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ORIGINAL ARTICLE

Functional characterization of *LkERF-B2* for improved salt tolerance ability in *Arabidopsis thaliana*

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Abstract

The ethylene response factors have been reported to play critical roles in developmental and environmental responses in plants. In the present study, an ERF transcription factor gene was aimed to be identified from *Larix kaempferi*. Molecular characteristics and function of this gene were further explored. The result showed that a 1344 bp ERF transcription factor gene containing initiation and termination codon was obtained by RT-PCR and named *LkERF-B2*. *LkERF-B2* gene encoded 447 amino acids containing a typical AP2/ERF domain. Alignment of predicted amino acid sequence of *LkERF-B2* in various plant species showed that this ERF transcription factor was highly homologous (79.0%) with that of *Picea sitchensi*. To elucidate the function of *LkERF-B2*, *LkERF-B2* overexpression vector was successfully constructed and transformed to *Arabidopsis thaliana* via dip flower. Compared with control plant, *LkERF-B2* overexpressed transgenic *A. thaliana* showed a significantly higher survival rate under cold, heat, NaCl and drought stresses. NaCl stress analysis revealed that control and transgenic *Arabidopsis* were both flowering earlier under 100 and 150 mM/L NaCl treatment. While under 200–300 mM/L NaCl treatment, the growth of control plant was significantly inhibited compared with transgenic *A. thaliana*. Salt injury rate and salt injury index of transgenic *Arabidopsis* were lower than those of the control. Further investigation showed that transgenic *Arabidopsis* exhibited much higher content of chloroplast pigments under different NaCl concentration. Meanwhile, the activity of SOD and POD was also enhanced in transgenic *A. thaliana*. These results suggested that *LkERF-B2* was a key transcription factor and could lead to enhanced salt stress tolerance.

Keywords *Larix kaempferi* · *LkERF-B2* · NaCl tolerance · Functional characterization

Abbreviations

GUS	*β*-Glucuronidase enzyme
MS	Murashige and Skoog
NBT	Nitroblue tetrazolium
SOD	Superoxide dismutase
POD	Peroxidase
ROS	Reactive oxygen species

Introduction

In long-term evolution, plants have formed a complete and complex mechanism to adapt and resist a variety of abiotic stresses (Liu et al. 2018). Plants resist abiotic stresses at the molecular, cellular, tissue, and whole-plant levels (Bohnert and Jensen 1996; Wang and Altman 2003). At the molecular level, ABA-dependent and -independent pathways participate in stress-responsive (Zhu 2002). However, some scholars believe that the two pathways function either alone or synergistically (Lee et al. 2010). Many genes involved in stress responses have been identified and validated, including functional genes and regulatory genes (Shinozaki et al. 2003; Shinozaki and Yamaguchi-Shinozaki 2007). Transcription factors (TF), which regulate gene expression, are classified into five types: NAC (NAM, ATAF1, ATAF2 and CUC2), MYB (v-myb avian myeloblastosis viral oncogene homolog), WRKY (tryptophan, arginine, lysine and tyrosine), bZIP (basic region/leucine zipper motif) and AP2/ERF

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(APETALA2/ethylene responsive factor) family (Wang et al. 2016).

AP2/ERF transcription factor plays an important role in plant biotic and/or abiotic stresses (Muhammad et al. 2012; Shu et al. 2016) and plant growth and development (Zhang et al. 2012). AP2/ERF transcription factors are involved in flower development (Elliott et al. 1996), spikelet meristem determinacy (Chuck et al. 1998), leaf epidermal cell identity (Moose and Sisco 1996), embryo development (Boutilier et al. 2002), stresses tolerance (Dubouzet et al. 2003) and so on. Members of AP2/ERF superfamily share a highly conserved DNA-binding domain known as AP2/ERF domain, which possess 60–70 conserved amino acid residues (Nakano et al. 2006). According to different numbers or structures of AP2 and other conserved domains, AP2/ERF family is classified into AP2, RAV (Related to ABI3 and VP1), ERF and Soloist families (Nakano et al. 2006). AP2 TFs contain two AP2 domains or a single AP2 domain, but the single AP2 domain is similar to an AP2 domain in the double-domain groups (Nakano et al. 2006). AP2 TFs were reported to regulate plant organ growth and development, such as flower development and determination of seed size (Elliott et al. 1996; Jofuku et al. 2005). RAV TFs, containing one AP2/ERF domain and a B3 domain, are involved in ethylene response pathway (Alonso 2003) and abiotic/biotic stress (Sohn et al. 2006; Li et al. 2011). Soloist family is a small group with MRG and HLG elements in the AP2/ERF domain (Ma et al. 2017); members of this family strongly diverge in gene sequence from other AP2/ERF members (Rao et al. 2016). ERF TFs, containing one AP2/ERF domain, are involved in both environmental stress responses and hormone regulatory pathways (Yu et al. 2017), such as the ethylene (Fujimoto et al. 2000), salicylic acid (Oñatesánchez and Singh 2002) and jasmonic acid pathways (Mantiri et al. 2008).

Based on differences of conserved residues in DNA binding domain, ERF family is further divided into ERF subfamily and the CBF/DREB subfamily (Nakano et al. 2006; Yu et al. 2017). The differences between CBF/DREB and ERF subfamilies are the 14th and 19th amino acids of AP2/ERF domain. The 14th and 19th amino acids of AP2/ERF domain in CBF/DREB subfamily are valine (V14) and glutamic (E19) acid, while in ERF subfamily the corresponding amino acid are alanine (A14) and aspartic (D19) acid, respectively (Riechmann et al. 2000; Sakuma et al. 2002). It has been reported that many DREB proteins bind to DRE/CRT (drought-responsive/C-repeat) element to activate or suppress gene transcription (Park et al. 2001; Zhao et al. 2012; Zhang et al. 2014). The ERF proteins mainly bind to AGCCGCC of the GCC-boxes (Ohme-Takagi and Shinshi 1995). Recent studies have shown that some ERF proteins also bind to DRE/CRT (Cheng and Lin 2013). In addition to binding to DRE and GCC-box, ERF TFs could also bind to TGG element (Wang et al. 2015). For example, ThERF1 from *Tamarix hispida* mainly binds to the TTG motif to regulate gene expression, and DRE and GCC box are rarely found in the promoters of ThERF1-regulated genes when exposed to salt stress conditions (Wang et al. 2015). Multiple modulating reactions could be due to their secondary binding to the promoter, which can mediate simultaneous regulation of multiple responses. However, the pathway remains unclear because of different regulatory pathways among plants (Phukan et al. 2017).

