Application of the LUminometric Methylatoion Assay for plant ecological researches: the study of global DNA methylation in leaves of *Elodea canadensis* under laboratory conditions and in leaves of fen orchid from wild populations

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**Abstract**

The epigenetic changes in the genome of plants are one of the important regulatory mechanisms in response to the environmental factors. The LUMinometric Methylation Assay (LUMA) requires a relatively small DNA amount, a short processing time and is easily adapted for species with a non-resolved genome. The LUMA has not been previously used for ecological research of plants. In this research, LUMA was used for the first time to investigate the changes of global DNA methylation under different environmental factors in the leaves of different plants. The influence of salinity on global DNA methylation was studied on aquatic macrophyte *Elodea canadensis* Michx., which grew in aquatic tanks under different NaCl concentrations. After the third week of growth, the *HpaII/MspI* ratio was measured by LUMA and global DNA methylation percentages were calculated. The results showed salt-stress-induced changes in the global DNA methylation level in *E.canadensis* leaves, compared to control. The response was salt dose-dependent. The changes of global DNA methylation in wildlife plant populations were analogically assessed on fen orchid *Liparis loeselii* (L.) Rich. It was shown that global DNA methylation level was higher in leaves of these plants in Engure Lake, where there are temporary changes in water regime, compared to leaves of plants from other places. It was assumed that global GC-DNA methylation plays an essential role in the survival of this plant. Therefore, we show the possibilities of using the LUMA method for epigenetic study of different plants ecological researches.

**Keywords:** DNA methylation; *Elodea canadensis* (Michx.); epigenetics; LUMinometric Methylation Assay; *Liparis loeselii* (L.) Rich.; salinity; wild populations.

**Abbreviations:** AFLPs\(_s\) analysis based on Amplified Fragment Length Polymorphisms after PCR, CTAB\(_s\) hexadecyltrimethylammonium bromide, ELISA\(_s\) enzyme-linked immunosorbent assay, HPLC-UV\(_s\) high-performance liquid chromatography in ultraviolet, LUMA\(_s\) The Luminometric Methylation Assay, LC-MS/MS\(_s\) coupled with mass spectrometry, MSAP\(_s\) analysis based on Methylation Sensitive Amplification Polymorphism, RFLP\(_s\) Restriction Fragment Length Polymorphism.

**Introduction**

Epigenetic mechanisms regulate high-order DNA structure and gene expression without changes in the underlying DNA sequence. A best-known epigenetic modification is methylation of cytosine. It is a chemical modification, which covalently involves the addition of a methyl group onto the position 5 of a pyrimidine cytosine ring (5mC). DNA methylation in plant is different from animals. In plants, DNA methylation (5mC) is species-, tissue-, organelle- and age-specific (Vanyushin and Ashapkin, 2011). A relatively high degree of nuclear DNA methylation is specific feature of plant genomes. In the plant genome DNA methylation at the cytosine C5 position is the most abundant modification with up to 25% of the cytosine (Steward et al., 2002). It occurs not only at CG dinucleotides as in mammalian, but also in CNG and CNN trinucleotide sites (where N is A, T or C). Because of their symmetrical nature, CG and CHG methylation can simply be copied after DNA replication, but non-symmetrical CHH methylation has to be established de novo following each DNA replication cycle (Bartels et al., 2018). Usually, two main functions is targeted: firstly, gene expression regulation by methylation or demethylation at gene promoter and/or body sites, and secondly, protection of genome stability by silencing repeated sequences, such as transposable elements (Chan et al., 2005). DNA methylation has been hypothesized as an underlying mechanism of temporary changes in the phenotype (Suzuki et al., 2008).

In recent years, it is known that pattern of DNA methylation in model plants is sensitive to various environmental stressors under laboratory conditions. The global DNA methylation is correlated with the response mechanism to various environmental changes such as salt stress, drought menace, and virus infection. The changes in DNA methylation occur under the influence of photoperiod and during fruit development and ripening (Dalakouras et al., 2010; Pan et al., 2009; Li et al., 2012; Kim et al., 2014). The level of DNA methylation varies in different ways depending
Results

