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Enabling the Spaceflight Methylome: DNA Isolated from Plant Tissues Preserved in RNAlater® Is Suitable for Bisulfite PCR Assay of Genome Methylation

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ABSTRACT

Spaceflight has a unique set of abiotic conditions to which plants respond by orchestrating genome-wide alterations to their transcriptome. The methods for preserving plants for RNA analysis are well-established and proven over multiple missions, but, methods for investigating the possible epigenetic mechanisms that may contribute to the transcriptome alteration are not well-developed for the confining limitations of the International Space Station (ISS). Currently, the methods used to isolate genomic DNA and to perform epigenetic analyses are ideal for frozen plants, as opposed to plants stored in RNAlater®—a high salt solution that chemically suspends all cellular activity and is typically used on the ISS. Therefore, we developed a method for extracting high-quality genomic DNA suitable for epigenetic analysis from *Arabidopsis thaliana* (Arabidopsis) plants that were preserved with the current preservation system aboard the ISS—fixation in RNAlater® using Kennedy Space Center Fixation Tubes (KFTs).

Key words: RNAlater®; DNA Methylation; *Arabidopsis thaliana*; Bisulfite; International Space Station

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INTRODUCTION

It is likely that alteration to the methylome of plants flown aboard the International Space Station (ISS) is one of the modalities engaged to cope with this unfamiliar environment (Paul et al., unpublished observations). Methylation occurs at

ACRONYMS

AmOAc	Ammonium Acetate
DIN	DNA Integrity Number
ELP2	Elongator Complex Subunit 2
EPEX	Epigenetic Expression
EtOH	Ethyl Alcohol
EVT	Experiment Verification Test
ISS	International Space Station
KFT	Kennedy Space Center Fixation Tube
KSC	Kennedy Space Center
MET1	Methyltransferase 1
PCR	Polymerase Chain Reaction
Veggie	Vegetable Production System

the 5 position of cytosine bases and coordinates chromatin modification by recruitment of chromatin remodelers, resulting in regulation of gene expression, X-chromosome inactivation, genomic imprinting, and embryonic development (Jaenisch and Bird 2003; Mohandus et al., 1981; Li et al., 1993; Finnegan et al., 1996), and has a vast amount of relevance to all kingdoms of life. DNA methylation on cytosine bases is utilized across the genome to withdraw the availability of DNA from interactions with transcriptional machinery, generating alterations in the plants' transcript profile and resulting physiology. Cytosine methylation is involved in a variety of biological processes in plants (Stroud et al., 2013), including development, pathogen response, temperature, and salt stress. Given that the environment of spaceflight is essentially unknown within the evolutionary history of plants, it is likely that a range of adaptation mechanisms, including DNA methylation, will occur as plants adapt to this novel environment. Therefore, the goal of this study is to establish a method for genomic DNA isolation suitable for the investigation of the methylation status, and that is consistent with accepted practices for plants that have been grown and harvested aboard the ISS.

APEX04-EPEX is an upcoming Arabidopsis plant growth experiment currently planned for launch on SpaceX-10. The focus of EPEX, which is an acronym for Epigenetic Expression, is to examine the spaceflight methylome of Arabidopsis. In preparation for that experiment, a ground-based Experiment Verification Test (EVT) was performed at Kennedy Space Center (KSC). The focus of this paper is to document the approaches used in the EVT, which tests the utilization of the resources and preservation techniques that will be available for the upcoming spaceflight experiment. In the design of this EVT, methylation mutants were included to give a base of evidence from which we could make conclusions as to the quality of the methods and approach for preserving the methylome and eventually for investigating the role of the methylome remodeling process as a response to spaceflight. The Methyltransferase 1 (MET1) and Elongator complex subunit 2 (ELP2) are key genes that encode regulators playing central roles in DNA methylation of plants (Kankel et al., 2003; Saze et al., 2003; Wang et al., 2013) and the *met1-7* and *elp2-5* mutants have been previously described (Kanno et al., 2008;Wang et al., 2013). Compared to the wild type control, the altered DNA methylation patterns in these mutants should reveal methylome differences, provided that the method preserves cytosine methylation across the genome. This paper describes the process that generates an examination of methylation states of two genes chosen from previous spaceflight data (Paul et al., 2013; Paul et al., unpublished data) in RNAlater™ -preserved Arabidopsis plants using a modified DNA extraction method followed by bisulfite conversion, polymerase chain reaction (PCR) amplification, TA cloning, and Sanger sequencing to determine methylation status.

