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CHARACTERIZATION OF PUTATIVE *RKD* HOMOLOGOUS GENE WITH MICROSPORE EMBRYOGENESIS IN THE LOCAL PIGMENTED RICE CULTIVAR 'SEGRENG'

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SUMMARY

The local pigmented rice cultivar 'Segreng' from Yogyakarta, Indonesia, has the potential to be a staple food, as well as medicine, because it contains a red pigment and high amounts of β-carotene. Compared with conventional methods, microspore embryogenesis is a shorter way for producing double haploid plants, which are important in plant breeding. The study of the mechanism related to this type of embryogenesis is required to increase its efficiency. *RKD* genes containing the RWPXRK motif (RWP–RK) domain protein play an important role in the initiation of embryogenesis at the very early stage in zygotic and somatic embryogenesis. Exploring the involvement of the *RKD* gene in microspore embryogenesis induction will be very helpful. This study, we isolated the putative *RKD* homologous gene of the local pigmented rice cv. 'Segreng' and analyzed its expression at the early induction stage of microspore embryogenesis. The partial sequence of the *RKD* homologue was isolated from this cultivar and found to consist of 563 bp encoding 75 amino acid sequences. Bioinformatics analysis by using BLASTP search showed that the putative RKD protein had high similarity with the RKD1 protein of *Oryza sativa* subsp. *japonica* cv. Nipponbare and the hypothetical protein of *O. sativa* subsp. *indica* that contained the RWP–RK motif. Protein analysis showed the presence of conserved amino acid sequences in N-terminal region of *Oryza sativa* RKD proteins. The fold-change in the relative expression level in *RKD* compared with that in the control was elevated from the first until the third day and decreased on the fourth day.

Keywords: *RKD* gene; relative expression; rice cv. 'Segreng'; microspore embryogenesis; *in vitro* stress treatment, doubled haploid

Key findings: The putative *RKD* gene was found to be expressed during the early stage of microspore embryogenesis in the local pigmented rice cultivar 'Segreng'. This *OsRKD* gene encoded a partial protein that was similar to the other proteins containing the RWP–RK conserved domain in *O. sativa* subsp. *japonica* and *indica*. This result provided evidence regarding the putative function of *RKD* genes in embryogenesis.

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INTRODUCTION

Rice (*Oryza sativa* L.) is the staple food for more than half of the world's population, and almost 90% of rice is grown in Asia (Miah *et al.,* 2013). Many rice cultivars were developed to produce plants with superior traits in terms of quality and quantity. One of these cultivars is pigmented rice, which possesses high contents of anthocyanins, flavonoids, and phenolic compounds. Indonesia has many local pigmented rice cultivars that can be act as genetic resources for developing new varieties (Kurniasih *et al.*, 2019). The local Indonesian red-pigmented rice cultivar 'Segreng' from Gunung Kidul, Yogyakarta, contains higher β-carotene levels than other local rice cultivars with red pigments (Kristamtini and Purwaningsih, 2009). This rice is an upland rice type that is tolerant to drought conditions and has shorter flowering time than other local pigmented rice cultivars (Supriyanti *et al.,* 2015). Therefore, this cultivar has great potential to be developed as an elite rice cultivar.

In plant breeding programs, pure lines, also called as double haploid plants, have genes in homozygote alleles and are very important. Conventional breeding methods that are commonly used to produce double haploids require relatively longer time than *in vitro* techniques via anther or microspore embryogenesis (Yan *et al.,* 2017; Germanà *et al.,* 2011). In this type of embryogenesis, stress treatments can reprogram the developmental pathway of the haploid microspore or young pollen from gametophytic toward embryogenesis (Bélanger *et al.,* 2018). This single-cell system has emerged as a model system for understanding the *in vitro* physiological and biochemical processes that occur during plant developmental processes and provides a faster way to produce important homozygous plants for breeding (Seifert *et al.,* 2016).

