



The transcription factor PjERF1 enhances the biosynthesis of triterpenoid saponins in *Panax japonicus*

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Abstract

The ERF-type transcription factors (TFs) play vital roles in plant secondary metabolism. ERF TFs simultaneously regulate the expression levels of key enzyme genes involved in the biosynthesis of secondary metabolites due to its “multi-point control” function. In this study, one gene of ERF TFs from *Panax japonicus* (*PjERF1*) was cloned. The open reading frame of *PjERF1* was 801 bp and encoded 266 amino acids. Phylogenetic analysis showed that PjERF1 belonged to ERF subfamily with a typical conserved domain. Subcellular localization found that PjERF1 protein might be located in eukaryotic cell nucleus. Yeast one-hybrid assay demonstrated that PjERF1 could bind to the promoters of *PjβAS*, *PjCAS*, and *PjSE* specifically and regulate the expression levels of such key enzyme genes involved in the triterpene saponins biosynthesis. Therefore, in the *PjERF1* overexpression cell lines, the expression levels of some key enzyme genes involved in the triterpenoid saponins biosynthesis were significantly increased compared with those in non-transgenic cell line. As a result of it, the biosynthesis of chikusetsusaponin IV and IVa, and other ginsenosides (Rd, Rb1, Re, and R0) were also promoted in the *PjERF1* overexpression cell lines. This study indicated that PjERF1 could regulate the biosynthesis of saponins in *P. japonicus* through controlling the expression levels of key enzyme genes related to the biosynthesis of triterpenoid saponins.

Keywords Transcription factor · *Panax* · Saponins · Triterpenoid · Promoter

Abbreviations

HPLC	High-performance liquid chromatography
MVA	Mevalonic acid
2, 4-D	2,4-Dichlorophenoxyacetic acid
Hyg	Hygromycin B
KT	Kinetin
CTAB	Cetyltrimethylammonium bromide

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Introduction

Panax japonicus is a kind of traditional Chinese herb used for hundreds of years. *P. japonicus* saponins (PJS) are the major bioactive components in *P. japonicus*. Up to now, more than 30 kinds of saponins have been isolated from the roots and leaves of *P. japonicus*, which were classified into dammarane-type saponins (ginsenosides Re, Rd, and Rb1) and oleanane-type saponins (ginsenoside R0, chikusetsusaponin IV, and chikusetsusaponin IVa) (Sz et al. 2020). Contrast to *P. japonicus*, the popular medicinal herbs, *P. ginseng*, *P. quinquefolium*, and *P. notoginseng* mainly contain dammarane-type saponins (Li et al. 2013; Yang et al. 2018). Such differences of saponins composition lead to the special pharmacological activities of *P. japonicus* (Leung and Wong 2010; Wei et al. 2014; Yang et al. 2014; Zhang et al. 2014).

PJS are triterpenoid saponins and synthesized via mevalonic acid (MVA) pathway (Tang et al. 2019). Mevalonic acid is catalyzed by geranyl pyrophosphate synthase (GPS), farnesyl pyrophosphate synthase (FPS), squalene synthase (SS), and squalene epoxidase (SE) to produce 2,3-oxidosqualene (Zhang et al. 2015; Zhao and Li 2018). 2,3-oxidosqualene is an important precursor of triterpenoid saponins

biosynthesis in *Panax*, which forms two kinds of saponin skeletons under the catalysis of dammarediol-II synthase (DS) and β -amyrin synthase (β -AS) (Ghosh 2017), respectively. Cycloartenol synthase (CAS) is a key enzyme gene in the biosynthesis of phytosterols which share the same precursor, 2,3-oxidosqualene, with β -AS and DS (Jin et al. 2017). β -amyrin and dammarediol could be modified by P450s and UGTs to form oleanane-type and dammarane-type saponins, respectively. Transcriptome sequencing showed that 18 UGTs might involve in the PJS biosynthesis but it is difficult to identify the exact function of such UGTs until yet (Zhang et al. 2015).

Transcription factors (TFs) widely involved in the process of plant growth and secondary metabolism, which would specifically bind to the promoters of genes to achieve the activation or repression of transcription. The artificial operation of TFs has become an effective strategy to regulate the biosynthesis of plant secondary metabolites through multi-point controlling the expression levels of key enzyme genes involved in the biosynthetic pathway. The transient expression of *AabHLH* in *Artemisia annua* increased the expression levels of *ADS*, *CYP71AV1*, and *HMGR*, which illustrated that the transcription factor *AabHLH* could positively control the biosynthesis of artemisinin (Ji et al. 2014). In *P. quinquefolius*, the transcription factor *PqWRKY* could regulate the transcription of related genes in triterpenoid saponins biosynthesis to improve the content of total saponins (Sun et al. 2013); The TSAR bHLH transcription factors have been reported to be involved in the regulation of saponin biosynthesis in *Medicago truncatula* (Mertens et al. 2016). Such studies proved that transcription factors could take part in the biosynthesis of secondary metabolism.

Among so many kinds of TFs, the ethylene-responsive factors (ERF) play important roles in both regulating the secondary metabolism and plant stress responses (Yang et al. 2012; Sun et al. 2014). ERF family includes a conserved domain consists of 60–70 amino acids, which is classified into five subfamilies according to the structure similarity and the number of conserved domains (Yao et al. 2020). It is known that ERF could specially bind to the GCC-box (AGC CGCC) and regulate the expression of genes (Sakuma et al. 2012). RNA interference of *NtERF32* in tobacco decreased the expression of related key enzyme genes *NtQPT2* and *NtPMT1a* to reduce the contents of nicotine and alkaloid (Sears et al. 2014). Jasmonate responsive ERF gene clusters control the biosynthesis of many important metabolites, such as nicotine, steroidal glycoalkaloids, artemisinin, vinblastine, and vincristine (Dai et al. 2009; Shoji and Yuan 2021). Furthermore, some studies have indicated that ERFs could regulate the biosynthesis of triterpenoids in medicinal plants, such as *Gynostemma pentaphyllum* and *Artemisia annua* (Xu et al. 2020; Ji et al. 2014). Base on such studies, we supposed that ERF TFs might also have the potential to

affect biosynthesis of *P. japonicus* saponins (PJS) because PJS belonged to triterpenoids.

