International Journal of Frontiers in Technology
An Open Access International Journal

Journal home page: https://frontierstechnologyjournal.com
ISSN: 0000-0000 (in progress)

Research Article

Analysis of SERK1 Expression associated with Somatic Embryogenesis in Rice

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Publication history
Received:20 November 2024
Revised: 25 November 2024
Accepted: 30 November 2024
Available online: 30 November 2024

International Journal of Frontiers in Technology, 2024, 01(01), 16-31

Abstract

Somatic embryogenesis (SE) is used for rice improvement where in vitro production of somatic embryos are generated from somatic cells which later converted into whole plants. Finding embryogenic regenerable callus in certain developmental stages is the main step to achieve successful and efficient regeneration. Many indica rice has been shown to have recalcitrant in regeneration capacity; hence identification of embryogenic marker for regenerable competent cell/calli would be beneficial and fasten the processes. This study analysed the SERK1 expression in different calli which confer embryonic competence using semi-quantitative PCR followed by gene expression studies by realtime PCR. In this study, amplification of SERK1 gene from cDNA aged 21-days was successfully in all four rice varieties at approximately 200bp under study. The neighbour joining tree analysis showed those SERK1 genes of all varieties were similar to the SERK1 of *Oryza sativa Japonica*. The real-time PCR analysis revealed that SERK1 expression was highest from callus on treated MS media with 2,4-D at 45°C pre-heat treated seed of MR220, MR220-CL2, MR232, Bario. The study was also found that expression of SERK1 were highest between 21 to 28 days of callusing response and decreased after that. The profile of SERK1 expression in callus, vegetative organ and immature seeds were different in each variety. Our results indicated that SERK1 expression was associated with the induction of SE and closely related to PGR/ preheat during tissue culture, and the gene might play an important role in inducing callus.

Keywords

Rice callus; SERK1 gene expression; Real time PCR; Pre-heat treatment; MS media, 2,4-D.

1. Introduction

Somatic embryogenesis (SE) pathway has been established to improve rice regeneration capability. During SE, various changes involving morphological, biological and biochemical were occurred from induced tissue which influenced by various parameters. Despite different explant used, supplementations of nutrients, optimization of carbohydrates, carbon sources or plant growth regulators in the tissue culture system, selection of developmental ages in embryogenic callus which serve an early indicator for competency plays a critical role for efficient regeneration. Furthermore the younger the explants, there was highly potential embryogenic cell for conducting successful regeneration [1]. Regeneration of indica rice varieties from different ages of calli has been reported such as eight-week age calli for MR219, MR232, while three-weeks for Super Basmati rice [3]. Since then, these duration has been used as benchmark for indica rice regeneration studies by several researchers [2,4–6, 20] with indication of nutrition regime should be modify to achieve good regenerable capacity. The regeneration capacity was started with good selection of potential embryogenic calli; therefore, the analysis of SERK1 gene in different developmental age for embryogenic capacity through gene expression analysis was importance. Among the several genes involved in the regulation of rice somatic embryogenesis (i.e. SERK1, SERK2) are proteins involved in transforming somatic cells into embryogenic cells and has been known to be a unique gene in a wide variety of plant species [12–16] however, the analysis of SERK1 profile only have been reported in Indonesian rice variety [11]. Even though the cells have the ability to regenerate but these were affected by external factors with complex mechanisms regulated by different genes [32]; therefore, signalling molecules coordinate the different stages of regeneration remains unclear. Hence; the aim of this study was to determine the SERK1 gene profile in competent embryogenic calli on different pre-heated and plant growth regulators media that showing high regeneration capacity in previous work [17].

The SERK1 receptor gene has been shown to confer embryonic competence of cells in rice [8]. Gene expression studies by using reverse transcriptase polymerase chain reaction (RT-PCR) could confirm the identification of a specific gene and its expression [9]. However, this RT-PCR could not confirm the quantity of gene expression in a specific cell. Real time PCR is known to measure the kinetics of the reaction in the early phases and provides advantages over traditional PCR. By real time PCR, it was possible to quantify the somatic embryogenesis receptor kinase gene expression which is responsible for somatic embryogenesis in many plant species [7,10]. Finding the suitable parameters influenced SERK1 gene profile in an embryogenic competent cell can only show the highest potentiality of regeneration [11].

2. Results

2.1 Analysis of Somatic Embryogenesis Receptor Kinase Gene (SERK1) Expression by Reverse Transcription Polymerase Chain Reaction (RT-PCR)

To detect the SERK1 gene expression in different rice varieties, semi-quantitative analysis by Reverse Transcription Polymerase Chain Reaction **(**RT-PCR) was conducted. Based on Figure 1**,** the gel electrophoresis result of the cDNA synthesised product from the 21 days old calli of *Oryza sativa* (MR220, MR220-CL2, MR232 and Bario) showed that the mRNA transcript of SERK1 was successfully amplified and produce a single distinct band (Lane 1–4) approximately at 200bp. The present study agrees with other studies where SERK1 transcriptase was amplified ranging from 190 bp [10,19]. In this present study, amplified SERK1 gene indicated that the calli used may have potential of embryogenicity in all four tested varieties. The negative control (lane 5) indicates that no contamination from other DNA was recorded during PCR. The SERK1 gene sequences were then sequenced and analysed its homology in Gene bank (NCBI).

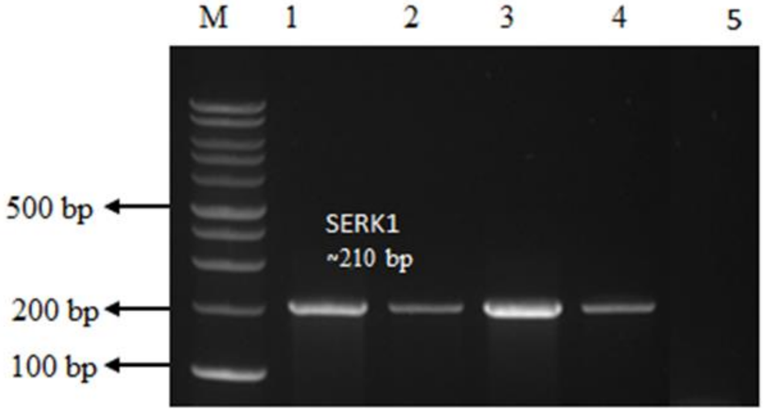

Figure 1. Agarose gel electrophoresis of SERK1 gene expression from cDNA of 21 days old calli of four *indica* rice varieties (MR220, MR220-CL2, MR232 and Bario). Product length with the 190 bp as a theoretically designed base pair. Lane 1: MR220 lane 2: MR220-CL2, lane 3: MR232, lane 4: Bario 5: negative control, M: 100 bp DNA ladder (Transgene).

2.2 Neighbour-joining Tree Analysis of SERK1 Gene

The evolutionary relationship between studies SERK1 (MR220, MR220-CL2, MR232 and Bario) and other SERKs were inferred by a Neighbour-joining tree (Figure 2) based on the alignment of nucleotide sequences.

