Cloning, transformation and expression of cell cycle-associated protein kinase OsWee1 in indica rice (Oryza sativa L.)

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Abstract

The development process of seed in plants is a cycle of cells which occur gradually and regularly. One of the genes involved in controlling this stage is the Wee1 gene. Wee1 encode protein kinase which plays an important role in phosphorylation, inactivation of cyclin-dependent kinase 1 (CDK1)-cyclin (Cyc) and inhibiting cell division at mitotic phase. The overexpression of Wee1 leads to delaying entry into mitotic phase, resulting in enlargement of cell size due to suppression of cell division. Accordingly, the cloning and overexpression of Wee1 in rice plant is important aim of this research in achieving better quantity and quality of future rice. The main objective of this present study is to cloning and generate transgenic rice plants overexpressing of Wee1 gene. Wee1 was isolated from cDNA of indica rice (Oryza sativa), called OsWee1. The full length of OsWee1 was 1239 bp in size and successfully inserted into plant expression vector pRH1010N. Seven-day-old rice seedlings were prepared for transformation of OsWee1 gene using Agrobacterium-mediated transformation method. Four positive transgenic lines were identified through the presence of kanamycin resistance gene (nptII) using genomic PCR analysis. Southern blot analysis result provides evidence that four independent rice transformants contained one to three rearranged transgene copies. Further screening in transgenic rice generation is needed in order to obtain stable expression of OsWee1.

1. Introduction

One of the parameters to increase the yield of rice is the seed sizes, which regulated by the seed development processes after pollination. The process is a part of cell cycle which occur gradually and regularly during its life cycle. The cycle is divided into four phases, the mitotic (M) phase which includes mitosis and cytokinesis, G1 (first gap) phase, S (synthesis) phase, and G2 (second gap) phase. The cell develops larger in the G1 phase, then in S phase, cell replicates the chromosome inside the cell. Furthermore, the cell will continue its growth in G2 phase, and divided. The daughter cells can repeat the cycle [1]. The cell cycle progression is controlled at distinct checkpoints which major checkpoints are synthesis phase (G1-S checkpoint), mitosis (G2-M checkpoint) and the spindle checkpoint [2].

In eukaryotes, the cell cycle is controlled by family of conserved cyclin-dependent protein kinases (CDKs). The activity of CDK fluctuates regularly during the cell cycle, triggering important processes such as DNA replication, cell growth, and cell division [3]. Phosphorylation and dephosphorylation of the CDK catalytic subunit, threonine 14 and tyrosine 15, are able to regulate and determine the timing of G2 and mitosis [4]. Previous study reported that phosphorylation of CDKs at tyrosine 15 in Schizosaccharomyces pombe is mediated by Wee1, which causes a delay in mitosis by phosphorylating the M-phase promoting factor on tyrosine 15 [5].

Wee1 is a gene encoding protein kinase located in the nucleus. The expression of this gene in plants is strongly induced by DNA damage which can be caused by radiation, ionization, chemicals and other stresses [6,7]. When DNA is damaged, ataxiatelangiectasia mutated (ATM) or ATM- and Rad3-related (ATR) kinases will be expressed depending on the genotoxic type of stress. Furthermore, the ATM and ATR signals will phosphorylate and activate Chkl and further phosphorylates the Wee1. Activation of Chkl caused cell cycle delay in G2-M phase by increasing Wee1 regulation and decreasing regulation of phosphatase (Cdc25) which
controls tyrosine-15 phosphorylation inhibitors on cyclin-dependent kinase (Cdc25) resulting in G2 phase arrest [8-10].

In fission yeast (S. pombe), loss of Weel1 activity causes insufficient growth cells early enter to mitosis phase and cytokinesis, therefore it causes cells to produce two abnormally small daughter cells [5,11]. However, increasing expression of Weel1 causes delayed entry into mitosis and increase in cell size, this indicates that the activity levels of Weel1 plays a role in ensuring the entry time of the mitotic phase and having strong effect on cell size [12]. Sun et al. [13] reported that expression of ZmWee1 was observed in endosperm tissue at 15 day after pollination, where this gene shows its role in endoreduplication in the endosperm and suppose to be a potential regulator of seed development. The similar result was reported in Weel1 tomato [14] and AtWee1 from Arabidopsis [15], that the expression levels of Weel1 gene was found higher in the generative organs such as seed, fruit and flower compared to that the vegetative organs. In previous study, the expression of rice OsWee1 was almost found in all the tissues; roots, stem, tiller, flowers, leaves and seeds. The highly expressed of rice OsWee1 was found in 5 day after pollination of the seeds [16]. These results revealed that besides having an important role in seed developments, Weel1 has also influence in the growth and developments of plants.

