Global Methylation of DNA Among Spartina Alterniflora Clones Differing in Age at North Inlet, SC

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GLOBAL METHYLATION OF DNA AMONG SPARTINA ALTERNIFLORA CLONES DIFFERING IN AGE AT NORTH INLET, SC

by

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DEDICATION

I am dedicating my thesis to my parents, Terry and Karen Agrelius, without whom I would not have been able to do it. Mom and Dad, you both have been amazingly supportive and awesome role models. You have helped me to grow and branch out to a new city, a new career, and a new way of life. I thank you both very much for all that you have provided and the constant support that you both gave throughout the years. No one could ask for a better set of parents. I love you both very much.  -T
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ABSTRACT

“Brown marsh” or “sudden marsh dieback” refers to the rapid death of patches varying in size of Spartina alterniflora. In 2000 following a 100-year record drought, approximately 8,000 hectares of S. alterniflora died along the Louisiana coast, which prompted Louisiana Governor Foster to declare a state of emergency. Other cases have been documented at North Inlet, SC as well as various other marshes along the southeastern United States. Currently, there is much dispute regarding the cause of “sudden marsh dieback” but environmental stress is one of the acknowledged constants across sites. We hypothesized an alternative mechanism in which methylation of the genome increases with plant age, reducing stress tolerance. DNA methylation occurs on the cytosine nucleotide and is an epigenetic modification that is crucial for stable gene regulation along with silencing of harmful transposable and repetitive elements (TE and RE respectively) in the plant genome. This modification is initiated and maintained through different pathways and methyltransferase families, but only one family of methyltransferases can propagate and maintain DNA methylation through a process denoted as de novo style methylation.

The primary focus of this study was to assess the level of global DNA methylation among Spartina alterniflora clones of variable age at North Inlet, South Carolina through a field survey of separate locations. After completing the field survey, a greenhouse study was conducted in an attempt to ascertain the effect of an environmental
stress, increasing salinity, on global DNA methylation. Finally, four isolated genets were located in the field and sampled to test the hypothesis of increasing global methylation with age within each genet. Our results show that although increasing environmental stress does impact survivorship, global methylation rates remain unaffected. This conclusion does not invalidate DNA methylation as a proposed mechanism for the brown marsh event; in fact it correlates with the gene-body methylation phenomena seen throughout various plant species in which a gene actively functions despite being heavily methylated. To further consider DNA methylation as a mechanism for brown marsh, it would be advantageous to look at individual genes, specifically those use for stress and defensive purposes, and their methylation status.
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CHAPTER 1

INTRODUCTION

1.1 Brown Marsh Overview

In the year 2000, Louisiana experienced a sudden and devastating dieback event that impacted 100,000 hectares (ha) of *Spartina alterniflora* along its coast and throughout the Mississippi River deltaic plain (Lindstedt and Swenson, 2006). This event was so devastating that it prompted Louisiana Governor Foster to declare a state of emergency across the state. During the seven months of May through October, intense yellowing and browning of *S. alterniflora* occurred resulting in bare flats while adjoining plant species were visually unaffected (Alber et al. 2008). Populations of *Juncus roemerianus* and *Avicennia germinas* (mangroves) and even *S. patens* remained largely unaffected while interior portions of the *S. alterniflora*-dominated saltmarsh deteriorated through the season. Marsh dieback causes large-scale reductions in ecosystem function, such as water filtration, habitat for other fish and crustaceans, and sediment retention, and thus is it imperative to investigate the causes of such dramatic losses to the ecosystem.

This thesis investigates a potential mechanism that could have played a role in the 2000 marsh dieback event in Louisiana.

Dieback events are not uncommon. Other dieback events, though smaller in scale, were reported in Florida, Georgia, South Carolina, California, Virginia, and various New England states. A five-year event occurred in the Florida panhandle from 1990-1995.
resulting in 1 ha of *S. alterniflora* becoming chlorotic and dying off within one month (Carlson et al. 2001). Similar events took place in 2001-2002 in Georgia and South Carolina (Ogburn and Alber 2006) have been reported resulting in the thinning of vegetation and eventual transformation of saltmarsh into bare mudflats. In other cases, reports of dieback events with the highly controversial hybrid (*S. alterniflora x S. foliosa*) found in the San Francisco, California bay have been noted (Neira et al. 2007).

Dieback events of plants have been described as cases of “cohort senescence,” and was initially defined in 1986 by Mueller-Dombois (1986) regarding *Metrosideros* trees in Hawaii. Mueller-Dombois described “stands of dead or dying trees whose dieback cause is not obvious and typically occurs in several locations of a large forest ecosystem.” The term “dieback event” is used to describe the unexplained phenomena of synchronized death among trees belonging to a single and spatially coherent age group, resulting in the rapid breakdown and destruction of the forest canopy (Odgen et al. 1993). Using this terminology, later studies described dieback events as a plant population phenomena as opposed to randomly occurring large-scale mortality events induced by disease or other environmental events (Odgen et al. 1993).

Environmental stresses and disease, such as drought and loss of fresh water availability resulting in unusually high soil salinities, can also cause large-scale dieback of vegetation. In 1985, the Charleston Harbor experienced a drought that coincided with the redversion of the Cooper River out of the harbor resulting in a wide loss of *S. alterniflora* populations. Likewise, the 2000 brown marsh event in Louisiana occurred in the midst of a record 100-year drought diminishing its natural freshwater inputs to the delta (Swenson et al. 2004). However, the dieback event in Louisiana was not ubiquitous
and occurred only in spatially limited areas, causing doubt as to the attribution of dieback to the extended regional drought.

Presently there is much dispute regarding the cause of brown marsh in Louisiana in 2000, but environmental stress is one of the acknowledged constants across sites. Wrack accumulation, such as the buildup of dead *S. alterniflora* stems, can smother populations of *S. alterniflora* causing local small scale diebacks (Bertness and Ellison, 1987; Valiela and Reitsma, 1995). Overgrazing from local fauna can create an “eat-out” within the marsh (Daiber, 1982), also resulting in local small scale diebacks. Invertebrates including the periwinkle snail, *Littoraria irrorata*, and certain crab species such as *Sesarma reticulatum*, have been reported to leave bare spots within the marsh where they occur in high density (Stiven and Hunter, 1976; Chrichton, 1960). Although these processes can cause local disruption of *S. alterniflora* population growth, there is no evidence to support that they caused the wide spread 2000 brown marsh event.