Embryogenesis involves the differentiation and development of an embryo and includes all the basic morphogenesis process, including cell division and expansion, cell–cell communication, and cell fate specification, thus allowing plant cells to develop a wide variety of organism architectures (Vikrant and Janardhanan, 2018; Wendrich and Weijers, 2013). The formation of the apical–basal axis during plant embryogenesis is one of processes of fundamental body pattern establishment and is followed by elongation and subsequently by cell specification and differentiation. The complex mechanism of zygotic and somatic embryogenesis has been well studied in plants, especially in the model plant *Arabidopsis thaliana* (Smertenko and Bozhkov, 2014). All the key regulators of zygotic embryogenesis are equally essential for somatic embryogenesis. Some of the key molecules that mediate embryonic patterning are transcription factors, hormones, and microRNAs (Mimura *et al.,* 2018). The possible cellular and molecular factors that control the transition process in microspore embryogenesis remain to be investigated. Studying cellular and molecular regulators in the reprogramming of microspores into embryos is an important factor to improve efficiency (Bárány *et al.,* 2010; Irikova *et al.,* 2016). The development of embryogenic microspores has been analyzed in the model species of microspore culture, including *Brassica napus* (rapeseed), *Hordeum vulgare* (barley), and *Triticum aestivum* (wheat) (Malik *et al.,* 2007; Seguí-Simarro and Nuez, 2008; Daghma *et al.,* 2014; Sánchez-Díaz *et al.,* 2013). Genes related

to stress hormones, cellular protection, starch metabolism, and proteolysis have been revealed to be involved in microspore embryogenesis induction.

The transcription factor of the RWPXRK motif (RWP–RK) gene family has been studied in many species (Chardin *et al.*, 2014). It is characterized by a conserved motif that is thought to be involved in DNA binding. On the basis of gene structures, RWP–RK proteins are classified into two groups, including the RWP–RK domain proteins (RKD) and the NODULE-INCEPTION-like protein (NLP) (Liu *et al.,* 2020). In contrast to RKD proteins, all NLP members contain a Phox and Bem1 motif in addition to RWP–RK. RKD proteins have been shown to function in gametophyte development, whereas NLP is involved in plant nitrate response. *RKD4* in *A. thaliana* affects the early polarization of the embryo (Waki *et al.,* 2011). It can trigger embryo-specific gene expression when it is overexpressed. The inability of *RKD4* genes to function in mutant *rkd4* causes the occurrence of defects in zygote elongation and suspensor formation. In maize, the RKD-type RWP–RK domain protein encoded by *Shai1* is an important factor of embryogenesis and endosperm development (Mimura *et al.,* 2018). The improper morphologies of embryos and the interruption of organogenesis occur because of the loss function of the *Shai1* gene in maize. Shimada *et al.* (2018) proved that the RWP–RK domain present in CitRKD1 plays an important role in the regulation of somatic embryogenesis in citrus nucellar tissues. Fifteen *RWP–RK* members have been identified in rice (*Oryza sativa* subsp. *japonica*) (Chardin *et al.,* 2014). However, the information on their relationships with microspore embryogenesis is limited. In this study, we isolated the putative *RKD* homologous gene and analyzed its expression during the early stage of microspore embryogenesis in the local pigmented rice cv. 'Segreng' from Yogyakarta, Indonesia.

MATERIALS AND METHODS

Plant materials and microspore embryogenesis induction

The local pigmented rice *O. sativa* cv. 'Segreng' was used as plant material in this research. Seeds that were collected from a local farmer were immersed in water for germination and sown in a plastic tray containing a mixture of soil and husk. Seedlings with four to five leaves were transplanted into bigger pots and grown under natural conditions. Periodic watering and fertilization were conducted to obtain plants with good growth. After 2–2.5 months or at the booting stage while spikes remained enclosed by sheath leaves, panicles were harvested to obtain microspores in the uninucleate stage. The incubation at 4 °C for 4-7 days was given to the panicle after the panicle harvesting. Microspore embryogenesis was induced by culturing anthers in B medium (Kyo and Harada, 1986) as starvation treatment and incubated at 33 °C for 4 days. Approximately 270 anthers from 45 spikelets were plated in 4 ml of B medium. The microspores were isolated from anther culture in accordance with Indrianto *et al.* (2014). The anthers were stirred in B medium by using a magnetic bar, filtered by using a 40 µm nylon filter, and then centrifuged at 1000 × *g* for 15 min.

Genomic DNA isolation and gene detection

Genomic DNA was extracted from the anthers of rice flowers by using 3% cetyltrimethyl ammonium bromide (CTAB) buffer in accordance with Murray and Thompson (1980) with modifications. Approximately 25 mg of anthers was ground by using a pestle with 500 µl of CTAB buffer added with 5 µl of β-mercaptoethanol in a 1.5 ml microtube.

