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Research Article

Molecular screening of rice (*Oryza sativa* L.) genotypes for submergence tolerance

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Abstract
Rice (*Oryza sativa L.*) is one of the most consumed staple food crops, providing food to about half of the world population. Submergence stress is an important constraint in rice cultivation faced by rice growers globally. In Kerala condition, flood prone areas like Kuttanad, Pokkali, as well as low lying paddy fields are mainly affected by submergence. *Sub1* is the major quantitative trait locus (QTL) that confers submergence tolerance to rice genotypes. Earlier researchers have already identified the presence of *Sub1* gene in rice varieties showing submergence tolerance. Such rice genotypes are characterized by a higher survival rates and better yield even after 2 weeks of submergence stress. The advancement of marker assisted selection (MAS) have a striking impact on developing improved *Sub1* varieties which can meet the increasing demand for submergence tolerant varieties. The present study was planned to ascertain the rice genotypes having submergence tolerance by *in vitro* phenotyping followed by marker assisted selection. Genotypes KAUM 7 and KAUM 19 showed a better survival rates than other test genotypes during *in vitro* phenotyping. Among the ten genotypes evaluated for submergence tolerance with the help of SSR markers, KAUM 7 and KAUM 19 were identified with *Sub1* QTL. Besides, this finding lays the foundation for further researches on breeding for submergence tolerance in rice.

Key words: *O. sativa*, submergence tolerance, *Sub1* QTL, SSR marker

INTRODUCTION

Rice (*Oryza sativa L.*) is one of the most consumed cereal crop worldwide. During the period 1990 - 2025, world rice requirement is expected to rise by 1.7 per cent annually, which demands an additional 13 million tonnes of rice. Major constraints faced in rice cultivation by the farmers are various abiotic and biotic stresses. Biotic stresses can be controlled to an extent by using pesticides, and following proper cultural practices while abiotic stress is beyond the control of man. Experts have already disclosed their opinion that, the major challenge for future agriculture is climate change. This reflects the importance of research needed in developing genotypes that can tolerate abiotic stresses. Among various abiotic stresses affecting rice, submergence is a major constraint to rice production in South and Southeast Asia. Every year around 1/3rd of Indian rice fields are affected by submergence (Sarkar *et al.*, 2006). As reported by Jayan and Sathyananthan (2010), submergence is considered as the third most important abiotic stress next to drought and salinity which influence rice production.

Flash floods and redundant rainfalls frequently act on rain-fed lowland rice (RLR) ecosystems in several parts of the country, where flood water remains for around two weeks. Currently, the frequency of flooding has been elevated on account of global warming and other unpredictable severe weather conditions such as cyclonic heavy rains and inundation of tidal water. Though rice is the only crop adapted to lowland or submerged situation, when it is fully submerged for more than three days, it will simply

die because of oxygen shortage around rizhosphere that restrict root respiration. Kerala is a state having a large extent of paddy fields situated below mean sea level and has reflective problems of water-logging particularly in the first crop season (*Kharif*) which gets coincided with a south west monsoon.

Traditional crop accessions are reservoirs of unique genes which confer resistance to various biotic and abiotic stresses. In the early 1960s itself submergence tolerant rice varieties were identified and since the 1970s breeding efforts to introgress this trait into rice cultivars have been undertaken. Some of the indica rice cultivars, like FR13A, BKNFR and Kurkaruppan (Mazaredo and Vergara, 1982; Mohanty and Chaudhary, 1986) are highly tolerant and can survive up to fourteen days of complete submergence due to a major quantitative trait locus (QTL) designated as *Submergence 1* (*Sub1*) present near the centromeric region of chromosome 9. Tolerant cultivars like FR13A lack agriculturally important traits. However, they are a reservoir of many valuable genes that have been lost in cultivated rice due to genetic erosion. When the *Sub1*from FR13A was introgressed into a susceptible cultivar and the near isogenic lines showed restricted shoot elongation similar to FR13A (Fukao *et al*., 2006, Kar *et al*., 2017). Swarna-*Sub1*, the first submergence tolerant variety (Sarkar *et al*., 2006, Neeraja *et al*., 2007) was released in India, Indonesia, and Bangladesh in 2009-10 (Bailey-Serres *et al*., 2010, Voesenek and Bailey-Serres ., 2015).