MATERIALS AND METHODS

Experimental Setup

In the process of attaining the requirements for the EVT for spaceflight experiment APEX04, nine 0.5x phytagel plates were sown 1.5 cm from the top edge of 10 cm square plates with 12-14 dry sterilized dormant seeds (Sng et al., 2014) of either Columbia (Col-0), *met1-7*, or *elp2-5* genotype, and were sent to KSC for growth in the Vegetable Production System (Veggie) hardware (Massa et al., 2013; Ferl and Paul, 2016). The plates were grown in a vertical position for 11 days before being harvested to 50 mL Falcon tubes containing RNAlater™. Samples were received from KSC and were stored at -80°C before processing for DNA extraction. Each tube contained the harvested Arabidopsis samples from one media plate. The Falcon tubes containing the samples were transferred to -20°C overnight and then defrosted at 4°C overnight. Samples were warmed to room temperature to dissolve RNAlater™ precipitate, and were subsequently photographed and dissected under a dissecting microscope, severing the root from the rest of the plant (shoot). Each 50 mL Falcon tube sample was divided and allocated to either RNA or DNA extraction. Consistent with previous reports (Finnegan et al., 1996; Jia et al., 2015), retarded growth was observed in *met1-7* and *elp2-5* seedlings, displayed in Figure 1, and had a large impact on the allocation of plant material for DNA extraction. We needed to get DNA sample of at least 10 ng/µL of DNA with a total amount of 200 ng DNA for the downstream bisulfite

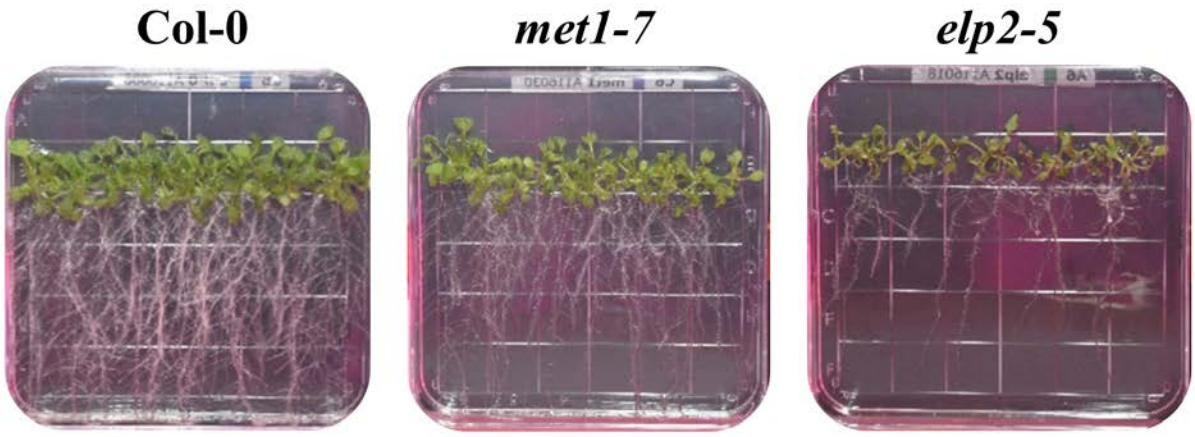

Figure 1. Plant mass varies among genotypes. 100 mm^2 Petri plates containing 11-day-old seedling of Col-0, *met1-7*, or *elp2-5* Arabidopsis plants grown on 0.5x phytagel media. These plants were grown under the Veggie hardware at Kennedy Space Center and were used in the EVT for APEX04.

conversion step. In determining the amount of plants required to get that concentration and amount of DNA, pilot experiments were run with an aim to conserve valuable spaceflight materials and to determine how many samples were necessary.

