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The cloning of the MPER1 and PX3 peroxidase genes conferring bacterial blight disease resistance genes in cassava (*Manihot esculenta* (L.) Crantz)

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Abstract

Cassava bacterial blight, CBB caused by *Xanthomonas ampestris pv. manihotis* pv. *Manihotis* is an important outbreak of cassava bacterial disease in Thailand. Plant peroxidases play major roles in many physiology processes both abiotic and biotic stress including plant defense response against a bacterial pathogen. The objective of this research was to clone the peroxidase genes homologs in cassava variety Rayong 60 (MTAI 8), which is a disease-resistant variety against cassava bacterial blight by using polymerase chain reaction method. According to our results, two peroxidase genes homolog named *MPER1* (cationic peroxidase; GenBank Accession No. EF645823) and *PX3* (secretory peroxidase; GenBank Accession No. EF645824) in cassava MTAI8 genome were isolated and determined. The *MPER1* contains 1,211 nucleotide 2 exons and 1 intron with 73% similarity to the peroxidase gene in *Arabidopsis thaliana which* was the first plant to have its genome sequenced, and is a popular tool for understanding the molecular biology of many plant traits. The *PX3* contains 945 nucleotides 3 exons and 3 introns with 57% high similarity to peroxidase gene in *Arabidopsis thaliana*. The predicted amino acid sequence revealed a 98 and 134-amino acid for *MPER1* and *PX3*, respectively. Both of the genes showed conserved a domain of peroxidase genes (gene superfamilies) with other plant species. These genes will be useful as a molecular marker in selecting disease-resistant varieties and for further used as plant genetic improvement approach.

Keywords: cassava bacterial blight, CBB, MPER1, PX3

Introduction

Cassava (*Manihot esculenta* (L.) Crantz) is one of the most important crops, being one of the main sources of calories for more than one billion people mainly in poor countries worldwide especially in sub-Saharan Africa, Southeast Asia, South America and some Pacific Island nations (Cock, 1985; Wydra et al., 2004; Johana et al., 2017). Due to its characteristic of high protein level, this causes the crop more to biotic stress including cassava bacterial blight (CBB) (Chittoor et al., 1997). Cassava bacterial blight caused by *Xanthomonas campestris* pv. *Manihotis* (*Xam*) is considered the most important foliar and vascular bacterial disease for cassava causing the yield loss up to 92% and is regarded as one of the most limiting diseases of cassava production worldwide (Umemura and Kwano, 1983). CBB is usually controlled through host resistance (Paula et al., 2018).

Peroxidases are enzymes that catalyze the oxidation of molecules at the expense of (H_2O_2) recognizing a broad range of substrates. Plant peroxidase genes have been mostly involved in activities during wounding, disease resistance, and physiological stresses such as radiation, salinity, and pollution (Campa, 1991). Pareira et al. (2000) reported that peroxidases can act as catalysts for the polymerization of phenolic compounds to form lignin and suberin in the plant cell wall, which can act as a barrier to block the spread of the pathogen in the plant (Fritig et al., 1987).

Peroxidases were suggested to play an important role in CBB (Flood et al., 1995). The identification of peroxidases has been demonstrated in interactions between cotton and *X. campestris* pv. *Malvacerum* (Dai et al., 1996); rice and *X. oryzae* pv. *Oryzae* (Chittoor et al., 1997). Rauyaree et al. (2001) reported the identification of peroxidases in Thai rice resistant to *Pyricularia grisea*. Pareira et al. (2003) reported the identification and initialization of a cassava peroxidase gene fragment by PCR, MEPX1, and its polymorphisms among different cassava cultivars.