Molecular markers have proven to be powerful tools in the assessment of genetic variation and in the clarification of genetic relationships within and among species. Molecular markers have been widely used for the identification of resistance genes and Marker Assisted Selection (MAS) has been applied for crop improvement by integrating different genes into rice cultivars lacking those (Hasan *et al.,* 2015). Among the DNA markers microsatellites or SSR sequences are particularly suited to discriminate closely related genotypes, due to their ability to detect a high degree of variability and polymorphism with the help of PCR. This study was carried out to identify the rice genotypes having submergence tolerance through *in vitr*o phenotyping and molecular screening by microsatellite markers. Entitled SSR markers (ART5 and Sub1 BC2) were used to identify the presence of *Sub 1* QTL in the selected rice genotypes. These SSR markers were reported to be closely linked to the *Sub1* locus which confers submergence tolerance (Neeraja *et al*., 2007).

MATERIALS AND METHODS

The present investigation was conducted at Rice Research Station, Vyttila, during the period 2017- 2018 to identify the field tolerant rice genotypes under submergence. The genotypes were also screened for the presence of *Sub*1 QTL using SSR markers.

The experimental materials used for the study comprises of ten genetically diverse genotypes (**Table 1**) of rice which were identified among the breeding lines at Rice Research Station, Monkombu, Kerala Agricultural University. Along with those ten genotypes one submergence tolerant and susceptible check were also included, they are Swarna-*Sub1* and Jyothi, respectively.

Table 1. List of rice genotypes used for the study

S.No.	Genotypes	S.No.	Genotypes
1.	KAUM1	7.	KAUM7
2.	KAUM2	8.	KAUM18
3.	KAUM3	9.	KAUM19
4.	KAUM4	10.	KAUM20
5.	KAUM5	11.	Swarna *sub 1*
6.	KAUM6	12.	Jyothi

A pot culture experiment was laid out in a completely randomised design (CRD) with 5 replications and each replication consists of 10 plants. Germinated seeds of 10 rice genotypes were sown in pots (length 17cm, diameter 18.5cm) filled with homogenized soil. The soil was filled up to a height of 13 cm within the pot. Plants were allowed to grow in normal growth conditions for two weeks. On the fourteenth day, the pots were transferred into a submergence tank (1m length x 3 m height x 1m depth) and submerged with 1 metre depth of tank water. Plant population, leaf number and plant height of 14 day old seedlings were recorded before submergence. The water depth was maintained at 1 metre height throughout the experiment. The fourteen days old seedlings were subjected to submergence of varying duration as 7 days, 14 days and 21 days and the following biometric observations like, pre submerged plant height, leaf count and post submerged recovery percentages were recorded. Survival percentage was estimated ten days after de-submergence. Statistical analysis was followed to interpret the results.

Molecular screening of rice genotypes using SSR markers

Table 2. List of primers used for molecular screening

S.No.	Primer	Forward primer	Reverse primer
1.	Sub1 BC2	AAAACAATGGTTCCATACGAGAC	CGCAACAAGGCAGAAAAATA
2.	ART 5	CAGGGAAAGAGATGGTGGA	TTGGCCCTAGGTTGTTTCAG

Ten test genotypes, submergence tolerant Swarna-Sub1 and susceptible Jyothi were raised in trays. For genomic DNA isolation leaves were collected from those plants when they reached about 21 days old. CTAB method developed by Murray and Thomson (1980) was followed for DNA isolation. Quantification of nucleic acids was performed by Nanodrop (NANODROP2000c Spectrophotometer, Thermo scientific) using the ND1000 spectrophotometer programme. The concentration of DNA was diluted to adjust the concentration to 25ng/µl for polymerase chain reactions. PCR analysis was carried out using 2 SSR primers (**Table 2**). PCR amplification was done by following the standard procedure in BIO RAD T100 Thermal cycler. To make out the amplicon size after PCR amplification, the PCR product along with 100 bp DNA ladder was allowed for polyacrylamide gel electrophoresis (PAGE). Finally, the silver nitrate stained gel was visualized in the BIO-RAD Gel Documentation System. The SSR allele sizes were determined by noting the position of bands relative to the DNA ladder.