Further research has investigated another potential mechanism for the 2000 brown marsh event via fungal pathogens traveling from across the Atlantic Ocean from Africa. Fungi are naturally occurring and important components of the saltmarsh ecology, making up the majority of the decomposer community (Kohlmeyer et al. 1999; Newell, 2001). Numerous *S. alterniflora* samples were tested for known fungal pathogens and in some cases manipulated to readily facilitate fungal infections (Smith, 2006). For the dieback events occurring in Florida and Louisiana, fungi from an African clade, *Gibberella fujikuroi*, were found, prompting the notion that there is a potential route for entry from Africa to North America via the Atlantic, which could transport African dust carrying fungal spores. Similar speculation has been made for proposed methods of coral
reef disease, though both are difficult to test (Garrison et al. 2003). In both cases, above- and below-ground *S. alterniflora* sample did not contain any debilitating infections nor did the induced infections result in dieback.

A final explanation of the cause of the 2000 marsh dieback event in Louisiana is the possibility of a genetic predisposition of particular groups of genotypes to be ill-adapted to adequately manage prolonged stress. Currently, the genome of *S. alterniflora*, a hexaploid species, has not been mapped nor have specific genes been located. Exact regulation pathways and the genes responsible for defensive properties are not known but work has gone into genotyping populations. Random amplified polymorphic DNA (RAPD) markers were used to assess genetic diversity in *S. alterniflora* populations invasively introduced in Willapa Bay, Washington (Stiller and Denton, 1995). Ayres et al. (1999) also used RAPD markers to measure the full extent of hybridization in California while still distinguishing between the native *S. foliosa* and *S. alterniflora*. Subudhi et al. (2008) used RAPD markers in addition to amplified fragment length polymorphism (AFLP) to measure the relatedness of *S. alterniflora* species in Louisiana. Several dieback locations were identified allowing Subudhi et al. (2008) to test plants currently undergoing a brown marsh event, those directly surrounding them, and the vast periphery that still maintained a healthy appearance. Although no specific genotype or group of genotypes could be identified as the ill-adapted subsets, the authors found an equally diverse set of genotypes between the three test groups suggesting a more complicated mechanism.

This research examines the global DNA methylation in *S. alterniflora* in order to determine if this process could play a role in large-scale dieback events of marshes, such
as the 2000 Louisiana event. Samples were gathered from sites at the North Inlet NERR in SC and analyzed using an ELISA-like method to quantify global methylation across the genome. We hypothesize an alternative mechanism in which methylation of the genome increases with plant age, reducing stress tolerance. We are proposing a generalized mechanism of increasing stress susceptibility with stand age in which intrinsic properties of a marsh stand breakdown gradually over time. The quality of the stand degrades, but the degradation has no outward effects other than increasing the susceptibility to environmental stressors. Eventually, a tipping point is triggered by the combination of the degraded marsh state and an environmental stress resulting in a brown marsh event.

1.2 DNA Methylation in Plants

DNA methylation on the cytosine nucleotide (5mC) is an epigenetic modification that is crucial for stable gene regulation and silencing of transposable elements (TE) along with other repetitive elements in the plant genome (Henderson and Jacobsen, 2007). This thesis tests the impact of global DNA methylation and potential accumulation of methylation in *S. alterniflora* to see if it affects the plant’s ability to deal with environmental stress. The sequence context of methylation can vary between the symmetrical CG and CHG patterns or the asymmetrical CHH sequence, being maintained by separate methyltransferase families (Chans et al. 2005; Henderson and Jacobsen, 2007; Goll and Bestor, 2005). The symmetric CG sequence is maintained predominantly by a subfamily of transferases, Dnmt1, known as METHYLTRANSFERASE 1 (MET1) in plants while the CHG (H being any nucleotide other than C or G) sequence is maintained by MET1 and a transferase unique to plants CHROMOMETHYLASE 3
The asymmetrical sequence is both established and maintained by the Dnmt3 homolog DOMAINS REARRANGED METHYLTRANSFERASE 2 (DRM2), the only methyltransferase capable of perpetuating \textit{de novo} style methylation of both symmetrical and asymmetrical methylation in plants (Matzke et al. 2009; Cahan et al. 2005). DNA methylation is essentially a two-fold process consisting of laying initial methyl marks on the cytosine nucleotide targeted as well as maintaining those marks during the process of replication and post-replication (Vanyushin and Ashapkin, 2011). This process, first observed by Wessanger et al. (2005), is referred to as RNA-directed DNA methylation (RdDM) due to it being propagated by small interfering RNAs (siRNAs). RdDM is a conglomerate of mechanisms involving the well know RNA interface machinery (RNAi) and DRM2 along with two plant specific RNA polymerases, PolIV and PolV, working in tandem with two chromatin remodeling factors (Huetterl et al. 2007; Matzke et al. 2009; Pikaard et al. 2008). The degree of specificity of DNA methylation is not restricted to the nucleotide or sequence context but also depends upon the accessibility and structural organization of the chromatin, hence why chromatin-remodeling factors are an essential part in RdDM (Vanyushin and Ashapkin, 2011).

The RdDM pathway begins with the transcription of single stranded RNAs from RNA Pol IV, which are used as substrates by RNA-DEPENDENT RNA POLMERASE 2 (RDR2) to form double-stranded RNAs (Matzke et al. 2009). The traditional RNAi machinery processes the newly produced dsRNA; DICER-LIKE 3 (DCL3) cuts the dsRNA into 21 to 24-nt siRNAs, which are then loaded onto argonaute (AGO) protein complexes, namely AGO4 and AGO6 (Henderson and Jacobsen, 2007; Matzke et al. 2009).
AGO4 is directed to target loci through base pairing of siRNAs and intergenic noncoding transcripts (IGN) produced by Pol V, where IGNs act as scaffolding for the recruitment of the silencing machinery (Wierzbicki et al. 2008; Haag et al. 2009).

