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Expression of cytokinin responsive and ethylene biosynthesis genes in rice callus with different regeneration rates

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ABSTRACT This study aimed to investigate the correlation between callus regeneration rate and the expression of several genes responsible for cytokinin response and ethylene biosynthesis in the Ciherang, Mentik Wangi Susu, Hwayoung and Tarabas rice varieties. The callus regeneration rate of each rice variety was in vitro tested using N6 media, while the gene expression during the callus regeneration stages was examined using quantitative real-time PCR (qRT-PCR). Our results showed that the callus of Ciherang and Mentik Wangi Susu showed earlier green spot formation that then turned brown at a later stage, resulting in a low regeneration rate. While Hwayoung and Tarabas showed late green spot formation, high shoot regeneration was observed in both calluses. Gene expression analysis of regeneration media-grown calluses showed that two cytokinin-responsive genes, *OsRR2* and *OsRR6*, were highly expressed in the Ciherang and Hwayoung callus, respectively. We also observed that ethylene biosynthesis genes such as *OsACS1* and *OsACO1* were highly expressed in the Mentik Wangi Susu and Hwayoung callus, respectively. Moreover, the expression of *OsBBM1* was high in Hwayoung and Tarabas. Thus, the positive correlation between the expression of cytokinin-responsive and ethylene biosynthesis genes with somatic embryogenesis activity likely depends on the induction level of *OsBBM1*.

KEYWORDS rice callus; regeneration rates; ethylene biosynthesis; cytokinin response

1. Introduction

Rice is a staple food for more than 3.5 billion people that provide at least 20% of their daily calorie intake (Fraiture et al. 2016). As rice demand increases during the current global climate change, increasing rice productivity has become a formidable challenge. Hence, applying biotechnological tools becomes an effective approach to face such challenging problems. Tissue culture is a biotechnological tool that has been recognized as a powerful method for in vitro plant propagation (Pais 2019). Tissue culture can be used in various biotechnological applications such as genetic transformation, synthetic seed production, cryopreservation, and assembling plants with superior genotypic properties to increase yields and be more productive (Martínez et al. 2019).

The initial stage of somatic embryogenesis in rice tissue culture requires the addition of exogenous auxin to induce and proliferate callus cells to some extent of growth level (Juárez-González et al. 2019). In regeneration media containing high cytokinin: auxin ratio, the initial stage of callus differentiation is visibly marked by the formation of green spots that are gradually transformed into shoots (Vercruyssen et al. 2015). The success of shoot regeneration in rice tissue culture is tightly correlated with the genotype of the explant source (Pais 2019). Rice subspecies japonica generally has a high frequency of callus to shoot regeneration, whereas the regeneration frequency of indica subspecies tends to be low due to the occurrence of callus browning during the regeneration stage (Yang et al. 2020; Tran and Sanan-Mishra 2015; Han et al. 2021).

It has been identified that several genes belonging to the response regulators (RR) gene family are the key point for cytokinin signaling in plants. The type-B RRs are transcription factors for regulating cytokinin primary-response gene expression, whereas the type-A of RRs are negative regulators that desensitize the plant to cytokinin (Kieber and Schaller 2014). In rice, *OsRR21*, *OsRR22*, *OsRR23*, and *OsRR24* have been identified as the most abundant type-B RRs, whereas *OsRR1*, *OsRR2*, *OsRR3*, *OsRR5*,

and *OsRR6* are categorized into the type-A of RRs (Duan et al. 2019; Yamburenko et al. 2020). In rice cytokinin-insensitive gnt1 mutant that was deficient in tiller bud formation and failure in calli differentiation, the expression of *OsRR2* and *OsRR6* were found to be down-regulated (Fanata et al. 2013).

In many studies, ethylene is another type of plant hormone that determines the success of shoot regeneration in rice tissue culture. The positive role of ethylene was illustrated by a significant increase in shoot regeneration rate in indica variety FR13A callus by the addition of ethylene synthesis inhibitor aminoethoxy vinyl glycine (AVG) into regeneration media, whereas the addition of ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC) decreased shoot regeneration (Yasmin et al. 2013). Moreover, the negative role of ethylene in rice tissue culture has been illustrated in experimental results that the addition of putrescine as another type of ethylene biosynthesis inhibitor increases shoot regeneration in rice anther culture (Arisandi et al. 2020; Dewi and Purwoko 2008). The conversion of methionine initiates the biosynthesis of ethylene to S-adenosyl methionine (SAM/AdoMet), which is subsequently converted into ACC by ACC synthases (ACS). At the last step of biosynthesis, ACC is further converted to ethylene by ACC oxidase (ACO), a member of the oxygenase superfamily (Wen 2015).

