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Epigenetic inheritance of spine formation in sago palm (*Metroxylon sagu* Roettb)

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Abstract

The formation of spine is a primitive evolutionary event in plants. In sago palm, studies reporting the results of RAPD and AFLP analyses showed lack of significant genetic variation among spiny and non-spiny plants. This observation led us to the hypothesis that spine formation may be an epigenetic event. The inheritance of spine formation is of high interest to plant breeders because spineless plants are preferred due to their more cost efficient handling. In this study, we analyzed the DNA methylation patterns of sago palm DNA by distinguishing methylated (5dmC) and non-methylated cytosine (dC) in HPLC analysis. We optimized HPLC conditions (nucleoside digestion, retention time of mobile phase and stop time) and identified the factors (growth environment and age of the palms) which may have affected DNA methylation levels. Our data show that a flow rate of 0.2 mL/min using C18 columns was most suitable in distinguishing between dC and 5mdC peaks. There was significant difference in methylation percentage between spiny (21.5%) and non-spiny (11.5%) palms at $P \leq 0.05$, indicating that the formation of spine was an epigenetic event. Further, there was an indication that the wet environment may have caused the epigenetic event and that spine formation was also age dependent. The influence of these factors will be confirmed using a more sensitive technique such as the methylation-sensitive amplified polymorphism (MSAP) analysis.

Keywords: sago palm; epigenetic inheritance; DNA methylation; HPLC analysis.
Abbreviations used: high performance liquid chromatography HPLC; thin layer chromatography TLC; retention time RT; non-methylated cytosine dC; methylated cytosine mdC; amplified fragment length polymorphism AFLP; methylation-sensitive amplified polymorphism technique MSAP; random amplified DNA polymorphism RAPD; polyvinylpolypyrrolidone PVPP; CTAB cetyl trimethylammonium bromide.

Introduction

Sago palm (*Metroxylon sagu*) is a plant of high socio-economic importance in many countries of Southeast Asia. The palm contains high amounts of starch and can yield from 15 to 25 t of dry starch/ha, making it an important basic food item for indigenous peoples of Melanesia, Polynesia and South Asia (Flach, 1977). Sago starch is also used in manufacturing some important food and beverage products such as cyclodextrins (which are carriers for spices and flavors), glucose, high-fructose corn syrup, maltodextrins, and monosodium glutamate. It also serves as a food processing aid by modifying color, flavor, taste and texture (Flores, 2008). The high amount of starch in the trunk also makes the sago palm a potential source of biofuel. Plants are currently being tapped both as sources of food and fuel and there is an increasing need to discover and improve novel plant sources. Sago palm is a unique source of starch and considered a highly potential source for both for food and fuel. For its full utilization, an extensive study of its agronomy, ecology, physiology, and economics must be carried out (Ehara, 2009). The genus *Metroxylon* is indigenous to Southeast Asia and inhabits freshwater swamps and moist rainforests (Flach, 1997). There are two recognized species of *Metroxylon* according to Beccari (1918): *M. sagu* without spines and *M. rumphii* with spines. In 1986 Rauwerdink merged the two species into *M. sagu* based on the fact that seeds from spineless palms can produce spiny seedlings (Rauwerdink, 1986; Ehara et al., 1998) or vice versa, spineless seedlings from spiny seeds (Jong, 1995). Furthermore, Sastrapradja (1986) reported that both spiny and spineless sago palm plants could be obtained from the same mother plant. In 2009, Ehara published results of a genetic variation study using RAPD analysis. Various types of sago palms from Malaysia, Indonesia and Mindanao, Philippines were used. A total of 77 PCR products were analyzed for polymorphism. Results showed that regardless of type (spiny or spineless), two sago palm populations from Mindanao along with three populations from neighboring Sulawesi, Indonesia belong to only one cluster in the dendogram. Thus, the original proposal that spiny and spineless sago palms are the same *M. sagu* proposed by Rauwerdink (1986) was confirmed. In Mindanao, seven morphotypes of sago palms (two spiny and five spineless) were characterized according to their morphological band patterns on the petioles and rachises (Adtoon, 2009). Using RAPD analysis of long and short primers as well as the RPBII gene, no genetic variation was found confirming the presence of only one species, *M. sagu.* Boonsermsuk et al. (1995) investigated isozyme variations of sago palm in Thailand based on zymograms of peroxidase, esterase, acid phosphatase and sorbitol dehydrogenase. The results showed that zymogram patterns of sago palm plants showed were less varied, i.e., no significant differences were observed among the zymograms, suggesting that cultivars of sago palm plants in Southern part of Thailand are identical. Employing AFLP (amplified fragment length polymorphism) analysis, Kjær et al. (2004) concluded that variation in vegetative morphological characteristics, including armature (presence of spines), is not correlated with the underlying genetic variation in the sago palm. The study also supported the presently accepted taxonomy of Rauwerdink (1986) and

Table 1. Analytical parameters evaluated for the standards dC and 5mdC.

