6, 7, 8 mg/mL) in 20 mmol/L acetate buffer (pH 5.0). After incubation at 37 °C for 5 min, 10 times the volume of methanol was added to stop the reaction. Then the products were analyzed by HPLC.

2.4. N-linked glycosylation analysis and site-directed mutagenesis of RDE

Since the fact that *P. pastoris* is able to add N-linked carbohydrate chains to the secreted proteins (Cereghino et al., 2002), and the deduced amino acid sequence of RDE contains 34 Asn residues which might act as potential N-linked glycosylation sites, PNGaseF (New England Biolabs, USA) was used to detect the presence of glycosylation modification in rRDE. To confirm the key amino acid responsible for RDE catalytic activity, site-directed mutagenesis was carried out using the QuikChange Site-Directed Mutagenesis Kit (Stratagene, USA). The original pPICZ(α)A-*RDE* plasmid was used as a template. Two conserved residues (E^{171} and E^{382}) of substrate binding pocket were mutated to Leu individually by four oligonucleotide primers (Table 1, mutation sites are underlined). The PCR product treated with *Dpn* I was then transformed into *E. coli* TOP 10 and the expected mutations were confirmed by sequencing. The constructs having the target mutant sites were transformed into *P. pastoris* for protein expression. Transformation and cultivation of *Pichia pastoris* and purification of mutant rRDE were conducted as described above.

2.5. FtRDE tissue-specific expression analysis

The leaves, stems, flowers, and seeds were harvested at grain filling stage for total RNA isolation and cDNA synthesis. For testing the tissue expression profile of *FtRDE* among different organs, semi-quantitative RT-PCR was performed with primers qFtRDE$_F$ and qFtRDE$_R$ (Table 1). The *FtGAPDH* was amplified as internal control with the primer set of GAPDH$_F$ and GAPDH$_R$. PCR reactions were conducted with the following protocols: 96 °C pre-incubation for 1 min; denaturation at 96 °C for 20 s, annealing at 60 °C for 20 s, extend at 72 °C for 15 s (28 cycles). The PCR products were detected by 1% (w/v) agarose gel electrophoresis.

Table 1
Primers used in this work (Underlined sequences are restriction endonuclease sites).

Primers	Application	Sequence (5′–3′)
E^{171}L-F	Site-directed mutation	CATTGGACAACAATGAACCTGCCGAATGTAATGACTG
E^{171}L-R	Site-directed mutation	CAGTCATTACATTCGGCAGGTTCATTGTTGTCCAATG
E^{382}L-F	Site-directed mutation	CAGCCGTCATTATAACTCTGAATGGGTTGTCGGATG
E^{382}L-R	Site-directed mutation	CATCCGACAACCCATTCAGAGTTATAATGACGGCTG
SP1	1 st of genome walking	TCGGCCACGTCTCCGTTACTTCCA
SP2	2 nd of genome walking	CCATCTATAAATGCAGCTCCTTCAGAC
SP3	3 rd of genome walking	AAGTCTTTTTGCACGTACTTGGTAGG
pF1	5′-deletion (−1829 bp)	GAGGGTACCCCTATATTTGTTATCGGACGCTATTTC
pF2	5′-deletion (−1077 bp)	GAGGGTACCGTGTGTCCAACTATCCATTCGTACACC
pF3	5′-deletion (−628 bp)	GAGGGTACCGCCTAGTGTTTTGGCGGTCC
pF4	5′-deletion (−489 bp)	GAGGGTACCAACGAGCTCAACCAAGAAAATAGC
pF5	5′-deletion (−393 bp)	GAGGGTACCGTCTCGAATTGCTTTGATGCTATATG
pR1	5′-deletion (reverse)	CAGGCTAGCACTATGAAGCTTTGATTGTCGCTTT
$qFtRDE_F$	(semi-) quantitative	GAATCAACCAAGCAGGGAT
$qFtRDE_R$	(semi-) quantitative	ATAGTCTCGGAAATCATCTACTATG
$GAPDH_F$	internal control	AGTTGCACTACCAACTGCCTTG
$GAPDH_R$	internal control	AGGTCAACCACGGACACATC

2.6. FtRDE promoter cloning and dual luciferase reporter vector construction

For *FtRDE* promoter cloning, genomic DNA was isolated from fresh young leaves and then used as TAIL-PCR (thermal asymmetric interlaced PCR) template. Three specific reverse primers (SP1, SP2, SP3) were designed to clone the *FtRDE* 5′ flanking DNA sequence using the Genome Walking Kit (Takara, China) based on the *FtRDE* coding sequence obtained in the previous step. The PCR products were cloned into a pBlunt vector for sequencing. Finally, the cloned sequence was analyzed for using online software PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html) and PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) for cis-acting elements prediction.

The pGL3, improved firefly (coleopteran) luciferase basic vector (Promega), was used as a carrier to construct the dual luciferase reporter vector (Gu et al., 2013). The 1829 bp *FtRDE* promoter and a series of *FtRDE* promoter fragments with different 5′ deletions were cloned using primer combinations of pF1 to pF5 with pR1, a universal antisense primer pR1 containing a *Nhe I* restriction site, the other forward primers containing a *Kpn I* restriction site (shown in Table 1). All these promoter fragments were digested with *Nhe I* and *Kpn I* and inserted into corresponding sites of the dual reporter expression vector to replace the 2 × 35S promoter (upstream of the Renilla luciferase). Each construct was identified by PCR, double enzyme digesting and sequencing, and the correct constructs were named FtRDEPR1-FtRDEPR5 respectively.

