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INFLUENCE OF CRYOPRESERVATION ON THE CYTOSINE METHYLATION STATE OF POTATO GENOMIC DNA

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Abstract

Shoot tips of *Solanum tuberosum* 'Désirée' were successfully cryopreserved by the DMSO droplet method and stored for almost 7 years, while control material was maintained *in vitro* for the same period of time. To analyse potential epigenetic changes, the DNA methylation status was assayed by methylation-sensitive amplified polymorphism (MSAP) analysis using restriction endonucleases *Msp*I and *Hpa*II. An amount of 93.6% of the analysed MSAP signals were stable among all cryopreserved and *in vitro* maintained samples tested, indicating extensive stability of DNA methylation. Only 0.9% of MSAP signals showed results that differed between the two treatments and at the same time matched for all three biological replications within each treatment. These can be seen as indicating directed effects of the two treatments on the DNA methylation. Cryopreserved samples displayed in comparison to *in vitro* stored samples consistent hypomethylation for 0.6% (3 of 469) of MSAP signals (Table 4, pattern 4) and consistent hypermethylation for 0.2% (1 of 469), respectively. For 5.6% of all MSAP signals, inconsistent results were observed among the three biological replications at least for one of the two treatments. These were interpreted as resulting from stochastic DNA methylation changes in individual samples. As results for two biological replications were identical and different from the result for the third biological replication, the direction of methylation change could be determined in those cases. Cases of stochastic loss of CG methylation in cryopreserved samples were most frequent among them, adding up to 3.4% of MSAP signals. Stochastic loss of CG methylation was also found in material maintained *in vitro*, only for 0.6% of all MSAP signals. In conclusion, methylation changes occurred in long-term cryopreservation of potato, in a random rather than directed fashion. Hence, cryopreservation and long-term *in vitro* maintenance both induce limited changes of DNA methylation status. The order of magnitude of methylation changes observed was consistent with other studies, where similar rates of DNA methylation changes have been found.

Keywords: DNA methylation, DMSO droplet method, genetic stability, *Solanum tuberosum*

INTRODUCTION

Conservation of plant genetic resources is an important issue because of degradation of natural environments and biodiversity loss. One option is the maintenance of *ex situ* germplasm collections. Whenever possible, genebank accessions are stored as mature seeds, which are the natural long term survival stage of flowering plants. For some species, such as cultivated potatoes, this is not possible, as the segregation of heterozygous alleles during sexual seed formation would hinder the aim to conserve clonal varieties. These crops are propagated vegetatively. This means that varieties need to be stored as tubers, *in vitro* propagated material or cryopreserved samples. Cryopreservation is the storage of biological material at temperature of liquid nitrogen (LN) (-196°C) in such a way that viability is retained after rewarming. Clear advantages of cryopreservation over field and *in vitro* culture are lower costs, reduced space and labour requirements, and prevention of pathogen infection of stocks (8).

In vitro propagation methods rely on the totipotency of plant cells. That is the ability of many somatic cell types to dedifferentiate and regenerate under suitable conditions in a spontaneous process into complete plants, which represent clones of the donor (9). Nevertheless, *in vitro* culture can also result in undesired genetic changes. Variations in chromosome numbers, accumulation of gene mutations, alterations in transcript and protein profiles, and changes in DNA sequences have been detected (14). In order to minimize genetic changes in the context of *ex situ* genebank operation, highly organized plant structures like nodal cuttings and shoot tips rather than undifferentiated callus cells are preferably used for *in vitro* propagation or cryopreservation.

Genetic changes are characterized by changes in DNA nucleotide sequences. In contrast, epigenetic modifications do not change the original DNA sequence (4), but may, nevertheless, result in heritable changes of gene expression patterns. Processes that can be altered epigenetically include regulation of gene expression, activity of transposable elements, defence against foreign DNA, and inheritance of specific gene expression patterns. Modifications of these processes can be caused by DNA methylation, modifications to particular histones and alterations in chromatin structure (4). DNA methylation is required for regular development of plants and can have an impact on vigour and morphogenesis (5).

It has been shown for several plant species that epigenetic alterations can be induced by environmental stimuli. Steward et al. (37) reported the maize gene *ZmMl1* to be more actively transcribed and hypomethylated at CG and CNG sites in plants subjected to a 4°C chilling treatment. The effect was persistent as methylation was not restored after the plants were returned to warmer growth conditions. In contrast, changes in heterochromatin-associated CNG methylation, which emerged in response to osmotic stress in tobacco cell culture, were reversible (27). Aluminium, heavy metal and drought are further environmental stressors that can lead to increase or decrease in cytosine methylation throughout the genome (28).

