

Characterization of β -1,3-GLUCANASE gene associated with resistance to anthracnose disease in mango (*Mangifera indica* L.)

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Abstracts: β -1,3-Glucanase plays key roles in plants and have been studied in a wide range of plant species. It has many functions including defense plants against fungal pathogens either alone or in association with chitinases and other antifungal proteins. The structure of β -1,3-Glucanase in mango has been incomplete. Thus, our study proposed to characterize this gene and compare DNA sequences in exon 2 of mango cultivars. The DNA was extracted from mango leaves and *Colletotrichum asianum* hypha, then this gene in exons 2, 3 and 4 were amplified by PCR and only sequences of exon 2 were sequenced and compared to database. The results presented that β -1,3-Glucanase gene is composed of 4 exons which exon 2 is the largest region. Exons 2 (1254 bp) and 3 (27 bp) were interrupted by intron 2 at about 180 bp while exons 3 and 4 (153 bp) were separated with intron 3 at about 16 bp in size. Sequences in exon 2 pointed that mango in Thailand is different with mango in database from Philippine. Moreover, there were 31 SNPs in exon 2 amplified from GLU2F and GLU2R. These SNPs may be the pathogenesis-related resistance against *C. asianum*.

Keywords: Beta-1,3-Glucanase Gene, Anthracnose, Mango.

1. INTRODUCTION

The β -1,3-glucans is carbohydrate that its monomer links together with 1,3- β -D-glucosidic bond. There are many organisms that produce the enzymes of β -1,3-glucanases (E.C. 3.2.1.39) to breakdown this linkage, for examples viruses [1, 2], bacteria, fungi, and plant species, such as *Arabidopsis* [3], rice [4], tobacco [5] and soybean [6]. Generally, β -1,3-Glucanase genes (*GLUs*) are complex depending on their specific activity and diverse gene families in plants, where they play major roles in physiological processes [3]. In plants, β -1,3-glucanase has focused primarily on their antifungal activity. For instance, β -1,3-glucanases in plant are classified as the PR-2 family of pathogenesis-related proteins against infection in two ways. Firstly, this enzyme will hydrolyze β -1,3-glucan in fungal cell walls and promote to release the immune elicitors of cell wall so that further stimulate defense mechanism [7, 8].

In previous studies, β -1,3-*GLU* plays a key role in *Colletotrichum gloeosporioides* pathogenicity on mango fruit. Several research show evidence of β -1,3-glucanase's role in *Colletotrichum* infection in various plants. In maize, there were nine *GLUs* to significantly upregulate in leaves during *C. graminicola* infection as a result of pathogen-associated molecular patterns (PAMP)- triggered defense response [9]. Similarly, strawberry infected with *C. acutatum* or *C. fragariae* induced the expression of two *GLUs* to over a thousand-fold [10]. In mango, Zhang et al. [11] reported that β -aminobutyric acid (BABA) treatment in mango fruits effectively suppressed anthracnose caused by *C. gloeosporioides* and significantly enhanced the activities of β -1,3-glucanases during storage at 25 °C. Moreover, when β -1,3-glucanase activities increased, the anthracnose after postharvest were also reduced and enhanced of disease resistance in mango fruit after exogenous nitric oxide (NO) treatment as well [12]. These reports indicated that β -1, 3-glucanases indeed significantly respond to *Colletotrichum* infection and possibly elicit defense response mechanisms that involve enhancement of other pathogenesis-related enzyme activities to confer resistance to the pathogen.