The homogenate was incubated at 55 °C–65 °C for 30 min. Then, 500 µl of chloroform was added, and the homogenate was shaken at 120 rpm for 30 min. Shaking was succeeded by centrifugation at 12 000 rpm for 10 min. The supernatant was transferred into a new tube. Then, an equal volume of isopropanol (1:1, v/v) was added. This step was followed by inversion for mixing. The samples were incubated at room temperature for 10 min, then centrifuged at 12 000 rpm for 10 min. The supernatants were removed, and the DNA pellets were washed by using 70% ethanol and subsequently centrifuged at 10 000 rpm for 5 min. The DNA pellet was dried for 15 min and resuspended in 50 µl of TE buffer (pH 8.0) with the addition of 1 µl of RNase A (10 mg/ml).

The *RKD* homolog was amplified from the genomic DNA of the rice cv. 'Segreng' (mentioned as *OsRKD*) by using a forward primer (5'-CGCATTGTGTTGCCTAAAATCG-3') and a reverse primer (3'-GCGTCTTCTTTCATGTCGCC-5') designed by using Primer-BLAST on the basis of *OsRKD1* sequence (XM_015771629.2), which was homologous to the *RKD4* of *A. thaliana*. PCR was performed by using KOD -Plus- Neo (TOYOBO, Japan) in a 25 µl total volume. The PCR components were as follows: 2.5 µl of 10× buffer for KOD -Plus- Neo, 2.5 µl of 2 mM dNTPs, 1.5 µl of 25 mM $MgSO_4$, 0.5 µl of KOD -Plus- Neo, and 0.5 µl of 10 pmol/µl forward and reverse primers. A total of 100 ng of DNA template was used for amplification. The PCR conditions were as follows: predenaturation for 2 min at 94 °C followed by 35 cycles of 10 s at 98 °C for denaturation, 30 s at 59 °C for annealing, and 10 s at 68 °C for extension. BIO-RAD T100™ Thermal Cycler was used. The *Actin* gene was used as the internal control with the forward primer 5'-CCCTCTTTCATCGGTATGGA-3' and the reverse primer 5'-TTGATCTTCATGCTGCTTGG-3'. The amplified DNA (amplicon) was checked on 1% agarose gel with ethidium bromide staining and visualized under a UV transilluminator. The positive result was sent to 1st BASE Apical Scientific Sdn., Bhd, Malaysia, via PT. Genetic Science Indonesia.

Bioinformatics analysis

The result of DNA sequencing was analyzed to identify introns, exons, and conserved domain and to build a phylogenetic tree. BLAST tools in NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi) were used to analyze sequence similarity on the basis of the database. Similar proteins were aligned by using MultAlin (http://multalin.toulouse.inra.fr/multalin/multalin.html). A phylogenetic tree was constructed by using exons translated into proteins via the Maximum Parsimony method with 1000 bootstrap replicates on MEGAX (Kumar *et al.,* 2018), which was used for alignment with the proteins of several species, including *O. sativa* subsp. *japonica* and subsp. *indica*, *Oryza brachyantha, Sorghum bicolor, Zea mays, T. aestivum, B. distacyon*, and *A. thaliana* retrieved from Plant TFDB (http://plntfdb.bio.uni-potsdam.de/v3.0/) or UniProt (https://www.uniprot.org/).

Total RNA extraction

Total RNA was extracted as previously described by Breitler *et al.* (2016) with major modifications. The pooled microspore from different time points of the stressed-anther culture was homogenized by vortexing with beads in 500 µl of RNAzol® RT (Sigma-Aldrich, R4533) and supplemented with 5 µl of β-mercaptoethanol. The homogenate then centrifuged at 13 000 × *g* for 15 min. The supernatant was transferred into a new tube and added with 200 µl of water to precipitate DNA, proteins, and polysaccharides by centrifugation at 13 000 × *g* for 15 min. This step was repeated twice. RNA was precipitated from the resulting supernatant through the addition of an equal volume of cold isopropanol in a new tube and incubated for 30 min at −21 °C after mixing by inversion. The centrifugation was

performed at 16 000 × *g* for 15 min to pellet RNA. The supernatant was discarded, and the pellet was washed two times with 1 ml of 75% ethanol and centrifuged at 6000 × *g* for 3 min. Finally, the pellet was dried out partially and dissolved in 30 µl of water. The purity and concentration of extracted RNA were assessed with a NanoDrop apparatus.