2.7. F. tartaricum protoplast preparation and transformation

The *Fagopyrum tartaricum* mesophyll protoplast isolation was performed according to the methods described by Yoo et al. (2007) and (Zhang and Chen, 2016) with some modifications. The enzyme digestion solution (1.5% (w/v) cellulase R-10, 0.4% (w/v) macerozyme R-10, 20 mM 2-morpholinoethanesulfonic acid (MES) and 0.5 M mannitol, pH5.7). After placing in a water bath at 55 °C for 10 min and cooling to room temperature, the solution was added with 10 mM $CaCl_2$ and 0.1% (w/v) bovine serum albumin (BSA) and sterilized with 0.45 μm filter. The well-expanded leaves from 4-week-old *Fagopyrum tartaricum* plants were surface sterilized with 75% (v/v) ethanol for 30-sec immersion, washed in deionized water immediately. Then, the lower surface of the epidermis was cut into 0.5–1 mm fine strips and rapidly transferred to the enzyme digestion solution (0.8 g/10 mL). The compounds were then digested on a rotary shaker (40 rpm/min) at 25 °C for 4 h in dark. After digestion, an equal volume of W5 solution (2 mM MES, 154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, pH 5.7) was added to the enzyme lysate compounds and mixed gently. The compounds were filtered through 200 mesh nylon membrane and centrifuged at 900 rpm for 4 min to collect the protoplasts. Then the protoplasts were washed once with W5 solution after incubating at ice bath for 30 min and re-suspended with 1 mL W5 solution for the next transient transfection.

PEG4000-Ca^{2+}-mediated plasmid transfection was performed according to the published protocol (Huang et al., 2013) with some modifications. The purified protoplasts were kept on an ice water bath for 15 min and centrifuged at room temperature, then the precipitate re-suspended with MMG solution (4 mM MES, 200 mM mannitol, 15 mM $MgCl_2$, pH 5.7) at a final concentration of 2 × 10^5 protoplasts/ml. The transfection was conducted with a mix of 20 μg plasmid DNA, 100 μL protoplasts, 110 μL PEG4000-Ca^{2+} solution (40% (w/v) PEG4000, 200 mM mannitol, 100 mM $CaCl_2$) at 25 °C for 20 min under darkness conditions and stop with the addition of 440 μL W5 solution, then the supernatant was removed by centrifuging at 900 rpm. The collected protoplasts were re-suspended with 600 μL WI solution (4 mM MES, 500 mM mannitol, 20 mM KCl, pH 5.7) and transferred to 6 well cell culture plates. For luciferase activity assay, the protoplasts were carefully transferred into an Eppendorf tube and centrifuged at 900 rpm for 2 min, and then re-suspended in 50 μL lysis buffer (100 mM K_3PO_4, 1 mM DTT, pH 7.8). After ice bath for 5 min and centrifuged at 12,000 rpm for 5 min, the cell supernatant were used for luciferase activity assess according to the Dual-Luciferase® Reporter Assay System (Promega, USA). All the experiments were repeated three times.

2.8. FtRDE promoter activity and gene expression response to ABA and MeJA treatments

To detect the promoter activity under ABA and MeJA treatments in protoplast, the protoplasts were treated with 100 μM ABA and MeJA (the control group added with an equal volume of WI solution) and incubated at 25 °C for 20 h for luciferase activity detection.

The ABA and MeJA treated seedlings were used for real-time RT-PCR, to detect *FtRDE* expression response to hormones. The first strand cDNA was synthesized from total RNA (1 μg) using Prime Script™ II 1st Strand cDNA Synthesis Kit (Takara), the reverse transcription products were diluted in triple and used for the real-Time RT-PCR of the gene expression. Real-time RT-PCR was performed in a total volume of 20 μL containing 2 μL of cDNA, 10 μL of 2 × SYBR Green II Mix, and 0.2 μM of each primer ($qFtRDE_F$ and $qFtRDE_R$; $GAPDH_F$ and $GAPDH_R$, Table 1). PCR conditions were conducted as follows: 94 °C pre-incubation for 3 min; followed by 40 cycles of denaturation at 94 °C for 10 s, annealing at 60 °C for 30 s. At the end of the amplification, a melting curve from 65 °C to 95 °C at 0.5 °C increments was performed. All the reactions were performed in a CFX96 real-time PCR detection system (Bio-Rad).

