Cryobiology 98 (2021) 103–111

Contents lists available at ScienceDirect

Cryobiology

journal homepage: http://www.elsevier.com/locate/cryo

ApSerpin-ZX from *Agapanthus praecox*, is a potential cryoprotective agent to plant cryopreservation

Guanqun Chen **, Ruilian Li, Xiaohui Shen *

School of Design, Shanghai Jiao Tong University, Shanghai, 200240, China

ARTICLE INFO

Keywords:
Plant serpin
Cryopreservation
cell viability
Programmed cell death
Agapanthus praecox

ABSTRACT

Cryopreservation-induced cell death is regarded as an important problem faced by cryobiologists. Oxidative stress and programmed cell death are detrimental to cell survival. Serine protease inhibitors (serpins) inhibit pro-cell-death proteases and play a pro-survival role in excessive cell death induced by abiotic stress. In this study, *ApSerpin-ZX* was isolated from *Agapanthus praecox* and characterized as a protective protein in plant cryopreservation. The mRNA level of *ApSerpin-ZX* was elevated under abiotic stress, such as salt, osmosis, oxidative, cold, and cryoinjury. The purified recombinant protein expressed in *E. coli* was added to the plant vitrification solution and used for *A. praecox* embryogenic callus cryopreservation. The concentration of 0.6–4.8 mg·L^{-1} of ApSerpin-ZX protein was beneficial to the survival of cryopreserved embryogenic callus of *A. praecox*. The most effective concentration was 1.2 mg·L^{-1}, which elevated the survival by 37.15%. Subsequently, the cryopreservation procedure with 1.2 mg·L^{-1} of ApSerpin-ZX protein was regarded as the treated group, compared to standard procedure, to determine the physiological mechanism of ApSerpin-ZX protein on cryopreserved cell. The MDA and H_2O_2 contents were significantly decreased in the treated group, along with reduced OH· generation activity in the recovery stage. After the addition of ApSerpin-ZX, the POD and CAT activities keep increased, while SOD activity increased only after dehydration. Besides, the caspase-1-like and caspase-3-like activities were lower than the standard procedure. This study indicated that ApSerpin-ZX was a potential cryoprotective agent that alleviated oxidative stress and cell death induced by cryopreservation.

1. Introduction

Vitrification-based cryopreservation is a commonly used technology for the storage of elite germplasms under ultra-low temperature (−196 °C), but cell death in the process is still a problem [6]. The main reason is that techniques used for cryopreservation impose compound stresses on cells or tissues. ROS-induced oxidative stress resulting from osmotic injury, desiccation, and temperature changes is thought to be a fundamental cause of cell death on cryopreserved samples [37]. Besides, programmed cell death (PCD) was induced by cryopreservation procedure, as indicated by the elevated caspases activities [6]. For plant cell cryopreservation, correlational studies were few. The caspase-3-like protease activity was detected after the rehydration of cryopreserved *Eucalyptus grandis* axillary buds [40]. Our earlier studies documented the ROS-induced oxidative stress, and PCD affected the cell viability of cryopreserved embryogenic callus in *Agapanthus praecox* [52]. Therefore, inhibition of PCD is a practical approach to improve cryopreservation efficiency.

Existing studies have found that PCD is blocked by channel blockers or inhibitors of different signaling steps, such as protease, kinase, and so on. A subset of the tested protease inhibitors (phenylmethylsulfonyl fluoride [PMSF], 4-[2-aminoethyl]-benzesulfonylfluoride [AEBSF] hydrocholride and leupeptin) blocked PCD [46]. It is noteworthy that both PMSF and AEBSF are serine protease inhibitors. In addition, these protease inhibitors all have inhibitory activity against cysteine proteases. Serpins, the most widely distributed family of serine protease inhibitors present in all higher eukaryotes, bacteria, viruses, and archaebacterial [20], possess cross-class inhibition. In other words, most serpin family members are indeed serine protease inhibitors, but several functioned as cysteine protease inhibitors [24]. Surprisingly, some are incapable of inhibiting proteases and serve other functions, such as ovalbumin and pigment epithelium-derived factor (SerpinF1) [3]. In general, the scissile bond (P1 and P1' residues) in the reactive center loop (RCL) mediates serpins inhibitory specificity against the target

* Corresponding author.
** Corresponding author.
E-mail addresses: chengq@sjtu.edu.cn (G. Chen), shenxh62@sjtu.edu.cn (X. Shen).

https://doi.org/10.1016/j.cryobiol.2020.11.018
Received 16 July 2020; Received in revised form 17 November 2020; Accepted 19 November 2020
Available online 11 December 2020

protease [27].

Serpins play crucial roles in regulating diverse physiological processes in animals and insects. Unlike animal serpins, very little is known about plant serpins' physiological role and their cognate proteases. Although more than 20 serpins have been isolated or cloned from plants, only a few serpins have been characterized. *AtSerpin1* is the best-characterized plant serpin to date. According to its feature of RCL, it is regarded as an LR-type plant serpin [6,29,30]. It has been shown to inhibit several PCD-related proteases, such as metacaspase AtMC9 and AtMC1, the endogenous papain-like cysteine protease RD21 [33,34,47]. Except for inhibitory properties, evidence also suggested that serpins respond to biotic and environmental stress in transcriptional and protein level. Down-regulated rice serpin *OsSRP-LRS* show enhanced cell death upon *Rhizoctonia solani* inoculation, UV, and salt treatments [5]. The protein level of Serpin-ZX from cashew was higher in a *Lasiodiplodia theobromae*-resistant plant than a susceptible plant [17]. In conclusion, serpins inhibit pro-cell-death proteases and play a pro-survival role in excessive cell death induced by biotic and abiotic stresses. Experiments showed that protease inhibitors added to the cryoprotectant agent improved the efficiency of cryopreservation, including well-known PCD related protease inhibitors, such as caspase I Inhibitor V [2], caspase-9 inhibitor [8], ROCK inhibitor [48], and other protease inhibitors and plant extracts, such as Kazal type serine protease inhibitor SPINK3 [51], winter wheat soluble protein extracts [15] and wheat enolase [25]. In our previous study, we identified a *serpin-ZX* gene from *Agapanthus praecox*, shared similar gene structure and protein domain features to AtSerpin1 and other plant serpins. Accordingly, we attempt to identify the protein properties of ApSerpin-ZX and assess its potential cryoprotective function in cryopreservation.

In the present study, we characterized the gene expression of *Apserpin-ZX* under abiotic stress and cryopreservation. For exploring its potential cryoprotective function, we purified the recombinant ApSerpin-ZX protein and added it to the cryoprotectant agent. The embryogenic callus of *A. praecox* was chosen as cryopreserved materials. The relative survival and regrowth of *A. praecox* were regarded as assessment criteria. For further revealing the physiological mechanism of ApSerpin-ZX, the change of oxidative and cell death-related physiological response in modified cryopreservation of embryogenic callus of *A. praecox* was carried out to reveal the theoretical basis and mechanism of ApSerpin-ZX.

2. Materials and methods

2.1. Plant materials and abiotic stress treatments

The embryogenic callus of *Agapanthus praecox* ssp. *orientalis* was obtained and cultured on standard MS medium with 1.5 $mg \cdot L^{-1}$ picloram [12]. Abiotic stress tests were performed on embryogenic callus on MS medium with NaCl (100, 200 or 300 mM), mannitol (200, 400 or 600 mM), methyl viologen (10, 20 or 30 μM). Control was transferred to MS medium. All the above treatments were cultured at 25 °C under dark for 12 h or 24 h. Cold treatment was performed in MS medium at 4 °C in the dark for 12 h or 24 h [14]. All samples from the described treatments were repeated three times and frozen in liquid nitrogen immediately after harvesting.

2.2. RNA isolation and cDNA synthesis

Total RNA was extracted from embryogenic callus using TaKaRa MiniBEST Plant RNA Extraction Kit according to the manufacturer's instruction (TaKaRa, China). The purified RNA was quantified by NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, USA). The synthesis of the first-strand cDNA was carried out based on the manufacturer's instruction of the PrimeScript™ II 1st strand cDNA synthesis Kit using the DNase I-treated RNA as template and oligo(dT)-adaptor as primer. The synthesis reaction was performed at 42 °C for 1 h, terminated by heating at 95 °C for 5 min. The cDNA mix was stored at −20 °C for subsequent use.

