

## Effect of cryopreservation on the efficiency of exogenous gene, genetic transformation and expression level of *Arabidopsis thaliana*

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### Abstract

**Background:** Cryopreservation refers to the storage of a living organism at ultra-low-temperature for long-term preservation of plant germplasm. The effect of cryopreservation on the efficiency of exogenous gene genetic transformation and expression level were studied herein. In this work, transgenic *Arabidopsis thaliana* were successfully conserved in vitro by cryopreservation methods.

**Results:** The effects of osmotic stress due to cryoprotectants during pretreatment and of storage at -196°C on the stability, the efficiency of genetic transformation and the expression level of exogenous gene were analyzed in *Arabidopsis*. The results showed that there had not any significant increasing in the efficiency of genetic transformation after cryopreservation, and our observation was not in agreement with earlier reports. Transgenic *Arabidopsis* lines over-expressing ATOST1 gene were used for the real-time PCR analysis, and the result indicated that the expression of the ATOST1 gene was up-regulated about 2.4-fold in the transgenic seedlings tissues retrieved from cryopreservation than those non-cryopreserved counterparts.

**Conclusions:** Cryopreservation could improve the expression of exogenous gene, however, could not promote the genetic transformation obviously.

**Keywords:** *Arabidopsis thaliana*; cryopreservation; real-time PCR; transformation efficiency.

### INTRODUCTION

Cryopreservation strategies could be successfully employed to reduce genetic variation and could be an important tool for the long-term preservation of germplasm (Elleuch et al. 1998). However, the rapid plunge into liquid nitrogen of unprotected cells could cause selection of physiological, epigenetic or genetic variants (Moukadiri et al. 1999).

Many studies have been conducted to test the genetic stability of cryopreserved plant materials. SSCP analysis and transient gene expression experiments indicated the stable maintenance and normal expression of the GUS gene in transgenic pear shoots retrieved from in vitro conservation (Hao et al. 2005). Rice calli that had been directly stored in liquid nitrogen also demonstrated a higher competence for genetic transformation than their unfrozen counterparts, as indicated by the transient gene expression levels obtained after particle bombardment (Moukadiri et al. 1999). Cryopreservation may usually induce DNA alterations, especially at the epigenetic level accomplished by altered DNA methylation status. Hao et al. (2001) and Hao et al. (2002) found some demethylated sites after cryopreservation by vitrification in strawberry and apple.

In eukaryotes, particularly, higher plants, the DNA methylation of promoter regions usually inhibit transcription. But methylation in coding regions does not generally affect gene expression (Chan et al. 2005). The DNA methylation status of the treatment samples varied during the processes of

cryopreservation, and both *de novo* methylation and demethylation were observed. Furthermore, the samples treated in liquid nitrogen showed more demethylation sites than those not treated in liquid nitrogen determined by methylation-sensitive amplification polymorphism technique in *Arabidopsis* (Wang and He, 2009).

The present study aimed to determine whether the cryopreservation methods affecting the efficiency of genetic transformation and the level of exogenous gene expression in *Arabidopsis thaliana*.

## MATERIALS AND METHODS

### Plant materials, growth conditions, and transformation

The *OST1*-pGEX-2TK constructs were introduced into *Agrobacterium tumefaciens* strain GV3101, *Arabidopsis thaliana* ecotype Columbia was used as the wild type for transformation employing the standard *Agrobacterium*-mediated floral dip method (Clough and Bent, 1998), and seeds later were harvested (for gene overexpression assays). The constructs were obtained from the Laboratory of Plant Stress Biology (Henan University). Putative transformants were selected by planting seeds on Murashige and Skoog (1962) (MS) basal medium supplied with 30 mg ml<sup>-1</sup> of hygromycin, then the hygromycin -resistant seedlings were transferred to soil at 12 days after germination and placed in a growth chamber at 22°C under a 16-hrs-light/8-hrs-dark photoperiod and 70% RH (Jiang et al. 2006). Seeds were harvested separately from individual plants.