However, the recruitment of DRM2, thought to be held in place by IGNs, is still poorly understood. Recent studies point to two newly discovered proteins that potentially aid in the signaling of DRM2 bridging the gap between siRNA bound AGO4 and IGN transcribing pathways. SUPPRESSOR OF TY INSERTION 5-LIKE (SPT5-like), a homolog of the protein SPT5 in yeast has been identified as a downstream effector of RdDM by interacting with AGO4 through a conserved glycine/tryptophan motif found in their carboxy-terminus (Huang et al. 2009; He et al. 2009). Likewise, INVOLVED IN DE NOVO 2 (IDN2), the second protein identified, shares homology with SUPPRESSOR OF GENE SILENCING 3 (SGS3) in that it maintains an XS domain (Austin et al. 2009). The XS domain is functionally important because it contains an aspartate residue that is capable of recognizing dsRNAs with 5’ overhangs (Austin et al. 2009). A potential substrate for the XS domain is the AGO4 bound siRNAs and IGN, which could be the signaling factor for DRM2.

DNA methylation has been mostly characterized as a developmental process for eukaryotic organisms, particularly showing dynamic changes in methylation status during embryogenesis or gametogenesis in plants. For both male and female gametogenesis, a genome-wide loss of methylation occurs in the tricellular pollen grains and ovule respectively (Huh et al. 2008; Slotkin et al. 2009). The pollen grains consist of two sperms cells and one vegetative nucleus; the later undergoing hypomethylation leading to reactivation of many previously silenced transposons (Slotkin et al. 2009). Genetic
information is only being passed on from the sperm cells, meaning that their genetic integrity must be kept in tact unlike the vegetative nucleus. Recent studies conducted by Slotkin et al. (2009) have depicted an interesting theory as to the purpose of the reactivation of transposons within the vegetative nucleus. Sequencing of the sperm cells has revealed high levels of siRNAs corresponding to silenced transposons, these particular siRNAs are thought to be products of the vegetative cell that travel to the sperm cell and reinforce the RdDM already occurring. By undergoing hypomethylation, the vegetative cell is affectively allowing potentially harmful TEs to be expressed, which triggers the production of siRNAs theoretically initiating RdDM in the sperms cells thus ensuring a deeper level of genetic security (Law and Jacobsen, 2010). Similarly, the female gametophyte DNA undergoes hypomethylation at discrete loci in the endosperm while the embryo remains predominantly methylated (Mosher et al. 2009; Hsieh et al. 2009). It is important to note that the 21-nt siRNAs produced from the vegetative cell and endosperm act in \textit{trans} on their subsequent targets, being the sperm cells and embryo respectively (Anges et al. 2013).

Dynamic changes in methylation status also occur during cell proliferation and differentiation within meristematic regions. During proliferation multitudes of cells are produced, all with heavily decreased methylation counts while the process of cell differentiation reestablishes lost methylation marks along with new ones to generate specific cell types (Tesillano et al. 2013). Agnes et al. (2013) demonstrated that active movement of methylation groups aid in restricting vascular propagation of \textit{Pto} DC3000, \textit{Pseudomonas syringae pv tomato}, in \textit{Arabidopsis}. This defense mechanism is active in regions where cell division is still occurring and maintenance methylation is ongoing,
silencing targets of RdDM. Once the plant has detected a microbe, a MAMP-triggered response is activated (microbe associated molecular pathway) that inactivates transcriptional gene silencing (TGS) by downregulating key components of RdDM, most importantly AGO4, AGO6, and MET1, while derepressing a subset of immune-response genes in cis, namely RESISTANCE METHYLATED GENE 1 (RMG1) (Agnes et al. 2013). Much like with the pollen grains, an accumulation of siRNAs acting in a trans manner was noted during MAMP-triggered release of TGS. Agnes et al. (2013) proposes that the release of TGS through both PAMP (pathogen associated molecular pathway) and MAMP-triggered responses leads to the production of TE-based substrates for DICER LIKE (DCL) proteins that initiate the biogenesis of 24-nt siRNAs. These trans-acting siRNAs then work on the regulators of the defense genes in the cells surrounding the areas of TE activation, ultimately silencing them. Once the regulators of the defense genes are turned off, the expression of genes like RMG1 form an immune cell layer around the vasculature, preventing the propagation of Pto DC3000 from xylem cells to neighboring mesophyll cells (Agnes et al. 2013). These findings agree with Slotkin et al. (2009) and his proposal of enhanced sperm cell genetic integrity. By undergoing a MAMP-triggered repression of TGS, the pollen grain is ensuring that the sperm cell will have a constitutively expressed antimicrobial immune response, which will limit the transmission of pathogens from the pollen grain (Agnes et al. 2013, Mink, 1993).

