# Effect of cryopreservation on the development and DNA methylation patterns of *Arabidopsis thaliana*☆

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**Abstract**

Seedlings of *Arabidopsis thaliana* were used to assess its recovery ability, DNA methylation alteration and exchanges after cryopreservation. The survival rate and the level of DNA methylation were evaluated after pre-treatment (loading and cryoprotection), unloading and recovery from cryopreservation of the plants. Over 98% of the plants were survived after pre-treatment and unloading without cryostorage in liquid nitrogen, whereas the survival rate decreased to 93.8% after pre-treatment, cryostorage and then unloading. The results suggest that cryostorage and cryopreservation of seedlings obviously impact on the change of levels in the DNA methylation determined by methylation-sensitive amplified polymorphism technique in *Arabidopsis*. [Life Science Journal. 2009; 6(1): 55 – 60] (ISSN: 1097 – 8135).

**Keywords:** *Arabidopsis thaliana*; cryopreservation; survival rate; MSAP; DNA methylation

## 1 Introduction

Among the long-term conservation ways of plant germplasm, cryopreservation is actually the most valuable because it needs very limited space and low maintenance, can be protected from contamination[1]. As a simple alternation method of cryopreservation, vitrification has been successfully applied to plant shoot tips, embrogentic, plasterm and so on[2–5].

The assessment of plant genetic integrity after cryopreservation has been a subject of increasing interest, especially with the fast development of DNA-based techniques[6]. In cryopreservation, plant materials are stored in liquid nitrogen (LN) where both cell division and metabolism are arrested, and the materials can be thus preserved theoretically without genetic alteration for an unlimited period of time. However, various factors associated with cryopreservation and recovery procedures underlie the production of cryo-selection and somaclonal variations[7]. Cryoconservation may usually induce DNA alterations, especially at the epigenetic level accomplished by altered DNA methylation status. Hao *et al* found some demethylated sites after cryopreservation by vitrification in strawberry and apple[8–9].

In eukaryotes and particularly in higher plants, 5-methylcytosine is the predominant modified base[2]. Variation in DNA methylation can lead to alterations in chromatin structure and changes in gene expression. Furthermore disturbance of intrinsic DNA methylation patterns may have structural and functional consequences to the organisms with this epigenetic code[10]. In *Arabidopsis*, DNA methylation levels are critical for embryogenesis, seed viability[11], and drastic global reduction of cytosine methylation due to loss-of-function mutation of the *Met*1 gene (counterpart of the mammalian Dnmt1) or *DDM*1 (decrease in DNA methylation1) gene, albeit non-lethal, produces pleiotropically defective phenotypes and developmental abnormality[12–14].

Two main methods are routinely used for the investigation of DNA methylation in the tissues of the eukaryotic organisms, and bisulfites or methylation-sensitive restriction enzymes are applied. In methylation-sensitive amplified polymorphism (MSAP) analysis, isoschizomers are used to detect the of DNA methylation. The amplified fragment length polymorphism (AFLP) technique was adapted, in which the isoschizomers *Hpa*

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II and *Msp* I are employed as "frequent-cutter" enzymes for AFLP instead of the usual *Mse* I[15]. This method has been proven reliability and used to study DNA methylation of several corps[16–22].

Although many studies have been conducted to test the genetic stability of cryopreserved plant materials, the research using the *Arabidopsis thaliana* seedlings as materials has not been done. In this study, the procedures of vitrification were modified, and high rates of survival had been evaluated. Although, minor alterations of methylation pattterns were observed by MSAP technique after the plants were treated for cryopreservation, the development of all the plants (including those treated with cryoprotection and cryostorage in LN) were normal.

## 2 Materials and Methods

### 2.1 Plant material and growth conditions

*Arabidopsis* seeds (Landsberg) used in the experiments were from Henan Laboratory of Plant Stress Biology. The seeds were sterilized for five minutes with 0.1% mercuric chloride and rinsed with sterile water 4 – 5 times, then sown on Petri plates (9 cm diam) containing solidified MS medium[23] with agar 0.7%. The seeds were kept in 4 ºC for 72 hours, then incubated at 22 ºC under cool-white fluorescent lights, 100 μmol/$m^2$ with 10 hours light per 14 hours dark photoperiod.