For screening of the susceptible and resistance characterization among cassava varieties compared with conventional breeding, peroxidases

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could be used as potential molecular markers that would provide the benefit of cost, rapid results and accurate. (Flood et al., 1995). Cassava variety used in this study was Rayong 60 (MTAI 8 in the germplasm collection) which was resistant to CBB. MTAI 8 in the germplasm collection is resistant to CBB, based on Flood et al. (1995), that classified MTAI 1 and MTAI 8 from Thailand are resistant cultivar to CBB at 4 scales. The objectives of this study were to isolate two peroxidase genes homologs in cassava bacterial blight disease (CBB) from Thai cassava Rayong 60 variety by PCR method.

Materials and methods

Cassava variety

Cassava variety used in this study was Rayong 60 (MTAI 8 in the germplasm collection) which was resistant to CBB. The cassava variety was developed by Rayong Field Crops Research Center, Department of Agriculture, Rayong Province, Thailand. Plants were grown for vegetative propagation and 4-week-old; leaves were collected and used for DNA isolation (NucleoSpin Plant L Kits, CLONTECH, Palo Alto, CA)

Polymerase chain reaction (PCR)

The deduced amino acid sequences of seven peroxidase genes from seven species including *Solanum tuberosum, Nicotiana tabacum, Hordeum vulgare, Oryza sativa, Pisum sativum, Spinacia oleracea,* and *Minihot esculenta* used in this study were compared using the Clustal W program (http://ebi.ac.uk/clustalW) to identify highly conserved regions. The two primers designed from highly conserved regions of the retrieved sequences for cationic peroxidase and secretory peroxidase genes in sense and antisense direction, respectively. PCR was carried out in 50 ul and contained genomic DNA, 2 mmol/l Mg2Cl, 10 mmol/l Tris-HCl (pH 8.3), 50 mmol/l KCl, 1.5 U Taq polymerase, 2 mmol/l of each dNTP, 0.5 umol/l of each primer (Ward medic Thailand). Primers sequences used in this study were indicated in tables 1 and 2. After an initial denaturation step for 2 min at 94°C, 30 amplification cycles were performed. Each cycle included denaturation at 94°C for 2 min, annealing 50°C for 1 min, extension 72°C for 1 min and a final extension 72 °C for 5 min, modified from Pareira et al. (2003).

Cloning and sequence analysis

PCR products of the expected sizes were cloned into pDrive® Cloning Vector according to the protocol described by the manufacturer (Qiagen, USA) (TransformAid™ Bacterial transformation kit, Life Science, USA). Plasmid DNA was obtained from QIAprep spin miniprep kit, Qiagen, Valencia, USA. Plasmid DNA harboring insert was analyzed and digested by *Eco*RI restriction enzyme (Figure 2); and sequenced using the BigDye® Terminator v3.1 Cycle Sequencing (Perkin-Elmer, CA, USA) on an ABI PRISM™ model 377 DNA sequencer. The primers used for sequencing reactions were SP6 (5'-CATTTAGGTGACACTATAG-3') or T7 (5'-GTAATACGACTCACTATAG-3') at the concentration of 3.2 pmole. The sequences were analyzed with DNAStar expert sequence analysis software, DNAStar, Inc, USA and compared against GenBank NCBI non-redundant sequence database (BlastN program) (Gapped BLAST VERSION 2.0, Altschul et al., 1997).

Gel electrophoresis

Following amplification, 5 µl of each PCR product was analyzed by electrophoresis in a 1% agarose gel and stained with ethidium bromide. The gels were visualized with UV light and photographed (Figure 1).

Table 1. Gene Specific Primers used to amplify Cationic peroxidase in PCR.

Primer Names	Nucleotide sequences
(1) PXC-1 **Forward Primers**	GC(AGCT)(AC)G(AGCT)GA(CT)(AT)(GC)(AGCT)GT
(2) PXC-1 **Reverse Primers**	(AG)TG(AGCT)A(AG)(AGCT)A(AG)(AGCT)CC(CT)TT
(3) PXC-2 **Forward Primers**	(CT)T(AGCT)GG(AGCT)(AC)G(AGCT) (AC)G(AGCT)GA(CT)
(4) PXC-2 **Reverse Primers**	CAT(CT)TT(AGT)ATCAT(AGCT)GC
(5) PXC2-1 **Reverse Primers**	(AG)TT(AGCT)CCCAT(CT)TT(AGT)ATCAT(AGCT)GC

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Table 2. Gene Specific Primers used to amplify the secretory peroxidase in PCR.

