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Partial Sequence Analysis of Cellulose Synthase *OsCESA4* and *OsCESA9* Genes in Native Upland Rice, Thailand

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Abstract Cellulose is a major component of plant cell which found in both primary and secondary cell wall and synthesized by cellulose synthase (CESA) complexes. The partial sequences of *OsCESA4* and *OsCESA9* genes in seven varieties of native upland rice were determined.. The results found that *OsCESA4* sequences showed the similarities to *Oryza sativa* Japonica (98.7-99.5%) and *Oryza sativa* Indica (97.5-99.5%). While all seven *OsCESA9* sequences revealed the same identity to both *Oryza sativa* Japonica (98.46%) and *Oryza sativa* Indica (98.46%). Since, lodging problems in the native upland rice which has been cultivated in Prachuap Khiri Khan Province (Pala U village) still unsolved. Therefore, the analysis of cellulose synthase genes of these varieties would be used to fulfill the genetic information for further upland rice breeding improvement.

Keywords: Cellulose synthase, *OsCESA4*, *OsCESA9*, Upland rice

Introduction

Rice (*Oryza sativa* L.), the most important staple food crops, is consumed as human main meal more than half of the world. Production system of rice can be classified to lowland and upland rice. Upland rice typically cultivated on slop area and open fields. Trends on upland rice cultivation over the last 30 years in Asia, Africa and Latin America have been reported the reduction of the upland rice cultivated area in Asia and Latin America, while the upland rice cultivated area in Africa has increased (Saito *et al.*, 2018). In Thailand, native upland rice has been cultivated by the ethnic minority farmers in both Northern and Prachuap Khiri Khan province. Low productivity (1.16t ha^{-1}) of this landrace upland rice is normally found since highland area planting, insufficient water supporting, and rice population heterogeneities (Karladee *et al.*, 2012).

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Paganyaw, the largest ethnic minority group stay at Pala U village, Prachuap Khiri Khan province, have been grown upland rice with their wisdom for a long time to dairy consuming and traditional ceremonies (Vechpong *et al.,* 2015). Previous researches attempted to understand and improve the breeding quality of these native upland rice varieties such callus induction responsibility to various media formulations (Na C*hiangmai et al.,* 2019) and effect of inorganic phosphorous level (Na Chiangmai *et al.,* 2021). Nowadays, a high potential variety of North and Northeast indigenous upland rice Thailand have been evaluated the yield stability and grain yields for further breeding program improvement (Phapumma *et al.,* 2020). Many factors cause low production yield of upland rice, including sub-optimal crop management practices, abiotic and biotic stresses, insufficiency water support, climate pattern and genetic rice varieties (Saito *et al.,* 2018).

One of reason in low-production yield of local rice is lacking of lodging resistant. Previously, lodging resistance rice varieties were identified and developed to reduce plant stem bending or breaking which increasing grain yield (Okuno *et al.,* 2014; Ookawa *et al.,* 2010; Zhu *et al.,* 2016). Recently, local upland rice in Indonesia has been studied and selected the lodging resistant varieties for solving the low yield production problem (Mustikarini *et al.,* 2021). In wheat and rice, major factors associated with lodging resistant in rice involves morphological (e.g. cell wall thickness or width of culm wall) and morphological (e.g. plant height, culm diameter, or stem length) traits together with the biochemical composition (lignin and cellulose) of stem (Shah *et al.,* 2019). Lignin and cellulose contents, are main components of the cell wall, which effect on wheat and rice culm strength, secondary cell wall strength, and lodging resistance (Chen, X.-G. *et al.,* 2011; Kong *et al.,* 2013). The coordinated of cellulose and lignin biosynthesis pathway in rice have been reported which aim to improve the economic viability of lignocellulosic crop utilization for biofuels ((Ambavaram et al., 2010). Trends of the productivity improvement of upland rice to enhance rice yield still have been proceeded. For example, in Africa, New Rice for Africa (NERICA) varieties were developed from crosses between improved tropical *japonica* and *Oryza glaberrima* (Saito *et al.,* 2018).