Standard	Concentration (µg/mL)	Peak Area	RT	Average RT	3% Window	Range of RT
dC	0.0001	716	13.608	13.69675	0.410903	13.28585
	0.0010	1757	13.683			to
	0.1000	38203	13.743			14.10765
	10.0000	3303108	13.753			
	Linear Regression: y = 330071x + 2426.1					
	R^2: 1.0000					
5mdC	0.0001	407	18.558	18.55325	0.556598	17.99665
	0.0010	994	18.542			to
	0.1000	28251	18.493			19.10985
	10.0000	2939583	18.620			
	Linear Regression: y = 293959x - 18.691					
	R^2: 1.0000					

Table 2. Analysis of variance of DNA methylation between compared parameters.

Compared parameters	Significance	P-value
Spiny vs. Non-spiny sago palm	0.008	$P \leq 0.05$ = significant
Wet vs. Dry environment	0.171	
Mature vs. Juvenile sago palm	0.495	

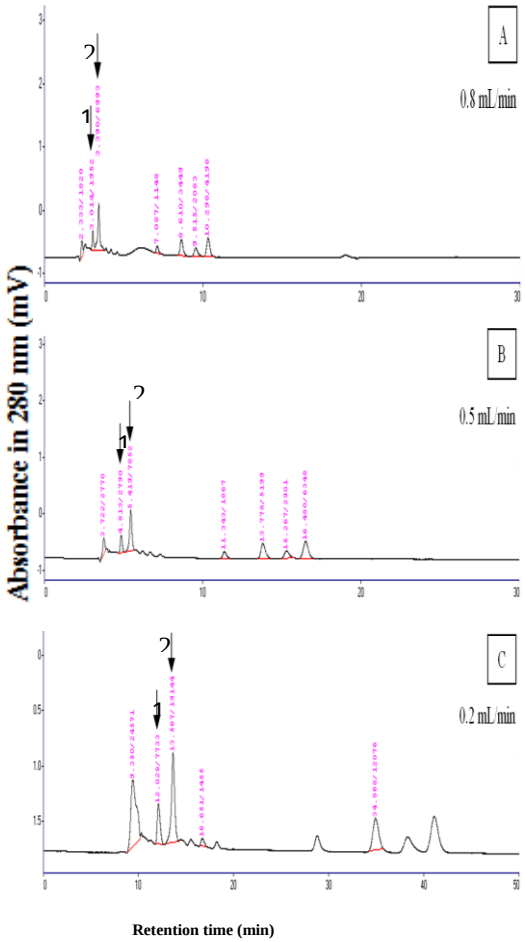

Fig 1. Effluents of the DNA samples from non-spiny juvenile sago palm in wet environment at different flow rates; A) 0.8 mL/min; B) 0.5 mL/min; C) 0.2 mL/min. Arrows 1 and 2 show peaks closest to one another whose retention times were used to optimize peak resolution.

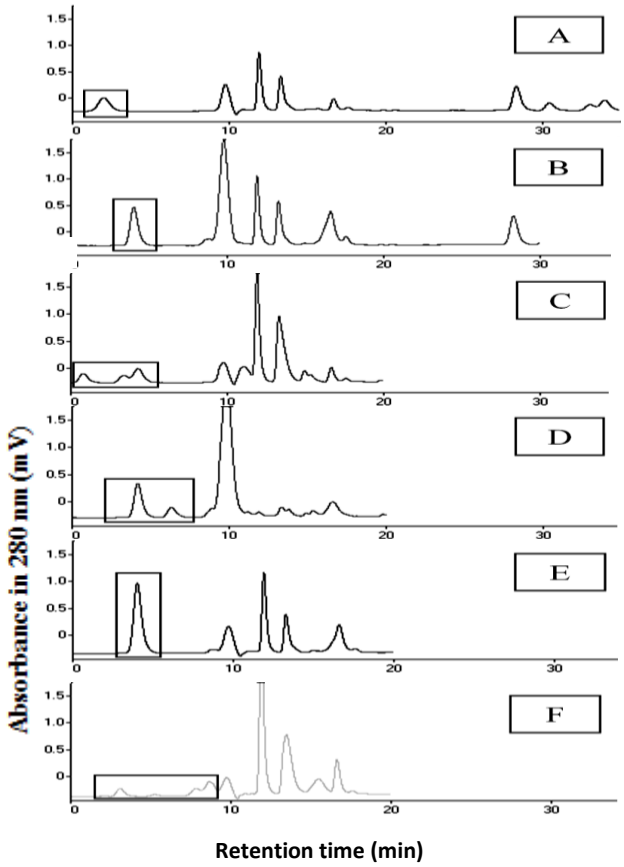

Fig 2. Chromatogram of DNA samples of spiny (A, B, C, and D) and nonspiny (E and F) juvenile sago palms in wet environment showing residual peaks (the boxed peaks are the residual peaks).