Considering the important role of Weel1 in the development of seed, cloning and transformation of OsWee1 was conducted in order to have understanding of the superior potential of OsWee1 overexpressing in rice. In this study, we present results of OsWee1 cloning and overexpression of this gene in rice.

2. Materials and methods

2.1. Plant materials

The mature seeds of indica rice (cv. Melongga) were used in this research. Dehulled seeds were sterilized with 70% ethanol for 2 min followed by 5.25% sodium hypochlorite for 10 min and then washing with sterile distilled water for 3–5 times. The sterilized seeds were placed on MS basal salt media (Table 1) pH 5.8, supplemented with 3% (w/v) sucrose, 100 mg/L L-glutamine, 0.25% phytagel, and cultured under continuous light at ±22 °C within a period of 7 days.

2.2. Plasmid construct

Cloning of OsWee1 consists of 2 steps, first step was cloning OsWee1 into pGEMT easy vector (Promega), and the second was cloned into plant expression vector pRI101ON vector (TaKaRa) (Fig. 1A). The amplification fragment of OsWee1 was conducted from DNA recombinant pGEMT; OsWee1 which obtained from previous study [16] and deposited in Genebank under Accession no. KX758541. PCR analysis were performed using the following a set of primer contain Ndel and BamHI sites (Table 2) overhang to ensure compatibility with pRI101ON vector. The fragment of OsWee1 was amplified as follows PCR Core kit (Roche) manufacturer’s procedure, initial denaturation at 94 °C for 2 min, each with 25 cycles of denaturation at 94 °C for 15 sec, annealing at 57 °C for 20 sec, extension at 72 °C for 2 min, and a final extension at 72 °C for 7 min. Fragment obtained was then purified using GeneAll ExpiMax™ Combo GP and quantified using nanodrop (NanoVue Plus spectrophotometer, BioLab). The DNA fragment of OsWee1 was ligated into pRI101ON and the recombinant pRI101ON:OsWee1 was then transformed into E. coli XL10 gold competent cells through heat shock method [15].

2.3. Flanking analysis of OsWee1

A recombinant of pRI101ON: OsWee1 was amplified and confirm the correct size by digestion using HindIII, EcoRI, Ndel and BamHI restriction enzymes (NEB Inc.). The flanking frame of OsWee1 in pRI101ON was checked and analyzed using Sanger dideoxy sequencing technology (The 1st BASE, Malaysia). The sequence was then analyzed using BLAST (www.ncbi.nlm.nih.gov/blast).

2.4. Transformation into Agrobacterium

DNA recombinant of pRI101ON: OsWee1 was transferred into Agrobacterium cells by heat shock method [17]. Aliquot of 100 μL freshly prepared competent cells and 1 μL of DNA recombinant were mixed, kept on ice for 5 min and chilled into liquid nitrogen. Heat shock was immediately conducted by heated at 42 °C in waterbath for 90 sec. Added 1 ml of YEP medium (10 g/L yeast extract, 10 g/L peptone, 5 g/L NaCl) and gently shaken at 28 °C for 1 h to allow bacteria and harboring DNA replication. Cultured bacteria was then collected by centrifuge at 5000 rpm for 3 min, collected pellet was dissolved and spread on YEP agar medium supplement with 50 mg/L kanamycin, 100 mg/L rifampicin and 12.5 mg/L gentamicin. The bacteria cells were incubated at 28 °C for 2 d and the colony grown was identified using PCR with several specific primers (Table 2).

