MOLECULAR SCREENING OF RICE GENOTYPES FOR SUBMERGENCE TOLERANCE

by

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THESIS

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DECLARATION

I hereby declare that the thesis entitled "**MOLECULAR SCREENING OF RICE GENOTYPES FOR SUBMERGENCE TOLERANCE**" is a bonafide record of research work done by me during the course of research and the thesis has not previously formed the basis for the award to me of any degree, diploma, associateship, fellowship or other similar title, of any other University or Society.

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Aryalekshmi A. S.

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LIST OF ABBREVIATIONS AND SYMBOLS USED

%	Percentage
µg	Microgram
µl	Microlitre
A_{260}	Absorbance at 260 nm wavelength
A_{280}	Absorbance at 280 nm wavelength
bp	Base pair
cm	Centimetre
cM	Centi morgan
CTAB	Cetyltrimethylammonium bromide
DNA	Deoxyribo nucleic acid
EDTA	Ethylene diamine tetra acetic acid
g	Gram
M	Molar
mg	Milligram
min	Minute
ml	Millilitre

mM	Milli molar
mRNA	Messenger ribonucleic acid
ng	Nano gram
°C	Degree Celsius
OD	Optical density
PCR	Polymerase chain reaction
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
rpm	Revolution per minute
sec	Seconds
SD	Standard Deviation
sp.	Species
SNP	Single Nucleotide Polymorphism
SSR	Simple sequence repeat
TBE	Tris-Borate EDTA buffer
TE	Tris-EDTA buffer
Tm	Melting temperature

via	Through
V	Volt
A	Adenine
ADH	Alcohol dehydrogenase
ANOVA	Analysis of variance
APS	Ammonium per sulphate
ATP	Adenosine triphosphate
C	Cytosine
Cd	Critical difference
CO2	Carbon dioxide
ERF	Ethylene Responsive Factor
FR	Flood Resistance
G	Guanine
GA	Gibbberellic Acid
LDH	Lactate dehydrogenase
MAS	Marker Assisted Selection
NADH	Nicotinamide adenine dinucleotide

O_2	Oxygen
PDC	Pyruvate decarboxylase
Pro	Proline
QTL	Quantitative trait loci
ROS	Reactive Oxygen Species
Ser	serine
SLR1	Slender rice1
SLRL1	Slender rice 1 Like
Sub1	Submergence 1
T	Thymine
TCA	Tricarboxylic acid
TEMED	Tetra methyl ethylene diamine

INTRODUCTION

1. INTRODUCTION

Rice (*Oryza sativa* L.) is a primary food crop and an excellent genetic monocot model amongst the cereal crops (Cantrell *et al.*, 2002). Rice production is concerned by various abiotic stresses and climate change. Submergence is considered to be the third most important abiotic stress influencing rice production, next to drought and salinity. About 1/3rd of India's rice area is affected by submergence (Sarkar *et al.*, 2006). Flash floods and redundant rainfalls frequently act on rain-fed lowland rice (RLR) ecosystems in several parts of the country, where flood water remains around two weeks. *Kharif*season rice grown in India, recurrently gets submerged during seedling and vegetative stages, and goes through ample yield losses. However rice is the unique cereal crop that is well adapted to the conditions of water logging, immoderate flooding confines growth and yield. Currently the frequency of flooding have been elevated on account of the global warming and other unpredictable severe weather status 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. Water logging hampers root growth and function because of oxygen shortages that restrict root respiration. The concentrations of potentially toxic compounds increase in anoxic soils, and these can enter through roots, damaging both root and shoot tissues. Upon re aeration after a period of oxygen deprivation, ethanol trapped in tissues will be converted to acetaldehyde, causing post-anoxic cell injuries. Reactive oxygen species (ROS) also accumulate excessively upon oxygen deprivation or re-oxygenation under light conditions (Kirk *et al.*, 2014).

In Kerala, significant range of paddy cultivation situated below mean sea level and has reflective problems of water-logging (Jayan and Nithya., 2010). Kuttanadu is a unique wetland agriculture system in Kerala,that favours rice cultivation below sea level. It is a delta region of about 900 sq. km situated in the west coast of Kerala, which is prone to submergence by the flash floods during monsoon. Normally, rice farmers in flood prone area usually loss their complete

crop to flood and thereby their live hood, thus submergence tolerance in rice varieties is essential for sustain stable yield.

Submergence-tolerant rice varieties were identified in the early 1960s and breeding efforts to introgress this trait into rice cultivars have been ongoing since the 1970s. A major quantitative trait locus (QTL) called *SUBMERGENCE- 1 (SUB1)* is responsible for conferring submergence tolerance to the rice landrace Flood Resistant 13A (FR13A). Detailed sequence analysis revealed that the locus encodes a variable cluster of Ethylene-Responsive Factors (ERF): *Sub1A, Sub1B and Sub1C* (Fukao*et al.,* 2006; Xu *et al.,* 2006). FR13A lacks agriculturally important traits. However, they carry many desirable genes that have been lost in cultivated rice. 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 and Voesenek, 2010). Molecular mapping allowed the identification of the major QTL *SUBMERGENCE 1 (Sub1)* on chromosome 9, contributing up to 70% of phenotypic variation in tolerance (Xu and Mackill, 1996).

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. The most striking progress was the discovery and deployment of the *SUBMERGENCE 1 (Sub1)* locus in rice conferring tolerance to complete submergence (Xu *et al.,* 2006; Ismail *et al.,* 2006). At the molecular level, *Sub1* is a variable polygenic locus encoding two or three ethylene responsive factor (ERF) DNA binding proteins. All *Oryzasativa* accessions encode *Sub1B* and *Sub1C* at this locus. An additional ERF, *Sub1A*, is present at *Sub1* in FR13A and other tolerant accessions. The induction of *Sub1A* expression by ethylene during submergence disrupts the elongation escape strategy typical of lowland and deepwater rice, by limiting ethylene-induced gibberellic acid-promoted elongation. (Bailey-Serres*et al.,* 2010).

Molecular markers have been used widely for the identification of resistance gene and Marker Assisted Selection (MAS) have applied for integrating different genes in to rice cultivars lacking them. Among the DNA markers microsatellites or SSR sequence are particularly suited to discriminate closely related genotypes, due to the high degree of variability and the polymorphism can be detected by PCR. In this study, entitled SSR markers were used to identify the presence of *sub 1* QTL conferring submergence tolerance in the selected rice genotypes using functional markers. In the present investigation, SSR markers, namely ART5 and SUB1BC2, were used. These SSR markers were reported to be closely linked to *sub1* locus (Neeraja *et al.*, 2007).

