

Polymorphic Marker Designed from Bioinformatics Sequences Related to Cell Wall Strength for Discrimination of Mangosteen (*Garcinia Mangostana L.*) Clones Resistant to Gamboge Disorder

E. Mansyah, Sobir, E. Santosa, A. Sisharmini, and Sulassih

Abstract—Gamboge disorder (GD) or fruit damage by the yellow sap is a major problem in mangosteen. Mangosteen plants varied in the level of GD, from very low or non GD to low, moderate and high GD. However it was difficult to differentiate between GD and non GD plants because evaluation of the disorder is strongly influenced by environment. In this study we investigated the usefulness of primer designed from bioinformatics related to cell wall strength, termed as MCWS, to predict GD. Plant materials used were 28 mangosteen plants selected based on percentage of GD categorized as high, moderate, low and very low or non GD. The result showed that the specific DNA fragments were absent in the high GD accessions. The MCWS marker suggests as a novel polymorphic marker for GD in mangosteen as well as a marker for detect variability in mangosteen as apomictic plant.

Keywords—Bioinformatics, cell wall strength, gamboge disorder, mangosteen, polymorphic marker.

I. INTRODUCTION

MANGOSTEEN is prospective fruit of Indonesia as export commodity. Volume of fresh fruit export increases gradually particularly to European countries, China, Hongkong, and United Arab Emirates [1]. Of the total Indonesian mangosteen production only about 25% are eligible for export. One of the factors causing the low volume of mangosteen exports is fruit damage by yellow gummy, commonly called as gamboge disorder (GD). Losses caused by GD are very serious because the fruit with yellow gummy unfit for consumption or used for processed products.

[2] suggested that the GD is a physiological disorder which showed symptoms of yellow fruit aril. One of the factors caused of GD is suspected by the destruction of the epithelial

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cells that surrounding the yellow latex secretory ducts. The rupture epithelial cell of yellow latex secretory ducts can be seen in the longitudinal section of the mangosteen fruit endocarp [3]. The turgor pressure of the cells contributed to the breaking pressure. Rupture occurs when the combination of cell turgor pressure exceeds the strength of the cell wall [4]

This information suggest that interaction between the cell wall strength and the release of the yellow sap in mangosteen fruits is probably the most important mechanisms of gamboge disorder. Mangosteen trees showed differences in resistance to gamboge disorder both among individuals and locations. Percentage of gamboge disorder of individual plants ranged from very low (0-1%) to very high (up to 69%). Observations at six locations in West Sumatra showed that gamboge disorder varied from 15% to 46% [5]. Reference [6] reported several trees with heavy bearing in Burma and susceptible to gamboge disorder. However it has remained difficult to differentiate between GD and non GD on the basis of the morphological findings. Beside that evaluation of the disorder is strongly influenced by environment. Previous work [5] reported that high rainfall contribute to the high percentage of GD. High turgor pressure induced by high water absorption can directly destruction epithelial cells and also release yellow gummy from the duct. Breaking pressures above turgor pressure were remarkably variable among organisms [4].

The strength of cell walls that responsible for allowing the persistence of GD is also the environmental and genetic interaction. The mechanisms that regulate the mechanical strength and cell wall biosynthesis is complex and requires coordination of a number of metabolic pathways involving gene [7]. Detection of cell wall strength by specific molecular markers could potentially be helpful in overcome GD. It has been accepted that mangosteen exhibits apomictic, where all sibling are believe as similar or uniform genetically. However, according to recent assessment using RAPD [8,9], RAF [10], AFLP [11] and ISSR markers [12] and also morphological traits [13] showed variation that suspected as different genetic. Based on the existence of genetic variation in mangosteen it is possible to develop reliable markers to access the variation and to detect gamboge disorder as well.

In order to enhance and coop with time frame to develop molecular markers, the marker related to cell wall strength was designed from NCBI sequences data base. The

availability of specific markers for cell wall strength provides a useful tool to detect GD in mangosteen. This study aims to a) determine the relationship between GD and the cell wall strength, and b) obtain specific markers for the detection GD in mangosteen.