recognized only one species of *M. sagu* in Papua New Guinea. The formation of spines is suggested to be a Mendelian trait (Mora Urpi, as communicated to Clement, 2000) and because the presence of spines is a primitive trait, it is further considered dominant. The importance of spine for heart- of- palm ('pejibaye'*; Bactris gasipaes* Kunth) production was reviewed by Clement (2000). The heart-of-palm is an important gourmet vegetable in Hawaii and Brazil and spine formation in relation to yield required comparative cost analysis. Thus, it was important to define the superiority of either the spiny or spineless plant phenotype. To examine whether spineless palms manifested inbreeding depression (if less productive than spiny ones), Clement (1995) investigated whether there was a positive correlation between allozyme heterozygosity and spine density in eight spineless progenies of the landrace Benjamin Constant. There were no significant correlations found. Not much has been published about the genetics of spine formation in plants although it has long been suspected that there may be more than one gene involved. As early as 1940, Hutchins proposed that two genes govern the formation of spines on cucumber fruits. In *Daucus carota*, two dominant genes and some minor ones were suspected to affect formation of spines on seeds (Nieuwhof and Garritsen, 1984). A study on differential expression of genes associated with spine formation in *D. carota* revealed the presence of several genes. The genes included cell wall-associated hydrolase, tail-fiber assembly protein, transcriptional regulatory protein, berberine bridge enzyme, S-adenosyl methionine synthase, transketolase and phenylalanyl tRNA synthetase beta chain (Park et al., 2006). These genes were expressed only in spiny seeds. Though genetic variation studies on sago palm are few and far between, it appears that spine formation is a quantitatively controlled epigenetic event and the environment may have a significant effect on phenotypic expression. In sago palm, we found no significant differences in starch yield between four sago palm ecotypes sampled in Agusan del Sur (Novero, 2012). The many intriguing observations on spine formation and lack of genetic variation between different morphotypes led us to explore the epigenetic control of spine formation caused by ecological adaptation. In 'heart-of-palm' as in sago palm, the absence of spines, especially having no correlation with yield is a desired trait because the presence of spines entails additional burden and cost to plant cultural management and postharvest practices. DNA methylation is a type of chemical modification of DNA in eukaryotic organisms that involves addition of a methyl group to the carbon 5 position of the cytosine ring and is catalyzed by DNA methyltransferases in the context of the sequence 5'-CG-3' or CpG dinucleotide. In plants, cytosines are methylated both symmetrically (CpG or CpNpG) and asymmetrically (CpNpNp), where p denotes the phosphate group (Singal and Ginder, 1999) and where N is any nucleotide except guanine (Gruenbaum et al., 1981; McClelland, 1983; Finnegan et al., 1998). This modification of DNA can be inherited and can also be removed without any changes in the DNA sequence thus making DNA methylation a part of the epigenetic code. It is also the best characterized molecular mechanism that mediates epigenetic phenomena (Jaenisch and Bird, 2003). One role of DNA methylation is in the differential regulation of gene expression (Holliday and Pugh, 1975; Riggs, 1975). A number of previous studies have suggested a possible mechanism (i.e. DNA methylation) in which site specific cytosine methylation within or adjacent to genes was found to be correlated with transcriptional repression. This inverse relationship, i.e., transcriptionally silent genes are generally observed to be more methylated compared to active genes in promoter or certain coding regions, has been observed in a large number of genes already (Singal and Ginder, 1999; Guangyuan et al., 2006). In the last ten years, molecular techniques specifically immunoprecipitation via anti-5'methylcytosine and selective digestion with methylation-sensitive restriction endonucleases were employed to examine DNA methylation levels indirectly (Harrison and Parle-McDermott, 2011). In plants, the heritability of DNA methylation is often investigated using methylation-sensitive amplified polymorphism technique (MSAP) such as in adaptive genetic divergence analysis of *Viola cazorlensis* populations (Hererra and Bazaga, 2010). Although HPLC is the only tool currently available in our laboratory, it is also considered an effective method for assessing methylation of total DNA (Johnston et al 2005). Wagner and Capesius (1981) reported the first HPLC analysis procedure for DNA methylation in plants. Our study reports the optimization of HPLC conditions for assessing global DNA methylation patterns in sago palm as well as discusses the factors governing spine formation.