Members of ERF TFs were documented in many species, such as *Arabidopsis thaliana* (Lorenzo et al. 2003; Oñatesánchez et al. 2007; Vogel et al. 2014), *Artemisia annua* (Yu et al. 2012), *Glycine max* (Zhang et al. 2009; Hernandezgarcia and Finer 2016), *Gossypium barbadense* (Zuo et al. 2007), *Oryza sativa* (Zhao et al. 2015; Lee et al. 2016), *Triticum aestivum* (Na et al. 2010; Dong et al. 2012; Zhu and Zhang 2014), *Nicotiana benthamiana* (Todd et al. 2010). Meanwhile ERFs are involved in plant responses to salt (Schmidt et al. 2013; Makhloufi et al. 2014), cold (Ma et al. 2014; Zhuo et al. 2017), heat (Yao et al. 2017), drought (Gao et al. 2008; Yang et al. 2016), pathogen (Zhu and Zhang 2014; Liu et al. 2017) and abscisic acid (Zhu et al. 2010).

Larix kaempferi, one of the most important afforestation and timber species in China, has important economic and ecological value. *L. kaempferi* is characterized for its fast growth and rapid reproduction, strong adaptability, low vulnerability to pests and diseases (Li et al. 2014). However, owing in part to its long life cycle and complex genetic background, identification and functional characterization of ERF members in *L. kaempferi* remains largely unexplored. In the present study, a putative ERF gene, named *LkERF-B2*, was isolated and cloned from *L. kaempferi*. To investigate the function of *LkERF-B2*, this gene was transformed into *A. thaliana* via dip flower to evaluated tolerance ability to abiotic stress.

Materials and methods

Plant materials and treatments

Larix kaempferi planted in Dagujia Forest Farm, Liaoning Province, China, was used in this study. Fresh leaves of *L. kaempferi* were harvested and frozen in liquid nitrogen and stored at − 80 °C until use.

RNA extraction and cDNA synthesis

Total RNA was extracted from fresh leaves of *L. kaempferi* with EasyPure RNA Kit (TransGen Biotech, China) according to the manufacturer's instructions. The concentration and

quality of extracted RNA were analyzed by spectrophotometry (ThermoScientific NanoDrop-2000, USA) and 1% gel electrophoresis. TIANScript M-MLV (TIANGEN, China) was used to synthesize first-strand cDNA according to the manufacturer's instructions.

Isolation and cloning of *LkERF-B2*

Based on the transcriptome database of *L. kaempferi* that has been already assembled in our laboratory (Li et al. 2016), the full-length coding sequence of *LkERF-B2* was further assembled by the corresponding contigs. Forward primer and reversed primer were designed according to the open reading frame (ORF) of *LkERF-B2*. *LkERF-B2* F: 5′-ATAAGAATGCGGCCGCATGTGTGGAGGTGCTATC ATCTC-3′ and *LkERF-B2* R: 5′-CCGGAATTCTCAATA AGCAGAATCGGAAATAG-3′. (Underlined are *Eco*RI and *Not*I restriction sites). RT-PCR was carried out using first-strand cDNA as template. The RT-PCR cycling conditions were: 94 °C for 2 min, 30 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 2 min, followed by 72 °C for 5 min.

Sequence analysis of *LkERF-B2*

The homology of the *LkERF-B2* protein was identified using protein BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The second structure of *LkERF-B2* was predicted by PredictProtein (https://www.predictprotein.org/). For multiple sequence alignments, *LkERF-B2* was aligned with the amino acid sequences of other ERFs using the program Clustal X (Larkin et al. 2007). MEGA 5.0 was used to construct the phylogenetic tree through neighbor-joining method and bootstrap analysis with 1000 replications (Tamura et al. 2011). The theoretical molecular weight and isoelectric point of *LkERF-B2* were calculated using expasy (http://web.expasy.org/compute_pi/). The hydrophilicity of *LkERF-B2* was predicted using expasy. The transmembrane domains of *LkERF-B2* were predicted using TMHMM v2.0 (http://www.cbs.dtu.dk/services/TMHMM/).

Construction of expression vector and plant transformation

The full-length open reading frame of *LkERF-B2* was inserted into plant expression vector. Recombinant expression vector was transformed into *A. tumefaciens* LBA4404 cells by electroporation (conditions: 25 μF, 200 Ω, 2 kV). *A. tumefaciens* LBA4404 with *LkERF-B2* was further introduced into wild-type *A. thaliana* via floral dip method (Clough and Bent 2010). The seeds of the transgenic plants were seeded on MS medium containing 30 mg/L kanamycin and screened for T_3 generations. After screening for kanamycin resistance, positive plants were identified by GUS assay, genomic DNA PCR and RT-PCR.

Treatment of transgenic plants

Arabidopsis thaliana seeds were vernalized at 4 °C for 3 days, surface sterilized, and seeded in MS medium. The culture was carried out in a tissue culture incubator at 22 °C under 16 h light/8 h dark cycle.

Two-week-old *A. thaliana* seedlings were treated with cold (− 7 °C/5 h and 4 °C/12 h), heat (40 °C/4 h), salt (planted in new MS medium with 200 mM/L NaCl for 1 week), and drought (planted in new MS medium with 400 mM/L mannitol for 1 week) stresses. After cold and heat stresses treatment, the seedlings were transplanted to normal environment for 2 days and survival rate was calculated.

