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# Evaluation of the heat stress response of the invertase enzyme family among select rice genotypes

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## Abstract
Heat stress is one of the most detrimental abiotic stresses to crop plants and causes considerable damage to the cell membrane, affects processes such as photosynthesis, respiration and many more. An increase in temperature beyond the optimum could result in reduction in the enzymatic efficiency thereby affecting various processes catalysed by them. Normal functioning of enzymes is very much essential to maintain cellular homeostasis. Invertase is one such enzyme which plays a crucial role in maintaining cellular homeostasis. Invertase catalyses the conversion of sucrose into glucose and fructose, which serves as fuel for respiration in plants. In the present study (based on the cues obtained from RNA Seq analysis of the rice varieties, CO51 and N22) we assayed the invertase activity in six (6) different rice varieties, in control as well as high temperature stress treatment conditions to understand the effect of high temperature on plant invertases. The results of the assay revealed that, an application of heat stress brings about a significant difference in invertase activity in all the tested genotypes. In all varieties, except MDU6 there was a significant increase in invertase activity after heat stress. N22 a globally accepted heat tolerant variety and Anna (R) 4, a drought tolerant variety exhibited high invertase activity both in control as well as heat stress conditions with a moderate increase after heat stress. Assaying invertase activity could be used as a tool for evaluating rice genotypes with heat and drought tolerance.

**Keywords:** rice, high temperature stress, invertase, homeostasis

## Introduction
“Climate Change” is the buzzword among Plant Biologists, because, an increase in the earth’s average temperature as a result of global warming (one of the components of climate change) is posing a serious threat to agricultural production worldwide. According to the IPCC report, the earth’s average temperature is expected to rise by 1.5 °C by the end of this decade (https://www.ipcc.ch/). Many studies have reported that the increasing temperature beyond the optimum, affects crop productivity and production (Challinor *et al.*, 2005) [1]. A negative effect of increasing temperatures poses a severe threat to global food security (Curtis *et al.*, 2014) [2]. Hence, plant breeders are working to develop climate resilient varieties to ensure global food security.

Rice, being a staple food for a majority of the world population, is one of the vulnerable crops to high temperature stress (Nguyen, 2002) [8]. With an optimum growth temperature of 25 °C – 35 °C, temperatures above 35 °C, especially during the reproductive stage, cause significant reduction in rice yield (Fujino *et al.*, 2004; Krishnan *et al.*, 2011) [3, 6]. Maintenance of cellular homeostasis (requires energy in the form of ATP; expression of genes involved in homeostasis as well as assimilation of gene products for ensuring unhindered biological activity) is a key to overcoming the negative effects of both biotic as well as abiotic stresses (Gull *et al.*, 2019) [4]. Energy in the form of ATP is produced by aerobic respiration where glucose molecules are channelled into the glycolysis followed by the Krebs cycle. One glucose molecule produces on an average of 38 molecules of ATP that provides necessary energy for maintenance of cellular homeostasis. Temperatures above the critical average, causes morphological as well as anatomical changes; damages cellular proteins, RNA and DNA; alters various molecular, biological and biochemical processes happening in a cell, thereby affecting cellular homeostasis (Kantidze *et al.*, 2016) [5]. Enzymes are highly susceptible for damage by increasing temperatures as the majority of them are heat labile. Enzymes, especially invertases, play a key role in cellular homeostasis by converting the reserve sucrose to glucose as well as fructose (Ruan *et al.*, 2010) [9]. Very few reports are available that have studied the effect of heat stress on the activity of invertases.

Breeding for climate resilient crop varieties requires a thorough understanding of the genes involved in imparting resistance / tolerance to the abiotic stresses. With the current advances in sequencing technology, it is possible to identify core sets of genes and their products that are differentially regulated upon abiotic stress. To understand the role of such genes in high temperature stress, RNA Seq based differential gene expression study was carried out in two rice varieties namely N22 (a globally accepted heat tolerant line) and CO51. Based on the preliminary results, it was observed that the topmost up-regulated gene was similar to an invertase enzyme in the heat tolerant variety N22. Based on this cue, the invertase family of enzymes was evaluated for differential gene expression levels. It was observed that the heat tolerant variety exhibited enhanced levels of transcripts of the invertase family of genes whereas, in CO51, there was a significant reduction (data not shown). This made us hypothesize that heat tolerant lines of rice may exhibit an increased level of invertase activity compared to that of heat susceptible varieties. In order to verify the above hypothesis, an attempt was made to characterize the invertase activity among a set of selected rice varieties. Here we report our findings of invertase activity among the rice varieties studied.