### Cryopreservation procedures

Two days old seedlings were immersed in loading solution (MS liquid medium + 2 M glycerol + 0.4 M sucrose) for 20 min at room temperature (1.0 ml for 30-40 seedlings in one 2.0 ml cryovial). Loading solution was then removed from the cryovial and rapidly replaced by filtered sterilized cryoprotective solution PVS2 (30% w/v glycerol, 15% w/v ethylene glycol and 15% w/v Dimethylsulfoxide in liquid MS medium supplemented with 0.4 M sucrose) and left at 0°C for 50 min and we named those procedures as pre-treatment. For cryopreservation treatment, the cryovials were then rapidly immersed in liquid nitrogen for at least 1 hr. Thawing was carried out by immersing cryovials in a water bath at 40°C for 60-90 sec, and cryovials were shaken vigorously. Subsequently, PVS2 solution was removed and replaced with unloading solution (MS liquid medium + 1.2 M sucrose) for 40 min, and the unloading solution was replaced once every 10 min. The seedlings were then cultured on MS medium and maintained under growth conditions as above. The cryopreserved samples had been done with exactly as above steps. Some seedlings were treated only with the pre-treatment (loading and cryoprotection) and then employed unloading and re-culture, which hadn't been cryostored in liquid nitrogen and thawed in 40°C water bath, were used as parallel experiments, and non-treatment samples used as control.

### DNA extraction and PCR analysis

When all the seedlings grew at more than 8 leaves, 1-2 leaves of each seedlings were used to extract DNA with CTAB method (Dellaporta et al. 1983) for the next molecular analysis.

Hygromycin-resistant plants were reexamined to eliminate pseudo transgenic lines by PCR amplification with the following primer pairs: sense: 5' AAGATGTTGGCGACCTCGT ATTGG3', antisense: 5'TTCGACAGCGTCTCCGACCTGAT3', these primer pairs are expected to amplify a 598-bp fragment of the *OST1* gene encoding sequence. PCR amplification was performed in a 25 µl volume containing 50 ng template DNA, 2.5 µl 10 x PCR buffer, 2.0 mmol L<sup>-1</sup> MgCl<sub>2</sub>, 120 µmol L<sup>-1</sup> dNTPs, 1.25 U of Taq DNA polymerase, 0.2 µmol L<sup>-1</sup> Primer 1 and Primer 2 each. The PCR reaction was performed in a PTC-100 machine, samples were heated to 94°C for 5 min and then amplified in a DNA thermocycler by 30 cycles of 30 sec annealing at 56°C, 1 min elongation at 72°C and 30 sec denaturation at 94°C, followed by 1 cycle of 10 min at 72°C. Electrophoretic separation of the amplified DNA fragment was carried out in 1.0% agarose gel containing 0.5 mg mL<sup>-1</sup> ethidium bromide. The gels were visualized and photographed with UV light. Positive samples lines were used for the following analysis.

## Statistical analysis

Data of the transformation efficiency of the *Arabidopsis* exogenous gene after cryopreservation, pre-treatment and untreated were submitted to analysis of variance using SPSS v11.5 software.

## Reverse transcription PCR and real-time PCR

Total RNA was isolated from 200 mg 15 days-old samples seedlings (cryopreserved, pre-treatment and control) with Trizol reagent. 5 µg of total RNA were used in reverse transcription with Moloney murine leukemia virus reverse transcriptase (Takara). Reverse transcription conditions were as follows: at 70°C for 5 min, at 42°C for 1 hr, and at 95°C for 5 min. The cDNA thereby obtained was used for RT-PCR and real-time quantitative RT-PCR on a Rotor-Gene 3000 apparatus (Corbett Research) and repeated experiments three times. Real-time PCR amplification with the following primer pairs: the forward primer of *ost1*: 5'AGTTGCGAGATTGATGAG3' and the reverse primer: 5'GGCTAAATGGGTTGGTGT3', Actin2-F: 5'ATTACCCGATGGGCAAGT CA3', Actin2-R: 5'CACAAACGAGGGCTGGAACA3'. Cycling conditions were as follows: 30 sec at 95°C, 40 cycles of 15 sec at 95°C, 20 sec at 56°C, and 30 sec at 72°C; 1 min at 95°C, 30 sec at 55°C, 30 sec at 95°C, and the following melting-curve program (56 to 99°C, with a 5-sec hold at each temperature). Specific cDNA was quantified with a standard curve based on the known amounts of amplified target gene fragments. The mean value of three replicates was normalized using Actin2 as the internal control. Ct values were analyzed using Excel (Microsoft).