II. MATERIALS AND METHODS

A. Plant Materials

Plant materials used were 28 mangosteen accessions (Table I) from Leuwiliang and Tajur (Bogor, West Java), Tembilahan (Riau), Indonesian Tropical Fruits Institute (ITFRI) collection (West Sumatera), and Bulukumba, Bantaeng and Manado (South Sulawesi). The plants different in gamboge disorder percentage categorized as non GD or very low (0-1%), low GD (<20%), moderate GD (20-30%) and high GD (> 30%). An example of mangosteen fruits with GD and non GD presented in Fig. 1.

TABLE I
PLANT MATERIALS USED IN THIS STUDY

No	Accession /Origin	Percentage of Gamboge Disorder	Habitat/Altitude
1	B1/ ITFRI	30% (M)	Dry land /237 masl
2	B2/ ITFRI	40% (H)	Dry land /237 masl
3	B3/ ITFRI	30% (M)	Dry land /237 masl
4	B4/ ITFRI	30% (M)	Dry land /237 masl
5	B5/ ITFRI	11% (L)	Dry land /237 masl
6	K/ ITFRI	8% (L)	Dry land /237 masl
7	T1/ Tembilahan	0-1% (N)	Tidal swamp/16 masl
8	T2/ Tembilahan	0-1% (N)	Tidal swamp /16 masl
9	T3/ Tembilahan	0-1% (N)	Tidal swamp /16 masl
10	T4/ Tembilahan	0-1% (N)	Tidal swamp /16 masl
11	T6/ Tembilahan	0-1% (N)	Tidal swamp /16 masl
12	T7/ Tembilahan	0-1% (N)	Tidal swamp /16 masl
13	T13 Tembilahan	0-1% (N)	Tidal swamp /16 masl
14	T14/ Tembilahan	30% (M)	Tidal swamp /16 masl
15	T23/ Tembilahan	0-1% (N)	Tidal swamp /16 masl
16	T24/ Tembilahan	*	Tidal swamp /16 masl
17	T25/ Tembilahan	*	Tidal swamp /16 masl
18	T26/ Tembilahan	*	Tidal swamp /16 masl
19	L1/ Leuwiliang	24% (M)	Dry land /340 masl
20	L3/ Leuwiliang	10% (L)	Dry land /340 masl
21	L6/ Leuwiliang	8% (L)	Dry land /340 masl
22	L7/ Leuwiliang	18% (L)	Dry land /340 masl
23	L8/ Leuwiliang	20% (L)	Dry land /340 masl
24	L10/ Leuwiliang	5% (L)	Dry land /340 masl
25	Tj4/Tajur	*	Dry land /240 masl
26	BB1/ Bulukumba	*	Dry land /30 masl
27	MD/ Manado	*	Dry land 60 masl
28	BT Bantaeng	45% (H)	Dry land /10 masl

* = unknown, masl = meter above sea level, L = Low GD, M = Moderate GD, H = High GD, N = Non GD



Fig. 1 An example of mangosteen fruits with gamboge disorder (a) and non gamboge disorder (b)

B. Primer Designed

DNA sequences associated with cell wall strength are determined by using bioinformatics data base of NCBI (National Center for Biotechnology Information) at <http://www.ncbi.nlm.nih.gov>. The DNA sequences obtained were then analyzed by using online analysis tools consists of several software for genome analysis including GeneBee ClustalW 1.83 and GeneBee BLAST 2.2.8 and Services (<http://www.genene.msus.edu>). ClustalW analysis is used to obtain multiple sequence alignment data and BLAST analysis (Basic Local Alignment Search Tools) to determine the sequence similarity with the plant cell wall strength or other organisms. A pair of primer was designed based on conserve region of multiple sequence alignment data. The simple method for design of primer sequences are based on [14] as follows: 17-28 bases long, G + C composition of 50-60%, 3' end is G or C, or CG or GC, melting temperature between 55 -80 ° C, and avoid the three or more C or G at the 3' end of primer.