Gene expression analysis by reverse transcriptase-PCR

cDNA was prepared with 1 µg of total RNA by using ReverTra Ace™ qPCR RT master mix with gDNA remover kit (TOYOBO, Japan) in accordance with the manufacturer's instructions. PCR was performed with a total volume of 25 µl to detect gene expression by using KOD FX Neo (TOYOBO, Japan). A total of 100 ng of cDNA was mixed with 12.5 of µl of 2× PCR buffer for KOD FX Neo, 5 µl of 2mM dNTPs, 0.5 µl of KOD FX Neo (1.0 U/µl), and 0.5 µl of 10pmol/ µl primer (for each forward and reverse primer) and amplified under the following conditions: 2 min at 94 °C of pre-denaturation followed by 40 cycles of 10 s at 98 °C for denaturation, 30 s at 59 °C for annealing, and 10 s at 68 °C for extension. The PCR products were analyzed via electrophoresis in 2.0% (v/v) agarose gels. For the expression analysis of the *OsRKD* gene, real-time quantitative PCR (CFX96 Real Time PCR System- Bio-Rad) in a total volume of 10 µl was performed by using SensiFAST™ SYBR® No-ROX kit (Meridian Bioscience) with 800 ng of cDNA as the template, and the result was analyzed via the Livak method (Livak and Schmittgen, 2001). Data were statistically analyzed by using IBM SPSS Statistic 23 by univariate ANOVA followed by Duncan analysis for significant difference at $P < 0.05$.

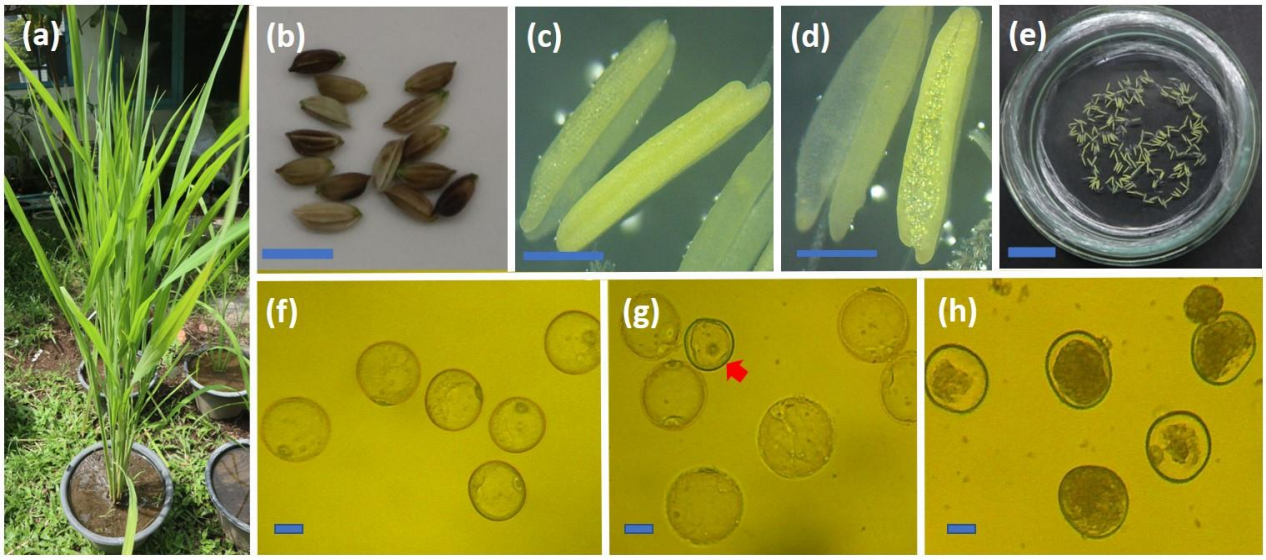

Figure 1. Morphology of the local pigmented rice cv. 'Segreng' from Yogyakarta. (a) Plant at the booting stage; (b) seeds; (c) fresh anther before culture induction; (d) anther dehiscence 4 days after culture induction; (e) plate containing anther culture; (f) isolated fresh microspore at the uninucleate stage before culture induction; (g) microspore with embryogenic potential after stress treatments (except for the non-embryogenic microspore with red arrow); (h) control culture after 4 days without stress treatment. Bars: 1 cm in (b)–(d), and 20 µm in (f)–(h).

