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Multiple Forms of Alcohol Dehydrogenase (Adh) Genes in Sago Palm: A Preliminary Study

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Abstract

Flooding is a worldwide phenomenon in wetland and river areas. Excess water in the soil could produce anoxic soil condition. Sago palms in Sarawak can be found on mainly waterlogged areas. These plants are able and possibly have evolved a system for overcoming the anoxic/hypoxic conditions especially in the root section. Here we report the detection and activity of *Adh* gene in sago palm. The *Adh* enzyme was isolated, analysed on polyacrylamide and agarose gel, and detected by specific *Adh* staining. We found that *Adh* is present in all sago tissues and three variants are present based on migration on gel. We also utilised the polymerase chain reaction method to generate the PCR products by using primers that are designed from other plant species. Genomic DNA was used for this purpose and four PCR fragments were generated and the nucleotide sequence were determined. Preliminary results of nucleotide sequence analysis indicated that at least three types of *Adh* genes are present in sago.

Keywords: Adh, anaerobic, genome walk, *Metroxylon sagu*, PCR

Abbreviations: M – molar
mM – milimolar
EtBr – ethidium bromide
rpm – rotation per minute

Introduction

Sago palms in Sarawak can be found on mainly swampy/ waterlogged areas. Plants living in this condition seemed to be able and subsequently have evolved a system of overcoming the anoxic/hypoxic conditions of the soil. As a way to survive, plants living in waterlogged areas develop various morphological, anatomical and physiological adaptations (Rozema and Verkleij, 1991). Previous researches have shown that plants in general are able to adapt to the low-/no- oxygen conditions by switching its metabolism from pyruvate to ethanolic/fermentative pathways.

Flooding is a phenomenon that is encountered worldwide, especially in wetland and river plain areas. The roots of plants that thrive in these areas eventually suffer from hypoxia or anoxia. Most plants are obligate aerobes and need a constant supply of oxygen to function. However, some plants are resilient and have evolved to overcome the flooding phenomenon. Various proteins can be produced in response to the low- or no- oxygen conditions by the roots. Among the proteins that are activated to respond are sucrose synthase and a amylase that are involved in breaking down sucrose (Springer et al., 1986; Martin et al., 1993; Perata et al., 1993). Another protein, namely 1-aminocyclopropane-1-carboxylic acid synthase biosynthesises ethylene, an adaptive molecule that is involved in various responses, such as in the formation of aerial shoots (Olson et al., 1995). Proteins that are involved in the glycolysis are also induced such as hexokinase (Fox et al., 1998), fructose-1,6-biphosphate, aldolase (Dennis et al., 1988) and glyceraldehyde-3-phosphate (Sachs et al., 1996). Subsequently, proteins that are involved in the alcohol fermentation pathway are also activated such as pyruvate decarboxylase and lactate decarboxylase (Kelly, 1989) that forms lactic acid as end-product, and alcohol dehydrogenase (Freeling and Bennett, 1985) that produces ethanol.

Sago palm thrives in flooded conditions and its adaptation to this condition may have involved the evolution of *Adh* gene. The phenomenon of flooding has been shown to increase the

alcohol dehydrogenase (ADH) activity of clover roots to as much as 30 times (Francis et al., 1974). Therefore *Adh* genes and its activity can be used as an indicator of anaerobic respiration in sago palm and the gene may have evolved over the years to overcome the anoxic/hypoxic conditions of the roots.

Materials and Methods

Plant material

The root and leaf samples were collected from the University of Malaysia Sarawak (UNIMAS) plant house to request sago palms growing under dry condition. For waterlogged and completely submerged conditions, samples were collected from sago palms in Padawan, Sarawak.

Protein extraction

Samples were ground in liquid nitrogen prior to addition of extraction buffer containing 75 mM of Tris-HCl buffer pH 7.5, 5% of sucrose, 5% of polyvinyl pyrrolidone (PVP)-40, 14mM of mercaptoethanol, 50mM of ascorbic acid, 10 mM of diethyldithiocarbamate (DIECA) and 0.1% of bovine serum albumin (BSA) (Wendel & Weeden, 1990). The samples were then centrifuged at 5,000 rpm for 15 min at 40°C. The supernatant was collected and re-centrifuged at 13,000 rpm for 4 min to get rid of the remaining debris. The extracted samples were stored at –80°C until further use.