BABY BOOM (BBM) is another essential transcription factor encoding genes that play a crucial role in somatic embryogenesis by regulating cell totipotency, proliferation, and regeneration ability (Jha and Kumar 2018). In *Arabidopsis thaliana*, BBM1 is responsible for the up-regulation of hundreds of morphogenesis-related genes that collaborate to produce somatic embryos, including *LEC, SERK*, and *YUCCA* (Khanday et al. 2019). The most recent experiment showed that the somatic embryogenesis was induced in rice callus abundant in *OsBBM1* transcripts, and its shoot regeneration was completed if cytokinin was supplemented into the regeneration media (Khanday et al. 2020).

We previously reported that the Tarabas rice variety showed high somatic embryogenesis capacity with an equal shoot regeneration rate with the japonica variety Hwayoung (Fanata and Qudsiyah 2020). However, the underlying molecular mechanism that supports a high level of somatic embryogenesis in those two rice varieties has not been described. In this study, we characterized the molecular response of Tarabas and Hwayoung callus as well as Ciherang and Mentik Wangi Susu as the low somatic embryogenesis counterpart by analyzing the expression of cytokinin responsive, ethylene biosynthesis, and somatic embryogenesis related genes. We found that the expression of cytokinin responsive genes *OsRR2* and *OsRR6* were high in Ciherang and Hwayoung callus, respectively, and the ethylene biosynthesis genes *OsACS1* and *OsACO1* were highly expressed in Mentik Wangi Susu and Hwayoung, respectively. Moreover, the high expression of genes related to the somatic embryogenesis *OsBBM1* in Hwayoung and Tarabas callus suggests the positive role of cytokinin responsive and ethylene biosynthesis genes in the rice somatic embryogenesis might depend on the high expression of*OsBBM1*.

2. Materials and Methods

2.1. Plant Materials and Culture Conditions

The seeds of Ciherang, Mentik Wangi Susu, Tarabas, and Hwayoung rice varieties were used in this study. Tissue culture for callus induction and shoot regeneration was performed according to the experiment of Fanata and Qudsiyah (2020). Seeds were sterilized and cultured for 14 days on solid callus induction media (CIM) pH 5.8 containing N6 basal salt with vitamin (Phytotech), supplemented with 3% sucrose, 0.1% casein hydrolysate, 0.05% proline, 0.05% glutamine, 0.4% gellan gum, 2 mg/L 2,4-Dichlorophenoxyacetic acid for two weeks in an incubator under the dark condition at 28°C. The growing callus was transferred onto solid shoot induction media (SIM) pH 5.8 containing N6 basal salt with vitamin (Phytotech), 3% sucrose, 0.03% casein hydrolysate, 0.5 g/L proline, 0.5 g/L glutamine, 0.4% gellan gum, 2 mg/L Kinetin, 2 mg/L BAP, 0.5 mg/L NAA in a growth chamber with supplemental lighting (8 h dark/16 h light; 120 µmol m^{-2} $^{-2}$) at 28 °C.

2.2. Measurement of Chlorophyll Content

Total chlorophyll content was measured based on (Jiang et al. 2017) with a brief modification. Two hundred milligrams of CIM grown 2-week-old and SIM grown 3-week-old callus were transferred into 2 mL microtube containing 1.5 mL of absolute ethanol. The mixtures were then shaken using a vortex and incubated in dark conditions for 24 hours. Absorbance readings were performed using a microplate reader (Corona SH-1000) at 649 and 665 nm wavelengths. Total chlorophyll was calculated using equation as follow: Chlorophyll a = $13.95 \times A_{665} - 6.88 \times A_{649}$ Chlorophyll b = $24.94 \times A_{649} - 7.32 \times A_{665}$ Total Chlorophyll = Chlorophyll a + Chlorophyll b. The results were expressed as mg of chlorophyll g^{-1} fresh weight.