Global DNA methylation in leaves of model plant E. canadensis

The E. canadensis is one of the fresh-water macrophytes species, which grows in brackish water occasionally only. Promotion of plant genome methylation might be one of the mechanisms that are used for developing tolerance to salt stress (Dyachenko et al., 2006). The global DNA methylation in leaf of elodea under different water salinity was studied by LUMA. The obtained data is shown in Fig. 4. It was demonstrated that E. canadensis control samples in water tank without salt (0.00 M NaCl), the HpaII/MspI ratio was 0.79. But the HpaII/MspI ratio was 0.64-0.66 in samples of E. canadensis in water with moderate salinity (0.025M (~1.45 %) and 0.05M (~2.9 %). However, HpaII/MspI ratio was 0.30 in samples of E. canadensis, grown in water under the 0.1M (~5.8 %) salinity (Fig 4, A). The data show that the relative global GC-DNA methylation increased in E. canadensis leaves under the increasing water salinity. This relation was well described by a linear equation (Fig 4, B). The concentration 0.05M NaCl corresponds to the water salinity in the Baltic Sea. Our data demonstrate that HpaII/MspI ratio not substantially varies in samples of E. canadensis at this water salinity level (0.025M NaCl and 0.05M NaCl). We can assume that enzymatic GC-methylation of E. canadensis genome is stable for this water salinity level.

Global DNA methylation in leaves of fen orchid L. loeselii wild populations

The orchid family is one of the most threatened plant families in Latvia. Plant samples of L. loeselii, were taken from six habitats in Latvia: at the seaside area, and at the more continental region, at the east part of Latvia. The HpaII/MspI ratio in DNA from leaves of L. loeselii measured by LUMA was shown in Fig 4. The HpaII/MspI ratio differed from 0.54 in leaves of L. loeselii from Lake Engure to 0.73 from Lake Pēlece. However, this ratio (0.69 ± 0.03) varied insignificantly among all these lakes (except Lake Engure). Accordingly, the HpaII/MspI ratio in DNA from leaves of L. loeselii, grown in Lake Engures differed from others by 21% (Fig 4, A). It was found that the relative global DNA methylation level of DNA varied also insignificantly between the habitats in seaside and continental regions from 35% to 27% (31±1.5%) (Fig 4, B). However, a relative global methylation level of DNA was significantly higher (46%) in leaves of L. loeselii from Lake Engure.
Fig 1. The location of sampling zones of *Liparis loeselii* (L.) Rich. in Latvia

![Map of Latvia showing sampling sites for *Liparis loeselii*](image)

Fig 2. Typical LUMA read-out of the Pyrosequencing reactions, using genomic DNA isolated from leaves of *Elodea canadensis* (A) Digestion reaction with restriction enzymes HpaII and EcoRI, (B) Digestion reaction with restriction enzymes MspI and EcoRI.

![LUMA read-out diagram](image)

Fig 3. The global DNA methylation changes of *E. canadensis* leaves under different salinity levels: (A) HpaII/MspI ratio in DNA. The results presented as mean values ± standard error, with three replications, (B) Regression between the level of salt (NaCl) concentration (mM), and global DNA methylation level (%).

![Graph showing DNA methylation changes](image)
Discussion

**Global DNA methylation in leaves of model plant *E. canadensis***

Plants can use several strategies to cope with high water salinity that all require a significant modulation in gene expression through different epigenetic processes (Yaish et al., 2018). Global DNA hyper-methylation was also detected in pea root tip (*Pisum sativum* L.) under the water deficit conditions by MSAP method. It was observed an increase in the methylation level of both cytosine residues in CCGG motifs, especially for the inner cytosine (Labra et al., 2002). An increase in cytosine methylation at CCGG sites was detected in different plants, which include both *de novo* methylation and demethylation events. However, salt stress can also cause demethylation at specific loci (Guangyuan et al., 2007). The MSAP was performed in oil seed rape plants subjected to salt stress and three types of bands were defined. Extensive changes in the types of MSAP bands were observed after treatment with 100 mM NaCl. These included the appearance and disappearance of all types of band, as well as exchange between band types. However, there was no change in the methylation of the cytosine of CG dinucleotides, as revealed by digestion with *MspI* and *HpaII* (Kovarik et al., 1997), which may be due to the loci that located in a heterochromatic region, having a high background level of methylation.

