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EARLY ISOLATION OF CELL CYCLE-ASSOCIATED PROTEIN KINASE (*OSWEE*) GENE IN RICE (*ORYZA SATIVA* L.)

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ABSTRACT

The developments of grain are regulated by specific genes that control the size and filling of nutrients during the period of post-fertilization, those specific genes are often known as the cell cycle genes. This study aimed to isolate and characterize genes that are involved in regulation of cell cycle. Using yeast two-hybrid screening, we isolated a gene and identified as a *OsWee* which belongs to a member of cell cycle genes. This gene has an importance role in regulation of cell division during mitosis, based on the expression level analysis using RT-PCR, and highly expressed in kernels 5 days after pollination (5DAP). The sequence analysis showed, a full-length *OsWee* gene 1239 bp in size encodes for 413 amino acids. Database searches revealed that *OsWee* contains protein kinase C-like superfamily (PKc-domain), which is known to play a role in phosphorylation of the hydroxyl groups of serine and threonine amino acid to control the function of proteins. Detailed functional analyses of the *OsWee* may provide more information concerning the complex regulation of protein kinase in rice and for the molecular breeding leading to improve productivity of various agricultural crops.

INTODUCTION

The eukaryotic cell cycle involves duplication and segregation of genetic materials in the chromosomes, which occur in this process of inheritance from the mother cell into two daughter cells (Gonzales *et al*., 2004). The cycle composes four distinct phases: the G1 phase, is the pre-synthetic DNA phase in which the cells are unable to replicate DNA, and the DNA is in a state of diploid (2n). In the S phase or synthesis: DNA replication occurs in the nucleus (doubling the number of copies) 2 copies (2C) of the DNA and produce diploid (2n); the G2 phase: DNA replication has been completed, and the cells prepare for division; and the last is the M phase or mitosis: is the division stage of the cell nucleus (Joubes and Chevalier, 2000). The regulation of cycles is controlled by a family of conserved cyclin-dependent protein kinases (CDKs). The regularly activity of CDKs play a critical role in regulating the cell cycle and trigger other important mechanisms in the cells, wherein the regulation is maintained by sophisticated regulatory networks that involve transcriptional and post-translational (phosphorylation/ dephosphorylations) mechanisms (Sun *et al*., 1999).

The development of seeds in paddy and cereal crops generally comprises into several steps: 1) *shortly after pollination*, endosperm holds syncytium and the division occurs in 3 days after pollination (DAP). 2) *Cell proliferation*, it develops from 4 DAP to 20 DAP and the most effective is in 7 DAP. 3) *Endore duplication*, this step starts from 8 DAP to 15 DAP, and 4) *Cell death,* characterized by the cessation of grain enlargement followed by a hardening of seeds (Sabelli and Larkins, 2009). This development seed process is controlled by two groups of genes known as cell cycle genes, composed of Cyclin and Cyclin-dependent Kinases (CDKs). CDK is Serine/ Threonine protein kinase pivotal regulator in cell cycle of eukaryotic cells (Bisbis *et al*., 2006).

Previous study was revealed that the size of the fruit and seeds is a combination result of cell number and cell size which primarily determined by the process of cell division and expansion (Sun *et al*., 1999). It shown by the growth of the fruit which is mainly supported by the expansion and enlargement of cells, developing of pericarp and locular tissues accompanied by a decreasing in mitotic activity and inhibit the Cyclin-Dependent Kinases (CDKs) activity, and number of cycles endore-duplication which induces an increase in ploidy DNA and cell size (Gonzales *et al.,* 2007).

Wee is the gene encoding protein kinase located in the nucleus. This gene was first discovered in fungi cells encoded by the protein 96 kDa in size, and predicted as a key regulator in the cell cycle (Nurse and Thuriaux, 1980). This gene effects on the cell size through inhibition time point when entering the phase of mitosis. In eukaryotic cells, the inactivation of *Wee* occurs either by phosphorylation or degradation

(Watanabe *et al*., 2004). Wee1 inhibits *Cdc2* through phosphorylation in two ways, through Tyr15 and Thr14. *Cdc2* reported have important role in cyclin-dependent through the various stages of the cell cycle. At least three phases inhibition of *Cdc2* by Wee1; G2/M phase; where WEE1 is phosphorylate Tyr15 and Thr14 from *Cdc2* and maintain a low activity of *Cdc2* kinase, thereby inhibiting entry to mitosis phase. During mitosis activity decreased by some regulators and thus increases the activity of *Cdc2*. A decrease in the activity of Wee1, cyclin synthesis and activation of phosphorylated by cyclin (CAK) is required to enter mitosis phase. The extension of G2 phase depends on Wee1 (De Schutter *et al*., 2007).