2.3. Molecular cloning of ApSerpin-ZX

RACE cDNA library was synthesized from total RNA using the SMARTer™ RACE cDNA amplification kit (Takara Bio., USA). 3′ and 5′ -RACE reactions were performed using gene-specific primer designed by existed sequence (extracted from *de novo* sequencing) [53] and the common anchor primer provided by the kit. All primers are listed in Supplementary Table S1. 3′ and 5′-RACE PCR product was run on agarose gels and selected single bright band to sequence. The cDNA sequence of *ApSerpin-ZX* was obtained from splicing of existed sequence and sequences of 3′ and 5′RACE.

2.4. Qualitative real-time PCR analyses

qualitative Real-time PCR (qPCR) was performed using the fluorescent dye SYBR-Green (Takara, Dalian, China) and the LightCycler 2.0 system (Roche Diagnostics, Germany). The reactions were carried out as follows: 60 s at 95 °C for denaturation, followed by 40 cycles of 10 s at 94 °C, 20 s at 55 °C, and 30 s at 72 °C for amplification. All the reactions were repeated at least thrice, and no-template controls were included. Primers are designed based on *de novo* sequencing of *A. praecox* [53]. Primers are listed in Supplementary Table S1. Gene expression quantification was calculated by $2^{-\Delta\Delta Ct}$ ($\Delta\Delta Ct = \Delta$ [($Ct_{targe\ gene}$ - Ct_{actin}) $_{treated}$ - ($Ct_{targe\ gene}$ - Ct_{actin}) $_{control}$]). *ApActin* (CL5322.Contig7_AP_BB) gene was used as a housekeeping gene [54].

2.5. Expression and purification of recombinant ApSerpin-ZX protein

The restricted enzyme sites of *Bam*HI and *Hin*dIII were introduced to the ORF fragment of *ApSerpin-ZX* by PCR amplification. The PCR product was digested and ligated into pET-30a vector. The recombinant plasmid pET-30a-*ApSerpin-ZX* was introduced into *Escherichia coli Transetta* (DE3) and cultivated in LB medium containing 50 $\cdot mg\ L^{-1}$ kanamycin. One positive colony was produced in 200 mL LB medium (containing 50 $\cdot mg\ L^{-1}$ kanamycin) at 37 °C to an optical density ($OD_{600} \approx 0.6$). Fusion protein expression was induced by the addition of 0.1 mM to 1 mM IPTG. The crude protein solution was prepared from the pET-30a-*ApSerpin-ZX* recombinant strain after 1–6 h of induction at 16 °C to 37 °C. The targeted protein was detected by 12% SDS-PAGE and Western blotting analysis. The uninduced was utilized as control (NC).

In optimized conditions, the bacteria were suspended and washed twice in washing solution (10 mM Tris base, 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) and disrupted by ultrasonication. After centrifuged for 30 min at 4 °C at 5000 rpm, the insoluble fraction was retained and solubilized in 40 mL of dissolving solution (washing solution containing 6 M Urea). The solubilized protein was purified by affinity chromatography using His-trap Ni-column (Transgene, China). The column was washed with a 5-fold volume of solubilized protein solution by dissolving solution and eluted with a pH 4.5 of dissolving solution. Adjusted the concentration of eluted protein to approximate 100 $\mu g \cdot mL^{-1}$, refolding was performed by gradient dialysis to lower urea concentrations. The refolding buffer was 50 mM Tris base, 100 mM NaCl, 1 mM EDTA, 1 mM GSH, 0.1 mM GSSG containing different urea concentration (6-4-2-0 M). Finally, the refolded protein was enriched by an ultrafiltration tube for higher purity.

2.6. Western blotting analysis

After separation on 12% SDS-PAGE, the proteins were electro-transferred onto a PVDF membrane, which then was blocked overnight at 4 °C in TBS buffer (pH 7.4) containing 5% (w/v) powdered milk. After being incubated with 1:2000 diluted mouse anti-His monoclonal antibody (Abcam) for 3 h, the membrane was washed 3 times in TBS

buffer with 0.1% Tween-20. And then, it was incubated with 1:10000 diluted HRP-conjugated goat anti-mouse IgG secondary antibody (Abjunt). The blotting detection was used EasySee® Western Blot Kit (TransGene, Beijing, China).

2.7. Protease inhibition activity assay

Protease inhibition assay was performed to check the inhibition activity of the purified protein to trypsin, as described by Shah et al. [45]. Briefly, ApSerpin-ZX protein solution was diluted to different concentrations (50, 100, 150, 200, 250, 300 $\mu g \cdot mL^{-1}$) and 100 μL of diluted solution (distilled water as control, 30% acetic acid as blank) together with 200 μL (20 $\mu g \cdot mL^{-1}$, diluted by HCl) of trypsin and 100 μL of distilled water were incubated at 37 °C for 10 min. 500 μL (0.4 $mg \cdot mL^{-1}$, diluted by Dimethyl sulfoxide) of Nα-benzoyl-DL-arginine ρ-nitroanilide hydrochloride (BAρNA; pre-warmed to 37 °C) was added to the mixture and then incubated for 10 min. The reaction was then terminated by the addition of 100 μL of 30% acetic acid (v/v). The optical density of the obtained supernatant was measured at 410 nm. The inhibition ratio (%) was calculated by $(A_{bs}-A_s- A_b)/A_{bs}*100$ (A_s indicates OD of sample; A_{bs} indicates OD of sample blank; A_b indicates OD of blank).

2.8. Cryopreservation procedure

A. praecox embryogenic callus cryopreservation is used as an assessment model. The cryopreservation procedure was described previously [12]. Briefly, the embryogenic callus was precultured in MS medium supplemented with 171 $g \cdot L^{-1}$ sucrose and 3 $g \cdot L^{-1}$ phytagel at 4 °C for 48 h (preculture). The embryogenic callus was transferred into cryovials, which contained 2 mL loading solution (liquid MS medium with 136.92 $g \cdot L^{-1}$ sucrose, 184.18 $g \cdot L^{-1}$ glycerol and 1 $g \cdot L^{-1}$ KNO_3), and incubated for 60 min at 25 °C (osmoprotection). Then the embryogenic callus was transferred into PVS2 solution (liquid MS medium with 136.92 $g \cdot L^{-1}$ sucrose, 30 $g \cdot L^{-1}$ glycerol, 15 $g \cdot L^{-1}$ ethylene glycol, and 15 $g \cdot L^{-1}$ DMSO) for 40 min in an ice bath (dehydration). Then the cryovials were immediately plunged into liquid nitrogen for at least 1 h. After rapid warming in a 40 °C water bath for 90 s (rapid warming), embryogenic callus was washed with unloading solution (liquid MS medium with 410.75 $g \cdot L^{-1}$ sucrose and 1 $g \cdot L^{-1}$ KNO_3) for 30 min (dilution). Finally, the embryogenic callus was recovered on the proliferation medium under the same conditions as for subculture above (recovery).

2.9. Cell viability assessment

The survival of *A. praecox* embryogenic callus was detected by the TTC staining method as described previously [12]. After recovery for 24 h, embryogenic callus was placed into a black-capped vial with 2 mL of the 8 $g \cdot L^{-1}$ TTC solution (dissolved in 50 mM phosphate buffer, pH 7.4). The vials were then incubated at 25 °C for 24 h. The embryogenic callus was washed by sterile water three times. Then, they were treated with 5 mL of 95% alcohol to fade the red color in a 60–85 °C water bath for 60 min. After the samples were centrifugated at 3000×*g* for 5 min, the supernatant was measured at A485 using a visible spectrophotometer. The ratio of absorbance value of cryopreserved (sample) and non-cryopreserved (check) samples was taken as the survival.