After 25 days of seed germination, *A. thaliana* plants were treated with different concentrations of NaCl (0, 100, 150, 200, 250, 300 mM/L) for 1 week. Then the physiological indexes in leaves were determined.

Method for determination of physiological indexes

Survival rates are measured by whether plants are alive or not. Survival rate calculation formula:

$$\text{Survival rate} = \text{Survival quantity}/\text{Total quantity} \times 100\%.$$

Salt injury was classified into the following grades: 0, no symptom of salt injury; 1, about 1/5 leaves yellowing; 2, moderate salt injury, about 1/2 leaves yellowing; 3, severe salt injury, most leaves yellowing; and 4, extremely severe salt injury, leaves burning and shedding death. The calculation formula of salt injury rate and salt injury index:

$$\text{Salt injury rate} = \text{Number of symptoms of salt injury}/\text{Total quantity} \times 100\%$$

$$\text{Salt injury index} = \sum (\text{Grade of salt injury} \times \text{Number of corresponding salt injury grades})/\text{Total quantity}/\text{The highest number of salt injury} \times 100\%.$$

Superoxide dismutase (SOD) activity was determined by the NBT method (Zang et al. 2015). Peroxidase (POD) activity was determined by guaiacol method (Podazza et al. 2012). Soluble protein content was measured through coomassie bright blue colorimetric method G-250 method (Grintzalis et al. 2015). Soluble sugar content was measured

using anthrone method (Ibrahim et al. 2012). Chlorophyll content was determined by colorimetry (Pápista et al. 2002).

Statistical analysis

SPSS 17.0 was used in the statistical analyses in the study. One-way analysis of variance (ANOVA) was conducted to determine statistical significance. $P < 0.05$ was considered statistically significant. All data are shown as mean ± standard error of the mean.

Results

Molecular characterization of *LkERF-B2* from *L. kaempferi*

LkERF-B2 gene was isolated from *L. kaempferi* by RT-PCR based on transcriptome database. Sequence analysis showed that the ORF of *LkERF-B2* was 1344 bp, encoding a protein of 447 amino acids. Multiple sequence alignment analysis showed that *LkERF-B2* was highly conserved with ERF transcription factors of several other species. In particular, the amino acid sequence of *LkERF-B2* showed the highest homology (79.0%) with that of *Picea sitchensi* (Fig. 1). Phylogenetic analysis indicated that *LkERF-B2* mostly closely related to ERF transcription factors products of *Picea sitchensis*, implying that they have similar origins (Fig. 2).

The predicted protein had a calculated molecular weight of 49 KD and an isoelectric point of 4.87. *LkERF-B2* was an unstable protein. The amino acid sequence of *LkERF-B2* contained a highly conserved 56-residue AP2 domain, which had an YRG and a RAYD element, at the 122nd–167th amino acids. The protein had a three anti-parallel β-sheet and an α-helix. The amino acids in the second β-fold at 14th and 19th were found to be alanine (A) and proline (P), consistent with typical AP2/ERF transcription factors. Secondary structure prediction suggested that the *LkERF-B2* contained 5.59% α-helix, 3.58% β-sheet and 90.83% loops and has no transmembrane structure.

Production and identification of transgenic plants

Five positive transgenic plants, named transgenic plants L1–L5, were selected and identified in T_1 generation. Each plant was seeded and harvested separately until T_3 generation, which is the homozygous plant (Fig. 3a–c). GUS assay showed GUS activity in the leaf tips and root of transgenic plant. In particular, the GUS activity was very strong in the whole root (Fig. 3e, f). RT-PCR method was used to validate the expression of corresponding transgenic plants (Fig. 3d). This indicates that *LkERF-B2* has been expressing into *A. thaliana* transgene system.

Analysis of survival rate of transgenic plants

Survival rate could effectively reflect the resistance of plants to adverse environments. Transgenic *A. thaliana* (lines L1, L3 and L5) and control plant were subjected to four abiotic stresses (drought, cold, heat and salt) treatments to analyze their adaptability to environmental stress. Survival rates of L1, L3 and L5 under drought stress were 22.22, 25.00 and 22.22%, respectively, which are significantly higher than that of the control (2.77%) (Fig. 4a). Under cold treatment, the average survival rates of transgenic *A. thaliana* plants were 14.82%, while all seedlings of control were died (Fig. 4b). Survival rates L1, L3, and L5 after heat stress were 47.22, 41.67, and 44.44%, respectively, which are significantly higher the control (27.78%) (Fig. 4c). The average survival rate of the *LkERF-B2* overexpressing plants was 52.77% after salt stress, whereas the control plant rate was only 11.11% (Fig. 4d). These results revealed that overexpression of *LkERF-B2* could enhance the adaptability and resistance of plant in various abiotic stresses.

Performance of transgenic plant against salt

The control and transgenic L3 line plant were further treated with different NaCl concentrations (0, 100, 150, 200, 250 and 300 mM/L) to explore the effect of salt on the growth of transgenic *A. thaliana*. 100 and 150 mM/L NaCl treatment could promote flowering than NaCl-untreated group both in control and transgenic L3 plant (Fig. 5a–c). The earliest bolting was detected in transgenic L3 plant under 150 Mm/L salt stresses (Fig. 5c). While flowering was delayed in both control and transgenic L3 plant and their leaves became yellow or even withered under 200, 250, and 300 mM/L NaCl stresses (Fig. 5d–f). Transgenic L3 line *A. thaliana* exhibited less damage, larger leaf area and better growth condition than control group. Yellowed leaves were detected earlier in control group than in transgenic L3 line. These results showed that the damage gradually increased with the increase of NaCl concentration (Fig. 5). Interestingly, low concentrations (100 and 150 mM/L) of NaCl promoted the growth of plants, but high concentrations (200, 250 and 300 mM/L) inhibited plant growth and development.