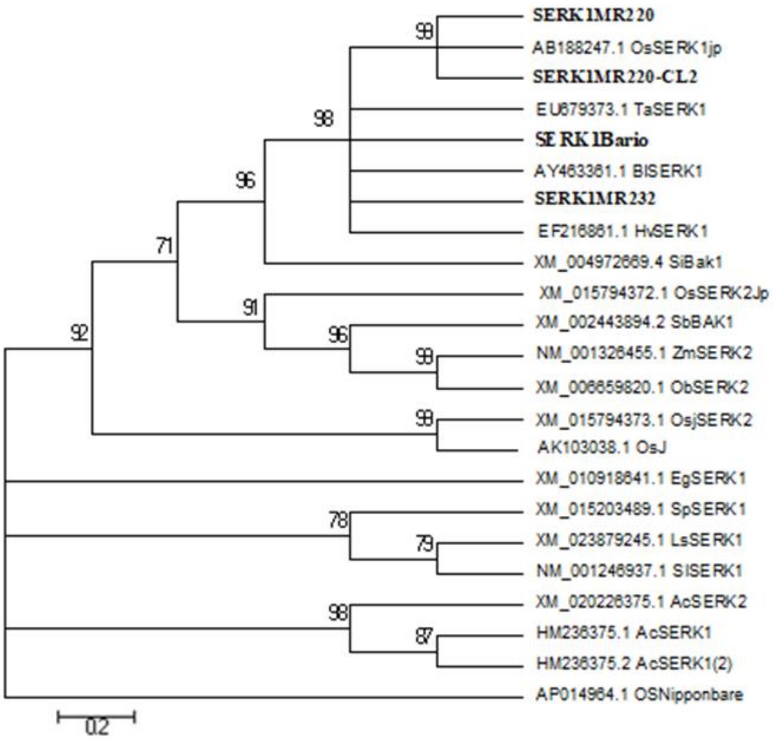

Figure 2. The Neighbour-joining tree (NJ) of SERK1 (MR220, MR232, MR220-CL2 and Bario) with other reported SERK1 sequences depicting the interrelationship with other SERKs. Bootstrap analysis with 1000 replicates was conducted in order to estimate the statistical supports of the topology of the consensus tree. % bootstop values are represented along the branch length and the values are shown next to the branches. Bold text indicates varieties tested from this present study.

In this study, alignment of the partial SERK1 gene sequence were similar to other known somatic embryogenesis receptor kinase genes from *Oryza sativa* species (Japonica, indica,) and other cereals (*Triticum aestivum*, *Hordeum vulgare*). In the present study, it was also revealed that the SERK1 shared the highest homology (99%) with that of SERK1 *O.sativa* japonica (Accession no. AB188247.1) and B1SERK1 indica (Accession no. AY463361.1) [8,61]. Within the tested varieties, MR220, MR220-CL2 were closely related with 99% homology while MR232 and Bario CL2 were closely related with 98% homology with each other. In this study, 18 classes of conserved SERK families were identified in monocot, whereas 11 classes were SERK1 families and 5 classes were SERK2 families based on previously reported SERK sequence-similarity by computational methods. The generated sequence established from this work was compared with published sequence taken from Genebank in terms of high similarity nucleotide for SERK1 (Figure 3). From the Figure 3, it was found that somatic embryogenesis receptor kinase gene1 of *Oryza sativa* japonica, *Oryza sativa* indica, *Triricum aestivum* were highly similar in term of percentages (98-99%) with studied sequence from MR220, MR220-CL2, MR232 and Bario varieties. Among the studied sequence, MR220 and MR220-CL2 showed 99% similarity where MR232 and Bario showed 98% similarity with each other. High sequence identity to another SERK1s family further confirmed SERK1 identity to present studied varieties (Sucharitakul et al, 2014).

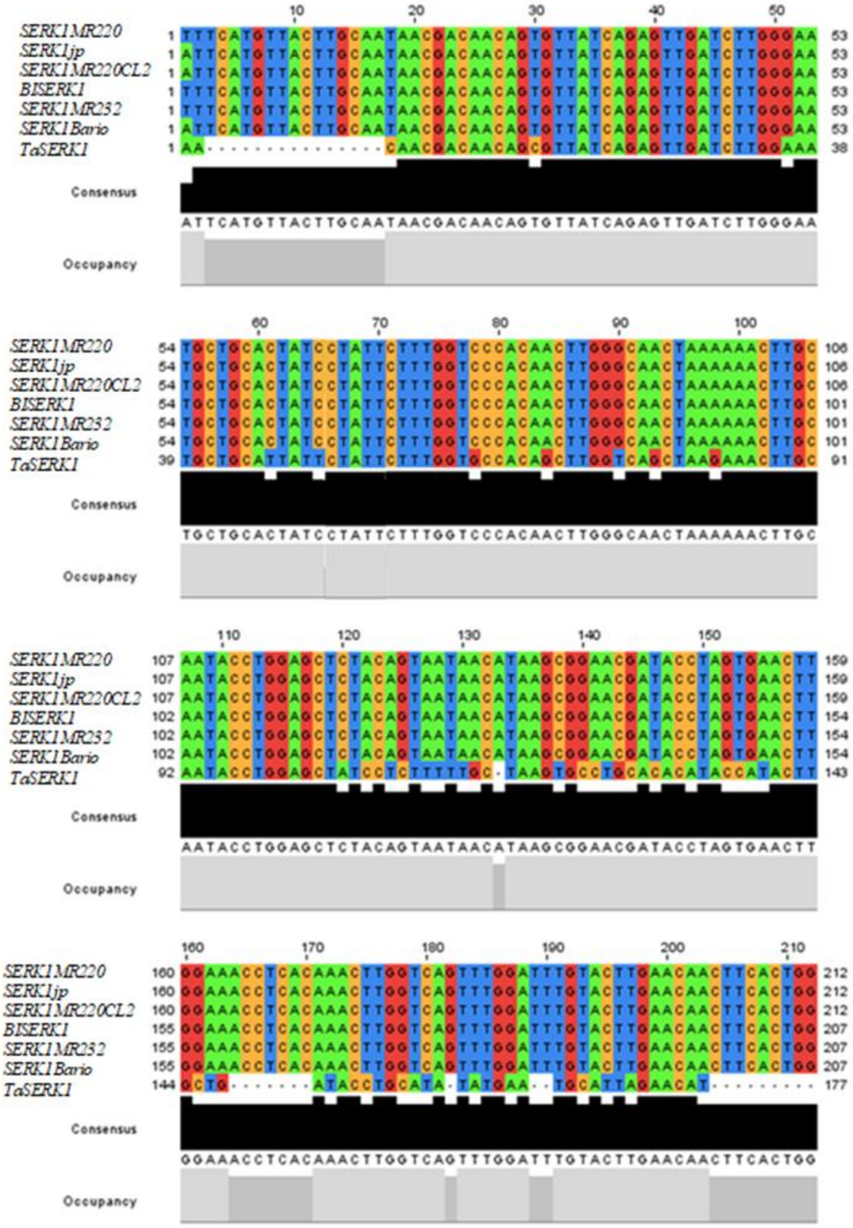

Figure 3. Comparison of pairwise sequence alignment of somatic embryogenesis receptor-like kinase (SERK1) gene of MR220, MR220-CL2, MR232, Bario to *Oryza sativa* japonica, *Oryza sativa* indica, *Triricum aestivum*.