In this study, one gene of ERF TFs from *Panax japonicus* (*PjERF1*) was cloned and the interaction between *PjERF1* and promoters of key enzyme genes involved in PJS biosynthesis was explored. Furthermore, *PjERF1* overexpressed cell lines were constructed to ensure the function of *PjERF1* in PJS biosynthesis.

Materials and methods

Plant materials and growth conditions

The callus was induced from the roots of *P. japonicus* and subcultured on Murashige and Skoog (MS) medium with 2.0 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D) and 1.0 mg/L kinetin (KT) every 45 days. The standard compounds were derived from the National Institute for the Control of Pharmaceutical and Biological Products (Beijing, China). All other chemicals were purchased from Sangon Biotech Co. Ltd. (Shanghai, China).

Cloning and sequence analysis of *PjERF1*

According to the study of (Deng et al., 2017) the total RNA of *P. japonicus* was extracted through guanidine isothiocyanate method and the first-strand cDNA was prepared for RT-PCR. In this study, the degenerate primers were designed according to the conserved domain of the *Catharanthus roseus* ORCA family, and the core sequence of the *PjERF1* transcription factor gene was amplified by homologous cloning. Then using the rapid amplification of cDNA ends (RACE) and taking the RACE-Ready cDNA as a template, the 5' and 3' end sequences of *PjERF1* were cloned. Next, the cloned sequences above were used as the basis for designing the primers to amplify the full-length cDNA of *PjERF1*. The PCR products of *PjERF1* full-length cDNA were selected by 1% agarose gel electrophoresis and cloned into the pGEM-T Easy vector (Promega, USA) for sequencing. Homology analysis of amino acid sequence was performed by DNAMAN. Phylogenetic tree of *PjERF1* was built-in MEGA 6.0 with neighbor-joining methods. The homology modeling of *PjERF1* was performed with the tool of SWISS-MODE (<http://swissmodel.expasy.org/>).

Yeast one-hybrid assay

To confirm whether *PjERF1* could directly bind to the promoters of key enzyme genes involved in PJS biosynthesis, *PjASP*, *PjCASP*, *PjSEP*, and *PjSSP* were selected and investigated such interaction by yeast one-hybrid assays. According to the requirements of Universal Genome Walker 2.0 kit,

promoters of the key enzyme genes were cloned. For the first time, the universal primer AP1 provided by the Universal Genome Walker 2.0 kit and the specific primer GSP1 was designed according to the instructions, which were used for PCR amplification of the key enzyme promoters. Since the amplified promoter fragments were based on Genome Walker Libraries, the sensitivity of the first PCR reaction is not enough to obtain specific promoter fragments. The primers used in the second PCR reaction were AP2 and GSP2, and the product of the first PCR was used as the template for the second PCR. The second PCR product all showed specific bands (Figure S1). The recovered specific bands were subjected to TA cloning, transformed into *Escherichia coli*; and the positive single clones were screened and sent for sequencing. Finally, the sequences of the promoters of *PjAS*, *PjCAS*, *PjSE*, and *PjSS* were obtained and the lengths of which were 2.52, 1.40, 1.54, and 0.88 kb, respectively.

Matchmaker™ Gold Yeast One-Hybrid Library Screening System (Clontech) was used for identifying promoters that bind to *PjERF1*. DNA was extracted and purified to construct the cDNA library and clone the promoters of key enzyme genes by Universal Genome Walker 2.0 (TaKaRa). Primers used in this assay are listed in Table S1. After sequencing, the promoters of related genes (*PjASP*, *PjCASP*, *PjSEP*, and *PjSSP*) were inserted into pAbAi vector and the full-length CDS of *PjERF1* was inserted into the pGADT7 vector to get pGADT7-*PjERF1* (Figure S2). The recombinant plasmids containing *PjERF1* and promoter fragments were co-transformed into Y1HGOLD yeast strain to examine the interaction on medium lacking Leu with the optimal AbA screening concentration. The plasmid p53-AbAi could specifically bind to GAL4 to be the positive control. The empty vector pAbAi was used as the negative control.

Construction of *PjERF1* overexpression vector

PjERF1 was amplified with specific primers (Table S2). The procedure of PCR was as follows: 95 °C for 5 min, 32 cycles of 94 °C for 30 s, 56 °C for 30 s and 72 °C for 50 s, a final cycle 10 min extension at 72 °C. After sequencing, PCR products digested by *Sma I*/*Xba I* were inserted into pCAMBIA2300S. The pCAMBIA2300S-*PjERF1* overexpression vector was constructed. The recombinant vector pCAMBIA2300S-*PjERF1* was transformed into *Agrobacterium tumefaciens* EHA105 by the freeze–thaw method (Jin et al. 2017).

Establishment of *PjERF1* transgenic cell lines

Agrobacterium tumefaciens EHA105 contained *PjERF1* was cultured on MGL liquid medium until the OD₆₀₀ reached 0.6 to 0.8. The wild-type (WT) cells of *P. japonicus* pre-cultured on MS medium with 40.0 mg/L

acetosyringone for three days were suspended in *A. tumefaciens* liquid to obtain *PjERF1* transgenic cell lines. After washing away the *A. tumefaciens* by sterile water containing 400.0 mg/L cefotaxime, the *PjERF1* transgenic cells were incubated on the MS agar medium with 400.0 mg/L cefotaxime for 15 days. MS agar medium containing 50.0 mg/L kanamycin sulfate was used for selecting *PjERF1* transgenic cell lines.