## 2. Materials and Methods

### 2.1 Growing of rice varieties

Seeds of six rice varieties namely, CO51, N22, MDU6, ASD16, Anna 4, and IR50 were sown on moistened germination paper in a Petri dish and placed in dark. Germinated seedlings were transferred to soil after 10 days (maintained in a pair of pots) and grown in an in-house greenhouse facility at the Department of Plant Biotechnology, CPMB & B, TNAU, Coimbatore. Then, after five days [15 days after sowing (DAS)], the potted plants were moved to the first environmental chamber (M/s. Genesis Technologies, Thane, Maharashtra, India) which was used as a control chamber. The settings adopted for growing the potted plants are as follows: 8/16 hours day-night cycle, 25/18 °C day/night temperature, 35 Klux of photosynthetically active radiation at the canopy level and 70-80% relative humidity. Plants were watered on a daily basis with Hoagland solution.

### 2.2 Heat stress experiment

On the day of heat stress (20DAS), one potted plant of each variety was shifted from the first to the second growth chamber, to give heat stress. The first chamber settings were not altered and the temperature was maintained as usual at 25 °C (Control condition - CC). The temperature settings of the second chamber were altered. The temperature was raised from 25 °C at 6.00 a.m. to 37 °C at 12.00 p.m. with an increment of 2 °C every one hour. Highest temperature of 37 °C was maintained for one hour (12.00 p.m. to 1.00 p.m.) and then the temperature was brought down to 25 °C by 6.00 p.m. by reducing 2 °C after every hour (Heat stressed condition - HS). The above temperature settings were adopted to simulate the natural environmental conditions where the highest temperature settings are expected to be around the mid day.

### 2.3 Collection of leaf samples for Invertase Assay

First two fully opened leaves from the top of every plant were collected from all the varieties at 6.00 p.m. from the controlled chamber (served as control samples) as well as heat treated chamber (Treatment samples), transferred in liquid nitrogen until storage at -80 °C. The collected leaf samples were subjected to invertase enzyme assay, the very next day after sampling. To verify the effect of heat stress on the activity of invertase family of enzymes, invertase enzyme assay (before and after heat stress) was carried using an invertase activity assay kit (Cat#: MAK118 - M/s. Sigma - Aldrich Chemicals, USA). Replicate assay (3 technical replicates per sample) was taken up for the analysis. The protocol described by the manufacturer was followed and the invertase activity was calculated by measuring the OD value.

### 2.4 Statistical analysis

Results of the invertase enzyme activity, under control (25 °C) and heat stress conditions (37 °C) from three technical replicates of all the varieties were subjected to statistical analysis as follows: Firstly, Levenes test for homogeneity of variances was performed for all the test varieties by comparing the absolute values of technical replicates at under control (25 °C) and heat stress conditions (37 °C). Then, an independent t-test was performed for comparing the mean values of invertase enzyme activity under control (25 °C) and heat stress conditions (37 °C). This was followed by all possible pair-wise comparisons using Tukey-Kramer Honestly Significant Test (HSD) to identify varieties that (which were exhibiting enhanced invertase activity) could be used for breeding for heat tolerance, using the globally accepted heat tolerant variety N22 as a check. The above tests were performed using the software SAS JMP Statistical Discovery V 10.0.

## 3. Results and Discussion

High temperature causes significant changes in the activity of enzymes that affect various molecular as well as cellular processes, in all living organisms. Invertase family, one of the key enzymes essential for maintaining cellular homeostasis, is also affected by high temperature stress. In order to study the effect of high temperature on the invertase activity, six rice varieties were evaluated at both control (25 °C) and high temperature (37 °C) conditions. Leaf samples of the rice varieties namely CO51, N22, MDU6, ASD16, Anna (R) 4, and IR50 were collected from both control as well as high temperature conditions (20 DAS) and were subjected to invertase assay followed by statistical analysis. The results of the invertase assay are reported in enzyme units where, “one unit of invertase is the amount of enzyme that generates 1.0 μmol of Glucose per min. at pH 4.5 at 37 °C”. The results of the assay are given in Table 2. Subsequently, an Independent t-test for comparing the means of invertase enzyme activity at 25 °C and 37 °C for every tested variety was performed. The assumptions underlying the t-ratio is that, higher the ratio, higher is the significant differences in the means of the tested groups viz., 25°C and 37 °C. The results are given in Table 2.

