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Research article

Potassium fertilisation reduced late embryogenesis abundant (*LEA*) gene expression in Malaysian rice (MR220) under water stress condition

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Abstract: The application of potassium fertiliser might mitigate water stress effects in developing rice, thus influencing *Late Embryogenesis Abundant* (*LEA*) gene expression and growth in the plant tissue. This study was conducted to examine *LEA* gene expression in its drought tolerance mechanisms and the growth of Malaysian Rice (MR220) when exposed to water stress and potassium fertilisation. Three treatments were developed, namely the control (CF; Continuously flooding + 80 kg K_2O/ha), water stress under standard potassium fertilisation (WS; Water Stress 25 days + 80 kg K_2O/ha) and water stress under high potassium fertilisation (WSK; Water Stress 25 days + 120 kg K_2O/ha). The plant growth and yield components were measured for each treatment with randomly tagged plant by 3 replicates. The result showed that *LEA* gene expression on WSK was 36% less than on WS, thus indicating that the application of additional potassium fertilisation on MR220 rice might mitigate the water stress effect imposed on this plant. The study showed that high *LEA* gene expression in WS was accompanied by a reduction in plant growth and yield performance, such as plant tillers, height, number of leaves and grain yield compared to the control and WSK.

Keywords*:* rice (*Oryza sativa*); *LEA* gene; water stress; potassium; drought tolerance

1. Introduction

Rice (*Oryza sativa*) is a crop which is exposed to many environmental stresses. Inadequate water leading to drought stress is a common problem in upland cultivation systems. On average, rice needs 5,000 L of water to produce 1 kg of grain [1]. More than half of the 40 million hectares of rain-fed lowland rice worldwide suffers from water scarcity at some growth stage [2]. Drought stress reduces the rice growth, and severely affects the seedling biomass, photosynthesis, stomatal conductance, plant water relations and starch metabolism [3]. Depending on timing, duration and severity of the plant water deficit, the grain yield of some rice genotypes could be reduced by up to 81% under drought [4]. The application of potassium fertilisation has been reported to induce tolerance of plants to osmotic stress [5]. Potassium increases the plants' drought resistance through its functions in stomatal regulation, osmoregulation, energy status, charge balance, protein synthesis, and homeostasis [6]. In plants coping with drought stress, the accumulation of K^+ may be more important than the production of organic solutes during the initial adjustment phase, because osmotic adjustment through ion uptake (such as K^+) is more energy efficient [7].

One of the strategies used by plants to overcome water stress is producing Late embryogenesis abundant (LEA) proteins. LEA proteins were initially discovered accumulating late in the embryogenesis of cotton seeds 25 years ago. LEA proteins were divided into several groups based on their different sequence/patterns or biased amino acid composition [7–10]. In addition, it was assumed that the majority of LEA proteins were hydrophilic due to biased amino acid composition [11] and natively unstructured, while some were in a folded structure. These proteins were also found accumulating in plant tissue that was exposed to environmental stress. LEA proteins play an important role in the dehydration tolerance mechanism as well as responding to cold and salinity stress [4,12–15]. The expression of the *LEA* gene is generally induced by accumulation of ABA and abiotic stress conditions such as cold and salinity stress, either in reproductive or vegetative tissues [8,16–19].

Previous research studies have shown a small gap overlap between the gene's expression in vegetative tissues and in seeds, with a high level of expression found in seed tissues. A few papers have studied the functional properties of LEA proteins and shown that Arabidopsis dehydrin *ERD10* binds to more water during drying than non-LEA control proteins [20,21] and the LEA proteins stabilise the dry sugar glasses on *Typha latifolia* [22] and Soybean [23]. These data indicate that LEA proteins are involved in cellular stabilisers during stress conditions. There exist a few reports on the expression of *LEA* genes of rice and other plants under environmental stress. However, there has been no documentation of *LEA* gene expression of rice under water stress, especially under potassium fertilisation to mitigate the drought effects. This information is important, and will be useful in rice cultivation under limiting water resources. It was hypothesised that the use of additional potassium fertilisation could mitigate the water stress effects by increasing rice tolerance to water stress by reducing the expression of the *LEA* gene and that this fertilisation would lead to better growth. A total of three treatments were involved, and these were arranged according to a completely randomized design (CRD), namely control (CF; Continuously flooding + 80 kg K_2O/ha), water stress under standard potassium fertilisation (WS; Water Stress 25 days + 80 kg K_2O/ha) and water stress under high potassium fertilisation (WSK; Water Stress 25 days + 120 kg K_2O/ha). The aim of the present work was to study the *LEA* gene expression in,