C. DNA Extraction, Purification and Amplification

DNA was isolated from 28 mangosteen accessions (Table 1). About 0.1 mg fresh leaflets were grind for DNA extraction. Total DNA was extracted according to the modified CTAB protocol [15] by addition 1% of PVPP (polyvinyl polypyrrolidone). DNA concentrations were determined with electrophoresis in agarose gel, ethidium bromide staining solution and visualization on UV transilluminator. DNA was then PCR-amplified by using the primer designed from bioinformatics sequences in a 96-well Applied Biosystems 2720 thermal cycler. Reactions were carried out in a total volume of 25 µl consisting of 2 µl (20 ng) of template DNA, 12.5 µl Go Taq Green Master Mix (Promega M7122), 1 µl primer (20 µM), and 9.5 µl free nuclease water. Amplification was performed under the following conditions: 4 min at 94°C for 1 cycle, followed by 0.5 min at 94°C, 0.5 min at 42°C and 1 min at 72°C for 35 cycles, and 5 min at 72°C for final extension. PCR amplification was repeated twice with similar conditions to ensure the stability of the DNA banding patterns produced. PCR products were then separated on 1.2% agarose gel and 1X TAE buffer solution, stained with ethidium bromide and visualized with UV light.

D. Data Interpretation

Genotypic data obtained from the gel compared with a molecular weight standard 1 kb DNA ladder. Observations made on the level of polymorphism and variability of DNA banding patterns produced. Presence or absence of the target trait (cell wall strength) in the plant samples estimated by the presence of specific bands generated and the degree of GD

III. RESULTS AND DISCUSSION

A. Primer Design

The results of BLAST analysis obtained five GenBank accessions having high homology with the genes related to the cell wall strength. The five accessions were AY158083.1 from *Arabidopsis thaliana* kinesin-like protein (FRA1) mRNA

complete cds, NM_180820.1 from Arabidopsis thaliana FRA1 (Fragile FIBER 1); microtubule motor (FRA1) mRNA, complete cds, NM_124156.4 from Arabidopsis thaliana FRA1 (Fragile FIBER 1); microtubule motor (FRA1) mRNA, complete cds, and AY158084.1 from Arabidopsis thaliana kinesin-like protein (FRA1) gene, complete cds [16], and XM_002510131.1 from Ricinus communis Kinesin heavy chain, putative, mRNA [17]. The sequences were then analyzed using ClustalW 1.83 Genebee and the results were used to design a pair of reverse and forward primers associated with the cell wall strength termed as *MCWS* (Mangosteen Cell wall Strength). Part of multiple sequence alignment of five DNA sequences presented in Fig. 2.

AY158083.1
AAGGTTGCTGTTACATCAGACCACTCATCGGCGAC
GAAAGGATTCAAGTTGTCAAGAT
NM_180820.1
AAGGTTGCTGTTACATCAGACCACTCATCGGCGAC
GAAAGGATTCAAGTTGTCAAGAT
NM_124156.4
AAGGTTGCTGTTACATCAGACCACTCATCGGCGAC
GAAAGGATTCAAGTTGTCAAGAT
AY158084.1
AAGGTTGCTGTTACATCAGACCACTCATCGGCGAC
GAAAGGATTCAAGTTGTCAAGAT
XM_002510131.1
AAAGTTGCTGTTATGTCAGACCACTCATCGGCGAT
GAACGTGCTCAAGGCTGTCAAGAT
* * * * *
* * * * *
AY158083.1
CAAGGCTACAATGCTACCGTCTTGCTTATGGACAAA
CGGGTTCCGGTAAAACATATACA
NM_180820.1
CAAGGCTACAATGCTACCGTCTTGCTTATGGACAAA
CGGGTTCCGGTAAAACATATACA
NM_124156.4
CAAGGCTACAATGCTACCGTCTTGCTTATGGACAAA
CGGGTTCCGGTAAAACATATACA
AY158084.1
CAAGGCTACAATGCTACCGTCTTGCTTATGGACAAA
CGGGTTCCGGTAAAACATATACA
XM_002510131.1
CAAGGATATAATGCGACAGTCTTGCTTATGGTCAGA
CGGGATCAGGGAAGACGTATACA
* * * * *
* * * * *
AY158083.1
ATGGGTACTGGCTGCGGAGACAGCAGCCAGACTGGT
ATCATCCCTCAAGTTATGAACGCT
NM_180820.1
ATGGGTACTGGCTGCGGAGACAGCAGCCAGACTGGT
ATCATCCCTCAAGTTATGAACGCT
NM_124156.4
ATGGGTACTGGCTGCGGAGACAGCAGCCAGACTGGT
ATCATCCCTCAAGTTATGAACGCT