Wee is reported to have an important role in the development of seeds in maize (Sun *et al*., 1999), Arabidopsis (Sorrell *et al*., 2002) and tomato (Gonzales *et al*., 2007). Mutant *wee1* was reported not able to prolong the G2 phase after gamma irradiation (Rowley *et al*., 1992). Deletion of Wee produces daughter cells smaller than normal cells, because cell division happening faster and it causes in premature cells (Russell and Nurse, 1987).

Previously, our study has characterized several genes involved in endosperm development using yeast two hybrid screening in cDNA library of rice using *OsCCS52A* (Suudi *et al*., 2012) which reported involved in mitotic cell division and endoreduplication cycles in rice endosperm. Database searching that one of the genes was identified as *Wee*. Considering *OsWee* might be significantly involved in determining the final size of the endosperm cereals as well as OsCCSs, we here describe the characterization of *OsWee* gene isolated from indica rice cv IR64.

MATERIALS AND METHODS

Yeast Two-hybrid Screening: Yeast two-hybrid analysis was performed using GAL4-based two hybrid system (Invitrogen, CA, USA). Screening was conducted by cloning the full-length of *OsCCS52A* into the GAL4 DNA binding domain (BD) of pDEST32 vector to obtain the *pBD-OsCCS52A* construct. The construct was then transformed into pJ69-4A yeast strain, which contains *His3* and *LacZ* reporter gene by Lithium acetate method (James *et al*., 1996). To screen the interaction partner of OsCCS52A protein, a rice cDNA library which contains full-length cDNA of approximately 1200 genes (constructed in pAD-GAL4) was transformed into yeast bait containing *BD-OsCCS52A* fusion. Yeast cells bearing both AD and BD plasmids were spread on synthetic defined (SD) medium lacking histidine, tryptophan, leucine, or adenine, and incubated at 30°C for 4 days. Transformants grown on medium lacking histidine or adenine were then examined for β-galactosidase activity using X-gal as a substrate.

RNA Isolation from Various Tissues of Rice: Sample preparation for RNA: Indica rice cv. IR64 was germinated in the growth chamber and green house at Laboratory for Biosciences, State Polytechnic of Jember. The tissues were then periodically harvest and separated in different parts of tissues, e.g., seeding 3 days after germination (DAG), seedling 7 DAG, 5 Days After Pollination (DAP) kernels, 10 DAP kernels, roots, tillers and flowers. Harvested tissues were then first frozen quickly in liquid nitrogen before stored at -80°C. RNA Isolation: RNA was isolated from several parts of the rice plant organs, as mentioned above, using two methods: phenol/LiCl extraction and precipitation with LiCl (Sambrook, 1989), and using Plant mini kit for preparation of mRNA synthesis (Qiagen).

cDNA Synthesis: cDNA was synthesized from 2 μg of total RNA of each tissue with 1 μl oligo (dT) primer were then heated at 65°C for 10 minutes, then immediately put on ice. Reverse Transcriptase was performed using cDNA synthesis (Roche, USA) at 50°C for 50 minutes according to the producer's protocol. Aliquots 1 μl of synthesized of cDNA was used as templates for PCR. *Isolation of cDNA fragment specific*: To determine the transcript expression of *OsWee*, PCR amplification were performed using the following a pair of primer: 5'-ATGGCACTTGGAATTAGTTGTGGTC-3' as a Forward primer, and 5'-TTATCGTGGCAAACCA-ACTGAGG-3' as a Reverse primer. The rice *actin* was used as a loading control, primers used as described previously (Suudi *et al*., 2012). PCR was performed using *pfu polymerase* (Solgent), with 37 cycles at 95°C for 15 seconds, 57°C for 15 seconds, and 72°C for 1 minute. PCR products were subsequently viewed by loading on 1% agarose gel and cloned into pGEMT-vector.