Levene’s test, a precondition for performing Independent t-test, was performed to verify the homogeneity of variances of the invertase enzyme activity at 25 °C and 37 °C across all the genotypes. Based on Levene's test, if the calculated p-value is greater than $\alpha = 0.05$, an assumption of equality of variances is made. The results revealed that all the tested genotypes, but for IR50, exhibited equal variances for enzymatic activity within technical replicates. Hence, IR50 was excluded from further data analysis.

Subsequently, an Independent t-test for comparing the means of invertase enzyme activity at 37 °C and 25 °C for every tested variety was performed. The assumptions underlying the t-ratio is that, higher the ratio, higher is the significant differences in the means of the tested groups viz., 25°C and 37 °C and vice-versa. We hypothesize that, in the context of plant breeding for heat tolerance, a variety with a lower t-ratio for invertase activity, i.e., stable expression of invertase enzyme at both 25 °C and 37 °C is preferred. The results are given in Table 2. Based on the t-ratio, it was observed that an application of heat stress brings about a significant difference in invertase activity in all the tested genotypes. Significant differences in enzyme activity included both up-regulation as well as down-regulation of invertase activity. It was observed that the invertase activity was increased to 14 - 16 Units per litre in all the tested varieties, upon high temperature stress, but for MDU6, the activity was reduced to 6.00 units per litre. Among the tested varieties, CO51 exhibited a very high invertase enzyme activity at 37 °C which was followed by ASD16. Though CO51 exhibited a very high invertase activity at 37 °C, it exhibited a very low invertase activity at 25 °C. On the other hand, the rice varieties, Anna 4, a popular short duration drought tolerant variety and N22, a globally accepted heat tolerant variety, exhibited only a very moderate increase of invertase activity at 37 °C. A comparison of invertase activity in the varieties Anna 4 and N22 revealed that even at 25 °C, invertase enzyme activity was on the higher side revealing the fact that invertases could be playing a major role in cellular homeostasis that confers drought and heat tolerance, respectively. A reduction in invertase activity in MDU6 makes us conclude that MDU6 could possibly be a highly heat sensitive variety that needs to be confirmed experimentally.

In order to identify the rice varieties that were performing on par with N22 and Anna (R)4, all possible pair-wise comparison were made using Tukey-Kramer Honestly Significant Differences test. The results of Tukey-Kramer HSD are given in Table 3. A cut-off mean value of 10.00 Units per litre was set and used to evaluate and identify varieties that were similar to N22 and Anna 4. Based on all pair-wise comparisons made, it was observed that only the rice variety ASD16 was performing on par with N22 and Anna 4 both at control (25 °C) and heat stress conditions (37 °C). This makes us hypothesize that ASD16 could be a potential rice variety besides N22 and Anna R4 for breeding rice varieties that are heat tolerant.

Under conditions of high temperatures, all the biological processes happening in a cell are altered resulting in an imbalance of various processes. Under such conditions, maintenance of cellular homeostasis is of utmost importance. Several enzyme families play role in maintaining cellular homeostasis and one such enzyme is the invertase family of enzymes that converts sucrose into glucose and fructose. Sucrose metabolism in plants provides energy for the cells to survive under heat stress conditions. The role of invertase in maintaining cellular homeostasis in rice spikelets under heat stress has been previously reported. In the present study, a similar observation has been made wherein increase of invertase activity after heat stress. The results of the present study reveal the possible role of invertase in maintaining cellular homeostasis during high temperature stress conditions.