Genomic DNA of *PjERF1* transgenic cells were extracted by CTAB method (Vzae et al. 2010). The kanamycin resistance gene (*npt II*) in T-DNA was detected to ensure the positive cell lines by PCR, and it had no effects on the expressions of other key enzyme genes or biosynthesis of secondary metabolites (Han et al. 2014; Jo et al. 2017). The PCR performed was as follows: 94 °C for 5 min; then 32 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s; and a final 2-min extension at 72 °C. The PCR products were checked by 1% agarose electrophoresis.

Subcellular localization analysis

The pMD18-T-*PjERF1* was digested by *Nde I*/*Sma I* and inserted into pBIN m-gfp5-ER vector. The specific primers are listed in Table S3. The recombinant vector pBIN m-gfp5-ER-*PjERF1* was expressed transiently in onion epidermal cells by *A. tumefaciens* infiltration (EHA105) using the same method as described above (Li et al. 2021). After being cultured in dark, different stages of onion epidermal cells were prepared for observing under confocal microscopy. The fluorescence was detected at 395 nm.

Expression analysis by qRT-PCR in *PjERF1* transgenic cell lines

Total RNA was extracted and reverse-transcribed into cDNA in *P. japonicus* cells by the method described above. The *18S rRNA* was selected as the reference gene. The qRT-PCR of GoTaq®2-Step RT-qPCR System (Promega, USA) was as follows: 95 °C, 2 min; next 40 cycles of 95 °C for 15 s, 57 °C for 30 s, and 72 °C for 30 s. Each sample was prepared in triplicate. The relative expression levels of key enzyme genes were analyzed by 2^{-ΔΔC_T} method and all the primers are listed in Table S3 (Livak and Schmittgen 2001).

Determination of *P. japonicus* saponins content

All the sample fresh cells were dried to the constant weight at 55 °C and grinded into powder. Samples soaked in methanol overnight were ultrasonically extracted (60 W) for 2 h. Liquid supernatant was collected after centrifugation to get the solution of PJS. According to the coloring reagents by vanillin and perchloric acid at 60 °C, the concentration of

total saponins was detected. The absorbance of the reaction products was determined at A_{550} , and the content of total triterpene saponins was calculated based on standard curve.

HPLC analysis of monomer saponins

Monomer saponins of ginsenoside Rd, Rb1, Re, R0, and chikusetsusaponin IV, IVa were detected by Agilent 1260 (Santa Clara, CA, USA) with a Waters XTerra MS C18 column (5 μ m, 250 mm \times 4.6 mm). The mobile phase was water-phosphoric acid (A)/acetonitrile (B). The flow-rate was 1.0 mL min⁻¹ and the column temperature was maintained at 30 °C. The gradient elution was as below: 0–20 min, 20% acetonitrile; 20–30 min, 20–35% acetonitrile; 30–40 min, 35% acetonitrile; 40–50 min, 35–40% acetonitrile; 50–60 min, 40% acetonitrile; 60–65 min, 40–100% acetonitrile; 65–75 min, 100% acetonitrile. The wavelength of detection was set at 203 nm. All standards were purchased from National Institutes for Food and Drug Control (China).

Statistical analysis

All analyses were performed with three replications. According to Student's *t* test, the error bars indicate the standard deviations from the means of triplicates. The asterisk indicates a significant difference in measured parameters (* $P < 0.05$; ** $P < 0.01$).

Result

Cloning and bioinformatics analysis of *PjERF1* from *P. japonicus*

The transcription factor gene *PjERF1* was obtained according to the ERF cDNA sequence in *P. japonicus* (GenBank Accession Number KP890784). The open reading frame (ORF) of *PjERF1* was 801 bp encoding 266 amino acids with the molecular weight of 30.068 kDa and a PI of 5.40. NCBI-Protein BLAST predicted that *PjERF1* covered a conserved domain which located in 74–134 amino acids. Based on the deduced amino acid sequence, multiple sequence alignment showed that *PjERF1* had the same conserve domains that covered YRG and RAYD elements with the ERF TFs in other plants. Furthermore, the 37th amino acid (alanine) could bind to cis-acting element GCC-box which proved that *PjERF1* might belong to ERF transcription factors (Figure S3).

Nine ERF amino acid sequences were selected from NCBI to construct a phylogenetic tree. *PjERF1* showed high homology with other ERF transcription factors (Fig. 1). Phylogenetic analysis proved that *PjERF1* belongs to ERF transcription factor family. To some extent, the function of

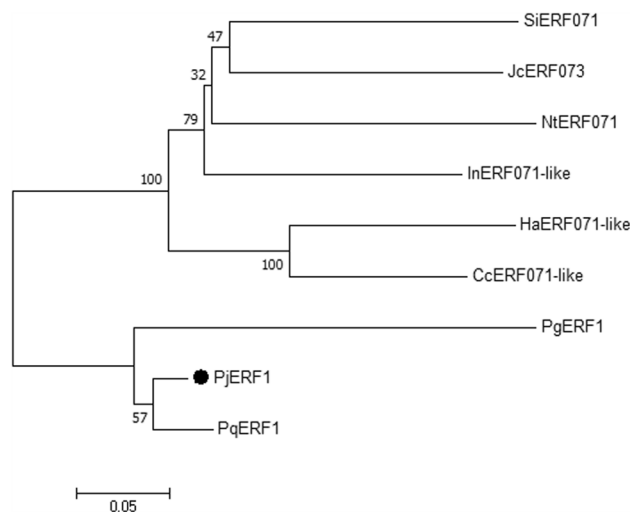


Fig. 1 Phylogenetic tree analysis of *PjERF1*. The sequences used were *PqERF1* (*Panax quinquefolium ERF1*), *PgERF1* (*Panax ginseng ERF1*), *SiERF071* (*Sesamum indicum*, XP_011079624.1), *JcERF073* (*Jatropha curcas*, XP_012082821.1), *NtERF071* (*Nicotiana tabacum*, XP_016475776.1), *CcERF071-like* (*Cynara cardunculus* var. *scolymus*, XP_024966446.1), *HaERF* (*Helianthus annuus*, XP_022035166.1), and *InERF* (*Ipomoea nil*, XP_019163870.1). The neighbor-joining method phylogenetic tree was constructed using the bootstrap method of MEGA 6.0 with 1000 replications and the respective plant species of the above proteins were shown in the tree. The black circular symbols indicate the *PjERF1* protein

the protein was determined by the tertiary structure. The 3D structure of *PjERF1* was predicted with the SWISS-MODE algorithm to comprehend the structure–function relationship of plant AP2/ERF TFs. Using the crystal structure of *AtERF096* (5wx9.1.A) as a template, the 3D model of *PjERF1* was built and shared 72.88% identity with the *AtERF096* (Fig. 2).

