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Screening of QTL pyramided rice lines for thermotolerance by thermal induction response (TIR) technique

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Abstract
High temperature resulted from global warming will be one of a major issue for sustainable rice productivity across the world. Varietal improvement through marker-assisted QTL pyramiding and precise phenotyping are alternatives for supplying future rice demand. In this study, 29 rice QTL pyramided lines with *qSSP$_{F}$10* and *qHT6* derived from IR64/N22 cross was screened by temperature induction response (TIR) technique for their thermotolerance. Of these PLs, PL405, PL352, PL81, PL425 and PL76 had shown better thermotolerance. QTL pyramiding with a precise screening of segregants by TIR can be used as techniques for improvement of thermotolerance rice varieties.

Keywords: rice, thermotolerance, pyramiding, temperature induction respons

Introduction
Rice (*Oryza sativa* L.) is the primary source of food, nutrients, energy, and livelihood for more than 3.5 billion people which accounts to 60% of the world's population accommodating in Asia, Africa, and Latin America. Rice is being provided 27% of the world nutritional energy and 20% of overall nutritional protein for human (Bashir *et al*., 2007) [3]. This is the world's second most widely grown cereal crop after wheat (Ashikari *et al*., 2005) [2] that is being grown over an area of about 161.61 million ha in the world with an annual production of 728.06 million tons of paddy which provides 490.3 million tons, milled rice along with the productivity of 4.51 tons/ha in the world in 2018 (USDA, 2018) [21]. It is being cultivated under diverse agro-ecological conditions ranging from irrigated to rain-fed uplands, rainfed lowland and deep water. To fulfil the demand for increased population in 2025, rice production should be increased by nearly 40% of the present status (Sandhu *et al.*, 2014) [15]. Factors like soil moisture and favorauble day and night temperature is essential for the growth and productivity of all crops including rice cultivation. The climate change-related risks along with the stagnating yield of rice varieties are major current issues in the rice cultivation.
Climate change might be the result of increased atmospheric concentration of greenhouse gases and it will increase the earth surface temperature (IPCC, 2007) [8]. Increase in global average temperature projections ranges from 2-4 °C by the end of twenty first century (Vijayalakshmi *et al*., 2015) [25]. The day time temperature and night time temperature is directly and indirectly affected for reducing yield capacity of the crop (Sheehy *et al*., 2005) [18]. Temperatures greater than 35ºC at anthesis and lasting for more than 1hour can lead to high sterility in rice (Jagadish *et al*., 2007) [9]. International Rice Research Institute (IRRI) showed that rice grain yield declined by 10% for each 1ºC increase in growing season (Peng *et al*., 2004) [14]. About 41% of the rice yield reduction is expected at the end of 21st century due to high-temperature stress (Ceccarelli *et al.*, 2010) [4].
As a solution for climate change, climate resilience rice varieties should be improved through breeding. The most well-known method for improving the thermo tolerant varieties is to transfer superior alleles from thermo tolerant wild relatives. At present, this traditional selection approach combining with the molecular based breeding approach is being used for many of rice breeding programmes throughout the world for identification of genes, quantitative trait loci (QTL), marker assisted selection (MAS) (Liu, 1998) [10]. In the past, several QTLs were recognized for heat tolerance such as *qHTSF4.1*, *qHTSF9.1*, (Ye *et al*., 2015) [26], *qSF4* and *qSF6* (Cheng *et al*., 2012) [6]. Once the relevant QTLs are pyramided, a precise screening method is required to measure the variability in thermotolerance.

Various screening techniques are available based on specific physiological parameters such as single leaf photosynthetic capacity and quantification of chlorophyll fluorescence etc screen thermo tolerance at field level (Selmani and Wasson, 1993) [16]. The major limitation of these measurements is that they are highly influenced by environmental changes. As an alternative, scientists develop a laboratory technique for screening acquired thermotolerance of rice genotypes. From this view, a protocol called temperature induction response (TIR) technique has been developed and standardized for rice. The current study was conducted to screen a set of QTL pyramid rice lines by standardized TIR technique in order to develop thermotolerent rice lines.