DNA Extraction

Root and shoot samples were blotted with kimwipes, weighed, and rinsed twice in 3 mL of wash buffer (50 mM EDTA, 25 mM EGTA) for 10 minutes. Samples were blotted again on kimwipes and transferred to mortar and pestle and ground in liquid nitrogen. 900 µL of lysis buffer (10 mM EDTA, 50 mM EGTA, 50 mM Tris), 80 µL of proteinase K (80 mg/mL), and 100 µL of 10% SDS were added and further ground. Samples were incubated at 65°C overnight. 300 µL of 5M potassium acetate was added to each sample and put on ice for 30 minutes. Each sample was then spun in a microcentrifuge at maximum speed for 5 minutes, supernatant was transferred to a new 1.5 mL microcentrifuge tube and spun for an additional 5 minutes. The supernatant was then split between two 1.5 mL microcentrifuge tubes and 800 µL isopropanol was added and the tubes were incubated at room temperature for 1 hour. Tubes were then spun at maximum speed for 10 minutes, the liquid was poured off, and the resulting pellet was air-dried for 15 minutes at room temperature. Pellets were suspended in 50 µL of TER buffer (10 mM Tris-HCl, 1 mM EDTA, 100 µg/µL RNase), split tubes were combined into one tube per sample and incubated at 60°C for 20 minutes. 100 µL of 25:24:1 phenol/chloroform/isoamyl alcohol was added to each tube, mixed, and spun at max speed for 10 minutes. The aqueous (upper) phase was transferred to a new tube and 40 µL of 7.5 M ammonium acetate (AmOAc) and 300 µL of 95% ethyl alcohol (EtOH) were added, the tubes were inverted to mix, and placed at -20°C overnight. Tubes were then spun at 4°C for 10 minutes, the EtOH and AmOAc solution was poured off, and the pellets were rinsed with 70% EtOH three times, dried for 15 minutes and suspended in 100 µL 0.25x TE buffer. This protocol is based in part on a previously developed method (Dellaporta et al., 1983).

Molecular analysis was performed to assess both the quality and quantity of DNA produced from these extractions. As an initial look into the DNA samples, 5 µL of each sample were run out beside standard DNA solutions of 50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78, 0.39, and 0.195 ng/µL on a 1% agarose gel, which generated bands within the DNA size, as visualized in Figure 2. In addition to gel electrophoresis, the DNA samples were also quantified by Qubit, generating the values displayed in Table 1, and TapeStation. For an intensive examination of DNA quality, the 2200 TapeStation instrument designed by Agilent Technologies was employed. Although there are no strict requirements upon DNA to have a certain

DNA Integrity Number (DIN), as displayed in the in silico gel electrophoresis image displayed in Figure 3, for bisulfite conversion and DNA methylation analysis, the values reported by this analysis could prove useful for other downstream applications.

Cytosine Methylation Analysis

Subsequently, PCR-based DNA methylation analysis was performed to further investigate the DNA extracted from the above method and to clone exonic regions from genes of interest for methylation analysis. In order to demonstrate that DNA methylation can be investigated in RNAlater® -preserved plant material, two genes were chosen: AT2G07698 (ATPase, F1 complex, alpha subunit protein) and AT2G07687 (Cytochrome C oxidase, subunit III). These genes were chosen due to their strong differential expression generated from transcriptome analysis by Paul et al. (2013), and differential methylation based on preliminary, unpublished pilot experiments. Three genomic DNA samples from shoots of each genotype with high concentrations were selected from the four biological replicates generated from APEX04-EVT. Approximately 500 ng of each DNA sample were subjected to bisulfite conversion using the Zymo EZ DNA Methylation-Lightning®, according to the manufacturer protocol. PCR products of exon regions from the genes AT2G07698 and AT2G07687 were generated using the Zymotaq PCR system, according to the manufacturer protocol (primers used are all listed in Table 2). PCR products amplified from shoot DNA samples of Col-0, *met1-7*, and *elp2-5* were performed in triplicate and run out on a 1% agarose gel displayed in Figure 4; the bands were excised according to their molecular weight, and gel was purified using the Thermo Fischer gel purification kit according to the manufacturer protocol. To generate clean sequences and to accurately quantify the percent of copies of DNA with cytosine methylation at a particular nucleotide site, the three replicates of PCR products from each sample were combined to one solution and ligated to the pCR 2.1 vector and transformed into chemically competent OneShot OmniMax *Escherichia coli* cells using the manufacturer protocol. Fifteen transformed colonies were selected by growing on LB media with 50mg/L kanamycin, 100 mg/L X-Gal, and 1mM IPTG for each plate. Ninety colonies in total were screened using PCR, and the colonies were submitted for Sanger sequencing. The final Sanger sequencing base calls were analyzed using the BioEdit software.

Table 1. Quantification of gDNA extractions. Qubit quantification of DNA extraction samples corresponding to Figure 2. Standard DNA with known concentration was used as positive control. The amount of roots or shoots used for DNA extraction and concentration of each DNA sample were listed.