According to Gonzalez and Pearson, [21; 22], similarity searching was considered reliable and effective only when the similarities of the sequences shared more than 70%, as it would represent the nucleotides that have common biological process and ancestry. In this perspective, the high similarity of SERK1 gene (99%) with that of SERK1 (BisSERK1 and OsSERK1 japonica) suggests that the amplification of the sequence of SERK1 from the cDNA of *Oryza sativa* indica (MR220, MR220-CL2, MR232 and Bario) were highly possible and reliable as well. The available SERK sequences of neighbour-joining tree were highly conserved. The significant number of plant species with SERK1 sequences confirming it as a SERK gene family (de Oliveira Santos et al, 2005). Several studies observed that most of the predicted SERK1 had regulatory roles during embryogenic development in different crop species such as; radish ([23], larch [24], 'Valencia' (*Citrus sinensis*) [16], cotton [25], peach [26] and barley [27]. Sindhujaa et al [7] reported that those SERKs were highly conserved in both monocot plants and dicot plants. In this study, neighbour-joining tree analysis shown that SERK1 analysis of tested varieties revealed that SERK1 was closely clustered with monocot plant species like wheat and barley [28,29]. However SERK1 expression of MR220, MR220-CL2 and MR232 and Bario were highly specific to *japonica* and *indica* rice subspecies [30]. So, in case of SERK1 in *Oryza sativa*, neighbour-joining tree analyses support its adjacent relation with other SERK1s rice varieties. High degree of sequence similarity suggested that SERK1 of MR220, MR220-CL2, MR232 and Bario could share similar function in somatic embryogenesis.

2.3 Expression of SERK1 gene by Real Time PCR

2.3.1 Effect of PGR on SERK1 Gene Expression

The relative expression of *Oryza sativa* of SERK1 gene from calli were obtained in MR220, MR220-CL2, and MR232 and Bario variety (Figure 4). In this present study, SERK1 was expressed in the embryogenic callus during induction on MS basal medium when supplemented with different plant growth regulators (2,4-D alone or combination of 2,4-D and NAA) but no expression found in control treatment as well as from MS media with NAA only treated calli in all four varieties tested. However, two peaks of expression of SERK1 transcript were occurred in 2,4-D alone or combination of 2,4-D and NAA treatments. The SERK1 expression was relatively higher in callus grew on MS media with 2,4-D compared to when 2,4-D combined with NAA in all varieties tested (Figure 4 a, b, c, d). However, there were no significant differences found in between 2,4-D and 2,4-D + NAA on SERK1 expression for MR232 (Figure 4 c). The first peak of SERK1 expression occurred in 2,4-D treated calli was observed in all tested varieties shown in Figure 4 (a, b, c, d). The second peak was observed in calli with 2,4-D + NAA containing a medium for MR220 (Figure 4 a), MR220-CL2 (Figure 4 b), MR232 (Figure 4 c) and Bario Figure (4 d).

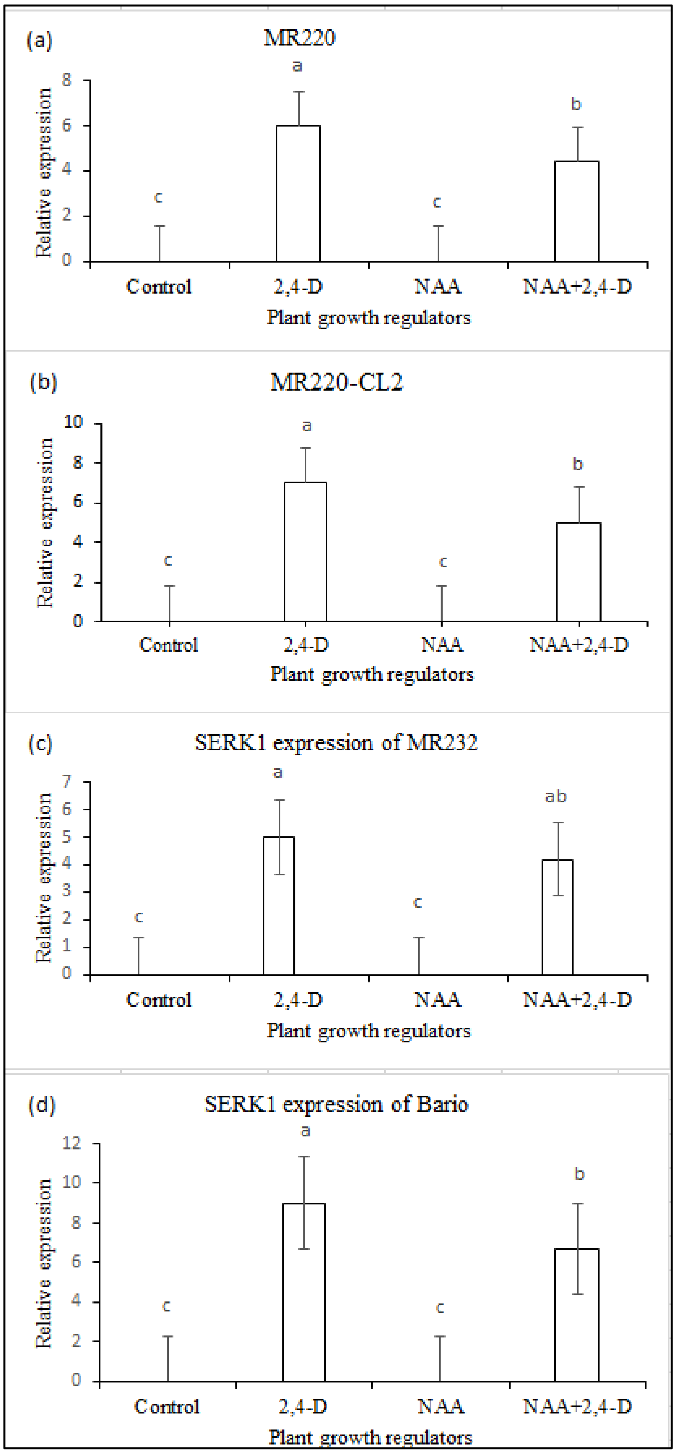

Figure 4: SERK1 gene expression (normalised relative expression-fold change) after 21 days age of calli cultured on MS media with different PGR (control, 2,4-D, NAA, NAA+2,4-D) in (a) MR220, (b) MR220-CL2, (c) MR232 and (d) Bario. The different letter indicates significant differences between treatments compared to control P≤ 0.005. (n= 3).