2.10. Determination of MDA content

Cryopreservation treated embryogenic callus (0.2 g) were homogenized in 5 mL of 10% (w/v) trichloroacetic acid (TCA) on ice with a mortar and pestle. The homogenate was centrifuged at 5000 ×g at 4 °C for 10 min, and then 2 mL of supernatant was mixed with 2 mL of 0.6% thiobarbituric acid (dissolved in 10% TCA). The mixture was incubated at 100 °C for 30 min and cooled rapidly with flowing water. Then, it was centrifuged at 5000 ×*g* for 10 min. The MDA content was measured at 532, 600, and 450 nm, according to Hao et al. [26] with modifications.

2.11. Determination of H_2O_2 content

H_2O_2 content was assayed according to Peng et al. [38]. Cryopreservation treated embryogenic callus (0.2 g) were ground in 1.8 mL of 100 mM cold phosphate buffer (pH 7.4) on ice. Extracts were then centrifuged at 5000 ×*g* at 4 °C for 10 min. To 1 mL of the supernatant were added 1 mL 16% of H_2SO_4, 0.2 mL of 20% KI, and 3 drops of 50 mM molybdic acid. After reaction for 5 min, the absorbance of the solution was measured at 405 nm. Standards were prepared by using known amounts of H_2O_2 in the same manner as the tested samples.

2.12. Determination of OH· generation activity

OH· generation activity was assayed by its capacity to oxidize bromopyrogallol red (BPR) as described by Wu et al. [49]. Cryopreservation treated embryogenic callus (0.2 g) were ground in 1.8 mL of distilled water on ice. Extracts were then centrifuged at 5000 ×*g* at 4 °C for 10 min. A 0.3 mL of 0.2 mM $FeSO_4$, 0.3 mL of 1 mM BPR and 0.3 mL of 1% (v/v) H_2O_2 solutions were added to 1 mL of the supernatant (sample) while 1 mL of distilled water served as the CK. After reaction for 1 min, the solutions were measured at 550 nm. The difference between the sample and the CK was expressed as OH· generation activity.

2.13. Determination of SOD, POD and CAT activities

For extract preparation, 0.2 g of embryogenic callus was ground in 1.8 mL of 100 mM cool phosphate buffer (pH 7.4). Mixtures were then centrifuged at 5000×*g* at 4 °C for 10 min. The supernatant was used for the assay of activities of SOD, POD and CAT. The method was referred to our previous article [12]. The protein concentration was measured using Bradford assay.

2.14. Determination of caspase-1-like and caspase-3-like activities

Preparation of cell extract and assay of Caspase-1-like and Caspase-3-like activities were performed as described by Biswas and Mano [7]. Cryopreservation treated embryogenic callus (0.2 g) were ground in 50 mM sodium acetate (pH 5.5, containing 50 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride and 0.1 mM E64). Extracts were then centrifuged at 14,000 ×*g* for 30 min at 4 °C. The supernatant was collected to measure the activities of Caspase-1-like and Caspase-3-like. Caspase-1-like and Caspase-3-like activities were measured with synthetic tetrapeptide fluorogenic substrates Ac-YVAD-AMC and Ac-DEVD-AMC, respectively. Fluorescence of AMC (excitation 380 nm; emission 445 nm) was determined with a spectrofluorometer. The fluorescence intensity difference between the absence and the presence of an inhibitor was considered as the activity of the protease. A standard curve was prepared with AMC in the range of 0–200 μM.

2.15. Data analysis

All data were mean values ± SE of three experiments. SAS 9.3 was applied for statistical analysis [9]. One-way ANOVA was used to compare one or two variables. An unpaired *t*-test was used to compare two samples (or treatments). Differences were deemed significant at $p < 0.05$ and extremely significant at $p < 0.01$.

3. Results

3.1. ApSerpin-ZX expression respond to abiotic stress

The complete cDNA sequence of *ApSerpin-ZX* gene was 1727 bp in length, with a full ORF of 1341 bp, encoding a predicted protein of 446

amino acids. The DNA sequence was 3089 bp in length with a single intron (supplemental Fig. S1A). This was similar to other plant serpin genes, e.g., barley *HorvuZ4* (*BSZ4*), maize *Zeama9* and Populus *PoptrZx*, suggesting that this represents the standard gene structure for plant serpins. The ORF encoded a polypeptide of 446 amino acids with the predicted molecular weight of 49.13 kDa and theoretical isoelectric point of 5.17. No signal peptide and transmembrane domain were predicted suggesting that ApSerpin-ZX were unlikely to be exposed to the N-

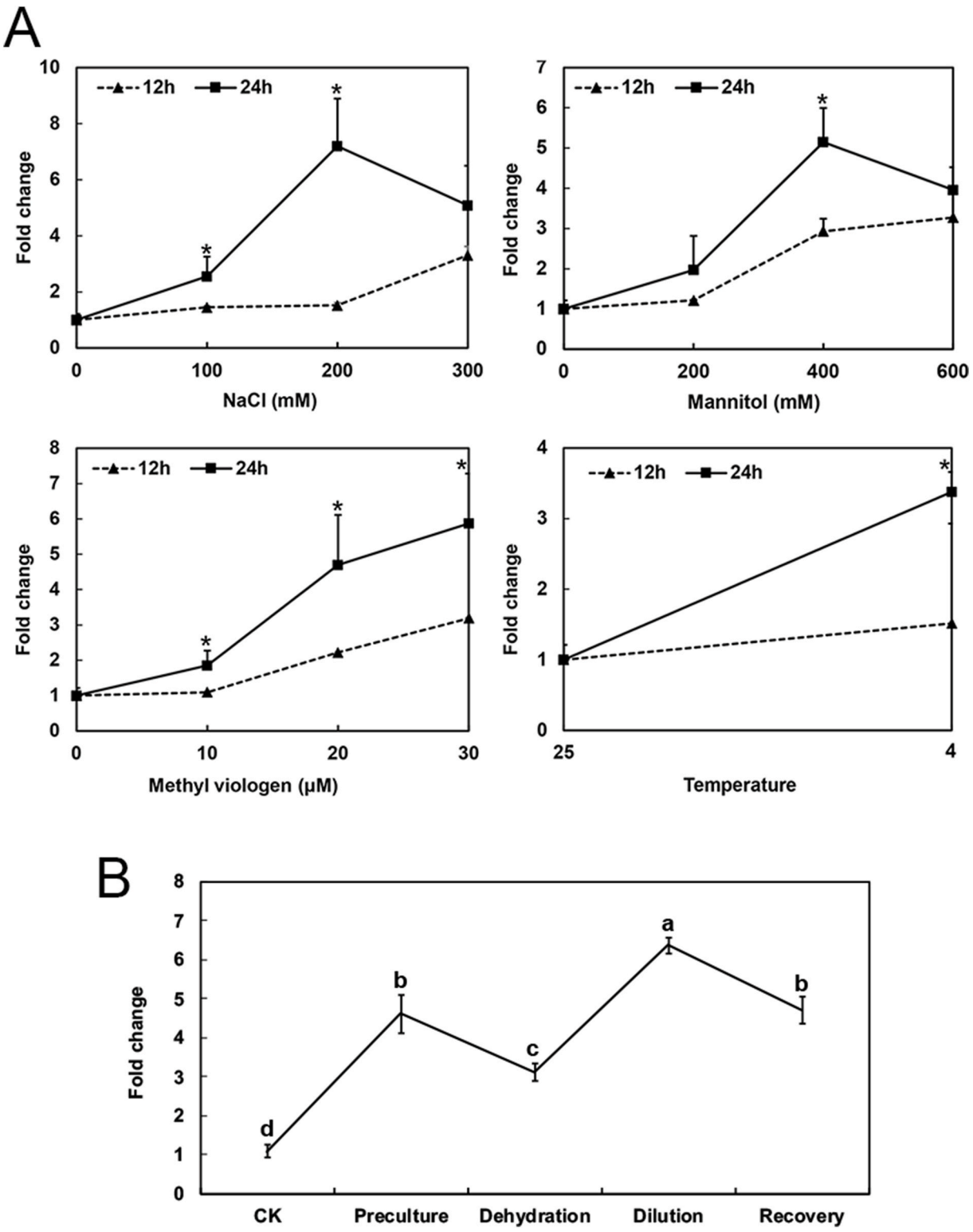

Fig. 1. Temporal expression of *ApSerpin-ZX* mRNA in *A. praecox* embryogenic callus under abiotic stress. (A) qPCR analysis of the expression of *ApSerpin-ZX* under different concentrations of NaCl, Mannitol, Methyl viologen and 4 °C. *ApActin* gene (CL5322.Contig7_AP_BB) was used as an internal control to calibrate the cDNA template for all the samples. The results are expressed as mean ± SD of three replicates. Significant differences evaluated by *t*-test are indicated as * ($P < 0.05$) between 12 h and 24 h in the same treatment. (B) qPCR analysis of the expression of *ApSerpin-ZX* during cryopreservation. The results are expressed as mean ± SD of three replicates. Different letters (a, b, c) indicate significant differences ($P < 0.05$) in the same group.