Materials and Methods

The experiment was conducted at Molecular Laboratory, Department of Genetics and Plant Breeding, S.V. Agricultural College, Acharya N.G. Ranga Agricultural University, Tirupati (13°54' N *latitude* and 79°54' E *longitude*), India.

Plant material

High yielding IR64 developed at IRRI, the Philippines in 1985 was used as recipient parent and drought tolerant Nagina22 (N22) which is an upland heta tolerant rice cultivar selected from landrace Rajbhog in Nepal and was released by the Nagina Zonal research station, Uttar Pradesh, India in 1978 was used as the donor parent. (Ye *et al*., 2012). IR64 is a semi-dwarf, medium duration (112-118 days) *indica* rice variety. The average grain yield is about 8.76 t/ha under favorauble conditions. It has better grain filling ability, but less number of spikelets per panicle are observed (Mackill and Khush, 2018; Vikram *et al*., 2015) [12, 22]. IR64 is considered as a susceptible variety to heat stress at anthesis period (Ye *et al*., 2012). A cross was carried out between IR64/N22 to obtain the F_1 and subsequent F_6 population was generated with phenotypic selection. Phenotypically selected 29 rice lines, parents were and susceptible check BPT5204 were used as entries for molecular characterization and phenotyping by TIR.

Molecular characterization of pyramided rice lines

Leaf samples were collected from 15 days old seedling of IR64, N22 and all the 29 selected individuals from F_6 generation. The DNA was extracted using the modified CTAB method (Murray and Thompson, 1980) [13]. The purity and concentration of the isolated genomic DNA samples were estimated by loading in 0.8% agarose gel and Nanodrop spectrophotometer (ND-1000, USA). The final concentrations of DNA were adjusted to 100 ng/µl for PCR amplification. Four polymorphic SSR (Simple Sequence Repeat) markers between parents, RM6100 and RM6132 flanked *qSSP-$_F$10* related to seed setting (Xiao *et al*., 2011) [23] and RM190 and RM225 flanked *qHT6* related to flowering stage heat tolerance (Liu *et al*., 2017 [11] were used for screening rice lines. PCR amplification (Eppendorf, Mastercycler®Nexux, Germany) reactions were carried out for 10 µL reaction mixtures containing 1X PCR buffer with 25mM $MgCl_2$, 1 mM dNTP mix, 5 µmol/ µL of each primer, 100 ng of template DNA, 5U/µL Taq polymerase and MiliQ water to make up the volume. The following PCR profile was used for marker amplification: initial denaturation step at 95 ºC for 5 min, followed by 34 cycles of denaturation at 95 ºC for 30 s, annealing at relevant degrees for 30 s and primer elongation at 72 ºC for 1 min and then a final extension at 72 ºC for 10 min. The amplified PCR products were analyzed on 3% agarose gel stained with 10 mg/ml ETB at the voltage of 5 V/cm for 45 min. The gel was documented by gel documentation instrument (Alpha Innotech Cooperation, U.S.A) and the product size was determined by comparing with 50 bp ladder (0.1 µg/µl). During the gel scoring, the plants with N22 type of alleles in homozygous condition were selected.

