Cloning and activity analysis of the promoter of nucleotide exchange factor gene \textit{ZjFes1} from the seagrasses \textit{Zostera japonica}

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After HSP70 binds to the J domain of the substrate and co-chaperone protein, ATP is hydrolyzed to ADP, and the nucleotide exchange factors (NEFs) promote the release of ADP. Under physiological conditions, the nucleotide exchange step is the rate-limiting step, which is accelerated by NEFs. In this study, the promoter of nucleotide exchange factor \textit{ZjFes1} was cloned, and its expression in tissues and under heat stress was studied to understand the regulatory mechanism of \textit{ZjFes1} and provide the molecular basis to study heat tolerance mechanism of seagrass. It was found that the promoter has common cis-acting elements in promoter and enhancer regions CAAT-box, as well as light response elements AE-box, Box 4 and TCCC-motif, a cis-acting regulatory element essential for the anaerobic induction of ARE, hormone response elements CGTCA-motif and TGACG-motif (MeJA response element), GARE-motif (gibberellin response element), TGA-element (auxin response element), a cis-acting regulatory element related to meristem expression CAT-box, and a cis-acting element involved in defense and stress responsiveness of TC-rich repeats. Two-week-old seedlings exhibited weak GUS activities in their cotyledons. In addition, the \textit{AtFes1A} promoter was constitutively active in the anthers. After exposure to 38 °C for 2 h, the root tips of two-week-old seedlings were stained a strong blue. Heat-inducible activities of GUS were also observed in the cotyledons, roots, leaves, anthers, sepals and siliques.

As is well known, molecular chaperones can help proteins fold in cells\textsuperscript{1}. NEFs are critical to the functional cycle of HSP70. Well-studied NEFs include GrpE in \textit{E. coli}\textsuperscript{2}, Bag-1\textsuperscript{3} and HspBP-1\textsuperscript{4,5} in animals, and Fes1p in yeast\textsuperscript{6}. The yeast Fes1 gene encodes a conserved NEF that acts on cytoplasmic Hsp70s. Mammalian HspBP1 is homologous to Fes1p. HspBP1 promotes the nucleotide dissociation of mammalian Hsc70\textsuperscript{4}. HspBP-1 was initially identified as a binding factor to human Hsp70 and was subsequently identified as a functional NEF of cytoplasmic Hsp70, which inhibits the CHIP ubiquitin ligase that directs Hsp70 to the 26S proteasome\textsuperscript{7}. When HspBP1 bound to Hsc70, the ubiquitin ligase activity of CHIP decreased\textsuperscript{7}. A protein quality control system protects cells from the accumulation of misfolded proteins by promoting selective degradation of misfolded proteins. Hsp70 combines misfolded proteins to facilitate their refolding. If the protein cannot be folded, Hsp70 interacts with ubiquitination enzymes to promote the degradation of misfolded proteins. Hsp70 NEF Fes1 is essential for the ubiquitination of cytoplasmic misfolded proteins, and Fes1 directs Hsp70 substrates to the degradation machinery\textsuperscript{8}. Fes1 selectively binds proteins bound to Hsp70 to facilitate their release from Hsp70\textsuperscript{8}. In the absence of Fes1, misfolded proteins cannot be polyubiquitinated\textsuperscript{8}. As a result, they aggregate and induce a strong heat shock response\textsuperscript{8}. Cells maintain protein homeostasis by selectively identifying and degrading misfolded proteins. In \textit{Saccharomyces cerevisiae}, Hsp70 NEF Fes1 is essential for the degradation of misfolded proteins using an ubiquitin–proteasome system. The cytoplasmic splicing variant of Hsp70 NEF Fes1 is essential for the degradation of misfolded proteins in yeast\textsuperscript{8}. Fes1 transcripts produce two active isoforms through 3’ alternative splicing\textsuperscript{8}. The C-terminus of these two isoforms differ and are referred to as Fes1L and Fes1S\textsuperscript{8}. Fes1L is located in the nucleus and was the...
first nuclear Hsp70 nucleotide exchange factor identified. In contrast, Fes1S is located in the cytoplasm, which is a necessary condition for maintaining protein stability. In the absence of Fes1S, the heat shock response was induced under conditions that were normally not stressful. In addition, when the temperature increased, the cells showed severe growth defects. Importantly, misfolded proteins cannot be degraded by the ubiquitin–proteasome system. Genes homologous to HspBP-1 have also been found in other eukaryotes, such as Fes1p in yeast. The deletion of Fes1 moderately damaged the growth of yeast at 37 °C. Three Fes1p homologues were identified in Arabidopsis thaliana, and AtFes1A plays an important role in heat response. The expression of AtFes1A was induced by high temperature, and AtFes1A prevented the degradation of Hsp70.