2.5. Agrobacterium-mediated transformation and putative rice transfronts selection

The Agrobacterium harboring the binary construct pRI101ON: OsWee1 was inoculated in YEP medium supplemented with 100 mg/L rifampicin, 12.5 mg/L gentamicin and 50 mg/L kanamycin, then incubate at 28 °C by gently shake at 110 rpm for 48 h. The growth of Agrobacterium was checked for its optical density by spectrophotometer (OD600nm = 0.3). Seven-day-old rice seedlings were soaked in the Agrobacterium suspension for 20 min. To reduce the growth of excessive bacteria, the infected seedlings were dried using sterilized filter papers for 5 min. The infected seedlings were grown into co-cultivation medium (MS basal salt (Table 1), 3% sucrose, 100 mg/L acetylsyringone, 0.3% phytagel; pH 5.2) and incubated for 2 d in the dark condition. The co-cultivated rice seedlings were thoroughly washed with 500 mL of cefotaxime followed by sterilized water for three times. Furthermore, the seedlings were cultured on selection medium (MS basal salt, 3% sucrose, 0.25% phytagel, 50 mg/mL kanamycin and 250 mg/L cefotaxime; pH 5.8) under a 16/8-h (day/night) light cycle at 22 °C and periodically sub-cultured every 4 weeks into fresh media. The screening

Table 1

| Component       | Concentration (mg/L) |
|-----------------|----------------------|
| NH₄NO₃          | 1650.0               |
| KNO₃            | 1900.0               |
| MgSO₄·7H₂O      | 370.0                |
| MnSO₄·4H₂O      | 22.3                 |
| ZnSO₄           | 10.6                 |
| Ca(NO₃)₂·4H₂O   | 0.025                |
| CaCl₂·2H₂O      | 440.0                |
| KI              | 0.83                 |
| CuSO₄·5H₂O      | 0.025                |
| KH₂PO₄          | 170.0                |
| H₃BO₃           | 6.2                  |
| Na₂MoO₄·2H₂O    | 0.25                 |
| FeSO₄·7H₂O      | 27.35                |
| Na₂EDTA·2H₂O    | 37.25                |
| Nicotinic acid  | 0.5                  |
| Pyridoxine HCl  | 0.5                  |
| Thiamine HCl    | 1.0                  |
| Glycine         | 2.0                  |
putative transformants were conducted by screening of rice explants on the selection media containing 50 mg/L kanamycin.

2.6. Molecular analysis of putative transformed plants by PCR and Southern blotting

Genomic DNA was isolated from 4 g of leaves of wildtype and putative transformants rice [18] with minor modification. To confirm the presence of transgene, the putative transformants and non-transformant rice samples were analyzed by PCR analysis using nptII-F and nptII-R primers (Table 2). About 50–100 ng of total genomic DNA from independent putative transformatn lines and non-transformant were mixed with 50 μl of reaction mix (KAPA Taq Extra HotStart ReadyMix) and subjected for PCR analysis under pre-denaturation condition at 95 °C for 3 min, followed by 30 cycles at 95 °C for 30 sec, 58 °C for 30 sec, 72 °C for 1 min and a final extension at 72 °C for 5 min. The results were visualized by loading on 1% (w/v) agarose gel electrophoresis.

Southern blot analysis was performed by method previously described [19]. Twenty μg of the genomic DNA was digested with the restriction enzymes BamHI (Promega) at 37 °C overnight. The digested DNA was separated on a 1% agarose gel and shifted to an Amersham Hybond N+ membrane (GE Healthcare, UK) using capillary transfer. The membrane was hybridized with a DIG-labeled DNA probe (Roche, Germany) and incubated overnight at 42 °C with gentle agitation. The DNA probe was prepared by amplification of pRI0101-ON_OsWeeI by PCR using FNdel and RBamHI primers and the PCR product was then labelled with DIG. The processes of probe preparation and washing of the membrane to remove the unbound probe were performed according to the manufacturer's instructions (Roche). Hybridization was visualized by exposing the membrane to X-ray (Fuji Film).

2.7. RNA isolation and reverse transcriptase PCR (RT-PCR)

The total RNA was isolated from 100 mg of young leaves of putative transformant lines and non-transformant 30-day-old rice plants using RNAprep pure plant kit (Tiangen, Beijing). The first-strand cDNA was prepared from 1 μg of total RNA using iScript™ cDNA Synthesis Kit (BIO-RAD) according to the manufacturer's instructions. The synthesized cDNA was then used as template to check the expression of OsWeeI using OsWsee-F and OsWsee-R primers (Table 2). The RT-PCR conditions were 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 sec, 56 °C for 30 sec, 72 °C for 1 min and a final extension at 72 °C for 5 min. The primer pairs of OsActin (Table 2) was used to amplify the OsActin reference gene, at 95 °C for 3 min, followed by 25 cycles at 95 °C for 30 sec, 48 °C for 30 sec, 72 °C for 1 min and a final extension at 72 °C for 5 min. The results were visualized by loading on 1% (w/v) agarose gel electrophoresis.