Mango (*Mangifera indica* L.) is belonging to the family of Anacardiaceae in the order of Sapindales grown in many regions of the world, particularly in tropical countries [13]. It is the national fruit of India and Philippines and the national tree of Bangladesh. Over 1000 mango varieties are cultivated and sold worldwide. Of the available

varieties, only a few are grown on commercial scales and traded [14]. Mango is one of the most popular in tropical fruits and is an economic plant of Thailand. In addition to fruit consumption, their leaves contain active compound, namely mangiferin, being a strong polyphenolic antioxidant and a glucosyl xanthone including anti-lipid peroxidation, immunomodulation, cardiotoxic, hypotensive, wound healing, antidegenerative and antidiabetic activities [15]. Unfortunately, many factors negatively affect to mango production including the difficulty of inducing the trees to flower and various diseases, particularly anthracnose. Anthracnose, an almost universal disease of mangoes, is caused by the fungus *C. gloeosporioides* [16]. All phenological stages of mango (vegetative, reproductive, and fruiting) are affected, resulting in losses of up to 90% in pre- and post-harvest, particularly when relative humidity and temperature are high [17]. This disease generates a phytosanitary problem that impairs the yield of various crops and causes economic losses in tropical and subtropical regions of the world. Mango crops are one of the most affected by anthracnose, which harms the fruiting stage. This disease is characterized by colonizing initially the shell of the fruit, until it causes necrosis or death of the plant tissue inside the fruit in its final stage of development [18].

Therefore, in this study aimed to characterize β -1,3-glucanase gene in exon 2, 3 and 4 that is defense-related gene associated against *C. asianum* infection in mango to extend additional information about this gene and compare sequences of β -1,3-glucanase2 (*GLU2*) exon (*GLUs*) in 5 mango cultivars in Thailand and databases. The advantage of this study is novel knowledge of β -1,3-glucanase gene structure and may relate between single nucleotide polymorphism (SNP) associated with pathogenic resistance.

2. MATERIALS AND METHODS

2.1. Plant Materials and DNA Extraction

Five samples of mango cultivars were collected from Phitsanulok province in Thailand, during April-May 2023. Their leaves were stored in 4 °C until use for DNA extraction. The genomic DNA was extracted followed by modified CTAB method of Agarwal et al. [19]. Young leaves of mango were ground to powder in liquid nitrogen. Leaves powder were incubated with 1X CTAB extraction buffer (2% CTAB, 1.4M NaCl, 20 mM EDTA, 100 mM Tris-HCl) and β -mercaptoethanol at 60 °C for 60 min and degraded proteins using chloroform. Then the DNA solution was precipitated using absolute ethanol. The DNA pellets were suspended in 50 μ L sterile DNase free water and kept at -20 °C until ready for use. The genomic DNA was determined using 0.8% agarose gel electrophoresis, and the genomic DNA concentration was measured using a UV spectrometer at 260 and 280 nm. Stock DNA was diluted to a working solution of 100 ng/ μ L.

2.2. Fungal Identifications

A small piece of mango (1x1 cm) that shows lesion of disease was disinfected by 10% Clorox for 5 min then washed with DI water and let it dry. The tissues were grown on Potato dextrose agar at room temperature for 7-14 days and subculture until the fungal strain is pure. Fungal morphological characteristics were identified under the microscope in color and shape of conidia.

2.3. Fungal DNA Isolation

The genomic DNA was extracted followed by modified CTAB method [20]. The DNA pellets were resuspended in 30 μ L sterile DNase free water and kept -20 °C until ready for use. The genomic DNA was determined using 0.8% agarose gel electrophoresis. The purity and concentration of DNA samples were determined by using UV spectrometer at 260 and 280 nm.

2.4. Primer Design for β -1,3-Glucanase Gene

The sequence of β -1,3-glucanase gene of four plants, namely *Mangifera indica* (XM_044622765.1), *Pistacia vera* (XM_031397038.1), *Durio zibethinus* (XM_022914255.1), *Prunus avium* (XM_021968120.1) and *Curcumis* sp.

(Csa_5G423880) from EnsemblPlants database (<https://plants.ensembl.org/index.html>) were collected and aligned in GeneDoc programme [21]. Next three primers were designed followed in Table 1.

Table 1 Primer sequences were amplified for β -1,3-glucanase gene in mango.