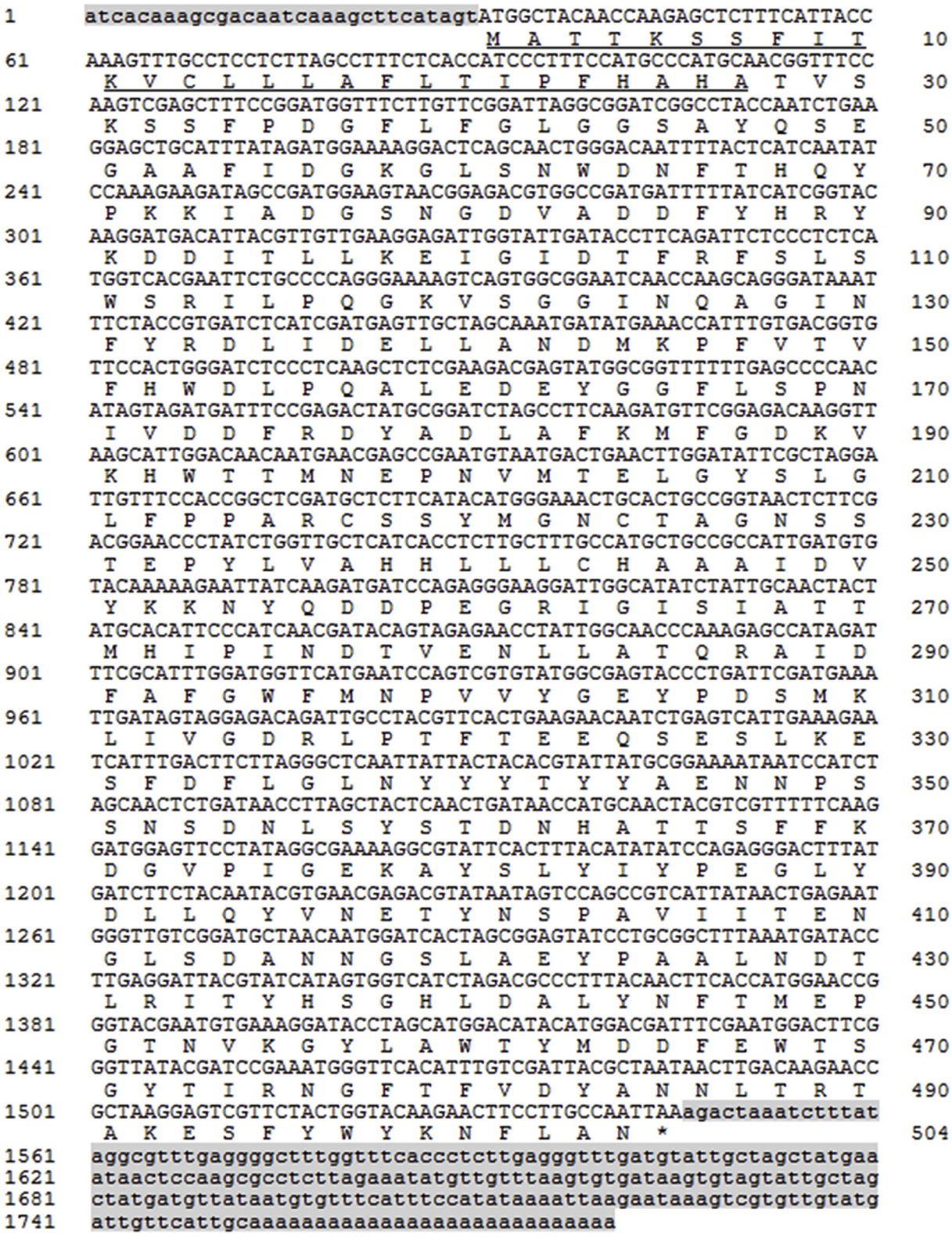

Fig. 1. Nucleotide and deduced amino acid sequences of FtRDE. Gray shading marked untranslated area. The signal peptide sequence was underlined.

There are three biological replicates for each sample and two technical replicates for each biological replicate. The relative expression levels were calculated using the relative $2^{-\triangle\triangle Ct}$ method (Livak and Schmittgen, 2001) and using *FtGAPDH* as the internal control.

2.9. Statistical analysis

The experimental data were statistically analyzed using SPSS12.0 software. Difference significance test was conducted with the least significant difference (LSD) tests at $P < 0.05$. All the results were expressed as mean ± standard deviations and plotted with Origin 8.0.

3. Results

3.1. FtRDE gene cloning and characteristic

After 5′ and 3′ RACE, we obtained a 1780-bp sequence of RDE, which was then deposited in GenBank under accession number MF383331. The cDNA consisted of 30-bp 5′ UTR, 235-bp 3′ UTR and 1515-bp open reading frame (ORF) encoding 504 amino acid residues, with a signal peptide containing 27 amino acids (Fig. 1). The molecular weight of the mature RDE was 53.8 kDa and the theoretical isoelectric point was 4.56. The genomic DNA sequences of *FtRDE* were also obtained in this work. The results showed that *RDE* had 3 copies in the genome named *RDE1* (1515 bp), *RDE2* (2681 bp) and *RDE3* (3151 bp). *RDE1* was intronless (GenBank: MF383331.1), while *RDE2* gene was interrupted by 11 introns that formed 12 exons, and *RDE3* was one intron more than *RDE2* (Supp. Figure 1A). There were single base mutations between the exons of *RDE2* and *RDE3*. Sequence identity between the coding regions of the three genes was 75%, and identity of putative peptide sequences was 83% (Supp. Figure 1B).

3.2. Expression of rRDE in Pichia pastoris and purification and enzymatic properties

Transgenic yeast was constructed for fermentation preparation of FtRDE, using the shuttle pPIC(α)A vector with the AOX1 promoter and α-factor secretion signal. After 72 h induction with 0.5% methanol, the culture supernatant was analyzed by SDS-PAGE, revealing the clear band at about 80 kDa (Fig. 2A lane 2). The gel-scanning analysis revealed that the rRDE accounted for more than 80% of the total protein content in the culture supernatant. rRDE was successfully purified from DEAE-Sepharose anion-exchange column (Fig. 2A, lane 3) with a yield of 21.5 mg/L. The molecular weight of rRDE (approximately 80 kDa) was apparently greater than nRDE (approximately 60 kDa), which might be caused by glycosylation modifications in the *Pichia* expression system (Macauley-Patrick et al., 2005). This prediction was further confirmed by the removal of N-linked glycans using PNGase F (Fig. 2B). However, the rRDE overexpressed in the prokaryotic system formed inclusion bodies and showed no activity although the solubilizing tags such as small ubiquitin-like modifier was applied (Supp. Figure 2) (Esposito and Chatterjee, 2006; Marblestone et al., 2006). This result indicated that the post-translational modification maybe essential for the solubility and activity of RDE when expressed in a heterologous system.

Hydrolyzed products of rutin by RDE were analyzed with HPLC system. According to the chromatographic profiles, a single peak with the same retention time as rutin substrate was detected in the control groups (Supp. Fig. 3A and B), in which both the nRDE and rRDE were heat denatured. While an additional quercetin signal was detected in experimental groups for both nRDE and rRDE (Fig. 3C and D). The results demonstrated that the rRDE had the same function as nRDE for hydrolysis of rutin. The purified rRDE from *Pichia pastoris* showed specific activities of 284 U/mg protein, the Km was 2.04 mmol·L^{-1} and Vmax was 4.19 mmol L^{-1}·s^{-1}. The optimum pH for nRDE was 5.0, and rRDE also followed the same pattern as nRDE. Both nRDE and rRDE presented catalytic activity over a narrow pH range of 3.0–6.0, but activities declined quickly above pH 6.0 (Fig. 4A). The optimum temperature of rRDE was found to be 50 °C with the same optimum temperature as of nRDE. Both rRDE and nRDE were stable over a broad temperature range of 30–60 °C (Fig. 4 B). nRDE maintained 60% activity at 70 °C while rRDE was completely inactivated, demonstrating that nRDE was more tolerant towards high temperature.