REVIEW OF LITERATURE

2. REVIEW OF LITERATURE

More than half of the world population used rice as a principal food. In Asia, over 90% of the world rice crop is produced and consumed. Rice cultivation areas are mainly classified into irrigated and rain fed. 47 million ha in Asia covers rain fed low land and flood prone area. Flash-floods are highly unpredictable and may occur at any growth stage of the rice crop and the yield loss may be anywhere between 10 and 100% depending on factors such as water depth, duration of submergence, temperature, turbidity of water, rate of nitrogen fertilization, light intensity and age of the crop (Setter *et al.*, 1997). It is essential to develop the productivity of this system with the impending food crisis and the recent inadequacy of rice in international markets. Flash-floods that cause transient, complete submergence are a consequence of various factors, including excessive heavy rains, overflow of rivers, high rains in upper catchments and high tides in coastal areas. (Mackill *et al.*, 1996). Submergence is a type of flooding stress and is defined as a condition where the entire plant is fully immersed in water (complete submergence) or at least part of the shoot terminal is maintained above the water surface (partial submergence). Under submergence and subsequent desubmergence, plants experience multiple external challenges continuously, which bring about various internal stresses that affect plant growth and survival. Rate of gas diffusion and oxygen uptake are considerably limited by submergence. It also compelling carbon inefficient anaerobic metabolism. (Bailey-Serres *et al.*, 2014).

2.1 EFFECTS OF SUBMERGENCE IN RICE

During submergence, flood water inhibitsdiffusive escape of ethylene from submerged tissues. It enhances ethylene accumulation in submerged plant. An increase in ethylene concentration in submerged plants promotes chlorophyll degradation and triggers leaf senescence (Eila *et al.*, 2003). Thus reducing photosynthetic carbon fixation during and after submergence. Ethylene also promotes elongation of shoots (Jackson *et al.*, 2000). This excessive elongation

triggered by ethylene accumulation is considered as an escape mechanism to allow plants to reach the water surface. However, these responses are not desirable when submergence is transient, as in flash-flood-prone areas. This is because the energy required for elongation competes with maintenance processes, thus hastening plant mortality. In addition, elongating shoots tend to lodge after the floodwater recedes (Das *et al.,* 2005). The variations in floodwater characteristics across the locations cause different responses in various rice cultivars hence flooding tolerance at one site cannot be extrapolated to other sites (Setter *et al.*, 1998). Flooding from rainwater usually results in clear water and causes less crop damage than that with turbid water. Therefore, comprehensive understanding of the relationship between floodwater qualities and plant survival is useful for developing a suitable package of management practices as well as crop improvement.

Light is an important factor, which affects plant growth and survival to a great extent. Light reaching to the leaves of submerged plants is attenuated by water, dissolved organic matter and phytoplankton suspended in the water. During flash floods, the floodwater is mostly turbid and only a less amount of solar radiation reaches to the plant. This condition limits the rate of photosynthesis. Underwater light availability is a major factor controlling the CO_2 and O_2 concentration in floodwater and affects greatly the physiological status of submerged rice plants (Sarkar *et al.*, 2006). In darkness, build up of CO_2 level occurs in flood water and it depleted during day time when light is available for photosynthesis (Setter *et al.*, 1998). Thus, increased concentration of O_2 determines the lowering of CO_2 level in floodwater. Under shaded condition, plants received much lower light intensity and temperature of the floodwater was lower by 1.3-1.6°C compared to the open water conditions (Das *et al.*, 2009). In open condition, water with higher O_2 concentration coupled with high light intensity, net photosynthesis might have decreased due to increased photorespiration, resulting in higher plant mortality compared to the shaded condition (Ramakrishnayya *et al.*, 1999).

2.2 PHYSIOLOGICAL BASIS OF SUBMERGENCE TOLERANCE

2.2.1. Elongation ability and duration of flood

Rice plants that exhibit limited elongation during submergence often show tolerance to complete flooding. If water level increases and remains at a level or recedes only partly then the ideal combination for adaptation to flooding is submergence tolerance together with some elongating ability. However, reduced elongation under flash flood condition (complete submergence for 1-2 weeks) is vital for survival because the elongating plants would tend to lodge as soon as the water recedes. Genotypes with limited elongation during submergence classifying as submergence tolerant because such genotypes likely to use only a small quantity of available carbohydrate for elongation, there by leaving carbohydrate reserve for survival after maintenance after submergence when flood water recedes. (Das *et al.*, 2005).

2.2.2. Carbohydrate status and submergence tolerance

Carbohydrates content before and after submergence is important for providing energy for maintaining the vital metabolic processes during submergence, regeneration and recovery of seedlings after submergence (Das *et al.*, 2001). Rice plants that survive under transient submergence differ from those which are susceptible to submergence in respect of the timing and duration of carbohydrate consumption and anaerobic metabolism (Das *et al.*, 2005).

2.2.3. Regeneration capacity

When rice plants are subjected to flash flooding, they need to adapt to two environmental changes: the shift from aerobic to hypoxic conditions during complete submergence and then again from hypoxic to aerobic conditions after the floodwater recedes. Therefore, it is pertinent to understand the physiological processes that are triggered during aerobic to anaerobic transitions. When the plant tissues are subjected to hypoxic or anoxic condition, the oxygen-dependent pathways especially the energy generating systems are suppressed and the

functional relationship between root and shoot is disturbed; both carbon assimilation and utilization of photosynthates are suppressed. Tolerant rice cultivars have shown higher regeneration growth in terms of emergence of new leaves and survival percentage (Panda *et al.*, 2008). Quick regeneration growth following submergence is a desirable trait under frequent flooding, as it can ensure production of sufficient biomass for achieving best possible productivity. The greater amount of sugar and starch before and after submergence seems to have considerable impact on plant survival and subsequent regeneration.