Salt injury status of transgenic L3 line against salt

The salt injury rate and salt damage index of the control and transgenic L3 line increased with increasing NaCl concentration. The salt injury rate was not significantly

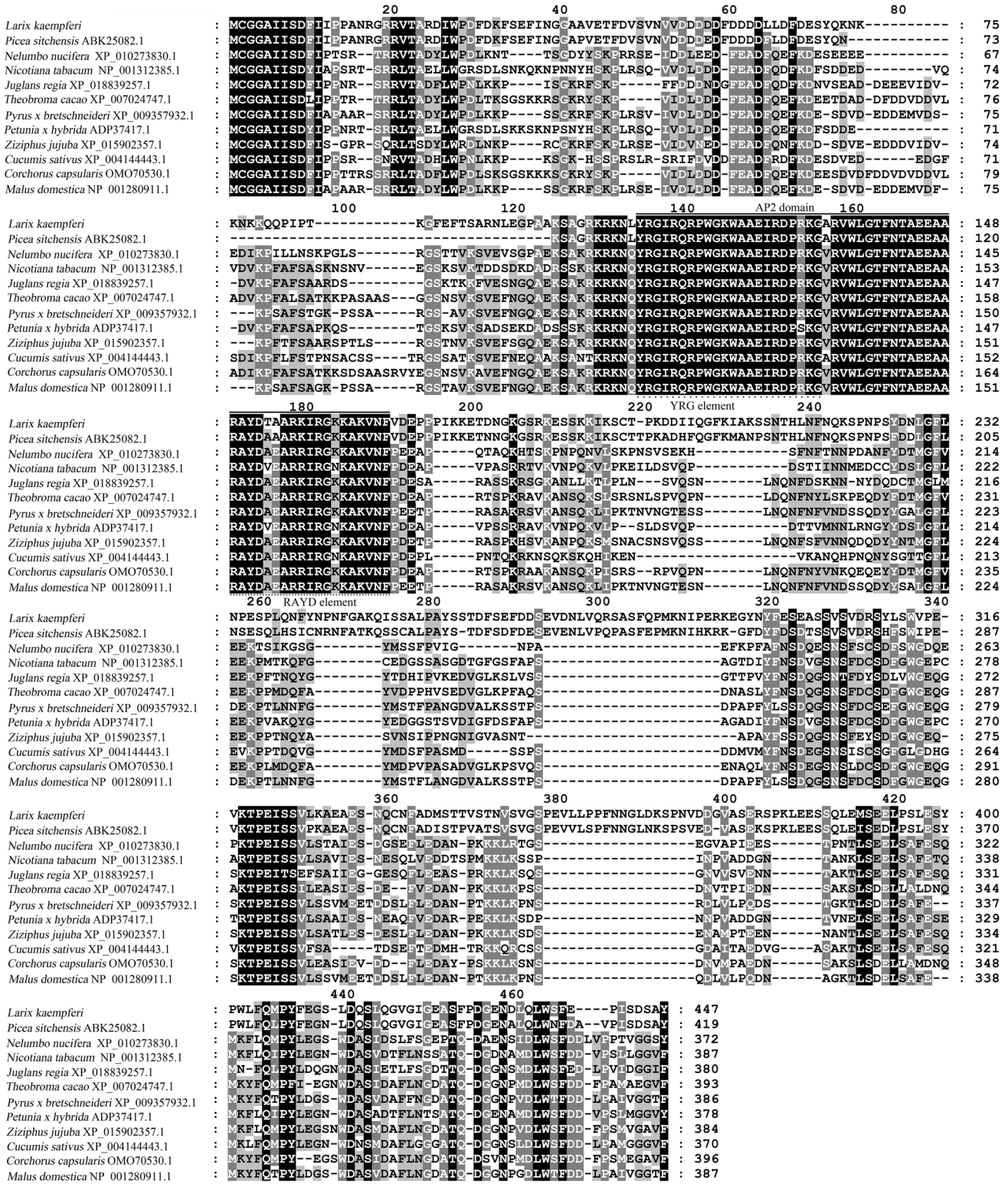

Fig. 1 Amino acid sequence alignment of *LkERF-B2* with ERFs from other species

different between the control and transgenic L3 (Table 1) subjected to high concentrations (200, 250 and 300 mM/L NaCl). The rate even reached 100% in both groups under 300 mM/L NaCl treatment. However, the rate is significantly higher in control group than in the transgenic L3 line under 100 and 150 mM/L NaCl. The salt injury index of the control plant is significantly higher than that of transgenic L3 (Table 2) line under 100–250 mM/L

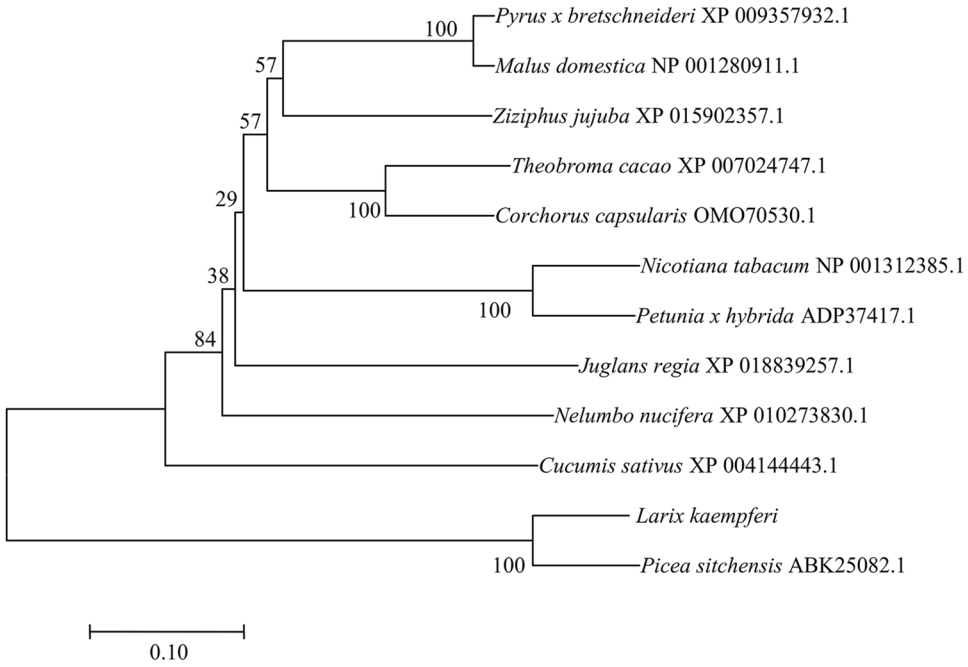

Fig. 2 Phylogenetic tree of *LkERF-B2* and ERF sequences from other species

NaCl. However, under 300 mM/L NaCl treatment, there was no significant difference. Under 100 mM/L NaCl, the salt damage rate and index of control plant were 2 and 1.99 times higher than those of transgenic L3 line, respectively.

