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Full Length Article

Gene Expression of Late Somatic Embryogenesis in Rice (*Oryza sativa*)

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Abstract

Rice *(Oryza sativa* L.) is a highly embryogenic reaction to its regeneration potential through somatic embryogenesis using mature rice embryos depending on the genotype. These biological variables may offer deep understanding into rice breeding programs. In somatic embryogenesis, rice requires expression of SOMATIC EMBRYOGENESIS RECEPTOR-LIKE KINASE (SERK), LEAFY COTYLEDON (LEC) and WUSCHELL (WUS). Early somatic embryogenesis was thoroughly studied through these genes, while late somatic embryogenesis was not. In this study, we discovered that only *OsSERK* was significantly expressed in late somatic embryogenesis in rice, whereas *OsWOX4* and *OsLEC1* were absent or expressed at low levels using modified combination hormones, T1: no hormones (control); T2: 2 mg/L kinetin + 1 mg/L NAA, and T3: 2 mg/L kinetin + 1 mg/L BAP + 1 mg/L NAA. The *OsSERK*, *OsLEC1*, and *OsWOX4* genes were expressed during late somatic embryogenesis on the days 28. GogoNiti II showed that combination of hormones responded positively to plant morphology after 45 days in plant regeneration. During late somatic embryogenesis research, rice regeneration is the most significant *in vitro* in specific rice cultivars and their epigenetic mechanisms. Also, callus cell sources can change rice to transform DNA inside cell growth.

Keywords: Hormones; *In vitro*; Morphological traits; Plant regeneration; Somatic embryogenesis

Introduction

Rice (*Oryza sativa* L.) is the world's most widely consumed grain crop. However, according to Gerten *et al.* (2020), rice cultivation is likely to face new challenges in future as the world's population is likely to approach over 10 billion people by 2050. Additionally, rice consumption was estimated to be increased by 5,2 million tonnes in 2018/2019 (FAO 2018). Furthermore, rice is highly embryogenic to its regeneration potential through somatic embryogenesis using mature rice embryos, which depends on the genotype (Siddique *et al.* 2014).

Somatic embryogenesis regulates gene expression and signal transduction pathways to activate or repress many genes (Mahdavi-Darvari *et al.* 2015). The exogenous application of hormones causes morphological changes by stimulating changes in tissues during callus development. Transcriptional and epigenetic factors regulate hormone activity through their biosynthesis and signaling effects, generating feedback networks. As a result, cell-fate transition and genome-wide gene expression changes as several primary transcription factors respond to exogenous hormone signals. Epigenetic pathways regulate them simultaneously. In addition, an increasing number of studies have revealed a strong link between plant hormone signaling and the epigenetic process (Yang and Zhang 2010).

Many types of stresses may induce plant cells to change their cellular and molecular programs. PGRs are one type of stress that can alter plant cell and molecular processes (Mariani *et al.* 1998; Debbarma *et al.* 2019). In addition, exogenous hormone exposure during the development of an organism can cause morphological changes in induced tissues (Méndez-Hernández *et al.* 2019). We believe these factors influenced the rice varieties in significant ways. Similar to the findings of Guo *et al.* (2013), environment signals increase endogenous PGR concentrations, and gene expression all trigger stages of somatic embryogenesis.

Among others, *SERK*, *WUS*, and *LEC* are totipotency markers expressed in several plants, including rice. In the early stages of embryogenesis, *SERK* gene expression begins by activating the embryogenic stage, which leads to the globular stage of somatic embryos but not in non-embryogenic stages (Santos and Aragão 2009). In addition, high expression of the *WUS* gene suggested that it may help initiate embryogenesis (Kumar and van Staden 2017). In comparison, *LEC* genes are essential in the early stages of

plant embryogenesis, especially during transcriptional encoding (Guo *et al.* 2013). Thus, these genes may be expressed at the early stages of somatic embryogenesis but have not yet been studied in late somatic embryogenesis; we thus explored their relevance.

In this research we performed the morphological analysis of late somatic embryogenesis on the 14th and 28th days associated with expressed genes in rice. Therefore, each rice variety had various genetic characteristics using molecular analysis of the epigenetic process in the somatic embryogenesis phase. However, it has not been studied thoroughly in Javanica rice. Previous findings by Gelvin (2003) showed that the cell source was a key to convert the cellular DNA to generate a new genetically modified rice to enhance cell transformation. Therefore, callus induction and rice regeneration are most significant in understanding the effectiveness of high-cell transformation, especially in some rice cultivars, related to their epigenetic process.

Many research initiatives have targeted rice genomic and transcriptomic data, which contributed to an understanding of the biology of rice. Differences in the developmental stage and regeneration capacity may be due to different active genes expressed during somatic embryogenesis detected in tissues (Mahdavi-Darvari *et al.* 2015). Here we intended to contribute to an understanding of rice breeding and rice biology by examining gene expression during late somatic embryogenesis associated with morphological analysis. This is the crucial reason why we were investigating late somatic embryogenesis genes in Javanica rice.