The method was modified by Liu *et al*[24]. Two days old seedlings were immersed in loading solution (MS liquid medium + 2 M glycerol + 0.4 M sucrose) for 20 minutes at room temperature (1.0 ml for 30 – 40 seedlings in one 2 ml cryovial). Loading solution was 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 DMSO in liquid MS medium supplemented with 0.4 M sucrose) and left at 0 ºC for 50 minutes. The cryovials were then rapidly immersed in LN for at least 1 hours. The thawing was carried out by immersing cryovials in a water bath at 40 ºC for 1 minute, and cryovials were shaked vigorously. Subsequently, PVS2 solution was unloaded by removing it out from the tube and replacing with unloading solution (MS liquid medium + 1.2 M sucrose) for 40 minutes, and the solution was replaced once 10 minutes. The seedlings were then cultured on MS medium and maintained under the exactly same condition as that for seed germination. The cryopreserved samples had been done with exactly as above steps, some seedlings were treated only with the pre-treatment (loading and cryoprotection), then employed unloading and reculture, which hadn't been cryostoraged in LN and thawed in 40 ºC water bath.

### 2.2 Plants survival and development

The survival rates were evaluated after 5 days of thawing. Seedlings were transferred into solid culture medium and placed in the same growth chamber after growing on MS about 15 days. Regular management was kept until new seeds were harvested.

**2.2.1 DNA extraction.** When all the seedlings begin to have stalks, the leaves of about 30 seedlings once treatment (from the same pool) were used to extract DNA using CTAB method. The DNA was used for the following analysis.

**2.2.2 MSAP analysis.** The MSAP was adopted from Cervera *et al*[25]. Aliquots (250 ng) of DNA were digested for 3 hours at 37 ºC, 3 Units (U) *Eco*R I in 20 μl of 2 μl 10 × buffer (500 mM Tris-HCl, pH 7.5; 100 mM $MgCl_2$; 10 mM Dithiothreitol; 100 mM NaCl). After digestion, DNA was precipitated and digested with 3 U *Hpa* II in 20 μl of 2 μl 10 × buffer (100 mM Tris-HCl, pH7.5; 100 mM $MgCl_2$; 10 mM Dithiothreitol) for 3 hours at 37 ºC. *Eco*R I/*Msp* I DNA digestion were carried out in a final volume of 20 μl with 3 U each of *Eco*R I and *Msp* I, 2 μl 10 × buffer (330 mM Tris-Ac, pH 7.9; 100 mM Mg-Ac; 5 mM Dithiothreitol; 660 mM K-Ac), 4.0 μl BSA and 250 ng of DNA for 3 hours at 37 ºC. The DNA fragments from the two reactions were added separately to an equal volume of the adapter/ligation solution, and the ligation reaction was allowed to proceed at 25 ºC for 2 hours, the ligation mixture was diluted five-fold for use as templates for the first selective amplification with *Eco*R I + A primers and *Hpa* II/*Msp* I + T primers. The PCRs were performed in a 20 μl volume of 10 × PCR buffer, 1.5 mM $MgCl_2$, 0.1 mM dNTP, 50 ng of each primer, 0.4 U Taq DNA polymerases and 5.0 μl of diluted fragments. The product was diluted 20-fold and used as the templates for selective amplification reaction. The selective PCR was performed in a final volume of 10 μl following the protocol of Vos *et al*[26]. All the sequences of the adapters and primers used have been list in Table 1. The PCR amplification was carried out using Zou *et al*[27]. The product of selective amplification were denatured by adding a 1/2 volume of fomamide-buffer (98% formamide; 10 mM EDTA, pH8; 0.05% bromophenol and 0.05% xylene cyanol) and heating for 3 minutes at 94 ºC, then moved into 0 ºC immediately. Electrophoresis was performed with aliquots of each sample on 6% polyacrylamide gel (acrylamide/bisacyylamide, 19 : 1) containing 7.5 M urea and 1 × TBE for 3.0 hours at 55 W.

Then the gel was stained with silver[28].

## 3 Results

The process of cryopreservation comprises three steps: pre-treatment (loading and cryoprotection), freeze-thawing (cryostorage in LN and bath in 40 ºC water), unloading and reculture. The survival rate, stability of DNA methylation were determined.

### 3.1 Plants survival and development

Seedlings were pre-treated, unloaded (not cryostorage in LN and thawing in water) and then recultured in the conditions described above (see methods). Almost all seedlings (96.8% – 100%) were survived and started to grow up within 5 days. Other seedlings after pre-treatments were followed by cryostorage in LN, then thawing in 40 ºC water, unloading and reculture on MS medium. After cryostorage, the survival ability of seedlings decreased a little, and the average survival rate reached 93.8%. Twenty days after re-culture, all the plants with treatments (involving freeze-thawing or not) did not show morphological alterations as compared with the untreated and unfrozen controls (Figure 1.4), but in the first, seedlings cryostoraged in LN grew more slowly than the controls and those treated without freeze-thawing (Figures 1.1 – 1.3).