RESULTS

Induction of microspore embryogenesis

Microspore embryogenesis in the local pigmented rice cv. 'Segreng' was induced by culturing anthers in starvation medium and incubation at 33 °C for 4 days. The shed microspores in the medium from the dehiscent anther showed characteristics that indicated that embryogenic potential was acquired (Figure 1g). The embryogenic microspores were bigger than non-embryogenic microspores. The other type showed the fragmentation of the cytosol that resulted in star-like structures. Isolated microspores from this anther culture were used for gene expression analysis during induction starting from the first until the fourth day.

Detection of the *OsRKD* gene in the rice cv. 'Segreng' and bioinformatics analysis

The prior step in *OsRKD* gene detection was performed by using the genomic DNA of the rice cv. 'Segreng'. The sequencing result from amplified DNA of this gene was 563 bp in length. This sequence consisted of three predicted regions of exons and two regions of introns after alignment with the sequence of the reference gene (mRNA transcript variant X2 of *O. sativa* subsp. *japonica* cv. Nipponbare, XM_015771629.2) (Figure 2.). A single-nucleotide difference at 551 bp was observed, wherein the nucleotide G in the rice cv. 'Segreng' corresponded to nucleotide A in *O. sativa* subsp. *japonica* cv. Nipponbare. However, the amino acid sequence showed no difference in amino acid product. The product of the amino acid was lysine (K), which was encoded by codon AAA or AAG at position 113 of the amino acid alignment (Figure 3).

The *OsRKD* sequence alignment to the protein database in NCBI showed that the *OsRKD* had high similarity with the RKD1 protein of *O. sativa* subsp. *japonica* (XP_015627113.1) and the hypothetical protein of *O. sativa* subsp. *indica* (EAY875121.1) (percent identity of 96.67%). Both of these proteins contained the RWP–RK domain. Other comparisons with the exon sequences as queries to the protein database revealed other similar proteins, including the RKD1 protein of *B. distachyon* (XP_014756209.2) and the predicted RKD1-like protein of *O. brachyantha* (XP_006649045.1). The result for the amino acid sequence from this alignment was then used to analyze the protein motif through comparison with similar related proteins (Figure 3). As shown in Figure 3, the other investigated proteins had the RWP–RK conserved domain. Meanwhile, the *OsRKD* of the rice cv. 'Segreng' was not been found because a small amplicon was produced. Comparison with the amino acid sequences in *O. sativa* (sequence a, b, and c in Figure 3) revealed a high similarity in the N-terminal region. A phylogenetic tree was built on the basis of the amino acid sequence of the RKD protein from other species to analyze the evolutionary relationship (Figure 4).

Expression pattern of the *OsRKD* gene during the early stage of microspore embryogenesis in the rice cv. 'Segreng'

Expression analysis was conducted on freshly isolated microspores (D0) from the rice cv. 'Segreng' and throughout the induction period of four days (D1–D4) to obtain insight into the role of the *OsRKD* gene. The result showed that *OsRKD* gene expression was detected from a 283 bp partial fragment of cDNA (Figure 5). Relative expression analysis with the control (D0) by real-time PCR showed an upregulated pattern in the treated sample after culture induction (Figure 6). The fold change increased until the third day of culture induction and then decreased on the last day of culture induction.