Phenotyping of pyramided lines by TIR

The procedure standardized by Sudhakar *et al*. (2012) [19] was followed for the experiment. Rice seeds of phenotypically selected 29 rice lines, parents and susceptible check BPT5204 were washed with distilled water 2-3 times and were kept in petri plates (11 cm diameter) for germination at 30 ºC and 60% relative humidity. After 42 hours, 50 uniform seedlings were selected in each line and sown in aluminium trays containing blotter paper wetted with water. These trays with seedlings were subjected to sub-lethal temperature with gradual temperature increasing for every half an hour from 38 °C to 55 °C for 4 hours in the environmental chamber (Seed germinator, model-NLSg-200, Nanolab, India). Later these seedlings were exposed to the lethal temperatures (55 ºC) (induced) for 2 hours. Another subset of seedlings was exposed directly to lethal temperatures (non-induced). Induced and non-induced rice seedlings were allowed to recover at room temperature for one week. A control tray was maintained at room temperature, without exposing to sub-lethal and lethal temperatures. Seedlings were kept for recovery at room temperature for 72 hours at 60% RH. The reduction in growth percent has been estimated. Recovered number of seedlings in induced condition and number of seedlings in the control condition were counted. Root and shoot length were measured in seedlings which were in induced and control conditions. The following parameters were calculated from the seedlings according to Sudhakar *et al*. (2012) [19]. The data analysis was performed with ANOVA by IBM SPSS20.

$$\text{a) Percent seedling survival} = \frac{\text{Number of seedlings survived after recovery}}{\text{Total number of seedlings tested}} \text{X100}$$

$$\text{b) Percent reduction of shoot} = \frac{\text{Length of shoots in induced}}{\text{Length of shoots in control seedlings}} \text{X100}$$

$$\text{c) Percent reduction of shoot} = \frac{\text{Length of roots in induced seedlings}}{\text{Length of roots in control seedlings}} \text{X100}$$

Results and Discussion

N22 is a heat-tolerant variety and we found that it had both the tested QTLs. IR64 is one of the heat susceptible variety, hence we couldn't find any tested QTLs in IR64 genome. Based on the genotyping, some pyramided lines contained both QTLs while some had only one QTL either *qSSP$_F$10* or *qHT6* (Table 1).

Table 1: List of QTL pyramided line and parents with their QTL constitution

Pyramided line/variety	HT QTLs		Pyramided line/variety	HT QTLs	
	qSSP$_F$10	*qHT6*		*qSSP$_F$10*	*qHT6*
PL12		+	PL25		
PL16	+	+	PL28	+	+
PL41	+		PL46	+	+
PL59	+	+	PL76	+	
PL61	+		PL81	+	
PL67		+	PL130	+	+
PL93		+	PL153	+	+
PL101		+	PL221		+
PL118	+		PL273	+	+
PL167		+	PL405	+	+
PL208		+	PL425	+	+
PL352	+	+	PL457	+	+
PL419	+	+	PL476	+	+
PL424			PL488	+	+
PL495	+	+	N22	+	+
			IR64		

HT- heat, + mark indicates the presence of relevant QTL

TIR technique has been using to screen thermotolerant varieties of pea (Shrikanthbabu *et al.*, 2002), groundnut (Gangappa *et al.*, 2006) [7], sunflower (Senthil *et al.*, 2001) [17] and rice (Sudhakar *et al*., 2012) [19]. Under the heat stress, loss of cellular membrane integrity and imbalance between photosynthesis, respiration and enzyme degradation can be observed (Vierling, 1991) [24]. Plants get thermotolerance based on the cell-autonomous phenomenon due to prior exposure to a conditioning pretreatment (sub-lethal high temperatures). With this theory, rice genotypes were screened for high temperature tolerant using temperature induction response (TIR) technique. The TIR technique was validated for rice by few scientists including Sudhakar *et al*. (2012) [19] and Vijayalakshmi *et al*. (2015) [25]. In this technique, temperature stress develops gradually from sub-lethal levels to lethal levels of stress. Different levels of response events are obtained during sub lethal temperatures and show cellular protection at lethal temperatures (Abdullah *et al*., 2001) [1]. In the present study, 29 PLs of F_6 generation, parents IR64 and N22 and susceptible check BPT5204 were screened by TIR technique. After the recovery period, the parameters such as percent survival of seedlings, percent reduction in root growth and percent reduction in shoot growth were calculated and presented in Table 2.