The *E. canadensis* have invaded numerous aquatic ecosystems in the Europe and is well-represented in coastal marshes, where they exhibit different tolerances to different water salinity. During the tides, salt water from the Baltic Sea (3.5-4.0 %) gets to the Lake Kisezers near Riga. Our data shows that the relative global GC-DNA methylation was increased in *E. canadensis* leaves under the increasing water salinity. The high concentrations of NaCl can affect the physiological processes in *E. canadensis* (Petjukevics et al., 2015). Promotion of plant genome methylation might be one of the mechanisms that are used to develop tolerance to salt stress (Dyachenko et al., 2006). DNA hypermethylation may be a plant protective mechanism that induces cell cycle arrest with the consequent reduction of plant growth and development (Labra et al., 2004). *E. canadensis* plants are genetically homogeneous populations and in Western Europe there are only female specimens. The same genotype in situations of external stress manifests a pronounced epigenetic plasticity. Changes in plant genome methylation might be one of the mechanisms that are used to develop tolerance to salt stress.

**Global DNA methylation in leaves of fen orchid *L. loeselii* wild population**

The orchid family grows eastwards in the temperate zone to central Siberia and temperate, boreal northern America except Alaska (Roze et al., 2014). *L. loeselii* range occurs in different moist habitats: wet and sandy lakeshores and meadows, fens, ditches, old fields, abandoned peat excavations and gravel pits, forested wetlands. The broad geographic distribution of the species indicates its adaptability. The genetic variations in most cases might be neutral. It might not impact genomic function, but the epigenetic variation could have a direct impact on genome function, and through this might affect the fitness of an organism to specific environmental condition. However, the lack of information about the heritable epigenetic variation and its influence on local adaptation in natural populations are observed. This orchid species is dependent on a constant hydrological regime (Roze et al., 2014). When temporary changes in water regime, such as flooding or a drop in the water level occurs, the species does not flower and can survive in a vegetative stage. When the changes are permanent, the species disappear after three or four years. The bogging of the shore of Lake Engure creates new suitable habitats for *L. loeselii* as an early successional species. The survival of *L. loeselii* in wet habitats with fluctuating water level is ensured by various adaptations. In this research, we found that DNA methylation plays an essential role in the survival of the *L. loeselii* in Lake Engures. Although LUMA is very sensitive to low-quality DNA and measures only CpG methylation within the recognition of sequences of the restriction enzymes (CCGG), this method is fast and convenient for different ecological researches of plants.

**Fig 4.** The global GC-DNA methylation changes in leaves of *L. loeselii* from different zones of Latvia: (A) Hpa II/MspI ratio and (B) global DNA methylation level (%). The results presented as mean values ± standard error with three replications.

**A.**

**B.**
Materials and methods

Objects of investigation

Leaves of Elodea canadensis (Michx.) were used for investigation of water salinity influence on global DNA methylation by LUMA method in laboratory conditions. The leaves of 3-year-old fen orchid Liparis loeselii (L.) Rich. from two different zones of Latvia were used to evaluate the possibility of using the LUMA method to understand influence of environmental factors on global DNA methylation in wild plant populations.

Plant growth conditions and sample collecting zones

Aquatic macrophytes E. canadensis were cultured in glass tank under laboratory conditions in climate chambers (POL-EKO, Poland), a photoperiod regime: 8h/16h (dark/light) cycle, light intensity: 50 μmol photons m⁻² s⁻¹, temperature: 18°C/15±1.0°C (day/night). The tap water after filtration in water tanks was prepared with following concentrations of NaCl: 0.00 M, 0.01M (0.58 %), 0.025M (1.45%), 0.05M (2.95%) and 0.1M (5.8%) NaCl. It was known that E. canadensis tolerates salinities up to 2.5% (Sand-Jensen, 2000). After the third week of growth in glass tank with different water salinity, the leaves of E. canadensis were cut and stored at -80°C.

Fen orchid L. loeselii (L.) Rich. samples were taken from six habitats in Latvia: Lake Kaniera, Lake Engure, Orchid trail (which are located at the seaside area, at the Gulf of Riga), Lake Grendzes, Lake Gaisezers, and Pelecu swamp (which are located at the more continental region of Latvia) (Fig 1). The leaves of 3-year-old L. loeselii were cut and stored at -80°C. Methylated DNA is very stable, but the DNA fragmentation can be problematic for LUMA (Head et al., 2014). Therefore, storage conditions of plants are very important for this method.