PjERF1 is located to the cell nucleus

The subcellular localization of *PjERF1* protein was determined by confocal microscope combined with GFP and propidium iodide (PI) markers. PI is a kind of staining reagent for nucleus. The *PjERF1-GFP* fusion gene was transiently expressed in onion epidermal cells transiently transformed by *A. tumefaciens*. The panels on the left of Fig. 3 show that the recombinant fusion green fluorescent protein was mainly concentrated in the nuclear region and thus confirmed that the *PjERF1* transcription factor was located to the nucleus. The panels on the right of Fig. 3 indicate that the onion inner epidermal cells was transfected with *GFP* vector without *PjERF1* gene; and the green fluorescence could not only be observed in the nucleus but also in the cytoplasm.

ERF TFs are considered as important regulators to control plant secondary metabolism. We speculated that *PjERF1* might be related to triterpenoid synthesis. Based on the

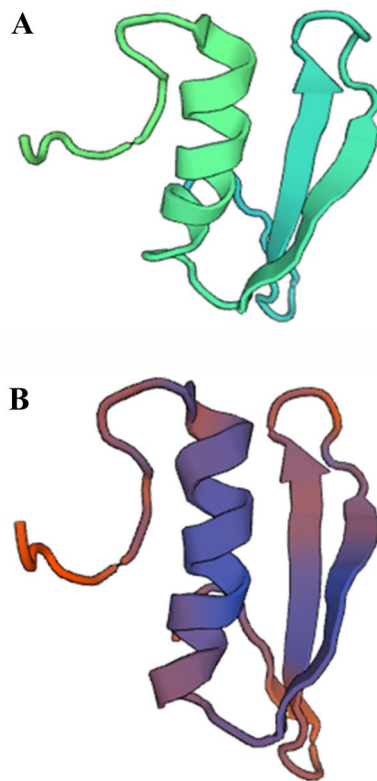


Fig. 2 Three dimensional structure analysis of PjERF1 protein from *Panax japonicus* (A: *Arabidopsis thaliana* ERF096; B: PjERF1)

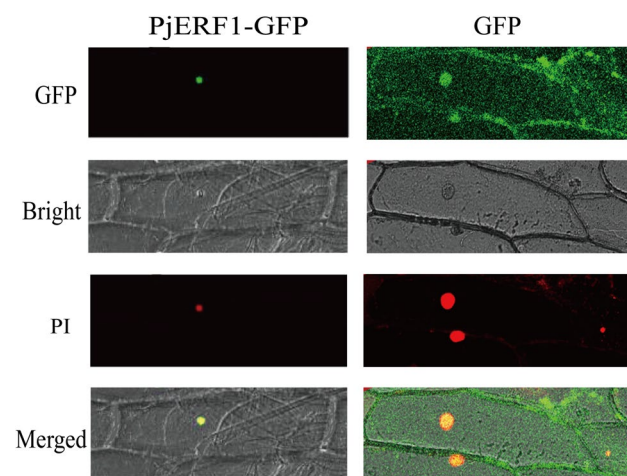


Fig. 3 Subcellular localization of the PjERF1-GFP fusion protein. The PjERF1-GFP fusion protein was detected in the nucleus after the genetic transformation mediated by *A. tumefaciens*. GFP, fluorescent light; Bright, white light; PI, propidium iodide (for staining nuclear DNA); Merged, overlay of the GFP and PI images

“multi-point control” character of TFs, the interactions between PjERF1 and the promoters of key enzyme genes related to PJS biosynthesis were discussed next.