Amino acid sequence alignment showed that the two glutamic acids (E^{171} and E^{382}) are conserved within 'TXNEP' and 'ITENG' motif (Fig. 5A), which had been identified as catalytic acid/base and nucleophile in β-Primeverosidase and Os4BGlu12, respectively (Saino et al., 2014; Sansenya et al., 2011). Mutations in either of the two E to L (E^{171}L and E^{382}L) could lead to complete loss of catalytic activity. This finding indicated that both the E^{171} and E^{382} are indispensable for RDE activity.

3.3. Tissue specificity of FtRDE gene in Fagopyrum tartaricum

The expression of *FtRDE* showed an obviously difference in different organs. Semi-quantitative RT-PCR results demonstrated that the *FtRDE* was highest expressed in buckwheat seeds (Fig. 6). The high expression level was also recorded in leaf, while lower in stem and flower. These results indicated that *FtRDE* plays a major role in seeds and leaves.

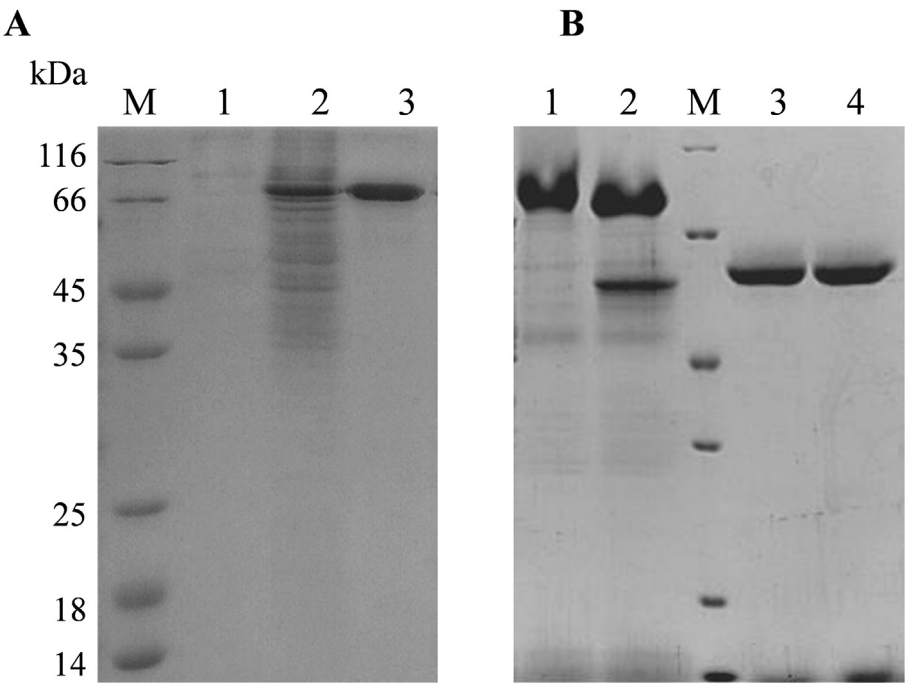

Fig. 2. Recombinant expression, purification, and glycosylation analysis of RDE. A, Recombinant expression and purification of RDE. Lane 1–2, SDS–PAGE analysis of the total protein in BMGY or BMMY culture medium, respectively. Lane 3, Purified recombinant RDE. B, SDS–PAGE analysis of recombinant (lane 2) and native RDE (lane 4) treated with PNGaseF.