	Primers	Base sequences
(6)	PXS1 Forward Primers	GAGTTGGCAGAGAAGGATGC
(7)	PXS1 Reverse Primers	TGTGCACCTGAGAGAACCAC
(8)	PXS2 Forward Primers	GAGTTGGCAGAGAAGGATGC
(9)	PXS2 Reverse Primers	GTGTGCACCTGAGAGAACCA
(10)	PXS3 Forward Primers	GAGTTGGCAGAGAAGGATGC
(11)	PXS3 Reverse Primers	TGTGTGCACCTGAGAGAACC
(12)	PXS4 Forward Primers	GTGGAGGGATGTGAGAGCAT
(13)	PXS4 Reverse Primers	TGTGTGCACCTGAGAGAACC
(14)	PXS5 Forward Primers	GTGGAGGGATGTGAGAGCAT
(15)	PXS5 Reverse Primers	GTGTGCACCTGAGAGAACCA
(16)	PX Forward Primers	CGTCTCCACTTTCATGACTGC
(17)	PX Reverse Primers	GAAACCTACCGTGTGTGCACC

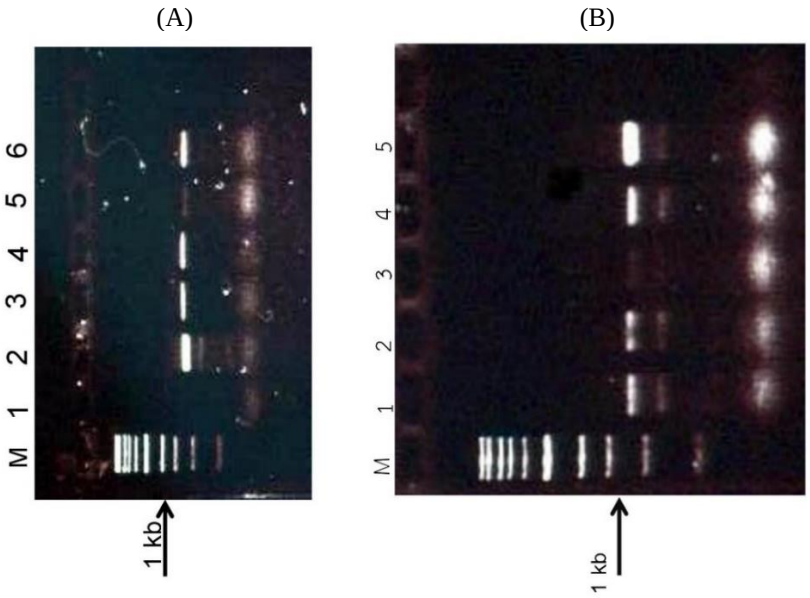

Figure 1. Agarose gel electrophoresis demonstrating the amplified PCR products derived from MTAI 8 (Rayong 60) as a template genomic DNA. (A) PCR bands obtained using gene specific primers cationic peroxidase (Lane 2 – 6); (B) PCR bands obtained using primers using gene specific primers secretory peroxidase in polymerase chain reaction (Lane 1 – 5). M = 1 kb DNA ladder.