Results

Optimization of peak resolution

The success of DNA digestion was determined through running one sample in HPLC at a flow rate of 0.8 mL/ min (the higher flow rate used in the study of Sandhu et al., 2009) before the rest of the samples were analyzed. It was also done to determine the possible behavior of the peaks and the chromatogram profile of the DNA samples indicating the success of DNA digestion. Successful DNA digestion was evident by nucleoside peaks registered by the samples. Peak identity was confirmed using standard nucleosides. The HPLC- analyzed samples were also used to determine the optimum flow rate to establish a high peak resolution. Fig 1A and Fig 2B show peaks detected before 5 min ((identified as dC and 5mdC peaks) with low resolution at 0.8 and 0.5 mL/min flow rate, respectively. Optimized peak resolution was obtained by decreasing the flow rate and calculating (1) the difference in retention times between peaks 1 and 2 which are the two peaks closest to each other among other adjacent peaks and (2) the peak resolution. A resolution of 1.0 or higher is considered a representation of an adequate separation. Also, the farther apart the peaks are in the chromatogram (i.e. the greater the difference between the retention times of two adjacent peaks), the higher the resolution. The flow rate 0.2 mL/min gave the highest peak resolution (Fig 1C) and the greatest difference between two adjacent peaks. The importance of this optimization step is that better resolution of peaks further aided in the clear cut detection of the target peaks dC and 5mdC in the sago palm DNA samples.

Modified stop time

With decreased flow rate, stop time was extended to 45 min to allow complete elution of all compounds in the sample; otherwise, residual peaks which are unwanted and unknown peaks would appear in the succeeding injected DNA samples as shown in Fig 2. The boxed peaks were determined to be residual peaks as shown in Fig 2C, were eluted not later than 10 min.

Retention Time

Retention times of dC and 5mdC were determined to be 13.69675 ± 0.410903 and 18.55325 ± 0.556598, respectively. Any peak in the chromatogram of the DNA samples that

appeared during these retention times were dC and 5mdC of that DNA sample. Any minor day-to-day fluctuations in the retention times of dC and 5mdC of the DNA samples from the determined retention times of the standards were due to the electric response of the equipment to the sample. In such cases, the 3% tolerance of the means of the retention times for dC and 5mdC were calculated. Table 1 shows the evaluated analytical parameters of the target compounds dC (non-methylated cytosine) and 5mdC (methylated cytosine) used to establish the calibration curves for each.

Analysis of DNA methylation in sago palms

Target compounds analyzed in this study such as dC and 5mdC eluted on the determined retention times of the standards or fell within the calculated 3% tolerance limit of the mean of the retention times of DNA samples were run in the same day. The two forms of cytosine were well distinguished from their adjacent peaks due to the modified flow rate which was 0.2 mL/min. No residual peaks which eluted earlier than approximately 10 min were observed except in Spiny Wet Juvenile sample no. 13. This, however, did not impede the identification of the peaks of dC and 5mdC of the said DNA sample. Fig 3 shows the mean percentage of DNA methylation between (1) spiny and non-spiny sago palm regardless of the kind of environment and stage of development, (2) sago palms in wet and dry environment regardless of armature and stage of development, and (3) mature and juvenile sago palms regardless of armature and kind of environment. Spiny sago palms were found to be more methylated (21.5%) than the non-spiny sago palms (11.5%). Sago palms in the wet environment have greater DNA methylation (18.3%) than sago palms in the dry environment (12.9%). Furthermore, juvenile sago palms showed more methylation (17.5%) than their mature counterparts (14.8%). Analysis of variance (ANOVA) was performed to determine which among the three compared parameters has significant difference in DNA methylation and therefore has significant association with the epigenetic inheritance in sago palm. Results of ANOVA are presented in Table 2.

DNA methylation between spiny and non-spiny sago palms

Among three parameters compared, only the DNA methylation between spiny and non-spiny sago palms showed statistically-significant difference (P = 0.008; Table 3). This connotes pronounced difference in the total genomic DNA methylation between the two phenotypes compared to other evaluated parameters.

DNA methylation between sago palms in wet and dry environment

While a significant difference in DNA methylation was elucidated between spiny and non-spiny sago palms (Fig 3), non-significant differences were observed between sago palms in two types of environment (P = 0.171) and stages of development (P = 0.495).

DNA methylation between mature and juvenile sago palms

Between the two developmental stages juvenile and mature, juvenile palms were more methylated (17.5%) than mature palms (14.8%). However, this difference was statistically insignificant (Table 3).