The result showed that SERK1 expressed was found only in calli that cultured on MS media with 2,4-D alone or 2,4-D combined with NAA treatments, this coincided performed in the appearance of calli with embryogenic potential, the peak of SERK1 can be initiated only in embryogenic cells (Rekik et al, 2013). The effect of plant growth regulators (PGR) such as 2,4-D, NAA and its combination on tissue specific expression and callus induction suggests the involvement of SERK1 in plant growth regulation pathway (Figure 4). The findings from the present study confirm that SERK1 exerts an intense effect on embryogenesis. This result was the same with MR219 and other rice types [10, 11], coconut [31] and passion fruit [26] where SERK1 expression only found in an embryogenic cell. Previous research indicated that PGR effected gene expression profile and the metabolic process in the plant tissue during calli induction of rice [11,32]. Other function of somatic embryogenesis receptor kinase (SERK1-SERK5) involve in the regulation of plant development and immunity [14]. Within these, SERK1 gene was expressed in the embryogenic cell under auxin treatment or other plant growth regulators stimulation during the process of somatic embryogenesis [29]. Nolan et al [33] observed that SERK1 expression induced by auxin and increased by cytokinin in the callus tissue of *Medicago truncatula*. In addition, Ma et al [34] also found that high level of SERK expression was effectively induced by 2,4-D and maintained at the early stage of somatic embryogenesis in pineapple. Also, SERK1 accumulation was triggered by 2,4-D supplementation studied in another monocot plant, *Cocos nucifera* [13]. This was in agreement with the present study where it showed that the peak of SERK1 accumulation was the highest from 2,4-D induced calli in all four tested rice varieties but lower when NAA was included as combined treatment. This showed that inclusion of NAA may supressed the SERK1 expression therefore the callus percentages. As stated by several researchers [7,35,36], auxin was responsible for embryogenic callus formation in both of monocot and dicot plant species. Within different types of auxin, 2,4-D was responsible for the formation of embryogenic calli in Malaysian indica rice variety [37]. Ito et al [8] also found that plant receptor kinase gene were involved in signal transduction pathway in somatic embryogenesis of rice which was induced by exogenous auxin 2,4-D. Moreover, another research stated that 2,4-D regulates embryogenicity of callus tissues during in vitro callus induction by the expression of embryogenic responsive gene SERK1 [38].

On the contrary, the relative expression of embryogenic calli of wheat was up-regulated in defence responsive gene TaSERK2 with the presence of 2,4–D rather than embryogenic responsive gene TaSERK1 [29]. The reason behind different statements could be the minimal information about the correlation between the molecular regulation of somatic embryogenesis and plant growth regulators. Another reason might be, though, several plant growth regulators e.g. 2,4-D, NAA, IBA, IAA, were responsible for callus induction and regeneration, their role somewhat different in different crops and species [39,40]. The molecular response to embryogenic callus formation have been studied intensively in dicotyledonous and monocotyledonous plant [41], while SERK1 gene was upregulated with the supplementation of auxin. Above findings also support the present studies as SERK1 accumulation occurred in media with auxin only treated calli. Moreover, the combination with 2,4-D and NAA induced calli showed SERK1 accumulation in Vigna sativa [7], which partially went in line with this work. In contrary, NAA treatment was upregulated the SERK gene in other plant of *M. truncatula* [42].

It was proven that in vitro callus induction was an important part for further plantlet regeneration [43]. The SERK1 gene was expressed in competent cells to form somatic embryos from early embryo development to globular stages in *Dactylis glomerata* L (Poaceae group) plant and subsequently formed an embryogenic cell from suspension culture where supplemented with 2,4-D [44]. Hecht et al [37] confirmed that SERK1 could enhance the ability of suspension cells to undergo embryogenesis by ectopic overexpression in Arabidopsis with auxin treatment [23]. Sah et al [45] found that different combination of 2,4-D and combination of 2,4-D and NAA were also responsible for producing competent embryos for rice regeneration. Auxin supplementation was a prerequisite to trigger embryogenic cell formation [18]. Zuraida et al [6] stated that only NAA supplementation on MS media could not produce any callus in Malaysian rice cultivar. The above statement was in agreement with the present findings, where no gene expression was found either in only-NAA supplementation calli or in control calli (without PGR).

Therefore, from this present investigation, it can be noted that SERK1 gene expression was strongly co-related with the supplementation of 2,4-D treated calli for Malaysian indica rice varieties (i.e. MR220, MR220-CL2, MR232 and Bario). Reasons behind this finding might be due to the presence of an embryogenic cell of calli that SERK1 expression occurred in an embryogenic cell in rice [38]. These embryogenic calli contain dense meristematic cells which involve in mitotic activity, this mitotic activity is responsible for for embryogenic calli production [13]. A previous research stated that embryogenic marker gene was specifically expressed in meristematic cells and proliferating tissues of oil palm [46]. Thus, it was well established that inclusion 2,4-D in culture medium was outweigh the benefit of NAA in inducing embryogenic callus in *Oryza sativa* cultivars. In contrary, the combination of NAA and 2,4-D enhanced SERK1 accumulation which not exceeds that of 2,4-D [19]. It was noticed that SERK1 accumulation was possible with the presence of NAA and 2,4-D, although this SERK1 accumulation was lower than that of only 2,4-D supplemented calli in the present study for MR220, MR220-CL2, MR232 and Bario rice variety.

2.3.2 Expression of SERK1 Gene on Pre-heat Treatment Seed Calli

Real Time PCR was performed with calli samples of different pre-heat treatments at 25 °C (control: without treatment), 35 °C, 40 °C, 45°C and 50 °C temperature with three days incubated seeds from MR220, MR220-Cl2, MR232 and Bario varieties. In this present study, relative expression (RA) of SERK1 showed significantly different ($P \leq 0.05$) peaks among all treatments for all tested varieties (Figure 5). The induction of calli from pre-heat treatment seeds at 45 ° C showed the highest peak compared to control (25 °C) as well as other treated seed-derived calli at 35 °C, 40 °C and 50 °C for all varieties except MR232 (Figure 5). For MR232, both of control and 45 °C showed higher peaks and 40 °C followed them. Within all treatments tested, 50 °C showed the lowest peak for SERK1 expression. These indicted that the expression of SERK1 were suppressed at high temperature. The high peaks (normalised relative expression of fold change) were tabulated at 45 °C (RA 34/45) for MR220, at 45 °C (RA 60/80) for MR220-CL2, at 45 °C (RA 12/14) and control (RA 11/14) for MR232, at 45 °C (RA 13/16) for Bario.