Materials and Methods

Sterilization of explants

The mature seeds of four varieties (Fig. 1a–d), Cigeulis, GogoNiti II, Ketan Hitam I and TN1, were dehusked by shaking for 30 min at 120 rpm in 50% Clorox bleach (5.25% hypochlorite, The Clorox Company, Indonesia), then rinsed with sterile distilled water five times, after which they were dried thoroughly on sterile filter paper.

The embryogenic callus induction

The seeds were then cultivated with 2 mg/L 2, 4-dichlorophenoxyacetic acid (2, 4-D), 30 g/L sucrose and 4 g/L gelrite to culture seeds in a sterile medium consisting of MS salts (Upadhyaya *et al.* 2015). Before autoclaving, the pH of the medium was adjusted to 5.8. After that, the seeds were cultured for three weeks at 27°C in dark conditions. The percentage of callus induction and callus size was determined after three weeks of incubation.

Plant regeneration

The treatments were utilized to obtain high-quality embryogenic calli using 1.0 mg/L BAP, 2 g/L casein hydrolysate, 2 mg/L kinetin, 0.1 g/L *myo*-inositol, 1.0 mg/L NAA, 5.2 g/L phytagel and 30 g/L Sucrose medium. In this research, the treatments for media regeneration were T1: no hormones (control), T2: 2 mg/L kinetin + 1 mg/L NAA (Toki 1997) and T3: 2 mg/L kinetin + 1 mg/L BAP + 1 mg/L NAA (Khanna and Raina 1998). Each treatment had three replications with four callus clumps per replication. The regeneration culture was carried out at 27°C on the 14th and 28th days with a photoperiod of 16/8 h (light/dark). The percentage of green spots and the percentage of plant regeneration were determined.

Fig. 1: Seed varieties. **a** TN1, **b** Cigeulis, **c** GogoNiti II and **d** Ketan Hitam I

Data recorded

The percentage of callus induction, green spot, and plant regeneration were determined accordingly:

$$\text{Percentage callus induction} = \frac{(\text{No. of seeds induced calli})}{(\text{No. of seeds cultured})} \times 100$$

$$\text{Percentage green spot} = \frac{(\text{No. of callus induced green spot})}{(\text{No. of seeds cultured})} \times 100$$

$$\text{Percentage plant regeneration} = \frac{(\text{No. of callus induced shoot buds (plants)})}{(\text{No. of seeds cultured})} \times 100$$

Data analysis

Data were evaluated by variance analysis (ANOVA). If the findings were significantly ($P<0.05$) different, then further analysis using the Duncan's multiple range test included in the SPSS V. 26 was used.

Isolation of RNA purification

The callus samples were taken on the 14th and 28th days of

Table 1: Gene-specific primers sequence

Genes	Sequence 5'–3'	Reference
OsLEC1	F- CAA GGA GAC GAT CCA GGA GT R- GGT AGC GGT GGA GGT AGA CG	Gruszczyńska A and M Rakoczy-Trojanowska (2011)
OsSERK	F- TTG CTG GAG GTG TTG CTG R- TAC ACC TTT CCA AAG CCA	Gruszczyńska A and M Rakoczy-Trojanowska (2011)
OsWOX4	F- CTA GCT TAT CGA TAC CGT CG R- CCT ATC TGT TCT TGA GTC GG	Kumar V and J van Staden (2017)
OsActin	F- GGT ATT GTT AGC AAC TGG GAT G R- GAT GAA AGA GGG CTG GAAG A	Gruszczyńska A and M Rakoczy-Trojanowska (2011)

medium regeneration and frozen immediately in liquid nitrogen. Then, total RNA was isolated with some modifications to the manufacturer's guide (All Ribospin Plant RNA Mini Kit, GeneAll Biotech, Korea). The 260 nm/280 nm measurement at a level between 1.8 and 2.2 for cDNA synthesis and RT-PCR was obtained using nanodrop (TECAN® Infinite M200 Multi-Detection Microplate Reader Part).

cDNA synthesis

Total RNA was used to design cDNA. The mRNA was amplified into cDNA. After verifying RNA quality, the GoTaq® Green Master Mix kit (Promega) enhanced the target cDNA. ReverTra Ace® RT Master Mix with gDNA Remover (Toyobo, Osaka, Japan) treats 0.5 μg of total RNA. This process eliminated and replaced genomic DNA with a single-stranded cDNA. Total RNA was incubated at 37°C for the DNase reaction for 5 min and reverse transcription reaction. Finally, THUNDERBIRD SYBR qPCR Mix (Toyobo) and gene-specific primers (Table 1) were used in real-time PCR applications.