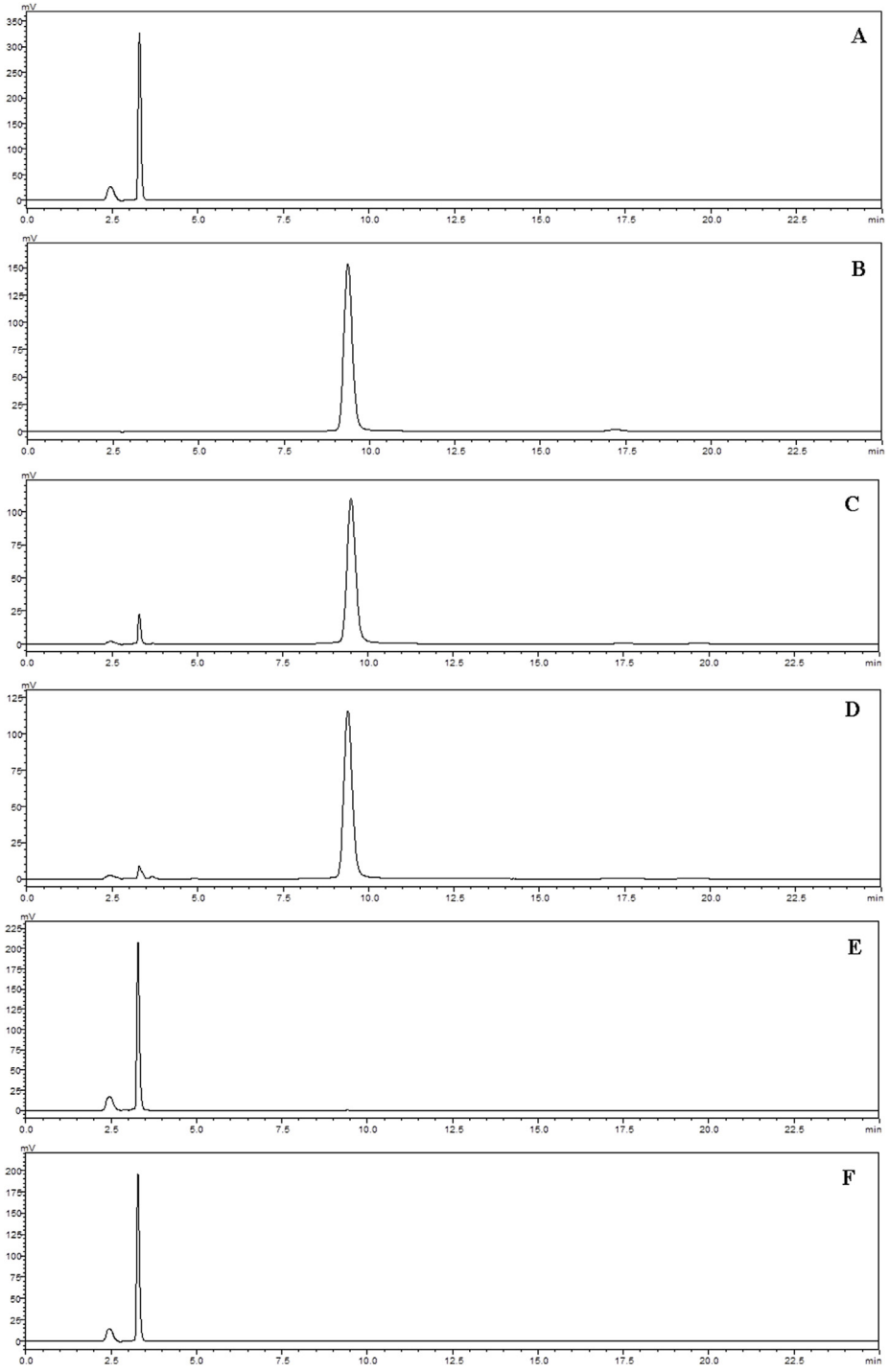

Fig. 3. HPLC chromatogram for rutin, quercetin and the hydrolyzates by RDE. HPLC for rutin (A), quercetin (B), the hydrolyzate by native RDE (C) or by recombinant RDE (D), and HPLC for the hydrolyzate by the mutant $E^{171}L$ (E) and by the mutant $E^{382}L$ (F).

3.4. Cloning and characteristic analysis of the FtRDE promoter

The 1829 bp *FtRDE* promoter fragment was cloned from *Fagopyrum tartaricum* genomic DNA using TAIL-PCR method (Supp. Figure 4). By the PLACE database (Higo et al., 1999), some putative *cis*-acting elements were predicted in *FtRDE* promoter, including a MeJA-response related element (−443 bp), three ABA response elements (ABRE, TGACGA-motif, −438, −535, −1145 bp). Notably, one seed specificity regulatory element (RY-repeat) was also predicted in the promoter region (Bobb et al., 1997)(Fig. 7).

3.5. FtRDE promoter activity under ABA and MeJA treatments

Promoter activity analysis in protoplast showed that the full-length 1.8 kb promoter fragment (FtRDEPR1) and its truncations (FtRDEPR2-FtRDEPR5) showed obviously different activities (Fig. 8A). FtRDEPR2 showed the highest activity compared to the other four fragments ($P < 0.05$), which suggested that there probably exist a (or several) negative element(s) among −1077 bp to −1829 bp that inhibited the *FtRDE* promoter transcriptional activity.

According to the bioinformatic prediction results, one MeJA response element and three ABA response elements might distribute in

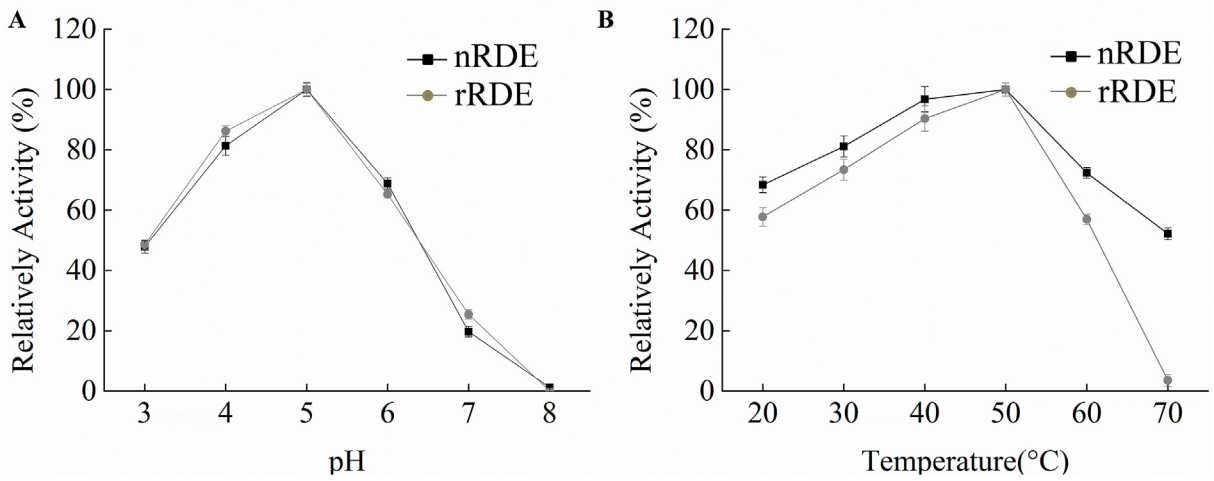

Fig. 4. Effect of pH (A) and Temperature (B) on native and recombinant RDE. the y-axis indicates the ratio of activity at respective pH (A) or temperature to the activity at pH 5.0 or 50 °C.

the promoter region. Therefore, the transformed protoplasts were treated with 100 μM MeJA and ABA for luciferase activity analysis. After treated with MeJA, the fragment FtRDEPR4 (contain the TGACGA-motif) showed two-fold activity than the control group, and there were no significant differences in the activity of fragment FtRDEPR5 (not contain the TGACGA-motif) (Fig. 8B), the results above indicated that the MeJA response element is functional. Meanwhile, compared with the control group, the fragments FtRDEPR1 to FtRDEPR4 (contain the ABRE) showed greater promoter activity under ABA treatment, and the fragment F5 (not contain the ABRE) did not show a significant difference (Fig. 8C), this result indicated that the ABRE elements are functional.