RESULTS AND DISCUSSION

In order to understand the performance of selected rice genotypes during submergence conditions *in vitro* phenotyping was performed. Results of different biometric observations made are presented as the mean over five replications in **Table 3** (plant height and leaf count) and **Table 4** (post submerged recovery).

Pre and post submerged plant height showed significant differences among genotypes. Plant height of KAUM 7 and Swarna Sub1 was found to be the same on pre and post submergence conditions while in other genotypes, plant height was found to be increased on submergence. The increase in plant height varies from 0.8 to 2.4 cm between genotypes. Statistical analysis of pre submerged plant height suggested that, no genotypes have a height less than tolerant check Swarna-*Sub1*. KAUM7 and KAUM 19 were on par height or similar at least height after Swarna-*Sub1*. The increase in plant height of the tolerant lines was the same under submergence and normal conditions, while others showed elongation or increase in plant height, indicating acceleration in elongation of the intolerant line under the stress. These data confirm the presence of ethylene-responsive factor (ERF) gene *Sub1* that is responsible for submergence tolerance. The results are in accordance with the findings of Singh *et al.*(2001); Das *et al.*(2005) and Sarkar and Bhattacharjee (2011), that the *Sub1* haplotype suppresses elongation during submergence but does not influence plant height under normal conditions. Das *et al*. (2005) reported that genotypes with limited elongation during submergence, likely to use only a small quantity of available carbohydrate for elongation, thereby leaving carbohydrate reserve for survival after maintenance after submergence when flood water recedes. Leaf count of 14 days old seedlings showed no significant difference between genotypes as well as before and after submergence. All genotypes were similar with respect to leaf count.

Table 3.Plant height and leaf count of 14 days old presubmerged plants

Genotypes	Plant height(Mean)		Leaf count(Mean)	
	Pre submerged	Post submerged	Pre submerged	Post submerged
KAUM1	20.2^{d}	21.000^{de}	2	2
KAUM2	19.6^{d}	20.600^{e}	2	2
KAUM3	17.3^{e}	19.400^{f}	2	2
KAUM4	25.4^{a}	27.200^{a}	2	2
KAUM5	24.2^{b}	25.400^{b}	2	2
KAUM6	22.7^{c}	24.20^{c}	2	2
KAUM7	15.1^{f}	15.100^{g}	2	2
KAUM 18	19.4^{d}	21.800^{d}	2	2
KAUM 19	14.2^{g}	14.000^{h}	2	2
KAUM 20	24.6^{ab}	25.600^{b}	2	2
Swarna -*sub1*	12.8	12.800^{i}	2	2
CD(0.05)	0.923	1.022	Not significant	

Table 4. Post submerged recovery percentage

Treatment	Recovery percentage (Mean)	
	7 days submergence	14 days submergence
KAUM1	32	0
KAUM2	46	0
KAUM3	34	0
KAUM4	30	0
KAUM5	38	0
KAUM6	26	0
KAUM7	76	66
KAUM 18	46	0
KAUM 19	76	58
KAUM 20	58	0
Swarna -*sub1*	94	84
CD(0.005)	8.9934	3.2839

In the case of post submerged recovery percentage, significant differences were recorded among different genotypes for 7 days and 14 days of submergence.No test genotypes survived on 21 days of submergence. Swarna-*Sub1* showed the highest survival percentage in both 7 and 14 days of submergence *ie*., 94 and 84 per cent, respectively. Among the listed genotypes KAUM 7 and KAUM19 had partial submergence tolerance of 66 and 58 per cent, respectively. Hence, it is inferred that none of the test genotypes has complete tolerance as Swarna-*Sub1*. The best recovery percentage of 66 was shown by KAUM7, suggesting its partial tolerance to submergence followed by KAUM 19 (58%). The tolerance level of KAUM 7 was significantly higher than KAUM 19. None of the other genotypes survived under 14 days of complete submergence.