glycosylation machinery and thus may not be glycosylated *in vivo*. ApSerpin-ZX contained a plant serpin domain with 385-amino acid (aa 21–405) and a conserved RCL structure, which possessed cleavage sites of Phe(F) and Glu (E) (supplemental Fig. S1B). Meanwhile, the sequence of RCL was conserved with other plant serpin. Further phylogenetic analysis showed that ApSerpin-ZX belonged to monocot serpin (supplemental Fig. S1C).

To investigate the transcriptional response of *ApSerpin-ZX*, the expression profiles of *ApSerpin-ZX* under different abiotic stress were analyzed. The *ApSerpin-ZX* was upregulated under various abiotic stresses (Fig. 1). The expression of *ApSerpin-ZX* treated for 24 h was much higher than that of 12 h in each abiotic stress treatment. After treated with 200 mM NaCl for 24 h, the expression level of *ApSerpin-ZX* was about seven times than untreated sample. Similarly, the quantity of *ApSerpin-ZX* transcripts increased by almost five and six times when it was grown in 400 mM mannitol and 30 μM methyl viologen for 24 h, respectively. The expression level of *ApSerpin-ZX* was also improved by cold stress. The expression level of *ApSerpin* in the entire cryopreservation procedure was higher than that of untreated (CK). It was increased 4 times after preculture treatment. After dilution treatment, the expression level of *ApSerpin* reached the top, which was 6 times to CK. These results showed that *ApSerpin* expression was highly induced by different abiotic stress and compound stress in cryopreservation.

3.2. Ectopic expression of ApSerpin-ZX in E. coli strain

To obtain the protein of ApSerpin-ZX, the recombinant expression of ApSerpin-ZX in *E. coli Transetta* (DE3) was performed. SDS-PAGE electrophoresis analysis of crude extracts from the transformed *E. coli Trans*etta (DE3) showed high amounts of polypeptide with the expected molecular mass (ca. 49.13 + 3 = 52.13 kDa) of the ApSerpin-ZX (Fig. 2A). For exploration of the optimized expression condition of pET-30a-*ApSerpin-ZX*, we examined the yield of recombinant protein in a full combination of final IPTG concentration (0.1, 0.5 and 1 mM) and induction temperature (16, 25, 30 and 37 °C) (supplemental Fig. S2). SDS-PAGE electrophoresis analysis showed that the optimized expression condition was 0.1 mM IPTG inducted for 3 h at 25 °C. The expression level of ApSerpin-ZX recombinant protein was confirmed by western blotting assay (Fig. 2B).

3.3. ApSerpin-ZX possessed trypsin inhibition activity

ApSerpin-ZX protein was expressed as a non-soluble protein. The inclusion body purification method was used by a nickel column. The purified His-tagged ApSerpin-ZX protein was refolded through gradient dialysis for further experiments. The inhibition ratio of ApSerpin-ZX was confirmed by incubating trypsin with varying concentrations of ApSerpin-ZX (Fig. 3). 30 μg of ApSerpin-ZX inhibited more than 95% activity of trypsin. The concentration of ApSerpin-ZX, which inhibited trypsin's total inhibition activity by 50%, was considered one unit of protease inhibitor, e.g., 8.3 μg.

3.4. ApSerpin-ZX protein improved the survival of cryopreserved A. praecox

The efficacy of the recombinant ApSerpin-ZX protein as a cryoprotectant was determined in the embryogenic callus of *A. praecox*. Firstly, we added ApSerpin-ZX directly to embryogenic callus for cytotoxicity test (supplemental Fig. S3). The cell viability has barely changed after incubation. Hence, we estimate that ApSerpin-ZX recombinant protein has no cell toxicity. Then, the purified ApSerpin-ZX protein was added into the plant vitrification solution (PVS), which was the crucially important step in cryopreservation [13,16,44]. The viability of cryopreserved embryogenic callus increased as a function of growing quantities of ApSerpin-ZX protein from 0.6 to 1.2 mg·L^{-1} (Fig. 4). Although the survival decreased slightly with a high concentration of

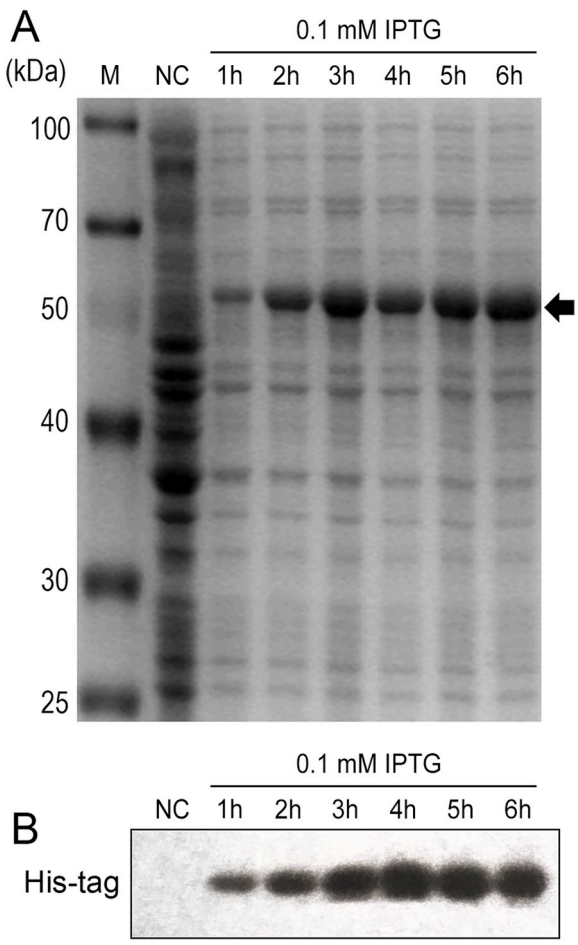

Fig. 2. Expression and western blotting analysis of ApSerpin-ZX on *E. coli*. (A) SDS-PAGE analysis of bacteria containing pET-30a-*ApSerpin-ZX* plasmid with or without induction by IPTG. Proteins were stained with Coomassie Brilliant Blue. Lane M, protein molecular mass marker (kDa); Line NC, negative control for ApSerpin-ZX (without induction); Line 1h~6h, 0.1 mM IPTG induced for 1~6 h. (B) Western blotting was performed using anti-6 × His-tag antibody. The inducing conduction was as same as (A) described.

ApSerpin-ZX protein, it was still higher than PVS without ApSerpin-ZX protein. The addition of 1.2 mg·L^{-1} ApSerpin-ZX protein achieved the most increased survival to 70.41%. The ApSerpin-ZX protein had a positive effect on the survival rate of cryopreserved *A. praecox* embryogenic callus. For a comprehensive evaluation of the ApSerpin-ZX protein on cryopreservation efficacy, Arabidopsis' regrowth geminated for 60 h was also tested (supplemental Fig. S4). The regrowth of seedlings from the procedure with ApSerpin-ZX protein was significantly elevated, especially for a concentration of 0.3 mg·L^{-1}. This result confirmed that ApSerpin-ZX protein had a positive effect on survival and regeneration.