Some studies have discussed possible effects of cryopreservation or *in vitro* propagation of stocks on genomic DNA stability and DNA methylation (15, 33). Modification of epigenetic features, due to stress occurring during *in vitro* culture, could result in altered phenotypes in the recovered plants. Alterations of epigenetic features were found within *in vitro* propagated hop, but no extensive analysis of possible physiological changes was performed (33). It is, therefore, of high practical and scientific interest to assess how genetic and epigenetic stability of *in vitro* propagated and cryopreserved plants is affected by parameters of *in vitro* culture procedures or by cryo-injury caused by physical, chemical and physiological stresses during cryopreservation (15).

For a crop species of such high economic importance as potato, surprisingly few studies of epigenetic changes in response to *in vitro* manipulation have been published. In 1994 a

solitary study reported CNG hypermethylation of ribosomal RNA encoding repeats in response to 'slow growth' in *in vitro* culture (13). Since then, there have been no further reports of epigenetic analyses on *in vitro* or cryopreserved cultures of potato, although genetic stability was confirmed on nuclear and chloroplast DNA for potato (16).

At IPK Gatersleben a collection of presently 1028 cryopreserved potato accessions has been established (1) using the DMSO droplet method. Regeneration percentages range from 10 to 100 % and are, on average, 58% (21, 35). This collection was started 1992 at the Institute of Crop Science of the Federal Agricultural Research Centre [FAL, Braunschweig (36)] in collaboration with the German Collection of Microorganisms and Cell Cultures (DSMZ) and, later in 1997, in a parallel action at IPK (24). The FAL and DSMZ collections were unified at IPK within the fusion of the German *ex situ* genebanks in 2002. During this fusion, a major part of all samples had been rewarmed and survival and regeneration percentages of explants were verified.

In a random sample of the collection of potato shoot tips cryopreserved using the DMSO droplet method, genetic stability was confirmed in regenerated plants using morphological parameters, flow cytometric measurements and restriction fragment length polymorphism (RFLP) analysis (36). The comparison of morphological parameters revealed only one abnormal individual and a few plants with low growth vigour in a set of approx. 1000 regenerated plants from 98 potato varieties. The vitality was not different from control plants, which did not undergo cryopreservation (36). No abnormalities were found in ploidy status or RFLP patterns within 161 samples of regrown plants tested (34, 36). Storage in LN for up to ten years was found to have no adverse effect on the regeneration rates (26, 30). Therefore, the stability of regeneration rates and morphological as well as yield parameters was confirmed (24-26) in contrast to other biometric studies (18). Nevertheless, no examination of genetic or epigenetic stability was performed at the molecular level.

The present study investigates DNA methylation states of *in vitro* cultivated and cryopreserved plants of *Solanum tuberosum* 'Désirée' shoot tips.

MATERIALS AND METHODS

Plant material

In 1996, tissue culture of *Solanum tuberosum* L. 'Désirée' had been established in Groß Lüsewitz in the Cultivated Potato Collection, situated in the Satellite Collections North of IPK, Germany. *In vitro* plants were maintained over cycles of microtubers until 2002 (38). For cryopreservation, plants from cultures initiated in 1996 were received at the cryo-unit at Gatersleben in 2002 and multiplied *in vitro*. Plants were grown in tissue culture on MS-medium (31) supplemented with 20 g/l sucrose and 10 g/l agar (MS2). They were maintained under a 16 h light / 8 h dark photoperiod at 22°C. Solely *in vitro* propagated plants from cultures initiated in 1996 were continuously cultured under the same conditions in the time period between 2002 until the harvest for DNA extraction in 2009 (Fig. 1). Every four to six months, plants were multiplied by nodal cuttings and transferred to fresh medium.

Cryopreservation

In 2002, the DMSO droplet protocol according to Schäfer-Menuhr et al. (35) was used with some modifications to establish cryopreserved samples. Briefly, apical shoot tips (approximately 3 mm long and 0.5 mm thick) were isolated and placed in liquid MS solution with 30 g/l sucrose, 0.5 mg/l zeatin riboside, 0.5 mg/l indole acetic acid and 0.2 mg/l gibberellic acid [MSTo medium (39)] overnight. On the second day the shoot tips were placed in cryoprotectant solution (MSTo medium with 10% DMSO) for 2 h. They were then

transferred into 2.5 µl drops of cryoprotectant solution placed on a small piece of heat-sterilized aluminium foil and quickly cooled in LN within cryovials.