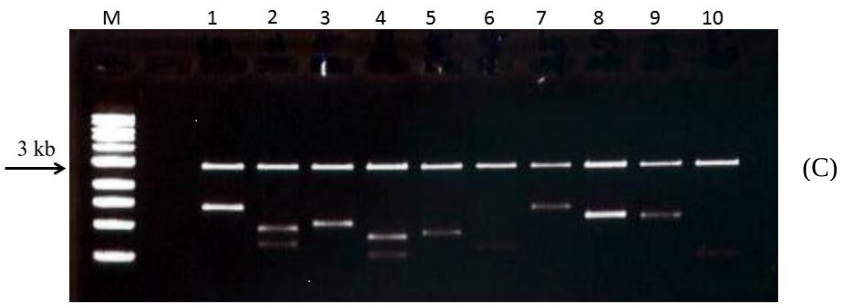

Figure 2. The cloned plasmid DNA harboring the DNA inserts was confirmed and by digestion with restriction enzymes *Eco*RI. (Lane 1 – 10); M = 1 kb DNA Ladder.

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Results and discussion

PCR reactions and Cloning

The PCR products were amplified from Rayong 60 genomic DNA using primer pairs (1-3), (6-7), (8-9), (10-11), (12-13) and (14-15). Two different sizes were cloned and cloned PCR product of 1,211 bp (*MPER1*, GenBank Accession No.EF645823) and 945 bp (PX3, GenBank Accession No.EF645824) showed sequence homology with 73% and 57% similarity to peroxidase gene in *Arabidopsis thaliana*, a small flowering plant that is widely used as a model organism in plant biology, respectively.

Nucleotide sequence analysis of the peroxidase genes

The nucleotide sequence of a 1,211 bp fragment containing the cationic peroxidase *MPER1* gene and a 945 bp fragment containing secretory peroxidase gene PX1 gene and its flanking regions were determined. For *MPER1*, a 293 bp open reading frame was obtained, encoding the enzyme which was comprised of 98 amino acid residues with a calculated molecular weight of 10.78 kDa. The deduced amino acid sequence of the Rayong 60 cationic peroxidase showed a high degree of identity to *Arabidopsis thaliana* peroxidase at 73% The fragment comprises of 2 exons and 1 intron. For *PX1*, a 403 bp open reading frame was obtained, encoding the enzyme which was comprised of 134 amino acid residues with a calculated molecular weight of 14.74 kDa. The deduced amino acid sequence of the Rayong 60 secretory peroxidase showed a high degree of identity to *Arabidopsis thaliana* peroxidases at 57%. The PX1 fragment comprises 3 exons and 3 introns.

Recently, Johana (2017) reported QTL that represents a first step in the dissection of the molecular mechanisms that govern CBB resistance in cassava and a new source of genes to be validated through different approaches. Furthermore, the resistance to CBB has been classified as a quantitative trait, the detection and localization on the genetic map of cassava QTL (Quantitative Trait Loci) conferring resistance to CBB would still useful for developing disease resistance lines. With the advance of gene methodologies like gene editing, the function of the interesting genes in CBB resistance would also be possible (Sander and Joung, 2014). Despite the fact that plants have evolved several mechanisms to defend themselves against pathogens, these mechanisms have solely been extensively studied in model plants. But the knowledge generated in cassava is relatively scarce. Further attempts might be involved with identifying new Thai cassava varieties that are more tolerance or resistance to CBB by both NBTs (New Breeding Techniques) and/or by transgenic approach. This result reported here might improve our understanding and knowledge regarding bacterial blight disease-resistant genes for CBB. *MPER1* and *PX1* could be useful as a marker for the identification of new resistant varieties.