3.5. Effect of ApSerpin-ZX protein on oxidative stress induced by cryopreservation

For further revealing the physiological response of ApSerpin-ZX protein in *A. praecox* embryogenic callus cryopreservation, the optimized concentration of ApSerpin-ZX protein (1.2 mg·L^{-1}) was applied as treated group, the standard procedure as control group. The degree of membrane lipid peroxidation of the cryopreserved cell had a significant relationship to the cell viability [39]. We tested the cell viability (Fig. 5A, B and 5C) and MDA content (Fig. 5D) during the procedure. It showed that addition of ApSerpin-ZX protein in the dehydration step elevated the cell viability and weakened the membrane lipid

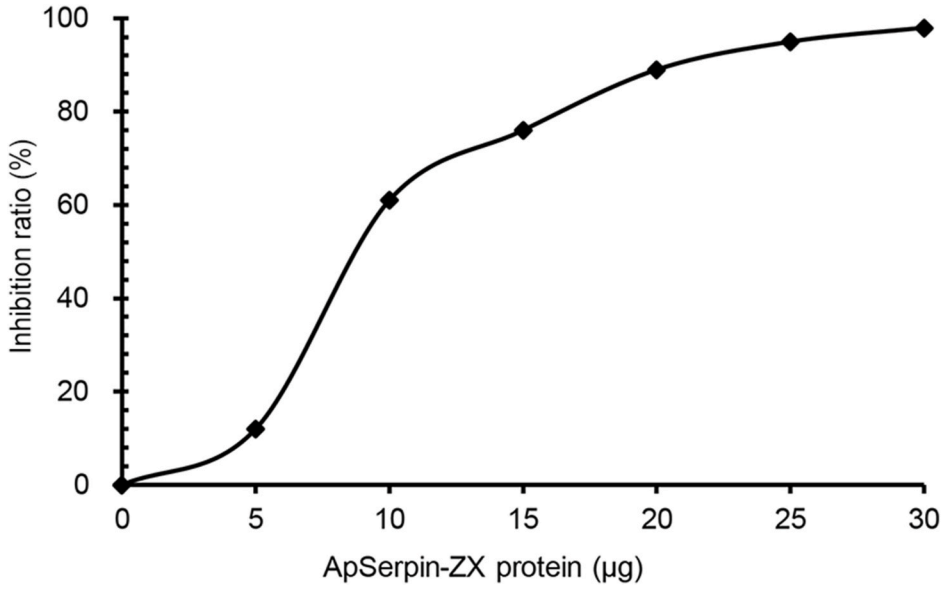

Fig. 3. Inhibitory activity of ApSerpin-ZX with trypsin. Inhibitory activity of ApSerpin-ZX was recorded as residual enzyme activity in the presence of inhibitor. The highest inhibition activity was regarded as 100% inhibition ratio.

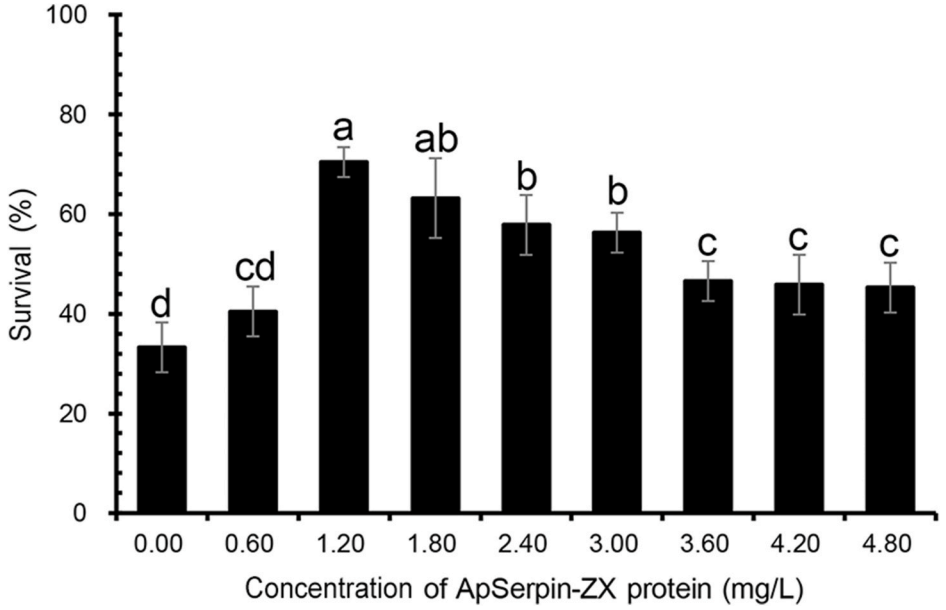

Fig. 4. The effect of different concentrations of exogenous ApSerpin-ZX protein on the survival of *A. praecox* embryogenic callus. The results are expressed as mean ± SD of three replicates. Different letters (a, b, c) indicate significant differences ($P < 0.05$).

peroxidation. H_2O_2 was the main ROS molecular inducing oxidative stress during *A. praecox* cryopreservation [12]. ApSerpin-ZX protein decreased H_2O_2 content (Fig. 5E). In the meanwhile, OH· generation activity was also reduced in dilution and recovery steps (Fig. 5F). The change of antioxidant enzymes' activities was also analyzed (Fig. 5G, H and 5I). POD and CAT activities strongly increased, while SOD activity showed different feedback. The scavenging activity of H_2O_2 was enhanced by ApSerpin-ZX protein. Consequently, ApSerpin-ZX protein interfered with ROS generation and then weakened the oxidative stress and improved cell viability.

3.6. Effect of ApSerpin-ZX protein on caspases-like activities in cryopreservation

Cryopreservation treatment would activate the caspase activity in animal cell and caspase-like activities in plant cells. We examined the caspase-1-like and caspase-3-like activities during cryopreservation procedure (Fig. 5J and 5K). Caspase-1-like and caspase-3-like activities were significantly reduced in the process with ApSerpin-ZX protein. The caspase-1-like activity was decreased by 43.77% in the dehydration stage and 24.87% in the recovery stage, respectively. ApSerpin-ZX protein also weakened the caspase-3-like activity. This result suggested that ApSerpin-ZX protein was related to inhibiting from PCD event during cryopreservation.

4. Discussion

In plants, more than 20 serpins were isolated or cloned from wheat [18,43], *Hordeum vulgare* [19], *Cucurbita maxima* [50], *Avena sativa* [28], and their inhibitory properties were examined. The serpin family