Global climate change is one of the major factors that affects seagrass meadows through its effects on sea level, temperature and CO₂ in the atmosphere, which can change the distribution and productivity of seagrass. Rising sea surface temperatures impose heat stress on seagrass and the changes in sea surface temperatures directly affect the maintenance of C balance and metabolism in seagrass. In addition, temperature is the main factor that controls the growth of seagrass by altering biochemical processes, where high temperatures inhibit the growth of seagrass.

Zostera japonica is a species of seagrass endemic to Asia and is primarily distributed in Japan, Korea and China. Z. japonica is the most widely distributed seagrass species in subtropical and temperate coastal areas of China. In the subtropical zone, Z. japonica often appears near mangroves or coexists with Halophila ovalis. In the temperate zone, Z. japonica often lives near Z. marina and in the intertidal zone where the water level is shallower than that in which Z. marina grows. Only Z. japonica can be found in both temperate and subtropical zones in China. Z. japonica is an intertidal seagrass, and intertidal seagrass species are more susceptible to heat stress at low tide during the summer months than subtidal seagrass species.

Fes1p has three homologous genes in Arabidopsis thaliana, which belong to the ARM (armadillo repeat) superfamily. The fes1p homologous genes of A. thaliana are designated AtFes1A (AT3G09350), AtFes1B (AT3G53800) and AtFes1C (AT5G02150). Microarray analysis (Genevestigator, https://genevestigator.com/gv/) showed that among the three AtFes1 genes, AtFes1A was the most significantly expressed by heat induction. A knockout of AtFes1A severely impaired acquired thermotolerance in seedlings. Furthermore, AtFes1A and Hsp70 interact in vivo and in vitro. However, AtFes1A has no NEF activity in vitro. Surprisingly, an AtFes1A knockout resulted in the down-regulation of Hsp70 protein in cytoplasm and increased transcription of Hsps and Hsfs.

Although the homologous genes of hspbp-1/fes1p in A. thaliana have been identified, little is known about their homologous genes in seagrass. Promoters control gene expression, and it is important to study the promoter of nucleotide exchange factor in Z. japonica to understand regulatory mechanism of the gene. We have previously studied the nucleotide exchange factor of Z. japonica and preliminarily verified the function of ZjFes1, but we did not study the promoter of the gene. In this study, the upstream promoter of ZjFes1 was cloned using a genome walking method; the promoter sequence was analyzed, and the expression vector was constructed to verify the promoter activity. The promoter activity under heat stress was studied by transforming A. thaliana and subjecting the transgenic A. thaliana to heat treatment. It is helpful to understand the regulatory mode of ZjFes1 in Z. japonica, and this study provides a theoretical basis to study related nucleotide exchange factors in the future.

Results
Cloning and analysis of promoters. According to the primers designed, the 2 kb promoter was obtained by genome walking. Primers were designed at both ends of the promoter, and 2 kb bands were amplified by PCR based on the primers designed. Sequencing results showed that the amplified sequence was identical with the promoter sequence obtained by genome walking, and the sequence could be used in the next experiment. The cloned 2000 bp sequence was predicted online, and the results showed that ZjFes1 promoter not only contained repeats (Fig. 1). According to the primers designed, the 2 kb promoter was obtained by genome walking. Primers were designed at both ends of the promoter, and 2 kb bands were amplified by PCR based on the primers designed. Sequencing results showed that the amplified sequence was identical with the promoter sequence obtained by genome walking, and the sequence could be used in the next experiment. The cloned 2000 bp sequence was predicted online, and the results showed that ZjFes1 promoter not only contained

Construction of the plant expression vector. The promoter fragment obtained (2000 bp) was added with tail A and connected to vector pCXGUS-P/pXcm1-GUS (only GUS, no promoter) to transform E. coli. The recombinant plasmid was identified by PCR, which indicated that ZjFes1 promoter had been inserted into pCXGUS-P/pXcm1-GUS (Fig. 2).