Mechanical strength of plant is controlled by cell wall, which is a complex fiber network and consist of primary and secondary wall. Major component that is found in both primary (30%) and secondary (90%) cell wall is cellulose (Taylor, 2008). Cellulose is an insoluble polysaccharide polymer, consisted of a linear, unbranched β-1,4-glucans chains. Cellulose microfibrils are made by 30 nm diameter plasma membrane complexes composed of approximately 36 subunits representing at least three types of related CESA

(Cellulose Synthase A) protein (rosette structure), using uridine diphosphate (UDP)-glucose as substrates (Somerville, 2006). Synthesized cellulose was passaged to plasma membrane and deposit on the cell wall of plant cell (Doblin *et al.,* 2002). Cellulose is the most abundant organic polymer in the world which also has valuable as raw material in various industrial sectors: paper, cellophane, rayon and biofuels.

Cellulose synthase (CESA), the catalytic subunit of the plasma membrane-localized cellulose synthase complexes (CSC), is used for cellulose synthesis in plants. This enzyme is encoded by a large gene family. In rice, mutational analysis and bioinformatics researches have been revealed that OsCesA1, 3, 8 and OsCesA4, 7, 9 are related in the cellulose biosynthesis of the primary and secondary cell wall, respectively (Wang, L. et al., 2010). Interestingly, missense mutation in transmembrane domain of OsCESA4 in *japonica* rice was found that effect on protein level in plasma membrane and leads to abnormal cell wall biosynthesis (Zhang *et al.,* 2009). More recently, the *OsCESA4* mutant rice (*fc17*) exhibited cellulose deposition and cell wall remodeling, which can enhance biomass digestion and lodging resistant (Li *et al.,* 2018). In case of *CESA-9* mutation, a mutant *S1-60* was mutagenized from *japonica* rice cultivar Nipponbare, revealed the cellulose reduction (44.7%) in culm which cause the defect in thickening of the sclerenchyma cell wall and brittle culm (Wang, D. et al., 2012). Cellulose features in rice influence not only lodging resistance but also biomass enzymatic saccharification in biofuel issue. The *OsCESA-9* gene mutant rice showed a slightly affected on plant growth but had a higher biomass yield compared with wildtype and decreasing in the degree of cellulose polymerization (DP) (Li *et al.,* 2017). Recently, a semi-dominant mutation in *OsCESA-9* in rice exhibited low cellulose content, reduction of cell wall thickness, and enhancing biomass enzymatic saccharification which can apply to using rice straw for biofuel and bioproducts (Ye *et al.,* 2021). Moreover, *CESA9* mutation could affect integrity of CESA4/7/9 complexes which may result in a rapid CESA proteasome degradation for low-DP cellulose biosynthesis (Li *et al.,* 2017).

Indigenous upland rice in Thailand have been evaluated and characterized to seeking for high yield potential, grain quality and stability in each variety, including tolerance to drought stress (Narenoot *et al.,* 2017; Phapumma *et al.,* 2020). Anyway, the genetic information of Thai native upland rice still needs to do more research to understand the basic genetic background in detailed. Previously, the seven varieties of Pala-U village upland rice were analyzed the lignin biosynthesis gene (*OsPAL* and *Os4CL3*). The results showed the *OsPAL* sequences more similarity to *O.sativa* Japonica than *O.sativa* Indica whereas all *Os4CL3* sequences had the 100% sequence identity

to both references varieties (Laosutthipong *et al.,* 2019). In this study, our objective was to investigate the cellulose biosynthesis gene (*OsCESA4* and *OsCESA9*) of seven Pala-U village rice varieties, Thailand. The genetic information of these genes may utilize to understand the character of these native upland rice group in detail which may further related to the lodging problem.

Materials and methods

Plant samples

Rice seeds were obtained from the farmers at Pala-U village, Hua Hin district, Prachuap Khiri Khan province, Thailand. All seven upland rice varieties were named *1. Aung Jerng Yai,* 2. *Nah San,* 3. *Bue Soo Sue La,* 4. *Kao Niew Pala U,* 5. *Bue Ke,* 6. *Bee Kor Bi*, and 7. *Rao Soo Ya*. Individual seeds were immersed in sterile water overnight and placed on moist paper in plastic box for germination. Seedlings were observed and collected at 2 weeks later for RNA extraction.