PCR analysis

The PCR analysis was performed in a total volume of 10 μL containing 5 μL of 2 × GoTaq® Green Master Mix, 1 μL cDNA templates, 2 μL Nuclease-free water and 1 μL forward (F) and reverse (R) primer to detect the presence of a specific nucleic acid sequence using the GoTaq® Green Master Mix kit (Promega). The PCR amplification profile consisted of an initial denaturation of 95°C for 2 min, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 53°C for 30 s, extension at 72°C for 1 min and a final extension at 72°C for 5 min. An electrophoresed 2% agarose gel in 1 X TAE buffer stained with GreenStar™ was used for PCR analysis. In addition, the UV transilluminator was used to visualize the banding patterns.

Results

Rice genetic backgrounds and callus growth and morphology

TN1 was used as a control in this study. This model produced thousands of embryogenic calli from 50 seeds in 10 weeks (Sivamani *et al.* 1996). On the other hand, local Indonesian rice varieties such as Cigeulis, GogoNiti II, and Ketan Hitam I were little studied, particularly *in vitro*. From observation under a light microscope, the TN1 callus showed light yellow to whitish color and had a compact cell after three weeks of media induction cultivation. However, GogoNiti II and Cigeulis callus turned yellow or cream, with nodular cells and a not similar compact cell-like TN1. Also, a hairy thread surrounding the callus appeared in Ketan Hitam I under media induction.

Furthermore, the hairy cells become thicker and more whitish during pro-embryo regeneration (Fig. 2a–d). According to the current study, both TN1 and GogoNiti II were classified as Type I, with an organized callus, a pro-embryo stage, numerous globular in small compact clusters, embryogenic potential, and the ability to regenerate. Cigeulis, on the other hand, is classified as Type II (organized yellow callus). In contrast, Ketan Hitam I was classified as a Type IV (rhizogenic callus) that was not able to regenerate into plantlets.

This study further determined the effect on callus induction and its size that reached to 2 mg/L of 2, 4-D on MS basal media for weeks on different rice varieties. Significant effects ($p \leq 0.05$) were shown for callus induction (%) and callus size (mm) (Table 2). Ketan Hitam I (88%) showed the best performance compared to TN1 (81%) and demonstrated a significant difference with GogoNiti II (70%) and Cigeulis (49%). Cigeulis, on the other hand, had the lowest callus induction percentage (Table 2). As regards callus size, TN1 (6.60 mm) and Gogo Niti II (6.76 mm) had significantly larger calluses than Ketan Hitam I (5.06 mm) and Cigeulis (4.93 mm). In addition, a significant genotypic response to 2, 4-D was noted in cultured tissue cells with varying genetic backgrounds. The present result indicated that genotypic variation exists in the ability to induce callus formation.

Somatic embryogenesis under hormonal combinations

This study showed the duration of developing somatic embryos between each treatment on different varieties (Fig. 3a–c). There was a significant effect ($p \leq 0.05$) of media regeneration on globular (Fig. 3a), scutellar (Fig. 3b) and coleoptillar (Fig. 3c). TN1 and GogoNiti II showed rapid growth from globular to coleoptillar phases compared to Cigeulis and Ketan Hitam

Table 2: The callus induction (%) and callus size (mm) of 3 weeks calli derived from MS media with 2 mg/L of 2, 4–D

Varieties name	Percentage of callus induction (%)	Callus size (mm)
TN1	81.00 ± 5.37^{ab}	6.60 ± 0.31^{a}
Cigeulis	49.00 ± 5.22^{c}	4.93 ± 0.29^{b}
GogoNiti II	70.20 ± 5.65^{b}	6.76 ± 0.34^{a}
Ketan Hitam I	87.80 ± 2.31^{a}	5.06 ± 0.19^{b}

$^{a-c}$ means ± standard deviations (n=3) followed by different superscript letters within the same column are significantly different at p≤0.05 according to Duncan's multiple range test

Table 3: Percentage of green spots and plant regeneration of 3 weeks calli derived from MS media transferred to the regeneration medium. That supplemented with **T1** control (no hormone), **T2** Kinetin 2 mg/L and NAA 1 mg/L, and **T3** BAP 1 mg/L, Kinetin 2 mg/L and NAA 1 mg/L on different genotypes

Varieties Name	Percentage of green spots (%)			Percentage of plant regeneration (%)		
	T1	T2	T3	T1	T2	T3
TN1	61 ± 10^{ab}	100 ± 0^{a}	78 ± 38^{ab}	29 ± 6^{d}	88 ± 18^{ab}	84 ± 23^{ab}
Cigeulis	80 ± 35^{a}	89 ± 19^{a}	83 ± 14^{a}	42 ± 12^{cd}	63 ± 18^{bcd}	71 ± 6^{ab}
GogoNiti II	72 ± 30^{ab}	92 ± 14^{a}	93 ± 12^{a}	71 ± 6^{abc}	$^{*}100\pm0^{a}$	84 ± 23^{ab}
Ketan Hitam I	37 ± 15^{b}	82 ± 16^{a}	71 ± 8^{ab}	33 ± 0^{d}	59 ± 12^{bcd}	63 ± 18^{bcd}