## RESULTS

### Survival and the plant development

After cryostorage, the ability of the seedlings to survive decreased a little, and the average survival rate reached 94.2%. Twenty days after re-culture, all the plants with treatments did not show morphological alterations as compared with the untreated and unfrozen controls, but in the early development, seedlings after cryostorage in LN grow more slowly than the controls, which are as same as our previous findings (Wang and He, 2009).

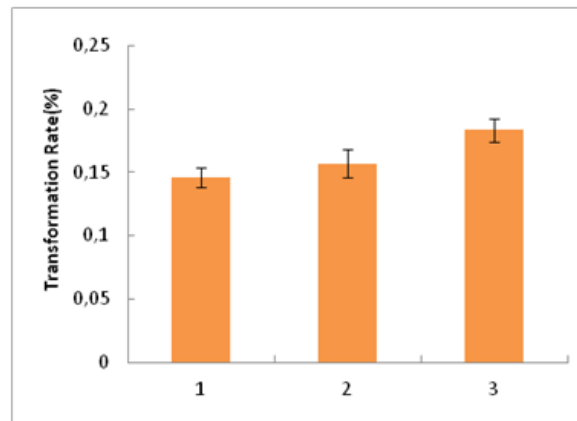
### Effect of cryopreservation on the efficiency of exogenous gene genetic transformation

Three *Arabidopsis* seedlings lines (the control, pre-treatment and cryopreservation) were used for transformation after grown 35 days employing the standard *Agrobacterium*-mediated floral dip method. Transgenic lines resistant to hygromycin B were phenotypically indistinguishable from wild-type lines. PCR amplification of genomic DNA was performed to detect the presence of the exogenous gene. The predicted 598-bp amplified fragment overlapping the major part of the OST1 gene coding sequence and part of the 35S promoter was detected with the same intensity when using DNA extracted from the three *Arabidopsis* transgenic seedlings lines (Figure 1). The positive control (plasmid 35S::OST1) gave a PCR product of the same size.



**Fig. 1 Amplification of ATOST1 sequence.** Amplification of ATOST1 sequence from genomic DNA of the control pre-treated and cryopreserved *Arabidopsis* transgenic seedlings lines. PCR products were separated by 1% agarose gel electrophoresis. The arrow indicates the size of the predicted amplified DNA fragment, 598-bp (Lane M, 2-kb DNA ladder; Lanes 3, 4: amplification from the control; Lanes 5, 6: amplification from the pre-treated seedlings lines; Lanes 7, 8: amplification from the cryopreserved seedlings lines; Lanes 1, positive control: amplification from the plasmid 35S::OST1 (20 ng) containing the *ost1* coding sequence; Lanes 2, negative control: amplification from genomic DNA of non-transformed seedlings lines).

The transformation rate, namely, the percentage of the positive seedlings to the total seedlings, was calculated. In the cryopreserved seedlings lines, the number of transgenic positive seedlings was 395 of the total 270175 and giving the transformation rate 0.167% (L-3); in parallel, the transformation rate was 0.150% from the number 440 of transgenic positive seedlings versus the total seedlings 280492 in the pre-treated seedlings lines (L-2); and the transformation rate was 0.141% in the control lines, the number of the transgenic positive seedlings was 463 against the total seedlings 252270 (L-1). (The mean  $\pm$  standard deviation of eight replicates) (Figure 2). The cryopreserved and pre-treated samples compared with the control, the amount did not allow a statistically significant comparison ( $p = 0.964$ ) (Table 1). These data showed the efficiency of the exogenous gene transformation was not significantly different among the three lines.