2.2.4. Survival Percentage

Survival after submergence seems to be strongly dependent on non-structural carbohydrate reserves remaining in the shoot after de-submergence, which is in turn equally dependent on the initial carbohydrate content before submergence as well as on the extent of stem elongation during submergence (Das *et al.*, 2005). Survival of seedling is positively correlated with stem starch along with chlorophyll concentration both in before and after submergence (Ismail *et al.*, 2006). Plant height did not increase much in *sub*1 introgressed cultivars, resulted significantly lower elongation compared to other genotypes (Bhattacharjee *et al.*, 2011). Maintenance of high levels of stored carbohydrates in the seedlings prior to submergence coupled with minimum shoot elongation and high retention of chlorophyll are all desirable traits for submergence tolerance (Panda *et al.*, 2014)

2.3 IDENTIFICATION OF SUBMERGENCE-TOLERANT GERMPLASM

International Rice Research Institute (IRRI) initiates the systematic effort to identify the submergence tolerant varieties (Vergara and Mazaredo, 1975). Screening under controlled submergence in tanks allow the scientists to assess thousands of accessions from the international germplasm collection. In 1975, Vergara and Mazaredo found that 10-day-old seedlings, submerged for 7 days revive after 5 days of de-submergence. They distinguish a number of remarkably tolerant varieties, with more than 75% survival, and most of these varieties were

from Sri Lanka. Goda Heenati, Kurkaruppan, Thavalu are some of these Sri Lankan varieties have been used extensively to breed improved varieties and for basic studies on submergence tolerance. FR13 A and FR43 B are the two varieties identified from India by subsequent submergence screening. Both of these varieties were formerly collected from the eastern state of India. These varieties were identified in advance by Indian scientists to be "Flood Resistant," hence they designated as FR. (Richharia and Govindaswami, 1966). FR13 A and FR 43 B were pure-line selections from farmer's traditional landraces. The strong tolerance of FR13A is confirmed by successive screening at several sites (HilleRisLambers and Vergara, 1982). Now, it has been the most widely used donor of tolerance in breeding programs.

FR13A and other tolerant donors were used to develop submergence tolerant varieties since 1970 nationally and internationally (Mackill *et al.,* 1996). In the beginning, the strong submergence tolerance of FR13A was combined with the semi dwarf plant type associated with high-yielding varieties, resulting in tolerant semi dwarf lines such as 'BKNFR 76106-16-0-1' and 'IR26702-111-1' (HilleRisLambers et al., 1986; Mackill et al., 1996; Mohanty and Chaudhary, 1986). However, these semi dwarf lines had low grain yields and poor grain quality. Subsequent breeding efforts using these semi dwarf lines led to the development of higher yielding breeding lines that retained the submergence tolerance of the donors (Mackill *et al.,* 1993). Most studies concluded that tolerance was a quantitative trait and was controlled by a number of genes (Haque et al., 1989; Mohanty and Khush, 1985; Mohanty et al., 1982; Sinha and Saran, 1988; Suprihatno and Coffman, 1981). However, it was acclaimed that there were reasonable environmental effects in measuring tolerance that complicated the genetic analysis, including floodwater conditions (Das *et al.,* 2009). Although these early genetic studies failed to identify a major gene controlling tolerance, breeders noted that it was not very difficult to recover progeny with high tolerance from crosses with these donors (Mackill *et al.,* 1996). These findings indicated

that the inheritance of submergence tolerance was controlled by one or a few genes with larger effect.

Fukao *et al.,* 2006 proposed a model for the ethylene- and GA-mediated transcriptional regulation of genes involved in acclimation to submergence by *sub1A* and *Sub1C.* Submergence-promoted ethylene production or entrapment, stimulates the accumulation of the *Sub1A* transcript in leaves of submergence-tolerant rice. *Sub1 A* transcription of tolerant indica enhances the levels of mRNAs associated with ethanolic fermentation and represses the accumulation of mRNAs responsible for cell elongation and carbohydrate catabolism. *Sub1A* also restricts ethylene production during submergence, possibly by feedback regulation. It has been shown that ethylene increases the endogenous GA content and sensitivity to this phytohormone in deepwater rice (Kende *et al.,* 1998; Peeters *et al.,* 2002). Although the level of *Sub1C* mRNA is increased by both ethylene and GA, the presence of *Sub1A* is correlated with limited accumulation of *Sub1C* mRNA. This suppression of *Sub1*C mRNA accumulation is also observed during submergence in M202 (*Sub1*) genotype and transgenic lines over expressing *Sub1A* (Xu *et al.,* 2006). The *Sub1A-1* allele of tolerant genotype is responsible for the repression of ethylene and GA-mediated gene expression involved in carbohydrate consumption and cell elongation as well as the enhancement of fermentation capacity during submergence. The coordination of carbohydrate consumption and energy economy by *Sub1A* influences the enhancement of submergence tolerance in lowland rice.

2.4 IDENTIFICATION OF THE *SUB1* GENE AND ITS FUNCTIONAL ROLE

The use of molecular mapping through DNA markers enhanced the genetic studies of submergence tolerance. In 1996, Xu and Mackill identified a major quantitative trait locus (QTL) for submergence tolerance on rice chromosome 9 using a parent that derived its strong submergence tolerance from FR13A through the use of RAPD and RFLP. This single QTL explained about 70% of the phenotypic variation in the F2–F3 population they evaluated. This

QTL, designated as '*Sub1*'. Further Sub1 was fine-mapped to a small enough interval to allow marker-assisted selection with tightly linked markers (Xu *et al.*, 2000, 2004). Toojinda *et al.* 2003 reported that, although a few minor QTLs were observed for submergence tolerance, *Sub1* locus was the major determinant of tolerance. Using a positional cloning approach found that the *Sub1* locus was constitute of three ethylene response-like transcription factors designated *Sub1A, Sub1B,* and *Sub1C* (Xu *et al.*, 2006). It was determined that *Sub1A* was the gene largely responsible for tolerance through complementation analysis. All japonica cultivars and some indica cultivars lack *Sub1A* gene. Strong expression of this gene seems to be a requirement for submergence tolerance (Septiningsih *et al.*, 2009; Singh *et al.*, 2010). The proximity of *Sub1A* and *Sub1C* on the chromosome made it difficult to retrieve recombinants between the two. Septiningsih *et al.*, 2009 were decisively confirmed that only the tolerant allele of *Sub1A* is required to confer tolerance. *Sub1* is used to refer to the *Sub1A* gene. *Sub1* is an ethylene response factor and its expression is induced by ethylene (Fukao and Bailey-Serres, 2008a). The build up of ethylene in submerged condition competently induces *Sub1* gene. Under submergence most plants meet with rapid leaf, sheath, and internodes elongation. This leads to accelerated consuming of energy and death of the plants. Plants having *Sub1*, showing an inhibition of elongation upon submergence and recover rapidly and continue growth by de submergence (Fukao and Bailey-Serres, 2008).