Table 2: Screening of pyramided rice lines of F_7 generation for thermo tolerance through TIR technique

Gene pyramided line/variety	Percent survival of seedlings	Percent reduction in root growth	Percent reduction in shoot growth
PL12	100.00^a	78.17^a	51.93^b
PL16	100.00^a	81.37^a	49.62^b
PL28	100.00^a	73.50^a	42.59^b
PL41	100.00^a	80.63^a	49.08^b
PL46	100.00^a	83.68^a	49.58^b
PL59	100.00^a	75.14^a	54.61^a
PL61	100.00^a	83.07^a	46.18^b
PL67	100.00^a	78.71^a	42.05^b
PL76	100.00^a	69.82^b	43.94^b
PL81	100.00^a	67.99^b	43.11^b
PL93	100.00^a	76.58^a	33.60^b
PL101	100.00^a	74.62^a	29.50^c
PL118	92.67^b	85.42^a	40.81^b
PL130	100.00^a	71.85^a	36.94^b
PL153	100.00^a	81.17^a	36.97^b
PL162	100.00^a	73.71^a	45.87^b
PL167	100.00^a	81.32^a	52.14^b
PL208	100.00^a	72.48^a	40.13^b
PL221	100.00^a	80.20^a	41.69^b
PL273	100.00^a	76.09^a	52.73^b
PL352	100.00^a	55.25^b	31.94^b
PL405	100.00^a	52.38^b	33.94^b
PL419	100.00^a	76.32^a	41.30^b
PL424	100.00^a	82.57^a	45.93^b
PL425	100.00^a	69.51^b	25.69^c
PL457	100.00^a	73.15^a	50.92^b
PL476	100.00^a	86.10^a	48.06^b
PL488	100.00^a	82.99^a	43.39^b
PL495	100.00^a	77.84^a	52.70^b
N22	100.00^a	70.79^b	28.97^c
IR64	79.33^c	81.14^a	38.59^b

BPT5204	65.67[d]	80.81[a]	42.83[b]
CV (%)	0.63	10.11	13.51

Mean values followed by a different letters are statistically different at P≤0.05 (Turkey's test)

A significant low seedling survival percentage had shown at P≤0.05 level by PL118, IR64 and BPT5204. IR64 and BPT5204 are known as heat susceptible varieties. A significant low percent reduction in root growth was observed in several PLs such as PL405, PL352, PL425, PL81, PL76 and the donor, N22 which heat-tolerant QTLs provided. When seedlings are survived under severe stress, those plants can be considered as thermotolerant plants (Sun *et al.*, 2001) [17]. Vijayalakshmi *et al*. (2015) [25] experimented to evaluate previously reported heat tolerant landraces. Among the landraces, Apo and Norungan showed a mortality of 12 %, coupled with a less reduction in percent root and shoot growth when subjected to induction treatments. Thus their intrinsic thermo-tolerance was found to be comparable with that of N22. These lines confirmed their thermotolerance by higher chlorophyll stability index and a strong antioxidant enzyme system with lesser lipid peroxidation in terms of malondialdehyde content values. According to the results, N22, Apo, Norungan, Ottadiayan and Vellaikudaivazhai can be used as donors in breeding programmes in order to improve heat tolerant varieties (Vijayalakshmi *et al*., 2015) [25].

Heat susceptible varieties, IR64 and BPT5204 had shown high root reduction under the heat treatment. PL425, N22 and PL101 had shown low percent reduction in shoot growth as 25.7%, 28.9% and 29.5% respectively. Other PLs showed a reduction in shoot growth in the range of 31.9% to 54.6%. Well known heat susceptible varieties, IR64 and BPT5204 had shown the shoot growth reduction as 38.6% and 42.8%. Sudhakar *et al*. (2012) [19] screened 72 rice genotypes by TIR technique and NLR-40066, NLR-40070 and NLR-40050 were reported as thermotolerance varieties with 100% seedling survival and no reduction in root and shoot growth. In the present study, most of the PLs survived even under the lethal temperature. During the induction period, accumulated heat shock proteins (HSPs) induce several important physiological and biochemical parameters (Chen *et al.*, 1990) [5]. These changes are helpful when the seedlings exposed to the lethal temperature.