$^{a-d}$ means ± standard deviations for percentage of green spot (n=3) and percentage of plant generation (n=2) * the highest percentage of plant regeneration followed by different superscript letters within the same column and row are significantly different at p≤0.05 according to Duncan's multiple range test

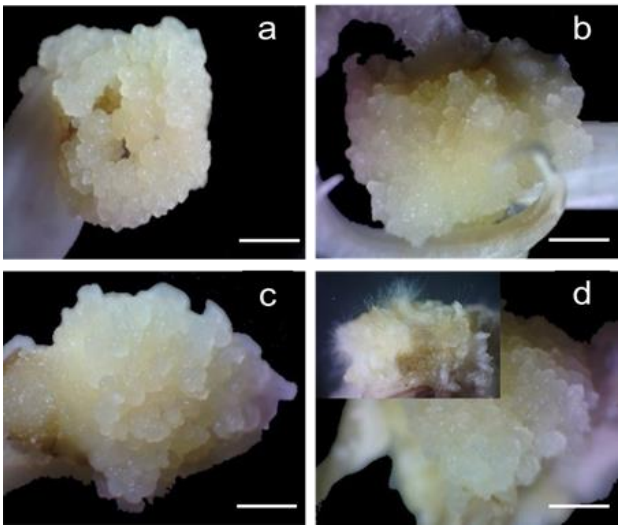

Fig. 2: The morphology of embryogenic callus of rice varieties on MS media supplemented with 2 mg/L of 2, 4–D after 3 weeks (Bars = 5 mm). **a** TN1, **b** Cigeulis, **c** GogoNiti II, and **d** Ketan Hitam I

I, with the best combination of horm ones T3. In contrast, T2 was second-best compared to T1 (control). Nevertheless, Ciguelis showed no significant differences between hormones on media regeneration in developing coleoptillar. While, in Ketan Hitam I, T3 led the best performance informing the coleoptillar compared to T2 and T1 (control). Next, a light microscope was used to observe the somatic embryogenesis process of rice varieties (Fig. 4a–d).

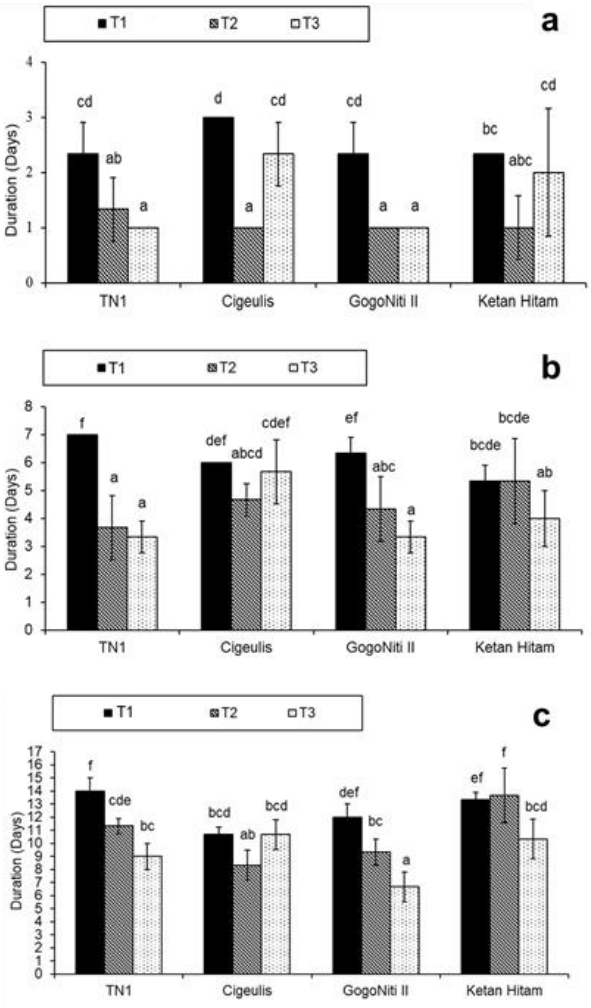

Fig. 3: The duration for development of somatic embryogenesis phases in different media regeneration hormones. Means with different letters indicate significant differences according to Duncan's multiple range test (p≤0.05) (n = 3). **a** duration to form globular stage, **b** Duration to form scutellar stage and **c** Duration to form coleoptillar stage. **T1** control (no hormone), **T2** 2 mg/L kinetin + 1 mg/L NAA, and **T3** 2 mg/L kinetin + 1 mg/L BAP + 1 mg/L NAA00