### 3.2 Analysis of the DNA methylation patterns

*Hpa* II and *Msp* I are isoschizomers frequently used to detect cytosine methylation. Both restriction enzymes recognize the tetranucleotide sequence 5'-CCGG-3'. *Hpa* II is inactive when one of the two cytosine is fully-methylated (both DNA strands are methylated), but it cuts the hemi-methylated 5'-CCGG-3' (only one DNA strand is methylated); whereas, *Msp* I cuts 5'-CmCGG-3', instead of 5'-mCCGG-3'. Therefore, type I bands have been previously associated with unmethylated DNA sequences; while, type II and type III bands have been associated with methylated and hemi-methylated DNA sequences, respectively. The DNA methylation of three samples (1: the control; 2: plants after cryopreservation; 3: plants treated by cryopreservation except freeze-thawing) has been analyzed using MSAP. Three types of MSAP bands were observed. Type I bands were present in both restriction enzyme combinations *Eco*R I/*Msp* I and *Eco*R I/*Hpa* II, while type II bands were present in *Eco*R I/*Hpa* II but absent in *Eco*R I/*Msp* I, and type III MSAP bands were present in *Eco*R I/*Msp* I but absent in *Eco*R I/*Hpa* II (Figure 2 ). According to Xu *et al*[29], disappearance of type I and type II MSAP bands is mainly due to methylation of the outer cytosine of the 5'-CCGG-3' and 5'-CmCGG-3' sequences, respectively. While appearance of type I and II MSAP bands is largely due to demethylation of the outer cytosine of the 5'-mCCGG-3' and 5'-mCmCGG-3' sequence, respectively. However, appearance and disappearance of type III MSAP bands involve both inner and outer cytosines. Methylation alteration of the inner cytosine is responsible for exchanges between type I and type II bands.

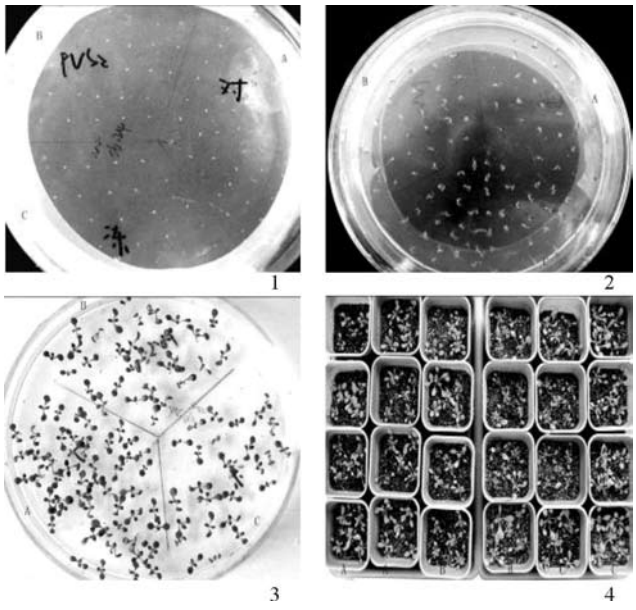


**Figure 1.** Developmental phenotypes of the plants (A: controls, B: treatment with cryopreservation expect cryostorage in LN and thawing in 40 °C water, C: treatment with the whole process of the cryopreservation). Figure 1.1: seedlings of the day just treatments; Figure 1.2: five days seedling; Figure 1.3: ten days old seedlings; Figure 1.4: seedlings of twenty days old, recultured in the soild from MS.

Sixteen primer combinations (Table 1) were used and a total of 619 distinct and reproducible bands were generated. In order to reveal the change among the three samples, all bands belonging to each of the banding patterns were scored (Table 2). Type I, II and III MSAP bands that were present in three samples were designated MSAP-banding patterns 1, 2, 3, respectively. Sixty-one changed bands were observed among all three samples and exhibited 27 MSAP-banding patterns. Various changes in banding types among the different samples were observed. Among these changes, both de novo methylation and demethylation states in sequence were observed (Table 3). Base on the statistics, alterations in DNA methylation of the controls were significantly

different from the plants treated without cryostorage in LN, while those of controls and treatment with cryopreservation were not significantly. Compared to the control, 50 DNA methylation patterns of the samples treated without LN and water were observed to be changed. 15 sites showed de novo methylation, and 6 sites were demethylated. 33 different patterns were seen between the controls and the samples after cryopreservation. The treatment samples after cryostorage in LN showed 11 sites de novo methylation and 7 sites demethylation contrasted with those without cryostorage in LN.