**Fig. 2 Analysis of exogenous gene transformation rate.** Analysis of the exogenous gene transformation rate of the three *Arabidopsis* seedlings lines. Data were analyzed by two-way ANOVA and Student-Newman-Keuls's test ( $p = 0.05$ ). The efficiencies of the exogenous gene transformation among the three lines were not significantly different. (Lanes 1, the control; Lanes 2, the pre-treated samples; Lanes 3, the cryopreserved samples). Values are means  $\pm$  SD of eight independent experiments (>3500 seeds per treatment).

**Table 1. Analysis of the exogenous gene transformation rate of the three *Arabidopsis* seedlings lines.**

		Subset for alpha = 0.05	
Tukey HSDa	TYPE	N	1
	Control	8	50.1250
	Pre-treatment	8	55.5000
	Cryopreservation	8	59.1250
	Sig.		0.964

Data were analyzed by two-way ANOVA and Tukey's test ( $\alpha = 0.05$ ). The efficiencies of the exogenous gene transformation among the three lines were not significantly different. ( $p > 0.05$ ). Means for groups in homogeneous subsets are displayed.

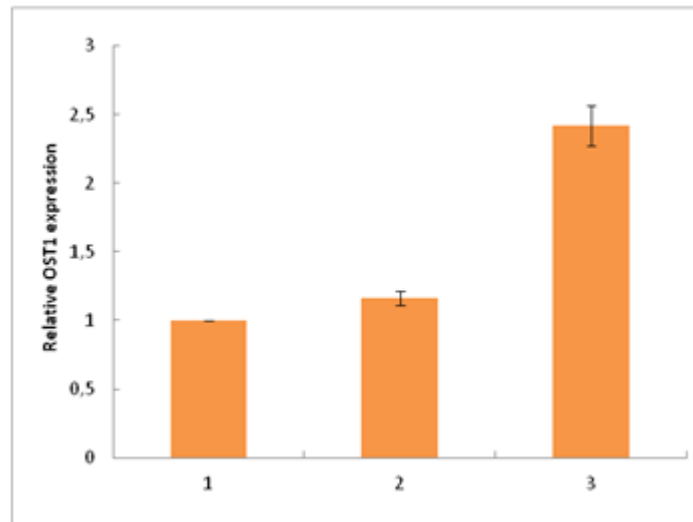
<sup>a</sup> Uses Harmonic Mean Sample Size = 8.000.

### The analysis of expression levels of the exogenous gene in the transgenic seedling tissues

The effect of cryopreservation on the efficiency of genetic transformation and the level of gene expression with respect to an exogenously introduced gene may depend on the locus where the T-DNA was inserted into the chromosome. Therefore, *Arabidopsis* transgenic positive seedlings lines without any treatment obtained by the standard *Agrobacterium*-mediated floral dip method were used for following analysis. Determination of the expression levels is conducted using ten independent lines of transgenic *Arabidopsis* separately. Each of these lines originated from one transgenic seedling, respectively. In each line, a portion of the seeds without any treatment was used as the untreated control, another portion of seeds was used as parallel experiments (with pre-treatment), and the third portion of seeds was cryopreserved in liquid nitrogen for more than 1 hr. Subsequently, total RNA samples were obtained after seeding 15-day from the three *Arabidopsis* seedlings lines (the control,

pre-treatment and cryopreservation) to confirm the level of gene expression. RT-PCR was performed with a pair of primers specific to the OST1 gene. The primer pair produced only one fragment from each cDNA sample.