Callus greening and plant regeneration under hormonal combinations

Table 3 shows the percentage of green spots (p≤0.05) and plant regeneration (p≤0.05) between each treatment on the different varieties. Moreover, there was no correlation between the percentage of green spots and plant regeneration in this study. Based on the percentage of green spots, T2 showed a high percentage of green spots, including TN1, GogoNiti II, Cigeulis and Ketan Hitam I (100, 92, 89 and 82% respectively). On the other hand, T3 also showed a high percentage of green spots only for Cigeulis and GogoNiti II (93 and 83% respectively). Next, most of the green spots will

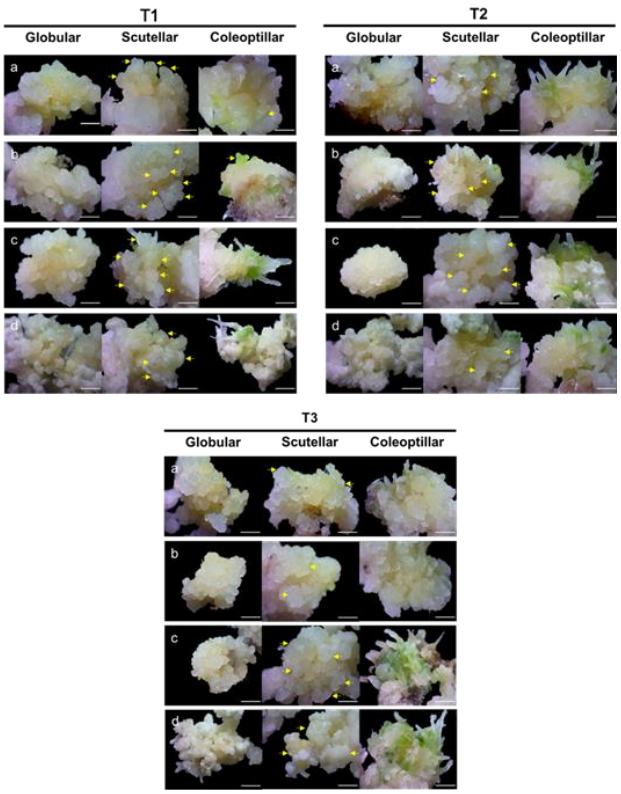

Fig. 4: Somatic embryogenesis stages with different treatments. (Bars: 5 mm). **a** TN1, **b** Cigeulis, **c** GogoNiti II, and **d** Ketan Hitam I. **T1** control (no hormone), **T2** 2 mg/L kinetin + 1 mg/L NAA and **T3** 2 mg/L kinetin + 1 mg/L BAP + 1 mg/L NAA

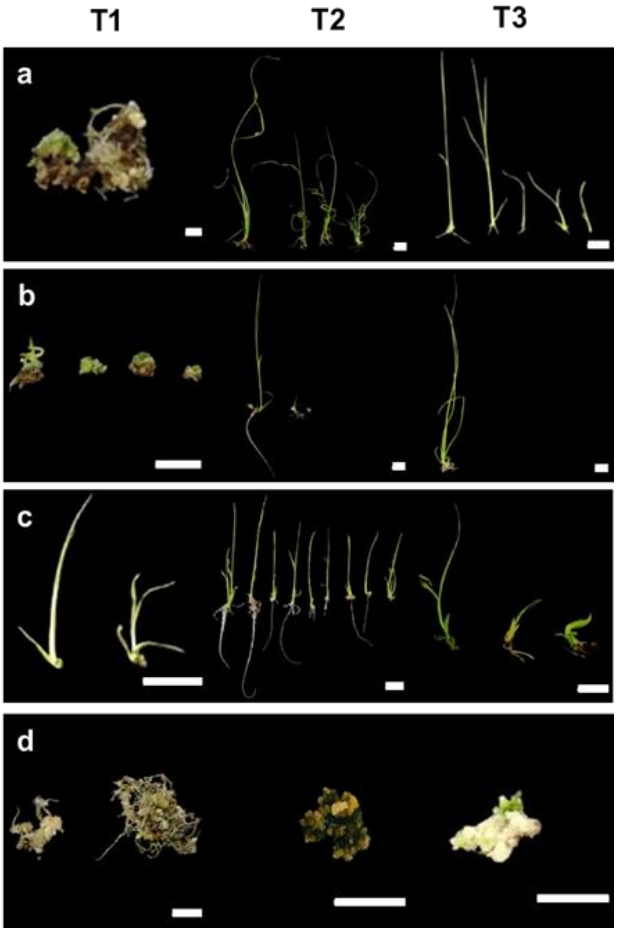

Fig 5: Plantlet on the 45th days after apply hormone in media regeneration. (Bars: 10 mm). **a** TN1, **b** Cigeulis, **c** GogoNiti II, and **d** Ketan Hitam I. **T1** control (no hormone), **T2** 2 mg/L kinetin + 1 mg/L NAA, and **T3** 2 mg/L kinetin + 1 mg/L BAP + 1 mg/L NAA