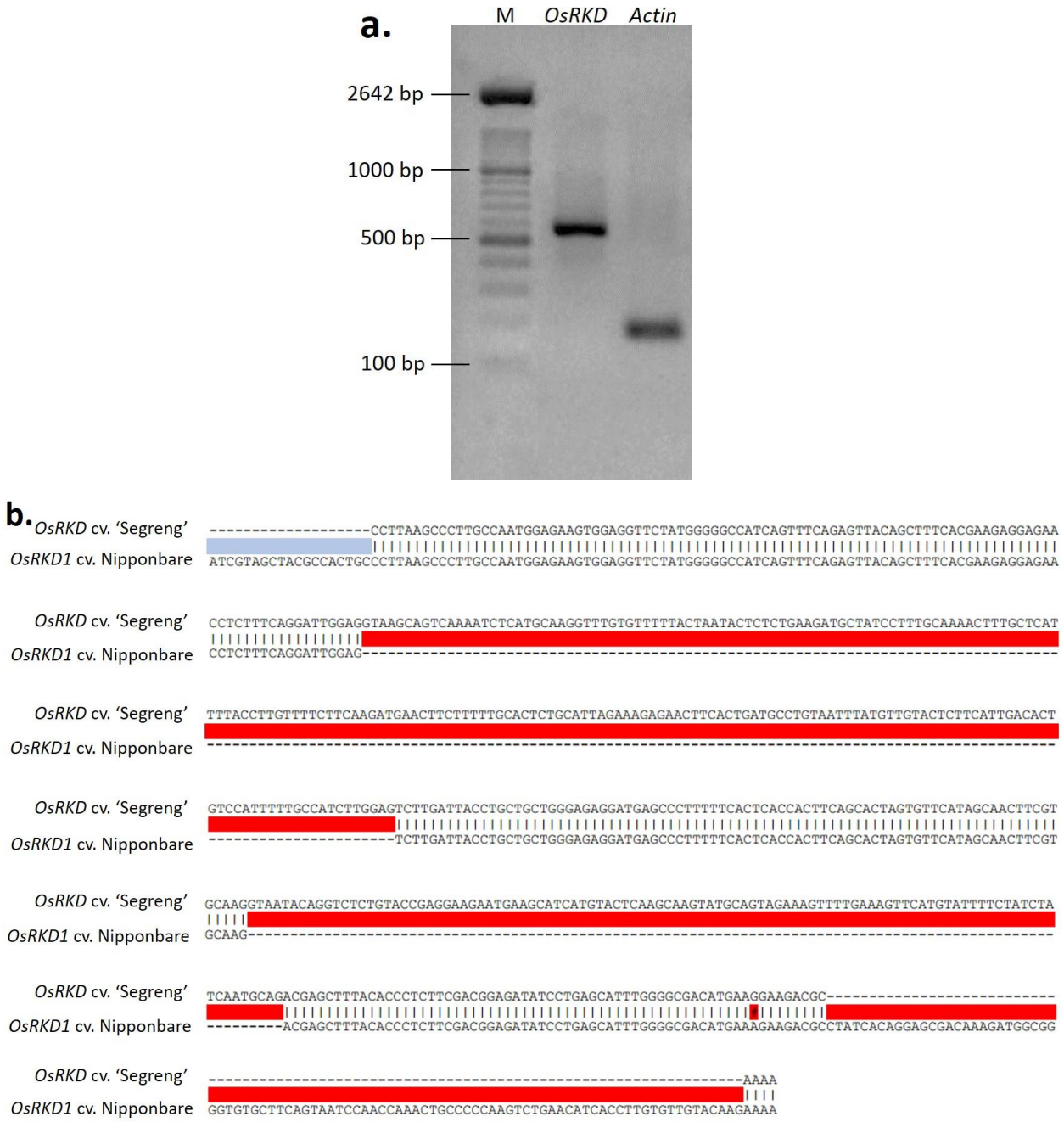

Figure 2. Detection of the *OsRKD* gene in the genome of the rice cv. 'Segreng'. a) 563 bp DNA fragment of *OsRKD* and 194 bp DNA fragment of *Actin* as the internal control; b) alignment of *OsRKD* sequence in cv. 'Segreng' to the reference gene of *OsRKD1* in *O. sativa* subsp. *japonica* cv. Nipponbare. Red boxes indicate introns.

Figure 3. Amino acid sequence alignment of the putative OsRKD protein in the rice cv. 'Segreng' with RKD1 from other species. (a) Putative sequence of OsRKD in the rice cv. 'Segreng'; (b) RKD1 of *O. sativa* subsp. *japonica* cv. Nipponbare; (c) hypothetical protein of *O. sativa* subsp. *indica*; (d) RKD1-like of *O. brachyantha*; (e) RKD1 of *B. distachyon*. Purple box shows the RWP–RK conserved domain.