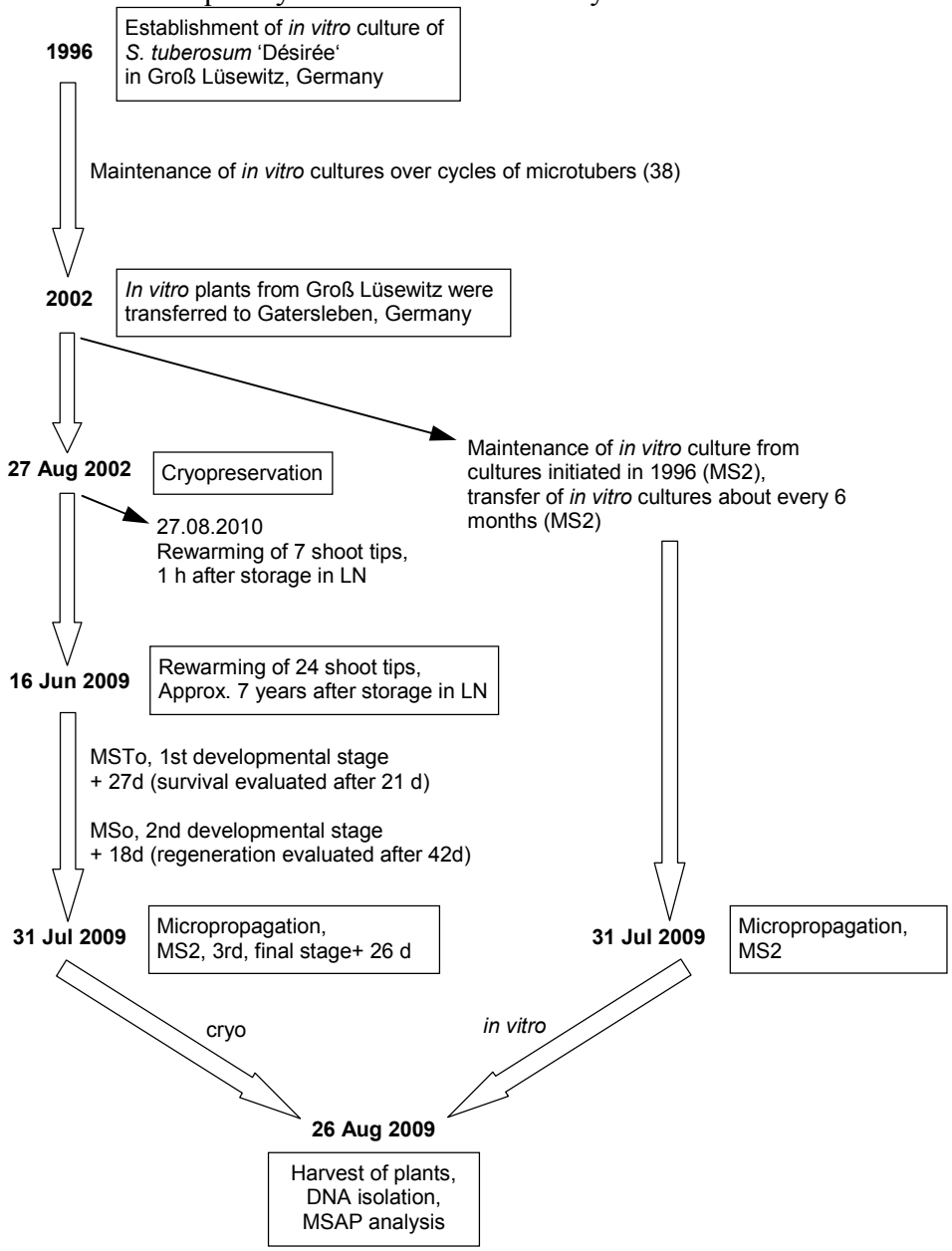

Figure 1. Time scheme for establishment and maintenance of *in vitro* culture of *S. tuberosum* 'Désirée', cryopreservation, regeneration and harvest of material. For abbreviations see text.

The cryovials with shoot tips on foil were transferred and stored in LN either for 1 h or for 6 years and 9 months. After one hour (in 2002) and after 6 years and 9 months (in 2009), formerly cryopreserved shoot tips were rewarmed by placing the aluminium foil in liquid MS medium at room temperature for 1 to 5 min. Regeneration protocols were slightly different in 2002 and 2009. In 2002, shoot tips were placed in drops of warmed agarose in small plastic Petri dishes for regeneration. After solidification of the drops, liquid MSTo solution was added and shoot tips were incubated in transparent plastic boxes with increased humidity for regeneration at 23 °C with 16 h photoperiod (light intensity about 50 µmol/s/m^2). In 2009, shoot tips were placed directly after rewarming onto solid medium (MSTo with 10 g/l agar) and Petri dishes with shoot tips were incubated for regeneration in a climate room with

25/20°C (day/night temperature) and 16 h photoperiod (light intensity about 50 µmol/s/ m^2). In 2002 only 7 shoot tips were rewarmed as control for the cryopreservation experiment, whereas in 2009 a number of 24 shoot tips were rewarmed to receive enough regenerated plants for MSAP analysis (Fig. 1).