Real-time PCR analysis (Pfaffl, 2001; Miao et al. 2006) was used to determine whether the expression of exogenous gene was affected by cryopreservation among the control, pre-treatment and cryopreserved samples. Transcript levels are expressed relative to the level of transcripts in the untreated control, which are assumed to be one (Devaiah et al. 2009). This result indicates that cryopreservation affects the level of exogenous gene expression. The level of OST1 gene expression shows enhancement about 2.4-fold after cryopreservation compared with the control and pre-treatment samples (Figure 3), and the level of ATOST1 expression among the three lines has no statistical significance (Table 2).



**Fig. 3 Analysis of expression levels of exogenous gene.** The level of ATOST1 expression measured in the control, pre-treatment and cryopreserved seedlings by RT-qPCR. Real-time PCR was performed in three independent experiments. Error bars indicate SD. An actin2 primer was used in the PCR as an internal control. Lanes 1, the control; lanes 2, the pre-treated samples; Lanes 3, the cryopreserved samples.

**Table 2. Analysis of Expression Levels of Exogenous Gene.** Data were analyzed using matching t-test ( $\alpha = 0.05$ ). The level of ATOST1 expression among the three lines has no statistical significance. ( $p > 0.05$ ). Means for groups in homogeneous subsets are displayed.

			Subset for alpha =0 .05
<b>T-test (A1-A2)</b>		N	1
	Control - Pre-treatment	10	0.052
	Control - Cryopreservation	10	0.137
	Pre-treatment - Cryopreservation	10	0.151
	Sig.		0.796

## DISCUSSION

Early reports have been found that the integrated NPT II gene was stably maintained in cryopreserved-regrown citrus cells without DNA alteration (Kobayashi et al. 1994). Similarly, cryopreservation has no impact on the existence, transcription, and expression of the *sam1* gene in *opium poppy* (Elleuch et al. 1998) and the NPT gene in *wheat* (Fretz and Lorz, 1995). Recently, the transgenic clones of *Beta vulgaris*, *Nicotiana rustica* (Benson and Hamill, 1991), *navel orange* (Kobayashi et al. 1994), *opium poppy* (Elleuch et al. 1998), *barley* and *wheat* (Fretz and Lorz, 1995), and some medicinal plant species (Yoshimatsu et al. 2000) have been conserved *in vitro* by cryopreservation.

In this study, transgenic *Arabidopsis* seedlings were preserved *in vitro* by cryopreservation, and these transgenic seedlings can be cryopreserved for an unlimited period Wang and He (2009) found DNA methylation status changed in the cryopreserved treatment samples but the phenotypic traits of those plants were normal, including the times of beginning to stalk and flowering, and yield-component traits etc. Our result indicates the efficiency of the exogenous gene transformation was not significantly different among the three *Arabidopsis* seedlings lines (the control, pre-treatment and cryopreservation) and the expression of the exogenous gene was enhanced in the transgenic seedling tissues retrieved from cryopreserved samples than the non-cryopreserved (Real-time Quantitative RT-PCR). We conclude from our study that none of the steps of the cryopreservation procedures, *i.e.* osmotic stress due to cryoprotectants, freezing and thawing, affect either the integrity or transcription of the transgene. The mRNA expression these results agree with the studies on transformed *Beta vulgaris* and *Nicotiana rustica* (Benson and Hamill, 1991) owing that the T-DNA and biosynthetic stability were maintained after cryopreservation. Some reports showed that cryopreservation induced DNA demethylation (Johnston et al. 2009; Wang and He, 2009), and methylation has effect on the expression of gene (Finnegan et al. 1996; Chan et al. 2005), and it was unknown whether the variation of DNA methylation status was one reason for the present results. Further experiments are required to examine the DNA methylation levels, using a bisulphite sequencing method, and elucidate the relation between the demethylation and the level of exogenous gene expression in cryopreservation plant materials. It is important to accumulate more data for the effect of cryopreservation on the stability of transgene expression with other system, that will contribute to broaden the future application of cryopreservation to diverse genetic resources.

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