Primer name	Sequence (5'-3')	Reference
Glu2-F	ACTGCAGCTAATTGGG	Felipe et al.
Glu2-R	GGTTTGTAGTATCATTGCT	(2022)
Glu2-1067F	TTAGATTGGGCATGTGG	In this study
Glu3-3R	GAACATACAGGAACCGTG	
Glu4-30R	TTCATAGATGGGGCTGTG	

2.5. PCR Amplification

PCR amplification was performed in a 20 μ L volume containing 100 ng of genomic DNA, 1X PCR buffer (2 mM MgCl₂), 0.2 mM dNTPs, 0.2 μ M forward primer, 0.2 μ M reverse primer, 1U Taq DNA polymerase (GeneDirex, Taiwan) and deionized water. The conditions of Glu2-F and Glu2-R primer was performed according to Felipe et al. [22]. The amplification of Glu2-1067F, Glu3-3R and Glu4-30R primer were performed by denaturing step at 94 °C for 5 min, followed by 30 cycles of denaturing step at 94°C for 30 sec, annealing step at 53°C for 30 sec and elongation step at 72°C for 30 sec, with final extension at 72°C for 5 min. PCR product were run on 1% agarose gel electrophoresis (stained with SafeView: ABM, Canada) at 100 volt for 30 min. The gel was visualized under a UV transilluminator.

2.6. Identification and Characterization of β -1,3-Glucanase

PCR fragment of β -1,3-glucanase were purified using PCR clean-Up & Gel Extraction Kit (PureDirex, Taiwan) and sequenced using Glu2-F and Glu2-R primers at 1st First Base (Malaysia). The obtained sequence was identified using nucleotide blast in NCBI database (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>). Finally, these sequences were aligned in GeneDoc programme [21] to find size of exons and introns.

3. RESULT AND DISCUSSION

3.1. The Amplification of β -1,3-Glucanase using Specific Primer for Exons 2, 3 and 4

The specific primers namely Glu2-1067F, Glu3-3R and Glu4-30R primer were developed in our study based on the sequences of *M. indica*, *P. vera*, *D. zibethinus*, *P. avium* and *Curcumis* sp. The size of PCR fragment for Glu2-F and Glu2-R primers in mango was at approximately 800 bp while there was no PCR product in DNA of *C. asianum* (Fig 1A in blue block). When the region between exons 2 and 3 was amplified using Glu2-1067F and Glu3-3R primers, both PCR products were produced in same size about 300 bp (Fig 1A in yellow block). For Glu2-1067F and Glu4-30R primers amplification, the PCR product were generated in different size in mango and *C. asianum* approximately 500 and 600 bp, respectively (Fig 1A in green block). The Glu2-F and Glu2-R primers was validated by amplifying the mango cultivars. All samples were produced a unique band in about 800 bp (Fig 1B).