3.6. Expression of FtRDE in response to hormone treatment

The expression level of *FtRDE* in leaves increased continuously within 24 h under 100 μM ABA treatment. The *FtRDE* expression level of ABA-treated seedlings was 18.79-fold high than the control group at 12 h, then reached the highest level at 24 h (41.89-fold) and decreased at 48 h (10.68-fold) (Fig. 9A). The expression pattern of the *FtRDE* gene showed a similar trend under the treatment of 100 μM MeJA. The expression level was 12.59, 25.76 and 7.16-fold that of the control group at 12 h, 24 h and 48 h, respectively (Fig. 9B).

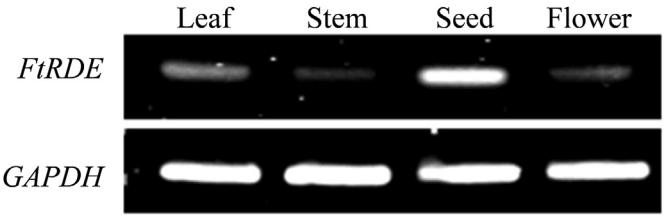

Fig. 6. Relative expression of *FtRDE* in different organs in tartary buckwheat. Semi-quantitative RT-PCR was performed to detect the expression of *FtRDE* in different tissue, the *FtGAPDH* was used as an internal control.

4. Discussion

Rutin is secondary metabolites with important pharmacological effects, widely distributed in plant species (Duric et al., 2009; Noldner and Schotz, 2002; Zu et al., 2006). RDE is a crucial enzyme that participates in rutin degradation and its history has been more than 25 years, from the first time that the enzymes with rutin degradation

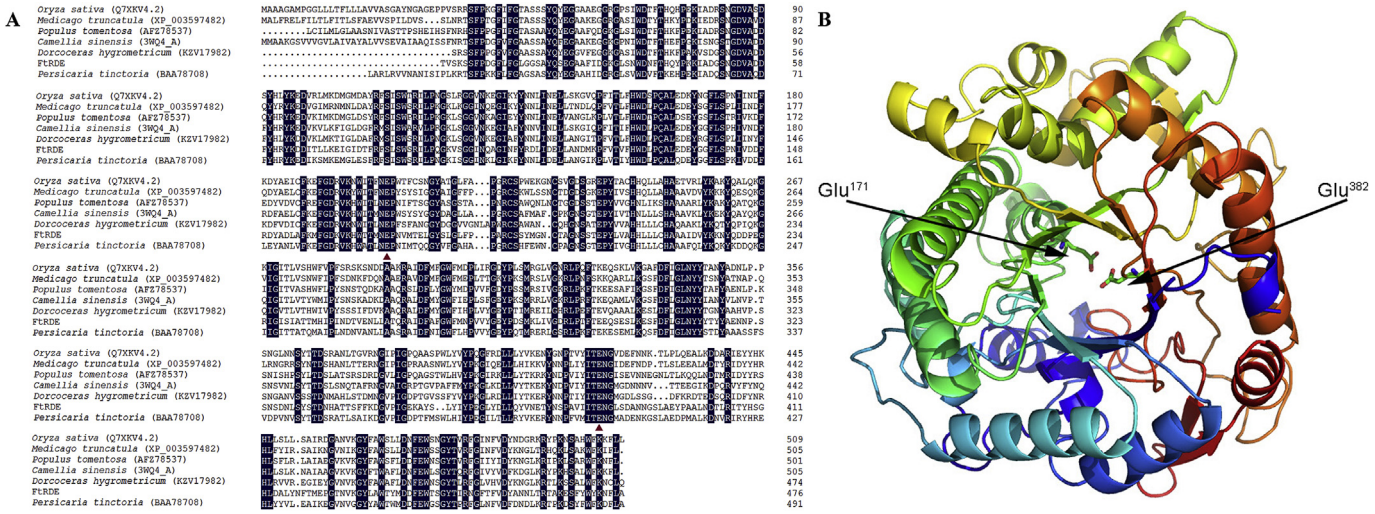

Fig. 5. Sequence alignment of FtRDE with six β-glucosidase (A) and the predicted 3-D structure model of FtRDE (B). The two red triangles indicated the catalytic acid-base Glu^{171} and nucleophile Glu^{382}, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Fig. 7. Nucleotide sequence of the *FtRDE* promoter and the putative cis-acting elements. Some basic cis-acting elements (such as TATA-box, CAAT-box) and plants stress-related elements: ABA response elements (ABRE) and MeJA response element (TGACGA-motif). A seed-specific regulatory element (RY-repeat) was also predicted in the *FtRDE* promoter.