Sample	# of plants	[DNA] (ng/µL)
Col-0 R1	10	30.8
Col-0 R2	10	14.9
Col-0 R3	10	26.0
Col-0 R4	10	23.6
Col-0 S1	10	27.2
Col-0 S2	10	25.6
Col-0 S3	10	27.2
Col-0 S4	10	29.4
met1-7 R1	16	19.8
met1-7 R2	16	15.2
met1-7 R3	16	20.6
met1-7 R4	16	9.06
met1-7 S1	16	37.2
met1-7 S2	16	31.8
met1-7 S3	16	30.8
met1-7 S4	16	22.4
elp2-5 R1	16	9.24
elp2-5 R2	25	9.24
elp2-5 R3	25	13.0
elp2-5 R4	17	12.3
elp2-5 S1	16	47.4
elp2-5 S2	25	49.2
elp2-5 S3	25	36.6
elp2-5 S4	17	45.4

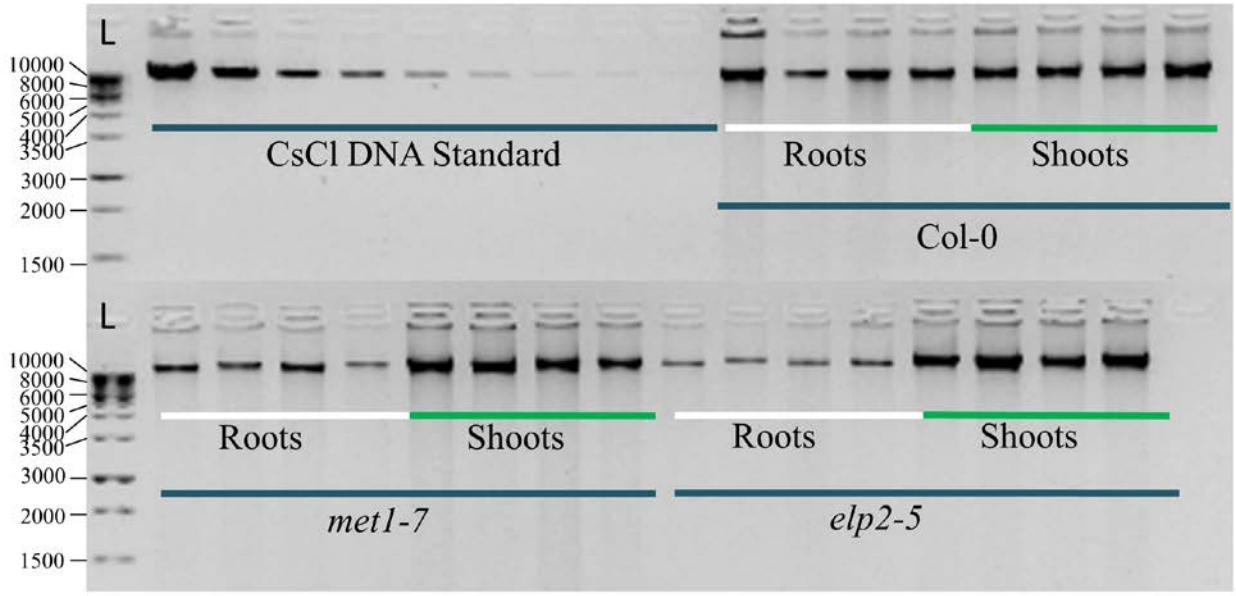

Figure 2. Agarose gel electrophoresis of gDNA extractions. Genomic DNA extraction from Col-0, *met1-7*, and *elp2-5* root and shoot samples obtained from the EVT at KSC. Known genomic DNA purified using the CsCl method was diluted to gradient concentrations (50, 25, 12.5, 6.25, 3.125, 1.5625, 0.78, 0.39, and 0.195 ng/µL) and used to generate a standard curve by which the DNA samples were quantified by band intensity. For each sample, 5 µL of DNA solution was loaded.