2. 5 MAPPING AND MOLECULAR CHARACTERIZATION OF *SUB1*

The genetic control of submergence tolerance remained unclear until the 1990s. Certain studies proposed that it was a typical quantitative trait (Suprihatno and Coffman, 1981; Mohanty *et al.* 1981; Mohanty and Choudhary, 1985; Haque *et al.* 1989). Several independent studies confirmed the major chromosome 9 QTL and identified other minor QTLs that accounted for less than 30% of the phenotypic variation in tolerance (Nandi *et al.*, 1997). The positional cloning of *Sub1* was accomplished by identifying a contiguous set of BAC and binary clones spanning the region, using the intolerant indica Teqing and the tolerant FR13A

derivative 'IR40931-26' respectively. This resolved a chromosomal integral, from marker CR25K to SSR1A that varies in length, structure, and gene content in the japonica and indica genomes, using Nipponbare and Teqing as representatives. The sequencing of the Teqing BAC contig in the *Sub1* region confirmed the presence of approximately 50% interspersed sequences of transposon or retro transposon origin (Xu *et al.*, 2006). Recombination suppression in this region was associated with an inversion and large deletion, between markers '101O9L' and '14A11-71K' in japonica relative to submergence intolerant Teqing and tolerant IR40931-26. This variant segment of *Sub1* contains a gene designated *Sub1A* that encodes a putative ERF DNA binding protein with a single ERF/APETELA2 domain (Xu *et al.*, 2006). Two additional ERFs, designated *Sub1B* and *Sub1C*, were identified proximal to the centromere within approximately 100 bp of *Sub1A* in Teqing. The genome of the submergence intolerant japonica cultivar Nipponbare encodes *Sub1B* and *Sub1C* in this chromosomal interval but lacks *Sub1A* (GenBank AP006758 and AP005705). Phylogenetic analysis of the *Sub1* ERFs indicates that *Sub1A* derive from a duplication of *Sub1B*, probably later the domestication of indica rice (Fukao *et al.*, 2006). *Sub1A* is recognized in two allele forms in submergence tolerant and intolerant indica and aus accessions, based on nucleotide variations in the protein-coding region (Xu *et al.*, 2006). *SubA1-1* is found only in tolerant lines such as FR13A, whereas the *Sub1A-2* allele is present in intolerant indica accessions. *Sub1A-1* and *Sub1A-2* encode identical proteins, with the exception of 'Ser186' in the tolerant allele and 'Pro186' in the intolerant allele. Another distinction is recognized at the level of gene expression. *Sub1A-1* promotes rapid, prolonged, and pronounced transcript accumulation in leaves of 14- to 28-day-old plants in response to submergence, whereas *Sub1A-2* promotes a lower level of transcript induction by the stress (Fukao *et al.*, 2006; Xu *et al.*, 2006). Strong ectopic expression of the *Sub1A-1* cDNA from FR13A in a highly intolerant japonica verified to be sufficient to confer substantial submergence tolerance (Xu *et al.*, 2006). An extensive analysis of diverse rice accessions with different *SUB1* haplotype showed that

submergence tolerance is associated with variable levels of *Sub1A* transcript in internodes and nodes at the heading stage (Singh *et al.*, 2010).

2. 6 STRATEGIES FOR SUBMERGENCE TOLERANCE

In the *Sub1* region, three similar genes encode the AP2/ERF domain: *Sub1A, Sub1B and Sub1C*. These are located on chromosome 9 in FR13A.(Nagai, 2010) In comparison, the submergence-intolerant varieties Nipponbare and M202 possess *Sub1B* and *Sub1C*, but lack *Sub1A*. The introduction of *Sub1A* into submergence-intolerant varieties confers submergence tolerance (Nagai, 2010)

Quiescence and elongation are two opposite strategies by which rice adapts to submergence depending upon the nature of flooding. The ethylene response factor genes Snorkel1 (*SK1*) and Snorkel2 (*SK2*) allow rice to adapt to deep water whereas Submergence1A-1 (*Sub1A-1*) allows rice to acclimatize under flash flooding (Xu *et al.*, 2006; Hattori *et al.*, 2007; Nagai *et al.*, 2010). The QTL on chromosome 12 was the most effective for deepwater response (Nemoto *et al.*, 2004; Hattori *et al.*, 2008) whereas the QTL on chromosome 9 was most important for flash flood response (Xu and Mackill, 1996). Both *SK*s genes and *Sub1A* encode ethylene-responsive factor type transcription factor and are connected to gibberellin biosynthesis, yet deepwater and submergence-tolerant rice seem to have opposite flooding response; namely, escape by elongation or remain stunted under water until flood recedes (Hattori *et al.*, 2009; Bailey-Serres and Voesenek, 2010). The genes at the *Sub1* locus, which confer submergence tolerance, were identified by positional cloning (Xu *et al.*, 2006). *Sub1A* negatively regulates the transcription of these genes, to reduce energy consumption during submergence. Fukao and Bailey-Serres (2008) showed the mechanism by which *Sub1A* regulates plant growth. Slender rice-1 (SLR1) and SLR1 Like-1 (SLRL1) are repressors of GA signaling. The amount of SLR1 protein is elevated after submergence in submergence-tolerant rice (M202-Sub1), but not in submergence-intolerant rice (M202). Additionally, more SLRL1 protein accumulated in submergence-tolerant rice (M202-*Sub1*) than in submergence-

intolerant rice (M202). These results suggest that the restriction of growth by submergence-tolerant rice is due to the accumulation of SLR1 and SLRL1 through *Sub1A*. On the other hand, *SK1* and *SK2* are up regulated by the submergence-induced accumulation of ethylene in internodes, consistent with the essential role of ethylene in GA-stimulated underwater shoot elongation (Jackson, 2008).

2.7 MOLECULAR MECHANISM OF SUBMERGENCE TOLERANCE

Submergence stimulates the biosynthesis of 1-aminocyclopropane- 1-carboxylic acid. In an oxygen dependent manner, 1-aminocyclopropane- 1-carboxylic acid is converted to ethylene. (Banga *et al.,* 1996; Kende *et al.,* 1998; Peng *et al.,* 2001). During submergence oxygen produced by the plant itself can be used for ethylene synthesis. (Mommer *et al.,* 2005). Ethylene level can also enhance as a result of physical entrapment by the surrounding aqueous environment, (Banga *et al.,* 1996). Ethylene production is a significant factor in the acclimation responses to submergence. It facilitate the programmed cell death that promote the formation of aerenchyma and adventitious roots under hypoxia (Drew *et al.,* 2000; Mergemann and Sauter, 2000; Gunawardena *et al.,* 2001) and enhance internode and petiole elongation that allows deepwater rice to extend leaves above the water surface (Kende *et al.,* 1998; Peeters *et al.,* 2002). By reason of ethylene mediated GA response, elongation occurs in submerged rice plants(Kende *et al.,* 1998; Peeters *et al.,* 2002). Ethylene entrapped during submergence aid to a reduction in endogenous abscisic acid (Benschop *et al.,* 2005). The variation in both of these hormones is a mandatory for petiole elongation. Fukao *et al.,* 2006 compared the acclimative responses to submergence of near-isogenic japonica lines that differ in haplotype of an ERF gene cluster at the *Sub1* locus. Their results demonstrate that the ERF domain genes *Sub1A* and *Sub1C* are differentially adjusted by submergence. The presence of *Sub1A-1* at the *Sub1* locus results in ethylene and GA mediated negative regulation of genes come along with carbohydrate catabolism and cell elongation as well as positive regulation of genes involved in ethanolic fermentation during

submergence. These data demonstrate that evolutionary divergence in an ERF domain gene cluster is responsible for the positive and negative gene regulation that controls multifaceted cellular and developmental responses to submergence and negotiate submergence tolerance in lowland rice.