Survival and regeneration

Survival and regeneration percentages were evaluated after 21 d and 42 d of tissue culture, respectively. Survival was expressed as percentage of shoots remaining green, and regeneration as percentage of the shoot tips producing plantlets. Both survival and regeneration percentages were calculated relative to the total number of shoot tips rewarmed.

In 2002, all explants were kept on MSTo medium for 42 d and survival and regeneration percentages were determined. In 2009, regenerated plantlets were transferred to MS medium without hormones (MSo, MS with 30 g/l sucrose and 10 g/l agar) and later to MS2 medium to receive fully grown *in vitro* plants. In detail, survival was determined after 21 d and after first development of regenerated shoots (27 d after rewarming) plantlets were transferred to MSo. After 42 d, regeneration percentages were determined. After 45 d, plants were multiplied by nodal cuttings and transferred to MS medium supplemented with 20 g/l sucrose and 10 g/l agar. Individuals were maintained in such a way that each plant could be traced back to the respective rewarmed shoot tip from which it was derived. Until harvesting, 71 d after rewarming, they were cultured in a climate chamber at 22°C and 16 h photoperiod (light intensity about 70 µmol/s/m^2).

Methylation sensitive amplified polymorphism (MSAP) analysis

In 2009, DNA was isolated from plants, which were cryostored for almost 7 years (from 2002 until 2009) and then rewarmed and regrown (each derived from one individual rewarmed shoot tip), or from plants maintained only *in vitro* from the cultures initiated in 1996, micropropagated and cultured as described under "Plant Material". Cryopreservation-derived plants and *in vitro* plants were about 5 to 7 cm tall and of similar structure at the time of harvest. DNA was extracted from 100 mg shoot material using a DNeasy Plant Mini Kit (QIAGEN) according to the manufacture's instructions.

DNA methylation patterns were compared by MSAP analysis according to Banaei Moghaddam et al. (2) in three biological replicates from randomly selected plants for each treatment. Genomic DNA was cleaved with restriction endonucleases *Eco*RI (recognition sequence GAATTC) and either *Hpa*II or *Msp*I. *Hpa*II and *Msp*I are isoschizomers that both recognize the tetra nucleotide sequence 5' CCGG 3'. However, cleavage by them is differently affected by methylation at the internal and external cytosines (Table 1). *Hpa*II will not cut if the internal cytosine is methylated in one strand or in both strands. In addition, it will not cut if the external cytosine is methylated in both strands, but it will cut if the external cytosine is methylated in only one strand. *Msp*I will not cut if the external cytosine is methylated in one strand or in both strands. *Msp*I is not inhibited at all by methylation at the internal cytosine. Thus, the combined use of *Msp*I and *Hpa*II allows distinguishing, in principle, two types of methylation states (underlined in Table 1). Methylation of the external cytosine in one strand (mCCGG) inhibits *Msp*I, but not *Hpa*II, and methylation of internal cytosines (CmCGG) in one strand or both strands inhibits *Hpa*II, but not *Msp*I cleavage (7). It should be noted that many other potential methylation states of the 5' CCGG 3', indicated in Table 1, recognition sites are cut neither by *Msp*I nor *Hpa*II and, therefore, escape detection by the applied method.

DNA samples were split in two parts and parts were digested by *Eco*RI/*Hpa*II or *Eco*RI/*Msp*I, respectively. After restriction cleavage, *Eco*RI and *Hpa*II / *Msp*I adapters (Table 2) were ligated using T4 DNA ligase. DNA fragments with ligated adapters served as

templates for primary PCR amplifications using primers complementary to the *Eco*RI and *Hpa*II / *Msp*I adapters with one additional selective nucleotide at the 3´end (Table 2). PCR products were diluted and used as templates for secondary selective amplification with combinations of primers complementary to the *Eco*RI and or *Hpa*II / *Msp*I adaptor, but this time with two or three selective nucleotides, respectively, at the 3' ends (Table 2).

In a second amplification step, primers complementary to *Eco*RI adapters (*EcoR*I-AA, -AC, -AG, - AT in Table 2) were 5'-end-labelled with IRD700 fluorescent dye. PCR products amplified with primer pairs P1, P2, P3, P4, P5, and P6 (Table 2) were visualized by electrophoresis on a *LI-COR* 4300 DNA Analyzer device. The achieved band patterns were manually analysed. Presence or absence of particular bands in samples from cryopreserved or *in vitro* maintained plants was considered as an indication for DNA methylation changes.

Table 1. Inhibition of *Msp*I and *Hpa*II cleavage by cytosine methylation in the 5´ CCGG 3´site. Differential *Msp*I and *Hpa*II cleavage for a methylation state is marked by underlining.