Protein detection

Proteins were separated on polyacrylamide gel with 5% stacking gel and 12% separating gel. Tris-glycine buffer (pH 8.8) was used as running buffer with a voltage of 120V at 40°C. Following electrophoresis, the gels were stained with Coomassie Blue for protein visualization (Bollag and Edelstein, 1991). Protein concentration was determined by Bradford assay (Bradford, 1976) using Coomassie (Bradford) Protein Assay Kit as specified by the manufacturer (Pierce).

Enzyme staining

Enzyme was analysed on 1% agarose gel electrophoresis for *Adh* and specific activity staining was conducted as recommended by Cho et al. (1999).

DNA and RNA isolation

Genomic deoxyribonucleic acid DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method described by Doyle and Doyle (1990) with some modifications. The DNA was stored in –30°C until needed.

DNA quantification

Extracted DNA was analysed on 1% agarose gel electrophoresis and then quantified using a spectrophotometer to assess for DNA quality, quantity, and also protein and polysaccharide contamination.

Primer design

Primers for *Adh* screening were designed by aligning various *Adh* sequences from other species using Primer3 software and synthesis from Morton et al. (1996). Sequence of primers used is listed in Table 1.

Table 1. The sequence of primers used in the PCR work.

Primer name and sequences	Tm °C
MorAdh-F 5′ GGG TGC TGT AGG CCT TGC - 3′	55
MorAdh-R 5′ - GAT ATC TGC ATT TGA ATG CG - 3′	48

Cloning of PCR fragments

Polymerase chain reaction (PCR) fragment was cloned into pGEM-T easy vector (Promega), and transformed into *E. coli* JM109 via standard heat shock method. Colony PCR and restriction endonuclease method have been undertaken to confirm the positive clones that carry the inserted gene of interest.

Sequencing and alignment

Fragments that were generated from the PCR were sent for sequencing to a sequencing company using ABI DNA sequencer. Sequencing was carried out either directly from gel purified PCR fragments or on cloned plasmid DNA preparations. The sequences obtained were aligned using European Bioinfomatics Institute's ClustalW multiple alignment software and searched for similar sequences using the National Center for Biotechnology Information's (NCBI) BLAST.

Results and Discussion

Protein detection

Proteins in the sago samples could be detected successfully with Coomassie blue staining. Bright protein bands were observed for all the samples (Figure 1). All samples displayed multiple banding patterns, indicative of intact proteins.

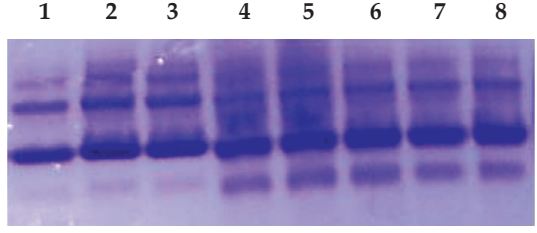

Figure 1. Coomassie Blue Staining for Protein Detection. Lanes 1-3 represents proteins from dry root samples (upper, middle and bottom parts respectively). Lane 4 is yong shoot whereas lane 5 is older leaf. Lanes 6-8 are proteins from submerged roots (upper, middle and parts respectively)

ADH specific staining

The ADH enzyme was subjected to agarose gel electrophoresis at 4°C with a voltage of 100V. Bands were observed after the specific staining for ADH was undertaken (Figure 2). White bands, indicative of superoxide dismutase

(SOD) expression were present when the samples were incubated under light exposed condition. SOD enzyme was used as control.

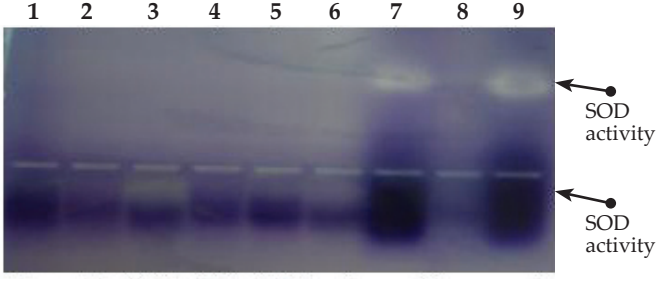

Figure 2. ADH & SOD Staining. Lanes 1-3 represents proteins from dry roots (upper, middle and bottom parts respectively). Lanes 4-6 are submerged root samples (upper, middle and bottom parts respectively). Lanes 7 and 9 are yound sago roots whereas lane 8 is an old leaf