PjERF1 is a direct regulator of *PjAS*, *PjCAS*, and *PjSE* promoters

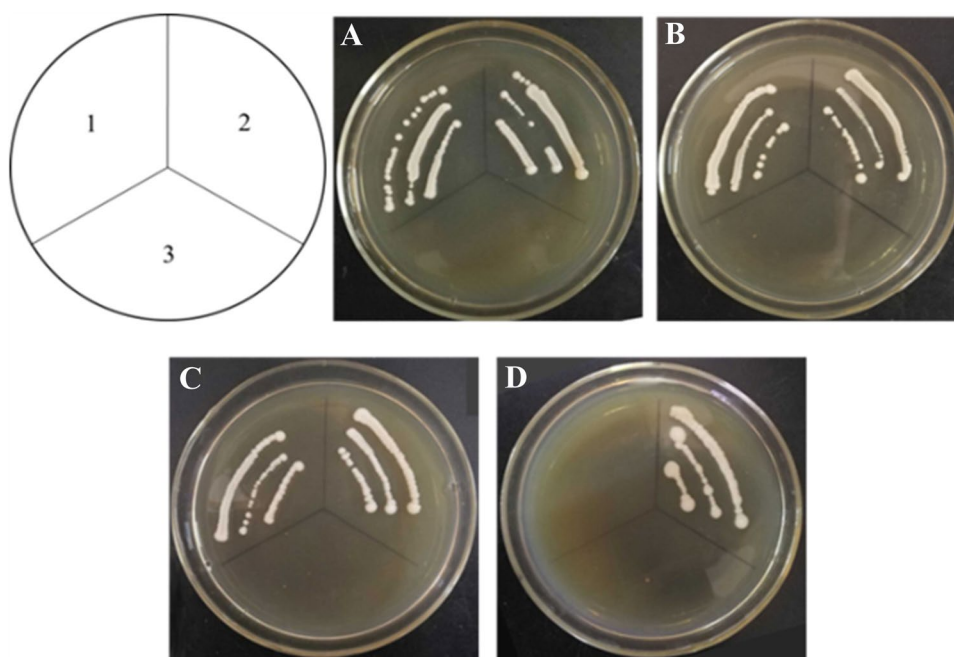
In the process of yeast one-hybrid assays, when PjERF1 was binding to the suitable site of the recombinant bait plasmid, the expression of downstream AUR1-C reporter gene was activated and Y1HGOLD could grow on the medium containing AbA. As shown in Fig. 4, Y1HGOLD could grow up normally on SD/-Leu/AbA solid medium and produced fusion protein GAL4-PjERF1. Through the analysis of the promoter sequence, it was found that the promoters of PjAS, PjCAS and PjSE contained GC-box (GCCGCC) which was considered as a classical binding box of ERF TFs. Moreover, the results of yeast one-hybrid assay also indicated that PjERF1 could bind to the promoters of PjAS, PjCAS, and PjSE (Fig. 4A–C). No yeast strain existed in SD/-Leu/AbA medium of Fig. 4D showed that PjERF1 did not interact with the promoter of PjSS. It was known that the ERF transcription factors could specific bind to GC-box (GCCGCC) and regulated the expression of downstream genes (Sakuma et al. 2002). In our study, sequence analysis confirmed that the GC-box was included in the promoters of PjAS, PjCAS, and PjSE which was consistent with result of yeast one-hybrid assay. Furthermore, PjERF1 might multi-point control the expression levels of some key enzyme genes involved in biosynthesis of PJS and then influenced the biosynthesis of PJS indirectly and effectively.

PjERF1 increased the expression levels of key genes involved in saponin biosynthesis

The *PjERF1* transgenic cell lines were produced by *Agrobacterium tumefaciens*-mediated genetic transformation. *PjERF1* was overexpressed in *P. japonicus* cells to explore whether PjERF1 could regulate the expression levels of key enzyme genes in PJS biosynthesis pathway. The *PjERF1* overexpressed cell lines were screened on the selective medium with kanamycin, and the *npt II* resistance gene was amplified to screen positive *PjERF1* transgenic cell lines. The amplified *npt II* products were in the positive cells and there was no signal in the WT (Figure S4). Four positive *PjERF1* transgenic cell lines (T1, T2, T3, and T4) were used for further studies.

In *PjERF1* transgenic cell lines, the expression level of *PjERF1* was increased significantly compared with WT cell line. We measured the saponins of *P. japonicus* cells in WT and transferred with empty pCAMBIA2300 load, and found that there was no difference between them (Figure S5). Especially in the T2 cell line, the expression level of *PjERF1* was approximately 9.5 times higher than that in control (Fig. 5A). In the T4 cell line, the expression level of *PjAS* was promoted even tenfold of that in control, which indicated that the content of oleanane-type saponins might

Fig. 4 Transcriptional activation assays of PjERF1. (1) The transcription activity analysis of pGADT7-PjERF1 interacting with PjASP (A), PjCASP (B), PjSEP (C), and PjSSP (D), respectively. (2) p53-AbAi (2) and (3) pAbAi were used as positive and negative controls, respectively



be increased remarkably (Fig. 5H). The expression level of key enzyme gene *PjDS* related to dammarane-type saponins biosynthetic pathway was also enhanced (Fig. 5G); therefore, the biosynthesis of dammarane-type saponins should be influenced. Besides, the expression levels of other key enzyme genes such as *PjSE*, *PjCAS* were also increased to some extent (Yin et al. 2017). To sum up, *PjERF1* overexpression in *P. japonicus* cells could regulate the expression levels of key enzyme genes involved in the biosynthesis of PJS.

Overexpression of *PjERF1* enhanced the biosynthesis of PJS

Compared with non-transgenic cell line, the contents of total saponins in the four *PjERF1* transgenic cell lines were significantly increased. In T2 transgenic cell line, the content of PJS was 1.53 times of that in control (Fig. 6). The result indicated that PjERF1 transcription factor played an important role in the regulation of PJS biosynthesis.

The major monomer saponins (ginsenoside Rd, Re, Rb1, R_g, chikusetsusaponin IV, and IVa) were detected by HPLC (Figure S6). In *PjERF1* transgenic cell lines, the contents of six monomer saponins were all enhanced (Fig. 7). The content of chikusetsusaponin IV and IVa were the highest in *P. japonicus* among six monomer saponins. In T2 transgenic cell lines, the content of chikusetsusaponin IV and IVa was 1.47 times and 1.32 times of those in control, respectively. In T4 transgenic cell line, ginsenoside Rd had the highest increase in content which was about 1.69 times of that in control; and in T2 cell line the content of ginsenoside Re

was 1.55 times of that in WT cell line. Overexpression of *PjERF1* could promote the biosynthesis of oleanane-type saponins and dammarane-type saponins in *P. japonicus*.

Discussion

Transcription factors have the advantage of “multi-point control” in the regulation of secondary metabolism. Up to now, ERF transcription factors were isolated from some kinds of plants; and the functions of them were explored comprehensively. Suppressing of *OpERF2* resulted in the reduced expression levels of genes which related to the biosynthesis of secologanin and strictosidine in *Ophiorrhiza pumila* (Udomsom et al. 2016). When the *GmERF3* from *Glycine max* was overexpressed in tobacco, the resistance to the pathogens such as *Ralstonia solanacearum*, *Alternaria alternata* and tobacco mosaic virus was also enhanced (Zhang et al. 2009). In *Catharanthus roseus*, ORCA3 (ERF TF) overexpression could regulate the biosynthesis of terpenoid indole alkaloid (Zhou et al. 2010). According to these studies, the regulation of secondary metabolism by ERF TFs might be a common phenomenon in plants. In this study, a new *PjERF1* gene from *P. japonicus* was cloned. The multiple alignment and phylogenetic analysis demonstrated that PjERF1 belongs to ERF subfamily and the 37th amino acid residue (alanine) in PjERF1 conserved domain was able to bind to GCC-box cis-acting element.