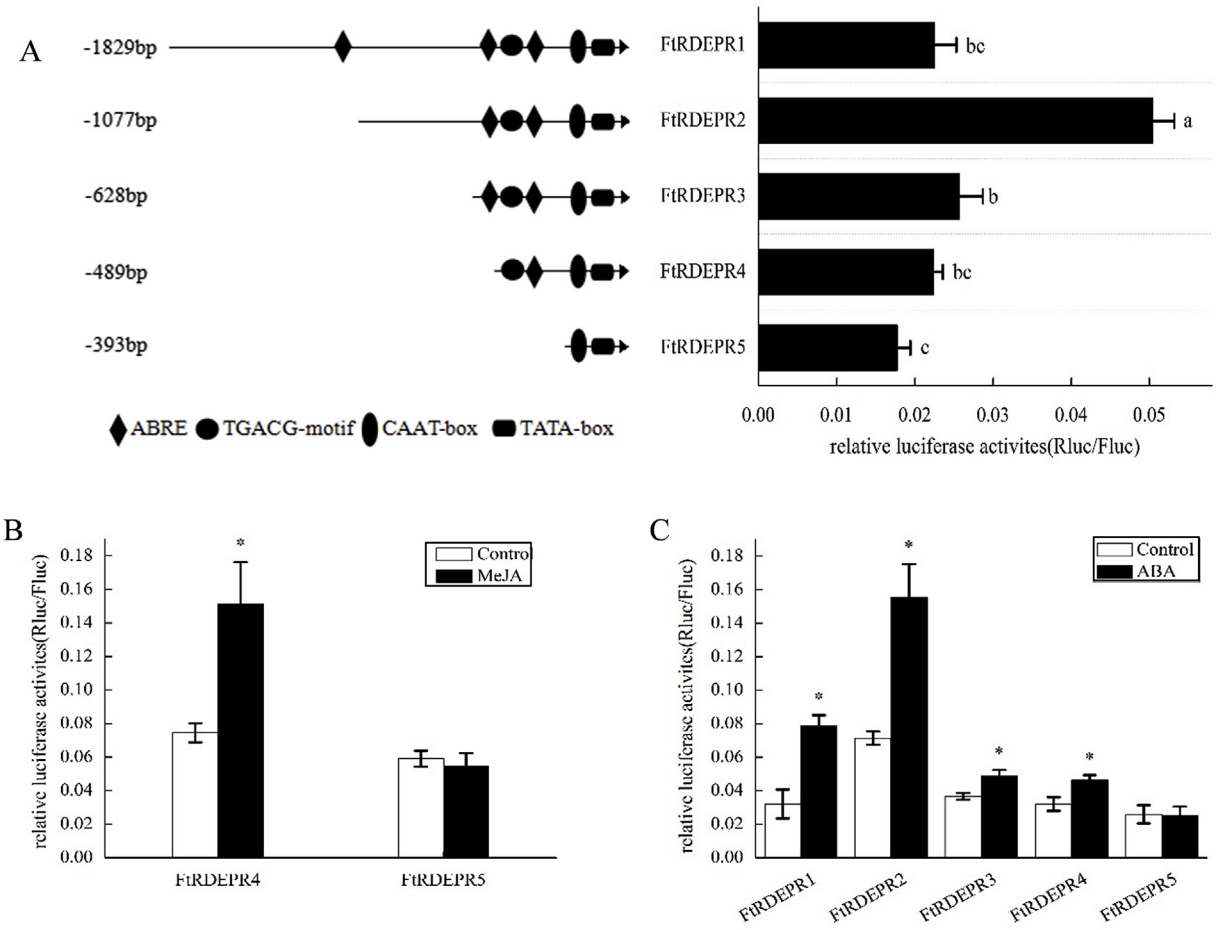

Fig. 8. The functional analysis of *FtRDE* promoter with different 5′-deletion. (A) Schematic structure of the *FtRDE* promoter with different 5′-deletion and the corresponding activity transient expression in protoplasts. (B) The activity analysis of *FtRDE* promoter with MeJA treatment. (C) The activity analysis of *FtRDE* promoter with ABA treatment. Fluc: Firefly luciferase; Rluc: Renilla luciferase. Data are means ± SD, n = 3. Different letters and * means $p < 0.05$ compare to control treatment.

activity had been found by Yasuda from tartary buckwheat seeds (Yasuda and Nakagawa, 1994). After that, several studies about the enzyme purification and enzymatic characteristics have been carried out (Baumgertel et al., 2003; Morishita et al., 1998; Suzuki et al., 2002; Yasuda and Nakagawa, 1994). In this study, we cloned the 1780-bp full-length coding sequence of FtRDE, using 5′ and 3′ RACE methods, based on the peptide sequences obtained previously. *FtRDE* had a 1515-bp ORF encoding 504 amino acid residues. FtRDE possibly existed in vacuole predicted with Softberry (http://www.softberry.com), where it might exhibit high intrinsic activity for physiological function at the

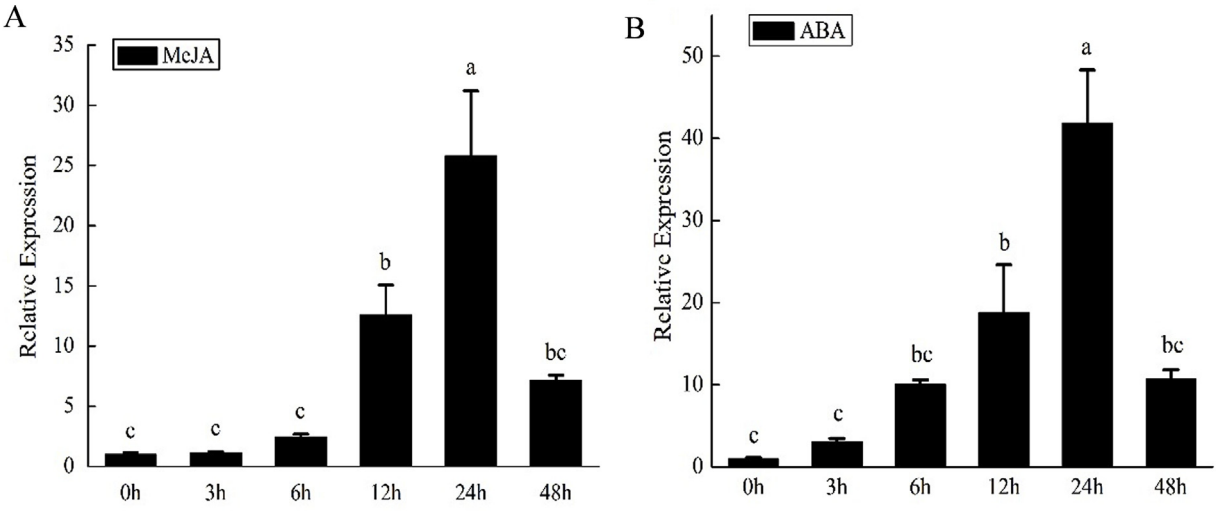

Fig. 9. Transcript accumulation of *FtRDE* in response to MeJA and ABA treatments, respectively.

appropriate environmental pH (close to the optimal pH 5.0 measured in this work). When transformed into *P.pastoris* for secretory expression, the rRDE showed high expression efficiency of 21.5 mg/L and its characteristics were similar to those of nRDE. Both could convert rutin into quercetin efficiently, suggesting that both can be used for the preparation of quercetin in vitro. The molecular weight of rRDE (about 80 kDa) is apparently greater than nRDE (about 60 kDa), which might be caused by glycosylation modifications in the *Pichia* expression system. This prediction was further confirmed by the removal of N-linked glycans using PNGase F (Fig. 2B). However, the rRDE over-expressed in the prokaryotic system formed inclusion bodies and showed no activity although the solubilizing tags such as SUMO (small ubiquitin-like modifier) was applied (Supp. Figure 2) (Esposito and Chatterjee, 2006; Marblestone et al., 2006). This result indicated that the post-translational modification is essential for the solubility and activity of RDE.