GenBank Accession No. 645823 (*MPER1*)

```
   1 GTGGAGGGAT GTGAGAGCAT TAGAAAGGCT AAGGCATTGG TGGAGAGCAA GTGTCCTGGT
  61 GTTGTATCCT GTGCAGATAT TCTTGCAATT GCTGCCAGAG ATTATGTCCA CCTGGTATGC
 121 CTCTGCATTT CAATTCTTGA TATCCCCTAC TCAATCCTTA ATTAACTATT TCAAACTCTA
 181 GATCTTATCC CACTCAATCA AAACTTATTA ACAATTTGGA ATATATTGAT GGTAACAAAG
 241 TCCTATAAAT AATCCAAAGC ATAGGGCTGG TTTGTTGATA TAAGGGAAAT CAAATTTCTT
 301 GACTGTAGGT GAAAATATAT GTTGGGGTGC TCATACTCAT AATGCTTCCA AAGTAGAAAG
 361 GTGGAAAAAG GAAGATTGGT TTTGTCATTT TTGACAAAGT ATTTATAACA AAACAAACTC
 421 TTCTAAAAGG GCAAGAAAGT ATAAAAAATC ATTAAGTCCA TGTGATTTGA ACAGCTAGGT
 481 TATTTGTCCT TTGCTAGAAT CAATATCTCT ATGAAAGTCA AGAATATTAA TCAATTAATC
 541 CTTCCAAAAA TAGGACCAAT GCTGTGAAAA ACCAAATGCC TCATTCACTG GTAACATGAT
 601 GAGAGAACTA ATAGACAATA AGACTGGCAT TTGACTTGTA TTGGTTTTCT AAATGTCTCA
 661 TTCATTGGTA ACTGGATGTG GTCAATGATT TTTATTTTCT CAAAACTGTA CTCTTTTAGT
 721 TATTTTCTGT TAGGATAACA ATATTATTAT ATGACCCCAA TAATAATCAT TTGTATTATT
 781 ATTAAATTAG TAATTTATAT TCAAATTTCT ATATAAATTT TAGAAAAATT AACTATTTAG
 841 ATCATGCTAA ATTCATTAAT CGATCGTTCA GTTTTGAAAA ATATATTAAA ATATTTTTAA
 901 AATATTAAAA TATTTTTCAT AAATCTCTCT AAATTTTAAT TTATAAAACT CCTTTTTATA
 961 GACCTCTATT TTCTTCTAAA TAATCCTATA ATGGAGTGCT CTAATTCCAT ATGCTACATT
1021 TTCATGACGC AGGCAGGGGG ACCTTATTAC CAAGTGAAGA AAGGGAGATG GGATGGCAAA
1081 ATATCAATGG CATCAAGGGT ACCCTATAAT CTACCTCAAG CAAATTCAAC CATTGATCAA
1141 CTACTGAAGC TTTTCAATTC CAAAGGATTA ACACCACAAG ATCTAGTGGT TCTCTCAGGT
1201 GCACACAATC T

TRANSLATION =
        "VEGCESIRKAKALVESKCPGVVSCADILAIAARDYVHLAGGPYY
        QVKKGRWDGKISMASRVPYNLPQANSTIDQLLKLFNSKGLTPQD
        LVVLSGAHNL"
```

Figure 3. Nucleotide and 98 deduced amino acid sequence of the Rayong 60 Cationic peroxidase (*MPER1*) (GenBank Accession No. EF645823). Underlined represent exon parts of the coding regions.

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GenBank Accession No.EF645824 (*PX3*) ; Nucleotide sequence.