2.7.1. Regulation of Three ERF Genes at the *Sub1* Locus under Submergence

The *Sub1* region on rice chromosome 9 accommodate three *Sub1*genes *Sub1A*, *Sub1B*, and *Sub1C*. Genotypic variation at this complex locus confers distinctions in submergence tolerance (Xu *et al.*, 2006). Submergence-intolerant japonica rice lacks *Sub1A* and possesses *Sub1B* and *Sub1C*. The near-isogenic line having *Sub1* was generated by introgression of the *Sub1* region from the submergence-tolerant indica cultivar FR13A (Xu et al., 2004). Based on molecular mapping and genome sequence analysis, found that the introgressed line contains all three *Sub1* genes of indica origin in a genomic region of 182 kb (Xu *et al.*, 2006). The level of *Sub1A* mRNA increased rapidly in abundance after 1 day of submergence in cultivars having *Sub1* gene and remained increased for up to 14 days of stress. The *Sub1B* transcript was detected in both genotypes and was slightly up regulated by the stress, with higher levels of the transcript consistently detected in *Sub1* genotypes. The distinctions in *Sub1B* and *Sub1C* mRNA levels under stress in the two lines may be inferable to the reflect the absence or presence of *Sub1A* in intolerant and tolerant genotypes respectively (Fukao *et al.*, 2006)

2.7.2. Differential regulation of elongation under submergence

Submergence sensitive lowland rice cultivars elongate more rapidly than tolerant cultivars under submergence stress (Singh *et al.*, 2001; Das *et al.*, 2005). To examine whether the *Sub1* haplotype controls growth rate, plant height was compared in M202 and M202 (Sub1) plants grown under normal or submerged conditions for 14 days. The height of 14-days old plants of these genotypes was nearly identical, and both lines grew uniformly under normal conditions. In response of 14 days of submergence stress, M202 displayed significantly greater

elongation than M202-*Sub1*. The increase in plant height of the tolerant line was the same under submergence and normal conditions, indicating acceleration in elongation of the intolerant line under the stress. These data confirm that the FR13A *Sub1* haplotype suppresses elongation during submergence but does not influence plant height under normal conditions.

2.7.3. Carbohydrate Consumption under Submergence

Plant cells consume carbohydrates through energetically inefficient coupling of glycolysis and anaerobic fermentation during anoxic condition (Drew *et al.*, 1997). Due to the diminished the light intensity, chlorophyll content and CO_2 diffusion, photosynthesis is withdrawn in submerged plants. Hence prolonged submergence causes extensive carbohydrate and energy starvation. A number of metabolic enzymes are involved in the catabolism of starch and soluble carbohydrates in plants. There is no starch degradation in wheat and barley due to the absence of α-amylase synthesis under anoxic condition (Guglielminetti *et al.*, 1995b). In contrast, rice α-amylases transcripts accumulate in the seed embryo during germination even under anoxia (Hwang *et al.*, 1999). In addition, α-amylase protein levels and activity were shown to be induced by anoxia in rice seedlings (Guglielminetti *et al.*, 1995b). Semi quantitative RT-PCR detection of the transcripts of three α-amylase genes, Rice Amylase-3C (RAmy3C), RAmy3D, and RAmy3E, revealed that their up regulation was controlled by the *Sub1* locus. The level of RAmy3C mRNA increased immediately under submergence stress, reached a maximum by day 6, and then decreased through day 14 in both genotypes. Overall RAmy3C transcript induction is greater in susceptible cultivar. RAmy3D and RAmy3E transcript increases occurred later than in RAmy3C; the increases in these transcripts were considerably lower in submergence tolerant varieties.

Sucrose, which is the major energy source and transport form of carbohydrates in rice, can be hydrolyzed via two distinct pathways: the more energy-efficient sucrose synthase pathway and the invertase pathway (Zeng *et*

al., 1999; Geigenberger, 2003; Fukao and Bailey-Serres, 2004). It has been shown that the transcript level and the enzymatic activity of sucrose synthase increase, whereas those of invertase decrease, during oxygen deprivation in maize (*Zea mays*) roots and potato (*Solanum tuberosum*) tubers, suggesting that sucrose synthase is the principal enzyme that converts sucrose to phosphorylated hexose sugars under low oxygen stress (Zeng *et al.*, 1999; Geigenberger, 2003). In susceptible leaves, the transcript levels of all three sucrose synthase genes, *Sus1*, *Sus2*, and *Sus3*, became increased by the middle of the submergence period and remained increased through day 14. By contrast, *Sus* gene transcripts were only transiently and much less dramatically increased in M202-*Sub1*. Thus, the Sub1 haplotype controls the regulation of the transcript levels of genes encoding α-amylase and sucrose synthase, which are required for carbohydrate catabolism during submergence.

2.7.4. Transcript levels of genes associated with ethanolic fermentation

Fermentation is a complex metabolic process that results from the series of chemical reactions catalysed by specific enzymes. Ethanolic fermentation is used only by specialized organisms or under special conditions. In plants, it has been studied because of its relevance to ATP production during flooding (Tadege *et al.*, 1999). Different fermentation pathways and products of anaerobic metabolism have crucial roles in surviving prolonged periods under anoxia. Lactic acid and ethanolic fermentation are the most common pathways that regenerate NAD+ for the continuation of glycolysis. Lactic acid fermentation is a one step conversion from pyruvate, which is catalysed by lactate dehydrogenase (LDH) with coupled oxidation of NADH. Ethanolic fermentation is a two step process in which pyruvate is first decarboxylated to acetaldehyde by pyruvate decarboxylase (PDC), and acetaldehyde is subsequently converted to ethanol by alcohol dehydrogenase (ADH), regenerating NAD+. Both lactate and ethanol are produced to a varying degree by most plants under oxygen stress. However, lactate is an acid and its accumulation in the cytoplasm could alter cellular pH and cause damage, whereas ethanol diffuses to the extracellular medium and poses no