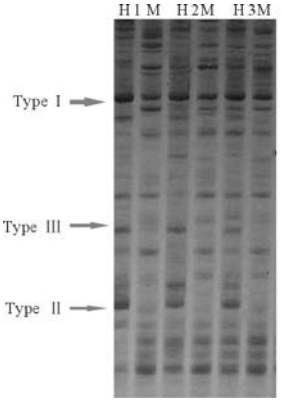


**Figure 2.** MSAP analysis of plants. DNA fingerprints were generated with the primer combination *Eco*R I + ACA/*Hpa* II + TCAA. H and M refer to digestion with *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I, respectively. 1: control; 2: cryopreservation without cryostorage and thawing; 3: cryopreservation. Arrows lead to the corresponding MSAP- banding patterns.

## 4 Discussion

### 4.1 Modification of experiment procedures

In our work, the method of cryopreservation was used from liu *et al*[24]. The thawing step they employed was at room temperature for 5 to 10 minutes, while we have changed it, rapidly warming in 40 ºC water bath for 1 minute. The average survival rate with our method reached 93.8%, this recover percentage is higher than that achieved by Liu *et al*. According to Martin and Gonzalez-Benito[30], rapid warming of plants (at 40 ºC instead at room temperatures) could increase thire recovery rate, and which was further bolstered by our results. Thawing as rapidly as possible could help to avoid the phenomenon of ice recrystallization of seedlings[31]. We found high rate of recovery after cryoprotection, but a low recovery rate after the cryostorage, the possible reason is damage resulting from ice-crystal growth during rewarming. Usually, during the cryopresevation procedure, materials cryostorage in LN at least for 24 hours. To keep the treated seedlings have same times of development with the controls, we have kept the seedlings in LN for only 1 hour. In our repeat experiments, the results showed that the time of cryostorage in LN had no impact on the survival rates (data not show).

During the MSAP analysis, fragment digestion and ligation condition were improved by reducing the DNA and restriction enzyme concentrations while optimizing separate digestion steps and increased the number of amplified fragments and improved fingerprint readability.

### 4.2 DNA methylation level change

In a MSAP analysis, only *Eco*R I/*Msp* I or *Eco*R I/*Hpa* II fragments were likely to be both amplified and visualized on the gels. *Msp* I and *Hpa* II showed no differences in cutting a nonmethylated 5'-CCGG-3' sequence and therefore generated type I MSAP bands.

**Table 1.** Sequences of adapters and primers

| | *Eco*R I | *Hpa* II/*MSp* I |
|---|---|---|
| Adapter 1 | 5'-CTCGTAGACTGCGTACC-3' | 5'-GATCATGAGTCCTGCT-3' |
| Adapter 2 | 3'-CTGACGCATGGTTAA-5' | 3'-AGTACTCAGGACGAGC-5' |
| Pre-selective primer | 5'-GACTGCGTACCAATTCA-3' | 5'-ATCATGAGTCCTGCTCGG-3' |
| Primer | (E+A) | (HM+T) |
| Selective primer | 5'-GACTGCGTACCAATTCAAC-3' | 5'-ATCATGAGTCCTGCTCGGTCAA-3' |
| | 5'-GACTGCGTACCAATTCAAG-3' | 5'-ATCATGAGTCCTGCTCGGTCCA-3' |
| | 5'-GACTGCGTACCAATTCACA-3' | |
| | 5'-GACTGCGTACCAATTCACT-3' | |
| | 5'-GACTGCGTACCAATTCACC-3' | |
| | 5'-GACTGCGTACCAATTCACG-3' | |
| | 5'-GACTGCGTACCAATTCAGC-3' | |
| | 5'-GACTGCGTACCAATTCAGG-3' | |

However, differential sensitivity occured when the inner cytosine in the 5'-CCGG-3' sequence was methylated. In this case, only *Msp* I can cut the 5'-CmCGG-3' sequence, and results in "standard" *Eco*R I/*Msp* I fragment without (an) internal 5'-CCGG-3' sequences. *Hpa* II could not cut the 5'-CmCGG-3' sequence until it recognized the next non methylated 5'-CCGG-3' sequences.