Agrobacterium tumefaciens-mediated transformation of Arabidopsis thaliana and GUS staining. The recombinant expression vector pZjFes1::GUS was transformed into A. tumefaciens GV3101. A. thaliana was transformed using an A. tumefaciens-mediated floral dip method. The homozygous plants of transgenic Arabidopsis thaliana were heat-treated and 2-week-old seedlings, leaves, flowers and siliques were stained with GUS. The expression products of GUS could be detected by A. tumefaciens transfected into pZjFes1::GUS in A. thaliana, which indicated that the cloned ZjFes1 promoter could drive the expression of downstream gene GUS, i.e., the promoter fragment had driving activity. The homologous gene of ZjFes1 in A. thaliana is AtFes1A (At3g09350). AtFes1A was constitutively expressed at low levels at 23 °C and was significantly induced by high temperature. Heat treatment was used to verify the expression of ZjFes1 promoter under heat stress. Two-week-old seedlings exhibited weak GUS activities in their cotyledons. In addition, the AtFes1A promoter was constitutively expressed in anthers. After exposure to 38 °C for 2 h, the root tips of two-week-old seedlings were strongly stained blue. Heat-inducible activities of GUS were also observed in the cotyledons, roots, leaves,
Figure 1. Sequence analysis of the ZjFes1 promoter. This promoter sequence data of ZjFes1 was registered in GenBank (No. MN161576). The blue arrow indicates primers used for genome walking and amplification of the full-length promoter sequence. The cis-acting elements are highlighted in green. The initiation codon is highlighted in blue.

-2000bp

AGCTAGCTAGAGAGAAACCAAGAGGATCATGAGTCTCCTATGACACACAAAAAGAAAACCATTT
ZjFes1-pro-R
TTACCTTCATGCGAGAGTGGGTTGTCATCTTTAGATCTAATGCCCCGACGAGATTTCCGGA
GTTGATCCATTTTTTGAACTATGAAAGAAGAGACCATGATGCTGTTGTTGTATATC
CAAT-box  ZjFes1-SP3-4th
TACAGGTTACCTTCATGCGAGAGTGGGTTGTCATCTTTAGATCTAATGCCCCGACGAGATTTCCGGA
GTTGATCCATTTTTTGAACTATGAAAGAAGAGACCATGATGCTGTTGTTGTATATC
CAAT-box  ZjFes1-SP3-4th
TGACG-motif
CCATGCGAGAGTGGGTTGTCATCTTTAGATCTAATGCCCCGACGAGATTTCCGGA
GTTGATCCATTTTTTGAACTATGAAAGAAGAGACCATGATGCTGTTGTTGTATATC
CAAT-box  ZjFes1-SPI-4th
CTGTGCGATCTTCATCATAAGACACAAAGAAAGAGACCATGATGCTGTTGTTGTATATC
CTACATTTTTTGAACTATGAAAGAAGAGACCATGATGCTGTTGTTGTATATC
TATA-box  ZjFes1-SPI-4th
GTAGAAGATCTTCATCATAAGACACAAAGAAAGAGACCATGATGCTGTTGTTGTATATC
TATA-box  ZjFes1-SPI-4th
TGACG-motif
CAGCAGCTTTCTGGAAGCTCTTACCCACCAAGAGGATCATGAGTCTCCTATGACACACAAAAAGAAAACCATTT
ZjFes1-SP3-3rd
ACAGGTTACCTTCATGCGAGAGTGGGTTGTCATCTTTAGATCTAATGCCCCGACGAGATTTCCGGA
GTTGATCCATTTTTTGAACTATGAAAGAAGAGACCATGATGCTGTTGTTGTATATC
CAAT-box  ZjFes1-SP3-3rd
TGACG-motif

Figure 3. Promoters of anthers, sepals and siliques (Fig. 3). This is similar to the expression of AtFes1A promoter in A. thaliana. In order to quantitatively detect the activity of ZjFes1 promoter, GUS activity assay was carried out (Fig. 3B). It can
be seen from Fig. 3B that the activity of ZjFes1 promoter under heat treatment is higher than that under normal temperature, which is consistent with the result in Fig. 3A.