RNA Extraction, RT-PCR and DNA Sequencing

RNA Extraction

The fresh rice seedlings (50 mg) were prepared for total RNA extraction using the Plant Total RNA Mini Kit (Geneaid Biotech Ltd., Taiwan) by following the commercial kit's procedure. Rice seedling samples were cut in small piece before grinding with micropestle in 500 µl RB Buffer and followed by adding 5 µl of ß-mercaptoethanol. After incubation at 60℃ for 5 min, the grinding sample were transferred to the Filter Column and spin downed. The clarified filtrate was transferred to a new 1.5 ml centrifuge tube. Next, the 250 µl absolute ethanol was applied to filtrate, followed by vigorous shaking. Then, the mixtures were transferred to RB column and centrifuged. The flow-through was discarded and the RB column was added 500 µl W1 buffer. After centrifugation, the RB column was washed twice with 600 µl of Wash Buffer and eluted using 50 µl of RNase-free Water. The total extracted RNA was quantified using a Nanodrop spectrophotometer (OD260/280) prior cDNA synthesis.

RT-PCR and DNA Sequencing

Rice cDNA synthesis was performed using iScript™cDNA Synthesis Kit (Bio-Rad Laboratories, USA) from 1 µg of total RNA. The reverse

transcription mixture consists of 5x iScript Reaction Mix, iScript Reverse Transcriptase, Nuclease-free water, and RNA template. After incubation of the reaction mix in thermal cycler, the cDNA was amplified by PCR. This study used two primer sets which involved in cellulose biosynthesis enzyme genes: *Oryza sativa Cellulose Synthase A4* (*OsCESA4)* and *Oryza sativa Cellulose Synthase A9* (*OsCESA9*). *OsCESA4* primers consisted of forward primer (*OsCESA4*_F:5'- CGTGACCACCCTGGAATGAT-3') and reverse primer (*OsCESA4*_R:5'- GGCATTTGTCAGAACTGCGG-3') which located on LOC4324625. *OsCESA9* primers were designed base on LOC4347093 sequence (*OsCESA9*_F:5'-GCGGAAGGGTGGATCATGAA-3' and *OsCESA9*_R:5'-CTTCTTGTGGTGCTGGAAGC-3'). PCR products size of two genes were 180 base-pairs.

PCR reaction was prepared by follow the commercial enzymes kit (Geneaid Biotech Ltd., Taiwan), that compose of 1x Ultra-pure *Taq* PCR master mix (1 U of Ultra-pure *Taq* polymerase, 2 mM $MgCl_2$ and 200 µM of each dNTPs), 0.8 µM of each primer, and 1 µl of cDNA template. The PCR profile was set up in the thermocycler (Biometra® T-gradient, Germany) with the initial denaturation at 94 °C (5 min), followed by 30 cycles of denaturation at 94 °C (30 s), annealing at 55 °C (30 s), and extension at 72 °C (1 min). After final extension at 72 ℃ (7 min), the PCR reaction were paused at 20 °C. The PCR products were determined using Ethidium bromide staining and 1.5% agarose gel electrophoresis. The correct DNA band size was excised under UV-light by UV-transilluminator and purified using the GenepHlow™ Gel/PCR Kit (Geneaid Biotech Ltd., Taiwan). Finally, the purified PCR products were sent to DNA sequencing service which analyzed in ABI Prism 3730XL DNA sequencer (U2Bio, Korea).

Data analysis

DNA sequencing results from both *OsCESA4* and *OsCESA9* genes were analyzed BioEdit program (Hall, 1999) and compared to reference sequences in GenBank database at website: http://www.ncbi.nlm.nih.gov/ using BLASTn tool for sequence alignment and the identity determination. Next, all sequences were deposited to GenBank database by using online BankIt program and established the accession number. Then, multiple sequence alignments were run on ClustalW tool in BioEdit program by using reference rice sequences from GenBank database. Reference sequences for *OsCESA4* genes comparison were *Oryza sativa* (Japonica) cellulose synthase A catalytic subunit 4 [UDP-forming]-like (LOC4324625), mRNA (accession no. XM_015765756.2) and *Oryza sativa* (Indica) clone OSS-97-192-F2 cellulose synthase cesa4, mRNA

(accession no. EF576384). For *OsCESA9* reference sequences, they were *Oryza sativa* (Japonica) cellulose synthase A catalytic subunit 9 [UDP-forming]-like (LOC4347093), mRNA (accession no. XM_015756793) and *Oryza sativa* (Indica) *CesA9* gene for cellulose synthase catalytic subunit, complete cds (accession no. AB527075).