Based on nucleotide composition and genome complexity, the genome of *Arabidopsis* is expected to contain an average of 45,000 *Eco*R I sites. Since two *Msp* I/*Hpa* II restriction sites should flank each of these *Eco*R I restriction sites, a total of 90,000 *Eco*R I/*Hpa* II or *Eco*R I/*Msp* I fragment could theoretically be detected using different selective nucleotide, although only CCGG sequences that lie close to *Eco*R I restriction sites can be detected. The probability that a methylated CCGG site will be digested by *Hpa* II is lower than that digested by *Msp* I due to the different sensitivities of the two isoschizomers to cytosine methylation. We should found more *Eco*R I/*Msp* I fragments than *Eco*R I/*Hpa* II fragments in MSAP profiles. However we generally observed that the number of the amplified fragments of *Eco*R I/*Hpa* II and *Eco*R I/*Msp* I had little difference, this might be that some *Eco*R I/*Msp* I fragments were shorter than 100 bp, which run out of the gel under our electrophoretic condition.

The DNA methylation status of the treatment samples varied in the study, and both *de novo* methylation and demethylation were observed. Furthermore, the samples treated in LN showed more demethylation sites than those treated not in LN. Interestingly, 29 fragments especially appeared in the cryoprotection samples, which represented 49.7% of those various bands among the total fragments. These special fragments were not observed in the controls and the cryopreservation samples. It was suggested that the processes of cryoprotection and cryostorage in LN had impact on DNA methylation status. And it was unknown whether the variation of DNA methylation status was a component of adaptation to the environmental conditions plants encountered. In plants, the DNA methylation of promoter regions usually inhibits transcription. But methylation in coding regions does not generally affect

**Table 2.** MSAP-banding patterns in three groups

| Banding pattern | 1 | 2 | 3 | No. of MSAP bands |
|---|---|---|---|---|
| No. 1 | I | I | I | 459 |
| No. 2 | II | II | II | 40 |
| No. 3 | III | III | III | 49 |
| No. 4 | – | II | – | 15* |
| No. 5 | III | I | III | 2* |
| No. 6 | I | I | III | 3 |
| No. 7 | I | III | III | 3 |
| No. 8 | I | II | II | 5 |
| No. 9 | III | III | I | 3 |
| No. 10 | III | – | – | 4 |
| No. 15 | I | II | I | 3* |
| No. 16 | – | I | – | 2* |
| No. 17 | I | – | I | 2* |
| No. 18 | – | III | III | 2 |
| No. 19 | I | III | I | 2* |
| No. 20 | – | – | I | 2 |
| No. 21 | – | – | II | 1 |
| No. 22 | – | – | III | 1 |
| No. 23 | II | I | I | 1 |
| No. 24 | II | I | II | 1* |
| No. 25 | – | I | I | 3 |
| No. 26 | III | I | I | 2 |
| No. 27 | I | I | II | 1 |
| No. 28 | III | – | III | 1 |
| No. 29 | II | – | I | 1 |
| No. 30 | III | II | II | 1 |

* refer to the specific patterns which present in the samples treated with the steps of cryopreservation except freezing in LN and thawing in 40 °C water bath. 1: control; 2: treatments except cryostorage and thawing steps; 3: treatments with whole process of cryopreservation.

**Table 3.** MSAP band type changes during the treatments

| Band type | controls→plants treated without freeze-thawing | controls→ plants after cryopre-servation | plants treated with-out freeze-thaw ing→plants after cryopreservation |
|---|---|---|---|
| I → – | 2 | 0 | 2 |
| II → – | 1 | 0 | 15 |
| III → – | 5 | 4 | 0 |
| – → I | 5 | 5 | 5 |
| – → II | 15 | 1 | 2 |
| – → III | 2 | 3 | 2 |
| I → II | 8 | 6 | 1 |
| II → I | 2 | 3 | 3 |
| I → III | 5 | 6 | 5 |
| III → I | 4 | 5 | 5 |
| III → II | 1 | 0 | 0 |
| Total | 50 | 33 | 40 |

I: type I band; II: type II band; III: type III band; –: absence; →: change.

gene expression[32]. In our study, DNA methylation status changed in the 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. Further experiments are required to isolate and sequence the variable fragments, and character whether these variations could be inherited to next generation and whether the change of DNA methylation caused during cryoprotection can accumulate as the materials being cryoprotected several times.

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