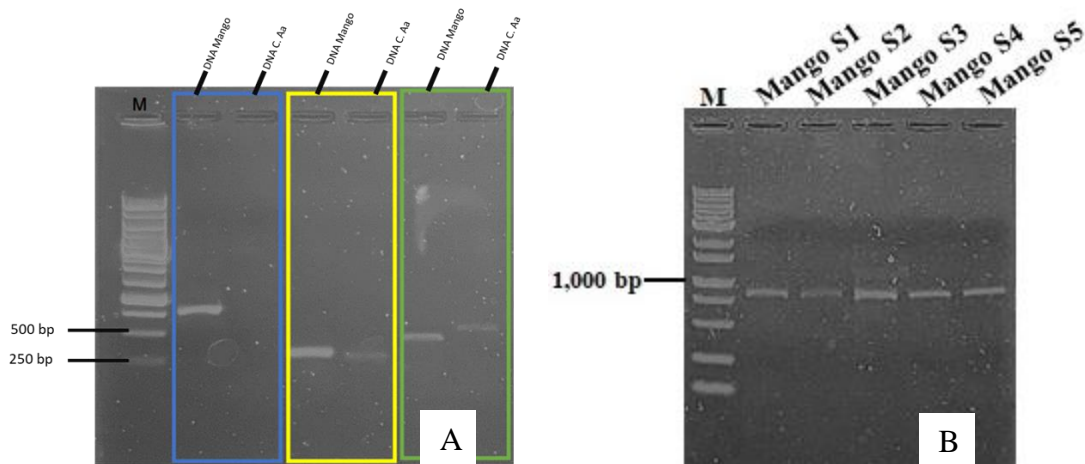


Figure 1 PCR amplification of mango and *Colletotrichum asianum* form 3 primer pairs (left) 5 mango cultivars (right), M is 1 Kb DNA ladder (GeneOn, UK)

3.2. Identification and Characterization of β -1,3-Glucanase

The sequence of *M. indica* from database was contained coding region ranged 1,497 bp (489 amino acid) consisting of four exons and three introns. The coding region ranged 64 bp, 1,254 bp, 28 bp and 151 bp, respectively. Among the sequence of five sequences, *M. indica*, *C. clementina*, *D. zibethinus* and *Curcumis* sp. contained four exons and three introns, while *P. vera* did not found coding region of exon 1. The first exon was highly variation ranged 52-79 bp. The coding region of exon 2 was the largest exon and highest conserved sequence (62.12%) ranged 1,213 to 1,254 bp. The variation of exon 2 included singleton site of 21.85% and parsim-informative site of 16.03%. The coding region of exon3 was smallest sequence ranged 28 bp. Their region comprised highly conserve sequences of 85.7%. The last exon contained 151-154 bp with conserve site of 39.74% and variation site of 60.26% (26.28% of singleton site and 33.97% of parsim-informative site)

Exon 2 in β -1,3-glucanase of mango cultivar was sequenced and compared with *M. indica* from database. The sequence of mango cultivars was ranged 777 to 782 bp (224 to 1006 in exon2) and contained conserve site 95.91%. The variation site of singleton site and parsim-informative site were obtained 1.79% and 0.5%, respectively. *M. indica* and mango cultivars had specific nucleotide 5 sites consist of 242 (base G), 257 (base G), 383 (base T), 408 (base C), 506 (base G) and 752 (base C) site (Fig 2). There were 31 SNPs in exon 2 which presented as both transition and transversion (Fig 3).