ERF TFs control the secondary metabolism mainly through regulating the expression levels of key enzyme genes in the biosynthetic pathway. When *AaERF1* and

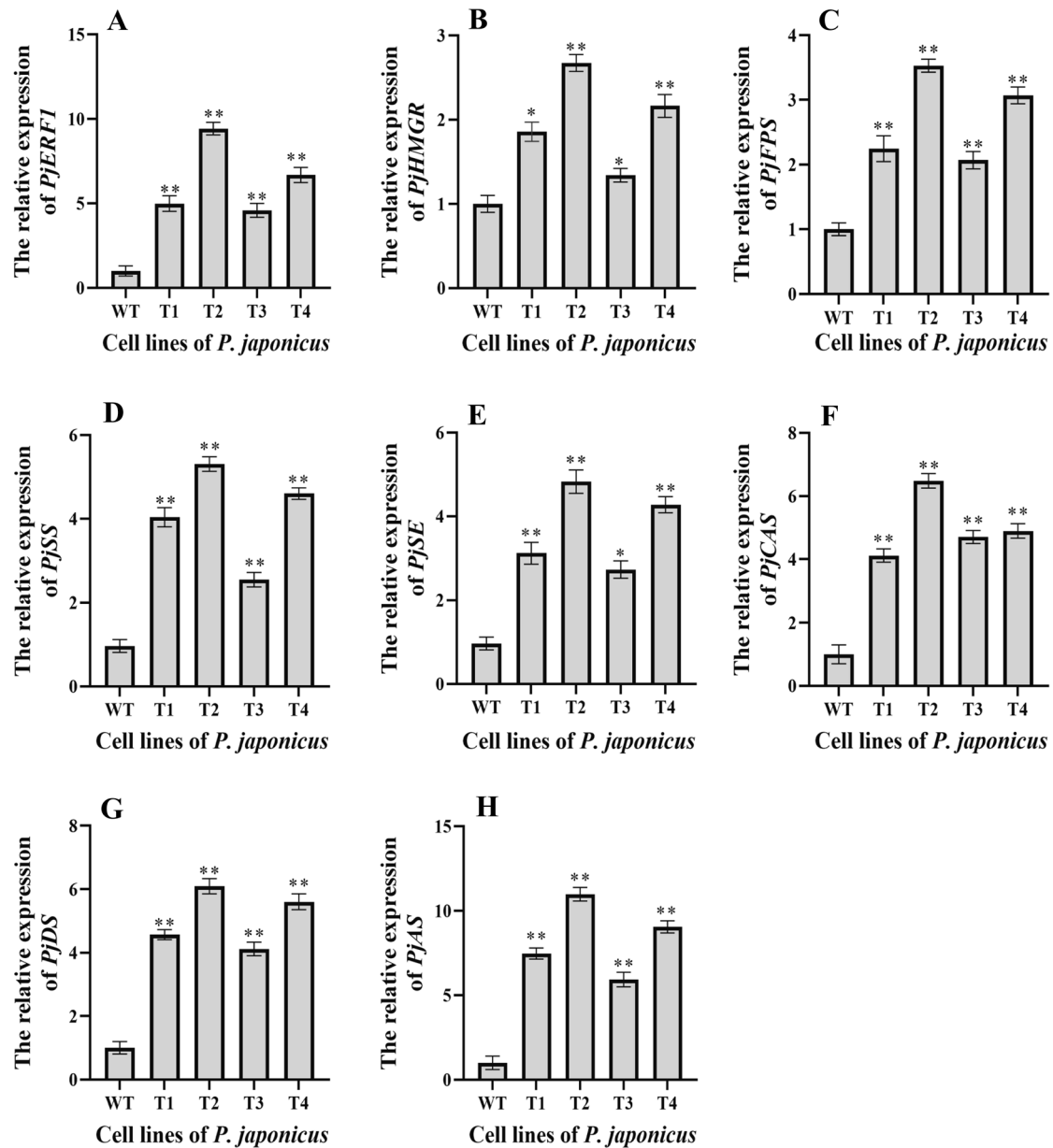


Fig. 5 Relative expression levels in four *PjERF1* transgenic cell lines. A–H represented the expression levels of *PjERF1*, *PjHMGR*, *PjFPS*, *PjSS*, *PjSE*, *PjDS*, *PjCAS*, and *PjAS*, respectively. Standard deviation (SD) of means from three independent experiments was indicated

by error bars. 18S rRNA was selected as the internal reference gene. $2^{-\Delta\Delta C_t}$ method was used to calculate the relative expression levels. Significant difference between the wild-type (WT) cell line and the transgenic cell line was indicated by asterisks (* $P < 0.05$; ** $P < 0.01$)

AaERF2 were overexpressed in *Artemisia annua* L, the expression levels of key enzyme genes *ADS* and *CYP71AV1* in artemisinin biosynthetic pathway were increased; and the biosynthesis of artemisinin was promoted (Yu et al. 2012). Transcription factor CitERF71 could enhance the content of *E*-geraniol in sweet orange fruit by activating the terpene synthase gene *CitTPS16* (Li et al. 2017). In this study, we demonstrated that *PjERF1* could bind to the promoters of *PjSE*, *PjAS*, and *PjCAS*, and regulate the expression levels of such genes. Moreover, although the expression levels of

some key enzyme genes, such as *PjFPS*, *PjDS*, and *PjSS*, were increased, there was no interaction between *PjERF1* and the promoters of these genes. The possible mechanism of such result might be that when the main metabolic flux was increased, the expression levels of relative key enzyme genes involved in biosynthesis pathway of saponins should be increased passively to process more metabolites.