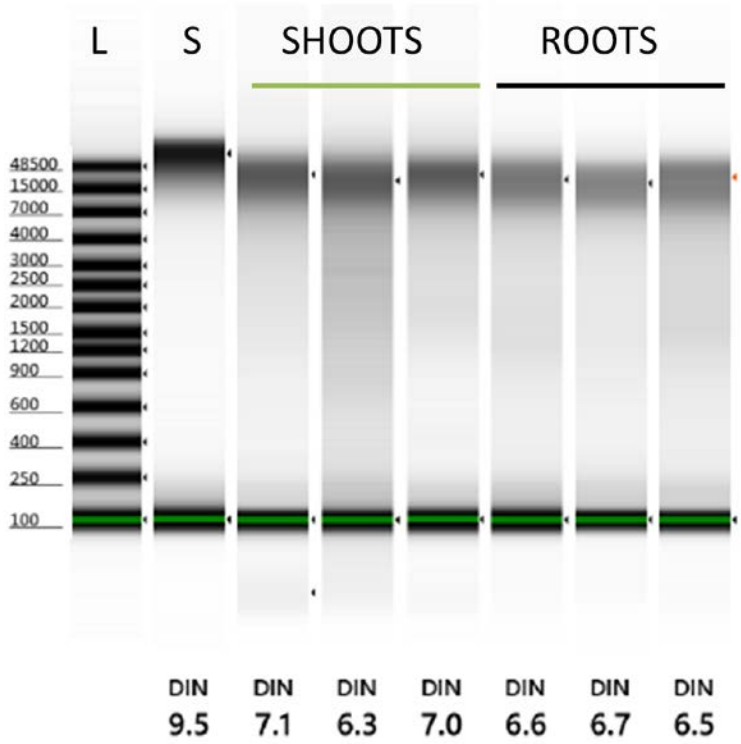

Figure 3. Exemplary DNA samples analyzed by TapeStation. Roots and shoots from the Col-0 ecotype EVT samples were analyzed on the Agilent 2200 TapeStation System using the Genomic DNA ScreenTape, generating concentration values and DNA Integrity Number (DIN). L–molecular weight ladder, S–CsCl purified gDNA standard.

Table 2. PCR primer sequences. The bisulfite primer seeker designed by Zymo Research was used to select regions to amplify from the exons of AT2G07698 and AT2G07687.

Primer	Sequence (5' to 3')	Tm (°C)	Product Size (bp)
AT2G07698-F	TAAATATTTTTTTTTATTTGTTTTTGGAG	50.1	254
AT2G07698-R	TAAAACRAAACTATAAAAAAAAAAAAAAAAAC	51.6	
AT2G07687-F	AGATAAAGTGGTTTATGATTGAATTTTAGAGG	55.6	337
AT2G07687-R	ATCTAAAACCTCAATCCCTTTTAAAAACC	55.9	

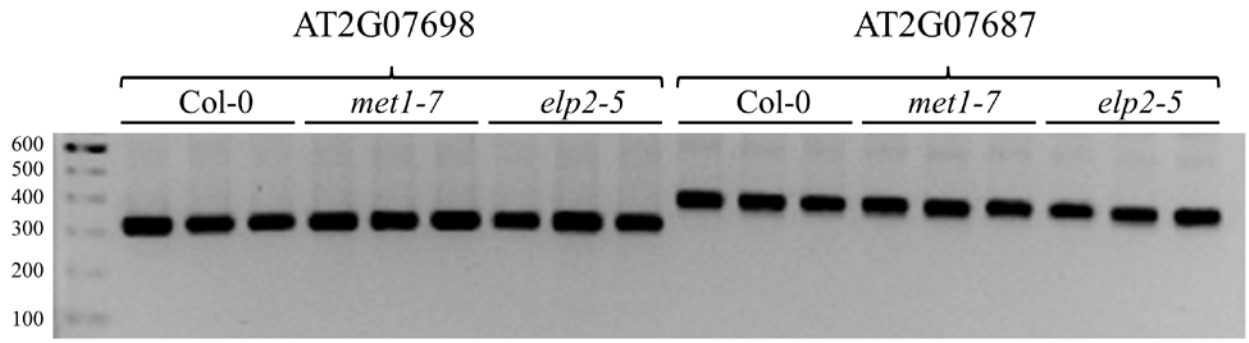

Figure 4. PCR amplification products from exonic regions of AT2G07698 and AT2G07687 shoots from Col-0, *met1-7*, or *elp2-5*. PCR-generated samples were run out on 1% agarose gel, and stained with ethidium bromide. The amplified region from AT2G07698 is expected to be 316 bases and from AT2G07687 is expected to be 398 bases (see Table 2).