1 GTGATTCGTC TCCACTTTCA TGACTGCTTT GTCAGGGTTA GTGTTTTATT GTTTCTTCTT
61 AAGATTTGAT TTTCTTTTAG TTTGTAATTA TTTATTTCTT GTTAATAGGG ATGTGATGCT
121 TCGTTGTTGC TATCTTCGCC GAGTAACAAT GCGGAGAAAG ACCACCCGGA TAATCTTTCG
181 CTCGCCGGAG ATGGTTTTGA CACGGTGATC AAGGCAAAGG CTGCCGTTGA TAGTGTTTCT
241 CAATGCAGGA ACAAAGTTTC ATGTGCTGAT ATTCTGGCTC TTGCTACTAG AGATGTTGTA
301 TCTTTGGTAA GCAAGGTTTT TTCCCTTTAT TTTCGTTAAT TTTTTTTTTT TTAAAAATTA
361 TGGATTTTTT TTATTTCAAT TTTATATCTG ATGAGTTTGA AAATGGGTTG GATATTTTGA
421 GTATTCTATT TAATTGAATT TTATTGAAAT TCATATAATA TATATATGGT AGGAAATACT
481 TTATCCTCTT TAGTAGGTAT ATTTGGTATA ATGAATAAAT TTTACAAGTA TAATAAAGTT
541 GTGGAGCATG TGCATGATTA ATTAAATGAA ATGGAAATGC AGGCAGGAGG ACCATTTTAT
601 GAAGTGGAAC TAGGGAGGCG TGACGGGAGA ATATCAACAA AGGCAAGTGT TCAACACAAG
661 CTGCCAAGTG CTGACTTCAA TTTAGACCAG CTCAATTCTA TGTTCGCTTC ACTTGGCCTT
721 ACCCAAACTG ACATGATTGC TTTATCAGGT

TRANSLATION :

"VIRLHFHDCFVRGCDASLLLSSPSNNAEKDHPDNLSLAGDGFDT

VIKAKAAVDSVSQCRNKVSCADILALATRDVVSLAGGPFYEVEL

GRRDGRISTKASVQHKLPSADFNLDQLNSMFASLGLTQTDMIALSG"