Ten rice genotypes were genotyped with SSR primer ART 5 and Sub 1 BC2 which were reported to be closely linked to the submergence tolerant QTL SUB1(Septiningsih *et al.,* 2009). The product amplified at 217 bp with ART 5 and 268 bp with Sub 1 BC2 as in the tolerant check Swarna-*Sub1* which was taken as the standard.

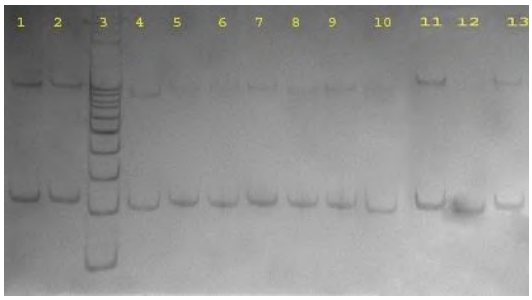

Fig. 1. Amplification pattern of 10 rice genotypes obtained by SSR marker ART5

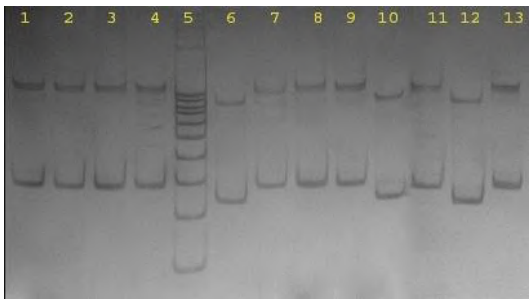

Fig. 2. Amplification pattern of 10 rice genotypes obtained by SSR marker Sub1 BC2

Lane 1- KAUM1, Lane 2- KAUM2, Lane 3- KAUM3, Lane 4- KAUM4, Lane 5- 100bp ladder, Lane 6- Swarna sub1, Lane 7 – Jyothi, Lane – KAUM5, Lane 9- KAUM6 , Lane 10- KAUM7, Lane 11- KAUM18, Lane 12- KAUM 19, Lane 13- KAUM20

ART5 and Sub1 BC2 were found to be polymorphic in test genotypes and checks used (**Fig.1**). Ten rice cultivars were genotypically screened out to determine the tolerance and susceptibility status of the *Sub1* gene using tightly linked Sub1BC2 *InDel* marker (**Fig.2**). The result indicates the presence of an approximately 268 bp fragment specific for Swarna-*Sub1* and approximately 230 bp fragments corresponding to the susceptible cultivar Jyothi. Then the rice genotypes were screened out by ART5 marker. Swarna-*Sub1* showed approximately 217 bp fragments in 8% poly acrylamide gel. Out of 10 rice genotypes, 2 genotypes, KAUM 7 and KAUM 19 have shown approximately similar band patterns of Swarna-*Sub1* for both the markers indicating the presence of the *Sub1* gene. In rest of the eight genotypes namely KAUM 1, KAUM 2, KAUM 3, KAUM 4, KAUM 5, KAUM 6, KAUM 18, KAUM 19 and KAUM 20 *Sub1*gene was absent.

From the current investigation to identify submergence tolerant genotypes it was revealed that, KAUM 7 and KAUM 19 showed a better survival rate than othertest genotypes during *in vitro* phenotyping. However, their performance was significantly different from the tolerant check Swarna-*Sub1*. Molecular screening revealed that two genotypes *viz.,* KAUM 7 and KAUM19 showed similar band patterns like that of tolerant check Swarna-*Sub1* and the remaining eight genotypes exhibited a similar banding patterns as that of susceptible check Jyothi. Hence, from the *in vitro* phenotyping and molecular screening, it was concluded that KAUM7 and KAUM19 were identified as submergence tolerant genotypes and the tolerance may be due to the presence of Sub1 QTL in their chromosome.

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