Among 29 PLs, PL405, PL352, PL81, PL425 and PL76 showed the highest thermotolerance in terms of 100% seedling survival, and less reduction in root and shoot growth ranged from 52.4-69.8% and 33.9-43.9%, respectively (Figure 1). Out of these lines PL352, PL405 and PL425 had both tested QTLs, *qSSP$_F$10* and *qHT6*. However remaining PLs also showed considerable level thermotolerance resulted from TIR. However, other theromotolerance PLs which does not has both QTLs may have another QTLs responsible for heat tolerance. These two tested QTLs are responsible for seed setting and flowering stage heat tolerance respectively. The TIR mainly tests the seedling stage heat tolerance and it might not directly correlate with flowering stage tolerance. However, in the present study, all the PLs were affected root and shoot growth by heat treatment with different percentages. The results are agreed with the results of Sudhakar *et al*. (2012) [19].

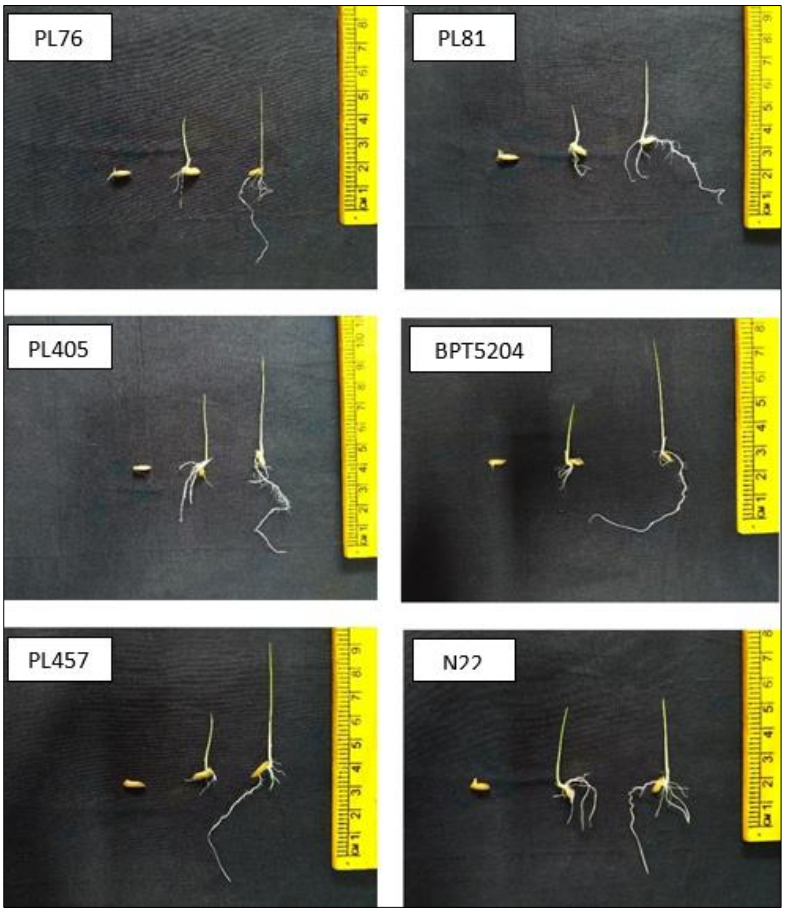

Plate 1: Root and shoot growth reduction under non-stress and heat stress conditions
Left- treated germinated seed under lethal temperature, Middle- Seedling formed from treated seed under sub lethal temperature, Right- Seedlings formed from treated seed under control condition

Conclusion

PL405, PL352, PL81, PL425 and PL76 showed the highest thermotolerance with 3 traits i.e. seedling survival, and less reduction in root and shoot growth. Therefore, heat tolerant related QTL pyramiding and precise screening by TIR can develop thermotolerant rice varieties.

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