major problem except at high concentrations (Tadege *et al.*, 1999). On the basis of *in vitro* LDH and PDC enzymatic activity, a self-controlling system for lactate and ethanol production called the 'pH-stat' hypothesis was proposed by Davies *et al.*, 1974. LDH has an alkaline pH optimum whereas that of PDC is acidic. At the onset of anoxia when oxidative phosphorylation is blocked, LDH is active at the alkaline pH of the cytoplasm and shunts pyruvate to lactate. The accumulation of lactate reduces cytoplasmic pH, which, in turn, inhibits LDH and activates PDC leading to ethanol production (Davies *et al.*, 1974). In rice shoots, cytoplasmic pH decreases immediately in spite of the low lactate production (Menegus *et al.*, 1991). In rice, the ability to use starch under anoxia is one of the major factors that contribute to anoxia tolerance (Perala *et al.*, 1992). It has been proposed that the different *K*m of PDH and PDC for pyruvate are the controlling factors that regulate the entry of pyruvate into the TCA cycle or the ethanolic fermentation pathway (Tadege *et al.*, 1998). The internal pyruvate concentration in plants is between 0.1 and 0.4 mM (Davies *et al.*, 1974; Rivoal *et al.*, 1990), which is too low for PDCs to compete with PDHs. Thus, at the aerobic pH even if PDC remains active, pyruvate preferentially enters the TCA cycle. But when respiration is blocked by inhibitors or lack of oxygen, pyruvate concentration increases considerably (Laber *et al.*, 1987; Good *et al.*, 1993) and pyruvate becomes available for the PDC reaction. In rice, where the lowest *K*m for PDC is reported (i.e. 0.25 mM at pH 6.5), the lag phase in enzyme activity is avoided by the presence of 3 mM pyruvate (Rivoal *et al.*, 1990) suggesting that pyruvate concentration is more important than pH.

2.8 IMPACT OF *Sub1* ON PLANT SURVIVAL AND PRODUCTIVITY

Recently, the *Sub1* QTL has been introgressed through marker-assisted breeding in 'Swarna' and other popular rice cultivars of South and Southeast Asia. Rice cultivars introgressed with *Sub1* QTL showed less elongation and better survival under submergence (Panda and Sarkar, 2012a, 2012b, 2012c, 2013). These cultivars maintained greater activities of alcohol de hydrogenase compared to the susceptible cultivars. The rate of chlorophyll degradation was significantly

lower in cultivars with *Sub1*. Chlorophyll fluorescence studies revealed that the genotypes with *Sub1* QTL maintained the chloroplast's structural and functional integrity in a better manner and could withstand submergence stress. Under mild stress, plant mortality in rice is generally very less but extensive damage of leaves occurs; however, the damage is almost nil in Swarna-*Sub1* due to the maintenance of higher activities of antioxidant enzymes. Whether the introgression of *SUB1* has any adverse effects on yield and yield attributes needs to be understood not only for commercial cultivation but also for future development. Introgression of *Sub1* QTL as such has no deleterious effects on yield and yield attributes. There was not much variation in the yields of Swarna and Swarna-*Sub1* under favourable conditions. The yield potential of different rice cultivars possessing *Sub1* QTL varies significantly. Therefore, the parent materials should be carefully selected before introgression of *Sub1*. Cultivars with greater initial vigour are considered better for this purpose.

MATERIALS AND METHODS

3. MATERIALS AND METHODS

The study entitled "Molecular screening of rice genotypes for submergence tolerance" was conducted at Rice Research Station, Vyttila, during 2017 October to 2018 July. The objective of this study was to identify the field tolerant rice genotypes under submergence and to screen them for the presence of *sub*1 QTL using SSR markers.

3.1 PLANT MATERIALS

The experimental material for this study contains of 10 genetically diverse genotypes of rice which were identified among the breeding lines at Rice Research Station, Monkombu , along with submergence tolerant Swarna *Sub1* and susceptible line Jyothi.

The investigation was carried out as two parts.

a. *In vitro* phenotyping

b. Genotyping

Table 1. List of rice genotypes used for the study

Sl. No.	Varieties	Sl. No.	Varieties
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

3.2 *IN VITRO* PHENOTYPING BY POT CULTURE EXPERIMENT

The experiment was conducted during 2017-2018 at Rice Research Station, Vyttila. A pot culture experiment was laid out in completely randomised design with 5 replications, 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, 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 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 genotypes were taken from the submergence tank after 7, 14 and 21 day. After complete submergence period plants were given 10 days recovery period under de-submerged conditions. Post submerged recovery percentages were recorded based on population and post submerged changes on leaf number and plant height were recorded.

3.3 MOLECULAR SCREENING OF RICE GENOTYPES USING SSR MARKERS

3.3.1 Plant materials

10 different rice genotypes, submergence tolerant Swarna *sub1* and susceptible Jyothi (Table 1) were raised in trays. About 21 days old plant was taken for further study.

3.2.2 Isolation of genomic DNA

Isolation of genomic DNA was done as per CTAB method (Murray and Thomson, 1980) as described below:-

About 500-750 mg leaves were collected in paper bags. Washed under running tap water and dried over filter paper. Frozen leaf tissues (about 100 mg) were ground to a fine powder with liquid nitrogen using mortar and pestle and

finely grinded powder was transferred to well label 2.0 ml autoclaved centrifuge tubes. 800 µl of CTAB buffer was added and incubated for 1 hour at 650C in water bath. During incubation, the contents were occasionally mixed four to five times by inverting the tubes gently. 800 µl of chloroform: iso-amyl alcohol (24:1) was added to the tubes and then put on the shaker for 30-40 minutes. 6. The mixture was then centrifuged in a microcentrifuge (Eppendorf Centrifuge 5415D) at 10,000 rpm for 15 minutes at room temperature. The upper aqueous phase was transferred to fresh tubes with the help of micropipette. Care was taken to avoid debris inclusion. About two- thirds volume of chilled isopropanol was added and mixed well by inverting the tubes. The samples were refrigerated at -20°C/-400C for 15 minutes followed by centrifugation at 10,000 rpm for 15 minutes to make a pellet. The supernatant was discarded. The DNA pellet was washed with 300 µl 70% ethanol and centrifuged at 10,000 rpm for 5 minutes. The ethanol was discarded and the pellet was retained. The pellet was air-dried and finally the purified DNA pellet was dissolved in 100µl of TE buffer. It was kept overnight at room temperature. DNA pellet was stored at -20^{0}C for long term stability.