Methylated cytosines	*Msp*I	*Hpa*II
5´ C C G G 3´ 3´ G G C C 5´	Cut	Cut
5´ CmC G G 3´ 3´ G G C C 5´	Cut	not cut
5´ mC C G G 3´ 3´ G G C C 5´	not cut	Cut
5´ CmC G G 3´ 3´ G G CmC 5´	Cut	not cut
5´ mC C G G 3´ 3´ G G C Cm 5´	not cut	not cut
5´ mCmC G G 3´ 3´ G G C C 5´	not cut	not cut
5´ mC C G G 3´ 3´ G G CmC 5´	not cut	not cut
5´ mCmC G G 3´ 3´ G G CmC 5´	not cut	not cut
5´ mCmC G G 3´ 3´ G G C Cm 5´	not cut	not cut
5´ mCmC G G 3´ 3´ G G CmCm 5´	not cut	not cut

Table 2. Adapters, pre-selective primers and selective primer pairs used in MSAP analysis.

		Nucleotide sequence (5' - 3')
Adapters	*Eco*RI – adapter I	CTCGTAGACTGCGTACC
	*Eco*RI – adapter II	AATTGGTACGCAGTC
	*Hpa*II / *Msp*I – adapter I	GACGATGAGTCTCGAT
	*Hpa*II / *Msp*I – adapter II	CGATCGAGACTCAT
Pre-selective primers	*Eco*RI -A	GACTGCGTACCAATTCA
	*Hpa*II / *Msp*I -0	ATGAGTCTCGATCGGA
Selective primer	*Hpa*II / *Msp*I -AAT	ATGAGTCTCGATCGGAAT
	*Hpa*II / *Msp*I -ATC	ATGAGTCTCGATCGGAAT
	*Eco*RI -AA	GACTGCGTACCAATTCAA
	*Eco*RI -AC	GACTGCGTACCAATTCAC
	*Eco*RI -AG	GACTGCGTACCAATTCAG
	*Eco*RI -AT	GACTGCGTACCAATTCAT

pair P1	**pair P2**	**pair P3**	**pair P4**	**pair P5**	**pair P6**
*Eco*RI –AA	*Eco*RI –AC	*Eco*RI –AG	*Eco*RI –AT	*Eco*RI –AA	*Eco*RI –AC
H / *M* -AAT	*H* / *M* -AAT	*H* / *M* -AAT	*H* / *M* -AAT	*H* / *M* -ATC	*H* / *M* –ATC

RESULTS

Survival and regeneration of plants

Twenty four shoot tips of *S. tuberosum* 'Désirée', which had been cryopreserved for almost 7 years, were rewarmed for molecular analysis. Twenty one days after rewarming most shoot tips had survived and remained green (Fig. 2). Forty two days after rewarming, surviving shoot tips had directly regenerated shoots (Fig. 2). A few shoot tips initially produced callus from which shoots were regenerated within 42 d after rewarming. The plantlets directly growing from rewarmed shoot tips were slightly faster growing than those which went through a callus phase. Total percentage of shoot tip survival was 67%, with a plant regeneration of 58% (Table 3). About 10% of the surviving explants did not produce shoots, so that the percentage of regeneration was lower than that of survival. In contrast, shoot regeneration percentage had been lower (14%) at the day of initial cryopreservation in 2002 and storage in LN for only 1 h (Table 3). In comparison to solely *in vitro* propagated plants, no morphological differences were found at the time point of harvest in plants regrown from cryopreserved material (71 d after rewarming, Fig. 2).

MSAP analysis

The MSAP assay was applied to examine the stability of cytosine methylation states of *S. tuberosum* at 5' CCGG 3´ sites in response to cryopreservation or to *in vitro* culture. Six primer combinations were used (Table 2, P1 to P6) on DNA of three biological replicates for cryopreserved and *in vitro* maintained samples. Two technical repetitions, performed with primer pairs P1, P2 and P3 using the same DNA preparations, gave fully reproducible results.