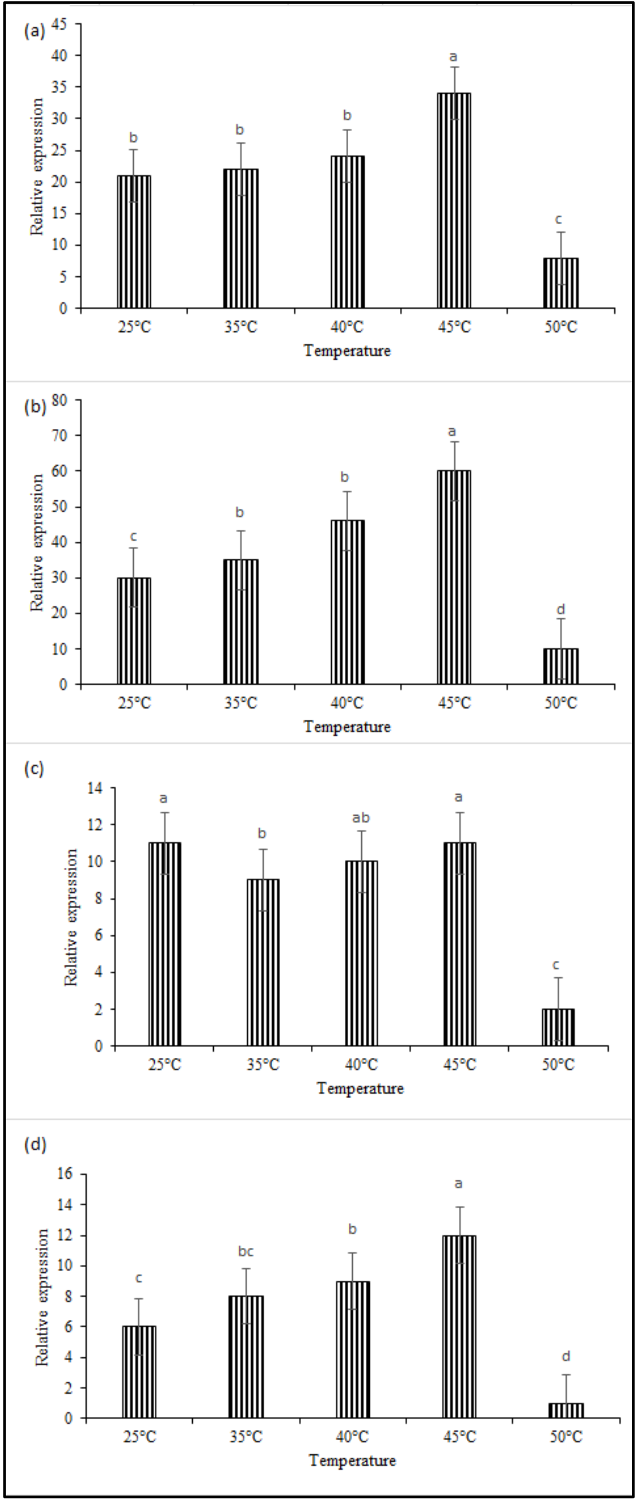

Figure 5: SERK1 gene expression (normalised relative expression-fold change) at 21 days of callus in (a) MR220, (b)MR220-CL2, (c) MR232 and (d) Bario in different temperature where 25°C used as control, 35°C, 40°C,45°C and 50°C of 3 mg/L 2,4-D. The different letter indicates significant differences between treatments compared to control $P \leq 0.005$. (n= 3).

To figure out the effect of pre-heat treatment on in vitro callus induction, SERK1 expression levels were measured by measuring fold changes of mRNA levels and to figure out cells which were more competent for SERK1 expression as several studies [10,16,29,31,47,48] and confirmed SERK1 as an embryogenic marker. Moreover, it was also observed that high SERK1 expression was found in embryogenic callus in rice [10,49]. This present investigation found that pre-heat treatment enhanced SERK1 accumulation in MR220, MR220-CL2 and Bario. However, accumulation of SERK1 showed the highest peak in parallel with both of 45 °C pre-heat treatment and control in MR232 with no considerable difference. Highly embryogenic callus can be formed by pre-heat treatment (35 °C) rather than that of non-embryogenic callus in a cereal crop spring wheat [50]. The above research was partially similar to MR220, MR220-CL2, and Bario in terms of pre-heat treatment. Although, the above studies were not carried out with SERK1 expression level. Singh and Khurana [29] also found that environmental condition was a major factor for embryogenic callus induction of wheat. Still, enough studies were not found on pre-heat treatment of rice seeds and SERK1 gene expression to figure out the relationship between heat treatment and embryogenicity, but SERK1 expression was highly influenced by 45 °C pre-heat treatment of tested rice seeds. More or less all of the pre-heat treatments of calli exhibit SERK1 accumulation in different levels, while 50 °C showed the lowest accumulation, thus higher temperature might have negatively affected on SERK1 accumulation.

It was found that the callus induction percentage (CIP) of MR220 (96%), MR220-CL2 (100%) MR232 (100%), and the Bario (95.7%) were significantly higher in pre-heat treatment seeds (45 °C at 3 days incubation) while 33-82% for control treatment after 21 days of culture [18]. The pre-heat treatments of 45 °C for all varieties, except MR232, were effective for SERK1 expression. The pre-heat treatment results of callus induction and SERK1 gene expression were in line with this present study. However, the result of expression of SERK1 for MR232 for both pre-heat treatment and control were identified as non-significant, which indicates that both treatment calli had possessed embryogenic potential. A calli sample can be considered as embryogenic potential according to the results of the SERK1 expression, callus induction percentages and callus morphology [17]. In that case, pre-heat treatment can be considered critical for further regenerable studies for MR232 variety.

In this study, it was found that there was a strong correlation between pre-heat treatment of callus and SERK1 accumulation. The SERK gene has been expressed during somatic embryogenesis during the formation of intact somatic embryos of maize [51]. Ma et al [34] confirmed that there was a SERK1 gene family in pineapple which could be used as a potential marker gene to monitor the acquisition of embryogenic competence. Previous research also stated that there was a strong correlation between the expression of SERK gene and somatic embryogenesis that had been proven in many other species, e. g. *Arabidopsis thaliana* [47], *Dactylis glomerata* [44], *Oryza sativa* [10], *Helianthus ananus* [52], *Ocotea catharinensis* [53]. Pre-treatment of the indica rice calli enhanced transformation efficiency by 77 % and transgenic shoots regenerated considerably faster upon pre-treatment of calli [54]. The present study on *in vitro* callus induction of rice in the aspect of pre-heat treatment reveals the effectiveness role for embryogenic calli formations of rice in MR220, MR220-CL2, and Bario variety. In this study, it can be a suggested that the pre-treatment of seed could be beneficial for successful callus induction and further regeneration potential upon SERK1 gene accumulation.