Until now, only the initial placement and maintenance of methyl marks have been discussed in depth but the process of removing methyl groups is just as complex, being either an active or passive process. Whether an active or passive process, specific enzymes ranging in distinctiveness depending upon the tissue type in which they are
found carry out DNA demethylation. The procedure of active demethylation is achieved by encoding DNA glycosylases, which possibly works with the BASE EXCISION REPAIR (BER) pathway (Zhu, 2009). The best-known members of the glycosylase family are DEMETER (DME) and REPRESSOR OF SILENCING 1 (ROS1), ROS1 being the counterweight of RdDM in the biotic stress response of *Arabidopsis* (Anges et al. 2013; Choi et al. 2002; Gong et al. 2002). The function of these enzymes is to recognize and remove methylated cytosines from double stranded DNA oligonucleotides when needed (Lister et al. 2008). As with methylatransferases, both DME and ROS1 share homology with the bifunctional glycoslase class of HELIX-HAIRPIN-HELIX-GLY-PRO-ASP (HhH-GPD), found in mammals (Baute and Depicker, 2008; Gehring et al. 2009). These enzymes are dubbed bifunctional because they break the N-glycosidic bond releasing the methylated cytosine as well as break the DNA backbone (Baute and Depicker, 2008; Gehring et al. 2009). An unknown pathway, perhaps BER, then repairs the DNA, though most notably BER is found in association with the removal of mutagenic substrates not with the repair of DNA from demethylation (Baute and Depicker, 2008). Though DME and ROS1 have similar roles, the latter is found in vegetative tissues while the former plays an active role in female gametogenesis by establishing imprinting (Huh et al. 2008). Already well-documented for establishing additional methyl marks on the DNA in mammals, imprinting for plants removes methyl marks from the DNA in the endosperm, thus reactivating previously silenced transposons (Huh et al. 2008). As previously stated, the endosperm during gametogenesis is hypomethylated, though this cannot be fully attributed to DME. The majority of methyl groups that are lost are thought to be removed by the passive demethylation process, such
as through the repression of MET1 (Jullien at al. 2008). Jullien et al. (2008) showed that MET1 is actively repressed during gametogenesis by two newly recognized proteins - MULTICOPY SUPPRESSOR OF IRA 1 (MSI1) and RETINOBLASTOMA RELATED 1 (RBR1). The repression of MET1, which is a maintenance methyaltransferase, and the active removal of methyl groups by DME are thought be to be the driving factor behind the activation of imprinted genes. Imprinting is still poorly understood in plants, with only six genes that have been effectively demonstrated to be materially imprinted in Arabidopisis.

Imprinting is an epigenetic mechanism by which genes are expressed in a parent of origin manner. For plants, this is perhaps most useful when considering the silencing of TE’s through successive generations. Imprinting ensures harmful and or repetitive transposons will remain epigenetically silenced throughout gametogenic reprogramming. Imprinting is restricted to only organisms with a “placental habit,” or an area that does not contribute directly to the genetic information (Granier et al. 2008). The endosperm is a product of fertilization and therefore does not contribute genetic information unto progeny, which makes the hypomethylation occurring an epigenetic modification rather than a genetic sequence alteration (Granier et al. 2008). Since it is an epigenetic change, parental genomic imprinting or unipaternal gene expression can be used to explain it. Expression of RNA Pol IV-dependent siRNAs, Pol IV being the initiator of siRNA biogenesis, is maternally specific in the endosperm or rather paternally imprinted (Mosher et al, 2009). This results in a genome-wide loss of CG methylation that coincides with an increase in CHH or CHG methylation as well as the expression of neighboring imprinted genes (Gerhing et al. 2009). This supports the findings of Jullien
et al. (2008) where MET1 activity is actively repressed in the endosperm, MET1 being responsible for the symmetric CG maintenance methylation. From here, the biogenesis of the 21 to 24-nt siRNAs due to paternal imprinting in the endosperm, leads to the proposed antimicrobial defense mechanism displayed in the embryo from the activation of TE’s within the endosperm propagating the enhanced silencing within the embryo.

Successful imprinting enables progeny to have a developmental advantage, though it is not the only way to influence the genome of offspring without changing the DNA sequence. DNA methylation is on a whole a more permanent change that lasts generations but alterations to histone and polycomb group proteins provide a more liable alternative for gene silencing (Vanyushin and Ashapkin, 2011). By altering the histone or DNA-histone relation, expression of genes can be regulated due to accessibility. Histone 3 lysine 9 (H3K9) is one particular histone tail that when methylated causes expression from genes wrapped around histone 3 to be silenced. The methyl group forces the DNA and histone to have an increased affinity for each other meaning that expression will not occur because the DNA will be inaccessible for replication. DNA-histone methylation patterns will not necessarily be passed onto progeny or even from cell to cell; rather modifications like these can be considered a local change. Akimoto et al. (2007) demonstrated that artificial changes in methylation status of the DNA could also create local changes that will induce adaptive responses to abiotic stress. Using rice and the rice pathogen Xanthomonas oryzae, Akimoto et al. (2007) noted three trends of local DNA methylation changes namely that an adaptive response will be initiated by the local change and have a possibility of lasting a few generations before ultimately reverting to wild type conditions. The ability to retain local changes in DNA methylation status
represents another layer of mitotic heritability for epigenetic changes. This heritability can also be seen in apomictic clones and their subsequent progeny. Verhoeven at al. 2009 found that local changes incurred from the parental plants still existed in the offspring when dandelion clones were exposed to a barrage of environmental stresses while progeny were left unstressed. This demonstrates that there is a transgenerational epigenetic plasticity of a single genotype though variations where found between the clones in the degree of local changes to the point that even new local changes were observed in the progeny.

1.3 Epigenetic Mechanism for Brown Marsh

If DNA methylation can be mitotically inherited and local changes in methylation status have the possibility to be transgenerational for apomictic clones, the probability of establishing and maintaining epigenetic changes due to environmental stresses in apomictic vegetative clones should be just as likely. Here, a differentiation must be established between apogamy and vegetative apomixis. The former undergoes a portion of sexual reproduction, either through selfing or spores, while the latter relies on a reduced internment sexual stage and prolonged expansion by vegetative means (Klekowski, 2003). Reproducing through a strictly vegetative process should allow for the transmission of local and global changes in DNA methylation status, perhaps even histone or polycomb variants. The gametic reprogramming responsible for resetting the epigenetic status of both pollen and the embryo is sidestepped and genetically identical plants are propagated through a network of underground stems known as rhizomes. Biotic and abiotic stress can influence the methylation status of DNA facilitating an adaptive response that has the potential to persist for generations. Vegetative clones can
be thought of as either a new plant, or perhaps more accurately, the branching of an existing plant that is genetically identical to the parental strand, which is how this thesis will treat them. Adaptive responses established in the parental clone are passed onto the vegetative progeny mitotically, perhaps in a much more successful manner than sexually.