Dendrogram was constructed using MEGA-X program (Kumar *et al.*, 2018). *OsCESA4* sequences from seven native upland rice varieties were compared with five reference *OsCESA4* DNA sequences from GenBank NCBI database: XM_015765756.2(*Oryza sativa* Japonica), EF576384.1(*Oryza sativa* Indica), XM_015842378.2 (*Oryza brachyantha*; grass rice), XM_037550740.1 (*Triticum dicoccoides*; emmer), and XM_025960233.1 (*Panicum hallii*; Hall's panicgrass).

For *OsCESA9* gene, MEGA-X program was utilized for dendrogram construction of seven sequences comparing with two *Oryza sativa* rice sequences (XM_015756793 and AB527075) and five outgroup sequences: XM_006660572.3 (*Oryza brachyantha*; grass in rice), XM_037585602.1 (*Triticum dicoccoides*; emmer), AK_254564 (*Hordeum vulgare*; barley), XM_025948581 (*Panicum hallii*; Hall's panicgrass) and XM_039939320 (*Panicum virgatum*; switchgrass).

Results

OsCESA genes RT-PCR amplification

Cellulose biosynthesis genes, *OsCESA4* and *OsCESA9* were amplified by RT-PCR using RNA templates which extracted from seven seedling upland rice. The RT-PCR result was determined using agarose gel electrophoresis and GelDoc UV-transilluminator (Figure 1 and 2). Both PCR product sizes were 180 base pairs in-length.

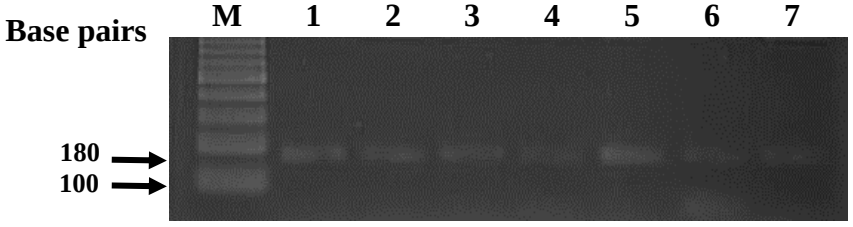

Figure 1. PCR products of *OsCESA4* gene run on agarose gel electrophoresis Lane M = 100 bp DNA marker, Lane number 1-7 = PCR products from 7 native rice varieties (180 bps)

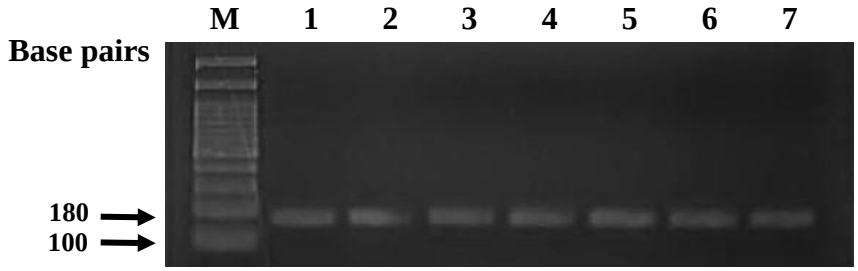

Figure 2. PCR products of *OsCESA9* gene run on agarose gel electrophoresis Lane M = 100 bp DNA marker, Lane number 1-7 = PCR products from 7 native rice varieties (180 bps)