Interaction of Factors that influence DNA methylation in sago palms

To determine which of the factors or interaction of factors had an influence over total genomic DNA methylation in sago palms sampled, factorial ANOVA was conducted. Table 3 shows that single factors A, B, and C have no significant effects on differences in DNA methylation. Among the four possible combinations of factors, A x C (armature x environment), B x C (environment x stage of development) and A x B x C (armature x environment x stage of development) showed $P > 0.05$ which means that these factors did not interact. Combination A x B showed $P < 0.05$ which means that, armature (phenotype) and type of environment (stimulus) interacted and that their interaction significantly influenced sago palm DNA methylation.

Discussion

Although the presence of spine in plants is a primitive morphological trait, not a great deal of information is known about the biological mechanisms and genes controlling spine formation. Several studies have indicated that the formation of spine is an epigenetic event (i.e. in carrot seed testa, cucumber fruit and 'heart-of-palm') and that such events are of great interest to plant breeders due to their heritability. There were many methods employed to analyze DNA methylation since the 1970's and these included HPLC and TLC (thin layer chromatography) methods, radiolabelling, the use of methylation-specific restriction enzymes and differential genome-wide screening. HPLC analysis of nucleosides has been found sufficient in analyzing global methylation changes of DNA that have resulted in relation to environmental and developmental stages. In this study, we report the optimization of conditions for HPLC analysis of sago palm DNA samples. Several factors were found critical in the optimization of HPLC conditions: the isolation of good quality DNA, successful digestion of DNA using nuclease P1, the choice of columns, optimum flow rate of sample in the columns, stop time and retention time of sample run. The DNA extraction method adopted in this study was a method devised by Angeles et al. (2005) for isolating high quality DNA from coconut leaves and endosperm. Because the coconut is high in lipids, polysaccharides and polyphenols, it poses quite a challenge in producing pure DNA suitable for HPLC analysis. The protocol involved the use of a higher salt (NaCl) concentration (2.0 M instead of the usual 0.5 M in typical DNA extraction methods). The addition of polyvinylpolypyrrolidone (PVPP) was effective in binding the polyphenols contained in the pelleted DNA sample after final centrifugation. Before the adoption of this PVPP method by Angeles et al. (2005), an attempt to extract sago palm DNA from the leaf using CTAB yielded brownish pellets. High polyphenol contents could prevent the success of DNA digestion. The PVPP method was also favored because it did not require the use of any organic solvent. Successful DNA digestion was also made possible by treatment of samples with nuclease P1. This was evident in the distinct nucleoside peaks produced in the chromatograms. The optimization of the mobile phase was achieved by determining the optimum peak resolutions of the nucleosides by adjusting the stop time and retention time. The use of a high flow rate of sample (0.8 mL/min) patterned after Baurens et al. (2004) for use in HPLC analysis of nucleosides hydrolysed from *Acacia mangium* DNA, was beneficial in the optimization of the flow rate of sago palm nucleoside samples. By decreasing the flow rate from 0.8 and 0.5 mL/min (Fig 1A and Fig 1B) to 0.2 mL/min (Fig 1C), the

Table 3. Factorial ANOVA of different factors or interaction of speculated to have an effect on sago palm DNA methylation.

Factors	Significance	P-value
A – Armature (Spiny and Non-spiny)	0.080	$P \leq 0.05$ = significant
B – Kind of environment (Wet and Dry)	0.220	
C – Stage of development (Mature and Juvenile)	0.333	
A x B	**0.006**	
A x C	0.734	
B x C	0.243	
A x B x C	0.123	

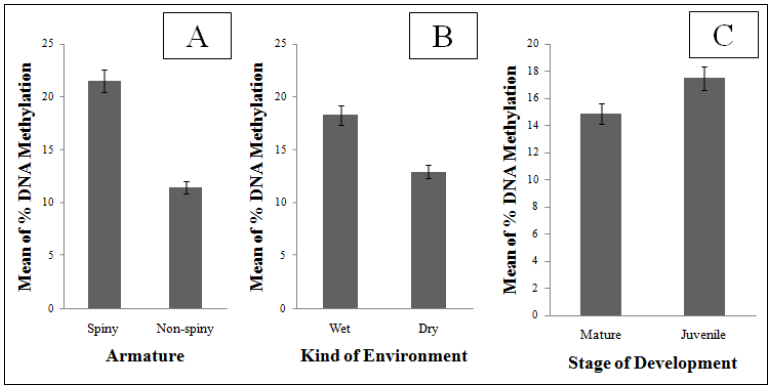

Fig 3. Means of percentage DNA methylation between spiny and non-spiny sago palms (armature i.e. presence or absence of spine), sago palms in wet and dry environment and mature and juvenile sago palms. Mean percentages between spiny and non-spiny sago palms showed significant difference ($P = 0.008$).