grow into multiple shoots then form plantlets. The results indicated that Ketan Hitam I had a high percentage of green spots but showed a small percentage of plant regeneration. However, many green spots are grown in somatic embryos, unable to develop into multiple shoots to form plantlets. This is because it may lose its capacity to regenerate by performing a dedifferentiation process. Based on the percentage of plant regeneration, GogoNiti II gave the best T2 (100%) performance among other varieties and treatments. On the other hand, the second-best performance was T3, shown in TN1, Cigeulis, and GogoNiti II (84, 71 and 84%, respectively) and T2 was shown only in TN1 (88%).

Morphology of plantlets

All varieties were analyzed for morphological analysis of the plantlet after 45 days of regeneration. GogoNiti II showed the most significant performance, treated with T2, which produced more plantlets (12 plantlets) than other varieties and treatments that responded positively to the percentage of plant regeneration (Fig 5a–d). In comparison, T3 only produced three plantlets, while T1 produced two plantlets. On the other hand, T3 in TN1 had the second-highest number of plantlets (5 plantlets). T2 produced four plantlets, compared to T1, which did not produce a plantlet or a slow response. However, Ketan Hitam I did not produce plantlets. Cigeulis had fewer plantlets than TN1 and GogoNiti II, in which T3 generated two plantlets compared to T2. After 45 days, the callus can produce shoots, roots, or entire plantlets from embryogenic calli. Non-embryogenic calli, on the other hand, can only produce shoots or roots and cannot regenerate plantlets.

The effect of different combinations of hormones on gene expression

The rice genome has an ortholog of the *SERK*, *WUS* and *LEC* genes expressed during somatic embryogenesis in this study. The *SERK* ortholog is referred to as *OsSERK*, the *WUS* ortholog is called *OsWOX4*, and the LEC ortholog is referred to as *OsLEC1*. In this study, on the 14th and 28th days of media regeneration, *OsSERK*, *OsWOX4* and *OsLEC1* displayed different expression patterns in TN1, Cigeulis, GogoNiti II, and Ketan Hitam I. Only one gene, *OsSERK*, was highly expressed on days 14 and 28 for all varieties (Fig. 6a–d, 7a–b).

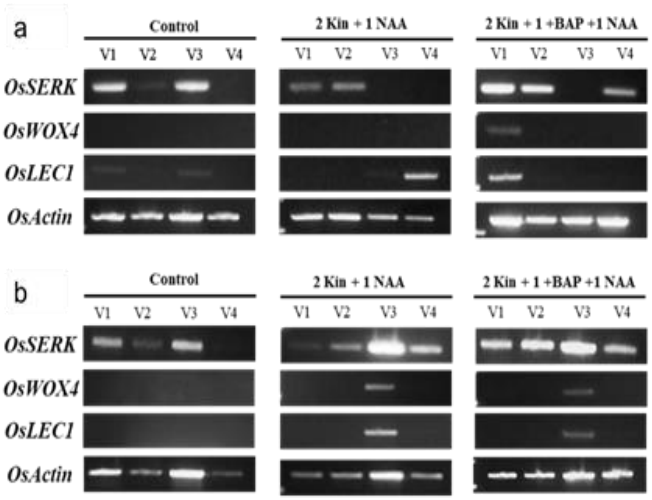

Fig 6: Expression of *OsSERK*, *OsWOX4* and *OsLEC1* were performed from total RNA samples for PCR analysis were isolated from callus under regeneration media with different hormones at 14 and 28 days. *OsActin* was used as a reference gene. **a** genes expression at 14 days, and **b** genes expression at 28 days. **V1** TN1, **V2** Cigeulis, **V3** GogoNiti II, and **V4** Ketan Hitam I. **T1** control (no hormone), **T2** 2 mg/L kinetin + 1 mg/L NAA and **T3** 2 mg/L kinetin + 1 mg/L BAP + 1 mg/L NAA