2.3.3 Expression of SERK1 Gene by Real Time PCR on Different Ages of Callus

In this study, SERK1 expression on various developmental ages of callus was shown on Figure 6. The normalized relative expression of SERK1 gene transcriptase from pre-heat treatment (45 °C) calli was conducted for MR220 (Figure 6 a), MR220-CL2 (Figure 6 b), MR232 (Figure 6 c) and Bario (Figure 6 d) which correlated with ages of calli. The profile indicates that SERK1 expression showed significant variations ($P \leq 0001$) among all tested ages for all rice varieties. It was also observed that all of the calli age were having potential of SERK1 expression with various fold change (0.3-3.6). Within these, 21 days old calli showed a significantly high peak in SERK1 expression in MR220 and MR232 varieties (Figure 6 a and c respectively), while both 21 days and 28 days old calli showed a significantly higher peak for SERK1 expression in MR220-CL2 and Bario. For MR220, the SERK1 expression was gradually decreased at the 35 days and 42 days. Also, 14 days old calli have shown a second higher peak for expression than 35 days and 42 days old calli.

According to Figure 6 b, high peak SERK1 expression was found in 21 days old calli of MR220-CL2 (3.4-folds) and 28 days (3.2- folds) old calli but decreased after that. While for MR232, 21 days calli showed significantly higher SERK1 expression and decrease started at 28 days onwards (Figure 6 c). For Bario, the expression level of SERK1 was the highest in 21 days and 28 days old calli which were significantly higher than others (Figure 6 d). In all varieties, SERK1 was the lowest in older calli (42 days).

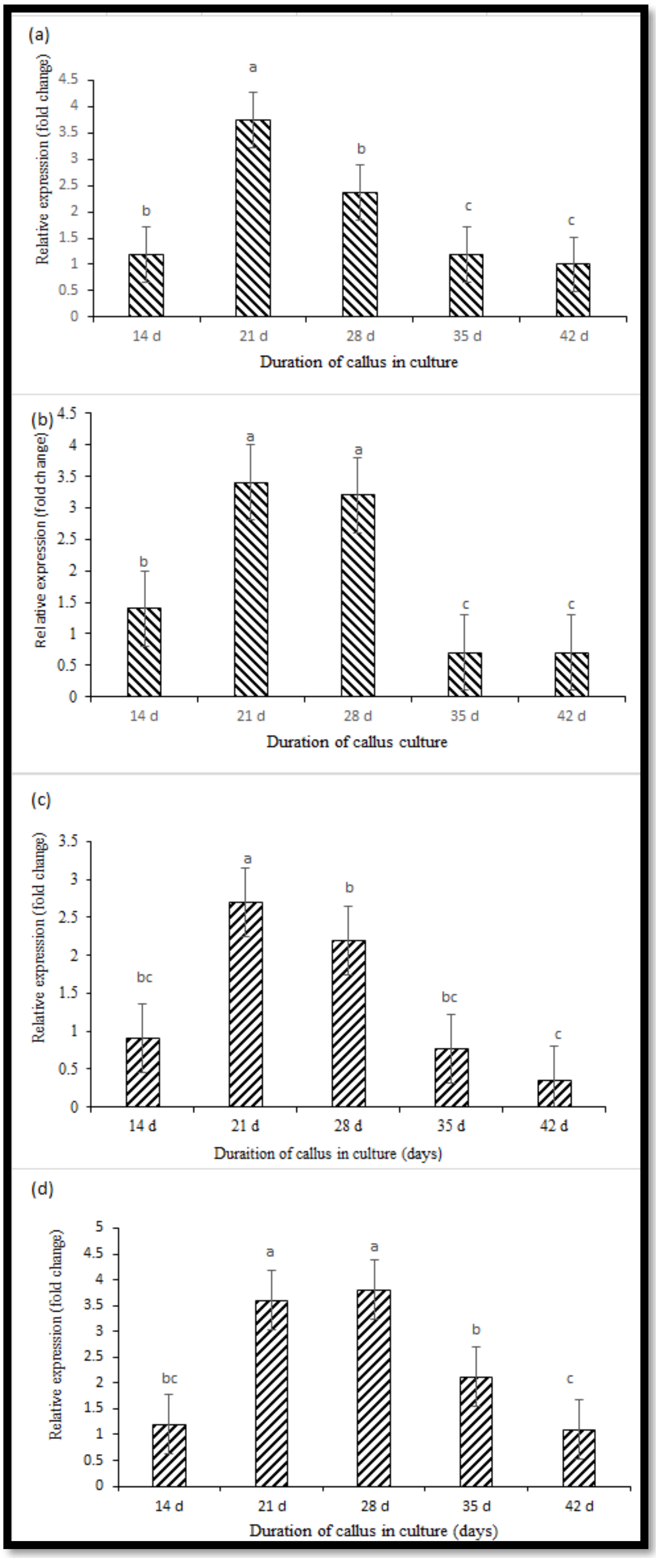

Figure 6: Real Time PCR expression profile of SERK1 expression at different stages of somatic embryogenesis of the variety (a) MR220, (b) MR220-CL2, (c) MR232, (d) Bario. Results are represented as the mean ±SEM.

In this study, SERK1 expression was started at 14 days of callusing, then increased at 21 days or 28 days depend on variety and later decreased with increasing days of callus culture. It was observed that accumulation of SERK1 expression was higher in 21 days old callus in all tested varieties while 28 days old were significant for MR220-CL2 and Bario. These indicated that highest SERK1 accumulation was only found during early callus induction stages of rice that indeed competent somatic cell. Previous research stated that SERK1 expression was started during the time of formation of globular somatic embryos which was happened between 21 days and 30 days of culture and during formation of proembryos at the very first step of callus induction of carrot according to Schmidt et al [15]. Singla et al [36] also observed the similar findings that SERK1 expression of wheat was upregulated in globular callus stage, it was evacuated that 21 days old embryogenic calli with white and globular in shape. Previous study had demonstrated that calli showed high expression of SERK1 were different according to monocot plant, e.g. between 15-21 days in *Cocos nucifera* [13], *Momordica charantia* at 28 days old calli [35] while 75 days old calli in *Coffea arabica* [11]. Other than monocot, SERK1 gene was only amplified from embryogenic globular and heart shaped callus of dicot plant *Vigna radiate* [7]. It was suggested that the early stage of calli were responsible to form competent cells for potential embryo formation during somatic embryogenesis compared to old aged callus tissue. The SERK1 gene have been revealed as an embryogenic marker for their direct role in induction of somatic embryogenesis for many plant species such as *Arabidopsis thaliana* [37], *Oryza sativa* [8], *Oryza sativa* subspp japonica [10], *Cocos nucifera* ([13], *D. carota* and *D. glomereta* [12]. Hecht et al [37] reported that a high amount of SERK1 accumulation was found in the embryogenic competence cells especially in the early somatic embryos. These results corresponded with the present observations for MR220, MR232, MR220-CL2 and Bario rice variety. On the contrary, Sucharitakul et al [56] stated that SERK1 can be able to express in stages during somatic embryogenesis but the high amount of SERK1 express in young calli or globular calli. The similar observations were also found in *Oryza sativa* Japanese rice [8]. In contrary, Karami [55] gave a different statement that SERKI expression was not strongly correlated with somatic embryogenesis because SERKI expression was also expressed in non-embryogenic callus of *Medicago truncatula*. The study showed SERK1 were expressed highly in those vegetative tissues (green spot, leaf) compared to immature seeds (Figure 7). For the green spot, results of relative expression analysis revealed high peaks of SERK1 expression in a green spot during somatic embryogenesis but not significance in all tested varieties.