Analysis of SOD and POD of transgenic L3 line

The altered activities of SOD and POD are physiological and biochemical indicators of deterioration conditions in plants exposing to environmental constraints conditions. SOD activity of transgenic L3 line was significantly increased compared with that of the control plant. SOD activity of transgenic L3 line under 300 mM/L NaCl treatment increased by 7.5% compared with that of control plant, but did not increase significantly at other concentrations (Fig. 6a). POD activity had no difference between transgenic *A. thaliana* and control under 0 mM/L NaCl treatment (Fig. 6b). However, POD activity of transgenic L3 line increased significantly at 200, 250 and 300 mM/L. Overall, SOD and POD activities of transgenic L3 line increased significantly in comparison with those of control plant under 300 mM/L NaCl.

Analysis soluble sugar and soluble protein content of transgenic L3 line

Soluble sugar content in transgenic L3 line is higher than that in control plant except for untreated (0 mM/L NaCl) plant. Meanwhile, the soluble protein content in transgenic L3 is higher than that in the control plant. The contents of soluble sugars and soluble proteins decreased with increasing NaCl concentration (Fig. 6c, d). However, the soluble protein content was not changed significantly when NaCl concentration reached more than 200 mM/L (Fig. 6d), and the soluble sugar content continuously decreased (Fig. 6c). Under the treatment of 150 mM/L NaCl, the contents of soluble sugars and soluble proteins in transgenic L3 line are 1.34 times and 2.74 times higher than those in the control plant, respectively (Fig. 6c, d).

Analysis of chloroplast pigment content of transgenic L3 line

The contents of chloroplast pigments (chlorophyll *a*, chlorophyll *b*, total chlorophyll, carotenoid) in transgenic L3 line are higher than those in the control plant (Fig. 7). The contents of chlorophyll *a*, total chlorophyll, and carotenoid decreased the increasing NaCl concentration (Fig. 7a, c, d). The contents did not decrease significantly under less than 200 mM/L NaCl treatment but decreased significantly under more than 250 mM/L NaCl treatment. Meanwhile, chlorophyll *b* content showed a trend of increasing first and then decreasing (Fig. 7b). Under 150 mM/L NaCl treatment, chlorophyll *b* content in control and transgenic L3 line reached the maximum values of 0.41 ± 0.02 and 0.42 ± 0.02 mg/g, respectively. Under 250 mM/L NaCl treatment, the contents of chlorophyll *a*, chlorophyll *b*, total chlorophyll, and carotenoids in transgenic L3 line are 1.17, 1.2, 1.18 and 1.15 higher than those in the control group, respectively. Also, under 300 mM/L NaCl treatment, the contents of chlorophyll *a*, total chlorophyll and carotenoids in transgenic L3