*Spartina alterniflora* is a vegetative, apomictically-reproducing species growing predominantly across marshes on the Atlantic coast of North America. Expansion of this species occurs primarily through the propagation of vegetative clones from underground rhizomes, though brief stages of sexual reproduction can occur to establish new colonies. Analysis of the DNA methylation of this species allows the examination of the heritability aspect of epigenetic modifications. Since this species is chiefly a clonal species, the mitotic heritability component and proposed memory capacity seen within progeny of dandelion clones can also be assumed to be present in *S. alterniflora* along with the mitotic heritability displayed through gametogenesis. The adaptations and alterations of the epigenome noted in *Arabidopsis* during a pathogenic infection, or biotic stress, can also be expected to be present in *S. alterniflora* during an abiotic stress.

Methylation may accumulate within a marsh stand in a clock-like fashion, with each passing year increasing the scale of genomic dysregulation. In addition, methylation may also be accumulating in response to environmental stresses themselves. In both cases, the accumulation of methylation will be synchronized across large sections of a stand since individual stems experience essentially the same stresses as their neighbors. We are proposing a generalized mechanism of increasing stress susceptibility with stand age in which intrinsic properties of a marsh stand breakdown gradually over time. The quality of the stand degrades, but the degradation has no outward effects other than
increasing the susceptibility to environmental stressors. Eventually, a tipping point is triggered by the combination of the degraded marsh state and an environmental stress resulting in a brown marsh event. We are proposing that DNA methylation and the potential accumulation of methylation from both time and environmental stress is the mechanism for brown marsh.
CHAPTER 2
FIELD SURVEY AND CRAB HAUL CREEK GENETS

2.1 Sample Location Selection and Genet Identification

2.1.1 Field Site- North Inlet

The North Inlet basin (Figure 2.1) is classified as a bar-built barrier beach estuary (NOAA, 2006) that is predominately populated by *Spartina alterniflora* and composed of largely of tidal channels (Gardner and Porter, 2001). North Inlet is located in Georgetown, South Carolina, and is one of the National Estuarine Research Reserve System (NERRS) sites that are sponsored by NOAA. Semidiurnal tides flush the basin resulting in approximately half of its water being discharged into the ocean (NOAA, 2006). The majority of the marsh is connected through winding tidal creeks that create an extensive hydrologic network. To the east, barrier islands surround portions of the marsh basin while the western barrier of the basin is forested (Gardner and Porter, 2001). The southern barrier is Winyah Bay.

2.1.2 Sampling Locations

To assess the level DNA methylation in *S. alterniflora* clones, samples were taken from five sites across the North Inlet marsh basin to test populations living in a variety of conditions (Figure 2.1). Both inland populations and various open water populations were selected to place emphasis on environmental stressors, like the accumulation of salinity.
from evaporation and the lack of regular tidal flushing. Crab Haul Creek (CBC), the most internal marsh basin in North Inlet, is predominantly composed of short-form *S. alterniflora* plants while tall-form populations line its tidal creeks. Due to its internal location, the CBC basin only receives a water input twice a day from the tidal creek. Without a constant influx of water, the evaporation of standing water leaves higher levels of salt in the soil forcing local *S. alterniflora* populations to combat a potentially more stressful condition.

Two open water locations were selected composed of tall-form populations, Old Man Creek (OMC) and South Town Creek. The interior portion of the South Town Creek site experienced a minor dieback event very recently, attributed to wrack accumulation, and was in the process of repopulation through seedlings. Finding a sexually produced plant provides a potentially unique look in DNA methylation in comparison to those produced through asexual cloning. Samples of the living periphery (STP) and the seedling were taken (STS).

In 2002, North Inlet experienced a minor dieback event resulting in the loss of a significant portion of the *S. alterniflora* populations composing the 60 Bass Creek. The interior portion of the 60 Bass Creek marsh died leaving a periphery of seemingly healthy plants behind. This presented a prime location for sampling plants directly involved in brown marsh event and those potentially re-reproducing from rhizomes. Healthy plants furthest from the event were labeled as 60 Bass No Dieback (SBND), those surrounding the dieback were labeled as 60 Bass Dieback Periphery (SBDBP), and the plants sampled from the exact dieback location were labeled as 60 Bass Dieback (SBDB). Lastly, samples were taken from open water location on the northern
Figure 2.1 Map of North Inlet, SC with an inlay of its location along the coast of South Carolina. Each sampling location has been marked with a yellow indicator.
edge of the NERR site closest to local inhabitants. Debidue Creek (D) is composed of tall-form populations.

2.1.3 Clonal Populations

*S. alterniflora* had not colonized the entire Crab Haul Creek basin leaving many open mud flats in 2014. In established portions of the marsh genets had grown together and have begun progressive lateral movements into unoccupied locations. Growing together makes it impossible to distinguish separate colonies in older sections of the CBC but newly forming independent genets can be seen in some areas (Figure 2.2). These identifiable genets provide a potential look into global DNA methylation across clones in which an arbitrary age can be associated. Older plants are expected to make up the interior of the genet while newly budding younger plants expand radially outward.

Using Google Maps time series progression, ideal locations for clonal sampling were narrowed down to Crab Haul Creek. GPS coordinates for isolated and clearly identifiable colonies were taken and used in the field. Each genet was measured and divided into four quadrants (Figure 2.3). Samples were taken from the outermost edges, midpoints, and centers of each transect on a North/South and East/West direction. The center was estimated to be halfway between the outermost edges and the midpoint was half way between the outermost edge and the center. Four genets were clearly identifiable and collected in total.

2.1.4 Sample Collection and Methylation Quantification Methods

The top 10 cm of every flag leaf was collected from visually healthy *S. alterniflora* plants in the field. Collected samples were kept on ice and stored in an -80°C freezer until it was processed. A 10% bleach solution was used to wash each sample,
Figure 2.2 Three Google Earth images illustrating clonal expansion into bare mudflats in Crab Haul Creek. The images range from (A) 2009, (B) 2011, and (C) 2014.
Figure 2.3 Identification of independent *S. alterniflora* colonies in Crab Haul Creek.
eliminating fungi and other bacterial components, and thoroughly rinsed with deionized water. After washing, leaf tissue was ground into a fine powder using liquid nitrogen and a mortar and pestle and weighed out to approximately 100 mg. Each sample was stored in a -80°C freezer until DNA extraction. DNA extraction was completed using Qiagen DNeasy Plant kits according to established Qiagen protocols. Extracted samples were tested for purity, 1.7-1.9 based on 260/280 ratio, and concentration using an Eppendorf Biophotometer. All samples were run on agarose gel for quality.