OsCESA4 and OsCESA9 sequence analysis

All DNA sequences from DNA sequencing were trimmed in BioEdit program and aligned by online BLASTn tool at GenBank database (http://www.ncbi.nlm.nih.gov). For *OsCESA4* gene, the seven 133 bp DNA sequences showed the similarities 98.7-99.5% to *Oryza sativa* Japonica cellulose synthase A catalytic subunit 4 [UDP-forming]-like (LOC4324625) (XM_015765756) and 97.5-99.5% *Oryza sativa* Indica cellulose synthase cesa4 mRNA (EF576384). While all seven 131 bp *OsCESA9* sequences revealed the same identity to both *Oryza sativa* Japonica cellulose synthase A catalytic subunit 9 [UDP-forming]-like (XM_015756793) (98.46%) and *Oryza sativa* Indica *CesA9* gene for cellulose synthase catalytic subunit (AB527075) (98.46%).

All *OsCESA4* and *OsCESA9* DNA sequences in this study were deposited to GenBank NCBI database via BankIt online submission platform. The accession number for all sequences were assigned in MZ835674 to MZ835680 for *OsCESA4* and MZ835667 to MZ835673 for *OsCESA9*. Next, comparison of all seven DNA sequences *OsCESA4* and *OsCESA9* with two rice reference sequences and other outgroups were performed using ClustalW analysis tool on BioEdit program. The multiple alignment results were illustrated in Figure 3 and 4. There were two nucleotide positions of *OsCESA4* from native upland rice varieties which differ from *O.sativa* Japonica and Indica. MZ835679 had nucleotide base T at position 7 as same as in wheat *CESA4* sequence (XM_037550740.1). Interestingly, nucleotide at position 10 which found that nucleotide base from six native rice varieties had similarity to *CESA4* sequences from grass in rice, wheat, and hall grass (T->C) (Figure 3).

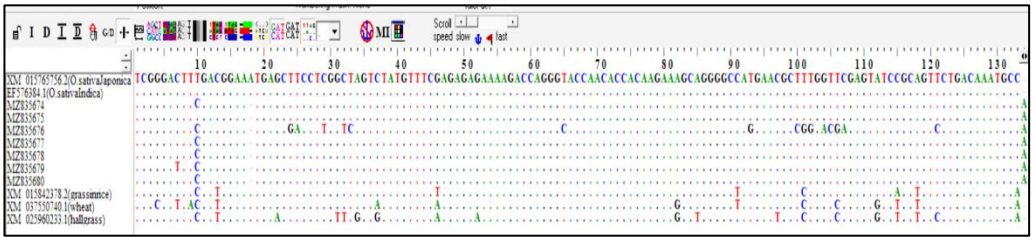

Figure 3. Multiple Alignment of *OsCESA4* sequences

Seven *OsCESA4* DNA sequences (MZ835674 to MZ835680) and five *OsCESA4* reference sequences from GenBank database were aligned using ClustalW on BioEdit program. The nucleotide identity base on XM_015765756.2 sequence was shown in dots (.).

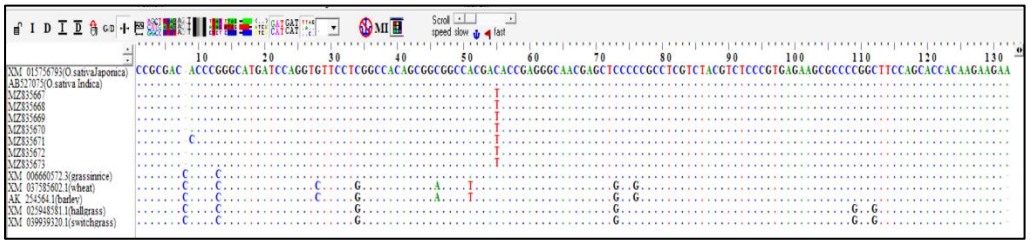

Figure 4. Multiple Alignment of *OsCESA9* sequences

Seven *OsCESA9* DNA sequences (MZ835667 to MZ835673) and seven *OsCESA4* reference sequences from GenBank database were aligned using ClustalW on BioEdit program. The nucleotide identity base on XM_015756793 sequence was shown in dots (.).