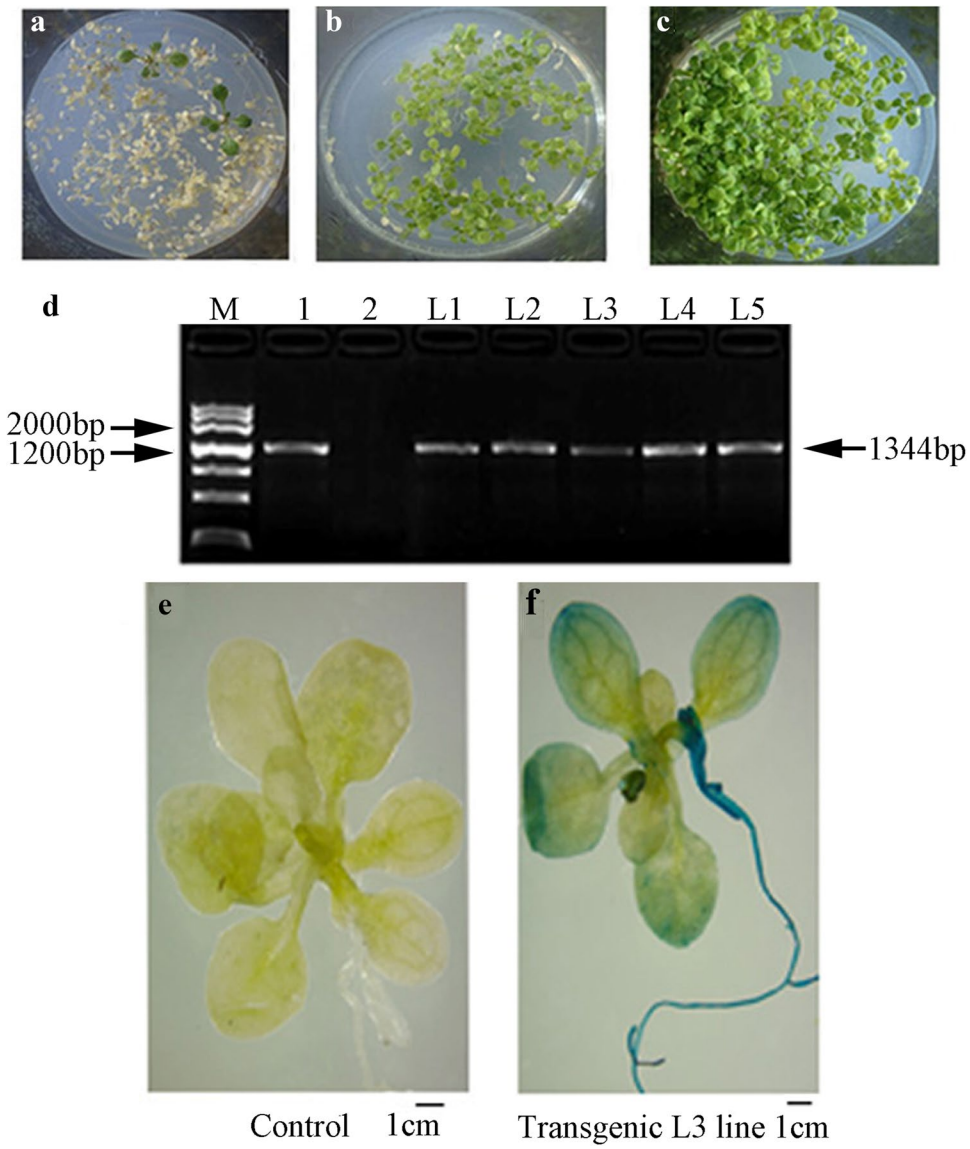

Fig. 3 Screening and identification of transgenic *A. thaliana*. **a–c** T_1, T_2, T_3 generation transgenic plants screening with Kanamycin, **d** RT-PCR identification of T_3 transgenic plants. M: Maker III, 1: RT-PCR products of positive control, 2: RT-PCR products of negative control, L1–L5: RT-PCR products of transgenic plants, **e** GUS expression in the control, **f** GUS expression in transgenic L3 line

plant are *A. thaliana* 1.6, 1.37, and 1.33 higher than those in the control group, respectively. At the same concentration, the contents of chlorophyll *b* in the control plant are higher than those in transgenic L3 line, but the difference was not significant.

Discussion

AP2/ERF family is one of the largest transcription factor families in plants. Among them, the ERF subfamily contained an AP2 domain with typical characteristics. The N-terminal is an alkaline hydrophilic region, and the C-terminal is rich acidic amino acids. The amino acid residues are all composed of three anti-parallel β-sheet and an α-helix (Allen et al. 2014). The 14th and 19th in the second β-fold are conserved, alanine (A) and aspartate (D), respectively. In particular, the 14th alanine plays a key role in determining the specific binding of ERF transcription factor to GCC-box (Ohmetakagi and Shinshi 1990). In this study, the ORF of *LkERF-B2* gene from *L. kaempferi* was obtained by RT-PCR. Analysis of AP2/ERF conserved region in *LkERF-B2* by bioinformatics indicated that *LkERF-B2* had an AP2 domain, which contained YRG and RAYD element. The secondary structure of *LkERF-B2* had three anti-parallel β-sheet and an α-helix. Meanwhile, the 14th is alanine (A) which is absolute conserved, but the 19th is proline (P) which had a slightly difference. These characteristics are basically the same as those of known AP2 conserved regions. Amino acid sequence alignment of *LkERF-B2* revealed that *LkERF-B2* had the highest homology with *P. sitchensis* (79%). The homology of ERF transcription factor protein with other species was 35–38%. Hence, *LkERF-B2* is a newly discovered

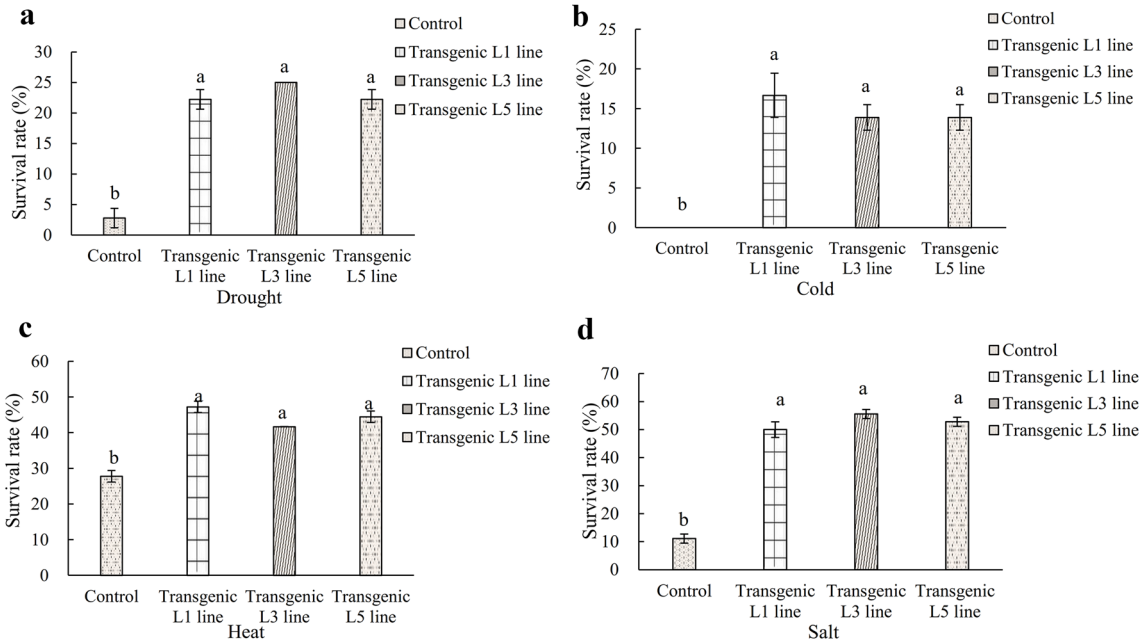

Fig. 4 Survival rate of control and transgenic *A. thaliana* seedling under various abiotic stresses. **a** 400 mM/L Mannitol for 7 days, **b** − 7 °C for 5 h 2 days later, **c** 40 °C for 3 h 2 days later, **d** 200 mM/L NaCl for 7 days

Fig. 5 Salt treatment of control and transgenic L3 line. **a–f** 0, 100, 150, 200, 250 and 300 mM/L NaCl