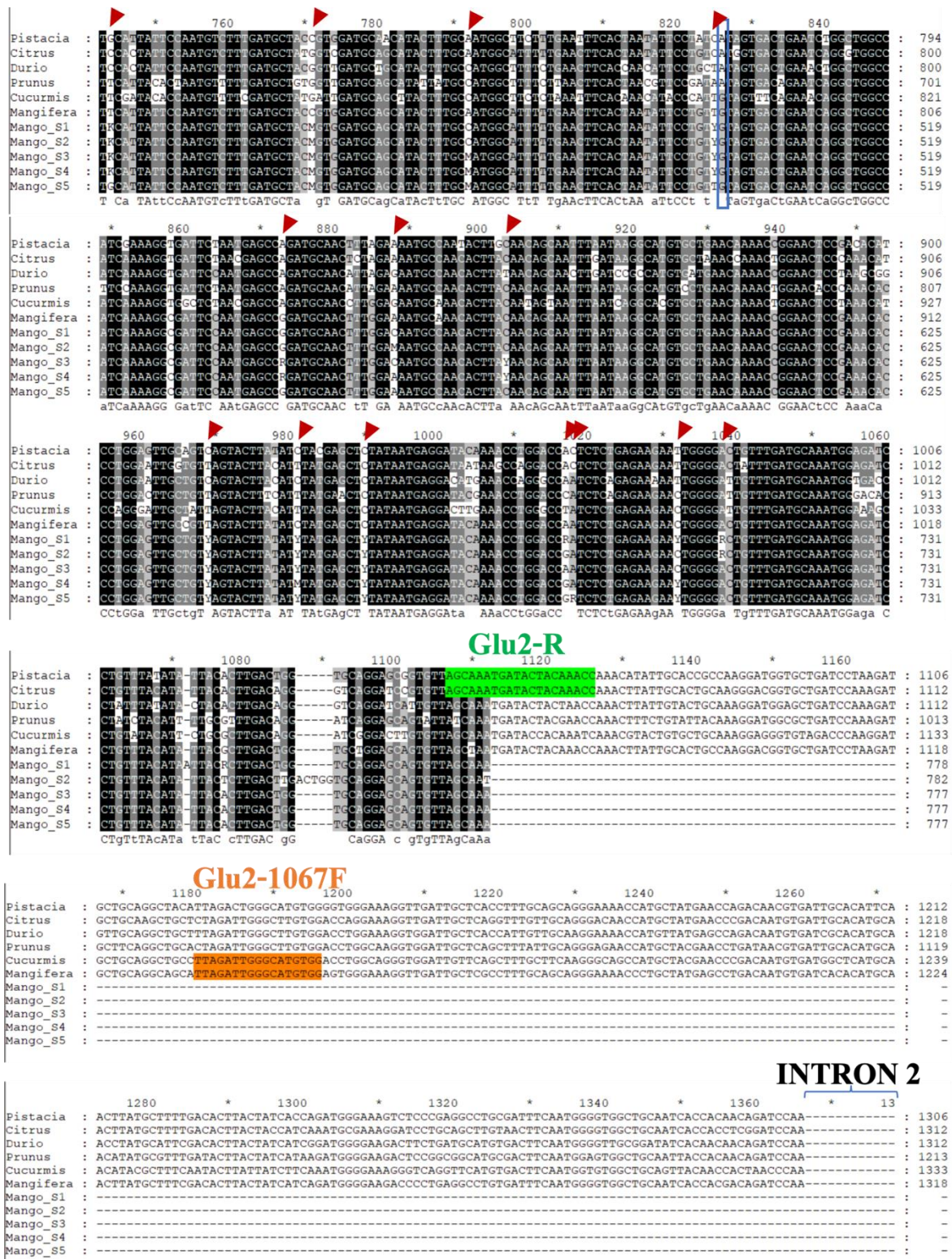


Figure 2 Sequences alignment in exon 2 of beta-1,3-glucanase and 31 SNPs positions (red arrow)