2.3. RNA Total Extraction and Quantitative RT-PCR

Total RNA was extracted from CIM and SIM grown callus using RNAprep pure kit (Tiangen) following the manufacturer's instruction. One microgram of total RNA was used for cDNA synthesis using a Revertra Ace qPCR RT Kit (Toyobo). Quantitative RT-PCR was performed using the CFX96TM Real-Time PCR Detection System (Bio-Rad) and SsoFastTM Evagreen® Supermix (Bio-Rad) qRT-PCR mixture under the following conditions: initial denaturation at 94 °C for 2 min followed by 40 cycles of 94 °C for 15 s, 60 °C for 30 s, and 70 °C for 1 min. Relative expression of *OsRR2, OsRR6*, *OsACS1*, *OsACO1*, and *OsBBM1* genes were normalized to OsActin1 as a reference gene.

2.4. Callus Morphology

The morphology of callus that was grown in CIM and SIM was analyzed using a stereomicroscope (Leica EZ4HD). The analyzed morphological parameters were callus structure, callus color, green spot, and shoot formation.

2.5. Statistical analysis

The somatic embryogenesis experiment data (n=4) were statistically analyzed using Analysis of Variance (ANOVA) with Duncan's Multiple Range Test (DMRT) for the posthoc test using Microsoft Excel software version 2019 (Microsoft, USA). The mean values were considered statistically significant if $p \leq 0.05$. The gene expression results were statistically analyzed using an unpaired Student t-test.

3. Results and Discussion

3.1. Callus Induction Rate

Rice mature seed has been known as an excellent explant for callus induction. Using CIM media containing 2 mg/L 2,4-D, we observed that callus were efficiently formed from the mature seeds scutellar tissue of Ciherang, Mentik Wangi Susu, Hwayoung, and Tarabas (Figure 1a). The microscopic observation of the two-week-old callus showed that the structure of each variety was compact with yellowish color and a dry appearance. Among the four rice varieties, Tarabas and Hwayoung produced the highest rate of callus formation (91% and 90%, respectively), which was significantly higher ($p<0.05$) compared to Ciherang (and Mentik Wangi Susu (85.6% and 85%, respectively) (Figure 1b).

3.2. Green Spot Formation

Green spot formation in callus has been considered as one of the indicators of the success of shoot regeneration in rice and wheat tissue culture (Amer and Borner 1997; Mostafiz and Wagiran 2018). In our result experiment, callus of four rice varieties was grown in SIM supplemented with 2 mg/L Kinetin, 2 mg/L BAP, 0.5 mg/L NAA, and the green spot formation were observed after one- and two-weeks incubation. Mentik Wangi Susu showed the fastest green spot formation among the four varieties, as most of the callus clumps started to show greenish color in the first week of incubation (Figure 2a). At two weeks of incubation, levels of green spot formation in Mentik Wangi Susu and Ciherang callus were 95% and 90%, respectively, which was significantly higher ($p < 0.05$) than Hwayoung and Tarabas (70% and 67.5%, respectively).

The results of green spot formation level were further confirmed by the analysis of chlorophyll content of three weeks SIM incubated callus (Figure 2b), which showed that the callus of Mentik Wangi Susu produced significantly the highest total chlorophyll ($p < 0.05$) (11.17 $mg.g^{-1}$ FW) compared to Ciherang (8.50 $mg.g^{-1}$ FW), Tarabas (6.24 $mg.g^{-1}$ FW), and Hwayoung (2.61 $mg.g^{-1}$ FW). These results suggested that the callus of Mentik Wangi Susu showed the fastest initial response against cytokinin treatment.

3.3. Shoot Regeneration

The shoot regeneration rate was analyzed at five weeks of incubation in SIM. The japonica rice Hwayoung and Tarabas callus showed the highest regeneration rate (100% and 85%, respectively), which was significantly different ($p < 0.05$) from Mentik Wangi Susu (17.5%), and Ciherang