Table 4. Conditions used in HPLC analysis of sago palm DNA samples.

Parameter	Optimized condition
Digestion buffer for sample preparation prior to HPLC	5 mM $ZnCl_2$; 50 mM Sodium acetate with volume ratio of 15 µl: 40 µl; pH 5.5
Wavelength at which effluents were monitored	280 nm
Flow rate	0.2 mL/min
Stop time	45 min

highest peak resolutions were attained, facilitating a better differentiation between the non-methylated (dC) and methylated (5mdC) nucleosides. The use of C18 columns following other protocols on HPLC analysis of other plant DNA samples was found suitable for sago palm DNA. There is generally an inverse relationship between DNA methylation and gene expression reported on plants (Burn et al., 1993; Gonzalgo and Jones, 1997; Singal and Ginder, 1999; Guangyuan et al., 2006, Akimoto et al., 2007; Hafiz et al., 2008; Law and Jacobsen, 2010). Such is not the case for sago palms reported in this study where hyper methylation was found associated with spine formation (Fig 3). It is possible that other genes associated with spine formation were the ones methylated. In a study on spine formation on carrot seeds (normally spiny using quantitative RT-PCR, 11 ESTs were found differentially expressed in spiny seeds but not in spineless mutants (Park et al., 2006). Another four genes were overexpressed in the spineless mutants. These four genes were speculated to be the transcription factors that repressed the spine formation genes. Results further indicated that many transcriptionally-regulated genes were differentially accumulated in the wild- type seeds with spiny testa. Because not much is known about the genes involved in spine formation in plants in general, we could only surmise that such case is similar for sago palm spine formation. There could be transcription factors associated with spine-forming genes and that these transcription factors are the ones which get methylated, thus, allowing spine formation. The results of this study also suggest that wet environment (continuously flooded) was the probable stress factor that caused differential methylation i.e., more methylation relative to the sago palms in the dry (intermittently flooded) environment. Armature may have been an epigenetic variation brought about by the environment. The high percentage of DNA methylation (26.79%; Fig 3A) in the spiny sago palms in the wet environment, though not statistically significant, suggests that the wet environment was a stress that caused presence of spines. A question that could be raised from this deduction is that if the wet environment is the speculated stress that triggered the appearance of spines, then why are there spiny sago palms in the dry environment and non-spiny sago palms in the wet environment? Although it is logical to deduce that

a certain phenotype exists only in a certain type of environment which induced such trait in the organism, Epigenetic Inheritance Systems (EISs, a domain of epigenetics) provide an explanation on how different phenotypes but identical genotype transmit their phenotype to their offspring even when the phenotype-inducing stimuli are absent. In the case of spiny sago palms in the dry environment, the spiny phenotype is manifested even if the environment is dry. This could be explained by steady-state systems where descendants of the cell in which the gene was expressed will inherit this activity even if the original gene-inducing stimulus is no longer present. In the case of non-spiny sago palms in the wet environment, the stimulus for spine-formation is present but the phenotype is non-spiny. This could be due to the faithfully copied pattern of DNA methylation for absence of spine through maintenance methylation, a chromatin-marking system, from the parental DNA to the daughter DNA. Lastly, in the case of the non-spiny sago palms in the dry environment, where the stimulus for spine-formation is absent, these sago palms maintained their hypo methylated state which is the pattern of methylation characteristic to spineless phenotype. Between two developmental stages: juvenile and mature, juvenile palms were found to be more methylated (17.5%) than mature palms (14.8%). However, this difference was statistically insignificant (Table 3). Greater methylation in the juvenile stage was also observed in *Acacia mangium* (Baurens et al., 2004). Such observed decrease in DNA methylation from juvenile to mature developmental stage might be caused by de-methylation of some key genes resulting to activation of sub sets of several genes related to the characteristics in the adult phase (Hafiz et al., 2008). However, there were also studies which showed greater methylation in the mature plant samples. Watson et al. (1987) reported low methylation in young pea seedlings while in the DNA obtained in immature buds contained high methylation. Messeguer et al. (1991) also reported that immature tissues and protoplasts possessed significantly lower levels of cytosine methylation compared to those of mature tissues in tomato. Moreover, (Fraga et al., 2002) found higher methylation levels in mature *Pinus radiata* trees (60%) than in the juvenile individuals (30%). Although there is no established general relationship between cytosine methylation and plant developmental stage, the changes in DNA methylation from juvenile to mature support the view, for several decades, that changes in DNA methylation are associated with differing range of maturational changes observed during plant development. A study on the assessment of DNA methylation changes during different developmental stages (embyo, boll stage and flowering) of the sago palm is currently underway in our laboratory.