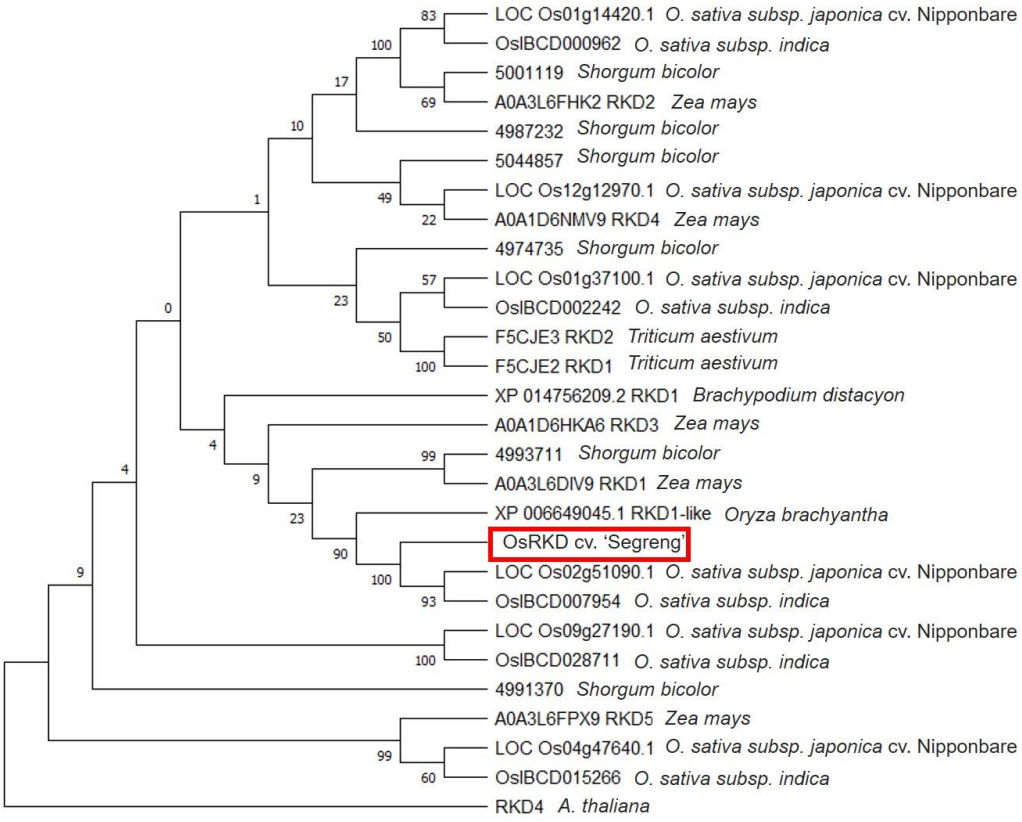

Figure 4. Phylogenetic tree of the OsRKD protein from the rice cv. 'Segreng' with RKD proteins from other species.

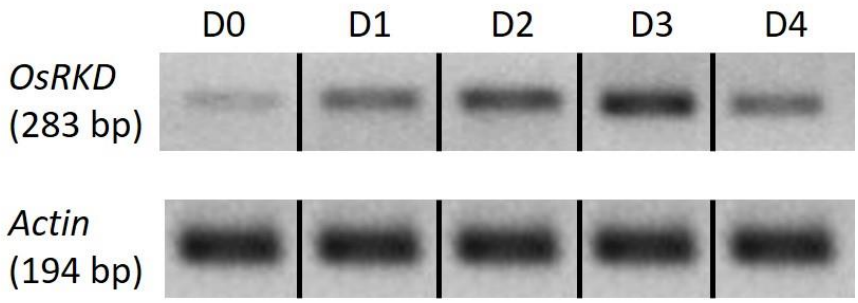

Figure 5. Expression of *OsRKD* in isolated microspore samples of the rice cv. 'Segreng'. D0, freshly isolated microspore before culture induction; D1–D4, microspore from stressed anther culture at 1–4 days in starvation medium and incubation at 33 °C.