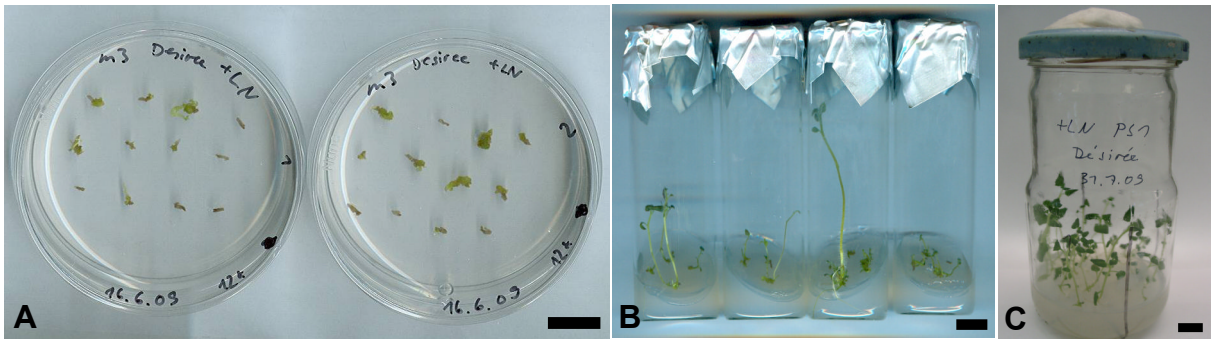

Figure 2. Survival and plant regeneration of cryopreserved *S. tuberosum* 'Désirée' shoot tips. (**A**) Shoot tip regeneration 27 d after rewarming (first developmental stage, MSTo medium). (**B**) Shoot tip regeneration 42 d after rewarming (second developmental stage, MSo medium). (**C**) Multiplied and regenerated shoots 71 d after rewarming (third developmental stage at time point of harvest, MS medium). For explanation of developmental stages see Fig. 1; for MSTo, MSo and MS explanation see text. Bar = 1 cm.

Table 3. Survival percentages and plant regeneration percentages of cryopreserved *S. tuberosum* 'Désirée' shoot tips from 2002/2009.

Duration of cryopreservation (year of rewarming)	Number of total shoot tips rewarmed	Number of survived explants	Number of regenerated explants
1 hour (2002)	7	1 (14.3%)	1 (14.3%)
6 years and 9 months (2009)	24	16 (66.7%)	14 (58.3%)

For the majority of analysed MSAP signals (443 of 469, i. e. 94.5%), results were consistent among all 3 biological replications for each treatment (Table 4). For most of these (439 of 469, i. e. 93.6%), results were identical for cryopreserved and *in vitro* maintained samples, indicating either stable absence (Table 4, pattern 1) or stable presence (Table 4, patterns 2 to 3) of methylation, respectively, at the analysed *Msp*I / *Hpa*II recognition sites. Only 0.9% (4 of 469) of MSAP showed results that differed between the two treatments and at the same time were consistent for the three biological replications within each treatment (Table 4, patterns 4 to 5). These were interpreted cases in which the methylation status of the respective *Msp*I / *Hpa*II recognition site changed in response to one of the two treatments in a directed way. Nevertheless, for these cases it was not possible to determine which was the native and which the derived state. Cryopreserved samples displayed in comparison to *in vitro* stored samples consistent hypomethylation for 0.6% (3 of 469) of MSAP signals (Table 4, pattern 4) and consistent hypermethylation for 0.2% (1 of 469), respectively (Table 4, pattern 5).

For 5.5% (26 of 469) of all MSAP signals, results were at least for one of the two alternative treatments inconsistent among the three biological replications analysed (Table 5, Fig. 3). These were interpreted as resulting from stochastic DNA methylation changes in individual cryopreserved samples, or *in vitro* maintained samples, or both. For these cases, the direction of DNA methylation change could be concluded by assuming that the result obtained for two of the three biological replications represented the native state, while the result obtained for one biological replication represented the derived state. For the majority of MSAP signals falling into this category, 3.4% (16 of 469) of total MSAP signals, a stochastic loss of CG methylation in cryopreserved samples was indicated (Table 5, pattern A to C). Only 0.6% (3 of 469) showed a stochastic loss of CG methylation in *in vitro* maintained samples (Table 5, pattern D). For 0.2% (1 out of 469), a stochastic gain of CG methylation was found for *in vitro* maintained material (Table 5, pattern E), but no corresponding case of stochastic gain of CG methylation was observed for cryopreserved samples.

An amount of 0.9% (4 of 469) of MSAP signals gave results that could only be interpreted as cases in which a directed change in response to one of the two treatments (compare Table 4, patterns 4 to 5) failed to occur in one of the three biological replications for the respective treatment (Table 5, patterns F to H). The respective *Msp*I / *Hpa*II sites seemingly in 0.4% (2 of 469) failed to lose CG methylation in cryopreserved samples (Table 5, pattern F), in 0.2% (1 of 469) failed to gain CG methylation in cryopreserved samples (Table 5, pattern G), and in 0.2% (1 of 469) failed to gain CG methylation in *in vitro* samples. Finally, for 0.4% (2 of 469) of all MSAP signals, no clear interpretation of the patterns was possible (Table 5, pattern 'unclear').

For illustration, Figure 3 provides an exemplary segment of an original output image from MSAP signal visualisation by the *LI-COR* 4300 DNA Analyzer. Primer pair P3 was used for amplification. The MSAP signals of 468 bp, 480 bp, and 486 bp show a pattern indicating the consistent presence of CG methylation at the respective *Msp*I / *Hpa*II sites (compare Table 4, pattern 2). The MSAP signal of 462 bp shows a pattern indicative for loss of CG methylation in one of three biological repetitions of cryopreserved material (compare Table 5, pattern B). Finally, the 478 bp MSAP signal is one representative of the two cases where patterns were too complex to allow a clear interpretation (compare Table 5, pattern 'unclear').