and the growth of, Malaysian Rice (MR220) exposed to water stress and potassium fertilisation; the purpose of this was to relate the expression of the *LEA* gene with the growth and yield of MR220 rice.

2. Materials and methods

2.1. Plant material and growth conditions

A particular Malaysian rice variety (MR220) was grown in pots in a glasshouse at 28 °C. At 15 Days After Sowing (15 DAS), the seedlings were transplanted into 9 new pots which were divided into 3 treatments, namely control (CF; Continuously flooding + 80 kg K_2O/ha), water stress under standard potassium fertilisation (WS; Water Stress 25 days + 80 kg K_2O/ha) and water stress under high potassium fertilisation (WSK; Water Stress 25 days + 120 kg K_2O/ha). Each pot contained 4 seedlings, and during the earlier transplantation days (15–29 DAS), the water in the soil media was maintained at 5 cm level. Later, at 30 DAS, all water stress treatments were applied. For water stress treatments (WS and WSK), the rice seedlings were exposed to 25 days without any watering. The potassium fertilisation and timing for potassium rate treatments are summed up in Table 1. Other major fertilisers, such as Urea (46% N) and Triose super phosphate (46% P) were added as normal fertilisation and applied at 120 kg N/ha and 70 kg P_2O_5/ha respectively. Standard procedures of rice growing culture were followed throughout the studies.

Table 1. Schedule of potassium fertilization during the experiment.

Treatments	Description	Potassium fertilisation schedule (muriate of potash; 60% K_20)
CF	Continuously flooding + 80 kg K_2O/ha (Control)	80 kg K_2O/ha fertilisation split into two phases: 30%—3 leaves stage (15 DAS) 70%—booting (55 DAS)
WS	Water stress 25 days + 80 kg K_2O/ha	80 kg K_2O/ha fertilisation split into two phases: 30%—3 leaves stage (15 DAS) 70%—booting stage (55 DAS)
WSK	Water stress 25 days + 120 kg K_2O/ha	120 kg K_2O/ha fertilisation split into two phases: 30%—3 leaves stage (15 DAS) 70%—booting stage (55 DAS)

2.2. Growth and yield

Determination of plant growth (plant height, number of tillers and number of leaves) and yield components (grain yield and straw biomass) was carried out on randomly tagged plants by 3 replicates for each treatment. Plant height was measured from the plant base to the tip of the plant leaves. Total tillers and leaves were counted on the same day that the plant height data were collected. Days to flowering was calculated from the days of sowing until the first appearance of a panicle. Grain yield per pot was obtained from the weight of filled grains calculated at 14% moisture. To find

the straw biomass, shoots were harvested by cutting about 2 cm above the soil, which involved excluding the grain. Following this, the samples were oven dried at 70 °C for 72 hours and weighed. Data were analysed via Proc ANOVA using SAS 9.2 (32). A mean separation test between treatments was performed using Least Significant Different. LSD and standard error of differences between means were calculated with the assumption that data were normally distributed and equally replicated.

2.3. RNA isolation and quality controls

With regards the isolation of total RNA, at 60 DAS, the fresh tissue samples from the plant leaves were pooled and homogenised under liquid nitrogen. Total RNA was isolated by using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Approximately 30 µg of total RNA was treated with Rnase-free Dnase. The purity and concentration of total RNA were measured using a spectrophotometer (Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies)). Only the RNA samples with 260/280 ratio (an indication of protein contamination) between 1.9–2.1 and 260/230 ratio (an indication of reagent contamination) greater than 2.0 were used for the analysis. The integrity of RNA samples was determined using agarose gel electrophoresis.