Figure 4. Nucleotide and 134 deduced amino acid sequence of the Rayong 60 Secretory peroxidase (*PX3*) (GenBank Accession No. EF645824). Underlined represent exon parts of the coding regions.

```
Solanum_tuberosum       ------------------------------------------------------------
Nicotiana_tabacum       MASLKINAIVLFILVSLLIGSSSAQLSTGFYSKSCPKLYQTVKSAVQSAINKETRASL
Hordeum_vulgare         ---MASSSYTSLLVLVALVTAASAQLSPTFYDTSCPRALATIKSGVMAAVTSDPRASL
Oryza_sativa            -----MASSLSVAVLLCLAAAAAAQLSPTFYDTSCPRALATIKSAVTAAVNNEPRASL
Pisum_sativum           ------------------------------------------------------------
Spinacia_oleracea       -------VFLAIAIAIAIVGFAEAQLKLGYYSESCPKAEAIVESFVHQHIPHAQSLAAPL
Manihot_esculenta       ------------------------------------------------------------

Solanum_tuberosum       ---HFHDCFVNGCDASILLDQTATIDSEKTARPNNN---SARGFEVIDRIKSEVDKVCGR
Nicotiana_tabacum       LRLFFHDCFVNGCDGSLLLDDTSSFTGEKRAAPNVN---SARGFEVIDNIKSAVEKVCPG
Hordeum_vulgare         LRLHFHDCFVQGCDASVLLSG-----MEQNAIPNAG---SLRGFGVIDSIKTQIEAICKQ
Oryza_sativa            LRLHFHDCFVQGCDASVLLADTATFTGEQNALPNKN---SLRGFNVVDSIKTQLEGICSQ
Pisum_sativum           ---------------VLLNKTDTVVTEQEAFPNIN---SLRGLDVINRIKTAVENACPN
Spinacia_oleracea       LRMQFHDCFVRGCDASVLLDRTEAGNNDTEKTANPN--LTLRGFGFIDGVKSLLEEECPG
Manihot_esculenta       -RLHFHDCFVEGCDASILISTKPGSKELAEKDAEDNKDLRVEGCESIRMAKALVESKCPG
                                      :*:             .: .     .*   :   *: ::  *
                         Myr/Pho
Solanum_tuberosum       PVVSCADILXAAARDSVVALHGPTWEVELGRRDSTTASRTTANNDIPTPLMDLPALIDNF
Nicotiana_tabacum       -VVSCADILAVTARDSVVILGGPNWNVKLGRRDSRTASQSAANSGIPPATSNLNRLISSF
Hordeum_vulgare         -TVSCADILTVAARDSVVALGGPSWTVPLGRRDSIDANENEANTDLPGFNSSRAELEAAF
Oryza_sativa            -TVSCADILAVAARDSVVALGGPSWTVGLGRRDSTTASMDSANNDLPPPFFDLENLIKAF
Pisum_sativum           -TVSCADILALSAQISSILAQGPNWKVPLGRRDGLTANQSLANTNLPAPFNTLDELKAAF
Spinacia_oleracea       -VVSCADIIALVARDSVWTIGGPWWPVTTGRRDGRISNETEALQNIPPPFSNFSSLQTIF
Manihot_esculenta       -VVSCADILAIAARDYVHLAGGPYYQVKKGRWDGKISMASRVPYNLPQANSTIDQLLKLF
                          .******:   *:      ** : *  ** *.  :    .  .:*       *   *
                                     Peroxidase domain
Solanum_tuberosum       KKQG-LDEEDLVALSGAHT-----------------------------------------
Nicotiana_tabacum       SAVG-LSTKDMVALSGAHTIGQARCTSFRARIYNETNN------LDASFARTRQSNCPRS
Hordeum_vulgare         LKKGGLNTVDMVALSGAHTIGQAQCSTFRARIYGGDTN------INAAYAASLRANCPQT
Oryza_sativa            GDKG-FSVTDMVALSGAHTIGQAQCTNFRGRIYN-ETN------IDAGYAASLRANCPPT
Pisum_sativum           AKQG-LTPTDLVALSGAHTFGRSHCSLFVDRLYNFSNTGKPDPSLNTTYLQELRKTCPK-
Spinacia_oleracea       ASKG-LDLKDLVLLSGAHTIGVAHCPSFSERLYNFTGRGYGQDPSLDSEYATNLMTRKCT
Manihot_esculenta       NSKG-LTPQDLVVLSGAHTVGF-----------------------------------------
                            * :   *:* ******

Solanum_tuberosum       ------------------------------------------------------------
Nicotiana_tabacum       SGSGDNNLAPLDLQTPNKFDNNYFKNLVDKKGLLHSDQQLFNGGSADSIVTS--YSNNPS
Hordeum_vulgare         VGSGDGSLANLDTTTANTFDNAYYTNLMSQKGLLHSDQVLFNNDTTDNTVRN--FASNPA
Oryza_sativa            AGTGDSNLAALDTTTPYSFDTAYYSNLLSNKGLLHSDQVLFNGNSTDNTVRN--FASNRA
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Figure 5. Deduced amino acid sequences of peroxidase genes indicating conserved domain from various plant species including *Manihot esculenta*. Greenbox represents peroxidase domain (DLVVLSGAHTVGF) from various plant species analyzed by Clustal W program (http://ebi.ac.uk/clustakw).

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Conclusion

The cassava variety Rayong 60 exited two peroxidase genes named Cationic peroxidase (*MPER1*) and Secretory peroxidase (*PX3*). Cationic peroxidase (*MPER1*) and Secretory peroxidase (*PX3*) which were considered as gene families contain 1,211 and 945 nucleotide in lengths, respectively. Both of the genes were deposited under GenBank Accession Numbers EF645823 and EF645824 respectively at the NCBI database. *MPER1* contains 2 exons and intron that showed sequence high homology with peroxidase gene in Arabidopsis (*Arabidopsis thaliana*) at 73% and *PX3* contains 3 exons and 3 introns that showed sequence high homology with peroxidase genes in *A. thaliana* at 57%. Both genes exhibited conserved domain with peroxidase gene superfamilies in other plant species. *MPER1* and *PX1* were confirmed from the total similarity of the peroxidase conserved sequence DLVVLSSGAHTV and GPVVSC for Myr/Pho (myristic acid/polyhydroxyoctanoate) peroxidase enzyme. The two peroxidase genes can be used as potential molecular markers for screening varieties against CBB.

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