3.2.3 Quantification and Estimation of quality of extracted DNA

Quantification of nucleic acids was performed by Nanodrop (NANODROP 2000c Spectrophotometer, Thermoscientific) using ND 1000 spectrophotometer programme. The absorbance reading at 230 nm and 280 nm should be about half of TE reading at 260. Clean the pedestal between each reading. The Optical Density (OD) values were taken at both 260nm and 280nm. The purity of the DNA was checked by calculating the ratio of absorbance at A_{260}/A_{280} and a ratio is 1.8 indicates good quality DNA. (If the ratio is 2 or above 2, it indicates high RNA contamination in the sample and if the ration is less than 1.8, protein contamination or phenol in the sample). Concentration of the DNA was calculated by the formula shown below.

174446

Concentration of the DNA µg /ml of sample = Optical density at 260nm X 50 X Dilution factor (i.e. 1 OD =50 µg/ml).

The concentration of DNA was diluted to adjust the concentration to 25ng/µl for use in polymerase chain reactions.

3.2.4 Dilution of genomic DNA

The isolated genomic DNA was diluted to adjust the concentration to 18-25 ng/µl for the use of polymerase chain reactions.

3.2.5 PCR Analysis

3.2.5.1 Selection of primers

The primer sequences were synthesized from Vision scientific as 25 nmol in dry form. These primers were diluted to 10 µM working concentration using TE buffer. For the present study 2 SSR markers (Table 2) are used.

Table 2. List of primers used for molecular screening

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

3.2.6.2 Dilution of primers

Add 1xTE buffer (10 mMTris, pH 7.5-8.0, 1Mm EDTA) to the lyophilized primer to make up the final working concentration 10 µM. The primers were diluted as per the following formula:

µM of oligo in 1001 of solution = OD/10

3.3 PCR Amplification

In-vitro amplification using Polymerase Chain Reaction (PCR) was performed in *BIO RAD T100* Thermal cycler. PCR analysis was carried out using 2 SSR primers. PCR analysis was carried out in the reaction volume of 20 µl

containing the 2µl template genomic DNA(16-25 ng), 1 µl of forward primer, 1 µl reverse primers, 10 µlof Thermoscientific 2X PCR master mix(which contains Taq DNA polymerase(0.05U/µL), reaction buffer, 4Mm $MgCl_2$ and 0.4 mM of dNTP) and 6 µl Nuclease free water. PCR reaction mixture and PCR profile is given in Table 4 and Table 5 respectively.

Table 3. PCR reaction mixture

Components	Stock concentration	Volume (µl)
Sterile water	-	6
PCR master mix	2 X	10
Forward primer	10µM	1
Reverse primer	10µM	1
DNA template	25 ng/µl	2
Total		20 µl

Table 4. Temperature profile used in PCR

Step No.	Cycling condition	Temperature	Time
I	Initial denaturation	94 °C	3 minutes
II	Denaturation	94 °C	1 minute
III	Annealing	58 °C	1 minute
IV	Extension	72 °C	1 minute
V	Go to step II	-	35 cycle
VI	Final extension	72 °C	5 minutes
VII	Store	4 °C	∞

3.4 PAGE AND SILVER STAINING OF GEL

3.4.1 Gel matrix preparation and gel casting

8% polyacrylamide solution was prerpared by using 30% acrylamide bis acrylamide , deionised water, 10 % ammonium persulphate (APS) ,10X TBE buffer solution and TEMED (Tetra methyl ethylene diamine).The plates were kept in slanting position in such a way that the gel matrix could flow freely into the plates without air bubble. The solution was poured between the plates. After the solution spread uniformly throughout the plate, the comb was placed. The plates were left for 30 minutes for polymerization.

3.4.2 Sample loading and gel running

After polymerization, the comb was removed and the gel setup was mounted on an electrophoresis apparatus. 4 µl gel loading dye was added to the 20 µl PCR product and from this 4 µl sample was loaded to the well. The electrophoresis was started and allowed to proceed at 120 volt for about 1 hours. Finally, plates were dismount from the electrophoresis apparatus for silver staining.

3.4.3 Silver staining and Visualization of gel

After careful removal of gel from the plates, they were subjected to staining process by washing treatments. First gel immersed in fixer for 5 minutes and then washed with deionized water then transferred to stainer. After 5 minutes incubation in staining solution gel again washed with deionized water. Then gel transferred to developer solution. After the devolpment of bands, the gel is transferred to stopper solution.

The silver nitrate-stained gel visualized in the BIO-RAD Gel Documentation System (Molecular imager, Gel Doc XR+).

The SSR allele sizes were determined by the position of bands in relative to DNA ladder. The amplicon size was made out from the 100 bp ladder run along with the PCR products on the gel. Score the genotypes by giving 1 for presence of allele and 0 for the absence of allele.

RESULTS

4. RESULT

In the present study, ten genetically diverse genotypes of rice were subjected to *in vitro* phenotyping for the field tolerant rice genotypes under submergence and to screen them for *sub*1 QTL using SSR markers in comparison with known susceptible and tolerant check varieties. The material for this study consists of the breeding lines which were received from Rice Research Station, Moncombu. The results were elaborated in this chapter.

4.1 *In vitro* phenotyping

In vitro phenotyping was done by pot culture experiment. The ten rice genotypes were grown as pot culture and fourteen days old seedlings were subjected to submergence of varying durations as 7 days, 14 days and 21 days and the following biometric observations were recorded.

i. Plant height

ii. Leaf count

iii. Post submerged recovery percentage

Table5 .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	14.0^{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	

Table6. Post submerged recover 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

Table7. ANOVA of post submerged recovery percentage after 7days of submergence

Source of variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between groups(Treatment)	28165	11	2560.4545	51.209091	1.068E-22	1.99458
Within groups(Error)	2400	48	50			
Total	30565	59				

Table8. ANOVA for post submerged recovery percentage after 14 days of submergence

Source of variation	*SS*	*Df*	*MS*	*F*	*P-value*	*F crit*
Between groups (Treatment)	55853.333	11	5077.5758	761.6336	6.7E-50	1.99458
Within groups (Error)	320	48	6.6666667			
Total	56173	59				

The analysis of variance for different biometrical characters was presented in Tables 5 to 8. In Table 5, it was revealed significant differences among genotypes for pre and post submerged plant height, while the lea count was found to be non significant among the genotypes as well as before and after submergence. Similarly the recovery percentages after 7 days of submergence (Table. 7) and 14 days of submergence (Table 8) were found to be significant among different genotypes studied.

The plant height of KAUM 7 and Swarna Sub1 were 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 cm to 2.4 cm between genotypes.