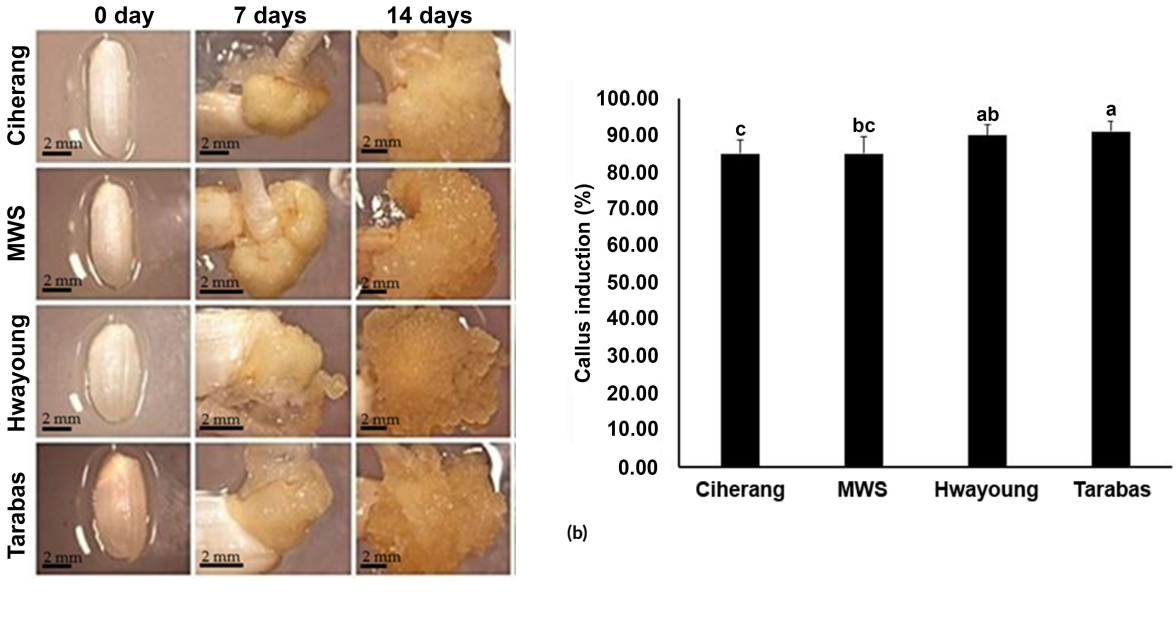

FIGURE 1 Callus induction rate of of Ciherang, Mentik Wangi Susu, Hwayoung, and Tarabas. (a) Callus morphology of four rice varieties on CIM at14 days. (b) Percentage of callus induction. Data are presented as mean (n=5) ± SD. Different letter are significantly difference at α 0.05 by DMRT

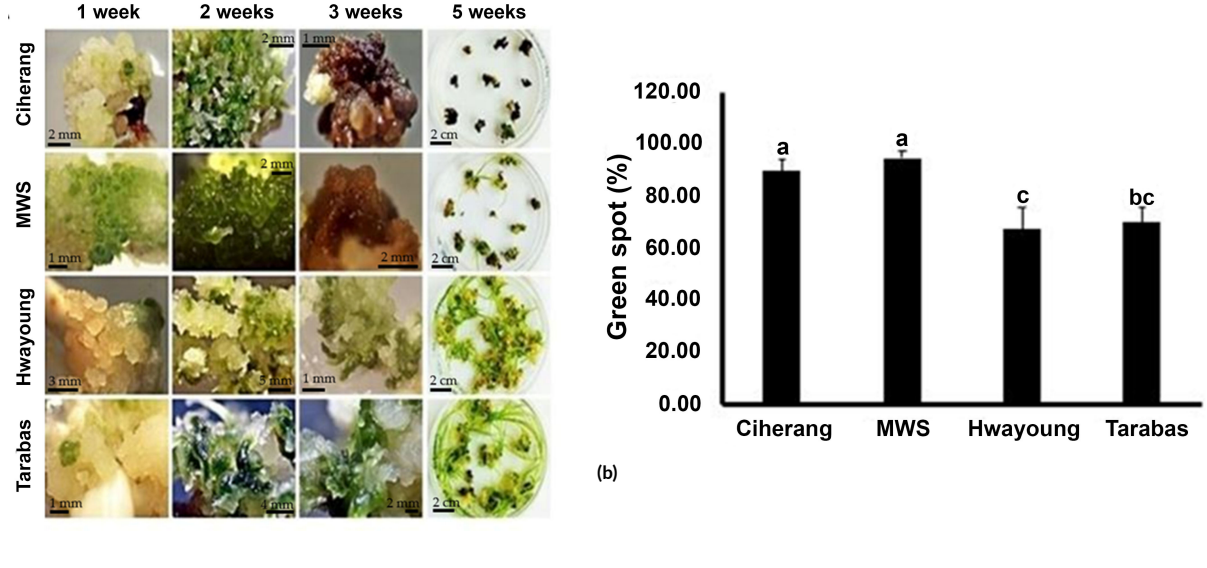

FIGURE 2 Somatic embryogenesis of Ciherang, Mentik Wangi Susu, Hwayoung, and Tarabas rice that have different regeneration rates. (a) Progress of shoot regeneration from callus at 1 to 5 weeks in SIM. (b) Green spot formation (%) of each rice varieties. Data are presented as mean (n=4) ± SD. (C) Chlorophyll content of rice callus on CIM and SIM for 2- and 3-weeks, respectively. Data are presented as mean (n=5) ± SD. Different letter are significantly difference at α 0.05 by DMRT