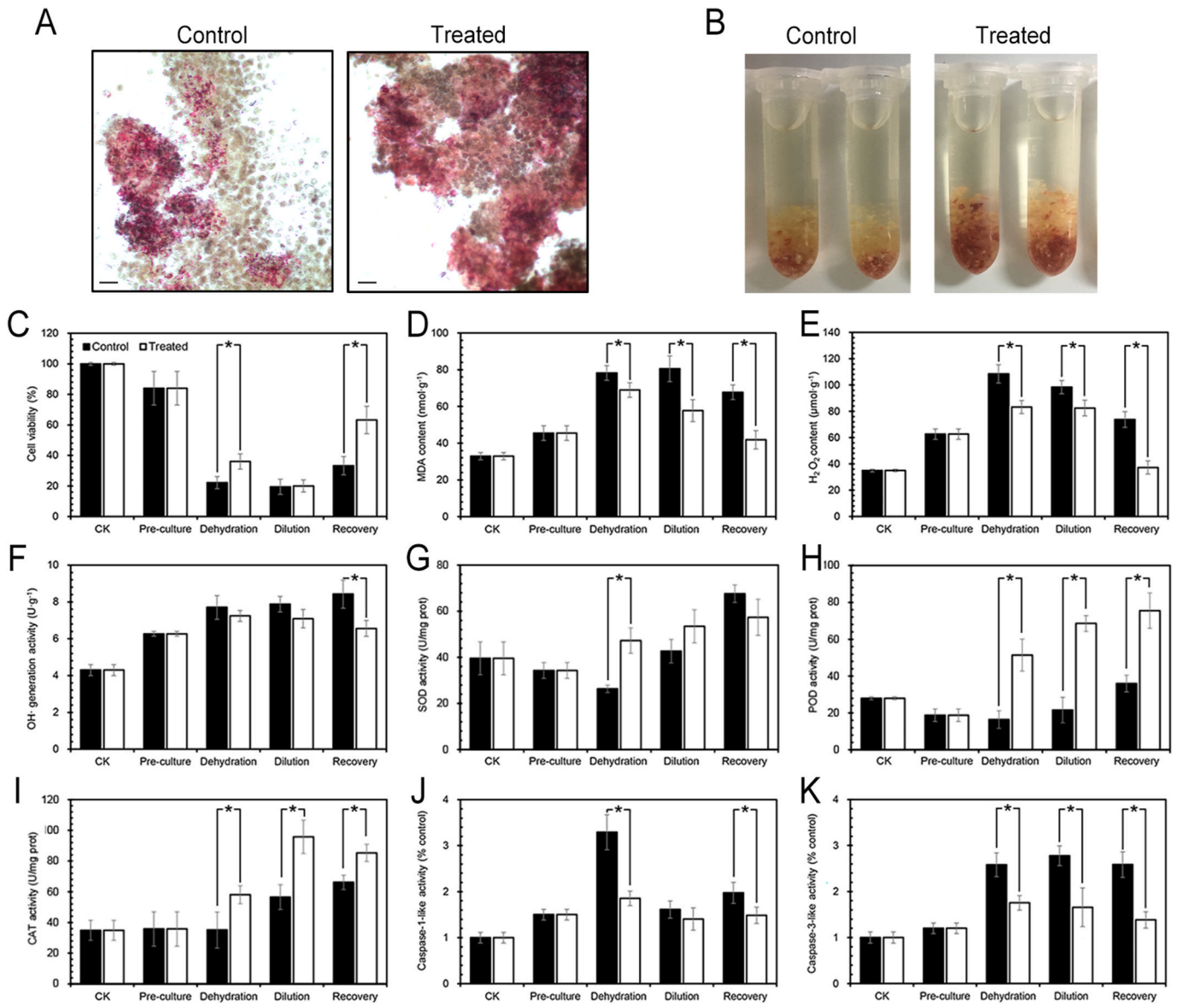

Fig. 5. The cell viability and physiological response of cryopreserved *A. praecox* embryogenic callus after the addition of ApSerpin-ZX protein. (A–B) TTC staining pictures of control and treated group. Bar = 200 μm. (C) Cell viability in the treated group. The cell viability was assessed by TTC staining method. (D–F) The change of oxidation level of control and treated group. The MDA content (D), H_2O_2 content (E) and OH· generation activity (F) were treated as the evaluation index of oxidation level. (G–I) The change of SOD (G), POD (H) and CAT activities (I) of control and treated group. (J–K) The caspase-1-like (J) and caspase-3-like (K) activities of control and treated group. Cryopreservation procedure with the optimized concentration of ApSerpin-ZX protein (1.2 mg L^{-1}) was regarded as a treated group, and the standard procedure was regarded as a control group. The results are expressed as mean ± SD of three replicates. Significant differences evaluated by *t*-test are indicated as * ($P < 0.05$) between control and treated.

has been characterized on a genome-wide scale in Arabidopsis [20], rice [23] and wheat [4]. The serpin family has gained attention in many plants for its role in disease response, i.e., maize [21], tomato [11], and abiotic stress response in *Arabidopsis* [1], rice [5], durum wheat [32] and tobacco [10], and even in cereal grain development in wheat [36] and barley [42]. In this study, we identified *ApSerpin-ZX* gene from *A. praecox*, and ApSepin-ZX protein was characterized as a potential cryoprotective.

The expression of ApSeprin-*ZX* responds to abiotic stress at transcriptional level. The mRNA of *ApSerpin-ZX* was increased after treated with NaCl, methyl viologen, mannitol and 4 °C for 12 h and further increased within 24 h treatment. This indicated that the expression of *ApSerpin-ZX* was induced by salt, oxidative, osmotic and cold stresses. That is to say, the function of ApSerpin-ZX was involved in abiotic stress response. This was consistent with other plant serpins. *AtSerpin1* was upregulated by cold stress, that it increased up to 25-fold in the leaves and 5-fold in the roots of plants exposed to 4 °C over a 24 h period [22, 31]. *AtSerpinZ4* (At2g26390) was increased by salt stress, with 16-fold change in roots after 6 h of salt stress and dropped back to a 2-fold increase after 24 h of salt stress [41]. Except for single abiotic stress, the transcription of *ApSerpin-ZX* was also regulated by cryopreservation procedure. The mRNA level of *ApSerpin-ZX* was increased at preculture, then declined slightly for dehydration, increased even more at dilution and declined slightly at recovery during the procedure. This indicated *ApSerpin-ZX* was upregulated by cryopreservation treatment and respond to cryoinjury. While for the relative protein level, ApSerpin-ZX was elevated almost 3-fold after preculture treatment and then fell back to the standard level in the following procedure (supplementary Fig. S5). The generation of cell-death proteases might explain this. Serpin activity was also inhibited and hydrolyzed by the protease when accentuated

cell death occurrence [33,34]. Hence, ApSerpin-ZX cannot be accumulated in the later stage of cryopreservation. This phenomenon offered the possibility for external ApSerpin-ZX to inhibit the activities of proteases induced by cryopreservation.

To investigate the cryoprotective property of ApSerpin-ZX protein, the effect of ApSerpin-ZX on the regeneration was assessed. It has been reported that serine proteases inhibitors had improvement to biological samples cryopreservation. Recombinant SPINK3 (Serine Protease Inhibitor Kazal type 3) is bound to the apical portion of both fresh and frozen ram sperm to improve sperm progressive motility [51]. For this study, we selected two well-researched plants in our lab as materials, e.g., *A. praecox* embryogenic callus and *Arabidopsis* seedlings germinated for 60 h. Our results indicated that proper concentration of ApSerpin-ZX could protect plant cells from cryoinjury, which include, but are not limited to osmosis stress, extreme temperatures, chemical toxicity, oxidative stress or programmed cell death. In animal cells cryopreservation, cell death was relative to the caspase activity, especially caspase-3 [6]. Although no homologous gene of caspase-3 was found in plants, the activity of caspase-3-like was detected when PCD took place [7]. The activity of caspase-1-like in the plant often reflects vacuolar processing enzyme activity, which is a certain regulator of PCD [35]. Many plant serpins are all efficient inhibits of mammalian serine proteinases *in vitro*, suggesting that they act as inhibitors *in vivo* [27]. AtSerpin1 inhibits the activity of proteases, such as RD21, MC1 and MC9 *in vivo*. In cryopreservation of *A. praecox* embryogenic callus, we also found that the caspase-3-like and caspase-1-like activities were elevated along with the rate of PCD. Given that, to further confirm the effect of exogenous ApSerpin-ZX protein, oxidative stress and PCD related physiological indexes were detected, such as MDA content, ROS level, antioxidant enzymes and caspase-like activities, which were proved to be the indicator of cell viability in cryopreservation. The conclusion was drawn that ApSerpin-ZX elevated H_2O_2 scavenging capacity through activating activities of POD and CAT. Therefore, the membrane lipid peroxidation was weakened. In the meanwhile, reduced ROS level decreased the activity of caspase-3 and caspase-1-like, suggesting that ApSerpin-ZX might mitigate the occurrence of PCD. However, more conclusive evidence is still needed. Based on this study, ApSerpin-ZX may find a place as a desirable biotechnological tool to achieve a higher proportion of surviving cells in plant cryopreservation.

Declaration of competing interest

The authors declare that they have no conflict of interest.

Acknowledgments

This work was supported by the National Natural Science Foundation of China (31901351 and 31670694).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cryobiol.2020.11.018.

References

[1] J.W. Ahn, B.J. Atwell, T.H. Roberts, Serpin genes AtSRP2 and AtSRP3 are required for normal growth sensitivity to a DNA alkylating agent in *Arabidopsis*, BMC Plant Biol. 9 (1) (2009) 52, https://doi.org/10.1186/1471-2229-9-52.

[2] J.M. Baust, Buskirk Van, J.G. Baust, Cell viability improves following inhibition of cryopreservation-induced apoptosis, In Vitro Cell. Dev. Biol. Anim. 36 (4) (2000) 262–270, https://doi.org/10.1290/1071-2690(2000)036<0262:cvifio>2.0.co;2.