RESULTS

We expected the DNA to be of lower concentration and amount in root samples, asthese tissues were of lower fresh mass and, in general, contain lower DNA concentration per gram of fresh mass. Given the small growth phenotype present in the *elp2-5* genotype, we expected DNA extractions of *elp2-5* roots to be of concern (Figure 1). Since the corresponding shoot DNA extractions had such a large fresh mass input, we expected the quality of shoot DNA to be qualitatively compromised. This also raised concern for *elp2-5* shoot extractions due to the comparatively large number of plants deemed necessary for adequate DNA quantity for bisulfite conversion. In general, four replicates of each material exhibited comparatively consistent DNA quality and quantity. The Col-0 roots and shoots provided DNA samples with similar concentration, while *met1-7* and *elp2-5* roots showed obviously lower DNA concentration compared with the corresponding shoot samples (Figure 2; Table 1), indicating the difficulty in handling root tissues of small mass. In all cases, no RNA or protein contamination or DNA degradation was observed in the gel (Figure 2), which suggested DNA quality of adequate levels for bisulfite conversion. Moreover, given the total DNA solution amount of 100 µL, most samples met the requirement we had set for bisulfite conversion (200 ng with 10 ng/ uL). The exceptions were one root sample of *met1-7* and two root samples of *elp2-5* (Table 2). However, total DNA amounts of these three samples were above 900 ng, which is much higher than the minimum suggestion for bisulfite conversion, and were suitable for bisulfite conversion after being subjected to standard concentration treatments. Three biological replicates from Col-0 root and shoot samples were analyzed by TapeStation, and generated DIN values above 6.3 on a 10-point scale (Figure 3). This extra step of qualification verified the level of quality displayed in the agarose gel electrophoresis (Figure 2) and also generated valuable information for a variety of downstream applications.