AY158084.1
ATGGGTACTGGCTGCGGAGACAGCAGCCAGACTGGT
ATCATCCCTCAAGTTATGAACGCT
XM_002510131.1
ATGGGCACCGGATTTAAAGATGGTTGCCAAACAGGA
ATAATTCCTCAAGTCATGAATGTT
* * * * *
* * * * *
AY158083.1
CTTTTCACCAAGATAGAGACTTTGAAGCAGCAGATT
GAGTTTCAGATTCATGTTTCTTTT
NM_180820.1
CTTTTCACCAAGATAGAGACTTTGAAGCAGCAGATT
GAGTTTCAGATTCATGTTTCTTTT
NM_124156.4
CTTTTCACCAAGATAGAGACTTTGAAGCAGCAGATT
GAGTTTCAGATTCATGTTTCTTTT
AY158084.1
CTTTTCACCAAGATAGAGACTTTGAAGCAGCAGATT
GAGTTTCAGATTCATGTTTCTTTT
XM_002510131.1
TTGTACAGCAAGATTGAAACTTTAAAGCATCAAAC
GAATTCCTCAAGTGCATGTTTCTTTT
* * * * *
* * * * *
AY158083.1
GGATCCTTGACTGTTAATAAATCAGACACTAATAAC
ACTGGACATGTTGGTAAGGTAGC
NM_180820.1
GGATCCTTGACTGTTAATAAATCAGACACTAATAAC
ACTGGACATGTTGGTAAGGTAGC
NM_124156.4
GGATCCTTGACTGTTAATAAATCAGACACTAATAAC
ACTGGACATGTTGGTAAGGTAGC
AY158084.1
GGATCCTTGACTGTTAATAAATCAGACACTAATAAC
ACTGGACATGTTGGTAAGGTAGC
XM_002510131.1
GGATCCGACTTCTTTGAACAAGCCAGATAC-
AGCAAA--TGGGCACACGGGAAAAGTAA-
* * * * *
* * * * *
AY158083.1
ACATGTCCCTGGGAAGCCACCGATACAGATTCGAGA
AACATCCAACGGTGTATCACACT
NM_180820.1
ACATGTCCCTGGGAAGCCACCGATACAGATTCGAGA
AACATCCAACGGTGTATCACACT
NM_124156.4
ACATGTCCCTGGGAAGCCACCGATACAGATTCGAGA
AACATCCAACGGTGTATCACACT
AY158084.1
ACATGTCCCTGGGAAGCCACCGATACAGATTCGAGA
AACATCCAACGGTGTATCACACT
XM_002510131.1 --
ATGTCCCTGGGAAGCCACCAATAACAAATTCGCGAAA
CATCAAATGGTGTGCATAACACT

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AY158083.1
AGCTGGATCCACAGAAGTAAGTGTAGTACGTTGAA
AGAGATGGCTGCTTGCCCTTGACCA
NM_180820.1
AGCTGGATCCACAGAAGTAAGTGTAGTACGTTGAA
AGAGATGGCTGCTTGCCCTTGACCA
NM_124156.4
AGCTGGATCCACAGAAGTAAGTGTAGTACGTTGAA
AGAGATGGCTGCTTGCCCTTGACCA
AY158084.1
AGCTGGATCCACAGAAGTAAGTGTAGTACGTTGAA
AGAGATGGCTGCTTGCCCTTGACCA
XM_002510131.1
AGCAGGATCTACTGAAGTTAGTGTACGTACGCTCAA
AGAAATGGCTGCTTGCTTAGAACA
** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** **
***** **