[3] S.P. Becerra, A. Sagasti, P. Spinella, V. Notario, Pigment epithelium-derived factor behaves like a noninhibitory serpin. Neurotrophic activity does not require the serpin reactive loop, J. Biol. Chem. 270 (43) (1995) 25992–25999, https://doi.org/10.1074/jbc.270.43.25992.

[4] H.R. Benbow, L.S. Jermiin, F.M. Doohan, Serpins: genome-wide characterisation and expression analysis of the serine protease inhibitor family in *Triticum aestivum*, G3 (Bethesda) 9 (8) (2019) 2709–2722, https://doi.org/10.1534/g3.119.400444.

[5] L. Bhattacharjee, P.K. Singh, S. Singh, A.K. Nandi, Down-regulation of rice serpin gene *OsSRP-LRS* exaggerates stress-induced cell death, J. Plant Biol. 58 (5) (2015) 327–332, https://doi.org/10.1007/s12374-015-0283-6.

[6] A. Bissoyi, B. Nayak, K. Pramanik, S.K. Sarangi, Targeting cryopreservation-induced cell death: a review, Biopreserv. Biobanking 12 (1) (2014) 23–34, https://doi.org/10.1089/bio.2013.0032.

[7] M.S. Biswas, J. Mano, Reactive carbonyl species activate Caspase-3-like protease to initiate programmed cell death in plants, Plant Cell Physiol. 57 (7) (2016) 1432–1442, https://doi.org/10.1093/pcp/pcw053.

[8] F. Boer, A.M. Dräger, H.M. Pinedo, F.L. Kessler, E. Wal, A.R. Jonkhoff, J. Lelie, P. C. Huijgens, G.J. Ossenkoppele, G.J. Schuurhuis, Extensive early apoptosis in frozen-thawed CD34-positive stem cells decreases threshold doses for haematological recovery after autologous peripheral blood progenitor cell transplantation, Bone Marrow Transplant. 29 (3) (2002) 249–255, https://doi.org/10.1038/sj.bmt.1703357.

[9] Inc Booksx, SAS Institute, Sas 9.1.3 Language Reference: Dictionary//SAS 9.1.3 Language Reference: Dictionary, SAS Institute, Incorporated, 2006.

[10] G.G. Brichkova, A.M. Shishlova, T.V. Maneshina, N.A. Kartel, Tolerance to aluminum in genetically modified tobacco plants, Cytogenetics 41 (3) (2007) 151–155, https://doi.org/10.3103/S0095452707030036.

[11] D.M. Carli, M.E. Villani, L. Bianco, R. Lombardi, G. Perrotta, E. Benvenuto, M. Donini, Proteomic analysis of the plant-virus interaction in *cucumber mosaic virus* (CMV) resistant transgenic tomato, J. Proteome Res. 9 (11) (2010) 5684–5697, https://doi.org/10.1021/pr100487x.

[12] G.Q. Chen, L. Ren, D. Zhang, X.H. Shen, Glutathione improves survival of cryopreserved embryogenic calli of *Agapanthus praecox* subsp. *orientalis*, Acta Physiol. Plant. 38 (10) (2016) 250, https://doi.org/10.1007/s11738-016-2271-y.

[13] G.Q. Chen, L. Ren, J. Zhang, B.M. Reed, D. Zhang, X.H. Shen, Cryopreservation affects ROS-induced oxidative stress and antioxidant response in *Arabidopsis* seedlings, Cryobiology 70 (1) (2015) 38–47, https://doi.org/10.1016/j.cryobiol.2014.11.004.

[14] G.Q. Chen, D. Zhang, X.H. Shen, Cloning and characterization of ApCystatin, a plant cystatin gene from Agapanthus praecox ssp orientalis responds to abiotic stress, Protein Expr. Purif. 149 (2018) 66–74, https://doi.org/10.1016/j.pep.2018.04.014.

[15] M. Chow-Shi-Yée, M. Grondin, F. Ouellet, D.A. Averill-Bates, Control of stress-induced apoptosis by freezing tolerance-associated wheat proteins during cryopreservation of rat hepatocytes, Cell Stress Chaperones 11 (2020), https://doi.org/10.1007/s12192-020-01115-y.

[16] S.P. Chua, M.N. Normah, Effect of preculture, PVS2 and vitamin C on survival of recalcitrant Nephelium ramboutan-ake shoot tips after cryopreservation by vitrification, Cryo Lett. 32 (6) (2011) 506–515, https://doi.org/10.7202/1017428ar.

[17] A.K. Cipriano, D.M. Gondim, I.M. Vasconcelos, J.A. Martins, A.A. Moura, F. B. Moreno, A.C. Monteiro-Moreira, J.G. Melo, J.E. Cardoso, A.L. Paiva, J. T. Oliveira, Proteomic analysis of responsive stem proteins of resistant and susceptible cashew plants after *Lasiodiplodia theobromae* infection, J Proteomics 113 (15) (2015) 90–109, https://doi.org/10.1016/j.jprot.2014.09.022.

[18] S.W. Dahl, S.K. Rasmussen, J. Hejgaard, Heterologous expression of three plant serpins with distinct inhibitory specificities, J. Biol. Chem. 271 (41) (1996) 25083–250888, https://doi.org/10.1074/jbc.271.41.25083.

[19] S.W. Dahl, S.K. Rasmussen, L.C. Petersen, J. Hejgaard, Inhibition of coagulation factors by recombinant barley serpin BSZx, FEBS Lett. 394 (2) (1996) 165–168, https://doi.org/10.1016/0014-5793(96)00940-4.

[20] R. Fluhr, N. Lampl, T.H. Roberts, Serpin protease inhibitors in plant biology, Physiol. Plantarum 145 (1) (2012) 95–102, https://doi.org/10.1111/j.1399-3054.2011.01540.x.

[21] I. Forieri, U. Hildebrandt, M. Rostas, Salinity stress effects on direct and indirect defence metabolites in maize, Environ. Exp. Bot. 122 (2016) 68–77, https://doi.org/10.1016/j.envexpbot.2015.09.007.

[22] S. Fowler, M.F. Thomashow, *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway, Plant Cell 14 (8) (2002) 1675–1690, https://doi.org/10.1105/tpc.003483.

[23] S.E. Francis, R.A. Ersoy, J.W. Ahn, B.J. Atwell, T.H. Roberts, Serpins in rice: protein sequence analysis, phylogeny and gene expression during development, BMC Genom. 13 (2012) 449, https://doi.org/10.1186/1471-2164-13-449.

[24] P.G. Gettins, Serpin structure, mechanism, and function, Chem. Rev. 102 (12) (2002) 4751–4804, https://doi.org/10.1021/cr010170+.

[25] M. Grondin, M. Chow-Shi-Yée, F. Ouellet, D.A. Averill-Bates, Wheat enolase demonstrates potential as a non-toxic cryopreservation agent for liver and pancreatic cells, Biotechnol. J. 10 (2015) 801–810, https://doi.org/10.1002/biot.201400562.

[26] Z.B. Hao, J. Cang, Z. Xu, Experimental of Plant Physiology, Harbin Institute of 567 Technology Press China, 2004, pp. 106–108.

[27] J. Hejgaard, Inhibitory serpins from rye grain with glutamine as P1 and P2 residues in the reactive center, FEBS Lett. 488 (3) (2001) 149–153, https://doi.org/10.1016/s0014-5793(00)02425-x.

[28] J. Hejgaard, S. Hauge, Serpins of oat (Avena sativa) grain with distinct reactive centres and inhibitory specificity, Physiol. Plantarum 116 (2) (2002) 155–163, https://doi.org/10.1034/j.1399-3054.2002.1160204.x.

[29] J. Hejgaard, T.H. Roberts, Plant Serpins. Molecular and Cellular Aspects of the Serpinopathies and Disorders in Serpin Activity, 2007, pp. 279–300.

[30] J.A. Huntington, R.J. Read, R.W. Carrell, Structure of a serpin-protease complex shows inhibition by deformation, Nature 407 (6806) (2000) 923–926, https://doi.org/10.1038/35038119.