To ascertain the percentage of DNA methylation, 100 ng/ul of each sample was used in an Epigentek MethylFlash Methylated DNA 5-mC Quantification Kit (Fluorometric) was used. Using Epigentek’s established protocols, an enzyme-linked immunosorbent assay (ELISA)-like format for fluorescence-based quantification of DNA occurs through binding DNA to strip wells in a microplate. Methylated DNA is detected using antibodies and enhanced for quantification from absorbance in a fluorescence microplate spectrophotometer at specific excitation.

2.2 Results

Preliminary sampling using an ELISA-like format for fluorescence based quantification of DNA methylation was taken from North Inlet, SC, and has shown global methylation for *S. alterniflora* clones to range from ~3-11% (Fig 2.4). Due to the finding of seedling at the South Town location, three separate ANOVAs were used to test for significance of DNA methylation. The three groups were created to test for significance based either on location, age, or previous history (Figure 2.5). The South Town seedlings were compared against the surrounding clonal populations of South
Town, the adult populations from each of the sample locations were compared against each other while the last test group consisted of the just the 60 Bass location.

As previously stated, various sections of the marsh were sampled including the 60 Bass dieback sight that occurred in 2002 but has since recolonized. Samples were taken from the center of dieback (SBDB), the periphery (SBDBP), and the outer most regions were no dieback event was recorded (SBND). Global methylation was analyzed using an ANOVA and showed no significance different between the three test groups in 60 Bass as seen in Table 2.1 (p=.0611).

![Global Methylation Across Varying Populations of Spartina alterniflora in North Inlet, SC](image)

Figure 2.4 Global methylation percentages from 5 isolated locations in North Inlet, SC.
Inland short form populations from Crab Haul Creek were also sampled (CHCA & CHCB), along with three open channel populations Debidue Creek (D), Old Man Creek (OMC) and South Town (ST). Comparing the adult populations using an ANOVA, including that of 60 Bass, CHC was significantly higher in methylation (Table 2.1). A population of seedlings was found at the South Town location (STS) and tested against surrounding adult colonies showing a significant difference in methylation (Table 2.1). Significant differences (ANOVA, p=3.38*10^{-6}) were found between the South Town seedlings and the adult populations from the same location as well as Debidue, and 60 Bass. Figure 2.5 shows the corresponding methylation percentages for the ANOVAs preformed in Table 2.1.

Figure 2.5 Division of the field survey into comparable test groups.
Table 2.1 Methylation significance from ANOVA analysis.

<table>
<thead>
<tr>
<th>Location</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Debidue</td>
<td>2.473e-7</td>
</tr>
<tr>
<td>Old Man Creek</td>
<td>3.38e-6</td>
</tr>
<tr>
<td>South Town</td>
<td></td>
</tr>
<tr>
<td>60 Bass ND</td>
<td>0.0611</td>
</tr>
<tr>
<td>60 Bass DBP</td>
<td></td>
</tr>
<tr>
<td>60 Bass DB</td>
<td></td>
</tr>
<tr>
<td>Crab Haul Creek</td>
<td></td>
</tr>
<tr>
<td>60 Bass ND</td>
<td>0.0001</td>
</tr>
<tr>
<td>60 Bass DBP</td>
<td></td>
</tr>
<tr>
<td>60 Bass DB</td>
<td></td>
</tr>
<tr>
<td>South Town Seedlings</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

At the CHC location, 4 separate genets were identified and sampled to test the hypothesis that global methylation percentages increase based on location or “age” within the genet (Fig 2.6). Findings from 3 of the genets have not shown a significant trend in genet position and global methylation percentages, however the third genet shows a trend along the north/south axis (Fig 2.7). The center of the genet has the lowest methylation total, ~5%, while the midpoints and outer edges range from 7 to 9% methylated, increasing in methylation radially away from the center. A regression of the North/South data gives an $R^2$ value of 0.869 while an ANOVA of the data delivers a p-value of $p=0.0352$, perhaps showing some significance to the methylation accumulation with age. The East/West data does not show the same trend.
Figure 2.6 Global methylation percentages from four isolated genets in Crab Haul Creek.

Figure 2.7 Global methylation percentage distribution in genet 3.
Global DNA Methylation and Clonal Genets

Global methylation is a quantification of all cytosines within the genome. It provides a broad interpretation of methyl groups without any reference as to location within the genome or if local accumulation is occurring, meaning grouping of methyl groups in specific locations or genes. DNA methylation is both a crucial and vital part of regulation and genomic stability namely by silencing transposable elements (TE) but its positioning within the genome is paramount. Unlike mammals, plant introns are almost completely free of TEs suggesting that methylation, or rather methylation of the gene body, is not necessary. Zhang et al. (2006) and Vaughn et al. (2007) both show this is not true; in fact, nearly 20-30% of genes in Arabidopsis show significant gene body methylation of CG sites. In this context the gene itself is not silenced, the majority of genes from Arabidopsis have unmethylated promoter regions and continue to produce normal levels of functioning protein products while remaining reasonably active. This suggests that although DNA methylation can be synonymous with gene silencing, the shear amount of methyl groups are not nearly as important as the placement of the methyl either on the gene, histone tail, or chromosome itself.