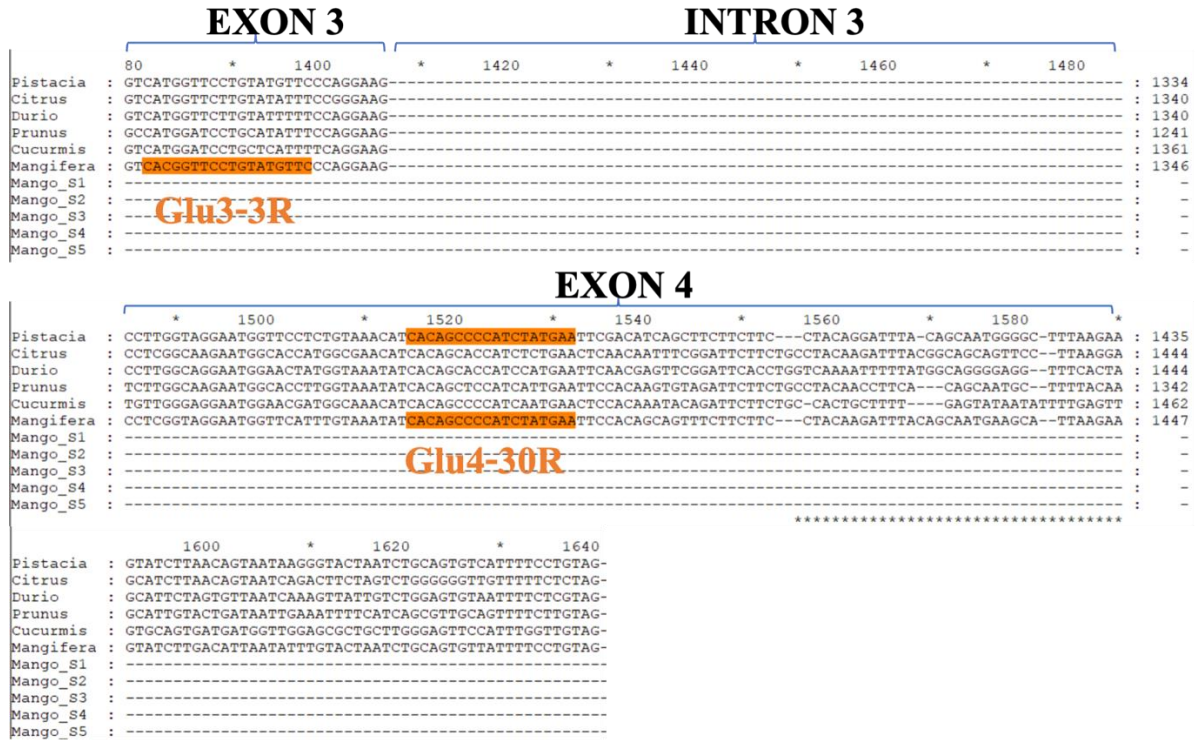


Figure 2 Sequences alignment in exon 2 of β -1,3-glucanase and 31 SNPs positions (red arrow)