The samples displayed three banding pattern; Fast, Middle and Slow, based on migration of ADH protein on agarose (Figure 3). Different tissues of sago also express the ADH enzyme with the highest expression located in young leaves while the lowest in older leaves. Three types of *Adh* enzymes were detected in dry and waterlogged root samples. The dry-root samples' upper and middle section contain only Adh-middle while bottom-root section contain both Adh-middle and Adh-fast. The waterlogged root samples contain the Adh-slow and Adh-middle in the upper- and middle-root sections, while Adh-middle and Adh-fast was observed to be present in the bottom-root sections. The young shoot samples seemed to contain all Adh forms and is highly-expressed, while for the old leaf sample, only Adh-middle was observed. These indicated that different sections of the root produce different Adh. This may indicate other stress factors that activate the *Adh* genes.

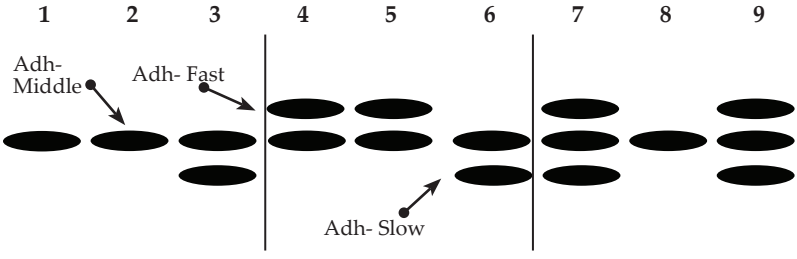

Figure 3. Zymogram representation of Figure 2 showing the Fast, Middle and Slow submit of ADH. Lanes 1-3 represent proteins from dry roots (upper, middle and bottom parts respectively). Lanes 4-6 are submerged root samples (upper, middle and bottom parts respectively). Lanes 7 and 9 are young sago shoots whereas lane 8 is an old leaf

DNA and RNA extraction

Good quality DNA have been successfully isolated and subsequently used in the optimisation of annealing temperatures for PCR work. The Mor-series primers produced four banding patterns. The bands were approximately 1,700bp, 1,200bp, 750bp and 300bp of molecular weight sizes (Figure 4). The PCR products were cloned into pGEM-T and sent for sequencing.

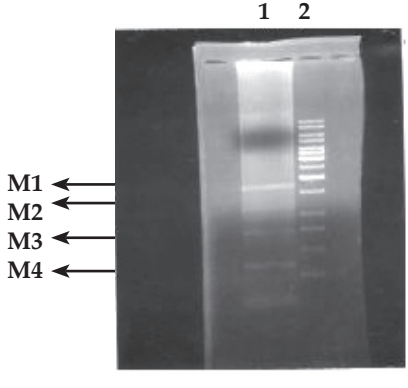

Figure 4. Agarose gel analysis of PCR product using primer set Mor-series. Lane 1 represents the PCR product M1, M2, M3 and M4. Meanwhile Lane 2 is 1Kb DNA ladder (Fermentas)

Sequence analysis

The sequences generated from the fragments are listed in figures 5-8 below. Similarity to other plant sequences was conducted using BLASTn (NCBI). All of the sequence resulted in similarity to other *Adh* genes such as that from *Washingtonia robusta*, *Calamus usitatus*, *Vitis vinifera*, *Arabidopsis thaliana*, *Fragaria x ananassa* (data not shown).