**Table 1:** Varieties subjected for invertase assay.

| Variety | Parentage | Year of Release | Salient properties | Citation |
|---|---|---|---|---|
| N22 | A selection from Rajbhog (Nepal land race) | 1978 | Resistant to drought, heat tolerant | (Vikram *et al.*, 2011)[10] |
| Anna (R) 4 | Pantdhan 10 x IET 9911 | 2009 | Drought tolerant | https://agritech.tnau.ac.in/ Accessed on 24 November 2021 |
| ASD 16 | ADT 31 x CO 39 | 1986 | Resistant to Blast | https://agritech.tnau.ac.in/ Accessed on 24 November 2021 |
| IR50 | IR 21153-14 x IR 28 Y | 1989 | Resistant to RTV, GLH, BLB, BPH & SB | https://agritech.tnau.ac.in/ Accessed on 24 November 2021 |
| MDU 6 | MDU 5 x ACM 96136 | 2014 | Moderately resistant to leaf folder, stem borer, GLH and WBPH | (Namasivayam *et al.*, 2017) [7] |
| CO51 | ADT 43 x RR 272 -1745 | 2013 | Moderately resistant to Blast, BPH and GLH | https://drdpat.bih.nic.in/ |

**Table 2:** Invertase activity of rice varieties at control (25 °C) and high temperature (37 °C) stress.

| Variety | Invertase activity (Units per litre) | | | | | | | | Levene's test for equality of variances (p>0.05) | Independent t-test# |
|---|---|---|---|---|---|---|---|---|---|---|
| | 25 C | | | | 37 C | | | | | |
| | N | Mean | Std Dev. | Std. Err. Mean | N | Mean | Std Dev. | Std. Err. Mean | Sig. | t-ratio (37-25) |
| Anna R4 | 3 | 10.38 | 0.70 | 0.40 | 3 | 14.63 | 0.23 | 0.13 | 0.09* | 10.03 |
| ASD16 | 3 | 11.39 | 0.17 | 0.10 | 3 | 16.19 | 0.37 | 0.21 | 0.16* | 20.51 |
| CO51 | 3 | 2.37 | 0.13 | 0.08 | 3 | 16.43 | 0.32 | 0.18 | 0.10* | 70.96 |
| IR50 | 3 | 12.16 | 2.41 | 1.39 | 3 | 16.04 | 0.12 | 0.07 | 0.02 | NA |
| MDU6 | 3 | 15.32 | 0.18 | 0.10 | 3 | 6.00 | 0.74 | 0.43 | 0.16* | -21.16 |
| N22 | 3 | 13.16 | 0.42 | 0.24 | 3 | 16.11 | 0.42 | 0.24 | 0.88* | 8.62 |

*Homogeneity of variances are observed among the sample means at 25°C and 37°C based on Levenne's test ($P \geq 0.05$)
#Positive values indicate enhanced invertase activity whereas negative values indicate a reduction in invertase activity
N=Number of technical replicates

**Table 3:** Effect of high temperature stress on invertase enzyme activity.

| Variety | Growth condition | Mean Invertase Activity (U) per litre | Level A | Level B | Level C | Level D |
|---|---|---|---|---|---|---|
| MDU6 | 25 C | 15.32 | A | | | |
| N22 | 25 C | 13.16 | | B | | |
| ASD16 | 25 C | 11.39 | | | C | |
| Anna R4 | 25 C | 10.38 | | | C | |
| CO51 | 25 C | 2.37 | | | | D |
| CO51 | 37 C | 16.43 | A | | | |
| ASD16 | 37 C | 16.19 | A | | | |
| N22 | 37 C | 16.11 | A | | | |
| Anna R4 | 37 C | 14.63 | | B | | |
| MDU6 | 37 C | 6.00 | | | C | |

*Varieties not connected by the same letters are significantly different based on Tukey-Kramer HSD

## 4. Conclusion

The increasing atmospheric temperature due to climate change (global warming) is posing a threat to crop production. In order to meet the demand for increased food production under shrinking cultivable areas, productivity of crops has to be improved. Though a detailed study at the molecular level could throw light on various genes involved in contributing to heat tolerance, a preliminary study on the activity of invertases could enhance the success rate in the selection of truly heat tolerant lines. In our study we have investigated the role of invertase under heat stress conditions, and its expression level in some varieties. When the plants were subjected to high temperature there was an increase in the invertase activity, as a result of which more sucrose would be broken down to supply more energy for the plants to maintain cellular homeostasis. Thus, assaying invertase activity in plants could be used as biochemical markers for heat tolerance in rice varieties.

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