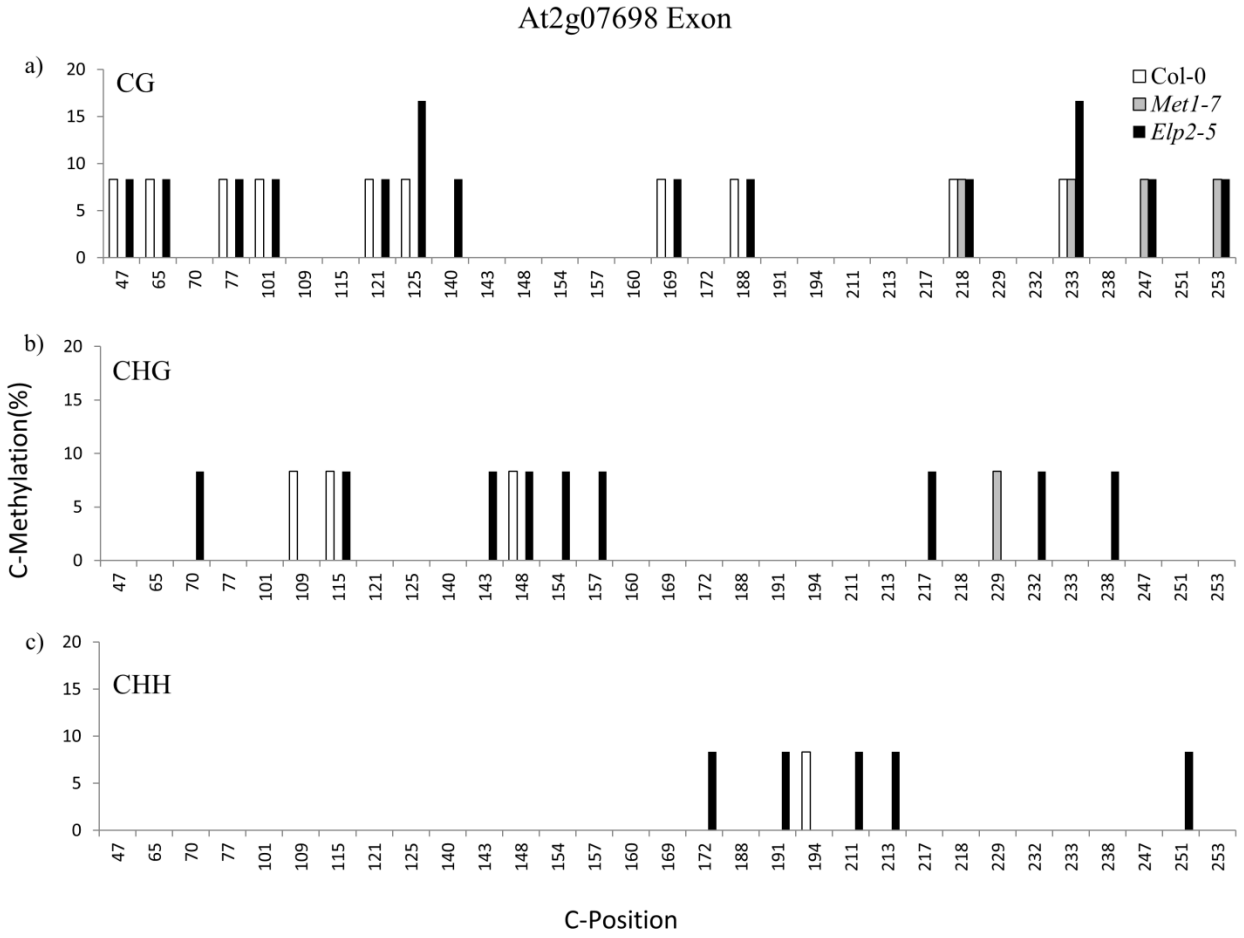

Figure 5. DNA methylation present in AT2G07698 from gDNA samples extracted for the EVT. DNA methylation levels in the CG (A), CHG (B), and CHH (C) context in a window of ~200 b.p. in PCR detected exonic regions of AT2G07698 (+253 to + 507 from ATG) from shoot DNA samples of Col-0, *met1-7,* and *elp2-5*. Each column represents the percent of 15 TA colonies that had a methylated cytosine at a given nucleotide site.

After the sequence information was produced, manual observations of the distribution of methylated cytosine bases were undertaken, generating the information displayed in Figure 5 and Figure 6. No mixed peaks were observed in the chromatogram peaks—a tribute to the uniformity of cloned amplicons in the TA colonies. For AT2G07698 and AT2G07687, the regions of +253 to +507 and +19 to +355 (the A of ATG start codon was designated +1) were analyzed, respectively. Methylation in AT2G07698 was much more bimodal and generally much lower than in AT2G07687, typically having only 0 or 1 out of 15 colonies methylated at a given position in the CG, CHG, or CHH context in wild type (Figure 5). In *met1-7*, no methylation was observed in most of the cytosine sites of AT2G07698 exon region (Figure 5), which is in line with the MET1 role of maintaining CG methylation (Cao and Jacobsen, 2002). It has been reported that loss-of-function mutation of ELP2 will lead to disorder in genome-wide DNA methylation (Wang et al., 2013). Indeed, in two CG sites and most of CHH/CHG sites of AT2G07698 region, *elp2-5* showed a higher methylation level than Col-0 (Figure 5). For AT2G07687, the methylation levels were somewhat consistent among three genotypes in most of CG and CHG sites (Figure 6a-b). For CHH context, most of the cytosine methylation was disrupted in both *met1-7* and *elp2-5* (Figure 6c). Interestingly, unlike AT2G07698,