Figure 5. The M1 sequence generated about 1167bp long using leaf DNA as template

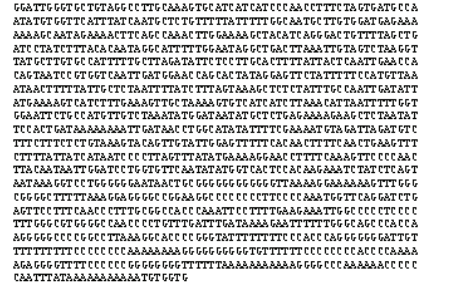

Figure 6. The M2 sequence generated about 1046bp long using leaf DNA as template

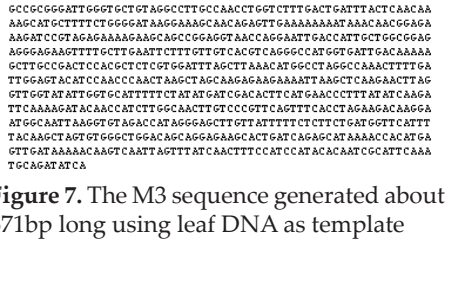

Figure 7. The M3 sequence generated about 671bp long using leaf DNA as template

Figure 8. The M4 sequence generated about 595bp long using leaf DNA as template

M1, M2 and M3 fragments were aligned to one another to look at the nucleotide similarities between the sequences. A conserved region of 23bp (sequences shown with asterix in Figure 9) was observed in all fragments. This region is highly conserved in *Adh* from other plant species. Alignment of these fragments indicated that the fragments generated are different in their 3′ nucleotide sequence (Figure 9). This indicates the possibility that three different *Adh* genes based on genomic sequences are present in sago palm.

Conclusion

We have demonstrated that the Adh gene is present and active in sago palm. This conclusion can be derived from the isozyme staining of Adh on agarose. The enzyme is present in all tissues but the activity varies depending on tissue type. The ADH zymogram indicated three different isozyme bands based on migration. PCR work have generated three unique sequences and analyses of these sequences showed the presence of a conserved region, similar to the Adh genes in other plant species. Therefore possibly three different Adh genes are present in sago palm based on the 3′ nucleotide sequences. Current work is in progress to further isolate the genomic full length of all three putative Adh genes of sago palm.

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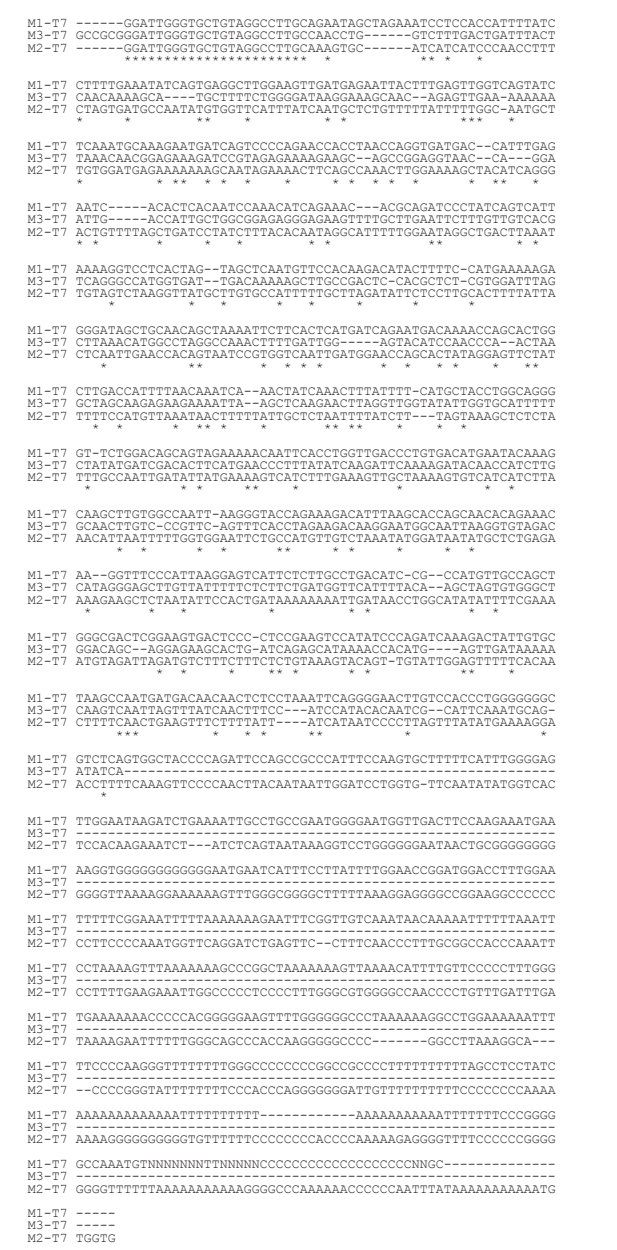

Figure 9. Multiple alignment of M1, M2 and M3 fragments using T7 sequencing primers. Asterix (*) indicate nucleotide that are similar in all sequences.

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