The Glu^{171} and Glu^{382} were conserved within the consensus sequences 'NEP' and 'ITENG' (Fig. 5A). Mutation of Glu^{171} or Clu^{382} to Leu caused complete loss of enzyme activity of RDE, demonstrating that the two Glu were indispensable for its activity in rutin hydrolysis. The identity of the active sites within β-glucosidase was already known and the two Glu were identified as catalytic acid-base and nucleophile, respectively. In many cases, the amino acid side chain involved as the nucleophile in this process has been shown to be an aspartic or glutamic acid. For example, the replacement of the active site nucleophile Glu in *Agrobacterium* β-glucosidase by Asn and Gln using site-directed mutagenesis results in essentially complete inactivation of the enzyme (Withers et al., 1992). Glu^{171} and Glu^{382} might serve as acid-base and nucleophile residue of RDE. Since there was no crystal structure available for RDE in PDB, homology modeling was applied to predict its three-dimensional structure (Fig. 5B). It showed that the Glu^{171} and Glu^{382} located in regions corresponding to the long loops connecting barrel strands and helices, and are adjacent to the cavity defined by the center of the barrel (Fig. 5B). Similarly, the 'NEP' and 'ITENG' motifs were conserved in ZMGlu1 and ZMGlu2, two β-glucosidase isozymes from maize, in which the former Glu was identified as an acid/base and the latter was nucleophile, and invariably located at the bottom of the active-site pocket (Czjzek et al., 2000). The hydrolysis of the β-glycosidic bond by a double displacement mechanism involves two steps and requires the participation of an acid/base catalyst and a nucleophile, which might be glutamic acids E172 and E^{382}, compared with other research results (Mizutani et al., 2002; Zechel and Withers, 2001). Overall, mutation of Glu^{171} or Clu^{382} caused complete inactivation of RDE.

Flavonoids and flavonoid metabolic enzyme genes were often involved in plant stress tolerance processes. Therefore, isolation of *FtRDE* promoter and detection of its expression pattern and response to different hormone treatments are important for revealing the biological function of *FtRDE*. Since the transcriptional regulation mechanism of *FtRDE* has not been reported, we cloned the promoter *FtRDE* 5′ flanking DNA sequence using the genome walking method and found some basic cis-acting elements (such as TATA-box, CAAT-box) and plants stress-related elements: ABA response elements (ABRE) and MeJA response element (TGACGA-motif). A series of 5′ -deletion fragments of *FtRDE* promoter activities analysis in mesophyll protoplast under ABA and MeJA treatments showed that the ABA and MeJA response element were functional. ABA and MeJA responses were further confirmed in buckwheat seedling by qRT-PCR (Fig. 9). Tartary buckwheat is an important coarse cereal that is mainly grown in the semi-arid and arid regions of the mountainous areas, where the adverse environmental factors such as cold (Su et al., 2016), drought, heat, salinity (Choudhury et al., 2017). Further, ABA metabolism and signaling transduction was likely to be related to seed germination, desiccation and dormancy (Li et al., 2019), and showed a significant positive correlation to the expression of flavonoid metabolism-related genes (González-Villagra et al., 2018) and flavonol contents (Sandhu et al., 2011). Flavonols have demonstrated the high capacity of dampening the ABA-dependent reactive oxygen species (ROS) (Watkins et al., 2017), which might reduce the harmful effects. Salt stress could elevate the MeJA level, and then MeJA could regulate other phytohormones and antioxidant activity to enhances salt tolerance (Tavallali and Karimi, 2019). MeJA pretreated seed showed the higher expression level of flavonoid synthase genes and increased the accumulation of the flavonols quercetin, which enables the seedlings grown from these seeds to reduce the attack of the soil-borne fungal pathogen (Król et al., 2015). The expression of *FtRDE* showed an obviously difference in organs. The highest expression level was recorded in buckwheat seeds (Fig. 6). *FtRDE* was also highly expressed in leaf, while lower in stem and flower. These results indicated that *FtRDE* might play a major role in seeds and leaves, and be involved in hormone-related response processes under adverse conditions.

Overall, large-scale preparation of FtRDE through heterologous expression in *P. pastoris* provides a new method to prepare quercetin by hydrolyzing rutin. Confirmation of the active sites of RDE established the basis for creating RDE gene knockout transgenic tartary buckwheat, to improve crop quality and taste. Promoter activity and hormone response analysis can further understand *FtRDE* function and expression regulation pattern in plants under adverse conditions.

5. Conclusions

The RDE coding sequence from tartary buckwheat was obtained and RDE was efficiently secretory expressed in *Pichia pastoris* system. Determination of the active sites of RDE provided the hint for creating RDE gene knockout transgenic tartary buckwheat, to improve crop quality and taste. ABA and MeJA hormone response analysis results suggested that the flavonoid metabolic enzyme FtRDE might also be involved in stress tolerance process. This study also offered a new approach for the large-scale preparation of RDE by heterologous expression and production of quercetin by hydrolyzing rutin. The transcriptional regulation analysis could be helpful for further study the *FtRDE* function under stress conditions.

6. Contribution

Peng Chen, Peng Jia and Yuan Wang conceived and designed the experiment. Peng Jia, Yuan Wang, Xiaowei Han, Yan Zhu maintained the experiment, annotated the plant's development and treatments. Yinan Niu collected samples, extracted protoplast and finished qRT-PCR. Yuan Wang and Peng Jia prepared constructs and purified protein. Quanle Xu and Yuhong Li operated HPLC. Peng Chen made an integrated analysis and discussion of the results. Peng Chen was involved in all experimental steps of the study, statistically analyzed the results and wrote the paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2019.08.016.

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