Table 4. Frequency of patterns indicating consistent stability of (1-3) or consistent changes in DNA methylation (4-5) detected by MSAP analysis within *in vitro* cultivated plants regenerated from cryopreserved shoot tips (cryo) in comparison to material always maintained *in vitro* (*in vitro*) of *S. tuberosum* 'Désirée'.

Pattern	M	H	M'	H'	P1	P2	P3	P4	P5	P6	Total	Percentage of total MSAP patterns (469) (%)
	in vitro	*in vitro*	cryo	cryo								
1	■	■	■	■	51	44	35	63	51	34	278	59.3
2	■		■		15	33	35	15	11	32	141	30.1
3		■		■	5	5	5	0	5	0	20	4.3
4	■		■	■	0	1	0	0	2	0	3	0.6
5	■	■			0	0	0	0	1	0	1	0.2
total					71	83	75	78	70	66	443	94.5

Footnote: Results from 3 biological replicates are summarized according to the presence (black bar) or absence (empty cell) of the corresponding signals in samples analysed. M – *Msp*I-*Eco*RI digest; H – *Hpa*II-*Eco*RI digest; P1 – P6 – primer pairs (see Table 2).

Table 5. Frequency of patterns indicating stochastic changes in DNA methylation (A-H) detected within *in vitro* cultivated plants regenerated from cryopreserved shoot tips (cryo) in comparison to material maintained always *in vitro* (*in vitro*) of *S. tuberosum* 'Désirée' by MSAP analysis.

Pattern	M	H	M'	H'	M	H	M'	H'	Total	Percentage of total MSAP patterns (469) (%)
	in vitro	*in vitro*	cryo	cryo	*in vitro*	*in vitro*	cryo	cryo		
A →	■		■		■		■	■	14	3.0
B →								■	1	0.2
C →							■	■	1	0.2
D →						■			3	0.6
E →	■	■	■	■	■	■	■		1	0.2
F →	■		■	■	■		■		2	0.4
G →	■	■	■		■	■	■	■	1	0.2
H →	■		■	■	■	■	■	■	1	0.2
unclear									2	0.4
total									26	5.5

Footnote: Results from two identical (left) and one divergent (right) biological repetitions are summarized according to the presence (black bar) or absence (empty cell) of MSAP signals. → indicates the concluded direction of change; M – *Msp*I-*Eco*RI digest; H – *Hpa*II-*Eco*RI digest; unclear – no interpretation of pattern possible.

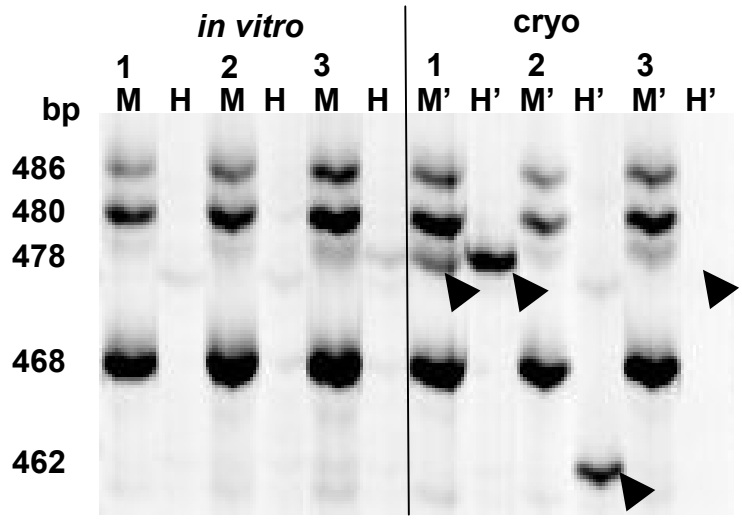

Figure 3. Enlarged segment of a MSAP pattern generated with primer combination E-AG_HM-AAT (primer pair P3) from genomic DNA incubated with *Msp*I (M) or *Hpa*II (H) of *in vitro* cultured (M, H) and cryopreserved (M', H') samples of *S. tuberosum* 'Désirée' in three biological repetitions (sample 1, 2 and 3). Arrowheads point to change in methylation pattern.

DISCUSSION

The plant regeneration success for potato *S. tuberosum* 'Désirée' was 58.3% after storage in LN for almost 7 years. Similar regeneration rates were found in previous applications of the method to the same cultivar (21, 22). In contrast, in 2002 the regeneration percentage was much lower after storage of shoot tips in LN for only 1 h. The lower regeneration percentage observed then could be due to the slightly different rewarming method applying agarose drops and liquid medium instead of solid regeneration medium. Another reason for apparently lower regeneration percentage for samples rewarmed in 2002 could be the low number of rewarmed samples (7 shoot tips) in comparison to 2009 (24 shoot tips).