2.4. Quantitative RT-PCR (qRT-PCR) analysis

First-strand cDNA was synthesised by reverse transcribing 5 µg of total RNA in a final reaction volume of 100 µL using a Bioteke Super RT Kit according to the manufacturer's instructions. The PCR mixture contained 2 µL of diluted cDNA, 10 µL of 2xSYBR Green PCR Master Mix (Applied Biosystem, USA), 8 µL of nuclease-free water and 1 µL of each gene-specific primer in a final volume of 20 µL. The primer sequences are listed in Table 2. PCRs with no template control (NTC) for each primer pair were also performed. The real time PCR was performed by employing CFX96 Touch System and software (CFX Manager) by BIORAD. The expression on *18S rRNA* and *GAPDH3* was determined by PCR and gel electrophoresis to normalise the data. The qRT-PCR was performed with the reference genes *18S rRNA* and *GAPDH3*, while the cycle was as follows: 3 min at 95 °C , 40 cycles of 3 min at 95 °C and 1 min at 56 °C, melting curve analysis by 65 °C to 95 °C and hold stage at 10 °C. The procedure was carried out according to the manufacturer's instructions (CFX 96 Real Time System, Biorad). Two biological replicates for each sample were used for qRT-PCR analysis and three technical replicates were analysed for each biological replicate. The quantitative variation between different treatments was evaluated using the 2(-Delta Delta C(T)) method [24], and the amplification of 18S rRNA and *GAPDH3* were used as internal controls to normalise the data.

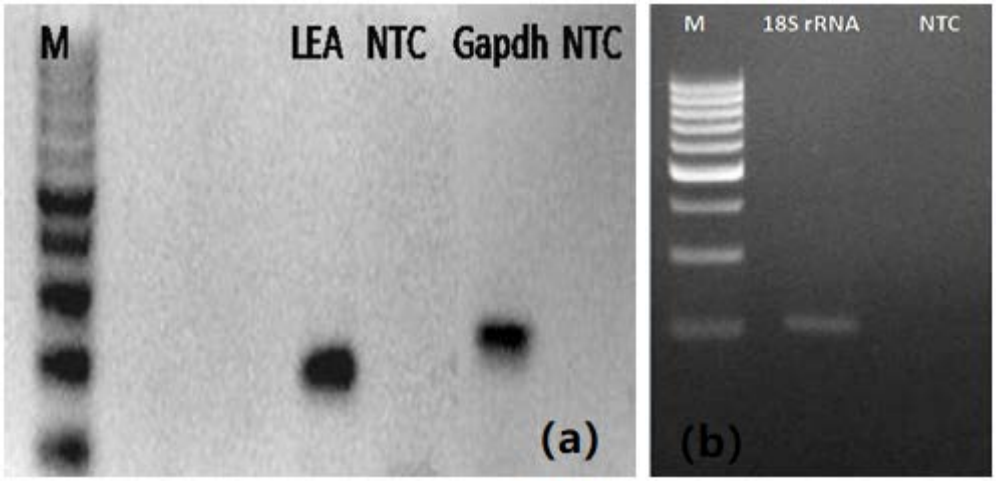

Figure 1. Agarose gel (1.5%) stained with Midori Green showing amplification of a specific PCR product of expected size for each gene in rice study. (a) on *LEA* and *GAPDH*; (b) *18S rRNA* (each sample lane is loaded with 20 µL of PCR product, while the marker lane is loaded with 2.5 µL of 50 ng/µL of DNA ladder) (NTC = No template control; *LEA* = *LEA 7*; *GAPDH* = *GAPDH3*; M = Fermentas 100bp DNA Ladder).

Table 2. Targeted gene (*LEA 7*) and reference genes (*18S rRNA* and *GAPDH3*) and their primer sequences used for real time PCR analysis.