The *OsCESA9* DNA sequences, position 8 of MZ835671 had nucleotide base C, whereas other six rice varieties showed the same nucleotide base A as two reference rice (Figure 4). At position 54, all seven native upland rice varieties in this study revealed nucleotide base T which differ to two reference rice varieties (C) and outgroup sequences.

Maximum Likelihood method and Kimura 2-parameter model (Kimura, 1980; Kumar *et al*., 2018) were used to analysis of *OsCESA4* and *OsCESA9* sequences and dendrogram construction on MEGA-X program (Figure 5 and 6). For *OsCESA4*, accession number MZ835674 to MZ835680 were all seven rice varieties *OsCESA4* DNA sequence that grouped in the same clade with two reference rice sequences (XM_015765756.2 and EF576384.1). As with the result of grouping by *OsCESA9* gene in phylogenetic tree, MZ835667 to MZ835673 sequences were shared the same branch with two *Oryza sativa* rice sequences (XM_015756793 and AB527075) and separate to the five outgroup sequences.

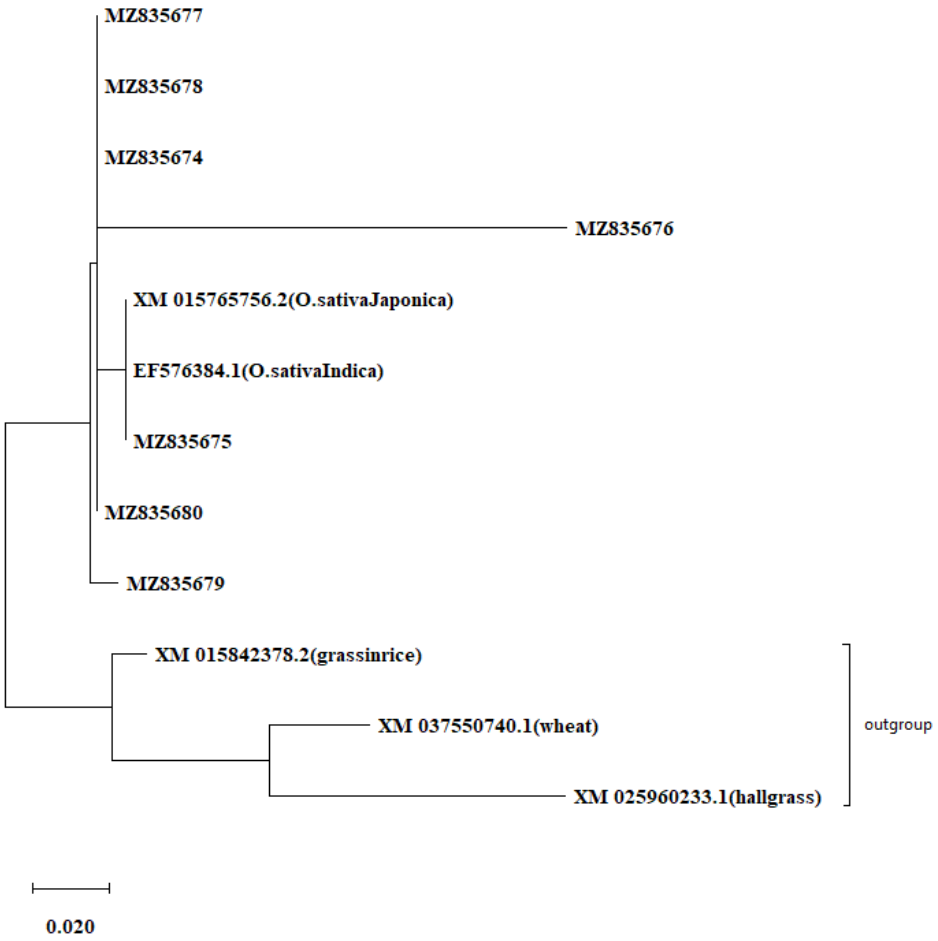

Figure 5. Dendrogram of *OsCESA4* sequences (MZ835674 to MZ835680), The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. MZ835674 to MZ835680 were all seven rice varieties *OsCESA4* DNA sequence. Two reference rice sequences were XM_015765756.2(*Oryza sativa* Japonica), EF576384.1(*Oryza sativa* Indica) and three outgroup sequences: XM_015842378.2 (*Oryza brachyantha*; grass in rice), XM_037550740.1 (*Triticum dicoccoides*; emmer), and XM_025960233.1 (*Panicum hallii*; Hall's panicgrass)