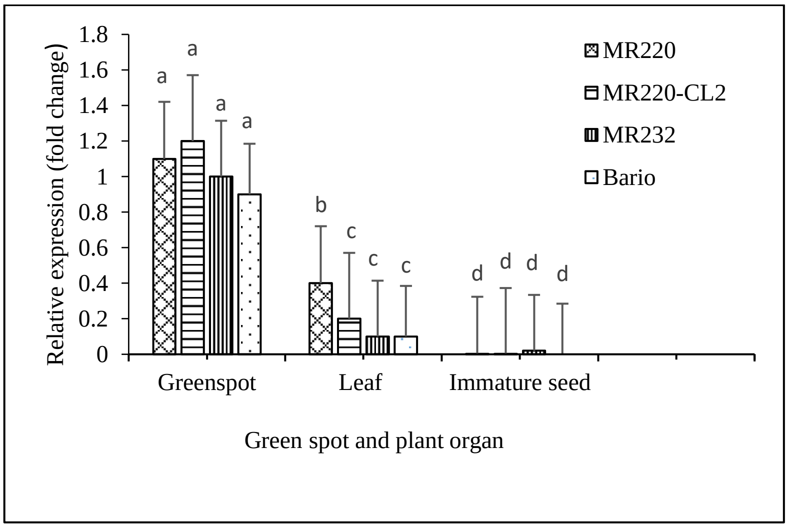

Figure 7: Real Time PCR expression profile of SERK1 transcript of different plant organs (i.e. green spot, leaf and immature seed) of different varieties of Malaysian *Oryza sativa*. Results are represented as the mean ±SEM.

As shown in Figure 7, SERK1 accumulation was found in green spot and leaf but very low in immature seed. Sindhujaa et al [7] showed that SERK1 can be expressed in different organs, but the expression level was only high in embryogenic callus and least with other rice organs [9]. The presence of SERK1 mRNA transcript was identified in the shoot apical meristem and seeds [44]. This statement supported this present study while the accumulation of SERK1 was found in a green spot which was later formed shoot apical meristem. However, very low accumulation or also ignorable amount of mRNA SERK1 was found in immature seeds of MR220, MR220-CL2, MR232 and Bario. The green spot that will developed into young leaf tissue might have a similar morphological characteristic, which triggered SERK1 expression in young leaf cell in the present study for MR220, MR220-CL2, and MR232 and Bario rice varieties. This was in agreement with the morphological characteristics of embryogenic competent cell evaluated by previous histology study [18]. This finding was also agreed with Ito et al [8] while low amount SERK1 expression was found in regenerating callus and panicle, however, it was high in embryonic callus in coconut [13]. The present study was in line with that of coconut plant but partially differed with Japanese rice. It can be summarized that SERK1 gene could be a potential marker for somatic embryogenesis in embryogenic callus tissue during in vitro tissue culture of Oryza sativa indica rice variety.

Table 1: Number of plantlet regeneration/ callus of four Malaysian indica rice on MS media with different regeneration media

Treatment (mg/L)	**Number of plantlet/callus**							
	MR220		MR220-CL2		MR232		Bario	
	Control	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**	**Control**	**Treatment**
RM1	4.0 c	4.0 c	4.0 b	4.0 c	4.2 b	4.2 c	4.6 b	4.6 c
RM2	5.0 b	6.0 b	4.1 b	4.1 b	5.1 b	5.1 b	5.4 a	5.4 b
RM3	4.5bc	6.0 b	5.3 b	5.3 b	4.2 b	4.2 c	3.9 c	4.2 c
RM4	4.0 c	7.0 ab	2.7 c	2.7 c	5.4 b	5.4 b	3.8 c	5.0 b
RM5	7.0 a	8.0 a	9.1 a	9.5 a	5.4 b	6.0 a	3.8 c	7.2 a
RM6	5.0 b	5.5 b	6.1 ab	6.1 ab	6.1 a	4.1 c	4.2 b	7.0 a
RM7	4.0 c	4.0 c	5.0 b	5.0 b	4.1 b	4.0 c	4.0 b	4.1 c
P (plantlet /callus)	0.002	0.001	0.001	0.001	0.042	0.043	0.033	0.045
P (Variety)	0.001	0.001	0.027	0.006	0.001	0.044	0.001	0.001

In the present study, the real time PCR analysis revealed that relative expression of SERK1 gene was significantly higher in 45 °C preheat treatment seed that formed callus when cultured on MS media for all four rice varieties. The SERK1 gene was detected as an embryogenic marker and these results were consistence with that of pre-heat treatment result in terms of callus induction percentage and morphological studies [18]. However, the result of different ages and plant growth regulators had shown that SERK1 accumulation proves to be an embryogenic marker for MR220, MR220-CL2, MR232 and Bario calli. The result revealed that calli induced from suitable PGR such as 2,4-D only showed high SERK1 expression compared to additional with another auxin, NAA. The gene expression studies of four tested Oryza sativa indica using the real time PCR analysis revealed that SERK1 accumulation was high in 21 days old calli of 45 °C pre-treated seeds cultured on MS media supplemented with 2,4-D. Finally, the results of the relative expression of SERK1 gene by using the callus of different ages were found to be highly significant expression at 21 days and 28 days.

3. Materials and Methods

3.1 Plant Materials and Media Preparation

Four Malaysian indica rice varieties (Oryza sativa L.) (i.e. MR220, MR220-CL2, MR232, Bario) were selected for this experiment and collected from MARDI, Penang, Malaysia. The rice seeds were cultured on the optimised MS media which was successfully identified [17]. The optimised media was MS media [57] with Gamborg vitamin [58] supplemented with 3 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) at 25 ± 2 °C in the dark condition, the above mentioned medium was used for pre-heat treatment seed culture.

2.2 Total RNA Extraction

All of the equipment used for the RNA isolation experiment were treated with diethyl pyro carbonate (DEPC) to remove the risk of Rnase (catalyses the degradation of RNA) and environmental contamination. A callus aged of 21 days was collected from pre-heat treatment at 45 °C at 3 days duration for all four varieties (i.e. MR220, MR220-CL2, MR232 and Bario). The calli were then used for RNA extraction and further cDNA synthesis. Total RNA of rice calli were extracted by using commercial kit (Qiagen Rneasy® Plant Mini Kit, Cat No./ID: 74904, USA) following manufacturer’s (Qiagen) instruction. The RNA was quantified by spectrophotometry by using Nano Drop 1000 spectrophotometer (ND1000, Thermo Scientific, USA). The data analyses of RNA quantity and purity (OD ratio) were conducted using SPSS software version 22.0 (SPSS, Chicago, IL, USA) where mean differences were evaluated using Tukey’s test. The differences were considered statistically significant when $P \leq 0.05$. Different means were marked with different letters (a, b, c) in order to represent the significant value of the differences.