**Discussion**

The release of ADP is facilitated by NEFs, and the substrate-binding domain (SBD) of Hsp70 returns to the open conformation and releases the substrate. Although there are some redundant functions and expression patterns, microarray analysis (Genevestigator, [https://genevestigator.com/gv/](https://genevestigator.com/gv/)) showed that among the three AtFes1 genes, AtFes1A was the most significantly expressed by heat induction. Recent studies have shown that AtFes1A may play a regulatory role in maintaining the stability of Hsp70 and abiotic stress tolerance.

Gene expression in higher plants is primarily regulated by complex factors at the transcriptional level, which are coordinated by many cis-acting elements and trans-acting factors. The promoter is an important cis-acting element in transcriptional regulation and is the center of transcriptional regulation, which determines the temporal and spatial sequence of target gene expression to a certain extent. Therefore, the study of structure and function of promoter is key to understanding molecular mechanism of plant gene expression regulation.

Z. japonica is an intertidal seagrass, and intertidal seagrass species are more susceptible to heat stress at low tide during the summer months compared with subtidal seagrass species.

In this study, the ZjFes1 promoter was cloned from genome of Z. japonica. A sequence analysis showed that the promoter not only contained common cis-acting elements in promoter and enhancer regions CAAT-box but also light response elements AE-box, Box 4, and TCCC-motif, a cis-acting regulatory element essential for the anaerobic induction ARE, the hormone response elements CGTCA-motif and TGACG-motif (MeJA response element), GARE-motif (gibberellin response element), TGA-element (auxin response element), a cis-acting regulatory element related to meristem expression CAT-box, and a cis-acting element involved in defense and stress responses of TC-rich repeats. The prediction of these cis-acting elements can provide a theoretical basis for the study of this gene regulation pattern. Since the GUS signals driven by the promoter of ZjFes1 as examined in the transgenic A. thaliana plants were induced by heat treatment, a typical heat-responsive element such as HSE could be contained in the promoter region. However, no such HSE element was found in the promoter region. In our previous study, to determine whether ZjFes1 expression was influenced by heat stress, we measured...
ZjFes1 mRNA levels 1 h after heat treatment at 40 °C and compared the measurements to plants maintained at the control temperature of 25 °C. Three independent biological replicates were conducted, and we found that the expression of ZjFes1 increased significantly (approximately 34.53-fold) at 1 h after treatment. The result of GUS staining was consistent with that of qRT-PCR. The promoter of Zjfes1 may contain a heat-responsive element that has not yet been identified.

This study determined the activity of ZjFes1 promoter and its expression in seedlings, leaves, flowers and siliques under heat stress. The results showed that ZjFes1 might play a role in the heat tolerance of Z. japonica.

Material and methods

Plant material. Z. japonica used in this study was collected from Fangchenggang, Guangxi, China.

DNA extraction and primer design. Leaves of Z. japonica were used as materials to extract genomic DNA from young leaves that had grown well. A MiniBEST Plant Genomic DNA Extraction Kit (TaKaRa, 9768) was used to extract genomic DNA from the leaves of Z. japonica following the manufacturer’s instructions. Based on the full-length cDNA sequence of ZjFes1 obtained by RACE, three identical and high annealing temperature specific primers (SP Primer) were designed, and four specifically designed degenerate primers, AP1, AP2, AP3 and AP4, were used for thermal asymmetric interlaced PCR (TAIL-PCR). Typically, at least one of these degenerate primers can react with specific primers by TAIL-PCR based on the difference of annealing temperature, and the flanking sequence of known sequence can be obtained by three nested PCR reactions. Because the length obtained in one experiment cannot meet the experimental requirements, we continue to acquire the flanking sequence according to the sequence information obtained in the first genome walking. Four genome walkings were conducted. Twelve SP Primers were designed. DNAMAN software was used to combine the four fragments described above into a consensus sequence by combining overlapping fragments. Specific primers were designed to amplify 2 kb sequences according to the results (Table 1), and the experimental results were verified.