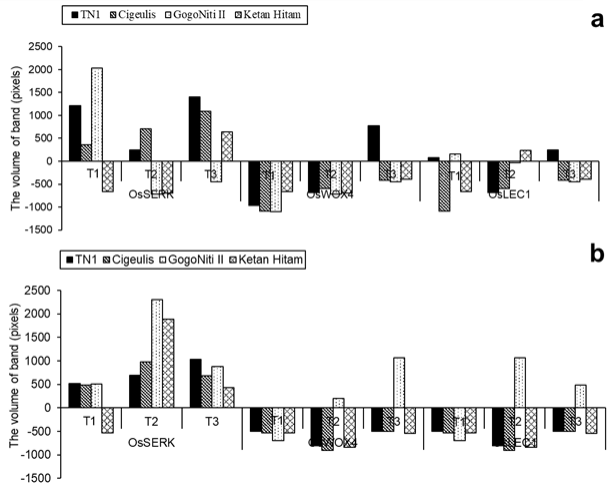

Fig 7: The volume of band was normalized to the constitutive gene *OsActin*. **a** genes expression at 14 days, and **b** genes expression at 28 days. **T1** control (no hormone), **T2** 2 mg/L kinetin + 1 mg/L NAA and **T3** 2 mg/L kinetin + 1 mg/L BAP + 1 mg/L NAA

Discussion

Monocotyledonous plants, like rice, have four embryogenesis stages: pro-embryo, globular, scutellar and coleoptilar. Each phase had a unique surface structure (Debbarma *et al.* 2019). These and previous studies by Mariani *et al.* (1998) established that pre-embryos remain globular until fibrillary material forms. Initially, pro-embryogenic masses on the surface of white translucent calluses were classified as embryogenic calluses that appeared smooth. During the globular stage, a mesh-like structure replaced the fibrillary material. Then, the development of the globular phase was completed, after which the apical scutellum region flattened, and the mesh-like structure ridge became low. The scutellum produces a signal after the coleoptile emerges, allowing the scutellar notch (Delgado-Aceves *et al.* 2021). Elongated cells on the coleoptile's surface were discovered later. Finally, the coleoptile and root growth. Moreover, embryos with a leafy scutellum and multiple shoot meristems, on the other hand, are classified as abnormal somatic embryos.

In this study, the development from globular to coleoptillar took 1–2 weeks, depending on the different treatments and genotype (Fig. 3). Zhao *et al.* (2017) explained that hormones influence somatic embryogenesis, due to external signals, endogenous concentration changes of various PGRs and gene expression. However, even without hormones, somatic embryogenesis can still develop because it depends on the genetic background of rice. Different varieties showed different responses to somatic embryogenesis. The induction of embryogenic callus leads to rice regeneration was different between rice genotypes and media composition similar to (Mohd Din *et al.* 2016; Binte Mostafiz and Wagiran 2018).

Prolonged exposure to light causes high percentage of green spots to grow in non-somatic areas, where the plant regeneration potential is significantly lower (ben Amer and Börner 1997). Therefore, the percentage of green spots were not correlated with the percentage of plant regeneration. Factors influencing the success of plant regeneration and the number of crops produced include genotype, degree of callus expansion and the composition of growth regulators in regeneration media (Heyser and Nabors 1982; Saharan *et al.* 2004). Treatments T2 and T3 showed significantly different responses as compared to T1 (without hormones) because auxin and cytokinins induce plant regeneration (Mohd Din *et*

al. 2016). This study used kinetin, BAP and NAA at different ratios. Kinetin plays a role in cell morphogenesis and somatic embryogenesis (Toki 1997; Khanna and Raina 1998). This can occur when sufficient nutrients support cell growth (G1 cell growth phase). In addition, kinetin can activate the transcription and translation, leading to cell proliferation (G2) and cell division (Umar *et al.* 2017). For T2 and T3 treatments, 2 mg/L kinetin was used because cytokinins are required for basal auxin biosynthesis in root and shoot tissues (Chang *et al*. 2015). In addition, biosynthesis is regulated interactively because developmental or environmental changes can affect auxin effects (Jones *et al.* 2010). However, auxin-cytokinin interactions are essential for plant growth and development because they control the growth of plant tissues and organs (Yamada *et al.* 1986; Saharan *et al.* 2004). In mass propagation, the total number of plantlets regenerations is the most critical factor. A previous study showed that increasing NAA concentration improves plantlet number (Tsukahara and Hirosawa 1992). However, the concentration of NAA in both combination hormones was the same in this research which was 1 mg/L NAA.