(0%) (Figure 3a). The low regeneration rate of Mentik Wangi Susu and Ciherang callus was correlated to the high-level callus browning of both varieties (82.5% and 100%, respectively) compared to 0% and 15% browning levels of Hwayoung and Tarabas, respectively (Figure 3b). Moreover, Hwayoung callus produced an average of 9.25 shoots number per clump, which was significantly highest ($p < 0.05$) compared to Tarabas (3.62 shoots), Mentik Wangi Susu (2 shoots), and Ciherang (no shoot formation) (Figure 3c). Although the shot number of Tarabas callus was lower compared to Hwayoung, the average shoot length of Tarabas was 6.75 cm which was significantly different ($p < 0.05$) compared to Hwayoung (1.97 cm), Mentik Wangi Susu (1.34 cm), Ciherang (no shoot formation). These results indicated that Hwayoung and Tarabas have a high regeneration rate despite the two varieties showing the late emergence of green spots.

3.4. Expression of cytokinin responsive, ethylene biosynthesis, and somatic embryogenesis genes

To deeply understand the molecular response for the different regeneration rates of four rice varieties, we further investigated the cytokinin signal perception and ethylene production in CIM and SIM grown callus by gene expression approach. We analyzed the expression of *OsRR2* and *OsRR6* to represent cytokinin responsive genes, *OsACS1* and *OsACO1* for ethylene biosynthesis genes, and *OsBBM1* for the somatic embryogenesis-related genes. Using qPCR analysis, the five analyzed genes generally were up-regulated in callus subcultured in SIM media (Figure 4). The expression of *OsRR2* was 3-folds induced in Ciherang callus after one week of subculture onto SIM, and the expression level was maintained until two weeks of subculture. We also observed that *OsRR6* was found to be up-regulated (7 folds) in the callus of Hwayoung after one week of subculture onto SIM, although the level was decreased after two weeks of subculture. The pronounced inductions of *OsRR6* were also shown by Ciherang, Mentik Wangi Susu, and Tarabas callus, but the level was lesser than Hwayoung.

The analysis of ethylene biosynthesis genes showed that *OsACS1* expression was 3.8-folds induced in the callus of Mentik Wangi Susu after one week of subculture in SIM, and the level was maintained at two weeks of subculture. The *OsACS1* basal expression level of Mentik Wangi Susu callus was two-fold higher than other varieties, indicating that the synthesis of ethylene precursor might be increased during callus formation and differentiation. Moreover, *OsACO1* expression was 2.25-folds induced in Hwayoung callus after one week of subculture onto SIM and slightly decreased at two weeks of subculture. The *OsACO1* expression was apparently induced in Mentik Wangi Susu and Tarabas, but the expression level remained unchanged in Ciherang callus.

The expression of *OsBBM1* was five- and two-fold induced in Hwayoung and Tarabas, respectively, but the levels were decreased at two weeks of SIM subculture. Interestingly, *OsBBM1* expression level was reduced in Ciherang and unchanged in Mentik Wangi Susu callus after two weeks of subculture in SIM. Thus, the positive correlation between the expression of cytokinin responsive and ethylene biosynthesis genes likely depends on the induction level of *OsBBM1*.

3.5. Discussion

In this study, we used the callus of Ciherang (indica subspecies), Mentik Wangi Susu (javanica subspecies), Hwayoung (japonica subspecies), and Tarabas (japonica

subspecies) to investigate the correlation between callus regeneration rate and the expression of several genes responsible for cytokinin response and ethylene biosynthesis. Callus of each rice variety were well developed in CIM containing 2 mg/L 2,4-D (Figure 1). The addition of cytokinin hormones such as kinetin is indispensable for the formation of green spots on the callus (Mostafiz and Wagiran 2018). Previous research suggests that green spot formation can be used as an indicator of the existence of embryogenic callus that can differentiate into coleoptiles in rice and wheat (Umar et al. 2017; Amer and Borner 1997; Mostafiz and Wagiran 2018).