Global DNA methylation of S. alterniflora clones in North Inlet, SC have shown to be variable ranging as low as 4%, 60 Bass, to 12%, South Town Seedlings. DNA methylation in plants can range from 4.6% of all cytosines to 37.2% depending on species (Messeguer et al., 1991). Typically, cytosine methylation has been negatively correlated with gene expression (Cedar, 1988) suggesting that higher methylation percentages are influencing either the gene itself or its protein product. There is evidence not only of DNA methylation blocking transcription factors from binding to DNA but
also protein products preferentially binding to methylated DNA thereby inhibiting transcription machinery (Boyes and Bird, 1991). Although exact mechanisms are not fully clear, DNA methylation has become synonymous with gene silencing. Our findings from the North Inlet field survey are not surprising for global methylation percentages in comparison to other plant species; in fact they fall perfectly into the range Messeguer et al. (1991) published. They do not however, support the hypothesis that increased age and the accumulation of DNA methylation globally result in the loss of phenotypic plasticity.

The sampling locations from the field survey included the 60 Bass site that experienced a dieback event. Presumably, because the interior experienced the dieback and was later recolonized, those plants should be the youngest and least heavily methylated making the surrounding periphery older and perhaps more methylated. Although the differences were not significant, there is a noticeable difference in global methylation between the SBND, SBDBP, and SBDB showing that exact trend. However, the highest degree of methylation was observed in plants that should epigenetically be the youngest, the South Town seedlings. Being that the seedlings were produced via sexual means, the seedlings would have been forced through the mitotic checkpoints. All DNA methylation would have been wiped clean and new ones added irrespective of parental patterns.

The four genets sampled in Crab Haul Creek exhibited clear variability in methylation but three of the four genets showed no visible pattern in relation to genet position (potentially age) and the degree to which it was methylated. Genet 3 however, fits a potentially significant trend between clonal age and methylation. The approximated center of genet should consist of the oldest plants while the outer edges are composed of
newly budding ramets expanding the colony. Knowing that vegetatively apomictic species essentially bypass mitotic check points designed to reestablish DNA methylation marks, it would follow that each progeny would contain parental DNA methylation patterns in addition to having the ability to add more methyl groups. This would mean outward clones could potentially be more heavily methylated. Preite el al. (2015) demonstrated that DNA methylation variation and successive accumulation of methylation can be passed unto progeny and remain stable for generations while not limiting the progenies ability to further methylate the genome.
CHAPTER 3
GREENHOUSE SALINITY STUDY

3.1 Greenhouse Salinity Experiment Methods

Samples taken from the preliminary field survey were used to test for potentially significant difference in DNA methylation based upon location. Significant differences in DNA methylation determined which populations (based on location) would be used in the greenhouse study. *S. alterniflora* plants were extracted in the field by digging out 15 cm² cores (in order to protect rhizome tissue and some root matter). 50 plants were taken from each of the 3 locations, Crab Haul Creek (CBC), South Town Seedlings (STS), and open creek bank populations from South Town (CB), and transported to the USC greenhouse in Columbia, SC.

A Hoagland’s nutrient solution ratio was used in conjunction with Instant Ocean to achieve a 30 ppt saline solution. Individual plants were placed in separate growth cups with their own nutrient media and covered with a Styrofoam lid (Figure 3.1). Above-ground portions of the plant were fed through a hole cut into the lid. Each week, 18 plants from each location were subjected to a 5 ppt salinity increase while their corresponding controls were kept at 30 ppt. Nondestructive biomass measurements were taken by measuring weekly growth of above ground biomass. Plant stress measurements were also taken by quantifying quantum efficiency (QE), commonly known as yield (Fv/Fm) with a
PAM-210 chlorophyll fluorometer. QE is a measurement of how well electrons are passed through photosystems II and I in active photoreaction centers. Progeny of plants were also measured and collected for DNA extraction. In some cases, stress to the parental clone was too great and only the progeny survived. All statistical analysis was completed using Matlab_R2015b. Sites of sampling were photographed through the summer of 2014 to watch growth and expansion of each population, so that a potential dieback, wrack accumulation, or clonal expansion would be documented for comparison to the greenhouse experiment. Since the South Town location was undergoing repopulation from seedlings, progression of their colonization and overall health was useful for comparison to greenhouse findings.

The top 10 cm of every flag leaf was collected from visually healthy *S. alterniflora* plants in the field. Collected samples were kept on ice and stored in an -80°C freezer until it was processed. A 10% bleach solution was used to wash each sample, eliminating fungi and other bacterial components, and thoroughly rinsed with deionized water. After washing, leaf tissue was ground into a fine powder using liquid nitrogen and a mortar and pestle and weighed out to approximately 100 mg. Each sample was stored in an -80°C freezer until DNA extraction. DNA extraction was completed using Qiagen DNeasy Plant kits according to established Qiagen protocols. Extracted samples were tested for purity and concentration using an Eppendorf Biophotometer.

To ascertain the percentage of DNA methylation, 100 ng/ul of each sample was used in an Epigentek MethylFlash Methylated DNA 5-mC Quantification Kit (Fluorometric) was used. Using Epigentek’s established protocols, an enzyme-linked immunosorbent assay (ELISA)-like format for fluorescence-based quantification of DNA
occurs through binding DNA to strip wells in a microplate. Methylated DNA is detected using antibodies and enhanced for quantification from absorbance in a fluorescence microplate spectrophotometer at specific excitation.

3.2 Results

With each week an increase of salinity by 5ppt was made for each of the test samples. To ascertain the level of stress each plant was under and measure the productivity of each plant, weekly biomass measurements were taken by measuring the growth of stems each week as well as measuring quantum efficiency (QE) of photoreaction center in the leaves. Results from the salinity stress tests in the greenhouse

Figure 3.1 Greenhouse salinity experiment setup. 18 duplicates were used for each test group along with corresponding controls.
show that increase salinity negative impacts growth of *S. alterniflora*. Figure 3.2 and Table 3.1 represent the normalized weekly growth and QE, respectively, in relation to increasing salinity concentrations. Weekly growth measurements averaged approximately .007-.013 cm for the first three weeks (week one has been omitted from graph), salinity ranging from 35-45 ppt, when there is a spike in growth from the Creek Bank (CB) test populations of .023 cm. After the third week, all three of the test populations, CB, STS, and CBC, begin a steady decline in growth while the controls continue to grow.