The significant difference in DNA methylation between spiny and non-spiny sago palms in this study suggests that one of the two phenotypes merely represents an epigenetic variant of *M. sagu*. Armature is likely caused by a change in gene expression as determined by changes in DNA methylation brought about by the environment. This change was stored in the epigenetic memory of the plant and was passed on to the next generation. The possibility of this sequence of events was proven in the MSAP (methylation-sensitive amplified polymorphism) analysis and bisulfite mapping of DNA methylation in rice (*Oryza sativa* spp. *japonica*) where the seeds before propagation were exposed to a demethylating agent (5-azadeoxycytidine). This treatment resulted to a completely erased methylation in the promoter region of *Xa21G* (gene for disease resistance). Wild types were determined to be disease-susceptible due to promoter methylation of *Xa21G*, while treated types were disease-resistant due to promoter demethylation resulting to expression of the disease-resistant gene. Furthermore, both hypo methylation and resistant trait were stably inherited showing a clear example of epigenetic inheritance and supporting the Lamarckian inheritance of acquired traits (Akimoto et al., 2007). MSAP analysis was also employed in the study of DNA methylation in Siberian ginseng (*Eleuterococcus senticosus*; Chakrabarty et al., 2003) and rapeseed (*Brassica napus*; Guangyuan et al., 2006). There are still many unknown mechanisms that govern DNA methylation including the mediation of signals into mainstream action such as the regulation of gene expression. One of the models proposed is a direct effect where transcription factors are prevented from binding their targets. In plants, there are homologs of the animal transcription factors CREB or E2F whose binding to the DNA targets can be blocked by DNA methylation (Scebba et al 2003). To fully understand the genetics/epigenetics of spine formation, differential gene expression levels and identification of spine formation and related genes such as transcription factors need to be elucidated.

Materials and methods

Collection of plant materials

Leaf samples were collected from sago palms in Prosperidad, Agusan del Sur, Philippines. Agusan del Sur has a Type II Climate where pronounced maximum rainy period occurs in the months of October-January and drier periods from March to May (Pajuelas, 2000; Provincial Board of Investment, Agusan del Sur, 2011). In 2010, Prosperidad had an annual rainfall of 268.83 mm. Leaf samples were taken in the months of least rainfall, March and April (around 194 mm). The average yearly rainfall in the sampling area was 240.4 mm from 1950-1996 (Pajuelas, 2000). Five grams of leaf samples were collected from mature (around 5 years old) and juvenile (around 3 months old) spiny and non-spiny sago palms in wet and dry environments during the drier months (month of least rainfall). The dry environment from which 'dry' samples were taken were therefore temporarily dry and the wet environment was continuously flooded.

Preparation of DNA samples

DNA modification of the protocol by Angeles et al. (2005) was used in this study. Polyvinylpyrrolidone (PVP; 0.1 g) was added to 1 g of leaf sample in a mortar. The leaf sample and was ground with liquid nitrogen rapidly into a fine powder. An aliquot of 2 mL DNA extraction buffer (2 M NaCl, 0.2 M Tris-HCl [pH 8.0], 0.07 M EDTA, 0.2 % β-mercaptoethanol) and 200 µL of 20% SDS was added to homogenize the mixture. Each of the homogenized leaf samples was placed into four 1.5 mL sterile Eppendorf tubes. Samples were then mixed in a vortex for 5 s and were incubated in a water bath at 65°C for 1 h. The homogenized mixtures were centrifuged at 21,000 x *g* for 15 min at 4°C, after which 500 µL of the supernatant was distributed to several sterile Eppendorf tubes. Then, to each Eppendorf tube, 125 µL of 3 M sodium acetate and 500 µL of absolute isopropanol were added. The resulting solution was mixed by gentle inversion and incubated at −80°C for 15 min. After incubation, the solution was centrifuged at 10,000 x *g* at 4°C for 15 min. The supernatant was discarded through slow and careful pipetting. DNA pellets were washed twice using 70% ethanol and then air-dried. After this, the DNA pellets were

re-dissolved with 50 µL of sterile ultrapure water. DNA stock solutions were kept at -80˚C until HPLC analysis.