The regeneration media containing 2 ppm kinetin, 2 ppm BAP, and 0.5 ppm NAA resulted in the highest regeneration rate using callus of indica rice (Ming et al. 2019). Using this hormone, we observed that the callus of Mentik Wangi Susu and Ciherang showed the fastest green spot formation compared to Hwayoung and Tarabas rice (Figure 2), but their shoot regeneration rate was low (Figure 3a). The high rate of callus browning in Mentik Wangi Susu and Ciherang (Figure 3b) was suspected as the leading cause of the low shoot regeneration rate. This result follows the previous findings that indica rice has low shoot regeneration due to the high rate of callus browning (Mostafiz and Wagiran 2018). Therefore, the green spot formation is not related to the success of shoot regeneration in Mentik Wangi Susu and Ciherang callus. Callus browning is tightly linked to the high accumulation and subsequent oxidation of phenolic compounds. The activity of phenylalanine ammonia-lyase (PAL) is a key factor for the conversion of phenylalanine into phenolic compounds and polyphenol oxidase (PPO) is responsible for the oxidation of phenol to quinones that generate the brown color pigment (Khosroushahi et al. 2011).

The gene expression analysis of cytokinin responsive and ethylene biosynthesis was conducted since cytokinin signal perception ability and ethylene biosynthesis in callus have been known as important factors for shoot regeneration in rice (Fanata et al. 2013; Arisandi et al. 2020; Dewi and Purwoko 2008; Yasmin et al. 2013). In vitro shoot formation is a precise tool for cytokinin response assay (Inoue et al. 2001; Kakimoto 1996). We observed that induction of *OsRR2* in Ciherang was maintained at a high level until two weeks in SIM, whereas *OsRR6* was highly induced in Hwayoung at one week in SIM, but the level was decreased after two weeks. Since both genes belong to the type A of RRs that negatively regulate the cytokinin response through cytokinin desensitization, the callus of Ciherang might be less sensitive to exogenous cytokinin treatment due to its high and stable expression level of *OsRR2* during the subculture period in SIM. Although Hwayoung callus showed the highest induction of *OsRR6*, the level decreased after two weeks in SIM, which caused the cytokinin insensitivity not to be present in Hwayoung callus. The constitutive overexpression of *OsRR6* in rice has been reported to abolish shoot regeneration, suggest-