Table 3.1 shows the plants QE at 30ppt and at 60ppt. The controls for each test group were kept at 30 ppt for all seven weeks and two of them, Control CBC & Control CB, have a QE of ~0. 7, indicative of healthy plants. Control STS however shows a very large drop in QE from week 3 to week 7 with one-third of their plants dying. Pictures from the field of the South Town seedlings also show large portions of the seedlings dying off (Figure 3.3). All three test populations (CB, STS, CBC) follow a dramatic decrease in QE with each increase in salinity. Mortality was seen in STS, similar to the

Table 3.1 QE measurements and the associated standard deviations.

<table>
<thead>
<tr>
<th>Location</th>
<th>Salinity 30 (ppt)</th>
<th>Salinity 60 (ppt)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>QE</td>
<td>SD (+/-)</td>
</tr>
<tr>
<td>CBC</td>
<td>0.719</td>
<td>0.011</td>
</tr>
<tr>
<td>STS</td>
<td>0.726</td>
<td>0.009</td>
</tr>
<tr>
<td>CB</td>
<td>0.719</td>
<td>0.008</td>
</tr>
<tr>
<td>Control CBC</td>
<td>0.728</td>
<td>0.007</td>
</tr>
<tr>
<td>Control STS</td>
<td>0.718</td>
<td>0.009</td>
</tr>
<tr>
<td>Control CB</td>
<td>0.719</td>
<td>0.004</td>
</tr>
</tbody>
</table>
control STS plans. CB plants did not suffer as dramatic a drop in QE until week 6 and continued to produce new ramets until week 7. In both graphs, progeny were measured for both weekly growth and QE and factored into each graph.

Although QE and growth were adversely impacted by increases in salinity, differences in global DNA methylation were not noticeable. An ANOVA of the data gives a p value of 0.0564, citing the difference between STS Control and CBC Control as the cause for significance. Methylation values range from 5-8% on average for both the controls and test groups for all 3 locations used (Figure 3.4). Using a two-way ANOVA

Figure 3.2 Normalized weekly growth measurements. Each bar represents an increase in solution salinity, with week 1 representing the first increasing in salinity of 5ppt, from 30 to 35ppt.
Figure 3.3 Four images of the South Town location. Images A and C show a high mortality rate among the newly colonizing seedlings while images B and D both depict a relatively healthy periphery of presumably older clonal genets.
to compare methylation percentages by test group and salinity stress revealed no statistically significant difference based on test group, whether the plant was subjected to stressed by salinity, or the test groups by stress (p= 0.5073, 0.5971, 0.6613 respectively).

3.3 Salinity Stress and Accumulation of Methylation

The increase in salinity per week led to a direct decline in plant fitness and in some cases mortality. Photoreaction centers began to close once salinity levels began increasing, as evident in QE yields, limiting the process of photosynthesis. Once photosynthesis has been either hindered dramatically or stopped, cellular respiration takes

Figure 3.4 Final global methylation percentages from the greenhouse study. Blue bars represent test groups while yellow bars represent their corresponding controls.
over within plant cells costing vital nutrients. This cost manifests itself in the plant’s ability to continue to grow. All of the test groups in which the salinity was increased saw a drop in weekly growth leading to chlorosis and in some cases death. Unexpectedly, a similar drop in both QE and growth was observed in the South Town seedlings controls. Kept at 30ppt each week, there was no increase in stress but plant productively declined none the less for a select portion. Similarly, significant portions of the South Town Seedlings were dying in the field while others remained seemingly healthy. This unexplained die-off could be explained by either the high methylation percentages seen from preliminary work or perhaps more readily through genotypic differences. Because the area was in the process of re-colonization from seed, each of those seedlings has the potential to carry a different genotype. Taking its location into account, South Town could have been repopulated with less-well adapted genotypes that were ill suited to compete in a continuously inundated (Fig 4.6) location with high salinity levels.

No accumulation of methylation was observed in the test groups after being subjected to higher levels of salinity for seven weeks. Given what is known about gene body methylation, random accumulation of methylation is not as potentially harmful as specific placement of methyl groups on targeted locations. Our results show that although increasing environmental stress does impact survivorship, global methylation rates remain unaffected. This conclusion does not invalidate DNA methylation as a proposed mechanism for the brown marsh event; in fact it correlates with the gene-body methylation phenomena seen throughout various plant species in which a gene actively functions despite being heavily methylated. Gene body methylation is a mechanism to ensure genomic stability by silencing harmful TEs and repetitive elements mandating the
need for high levels of DNA methylation. To further consider DNA methylation as a mechanism for brown marsh, it would be advantageous to look at individual genes, specifically those used for stress and defensive purposes, and their methylation status.

Quantifying the level of global DNA methylation allowed us to gain insight on a particularly complicated and intricate pathway of gene regulation. Global methylation is on a whole a representation of a numbers game, only offering information on the amount of methyl groups without any context as to their location within the genome. Increasing environmental stress did not lead to an accumulation of global methylation as we thought though it does open the door for new hypotheses. The sheer number of methyl marks may not amount to a mechanism for brown marsh but the location of a few strategically placed could bring about considerable changes in gene regulation and response. Due to the lack of a definitive mechanism, the brown marsh event of 2000 remains unexplained. DNA methylation remains a very probable contender and a likely candidate for future research.
CHAPTER 4

CONCLUSION

Work from both the field study and greenhouse experiment has shown us that there is a great range of variety in DNA methylation between not only geographical location but also within the genet structure itself. Quantifying the level of global DNA methylation allowed us to gain insight on a particularly complicated and intricate pathway of gene regulation. Global methylation is on a whole a representation of a numbers game, only offering information on the amount of methyl groups without any context as to their location within the genome. Increasing environmental stress did not lead to an accumulation of global methylation as we thought though it does open the door for new hypotheses. The sheer number of methyl marks may not amount to a mechanism for brown marsh but the location of a few strategically placed could bring about considerable changes in gene regulation and response. Due to the lack of a definitive mechanism, the brown marsh event of 2000 remains unexplained. DNA methylation remains a very probable contender and a likely candidate for future research.
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