Beside efficient plant regeneration, genetic stability is required for the successful conservation of plant genetic resources. In theory, all metabolic activities should be arrested at temperatures of LN, so that after rewarming from cryopreservation true-to-type plants are to be expected (32). Morphological, phenotypic, cytological, biochemical and molecular comparisons were conducted revealing that plant material was genetically stable after cryopreservation (3, 17, 30, 34, 36). Diverse diploid, tetraploid and hexaploid potato species exhibited normal developmental patterns (flowering, berry set and tuber formation) after regeneration of cryopreserved shoot tips (3). Cytological studies revealed that the ploidy status of the plant was maintained and chromosomal abnormalities were not observed (3). Harding (12) confirmed stability of the ribosomal RNA genes as well as of the nuclear-chloroplast DNA in potato plants regenerated from cryopreserved shoot apices (16). Plants regenerated from cryopreserved shoot tips of 'Brodick' and 'Golden Wonder' were compared in their microsatellite profiles to parental plants and their progenies and no differences were found (17). This demonstrates the stable somatic inheritance of genomic regions containing the measured simple sequences (17). Similarly, no indication for extended DNA sequence changes was obtained in our study. Only 0.6% (4 out of 469) of MSAP signals analysed showed results that could have indicated a change in DNA sequence alternatively to a change of cytosine methylation at the respective *Msp*I – *Hpa*II recognition site.

The analysed 469 MSAP signals represent only an infinitesimal part (469 times 4 bases equalling 1876 bases) of the potato genome. The method only tests treatment-induced changes in cytosine methylation status at those recognition sites specific to the methylation-sensitive restriction enzymes used. Nevertheless, due to the digital character of the MSAP signals, the method can detect rare changes in a background of highly stable DNA methylation. The 93.6 % of MSAP signals that were stable among all cryopreserved and *in vitro* maintained samples tested indicate extensive stability of DNA methylation following cryopreservation or *in vitro* culture of shoot tips of *S. tuberosum* 'Désirée'. Frequencies of events of consistent hypermethylation (0.2%) or consistent hypomethylation (0.6%) were low. In these cases, for which all three biological repetitions showed the same result, *de novo* methylation could have occurred during *in vitro* propagation before cryopreservation. But it cannot be excluded that the changes happened during cryopreservation or extended *in vitro* propagation. In the case where biological repetitions for the same treatment differed, methylation changes likely had occurred randomly during cryopreservation or *in vitro* maintenance, respectively. Most of them (3.4%) were stochastic events of loss of methylation in particular cryopreserved samples, but in some cases changes were also found in *in vitro* maintained samples. Thus, the difference between cryopreserved and *in vitro* maintained material lies in the frequency of stochastic changes. The changes, which occurred during *in vitro* propagation over the period of almost seven years, could be due to aging of the plants. Loss of cryopreservation ability, totipotency and vigour was reported for aged tissue cultures (19). Therefore, the epigenetic changes, found only in *in vitro* cultured plants in this study, could be related to this long period of development and metabolism under artificial conditions. Epigenetic changes after cryopreservation were also found in almond (6), papaya (23), chrysanthemum (29), strawberry (10), citrus (11) and *Ribes* (20). Hao et al. (10, 11) applied a MSAP protocol using *Msp*I / *Eco*RI and *Hpa*II / *Eco*RI cleavage to analyse DNA methylation in citrus callus and strawberry plants with and without cryopreservation. Cryopreservation led to hypomethylation in 0.8% (3 out of 358) of studied restriction sites for citrus (11) and in 0.6% (2 out of 314) of *Msp*I – *Hpa*II sites of strawberry (10). In conclusion, DNA methylation changes have been found after cryopreservation in previous studies (10, 11). Some hypomethylation and hypermethylation after cryopreservation were also described for *Ribes* (20). Notably, it was found that changes in DNA methylation detected after cryopreservation were reversible after the second subculture of plants (20). By discerning consistent and stochastic methylation changes, the resolution of analysis could be increased in the present study. Nevertheless, similar to previous studies, a higher percentage of analysed sites were found hypomethylated than hypermethylated after cryopreservation.

Thus, long term *in vitro* culture and cryopreservation seem to be intrinsically connected with limited epigenetic changes. These could be due to the chemical and physiological stresses occurring during cryopreservation and can be reversible (20). The changes in DNA methylation observed for potato with the cryopreservation protocol employed are well within the range of changes observed for other plant species. It needs to be elucidated in the future, if these changes on the epigenome have implications for long term storage in cryopreservation in *ex situ* germplasm collections.

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