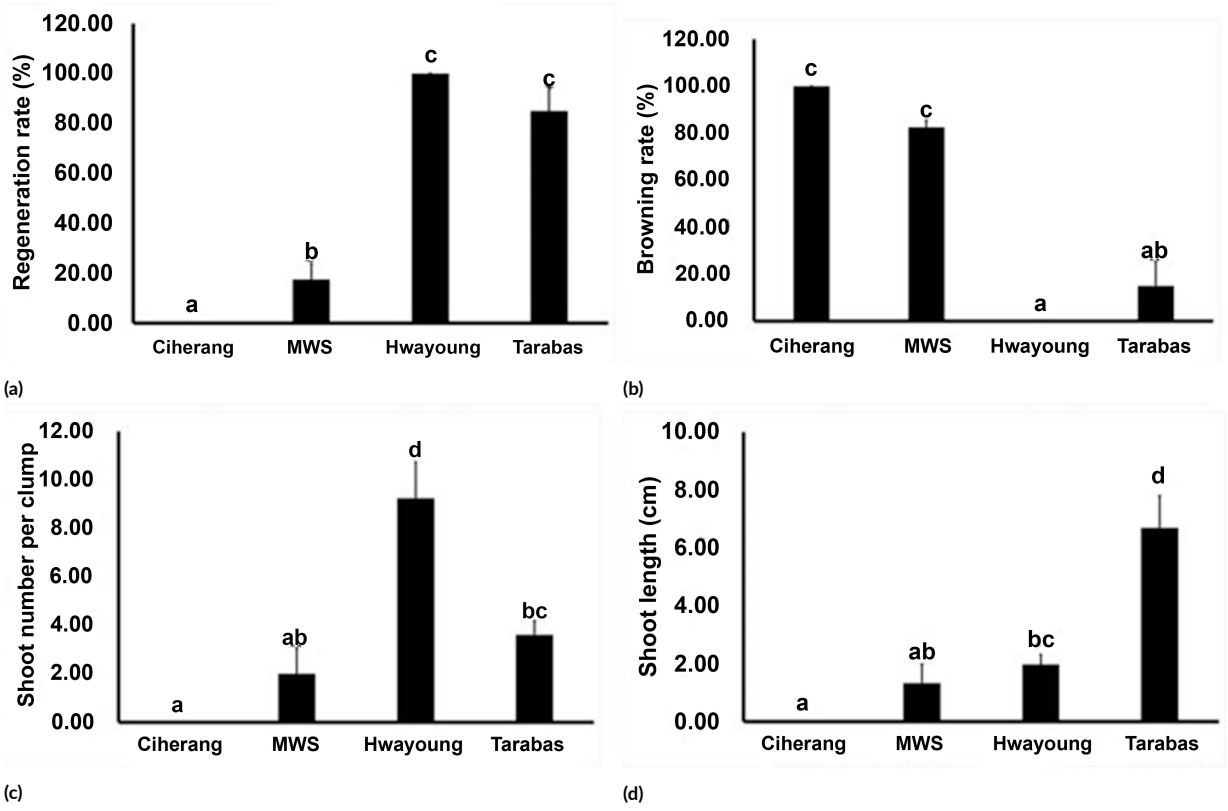

FIGURE 3 Quantification of regeneration rate, browning rate, shoot number, and shoot length. (a) Shoot regeneration rate; (b) Callus browning rate; (c) Shoot number per clump; (d) Shoot length. Data are presented as mean (n=4) ± SD. Different letter are significantly difference at α 0.05 by DMRT.