In the present study, the content of PJS was increased due to the promoted expression levels of key enzyme genes involved in PJS biosynthetic pathway, but the content of

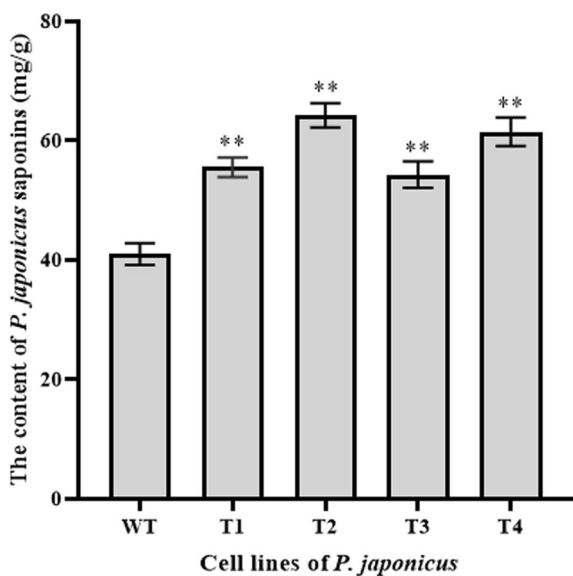


Fig. 6 The content of PJS in the cell lines. Standard deviation (SD) of means from three independent experiments was indicated by error bars. Significant difference between the WT cell and transgenic cells was indicated by asterisks (** $P < 0.01$)

diverse monomer saponins had different degrees of increase. We speculated that transcription factor PjERF1 may also regulate the expression levels of some genes of UDP-glycosyltransferases (UGTs) or P450s which related to the modification of triterpenoid skeleton. Up to now, several genes used for modifying the skeleton of saponins have been identified. Overexpressing of *UGTPg71A29* could lead to the increased contents of Rg1 and Rb1 in *P. ginseng* (Lu et al. 2018). Similarly, overexpressing *CYP716A52v2* could enhance the content of ginsenoside R0 in *P. ginseng* (Han et al. 2013). According to these studies, we considered that TFs might regulate the expression of UGTs and lead to the different increase of monomer saponins contents. As the *UGTs* and *P450s* are supergene families, most of them are difficult to be screened out and identified the exact functions. Such investigation will be carried out next.

Yeast one-hybrid technology could analyze the interaction between protein and DNA by detecting reporter genes (Wu et al. 2018). In this study, we performed yeast one-hybrid to explore whether PjERF1 could bind to the promoters of key enzyme genes related to the biosynthesis of

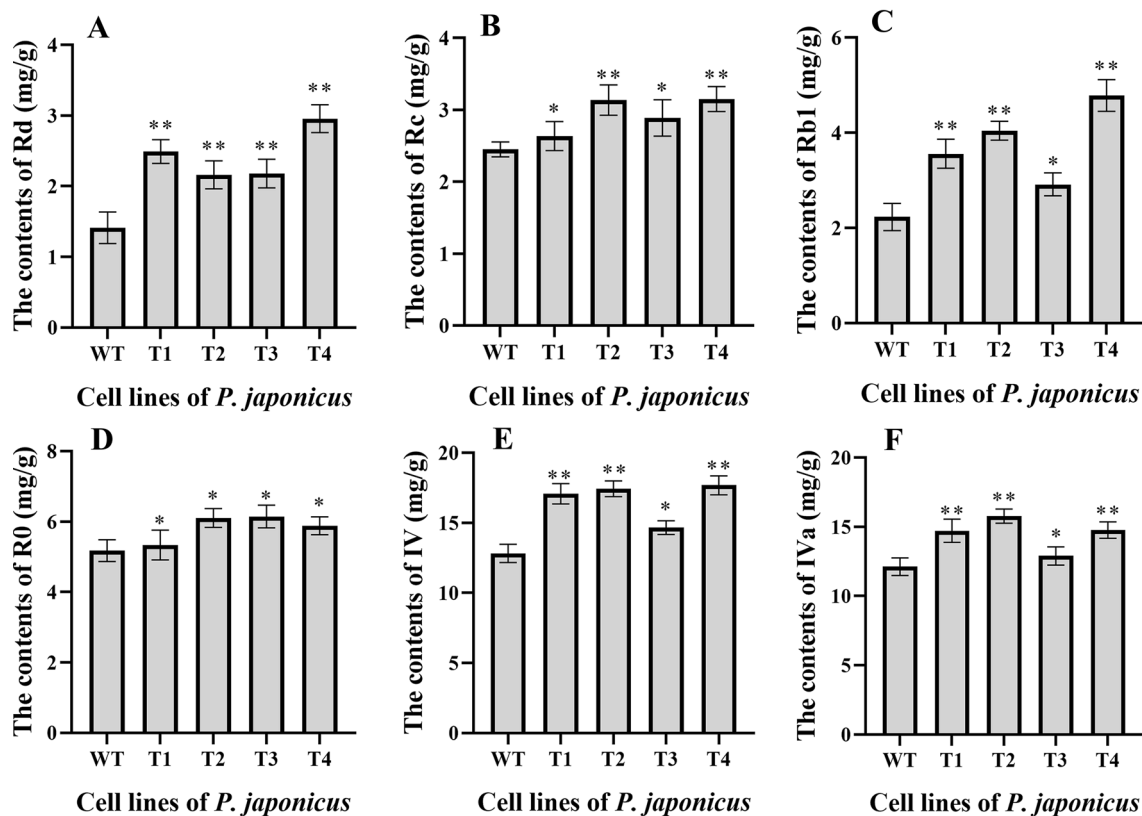


Fig. 7 The contents of major monomer saponins in *P. japonicus* cell lines. Concentrations of six monomer saponins in *PjERF1* transgenic cell lines and WT cell line. a-f stands for the Rd, Rc, Rb1, R0, IV,

and IVa six monomer saponins. Significant difference between WT and transgenic cells was indicated by asterisks (* $P < 0.05$; ** $P < 0.01$)