Table 1 Salt injury rate in control and transgenic L3 line under different NaCl concentration

Type NaCl (mM/L)	Control	Transgenic L3 line
0	0.00 ± 0.00 e	0.00 ± 0.00 e
100	75.00 ± 7.31 b	37.50 ± 7.31 d
150	87.50 ± 7.31 ab	50.00 ± 7.31 c
200	87.50 ± 7.31 ab	87.50 ± 0.00 ab
250	100.00 ± 0.00 a	87.50 ± 0.00 ab
300	100.00 ± 0.00 a	100.00 ± 0.00 a

Same letter means no significant difference according to Duncan's test at $\alpha = 0.05$

Table 2 Salt injury index in control and transgenic L3 line under different NaCl concentration

Type NaCl (mM/L)	Control	Transgenic L3 line
0	0.00 ± 0.00 g	0.00 ± 0.00 g
100	18.75 ± 0.02 e	9.38 ± 0.02 f
150	21.87 ± 0.02 de	12.50 ± 0.02 f
200	36.46 ± 0.04 c	25.00 ± 0.02 d
250	59.38 ± 0.02 b	38.54 ± 0.01 c
300	65.63 ± 0.02 a	62.50 ± 0.02 ab

Same letter means no significant difference according to Duncan's test at $\alpha = 0.05$

sequence that is relatively conserved in evolution and could be a member of ERF subfamily.

Survival rates of transgenic lines and control *A. thaliana* were analyzed. High survival rate of transgenic plants under abiotic stresses suggested that *LkERF-B2* might play an important role in plant abiotic responses. Previous studies revealed that ERFs could improve tolerance ability when it was expressed in transgenic plants (Makhloufi et al. 2014; Phukan et al. 2017). For example, overexpression of *ERF1* in rice improved its resistance to salt stresses (Schmidt et al. 2013). Also, overexpression of *SpERF1* enhanced drought tolerance of transgenic *A. thaliana* (Yang et al. 2016). In the present study, transgenic *A. thaliana* showed higher survival rate than the control plant. Moreover, physiological and biochemical analyses demonstrated that *LkERF-B2* could enhance the adaptability of plants to abiotic stress.

Salt stress could adversely influence plant growth and development (Hussain et al. 2017). Previous research reported that treatments with low salt concentration could promote plant growth and development, but high salt concentration could inhibit plant growth. In previous works, treatments with low NaCl concentrations (25 and 50 mM/L NaCl treatment) improved the growth of *Citrullus lanatus* seedlings. High concentrations of NaCl (75, 100 and 150 mM/L NaCl treatment) obviously inhibited seeding growth (Han et al. 2008). Moreover, low levels of salinity

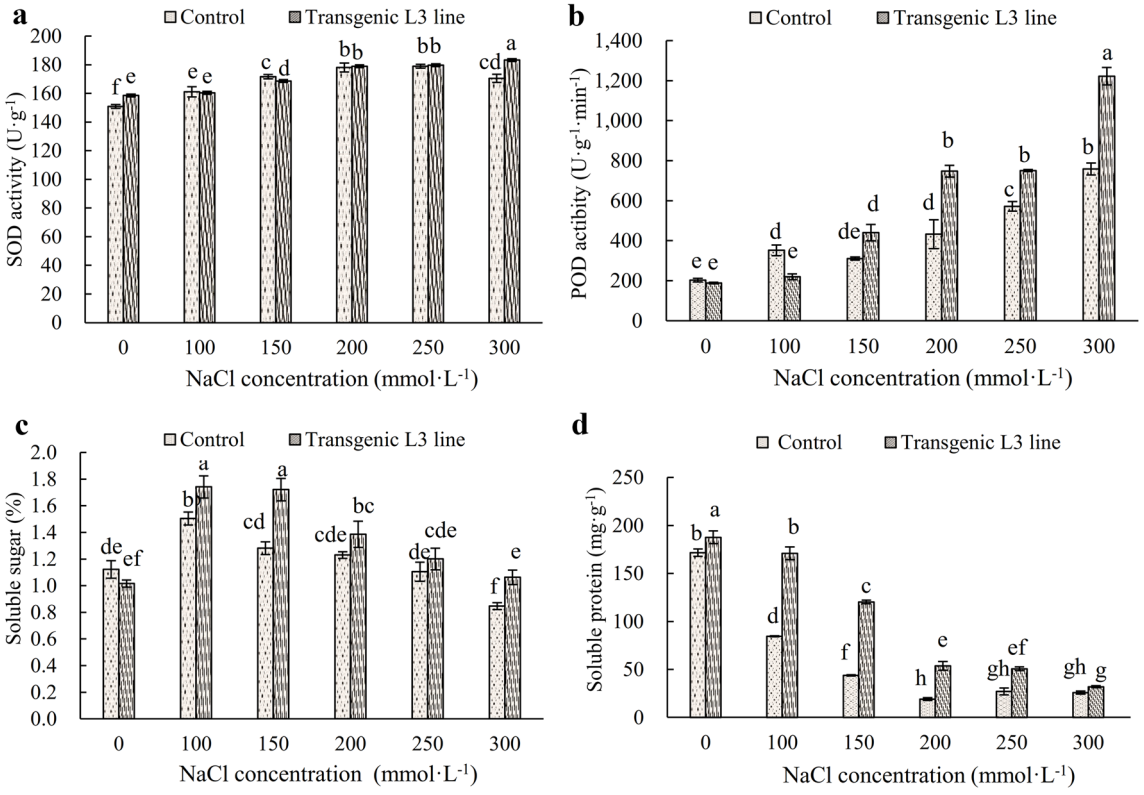

Fig. 6 Physiological and biochemical characteristics of transgenic L3 line under different NaCl concentration. **a** SOD activity, **b** POD activity, **c** soluble sugar content, **d** soluble protein content