Sequence Analysis: The purified recombinant DNA of *OsWee* was analyzed using Sanger dideoxy sequencing technology (The 1st BASE, Malaysia) to obtained the nucleotides sequence. The nucleotides and deduced of amino acid sequences of *Wee* were analyzed using NCBI Blast database (www.ncbi.nlm.nih.gov/blast). ClustalW-XXL(http:

//www.ch.embnet.org/software/ClustalW-XXL.html) and KOME database (http://cdna01.dna.affrc.go.jp/cDNA/) used as programs for the alignment of sequence.

RESULTS AND DISCUSSIONS

Isolation of Rice *OsWee*: To understand genes that are involved in the regulation of cell-cycle, we conducted yeast two-hybrid screening in cDNA library of rice using *OsCCS52A* as bait. An *OsCCS-52A* was reported involved in mitotic cell division and endoreduplication cycles in rice endosperm (Suudi *et al.*, 2012). A positive clone strongly interact with the bait were then isolated. The size of DNA clone was 1239 bp in length (Figure 1). Sequence and database searching revealed information that the clone-isolated was high similarity to the gene located in the locus no. LOC_Os02g04240 of rice chromosome. Using BLAST analysis, the resulting clone was highly identified as *Wee-kinase*. The information concerning of this gene in rice is still unreported. Furthermore, we designed the primers based on this sequence to amplify this *Wee* gene using mRNA of indica rice Cv. IR64 as a template.

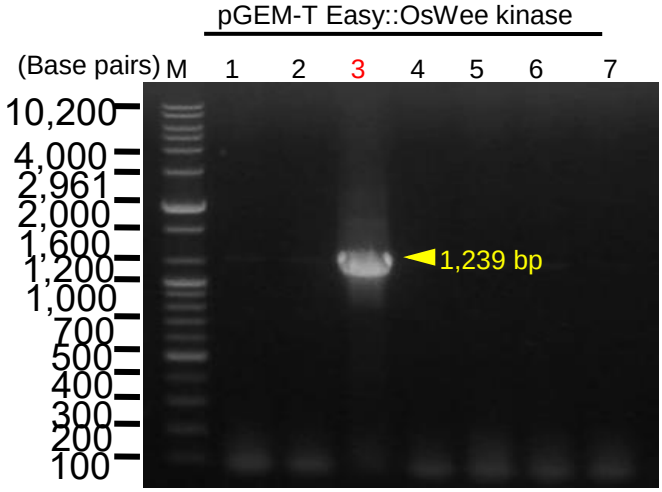

Figure-1: The *OsWee* was amplified from 5DAP of kernels rice using specific primers of *Wee*. The PCR product (1239 bp) was then cloned into a pGEMT-Easy vector. Aliquot 15 µl PCR products were subjected for electrophoresis on a 1% agarose gel. The high expression of *OsWee* shown in Lane 3. M: DNA Size Marker; Lane 1 – 7: *OsWee::pGEM-TEasy* clones.

The full-length of *OsWee* was composed of 1239 bp and it encodes 413 amino acids (Figure 2). We found that this gene comprises a conserved domain of protein kinase and that it indicates conformity to *Wee1* from Arabidopsis (Sorrel *et al*., 2002), tomato (Gonzales *et al.*, 2004), and maize (Sun *et al*., 1999) (*data not shown*). The domain of *OsWee* kinase protein that contained in between residues 85 to 270 is highly conserved and showed the characteristics of Wee-kinases sequence including an ATP binding site and protein kinase C-like superfamily (PKc-domain), which is known to play a role in phosphorylation of the hydroxyl groups of serine and threonine amino acid to control the function of proteins (Rudrabhatla *et al*., 2006). A putative Ser/Thr protein kinase active site (HRDVKSSN) was also found in the sequence.

Furthermore, identification of *OsWee* protein sequence was also revealed that the sequence contains a catalytically active enzyme site and substrate binding site (Figure 2) that in maize this domain sites are function to inhibit CDK (Sun *et al*., 1999). CDKs were implicated in the control of endoreduplication cycle. The Wee1 inhibitory kinase mediates inactivation of the CDK activity by phosphorylation and it regulations believed contribute to the endoreduplication process in endosperm, as well as in the endoreduplicating tissues of developing tomato fruit (Gonzales *et al*., 2004). The activity of *Wee1* can retard mitosis and causes cell elongation in yeast (Harvey *et al*., 2003). Based on these analysis, we conclude that this *OsWee* gene is a *Wee* homologue.