3. Results and discussion

3.1. Cloning of OsWeeI

The cloning of full length OsWeeI gene (1239 bp) in the expression vector (pRI0101-ON) was performed on the FNdel and BamHI restriction sites. Clone of OsWeeI that obtained in previous study [14] was amplified using a pair of FNdel and RBamHI primers (Table 2) in order to add those restriction sites on the open reading frame (ORF) of OsWeeI before cloning. The construct of OsWeeI was cloned into E. coli strain XL10 gold competent cells and selected using antibiotic kanamycin on the growth media. To verify the positive clones, 20 recombinant colonies were randomly selected and prepared for the colony PCR analysis using FNdel and RBamHI primers (Table 2). As shown in Fig. 1B, the presence of 1251 bp of OsWeeI gene was found in 12 clones which were confirmed by colony PCR and visualized on agarose gel. For further analysis, clone no. 7 was selected.

In order to evaluate the correct size and flanking of the OsWeeI clones, PCR analysis, restriction enzymes digestion and sequencing were performed. The PCR reaction was conducted using 4 pairs of primers, CaMV-F/RV-R, FNdel/RBamHI, nptII-F/nptII-R, CaMV-F/OsWsee-R (Table 2). The PCR result showed that expected bands appear at the appropriate sizes, 1868 bp, 1251 bp, 524 bp and 1504 bp, respectively (Fig. 2 A). Digestion was performed by single or combination of HindIII, BamHI and EcoRI restriction enzymes. Open reading frame of OsWeeI contains BamHI and EcoRI enzymes,
while HindIII was present two sites in the construct. Then, this clone was further conformed by sequencing (data not shown). The correct band sizes of OsWee1 after digestion was shown in Fig. 2B. The nucleotide sequence analysis showed a full length region of OsWee1 about 1239 bp and a 100% homolog with cDNA of OsWee1 in GeneBank (KX758541). It suggested that the desired recombinant pBI1010::OsWee1 was successfully prepared to be expressed in Agrobacterium.

3.2. Transformation of OsWee1 into Agrobacterium

The recombinant construct of pBI1010::OsWee1 was transformed into Agrobacterium strain GV3101 and selected on YEP solid medium supplemented with 50 mg/L kanamycin, 100 mg/L rifampicin and 12.5 mg/L gentamicin antibiotic (Fig. 3A). Twelve colonies were randomly selected for PCR analysis using F1 and R1 primers and 4 positive clones harboring OsWee1 were obtained by PCR (Fig. 3B). This result clearly confirm that recombinant pBI1010::OsWee1 was transformed into Agrobacterium, and it can be used for transformation in rice.

The successful Agrobacterium-mediated transformation in rice has been achieved using various methods. However, in monocot plants remains limited because it is not a natural host for Agrobacterium [29]. Many researchers have developed methods for Agrobacterium-mediated transformation in monocotyledons, especially in utilizing of different Agrobacterium strains [21,22], piercing method and vacuum infiltration [23], choosing of different cultivars [24] and type of explants [25,26]. The basic protocol of Agrobacterium-mediated transformation [27] was develop for induction of callus derived from scutella seeds. However, since most of indica rice genotypes have less regeneration potential [28], we conducted Agrobacterium-mediated transformation in rice using 7-day-old rice sprouts as explants.

A hundred explants were infected by Agrobacterium suspension for 20 min and co-cultivation in dark condition for 2 d followed by cefotaxime-antibiotic treatment. Transformation was carried out twice with 200 explants. However the percentage of rice transformation efficiency is very low (2%) (Table 3). Previous study reported [29] numerous factors that can improve transformation efficiency, was lighting condition temperature, co-cultivation periods and Agrobacterium-density during co-cultivation step. The most critical factor reported was the period of co-cultivation. The successful integration of target gene into the plant genome occurs mainly during co-cultivation. Sahoo and Tuteja [29] found that suitable method for rice transformation was infection for 20 min followed by co-cultivation for 2 d. An extension of co-cultivation period, caused arising of excessive bacterial growth which results in inhibiting explants growth, reduce the number of shoots generation and finally causing death of explant. Rashid et al. [30] reported that to anticipate those problems, reducing the density of bacteria into 0.1 – 0.2 OD prevents overgrowth bacteria during co-cultivation.