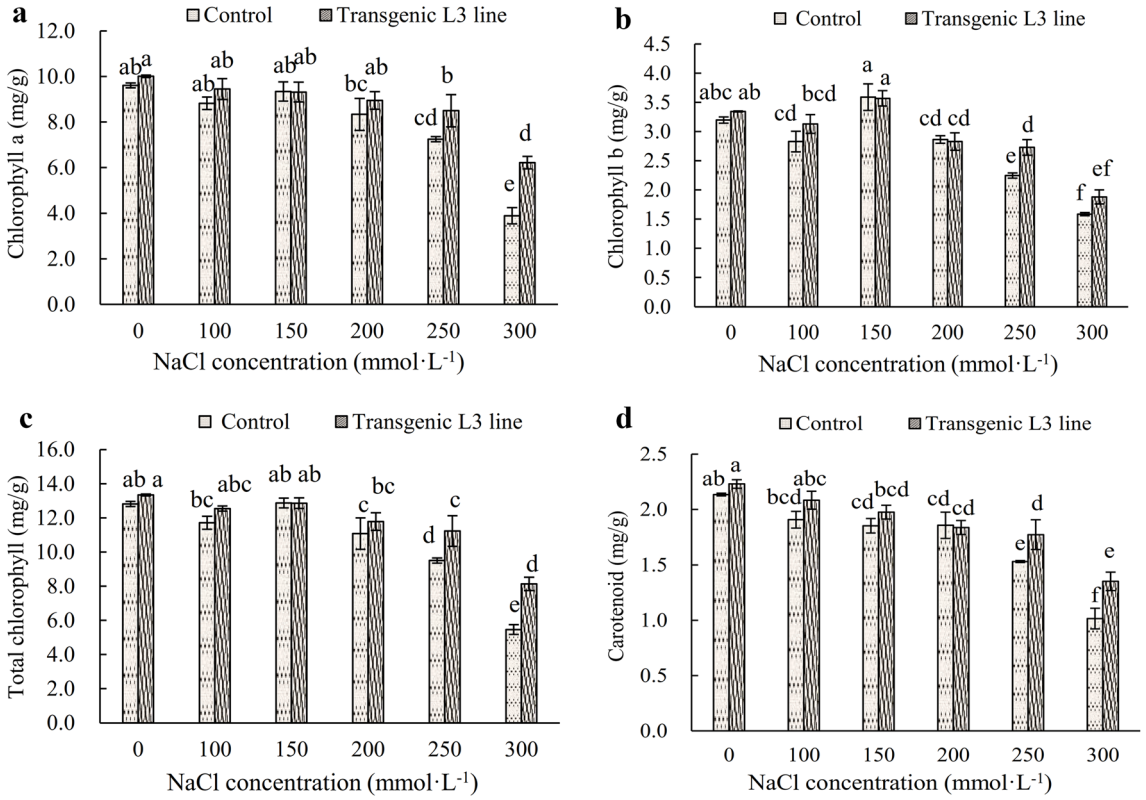

Fig. 7 Chloroplast pigment content in control and transgenic L3 line under different NaCl concentration. **a** Chlorophyll *a* content, **b** chlorophyll *b* content, **c** total chlorophyll content, **d** carotenoid content

stresses could improve the growth of *Medicago sativa*, but high levels inhibited seed germination (Gong et al. 2017). In the present study, the growth of *A. thaliana* (transgenic L3 line and control plant) was promoted by low NaCl concentrations (100 and 150 mM/L NaCl) but inhibited by high concentrations (200, 250 and 300 mM/L NaCl). Moreover, transgenic *A. thaliana* exhibited obvious growth advantage than control plant under the same NaCl concentration.

Abiotic stress may affect the balance between ROS production and removal in cells, leading to increased ROS. ROS could further destroy membrane lipid, proteins, DNA and RNA, consequently, plant growth and development were inhibited or even death in serious cases (Hossain et al. 2015; Jain and Gould 2015). Antioxidant enzymes (SOD and POD) can be used to effectively deal with ROS in plants. In this study, activities of SOD and POD in transgenic L3 line were significantly higher than those in the control plant. Therefore, *LkERF-B2* enhanced plant antioxidant ability in the present study.

Soluble sugars and soluble proteins are solutes that accumulate in plants under abiotic stresses. These substances could regulate osmotic potential and stabilize and protect the structure and function of biological macromolecules. In this study, the contents of soluble sugars and soluble proteins increased increasing NaCl concentration. Moreover, the concentrations of soluble sugars and soluble proteins in the transgenic L3 line plant are significantly higher than those in the control plant under 100 and 150 mM/L NaCl treatment. Therefore, *LkERF-B2* increased soluble sugar and soluble protein content in response to NaCl stress.

Content of chloroplast pigment decreased with increasing NaCl concentration. Carotenoids play an important role in plants growth and development. Carotenoids function in two ways: they function as antenna pigments and transmit captured light to chlorophyll; and they act as scavengers of free radicals in plant cells (Polívka et al. 2004; Polívka and Frank 2010). The study on carotenoid content of transgenic L3 showed that *LkERF-B2* could increase the carotenoid content and enhance the NaCl tolerance of plant.

Conclusion

The *LkERF-B2* was cloned from *L. kaempferi*. The ORF of *LkERF-B2* is 1344 bp, encoding 447 amino acids and containing an AP2/ERF domain. *LkERF-B2* has the closest relationship with *P. sitchensis* (79.0%). *LkERF-B2* is a hydrophilic protein with no transmembrane region. The

plant expression vector was constructed and *LkERF-B2* was transferred into *A. thaliana*. Five homozygous transgenic lines were obtained. Under various abiotic stresses (cold, heat, salt and drought), survival rate of transgenic *A. thaliana* was significantly higher than that of control. Under NaCl stress, salt injury rate and salt injury index of transgenic *A. thaliana* were lower than those of the control, while the activities of SOD, POD and contents of chloroplast pigments were higher than those of control. In conclusion, *LkERF-B2* plays a role in abiotic stress, especially salt stress. Further studies on stress tolerance genes are of great significance for improving the yield and quality of *L. kaempferi*.

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Compliance with ethical standards

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

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