The comparison amino acid sequence analysis of *OsWee* using KOME database revealed that *OsWee* derivated from indica rice cv. IR64 shares highly identity with that of *Wee* from japonica rice cv. Nipponbare obtained from the database. We found there were four amino acids apparently different from the both of *Wee* sequences (Figure 3). Differences in amino acid sequence is possible

because both *Wee* protein isolated from different cultivars, but this difference did not affect the specific domain of both *Wee* proteins.

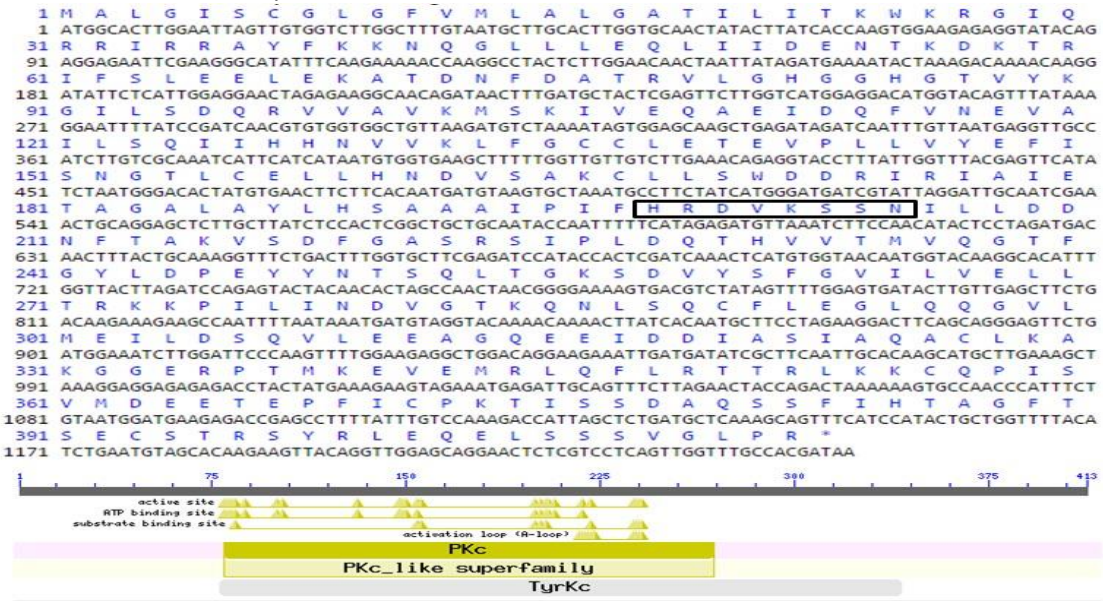

Figure- 2: Nucleotides and amino acid sequences of *OsWee*. A full-length *OsWee* gene was 1239 bp in size and encodes 413 amino acids. OsWee protein contains protein kinase C-like superfamily (PKc-domain). The Ser/Thr protein kinase active site is indicated in a box.

Figure-3: The alignment of amino acid sequence of *OsWee* with the homolog rice protein from a public database NCBI accession no. AK288287. The Nucleotide sequence of the *OsWee* was deposited in GenBank under accession no. KX758541 (BankIt1946132).

Expression Pattern of *OsWee:* To obtain further clues about the function of *OsWee*, we analyzed the expression pattern of *OsWee* in various tissues. As shown in Figure 4, the expression of *OsWee* gene found in seedlings 3 and 7 days after germination (DAG), 5 and 10 days after pollination (DAP) of kernel, root, flower and tiller. The relatively high expression of *OsWee* was detected in kernel 5 DAP and seedlings 3 DAG. However, the transcripts of *OsWee* in flower and root were almost undetectable. In tomato, *LeWee1*, the accumulation of RNA transcripts was related to mitotically active organs, e.g. the developing tissues of fruits, seeds and roots (Gonzales *et al.*, 2004). Sun *et al*., (1999) reported that corn Wee (*ZmWee1*) transcripts were found accumulated in endosperm tissue between 9 and 17 DAP, and highly expressed in seedlings and flowers in *AtWee1* (De Schutter *et al*., 2007).