The SERK is most likely connected to somatic embryogenesis and may help uncover rice's functions, roles, and processes, especially in morphogenesis and other developmental processes (Singla *et al.* 2009). In rice, SERK is a member of the LRR-RLK II subfamily of the receptor-like kinase superfamily. The extracellular domain of SERK includes a signal peptide, a leucine zipper domain, 4.5-5 LRR and a proline-rich region that includes the SPP motif. These two specific areas are found in the protein's intracellular domain (cytosolic domain), as catalytic serine/threonine or tyrosine kinase domains and the C-terminus (Gerten *et al.* 2020). SERK is a transmembrane receptor expressed in the early stages of somatic embryogenesis, embryogenic callus, the basal layers of embryos, the cotyledon of the embryo, and the vascular and provascular strands (Loyola-Vargas and Ochoa-Alejo 2016). In *Cocos nucifera* (L.), another monocot the *CnSERK* gene was expressed during embryogenic callus formation before embryo development. In dicots, *TcSERK* of *Theobroma cacao* was detected in somatic and zygotically mature embryos, while *DcSERK* expression of *Daucus carota* begins during the early globular stage, indicating a function in embryo development (Schmidt *et al.* 1997; de Oliveira Santos *et al.* 2005). *AtSERK1* in Arabidopsis increases the efficiency of somatic embryogenesis initiation by three to four-folds (Hecht *et al.* 2001). In most cases, *SERK* genes played a role in somatic embryogenesis, specifically in the early stages (Gruszczyńska and Rakoczy-Trojanowska 2011). However, these findings demonstrated the novelty of this study's findings. The *OsSERK* gene expressed during late somatic embryogenesis was discovered in rice.

On day 28, only GogoNiti II expressed *OsSERK*, *OsWOX4* and *OsLEC1* when treated with T2 and T3 as compared to T1 (Fig. 6b). This result can be related to the percentage of plant regeneration and the number of plantlets that responded positively. Besides, on day 14 under Ketan Hitam I, *OsSERK* was better expressed under T3 compared to T2 and T1. However, on day 14, under TN1, T3 better expressed *OsSERK*, *OsWOX4* and *OsLEC1* compared to when treated with T2 and T1. This indicated that different combinations of hormones had a differential effect on gene expression. During somatic embryogenesis, epigenetic regulation has been demonstrated to be significant in plants (Wójcikowska *et al*. 2020). Further a complex network of interactions regulates the expression of numerous genes, and several gene sets are activated or suppressed via different signal transduction pathways. The pattern of *OsSERK* gene expression increased with the combination hormone compared to T1 (control) in this study (Fig. 6).

In this study, the hormones were found to regulate endogenous or exogenous signals, including auxin, which upregulate the *SERK* gene. Additionally, expression of the SERK gene has been postulated to be involved in cellular reprogramming, the process by which a new developmental program is initiated in Arabidopsis (Schwessinger and Rathjen 2015). Adding auxin to the culture media causes high *SERK* expression in certain species, including *Medicago truncatula* (Nolan *et al*. 2003). In the presence of both NAA and BAP, *MtSERK1* expression is significantly increased. Additionally, BAP promotes NAA induction because BAP does not induce *MtSERK1* expression on its own. In addition, somatic embryogenesis and SERK transcription are induced in *M. truncatula* by adding auxin and cytokinin (Nolan *et al.* 2003). In *Cocos nucifera* (L.), cells detect the accumulation of 2, 4-D and activate a signaling pathway that induces the expression of *SERK* involved in somatic embryogenesis (Pérez-Núñez *et al.* 2009). In this study, the *SERK* family's function is possibly related to interactions within the complex network involved in somatic embryogenesis.

Meanwhile, *OsWOX4* and *OsLEC1* were absent or expressed at low levels (Fig. 6a–d, 7a–b). *LEC1* is a transcription factor and a master regulator of embryogenesis, and its expression is required for the development of somatic embryogenesis. Previously, it was shown that *LEC1* is active after embryonic development in Arabidopsis plants (Lotan *et al.* 1998). This gene may also be involved in embryo development because of reports of ectopic expression, the reason why the expression of this gene can influence plant growth in GogoNiti II on day 28 in T2 and T3. Moreover, several other studies have found that *ScLEC1* has not been detected in Secale L. after four weeks on regenerating medium (Gruszczyńska and Rakoczy-Trojanowska 2011). Previously it is reported that *LEC1* was highly expressed in somatic embryogenesis in *Coffea canephora* at day seven compared to days 14 and 21, which were low or almost undetectable. In addition, on days 14 and 28, *WOX4* was wholly absent or low in expression. This is because *WOX4* expression decreases during embryo maturation (Nic-Can *et al.* 2013).

Conclusion

The best combination hormones in this research was T2 (2

mg/L kinetin + 1 mg/L NAA), which produced more plantlets than other treatments. This combination of hormones also expressed the *OsSERK* during late somatic embryogenesis, whereas *OsWOX4* and *OsLEC1* were absent or expressed at low levels after the treatments in rice. These genes were expressed during late somatic embryogenesis on the days 28. The combination of hormones to plant morphology was relatively positive after 45 days in GogoNitiII in plant regeneration conditions.

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Author Contributions

MU and KMK: conceptualized the study, interpreted the results and responsible for the content and similarity index of the manuscript. MU and SN interpreted the result. SN planned the experiments and the practical study. SN, MAM and NR wrote the manuscript.

Conflict of Interest

The authors declare no conflict of interest.

Data Availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

Not applicable on this research.

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