AY158083.1
CACATGCCATTTTACTATCACCGTGGAGCAAATGCG
CAAAATTAATACGGATTCTCCTG
NM_180820.1
CACATGCCATTTTACTATCACCGTGGAGCAAATGCG
CAAAATTAATACGGATTCTCCTG
NM_124156.4
CACATGCCATTTTACTATCACCGTGGAGCAAATGCG
CAAAATTAATACGGATTCTCCTG
AY158084.1
CACATGCCATTTTACTATCACCGTGGAGCAAATGCG
CAAAATTAATACGGATTCTCCTG
XM_002510131.1
CGCATGCAATCTTACCATCACACTAGAGCAAATGC
GTAAGCTTAACCCTGTATTCCCTG
* ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** ** * **
* * * **
AY158083.1
AAAACGGTGCTTACAATGGGAGCTTGAAAGAAGAAT
ATTTGTGTGCCAAGTTGCACTTAG
NM_180820.1
AAAACGGTGCTTACAATGGGAGCTTGAAAGAAGAAT
ATTTGTGTGCCAAGTTGCACTTAG
MM_124156.4
AAAACGGTGCTTACAATGGGAGCTTGAAAGAAGAAT
ATTTGTGTGCCAAGTTGCACTTAG
AY158084.1
AAAACGGTGCTTACAATGGGAGCTTGAAAGAAGAAT
ATTTGTGTGCCAAGTTGCACTTAG
XM_002510131.1
GCGACAGCAGTCCTAATGAGGGCATGAATGAAGAGT
ATCTATGTGCCAAGCTGCATTTAG
* * * * ** ** ** ** * **
**** **
AY158083.1
TAGATCTTGCTGGTTCAGAACGAGCCAAAAGAAGCTG
GTTTCAGACGGTTTGAGATTTAAGG
NM_180820.1
TAGATCTTGCTGGTTCAGAACGAGCCAAAAGAAGCTG
GTTTCAGACGGTTTGAGATTTAAGG
NM_124156.4
TAGATCTTGCTGGTTCAGAACGAGCCAAAAGAAGCTG
GTTTCAGACGGTTTGAGATTTAAGG
AY158084.1
TAGATCTTGCTGGTTCAGAACGAGCCAAAAGAAGCTG
GTTTCAGACGGTTTGAGATTTAAGG
XM_002510131.1
TAGATCTTGCCGGTTCAGAGCGAGCTAAAAGAAGCTG
GTTCTGATGGTTTGCCTTTTAAGG
***** **
***** **
AY158083.1
GCTTGGTAATGTTATCAGTGCCTCGGTGATGAGAA
AAAGCGTAAAGATGGTGCCCATGT
NM_180820.1
GCTTGGTAATGTTATCAGTGCCTCGGTGATGAGAA
AAAGCGTAAAGATGGTGCCCATGT
NM_124156.4
GCTTGGTAATGTTATCAGTGCCTCGGTGATGAGAA
AAAGCGTAAAGATGGTGCCCATGT
AY158084.1
GCTTGGTAATGTTATCAGTGCCTCGGTGATGAGAA
AAAGCGTAAAGATGGTGCCCATGT
XM_002510131.1
GCTTGGTAATGTTATTAGTGCCTTGGTGTGAAAAA
AAGCGGAAAGAAGGTGTTACGTT
***** **
***** **
AY158083.1 -
ATTCCCTTGGTGGTAACAGCAGAAGTGTGATGATAG-
-----
NM_180820.1 -
ATTCCCTTGGTGGTAACAGCAGAAGTGTGATGATAG-
-----
NM_124156.4 -
ATTCCCTTGGTGGTAACAGCAGAAGTGTGATGATAG-
-----
AY158084.1
GATTCCCTTGGTGGTAACAGCAGAAGTGTGATGATA
GGTAACCTGCAGTTTTCTTTTTT
XM_002510131.1 -
ACTCACTTGGTGGTAACAGCAAAACTGTTATGATA
G-----
* ** **

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Fig. 2 Part of multiple sequence alignment of five DNA sequences for cell wall strength based on ClustalW 1.83 software (<http://www.genebee.msu.su>). The underlined sequences are MCWS Forward and Reverse primers

B. Polimorphysm of MCWS Primer

The MCWS primer was amplified successfully, produced 10 DNA bands ranged in size from 200 to 1200 bp. Amplified product consisting of 2 (20%) monomorphic bands and eight (80%) polymorphic bands (Fig. 3). This result inform that mangosteen shows diversity in genetic. Polymorphism and

genetic diversity in apomictic plants is the result of point mutation, insertion, deletion, inversion, non-disjunction, somatic recombination by transposon activity, and mutations or chromosomal changes in the maternal genome [18]. Previous study [8,9,10,11,12] have reported the variability of DNA banding patterns in mangosteen, and concluded that the mangosteen varied in genetics.