Cloning and construction of the plant expression vector and sequence analysis of promoter. The full-length promoter sequence was amplified using high fidelity polymerase 2× TransStart Fast-Pfu PCR SuperMix (-dye) (TRANSGEN BIOTECH, AS221-01) using the DNA of Z. japonica as a template following the manufacturer’s instructions. The PCR products were detected using 1% gel electrophoresis. The results showed that the size of the bands was the same as that of the target fragments, and the PCR products were recovered using a MiniBEST Agarose Gel DNA Extraction Kit Ver. 4.0 (TaKaRa, 9762). The pCXGUS-P plasmid is a vector designed to detect the activity of plant promoters. The promoter activity is detected by the dyeing intensity of GUS. We used XcmI to digest the empty vector to obtain T vector. After recovery, the product was recombined with T vector, and then the recombinant vector was transformed into E. coli DH5α Competent.
Cells (TaKaRa, 9057) following the manufacturer's instructions. The positive samples identified by PCR were verified by sequencing at the Guangzhou Sequencing Department of Invitrogen. The sequencing results were compared using DNAMAN software. The plasmid was extracted from the correct bacterial solution and designated pZjFes1::GUS. The sequence analysis of cis-acting elements that could possibly be found in the promoter was performed using the plant-CARE online prediction database (plant cis-acting regulatory element, https://bioinformatics.psb.ugent.be/webtools/plantcare/html/).20

Agrobacterium-mediated genetic transformation of pZjFes1::GUS into Arabidopsis thaliana. The fusion vector pZjFes1::GUS was transformed into Agrobacterium Rhizobium strain GV3101 chemically competent cells (Biomed, BC304) using the freeze–thaw method following the manufacturer's instructions. Transgenic plants of A. thaliana were obtained by floral dipping. Plants in nutrient soil were cultured to form a large number of immature flower clusters. The monoclonal of A. tumefaciens GV3101 was selected and inoculated in liquid LB medium containing kanamycin and rifampicin (50 µg/mL). The monoclonal was cultured overnight at 200 rpm and 28 °C. A volume of 2 mL bacterial solution was transferred to a 500 mL flask culture (containing 200 mL liquid LB with 50 µg/mL kanamycin and rifampicin added) and was cultured overnight at 200 rpm and 28 °C. The next day, the OD600 of Agrobacterium solution was 1.8–2.0. The solution was centrifuged at 5000 rpm for 15 min at 4 °C. The supernatant was discarded, and the precipitate of A. tumefaciens was resuspended in 1/2 volume (100 mL) osmotic medium (1/2 Murashige-Skoog, 5% sucrose, 0.5 g/L MES, 10 µg/mL 6-BA, 200 µL/L Silwet L-77, and 150 µM acetylene, pH 5.7), resulting in an OD600 of approximately 1.6. The bacterial solution was adsorbed on the transformed plants using the floral dip method (5 min), wrapped with film to keep it fresh, and cultured overnight, followed by the removal of the film. The plants were cultured until the seeds were ripe, and they were harvested. A mixed disinfectant consisting of 70% ethanol and 30% bleach-water was used to soak the seeds for 3 min, suspend them continuously, and wash them three times with anhydrous ethanol. The dried seeds were evenly dispersed on the surface of solid screening medium containing hygromycin (25 µg/mL). After stratification at 4 °C for 2 days, the seeds were germinated in a light incubator and cultured for 2 weeks at 21 °C and 16 h light/8 h darkness. The development of seedlings and length of roots were used to determine whether they were transformants.

GUS dyeing and activity analysis. The expression of GUS reporter gene in Arabidopsis tissues was determined using a GUS staining kit (Solarbio, G3060) following the manufacturer's instructions. The seedlings, leaves, flowers and siliques to be dyed were immersed in GUS dye solution and incubated overnight at 37 °C. The chlorophyll was removed with 75% ethanol until the background color disappeared completely. The results were documented by photography using a Canon 60d camera.