HPLC analysis

The protocol for cleaning the C18 column before HPLC analysis was adopted with some modifications from Johnston et al. (2005). At the flow rate of 1 mL min $^{-1}$, the column was washed in the following order until the baseline stabilized for 15 min: filtered ultrapure water (to remove traces of phosphate), 80% HPLC grade methanol (to clean the column), followed by filtered ultrapure water, then mobile phase. After this, flow rate was set at 0.2 mL min^{-1}. After use, the HPLC column was also washed with filtered ultrapure water then with 80% methanol for 15 min each at a flow rate of 1 mL min $^{-1}$. Standard stock solution was prepared through dissolving 5 mg of standard (dC, un-methylated cytosine and 5mdC, methylated cytosine) in 10 mL filtered ultrapure water thus giving a concentration of 0.5 mg ml^{-1}. This stock solution was filtered in 0.22 µm filter membrane. Four dilutions (10, 0.1, 0.001, and 0.0001 µg mL^{-1}) were made for each standard. Standard solutions were analyzed using HPLC and retention times of dC and 5mdC were determined. Quantitation was done using peak area. HPLC analysis was done according to the protocol of Chakrabarty et al. (2003) using HPLC apparatus LC-20 AD Prominence Liquid Chromatography (Shimadzu, Japan), with SPD-20AV detector. The column used was a Shimadzu shim-pack CLC-ODS with a guard column of the same packing material. The digestion reaction consisted of the following components: 20 µg genomic DNA, 10 µL of 0.5 U nuclease P1, 10 µL or 10 U alkaline phosphatase (since the concentration of alkaline phosphatase is 1 µL/U) and 10 µL of 10x alkaline phosphatase buffer. The total reaction volume was brought to 200 µL with digestion buffer (30 mM/L $NaCH_3$, 0.1 mM/L $ZnCl_2$, pH 5.3). Hydrolysis was performed at 37°C for 3 h and was stopped by addition of 490 µL absolute ethanol. The samples were centrifuged at 10,000 x *g* for 15 min, and supernatant was transferred to a new tube and vacuum-dried. The resulting nucleosides were re-suspended in 1 mL sterile ultrapure water. Samples were then filtered through 0.22 µM membranes prior to sample injection. HPLC analysis was performed on C18 column at 25°C for 20 min using isocratic elution buffer (50 mM/L KH_2PO_4, 8% methanol, pH 3.5) at flow rates of 0.2, 0.5 and 0.8 mL/min following the protocol of Sandhu et al., 2009. The effluent was monitored at a wavelength of 285 nm.
The content of 5mdC was calculated using the formula:

$$5mdC\ (5\%) = \frac{[5mdC]}{[5mdC]+[dC]} \times 100$$

Where, [dC] (non-methylated cytosine) and [5mdC] (methylated cytosine) are the respective concentration of two forms of cytosine, as deduced from the calibration curves for external standards of known concentration (Johnston et al., 2005). Conditions for HPLC analysis of sago palm DNA samples were modifications from the protocols of Shimelis and Giese (2006), Magaña et al. (2008) and Sandhu et al. (2009). Table 4 lists the HPLC parameters used in this study.
The difference between the retention times of the two closest adjacent peaks and peak resolution (R) was calculated using the formula,

$$R = \frac{(RT1-RT2)}{[0.5x\ (W1 + W2)]}$$

where RT_1 and RT_2 represent the retention times of peaks 1 and 2, and W_1 and W_2 represent the widths of the peaks taken at their bases which is the difference between the retention times where that particular peak completely eluted and began to appear. The necessary period to extend the stop time it was determined by running the sample for 45 min. The retention time of the last peak was noted and was used as a determining factor as to how much time was needed to extend the flow rate in such a way that all possible compounds have already eluted to prevent residual peaks from appearing in the succeeding injected samples.

Statistical analysis of DNA methylation

The HPLC data were statistically analyzed using SPSS for Factorial, Between-Subjects Analysis of Variance (Factorial ANOVA) to determine which factor or interaction of factors influence DNA methylation and thereby the epigenetic control in sago palm. ANOVA was also performed between spiny and non-spiny sago palms, sago palms in wet and dry environment, and mature and juvenile sago palms. A probability level of $P \leq 0.05$ was considered significant for all statistical analyses.

Conclusions

We have successfully optimized the conditions for HPLC analysis of DNA methylation in sago palm focusing on the distinction of peaks between dC and 5mdC. There was significant difference in DNA methylation levels between spiny and non-spiny palms. However, our results do not conform to the general observation that there is an inverse relationship between DNA methylation and gene expression. We speculate that there are activator/suppressor genes that influence spine formation in sago palm. Our data also show that although there is no significant difference in DNA methylation between palms grown in wet (continuously flooded) and dry (intermittently flooded) environments, there is reason to suspect that the wet environment is a stress responsible for changes in DNA methylation levels between samples. Further, there are changes in DNA methylation levels at different developmental stages of the plant. It is important that more sensitive molecular techniques like the MSAP analysis be utilized to confirm the results of the HPLC. The differential expression of spine formation- and other related genes must be investigated.

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