2.3 RNA Integrity through Gel Electrophoresis and (cDNA) Synthesis

The integrity of RNA samples was verified by electrophoresis gel at 1% agarose (w/v) stained with Ethidium bromide. For 1% agarose gel (w/v), 0.5 g of agarose (Bioline) were used. After RNA extraction and cDNA synthesis, SERK1 amplified product by RT-PCR was also verified by gel electrophoresis.For SERK1 amplified product, the gel was run at 72 V with a current of 400 mA for 50 minutes within 1 x TAE buffer. One μg of total RNA was used for cDNA synthesize with 1st strand cDNA synthesis kit (QuantiNova Reverse Transcription kit, Qiagen) according to manufacturer’s instructions. The cDNA was later used for both of reverse transcriptase polymerase chain reaction (RT-PCR) and quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

2.4 SERK1 Gene Expression Study

21 days old pre-heat treated calli were collected for conducting SERK1 gene expression by using reverse transcription polymerase chain reaction (RT-PCR) to perform Neighbour-joining tree analysis studies. Four different PGR treatment were selected to examine the effect of plant growth regulators (PGR) on SERK1 gene expression, i.e. control was without any plant growth regulators, 2,4-D was 3 mg/L 2,4-D, NAA was 2 mg/L and NAA+ 2,4-D was 3 mg/L 2,4-D. All of the calli were selected at 21-days old age. Also, five different treatments at 21-days old calli were collected from pre-heat treatment at 25 °C (control used as room temperature), 35 °C, 40 °C, 45 °C, and 50 °C temperature at 3-days duration for expression of SERK1gene by real time PCR.

The optimised media (45°C at 3-days duration calli) were used to study the effect of SERK1 gene from five different ages (i.e. 14 day, 21 day, 28 day, 35 day, 42 day) by real time PCR. In addition, green spot (greening callus in regeneration media) collected from somatic embryogenesis of regeneration media at 14 days, immature seeds after day 45 when grains have been filled in inflorescence while immature leaves were collected for all four tested variety from 28 days plant. To examine the expression of SERK1 gene of *Oryza sativa indica*, a set of primers were used (SERK1-F: ATCTATCCATCGGATGTCAA, SERK1-R: CCATCTTGGGGCGTTCTGTG) for RT-PCR and real time PCR listed in Table 1. For RT-PCR in the present study, actin primer (rice) was used as a normalizer in both RT-PCR and real time PCR.

2.4.1 Reverse Transcription Polymerase Chain Reaction (RT-PCR)

Total extracted RNA was subjected to reverse transcription PCR, and actin primer (rice) was used as a normalizer for Oryza Sativa indica rice variety. The primers were: Actin Forward: 5´AACTGGGATGATATGGAGAA3´, Actin Reverse: 5´CCTCCAATCCAGACACTGTA3´[8]. The actin amplification was carried out by using the following profile: pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 1 minute, annealing at 54°C for 1 minute, extension at 72°C for 2 minutes and final extension at 72°C for 5 minutes. After normalization, SERK1 amplification was done by using the normalised product using SERK1 primer. The SERK1 primers were Forward:5´AACTGGATGATATGGGAGAA3, Reverse:5´CCTCCATCCAGACACCTGTA3´ [8]. The thermal cycler (Applied Biosystems, USA) profiles were: pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 1 minute, annealing at 59°C for 30 second, extension at 72°C for 2 minutes and final extension at 72°C for 5 minutes, and the PCR product was assessed on 1% (w/v) agarose gel electrophoresis. For SERK1 expression studies, 21 days old calli were selected from optimal pre-heat treatment (45 °C at 3 days duration). This study was carried out to explore the Oryza sativa indica MR220, MR232, MR220-CL2 and Bario variety regulation of somatic embryogenesis through detailed computational analysis (Figure 1). The PCR product was sent to 1st BASE Laboratories (Malaysia) for sequencing and the result revealed that the retrieved sequence of SERK1 contained the proposed SERK1 gene sequence at nucleic acid positions 1-210 (Figure 1).

2.4.2 Quantitative Reverse Transcriptase Polymerase Chain Reaction by Real Time PCR (qPCR)

The gene expression was analysed by quantitative real time PCR (qPCR) using QuantiNova SYBR Green PCR kit (Qiagen, USA) by using Rotor Gene Q (Qiagen, USA). The SERK1 primer was used for PCR amplification for this experiment (Table 6.1). The PCR master mix (Qiagen, USA) containing 1 µL of cDNA (100 ng) template and 10 µM of each qPCR primer (1 µL), 10 µL of 2× PCR SYBR Green PCR master-mixture and sterile water (7 µL to make the volume up to 20 µL. The 2-step polymerase chain reaction was conducted by using Real time PCR for gene amplification using the following profile: an initial denaturation at 95 ˚C for 2 min, followed by 40 cycles of 95 ˚C for 10 sec, combined annealing/ extension 55 ˚C for 30 seconds and melt curve analysis for five minutes from 50 to 95 ˚C. A melt curve analysis was included in order to confirm the amplification of the desired fragment. The gene expression was presented using the CT method [59]. The SERK1 expression was analysed by using the 2-ΔΔCT method [60]. The embryogenic calli were transferred to regeneration media (RM) in order to investigate their potential for regeneration from the calli of different varieties. The regeneration media were Due to having significantly high callus induction percentage, calli of four rice varieties from MS media supplemented with 3 mg/L 2,4-D and 3% maltose were selected for further regeneration process.

5. Conclusions

Our study showed that high SERK1 expression was observed from callus varieties under study that treated with MS media contained with 2,4-D; similarly for pre-treated 45 °C treated derived calli while decrease when imbibition temperature increased. Younger callus showed high SERK1 gene expression compared to the old one while accumulation of SERK1 was found higher in vegetative tissues compared to immature seed that associated with somatic embryogenesis.

Author Contributions: S.B.M. carried out the experiment and wrote the manuscript, A.W. conceived the project, partially wrote the manuscript, and supplied all of the reagents and plant materials.

Funding: This research was supported by the Universiti Teknologi Malaysia (UTM-Tier 1) under grant number; Q.J130000.2545.09H22.

Data Availability Statement: All research data are presented in this research paper.

Acknowledgments: The RNA isolations, Nanodrop reading, gel electrophoresis, PCR works were performed in the Plant Biotechnology Laboratory and Analytical Service Laboratory at Faculty of Science, Universiti Teknologi Malaysia. This work was supported by the Universiti Teknologi Malaysia (UTM-Tier 1) under grant number; Q.J130000.2545.09H22.

Conflicts of Interest: The authors declare no conflict of interest.

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