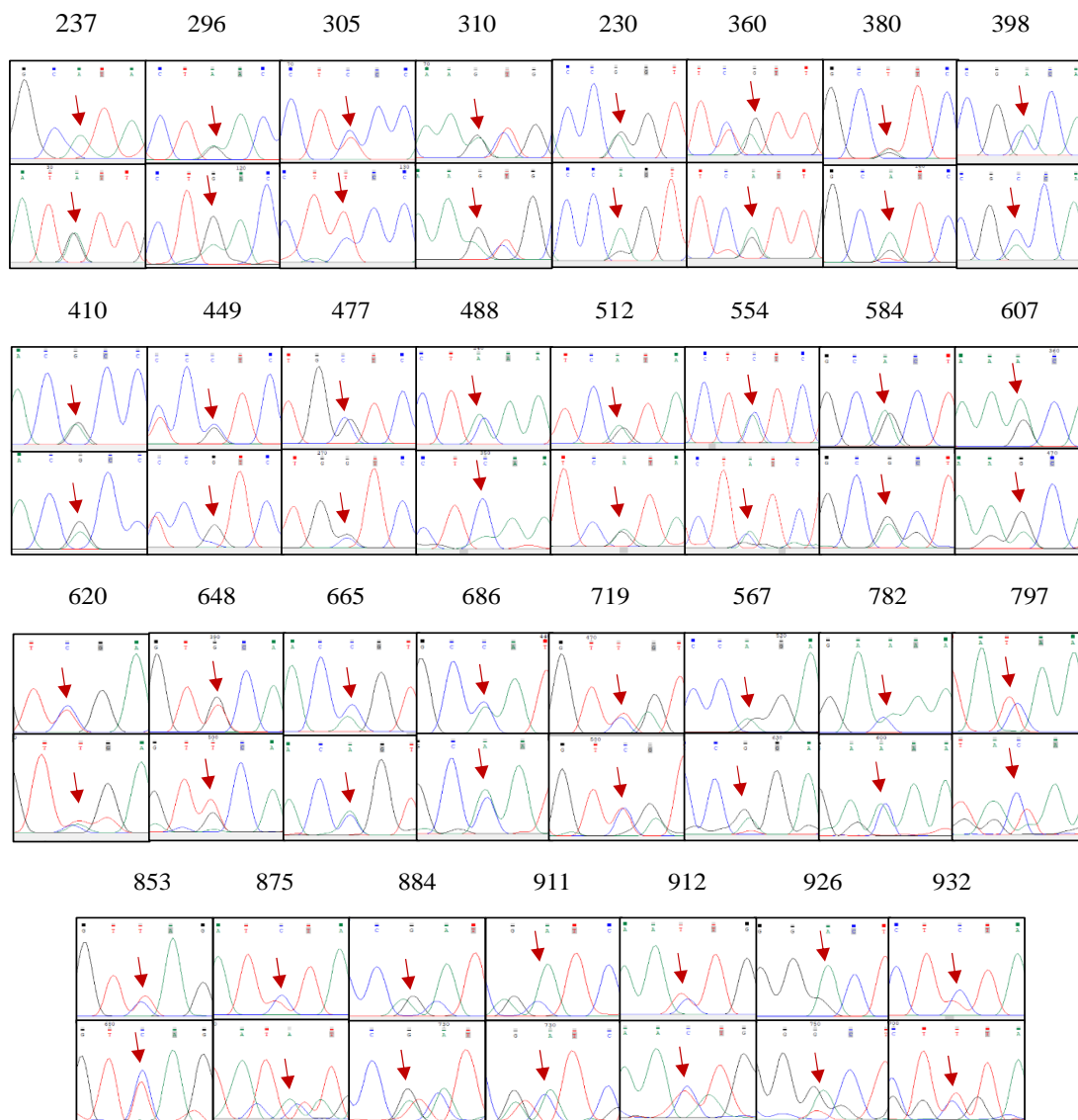


Figure 3 Profiles for 31 SNPs positions (red arrow) in exon 2 of β -1,3-glucanase

DISCUSSION AND CONCLUSION

Colletotrichum asianum is the fungal pathogen that was found in Asia, America, Africa, and Oceania. The primary cause of anthracnose disease affecting postharvest mango in Thailand lead to fruit quality. The development of new mango cultivars that resistant to disease were interrupted by lacking of molecular mechanisms. β -1,3-glucanases are enzymes that catalyze the hydrolysis of 1,3- β -D-glycosidic linkages between β -1,3-glucans, which is a major component of fungal and plant cell walls. β -1,3-GLU genes are found in bacteria, fungi, viruses including plants. It has an important duty in developmental processes and physiological, including pathogen defense mechanisms [23, 24, 25].

The β -1,3-GLUCANASE genes were isolated and sequenced to analyze changes that can occur due to the SNP mutation. At the molecular level, missense mutations caused by SNPs occurring at gene coding regions generally affect protein stability, protein-protein interactions, and critical components of biological reaction [26]. For example, SNP 21881933 located within the highly conserved Glycosyl hydrolases family 17 (GH17) domain, plays an important role in physiologically processes in plants, such as response to abiotic and biotic stresses, defense against herbivores, phytohormones activation, lignification, and cell wall remodeling [27]. Interestingly, the SNP is 816

situated near or within the active site region of the enzyme, and mutations within this domain could directly affect many functions in plants. Any conformational change altering the active sites of proteins and mutations close to it can affect biochemical reactions because catalytic reactions are very sensitive to the active sites for both of the products and reactants [26].

As demonstrated by these findings, it was a preliminary model for the possible mechanism of resistance against mango anthracnose involving β -1,3-glucanase. The thirty SNP alleles of β -1,3-*GLUCANASE* in exon 2 may be related directly to pathogenic resistance in mango due to all cultivar presence. However, there is no evidence to link to disease severity. In addition, when the β -1,3-glucanase activities were upregulated, other defense response mechanisms of mango would be against *C. asianum* infection. In other plants, oligosaccharide-elicitors such as β -glucans is released by β -1,3-glucanases activity and they interact with membrane receptors to activate a transduction cascade leading to a defense mechanism [25, 28]. In contrast, the SNP allele G in mango β -1,3-*GLU2* causes unfavorable changes in the structure of expressed β -1,3-glucanases. As a result, mango genotypes with this allele are more susceptible to anthracnose. This implies that the enzyme's catalytic activity was affected by the mutation, thereby hindering the primary defense response against *C. gloeosporioides* infection.

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