Data availability
All data generated or analyzed during this study are included in this published article. The promoter sequence data of ZjFes1 was registered in the GenBank (No. MN161576).
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References
1. Frydman, J. Folding of newly translated proteins in vivo: the role of molecular chaperones. Annu. Rev. Biochem. 70, 603 (2001).
2. Liberek, K., Marszalek, J., Ang, D., Georgopoulos, C. & Zylcz, M. Escherichia coli DnaJ and GrpE heat shock proteins jointly stimulate ATPase activity of DnaK. Proc. Natl. Acad. Sci. USA 88, 2874–2878 (1991).
3. Gassler, C. S., Wiederkehr, T., Brehmer, D., Bukauf, B. & Mayer, M. P. Bag-1M accelerates nucleotide release for human Hsc70 and Hsp70 and can act concentration-dependent as positive and negative cofactor. J. Biol. Chem. 276, 32538–32544 (2001).
4. Kabani, M., McLellan, C., Raynes, D. A., Guerriero, V. & Brodsky, J. L. HspBP1, a homologue of the yeast Fes1 and Sis1 proteins, is an Hsc70 nucleotide exchange factor. FEBS Lett. 531, 339–342 (2002).
5. Shomura, Y. et al. Regulation of Hsp70 function by HspBP1: structural analysis reveals an alternate mechanism for Hsp70 nucleotide exchange. Mol. Cell 17, 367–379 (2005).
6. Kabani, M., Beckerich, J. M. & Brodsky, J. L. Nucleotide exchange factor for the yeast Hsp70 molecular chaperone Ssa1p. Mol. Cell. Biol. 22, 4677–4689 (2002).
7. Alberi, S., Böhm, K., Arndt, V., Schmidt, A. & Hohfeld, J. The cochaperone HspBP1 inhibits the CHIP ubiquitin ligase and stimulates the maturation of the cystic fibrosis transmembrane conductance regulator. Mol. Cell. Biol. 15, 4003 (2004).
8. Gowda, N. C. K., Kandasamy, G., Froehlich, M. S., Dohmen, R. J. & Andressson, C. Hsp70 nucleotide exchange factor Fes1 is essential for ubiquitin-dependent degradation of misfolded cytosolic proteins. PNAS 110, 5975–5980 (2013).
9. Gowda, N. K. et al. Cytosolic splice isoform of Hsp70 nucleotide exchange factor Fes1 is required for the degradation of misfolded proteins in yeast. Mol. Biol. Cell 27, 1210–1219 (2016).
10. Zhang, J. X. et al. The role of Arabidopsis AtFes1A in cytosolic Hsp70 stability and abiotic stress tolerance. Plant J. 62, 539–548. https://doi.org/10.1111/j.1365-313X.2010.04173.x (2010).
11. Short, F. T. & Neckles, H. A. The effects of global climate change on seagrasses. Aquat. Bot. 63, 169–196 (1999).
12. Stillman, J. H. Acclimation capacity underlies susceptibility to climate change. Science 301, 65 (2003).
13. Rowan, R. Coral bleaching: thermal adaptation in reef coral symbionts. Nature 430, 742 (2004).
14. Zimmermann, R. C., Smith, R. D. & Alberte, R. S. Thermal acclimation and whole-plant carbon balance in Zostera marina L. (eelgrass). J. Exp. Mar. Biol. Ecol. 130, 93–109 (1989).
15. Lee, K. S., Sang, R. P. & Kim, Y. K. Effects of irradiance, temperature, and nutrients on growth dynamics of seagrasses: a review. J. Exp. Mar. Biol. Ecol. 350, 144–175 (2007).
16. Lee, K. S., Sang, R. P. & Kim, J. B. Production dynamics of the eelgrass, Zostera marina in two bay systems on the south coast of the Korean peninsula. Mar. Biol. 147, 1091–1108 (2005).
17. Yashwanti, M., Shin-Han, S., Stone, S. L., Salt, J. N. & Goring, D. R. A large complement of the predicted Arabidopsis ARM repeat proteins are members of the U-box E3 ubiquitin ligase family. Plant Physiol. 134, 59 (2004).
18. Chen, S. & Qiu, G. Heat-stress induced expression of stress-inducible nucleotide exchange factor Fes1 in seagrass Zostera japonica.Ecotoxicology https://doi.org/10.1007/s10646-020-02185-5 (2020).
19. Chen, S. & Qiu, G. Overexpression of seagrass nucleotide exchange factor gene zjfes1 enhances heat tolerance in transgenic Arabidopsis. Plant Signal Behav. 15, 1709719. https://doi.org/10.1080/15592324.2019.1709719 (2020).
20. Magali, L. et al. PlantCARE, a database of plant cis-acting regulatory elements and a portal to tools for in silico analysis of promoter sequences. Nucleic Acids Res. 30, 325–327 (2002).

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Author contributions
S.C. designed the study and performed the laboratory experiments. G.Q. designed the field work. S.C. and G.Q. wrote the main part of the manuscript. All the authors reviewed the manuscript and added details to it.

Competing interests
The authors declare no competing interests.

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