[31] E. Koh, R. Carmieli, A. Mor, R. Fluhr, Singlet oxygen-induced membrane disruption and serpin-protease balance in vacuolar-driven cell death, Plant Physiol. 171 (3) (2016) 1616–1625, https://doi.org/10.1104/pp.15.02026.
[32] P. Laino, D. Shelton, C. Finnie, A.M. De Leonardis, A.M. Mastrangelo, B. Svensson, D. Lafiandra, S. Masci, Comparative proteome analysis of metabolic proteins from seeds of durum wheat (cv. Svevo) subjected to heat stress, Proteomics 10 (12) (2010) 2359–2368, https://doi.org/10.1002/pmic.200900803.
[33] N. Lampl, O. Budai-Hadrian, O. Davydov, T.V. Joss, S.J. Harrop, P.M. Curmi, T. H. Roberts, R. Fluhr, Arabidopsis AtSerpin1, crystal structure and *in vivo* interaction with its target protease RESPONSIVE TO DESICCATION-21 (RD21), J. Biol. Chem. 285 (18) (2010) 13550–13560, https://doi.org/10.1074/jbc.M109.095075.
[34] S. Lema Asqui, D. Vercammen, I. Serrano, M. Valls, S. Rivas, F. Van Breusegem, F. L. Conlon, J.L. Dangl, N.S. Coll, AtSERPIN1 is an inhibitor of the metacaspase AtMC1-mediated cell death and autocatalytic processing in planta, New Phytol. 218 (3) (2018) 1156–1166, https://doi.org/10.1111/nph.14446.
[35] N. Hatsugai, K. Yamada, S. Goto-Yamada, I. Hara-Nishimura, Vacuolar processing enzyme in plant programmed cell death, Front. Plant Sci. 6 (2015) 234, https://doi.org/10.3389/fpls.2015.00234.
[36] H. Ostergaard, S.K. Rasmussen, T.H. Roberts, J. Hejgaard, Inhibitory serpins from wheat grain with reactive centers resembling glutamine-rich repeats of prolamin storage proteins. Cloning and characterization of five major molecular forms, J. Biol. Chem. 275 (43) (2000) 33272–33279, https://doi.org/10.1074/jbc.M004633200.
[37] B. Panis, B. Piette, R. Swennen, Droplet vitrification of apical meristems: a cryopreservation protocol applicable to all Musaceae, Plant Sci. 168 (1) (2005) 45–55, https://doi.org/10.1016/j.plantsci.2004.07.022.
[38] J. Peng, D. Wang, C. Xu, L. Chen, F. Deng, X. Zhang, Ammonium molybdate method for detecting the activities of rice catalase, Chin Agric Sci Bull 25 (16) (2009) 61–64.
[39] L. Ren, D. Zhang, X.N. Jiang, Y. Gai, W.M. Wang, B.M. Reed, X.H. Shen, Peroxidation due to cryoprotectant treatment is a vital factor for cell survival in *Arabidopsis* cryopreservation, Plant Sci. 212 (2016) 37–47, https://doi.org/10.1016/j.plantsci.2013.07.011.
[40] I. Risenga, P. Watt, D. Mycock, Programmed cell death and necrosis during cryopreparative drying of *in vitro* Eucalyptus grandis axillary buds, Cryo Lett. 34 (6) (2013) 583–597.
[41] T.H. Roberts, J. Hejgaard, Serpins in plants and green algae, Funct. Integr. Genom. 8 (1) (2008) 1–27, https://doi.org/10.1007/s10142-007-0059-2.
[42] T.H. Roberts, S. Marttila, S.K. Rasmussen, J. Hejgaard, Differential gene expression for suicide-substrate serine proteinase inhibitors (serpins) in vegetative and grain tissues of barley, J. Exp. Bot. 54 (391) (2003) 2251–2263, https://doi.org/10.1093/jxb/erg248.
[43] I. Rosenkrands, J. Hejgaard, S.K. Rasmussen, S.E. Bjørn, Serpins from wheat grain, FEBS Lett. 343 (1) (1994) 75–80, https://doi.org/10.1016/0014-5793(94)80610-1.
[44] A. Sakai, S. Kobayashi, Cryopreservation of nucellar cells of navel orange (*Citru sinensis* var. *brasiliensis* Tanaka) by vitrification, Plant Cell Rep. 9 (1) (1990) 30–33, https://doi.org/10.1007/BF00232130.
[45] K.R. Shah, D.K. Patel, A. Pappachan, C.R. Prabha, D.D. Singh, Characterization of a Kunitz-type serine protease inhibitor from Solanum tuberosum having lectin activity, Int. J. Biol. Macromol. 83 (2016) 259–269, https://doi.org/10.1016/j.ijbiomac.2015.11.068.
[46] M. Solomon, The involvement of cysteine proteases and protease inhibitor genes in the regulation of programmed cell death in plants, Plant Cell 11 (3) (1999) 431–444, https://doi.org/10.1105/tpc.11.3.431.
[47] D. Vercammen, B. Belenghi, B. van de Cotte, T. Beunens, J.A. Gavigan, R. De Rycke, A. Brackenier, D. Inze, J.L. Harris, F. Van Breusegem, Serpin1 of Arabidopsis thaliana is a suicide inhibitor for metacaspase 9, J. Mol. Biol. 364 (4) (2006) 625–636, https://doi.org/10.1016/j.jmb.2006.09.010.
[48] K. Watanabe, M. Ueno, D. Kamiya, A. Nishiyama, M. Matsumura, T. Wataya, J. B. Takahashi, S. Nishikawa, S. Nishikawa, K. Muguruma, Y. Sasai, A ROCK inhibitor permits survival of dissociated human embryonic stem cells, Nat. Biotechnol. 25 (6) (2007) 681–686, https://doi.org/10.1038/nbt1310.
[49] N. Wu, F. Ren, X. Wu, Assay of hydroxyl radical produced in Co^{2+}-H_2O_2 reaction system by spectrophotometry with bromopyrogallol red, J. Instrum. Anal. 20 (2001) 52–54.
[50] B.C. Yoo, K. Aoki, Y. Xiang, L.R. Campbell, R.J. Hull, B. Xoconostle-Cázares, J. Monzer, J.Y. Lee, D.E. Ullman, W.J. Lucas, Characterization of cucurbita maxima phloem serpin-1 (CmPS-1). A developmentally regulated elastase inhibitor, J. Biol. Chem. 275 (45) (2000) 35122–35128, https://doi.org/10.1074/jbc.M006060200.
[51] L. Zalazar, A. Ledesma, F. Hozbor, A. Cesari, Heterologous recombinant protein with decapacitating activity prevents and reverts cryodamage in ram sperm: an emerging biotechnological tool for cryobiology, Anim. Reprod. Sci. 164 (2016) 31–39, https://doi.org/10.1016/j.anireprosci.2015.11.007.
[52] D. Zhang, L. Ren, G.Q. Chen, J. Zhang, B.M. Reed, X.H. Shen, ROS-induced oxidative stress and apoptosis-like event directly affect the cell viability of cryopreserved embryogenic callus in *Agapanthus praecox*, Plant Cell Rep. 34 (9) (2015) 1499–1513, https://doi.org/10.1007/s00299-015-1802-0.
[53] D. Zhang, L. Ren, J.H. Yue, Y.B. Shi, L.H. Zhuo, L. Wang, X.H. Shen, RNA-Seq-based transcriptome analysis of stem development and dwarfing regulation in *Agapanthus praecox* ssp, *orientalis* (Leighton) Leighton, Gene 565 (2) (2015) 252–267, https://doi.org/10.1016/j.gene.2015.04.013.
[54] D. Zhang, L. Ren, J.H. Yue, L. Wang, L.H. Zhuo, X.H. Shen, A comprehensive analysis of flowering transition in *Agapanthus praecox* ssp. *orientalis* (Leighton) Leighton by using transcriptomic and proteomic techniques, J proteomics 80 (2013) 1–25, https://doi.org/10.1016/j.jprot.2012.12.028.