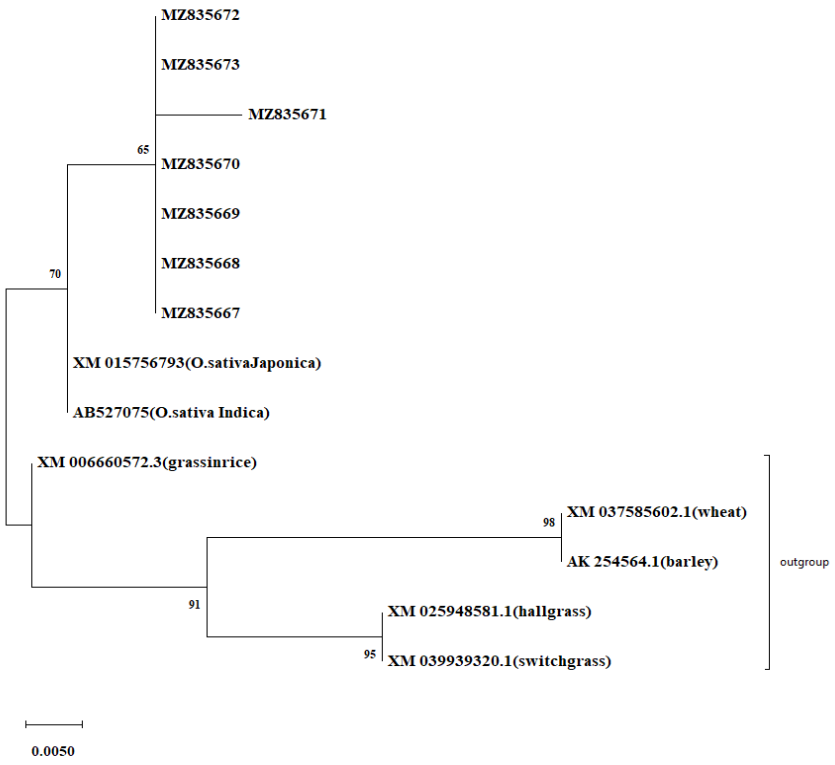

Figure 6. Dendrogram of *OsCESA9* sequences (MZ835667 to MZ835673), The evolutionary history was inferred by using the Maximum Likelihood method and Kimura 2-parameter model. MZ835667 to MZ835673 were all seven rice varieties *OsCESA9* DNA sequence. Two *Oryza sativa* rice sequences were XM_015756793 and AB527075) and five outgroup sequences: XM_006660572.3 (*Oryza brachyantha*; grass in rice), XM_037585602.1 (*Triticum dicoccoides*; emmer), AK_254564 (*Hordeum vulgare*; barley), XM_025948581 (*Panicum hallii*; Hall's panicgrass), and XM_039939320 (*Panicum virgatum*; switchgrass)

Discussion

Several studies have been investigated the genetic diversity and character of landrace upland rice in various countries such as Malaysia *(Tuhina-Khatun et al.*, 2015), Malaysia (Ahmad *et al.*, 2015), Indonesia (Kadidaa *et al.*, 2017), Brazil (Coelho *et al.*, 2017), Africa (Ndjiondjop *et al.*, 2018), China (Chen, X. *et al.*, 2018) and North east India (Vanlalsanga, 2019). In Thailand, twenty-two indigenous upland rice genotypes were characterized the high phenotypic and genetic coefficients of variation that can identified into three groups (Chuchert *et al.*, 2019). Recently, rice landrace in peninsular

Thailand was determined the putative-stress related genes using InDel markers analysis (Whankaew *et al.,* 2020). Even if previously information of native upland rice at Pala-U village, Prachuap Khiri Khun province has been reported about two lignin biosynthesis genes (Laosutthipong *et al.,* 2019), more genetic characters of these varieties should be identified. Therefore, this study was performed to investigate cellulose biosynthesis gene that involve in lodging resistance in rice. Two partial *OsCESA* genes were selected to DNA sequencing analysis (*OsCESA4* and *OsCESA9*).