PjS. As we have expected, PjERF1 bound to the promoters of *PjCAS*, *PjAS* and *PjSE* exactly. PjSE catalyzes squalene to form 2,3-oxidosqualene which is the precursor of phytosterols, dammarane-type, and oleanane-type saponins. Therefore, the biosynthesis of saponins would be affected in *P. japonicus* by regulating the expression of *PjSE*. Furthermore, *PjAS* is the first key enzyme gene in the branch of oleanane-type saponins biosynthesis pathway (Fig. 8). Overexpression of *PjERF1* could enhance the biosynthesis of oleanane-type saponins by up-regulating the expression of *PjAS* and the contents of chikusetsusaponin IV and chikusetsusaponin IVa were increased significantly in transgenic cell lines. In the meantime, the biosynthesis of dammarane saponins was also promoted because the up-regulation of *PjSE* led to the enhancement of primary metabolic flux. In addition, because the PjERF1 could bind to the promoter of *PjCAS* and increase the expression level of *PjCAS*, so we considered that the synthesis of phytosterols would be enhanced. Further result showed that in the four transgenic cell lines, the content of phytosterols was higher than that control, which suggested that PjERF1 could promote the biosynthesis of phytosterols through the interaction with *PjCAS* (Fig. 9).

Furthermore, with the unique “multi-point control” advantage, the operation of TFs is more efficient than directly regulate the key enzyme genes involved in saponins pathway. So far, few transcription factors involved in the regulation of triterpenoid saponin synthesis have been identified and functionally characterized in *P. japonicus*. This study is an interesting exploration. As the promoter sequence information of the key enzyme genes involved in saponin synthesis was insufficient, which limited the functional investigation of transcription factors. In the further work, more details about transcription factors on the regulation of triterpenoid saponins biosynthesis will be delineated.

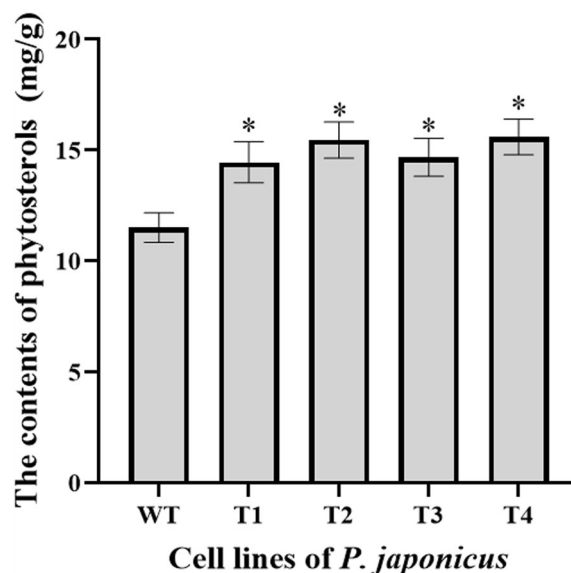
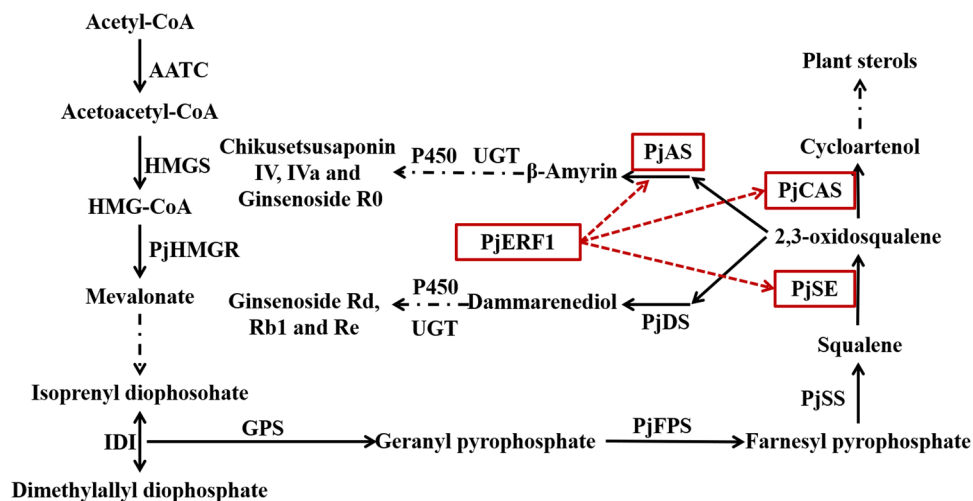


Fig. 9 The content of phytosterols in the cell lines. Standard deviation (SD) of means from three independent experiments was indicated by error bars. Significant difference between the WT cell and transgenic cells was indicated by asterisks (* $P < 0.05$)

Conclusion

In this study, a transcription factor *PjERF1* was cloned and analyzed from *P. japonicus*. PjERF1 could regulate the biosynthesis of saponins in *P. japonica* through controlling the expression levels of key enzyme genes related to the biosynthesis of triterpenoid saponins. The results might provide a theoretical reference for establishing efficient and stable regulation technology in the biosynthesis of triterpenoid saponins.

Fig. 8 The biosynthetic pathway of PjS. The dotted line indicates that PjERF1 transcription factor can act on the promoters of *PjAS*, *PjCAS*, and *PjSE*. PjHMGR: 3-hydroxy-3-methylglutaryl CoA reductase, *PjFPS* farnesyl pyrophosphate synthase, *PjAS* amyirin synthase, *PjSS* squalene synthase, *PjSE* squalene epoxidase, *PjDS* dammarenediol-II synthase, *PjCAS* cycloartenol synthase



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Author contributions FG: conceived and designed the experiments. QC and YY: performed the experiments. JZ and DL: provided technical guidance and support. QC, YY, and XZ: contributed to the data analysis. XC: Provided the experimental materials. QC, YY, and RZ: wrote this paper. QC and YY: contributed to the work equally and were regarded as co-first authors. All authors approved the final manuscript.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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