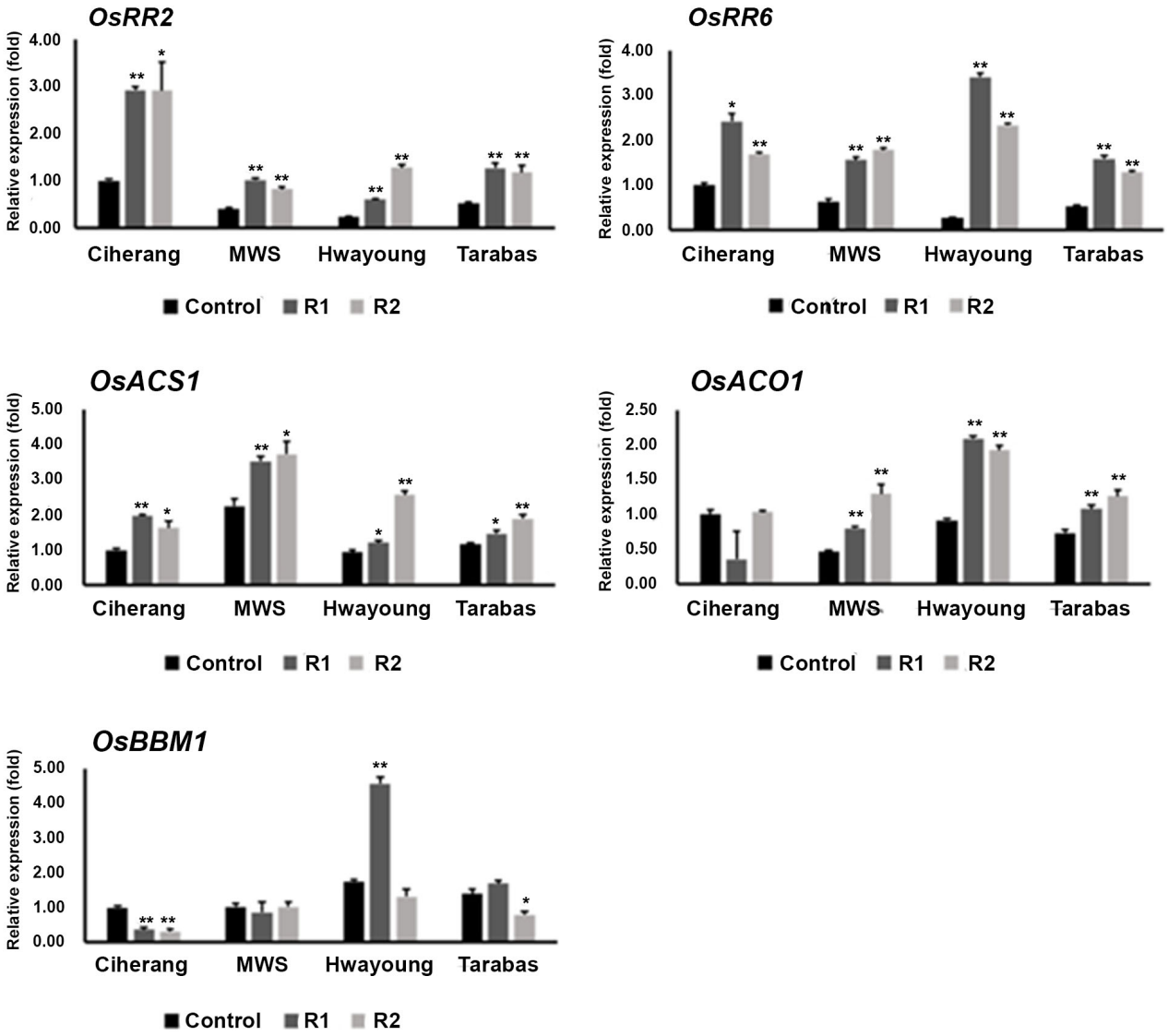

FIGURE 4 Analyses of the expressions of cytokinin-responsive and ethylene biosynthesis genes, in Ciherang, Mentik Wangi Susu, Hwayoung, Tarabas. Control : represent 2-week-old callus on CIM, R1: 1-week-old callus on SIM, R2: 2-week-old callus on SIM. OsActin was used as an internal control. Expression of the genes were analized by qRT-PCR (quantitative real-time polymerase chain reaction). * and ** significantly different (t-test at P < 0.05 or 0.01, respectively) compared with control (n=3).

ing that *OsRR6* acts as a negative regulator of cytokinin signaling (Hirose et al. 2007). The overexpression of several type-A RRs in Arabidopsis thaliana genes compromised the cytokinin-induced shoot regeneration capacity (Ren et al. 2009).

The ethylene biosynthesis gene expression revealed the high induction of *OsACS1* in Ciherang and the level was stably maintained until two weeks of culture in SIM (Figure 4). The induction of *OsACO1* was found to be high in Hwayoung callus and apparent induction was also shown by Mentik Wangi Susu and Tarabas. Moreover, *OsBBM1* expression was induced in Hwayoung and Tarabas but decreased in Ciherang callus. Ethylene hormone affects shoot regeneration differently, depending on species or even cultivar (Yang et al. 2015). The positive role of ethylene for shoot regeneration was shown by reducing of shoot regeneration rate in the ethylene insensitive mutant of Arabidopsis thaliana, while plants with constitutive ethylene response showed a high level of shoot regeneration (Chatfield and Raizada 2008). In rice, the addition of ethylene precursor into regeneration media increased shoot regeneration, whereas the opposite effect was shown by ethylene synthesis inhibitor addition (Yasmin et al. 2013). On the other hand, ethylene was identified as a browning inducer in plant cell culture that further represses shoot regeneration (Adkins et al. 1990; Kobayashi et al. 1991). Furthermore, somatic embryogenesis-related gen such as BBM is vital for shoot regeneration since the ectopic expression of BBM induces somatic embryogenesis in several plant species (Boutilier et al. 2002; Lowe et al. 2016). Therefore, both *OsBBM1* and ethylene induction might synergistically induce shoot formation in

Hwayoung callus, whereas a decrease of *OsBBM1* level and cytokinin desensitization might be the predominant factor for the absence of shoot regeneration in Ciherang callus.

4. Conclusions

Analysis of cytokinin-responsive gene expression showed that *OsRR2* induction was found to be highest in Ciherang callus, whereas *OsRR6* expression was found to be highly up-regulated in Hwayoung callus. Analysis of the ethylene biosynthetic gene showed that *OsACS1* was highly expressed in Mentik Wangi Susu callus. Thus, the positive correlation between the expression of cytokinin responsive and ethylene biosynthesis genes with the somatic embryogenesis activity likely depends on the induction level of *OsBBM1*.

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Authors' contributions

SFH performed the experiment, collected the data, and analyzed the data, PD and BS designed the analysis and analyzed the data, WIDF designed the analysis, analyzed the data and wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

The authors have declared that they have no competing interests.

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