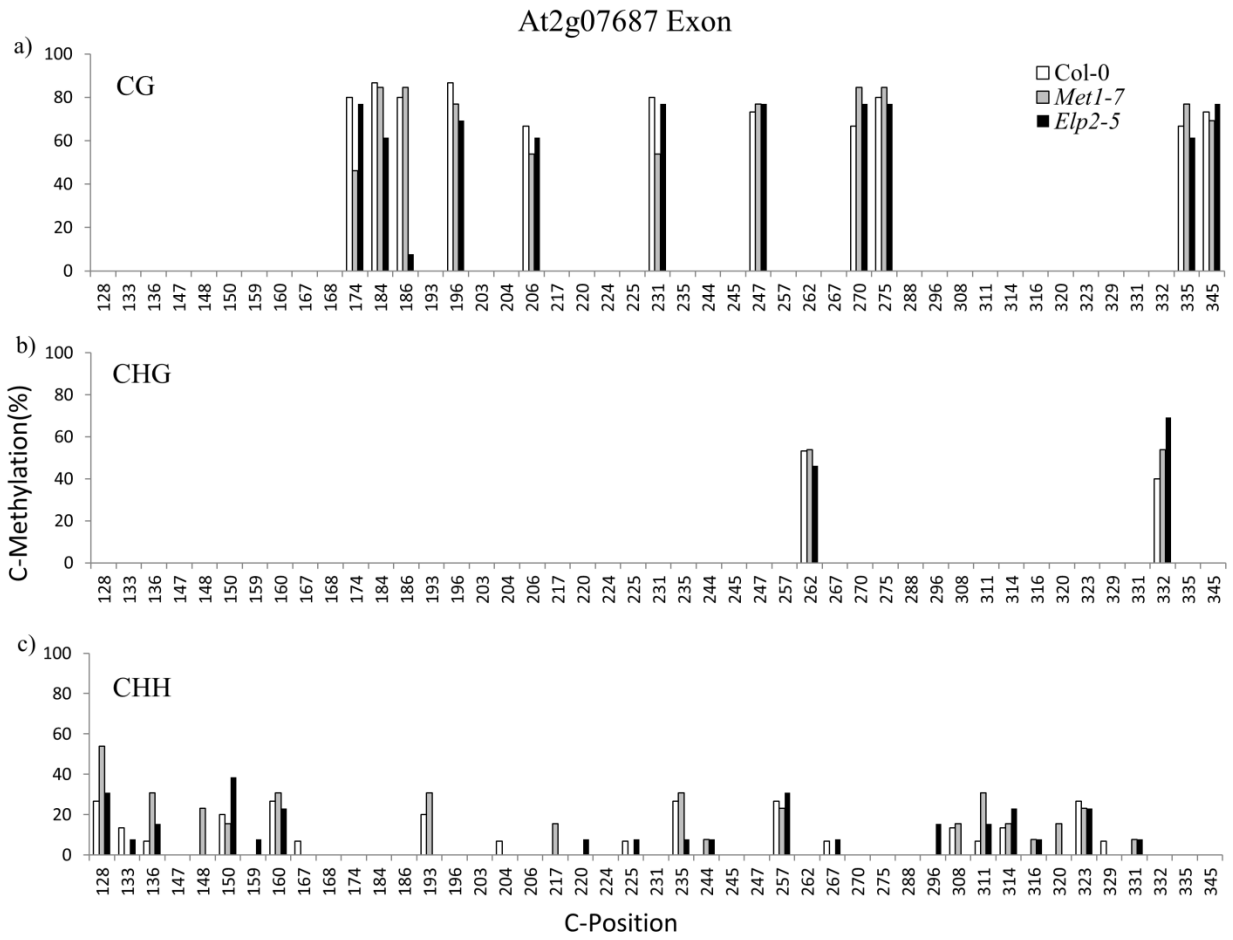

Figure 6. DNA methylation present in AT2G07687 from gDNA samples extracted for the EVT. DNA methylation levels in the CG (A), CHG (B), and CHH (C) context in a window of ~200 b.p. in PCR detected exonic regions of AT2G07687 (+19 to + 355 from ATG) from shoot DNA samples of Col-0, *met1-7*, and *elp2-5*. Each column represents the percent of 15 TA colonies that had a methylated cytosine at a given nucleotide site.

elimination of methylation in CG context was observed in AT2G07687 exon region in *met1-7*, suggesting the stability of CG methylation in this gene. Together, the Sanger sequencing results indicated that these extracted DNA samples were suitable for PCR-based cytosine methylation analysis.

DISCUSSION

In summary, we have generated a working method for isolating genomic DNA from RNAlater®-preserved material, and character-rizing the methylation status of DNA from RNAlater®-preserved plants. These data illustrate that novel approaches can be used to navigate some of the necessary limitations of spaceflight experiments, such as the reliance of RNAlater®-preserved material in many current ISS preservation scenarios. In addition, examinations of genotypes revealed genotype-specific differences in the methylation state of genes of interest. For DNA extraction, we deemed 10 of Col-0, 16 of *met1-7*, and the maximum available *elp2-5* (total minus 3 for RNA) plants to be necessary to get DNA solutions of adequate concentration for bisulfite conversion. We also found that the PCR conditions used to amplify bisulfite-converted DNA derived from RNAlater® samples were highly sensitive, and in our case it was necessary to use a polymerase kit designed specifically for amplifying bisulfite-converted DNA. In our

attempts to analyze the PCR products generated from our genes of interest, we adopted the TA cloning approach to not only clean up the Sanger sequencing chromatograms, but to also introduce the ability to quantify percent methylation of a locus within a diverse population of cell types. Results produced from the methylation analysis revealed that the levels of methylation varied widely between genotypes and the gene analyzed. This targeted approach adopted for the EVT will have wide application in space-related genomic research, and also revealed interesting gene-specific differences in methylation among genotypes. From the data that we collected during this experiment, we demonstrated that in addition to its intended purpose of RNA preservation, RNAlater®-preserved plant material is suitable for high-quality DNA extraction and methylation analysis among comparably preserved controls.

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