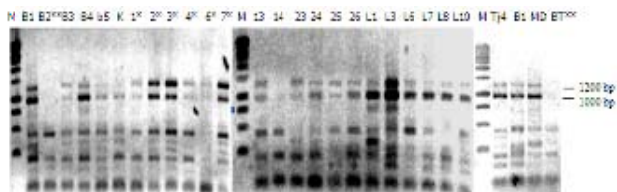


Fig. 3 Polymorphisms in 28 mangosteen clones by primer MCWS.
* = non GD accessions, ** = high GD accessions

C. Relationships between Cell Wall Strength and Gamboge Disorder

The polymorphism of MCWS primer indicated that the mangosteen not only has a diversity in genetics but also in the strength of the cell wall. The genetic diversity of cell wall strength has also been found in barley (*Hordeum vulgare* L.) which is known from the differences of its brittleness. Cell walls of brittle culms had 6 to 64% as much cellulose content as those of nonbrittle culms. Maximum bending stress correlated significantly with cellulose content of the cell walls ($r=0.93$), but not with the contents of noncellulosic compounds. The lower cellulose content of the brittle culm was significantly correlated with brittleness [19].

Among the polymorphic bands generated by MCWS primer there were two specific bands about 1200 and 1000 bp in size. The two DNA fragments present in non-GD, low and moderate GD accessions and absent in two high GD accessions (B2 and BT). Based on this result can be assumed that the two DNA fragments associated with the cell walls strength of mangosteen and suggest as a putative marker for mangosteen accessions resistance to GD. Accessions B2 and BT is suspected to have a weak cell wall indicated by the absence of 1200 bp and 1000 bp and the high percentage of GD. Accession T14 does not have a 1200 bp band but has a 1000 bp band with a moderate level of GD. This data suggested that 1000 bp fragment contribute more to the the cell wall strength.

The strength of plant cells is important traits to prevent the GD in mangosteen. Mechanisms regulated the mechanical strength and cell wall biosynthesis is very complex and requires coordination of a number of metabolic pathways that involved genes. Reference [16] showed that the cellulose content correlated with mechanical strength of the plant body. Fragile FIBER1 (FRA1), a kinesin-like protein, is essential for the deposition of cellulose microfibril orientation and cell wall strength. Gen FRA1 encodes kinesin-like protein with the *N-terminal microtubule binding motor domain*. Protein FRA1 concentrated around the periphery cytoplasm but absent in the

nucleus. Based on these findings concluded that the kinesin-like protein FRA1 involved in the microtubule control of cellulose microfibril. FRA1 showed dominant role in the orientation of cellulose microfibril deposition during secondary wall thickening of fiber cells. Since the MCWS primer designed from sequences associated with the sequences involved in cell wall strength, it is possible to use the primer in a suspected cell wall strength and gamboge disorder in mangosteen.

The interesting thing of this result is that there were several accessions having both the two bands but showed a different response to GD including non-GD, moderate GD and low GD. This is presumably due to the possible role of cell wall strength in response to unfavourable environmental conditions. Environmental factors that influence the strength and structure of the cell wall will determine the level of GD. Previous study [5].revealed that GD arise when stimulated by high intensity and fluctuation of rainfall and low soil Calcium content. Reference [20] stated that environmental conditions such as temperature, humidity, shade, and irrigation water salinity greatly affect the hardness of the fruit as a potential effect of cell wall structure and integrity. Other factors include the synthesis and metabolism of cell wall polysaccharides such as pectin. Increase in pH will stop the extension of the cells through the inhibition of protein and activation of enzymes involved in the relationship between cell wall by other factors.

Polymorphic marker for GD, here termed MCWS (Mangosteen Cell wall Strength), was detected in mangosteen accessions. The fragments 1200 bp and 1000 bp correspond to putative markers for cell wall strength and non GD to moderate GD accessions. Thus this study presents MCWS as a novel polymorphic marker for the cell wall strength as well as a marker for detects GD in mangosteen. The MCWS primer potentially is used as a tool for the detection of genotypes that are easily damaged by GD. This is the first study to differentiate GD from non-GD mangosteen, and as a marker of the severity of cell wall strength in causing GD on mangosteen fruits. The next activity needed is the sequencing analysis of the predicted fragments to determine its DNA sequence and then designed to more specific primers.

IV. CONCLUSION

Gamboge disorder in mangosteen fruits associated with the cell wall strength, indicated by the absence of two specific bands, about 1000 and 1200 bp in length, for high GD accessions by MCWS primer designed for cell wall strength. This results represent the initial information linkage between molecular markers specific to cell wall strength on mangosteen and its potential to be developed as a new approach for the identification of mangosteen accessions sensitive to gamboge disorder.

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