Gene Name	Gene description	Forward primer (5' → 3')	Reverse primer (5' → 3')
LEA 7	Late Embryogenesis Abundant	ATCTGCTCCGCGTCAAC TAC	AGAGGTGCACGAAGA ACCAG
18S rRNA	18S ribosomal RNA	CTACGTCCCTGCCCTTT GTACA	ACACTTCACCGGACC ATTCAA
GAPDH3	Glyceraldehyde 3-phosphate dehydrogenase	GGT GTC CAA GAA GAC CCT CG	GAG GAT GCC TTG AGC TCG TT

3. Results and discussions

3.1. Plant growth and yield

The result indicated that the treatments significantly influenced the growth performance and grain yield ($p \leq 0.05$; Table 3), but no significant on straw biomass. This was shown by the significant difference in plant height, number of leaves, number of tillers, days to flowering, and grain yield (Table 3). Generally speaking, it was found that WSK was not significantly different from WS in terms of days to flowering, although the application of WSK was shown to reduce the water stress effect by producing higher plant height, a greater number of leaves and a greater number of tillers compared to WS treatments. In addition, WSK obtained more yield, up to 63%, compared to WS, thus minimising yield reduction compared to CF. This result showed that increasing the

potassium rate from 80 kg K_2O/ha to 120 kg K_2O/ha could minimise the water stress effect and enhance plant growth by shortening the number of days of flowering, optimising plant height, increasing the number of leaves and tillers, and enhancing grain yield under water stress condition.

When comparing WS to CF, WS plants showed to have fewer days of flowering, thus illustrating that the imposition of water stress can enhance the efficiency of assimilate partitioning, which reduce time to flowering in rice. Although under water stress condition time to flowering was reduced this could also caused a reduction in plant height and number of tillers, which would have been unfavourable for plant growth performance. The reduction percentage of plant height was approximately 7.45% under WS, and 4% under WSK compared to the control. The present data showed that the WSK treatments could mitigate water stress effect and sustain plant growth performance. It was found that the imposition of water stress for 25 days with 120 kg K_2O/ha in MR220 rice was able to mitigate water stress effects and showed the best practice in tackling water scarcity of rice cultivation in Malaysia. Similar results were also found by Hara [25], Khan [26], Lindhauer [27], Mohd Zain et al. [28] and Aown et al. [29], who showed that the vegetative growth of rice under abiotic stress can be improved with the application of potassium fertilisation.

Table 3. Growth and yield performance of MR220 variety as influenced by the treatments.

Treatments	Plant Height, cm	Leaves No./hill	Tillers No./hill	Days to Flowering	Grain yield (g/pot)
CF	102.89 ± 0.44^{a}	22 ± 0.33^{c}	5 ± 0.17^{a}	80 ± 1.25^{a}	91.403 ± 1.99^{a}
WS	95.22 ± 0.45^{c}	25 ± 0.44^{b}	4 ± 0.29^{b}	74 ± 1.20^{b}	45.907 ± 3.19^{c}
WSK	98.78 ± 0.89^{b}	29 ± 0.22^{a}	5 ± 0.22^{a}	77 ± 0.67^{b}	75.163 ± 2.94^{b}

*Note: Data are means ± standard error of differences between means. Means not sharing a common single letter were significantly different at $p \leq 0.05$.

3.2. RNA isolation and gene expression

The primers in the current study were designed with amplicon lengths of 70 to 150 bp, yielding primers with an 18 to 24 base and a melting temperature of 58–62 °C. *18S rRNA* and *GADPH3* were chosen as reference genes based on their stable expression in rice. *18S rRNA* was found to be a reliable reference gene for normalisation of qRT-PCR data in rice [30]. *GADPH3* was a suitable reference gene in measuring gene expression in sugarcane [31] and was compatible with rice, since rice is homologous to sugarcane. High-quality total RNA for each sample was isolated from leaves' tissue samples and reverse transcribed. Following this, for each biological replicate, the same cDNA pool was used for qRT-PCR analysis of each gene using gene-specific primers. Real time PCRs were performed in triplicate for each cDNA sample along with no template control in parallel for each gene. Agarose gel electrophoresis showed that *LEA 7*, *GADPH3* and 18S *rRNA* amplified a single PCR product of desired size from cDNA pools (Figure 1).