The co-cultivated explants were transferred in the selection medium, to inhibit the formation of non-transformant explants and eliminate the residual of Agrobacterium. The first shoots produced from the explants were subjected to three successive propagation cycles with the same level concentration of antibiotic. This method is applied to select putative trans transformants and reduce false positive transformants or eliminate the chimeric of transgenic explants [31]. After three sub-cultures, the non-transformant plantlet were turned into white (chlorosis), while the putative transformants were able to survive and grow normally (Fig. 4A). The lacking of nptII gene in the non-transformic plants caused inhibiting of chlorophyll development and induce chlorosis at the shoot of rice plantlet. In contrast to the transgenic overexpressing OsWee1 showed normal green at the shoots. The absence of nptII gene will suppress the growth of non-transformant roots caused by inability of root to inactivated the kanamycin in the media (Fig. 4A). Similar results were also obtained in previous study [32] that increased concentration of kanamycin may lead to inhibition of root growth. Kanamycin inhibits the synthesis of protein in plastid and mitochondria. Kanamycin acts as an inhibitor, active destroy the function of ribosome following by inhibition of translational initiation. Another way, the response was automatically inhibit protein synthesis and effects on reduction the growth and development of plants.

3.3. Expression of OsWee1 in rice

The putative transgenic plants which survived in selection in vitro medium were then transferred to soil and placed in the greenhouse under agronomic conditions (Fig. 4B). These plants were then analyzed for their transgenic status by PCR analysis. PCR analysis showed that the nptII gene 550 bp in size was found in 4 of putative transgenic lines (Fig. 4C). To determine the expression of OsWee1 at the RNA levels, we performed a RT-PCR analysis. The quantity of RNA level is a reflection of the level of transcription. As shown in Fig. 5A, the transcript levels of rice overexpress-
Table 3
The percentage of rice transformation efficiency.

| Transformation number | Number of infected seedling | Number of selected plant | Number of transformant† | Transformation efficiency (%) |
|-----------------------|-----------------------------|--------------------------|------------------------|-------------------------------|
| 1                     | 100                         | 3                        | 3                      | 3%                            |
| 2                     | 100                         | 2                        | 1                      | 1%                            |
| Total                 | 200                         | 5                        | 4                      | 2%                            |

† Confirmation by PCR analysis.

Fig. 4. Transgenic rice overexpressing OsWeel gene. A. Phenotypic selection of transgenic rice overexpressing OsWeel, rice plantlet (upper) and root (lower) of wildtype (wt) and transgenic (lane 1-5); B. Transgenic plants after acclimation in green house; C. PCR analysis of the T0 transgenic plants (lane 1-5), wildtype (WT), pRR101ON (OsWeel (P)) as positive control, and DNA marker (M).

ing OsWeel were higher compared to the wildtype. The data indicates that OsWeel driven by CaMV35S was expressed in rice.

Southern blot analysis of transgenic rice was performed to prove integration of the transgene into the plant genome and to determine copy number of the T-DNA. Southern blot analysis was conducted using a gDNA isolated from leaves of four transgenic rice lines. The Southern blot analysis showed that the transgenic rice displayed one to three hybridized DNA copy with a difference in molecular size suggesting the independent transformation events. The hybridized DNA was not found in the genome of the WT plant (Fig. 5B). These results confirmed that the copy of the OsWeel gene was integrated into the genome of the transgenic rice.

The transgenic plants required stability of expression to be used in seed production [33], as well as OsWeel overexpression plants. In the present study, four among five independent transformation events have been shown to carry multiple copies of the T-DNA. Multiple copies of the transgene are prone for transgene inactivation, silencing, and likely to cause a high frequency of insertional mutagenesis [34]. To obtained stability of expression, the selection will be carried out through anther culture in the second generation...
of transgenic rice. Regeneration of haploid plants from another culture followed by chromosome doubling can produce double haploid or pure line of plants. This result will provide an opportunity to accelerate the time for the formation of inbred line which is normally through several inbreeding cycles [35].

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