DNA isolation

The correct method for DNA extraction is very important for quantitative global DNA methylation analysis using LUMA. Previous epigenetic studies, focused on DNA methylation, have assumed that methyl groups are not lost during routine DNA extraction, but this has not been empirically tested. Many different methods and technologies with different protocols are available for DNA isolation. The selection of method depends on several factors, such as DNA quality, purity and others. Regardless of the method used, DNA samples may be exposed to varying degrees of oxidative conditions. DNA oxidation could occur during isolation by the presence of oxidants in cells or by those produced by cell lysis (Kvam and Tyrrell, 1997; Guetens et al., 2002). Different levels of oxidation during the extraction procedure could decrease the methylation level. Our data demonstrate that LUMA is very sensitive to low-quality DNA and DNA fragmentation level. The extent of fragmentation of the DNA sample is influenced by the isolation method. The review of LUMA results from the studies of human blood shows differences around 20% between DNA chloroform extraction and column isolation kits (Soriano-Tarraga et al, 2013). As others have noted (Ammerpohl et al., 2009) LUMA is more sensitive to poor DNA quality than other methods for determining CpG methylation rates (such as HPLC or bisulphite sequencing) because the presence of single-stranded overhangs in degraded DNA can contribute to the LUMA signal.

Previously some DNA isolation methods were examined: CTAB, salting-out method, extraction with column isolation kits “DNeasy Plant Mini Kit” (data not shown). The column isolation kits “DNeasy Plant Mini Kit” (Qiagen) was the most appropriate method for high-quality DNA isolation. This isolation method is preferred by other researchers (Head et al., 2014). All samples also were treated with RNase A (100mg/ml, Qiagen).

DNA quality and quantity

DNA was quantified spectrophotometrically (Shimadzu BioSpec-Nano) at A260/A280 and A260/A 230, A260/A280 ~1.8, and A260/A 230 ~1.9, respectively (Wiltfinger et al., 1997). DNA quality was checked by agarose gel electrophoresis (1.5%) in TBE buffer and gels were stained with ethidium bromide (0.5μg/ml). The size of DNA fragments was determined by comparing with DNA ladders of known size (100bp DNA Ladder Plus, MBI Fermentas). For this study, only the high-molecular DNA was used. It was shown that even slightly fragmented samples can depress DNA methylation values when analysed by LUMA

Luminometric Methylation Assay (LUMA)

LUMA is a method to estimate genome-wide DNA methylation and requires a relatively small DNA amount (250–500 ng). It has short processing time and can be easily adapted to multiple species. The LUMA has the advantage that can be used with the DNA from various species without extra optimization. However, it is sensitive to a number of factors, such as DNA quality and the choice of isoschizomers used for cutting DNA in a methylation-sensitive manner. The method is based on a polymerase extension assay using the Pyrosequencing™ platform (PyroMark Q24), with PyroMark Gold Q reagents was used. All steps were performed based on the original method according Karimi et al. (2006).

Restriction enzymes (HpaII, MspI, and EcoRI) and Tango buffer for digestion reaction (final volume 20 μl) were used. PyroMark Gold Q24 Reagents (Qiagen) were used for luminometric methylation assay. The HpaII/MspI ratio can be defined as [HpaII/(HpaII+EcoRI)] / (MspI/EcoRI). Methylation percentages were calculated as: 1-[(HpaII(C) /EcoRI(A)) / (MspI(C) /EcoRI(A))] x100. The unmethylated DNA produces HpaII/MspI ratio close to 1.0. In the methylated DNA, the ratios are close to zero. The typical LUMA readouts from the Pyrosequencing reactions with genomic DNA from leaves of E. canadensis were shown in Fig 2. DNA was digested in parallel reactions with EcoRI (recognition site G/AATTCC) and isoschizomeric restriction enzymes, HpaII or MspI. Both cleave at the recognition site 5′...CGG...3′, but HpaII cuts only when the internal C is unmethylated, whereas MspI is insensitive to methylation status at the internal C. The A and T peaks denote the additions of dATPαS and dTTP respectively. The nucleotides of which are substrates for filling in all EcoRI generated T and A overhangs.

Thus, the dTTP-peak serves as a control for the dATP-peak, which in turn, is used as the internal control peak of the
assay, A and T peaks should be equal. dCTP and dGTP are added together, and the corresponding peak denotes HpaII or MspI cleavage. The second C peak is a control for completion of the first C peak and should be close to zero. The peak designated “S” is the substrate peak representing the starting point of Pyrosequencing reactions. Cells-to-CpG Methylated gDNA control kit (Applied Biosystems) was used as a high-methylated control, but sample without DNA as a control of nucleotide degradation.

Acknowledgments

This study has been partially supported by the National Research Programme 2014-2017 „EVIDEnt“ sub-project 4.6. “Freshwater ecosystems services and biological diversity”. Thanks Dr. D. Roze for orchid samples.

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