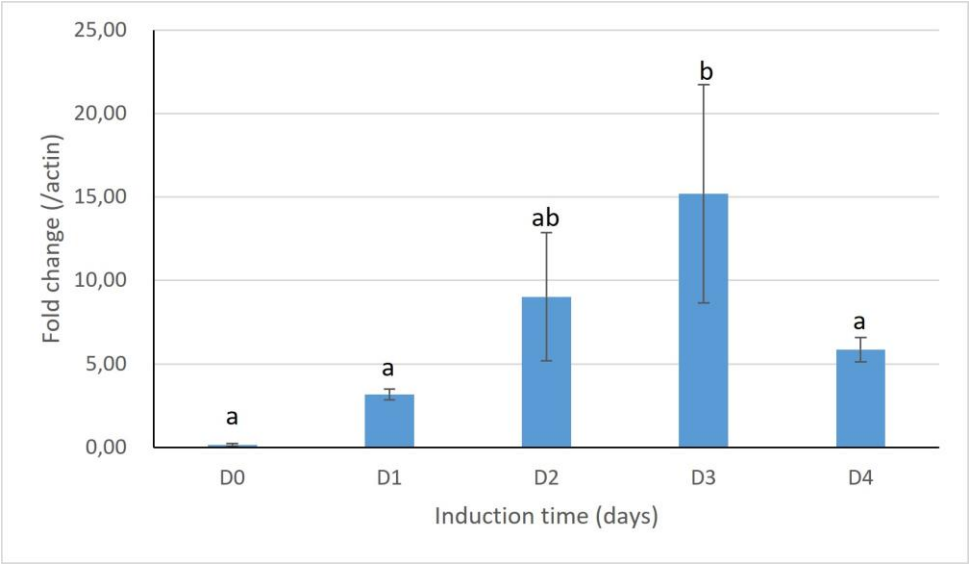

Figure 6. Relative genes expression of *OsRKD* during induction of microspore embryogenesis in rice cv. 'Segreng'. D0, fresh microspore before culture induction; D1-D4, microspore after induction 1-4 days, respectively. Data are reported as mean values ± SDM, n=4. Different letters (a and b) denote significant difference (P <0.05).

DISCUSSION

Stress treatments involving starvation in B medium and incubation at 33 °C for 4 days to induce microspore embryogenesis in the rice cv. 'Segreng' has been demonstrated (Nurbaiti *et al.,* 2019). This combined stress treatment also has been reported to provide the highest frequency of embryogenic microspores in other rice cultivars (Raina and Irfan, 1998). The characteristics of responsive microspores that have acquired embryogenic potential are different from those of nonresponsive ones. Indrianto *et al.* (2001) stated that embryogenic microspores increased in size and developed a star-like appearance with fragmentation in the cytoplasm. The initiation of cell division that produces a multicellular structure occurs after the acquisition of embryogenic potential and is then succeeded by pattern formation (Maraschin *et al.,* 2005). In our previous experiment (Nurbaiti *et al.,* 2019), multicellular structures were produced after the embryogenic microspore was transferred into medium for proembryo development (stress free-medium).

We identified the molecular mechanism in the early stage of

microspore embryogenesis on the basis of zygotic and somatic embryogenesis in which the interaction of key regulators has been identified. The putative RWP–RK transcription factor encoded by *RKD* genes is expressed in early embryogenesis to activate embryogenesis-related genes and to act in establishing embryo polarity cooperatively (Wendrich and Weijers, 2013; Waki *et al.,* 2011). Our results showed that the expression of the *OsRKD* gene was detected as early at the first day in the embryogenic microspore culture induction of the rice cv. 'Segreng' by starvation and incubation at 33 °C. The expression level of this gene increased and reached 15-fold on the third day compared with the control. These data supported the role of the involvement of *RKD* genes in embryogenic acquisition. Indrianto *et al.* (2001) reported that after *in vitro* stress treatment, embryogenic microspores were induced as indicated by the differences in cell characteristics that led to embryonic competence. The population of embryogenic microspores in the culture increased and developed further into multicellular structures. Similar to Febryanti *et al.* (2020) who analyzed the expression of *AtRKD4* gene in the transgenic Indonesian orchid *Dendrobium lineale* Rolfe found that *AtRKD4* can be detected at a very early stage, specifically, 1 day after induction with dexamethasone and thidiazuron.

Bioinformatics analysis revealed that the partial amplified cDNA of the rice cv. 'Segreng' used in this study encoded a deduced protein that was highly similar to the RKD1 protein of *O. sativa* subsp. *japonica* cv. Nipponbare with the RWP–RK conserved domain. Although a change in a single nucleotide of the mRNA sequence of the rice cv. 'Segreng' was found after alignment to the sequence reference, the produced amino acid was identical. Therefore, we assumed that the *OsRKD* gene also plays a role in microspore embryogenesis. However, the further investigation of this gene and other members of *RKD* gene via intensive functional analysis is needed to improve understanding and application.

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