A *Wee* gene was reported involved in regulation of the early stage of the tissues development (Gonzales *et al*., 2007). Therefore, we took a temporal analysis of this gene expression during vegetative and generative phases of development. The *OsWee* gene was expressed in every stage of development from the seedling to the mature plant with different of the levels expressions. The relatively high expression of *OsWee* was detected

in kernels 5 DAP and seedlings 3 DAG. The high expression of *OsWee* in the kernels 5 DAP suggests that *OsWee* might involve during endosperm development. A similar finding has already been demonstrated by Gonzales *et al*., (2004) in tomato. Overexpression of Arabidopsis *WEE1* affects morphogenesis and increase cell size as well as cell number in the root apical meristem (Spadafora *et al*., 2012). Taken together, analysis using the expression profiling data from Rice Genome Annotation Project (http://rice.Plant-biology.msu.edu/) shown that the expression levels of *OsWee* was higher in generative organs such as ovary and embryo compared to that the other organs.

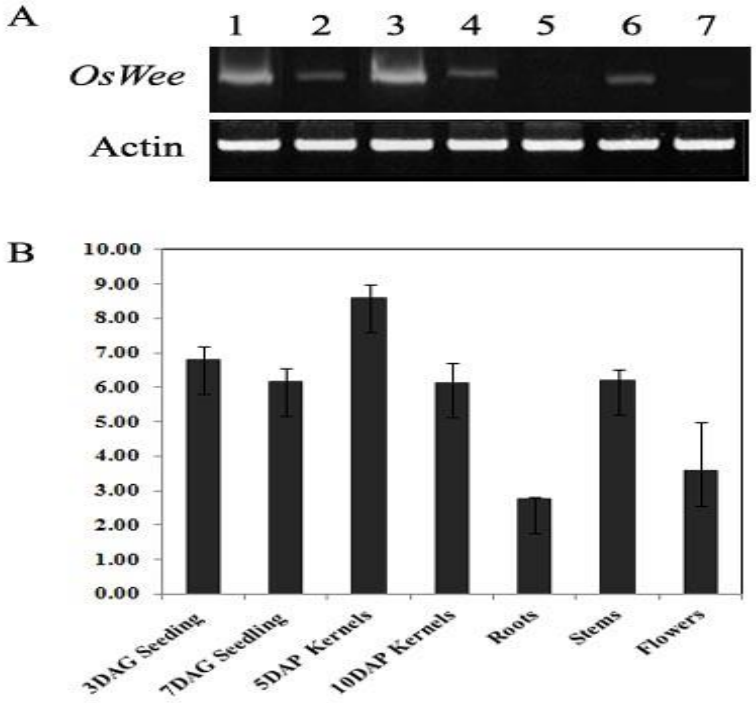

Figure-4: (A) Quantitative cDNA analysis of *OsWee* expression in 3DAG seedling (1), 7DAG seedling (2), 5DAP kernel (3), 10DAP kernel (4), root (5), tiller (6), and flower (7). Aliquot 20 µl of PCR products were subjected for electrophoresis on a 1% agarose gel. (B) Relative expression of *OsWee* in different rice tissues were determined by quantitative RT-PCR analysis. Bars show SD values of three separate experiments.

Previously reported, that during the development period of endosperm, nuclei initiates endoreduplication, which involves DNA synthesis phase with no intervening mitotic phase (Sun *et al.*, 1999), suggested that endoreduplication may require a high rate of *Wee* transcriptional. The *OsWee* gene expression analysis during rice kernels development thus resembles that accuring in maize endosperm (Sun *et al*., 1999) and tomato endosperm (Gonzales *et al*., 2004) during the period of endosperm developments and endoreduplication. *WEE1* in Arabidopsis reported to be an important target of the DNA replication and DNA damage checkpoints. Under optimal growth conditions *WEE1*-deficient plants grow normally, however plants are become hypersensitive to DNA-damaging agents (De Schutter *et al*., 2007), defects the response of DNA replication that triggers premature differentiation and symptomatic phenotype in cell death at the root meristem (Cools *et al*., 2011). Whether the differential pattern of *OsWee* gene expression associated with the endoreduplication process would be correlated with accumulation of *Wee* protein, or *Wee*- kinase activity, it remains to be observed. Further work will be conducting to examine the function of *OsWee* in the control of cell size during rice seed development by developing of transgenic rice overexpressing of *OsWee*.

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