Real time PCR analysis is very sensitive, highly specific, and has a large quantification range to seven orders of magnitude [32,33]. Thus, it has become the most common method in evaluating gene expression. The relative expression of the *LEA 7* gene with *18S rRNA* and *GAPDH3* as the reference genes was determined using the 2(-Delta Delta C(T)), $2^{-\Delta\Delta CT}$method [24]. The results are shown in Figure 2. Amplification efficiencies for the *LEA 7*, *18S rRNA* and *GAPDH3* genes were previously shown to be in the range of 99.5–100.5%. The relative expression of the *LEA 7* gene was found to be

10.63- and 6.82-fold in WS and WSK respectively compared to the control (Figure 2). The result indicated that the induction of the *LEA 7* gene in leaves tissue of rice was influenced by water stress and potassium rates. The increase of potassium fertilisation from WS (80 kg K_2O/ha) to WSK (120 kg K_2O/ha) under water stress 25 days condition was shown to reduce the expression of the *LEA* gene in WSK. The present result is in agreement with previous work focused on the alleviation of water stress with potassium fertilisation in rice, sunflowers, olives, sugarcane and fava beans [34–39].

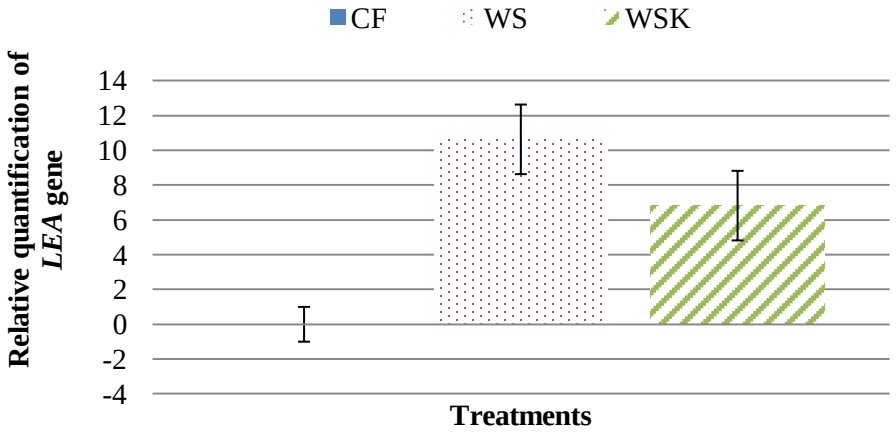

Figure 2. Relative quantification of *LEA 7* gene in the sample (WS relative to control sample and sample WSK relative to the control sample).

Under water stress, plants receive and transmit stress signals to a cellular defence mechanism. One of the mechanisms involved is induction of the LEA proteins, which play an important role in drought tolerance [40,41]. It can be concluded, based on the present result, that water stress does enhance *LEA* gene expression and reduce plant growth performance, although this can be minimised by applying additional potassium fertiliser to the plant, thus suggesting that potassium plays a role in minimising plant abiotic stress, and especially water stress.

4. Conclusions

The imposition of water stress for 25 days with additional 120 kg K_2O/ha (WSK) was proven to reduce *LEA* gene expression accompanied by high performance in plant growth and grain yield. Meanwhile, water stress for 25 days with 80 kg K_2O/ha (WS) was shown to lead to the highest induction of the *LEA* gene in tissues with a reduction in plant growth and grain yield. Therefore, increasing the amount of potassium fertiliser during water stress could reduce expressions of the *LEA* gene and thus act as one of the factors that could improve plant growth performance under drought condition.

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Conflict of interest

The authors declare no conflict of interest.

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