Cellulose biosynthesis process in higher plant utilizes the cellulose synthase enzyme complexes which are expressed from *Cellulose synthase* (*CesA*) and *Cellulose synthase-like* (*Csl*) genes (Brown *et al.,* 1996). Nowadays, researchers known that *OsCESA1, OsCESA3* and *OsCESA8* genes are expressed in rice primary cell wall (Wang, L. et al., 2010) whereas *OsCESA4*, *OsCESA7* and *OsCESA9* are mainly found in secondary cell wall (Wu *et al.,* 2017). Moreover, rice mutation in *OsCESA4* and *OsCESA9* genes revealed the effect on plant lodging resistance and biomass enzymatic saccharification *(Li et al., 2018; Ye et al.,* 2021). In this study, seven native upland rice varieties were identified partial of *OsCESA4* and *OsCESA9* genes by DNA sequencing. After alignment with reference sequences on NCBI database, 133 bp of *OsCESA4* sequence showed the similarity to both *Oryza sativa* Japonica (XM_015765756) and *Oryza sativa* Indica (EF576384). Nucleotide base changing was found two positions in *OsCESA4* gene from some native upland rice varieties which differ from *O.sativa* Japonica and Indica. Previously, rice (*Oryza sativa* Japonica cultivar *Nipponbare*) *OsCESA4* missense mutation in transmembrane domain affected protein abundance in plasma membrane and caused in cell wall biosynthesis abnormality (Zhang *et al.,* 2009). Cell wall remodeling to solving the lodging problem in FC17/CESA4 mutant rice (*Oryza sativa* Japonica cultivar *ShenNong265*) was constructed by conserved site substitution mutation (Li *et al.,* 2018). Recently, the C->T substitution in Bc19 mutant which obtained from the japonica rice cultivar *Nipponbare* by chemical mutagenesis and causing the P507S missense mutation in the *OsCesA4* protein (Xiaozhi *et al.,* 2021). When comparison of nucleotide substitution C->T in *OsCESA4* sequence in this work with Xiaozhi *et al.* (2021), it was not located in that key point mutation since our sequence was a partial nucleotide sequence.

For *OsCESA9* genes, all seven 131 bp DNA sequences in this study revealed the same identity (98.46%) to both *Oryza sativa* Japonica and *Oryza sativa* Indica. Interestingly, we found the nucleotide base T that differ to two

reference rice varieties (C) and outgroup sequences at position 54. Because of the one base pair substitution at ORF3, which changing GGC to GAC in *OsCESA9* gene, result in amino acid alteration (glycine to aspartic acid: at position 905aa) was found in S1-60 mutant rice (Wang, D. et al., 2012). Therefore, we interested to observe by using BLASTx analysis of our seven nucleotide sequences to protein database on NCBI. Result revealed 100%similarity to *Oryza sativa* Japonica and *Oryza sativa* Indica at position 489-529aa of OsCESA9 protein, which located at N-terminal of this protein sequence, while the S1-60 rice had mutation at C-terminal of OsCESA9 protein. The rice *Osfc16* mutant was identified as a single recessive gene, that encodes CESA9 protein with two amino acid substitutions at position 481(W->C) and 482(P->S) (Li *et al.*, 2017). This study, we designed partial *OsCESA9* gene which encodes amino acid at position 489-529 (41 amino acids), so we did not compare with *Osfc16* mutant at this moment.

In conclusion, this is the first report that attempts to investigate the cellulose biosynthesis gene in native upland rice varieties at Pala U village, Prachuap Khiri Khun province, Thailand. Our finding revealed that partial *OsCESA4* and *OsCESA9* DNA sequences from all seven rice varieties were identity to *Oryza sativa* Japonica and *Oryza sativa* Indica. However, characterization of more cellulose biosynthesis and other cell wall synthesis related genes in native upland rice will have chance for clearly understand lodging mechanism and variety improvement.

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