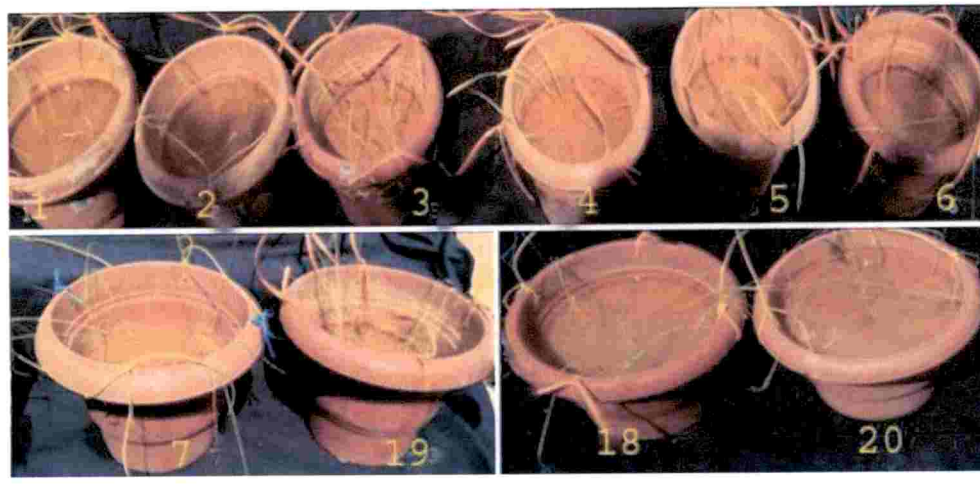

Plate 1: Rice genotypes after 7 days of submergence. (1-KAUM1, 2-KAUM2, 3-KAUM3 , 4- KAUM4, 5- KAUM5, 6-KAUM6, 7-KAUM7, 18- KAUM8 ,1 9- KAUM9, 20- KAUM10)

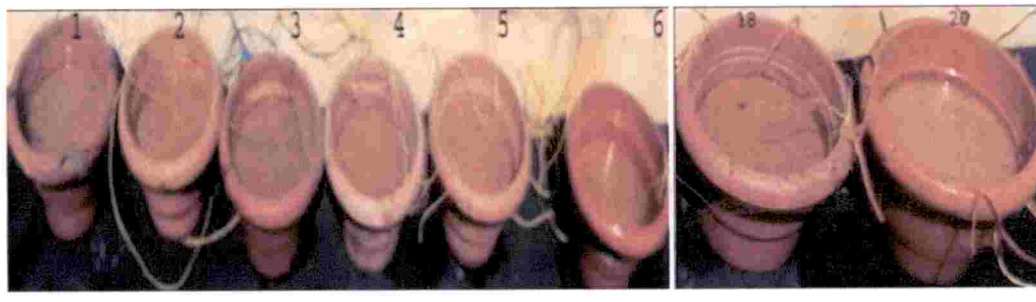

Plate 2: Submergence susceptible genotypes after 14 days of submergence (1-KAUM1, 2-KAUM2, 3- KAUM3 , 4- KAUM4, 5- KAUM5, 6-KAUM6, 18- KAUM8 , 20- KAUM10)

Plate 3: Submergence tolerant genotypes after 14 days of submergence (7- KAUM7, 19- KAUM 19)

4.2 Molecular screening for *sub1* QTL

The genomic DNA from the ten genotypes along with tolerant check Swrana- *Sub 1* and Jyothi was isolated.

4.2.1 Quality confirmation and Quantification of DNA

Quality and quantity of the isolated genomic DNA from 10 different genotypes Swarna-*sub1* and Jyothi were confirmed by Nanodrop ND 1000 Spectrophotometry. Quantity of DNA was calculated based on the absorbance of sample at 260nm are given in Table 9. The quality of DNA obtained ranged from 1.70 in KAUM 20 to 1.88 in Jyothi. The quantity of DNA obtained ranged from 1236 in KAUM 7 to 2112 in KAUM 1.

Table 9. Quality and Quantity of isolated DNA identified by Nanodrop (NANODROP 2000C, Spectrophotometer)

Genotypes	A260 (nm)	A280 (nm)	A260/A280	Quantity of DNA (ng/ µl)
KAUM1	0.058	0.031	1.87	2112
KAUM2	0.096	0.052	1.84	1667
KAUM3	0.079	0.042	1.88	1574
KAUM4	0.052	0.028	1.85	1245
KAUM5	0.080	0.046	1.73	1354
KAUM6	0.084	0.048	1.75	1645
KAUM7	0.062	0.033	1.87	1236
KAUM 18	0.086	0.049	1.75	1572
KAUM 19	0.060	0.033	1.81	1369
KAUM 20	0.098	0.055	1.70	1290
Swarna -*sub1*	0.0821	0.044	1.86	1567
Jyothi	0.086	0.044	1.95	1258

4.2 Visualization of PCR amplified product

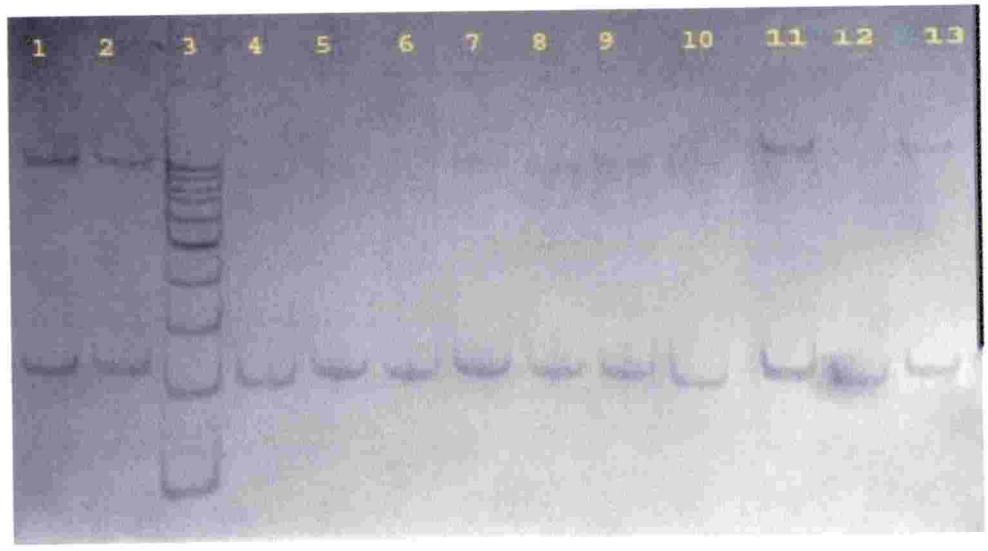

Plate 4: Amplification pattern of rice genotypes obtained by SSR marker ART5 (Lane 1- KAUM1, Lane 2- KAUM 2, Lane 3- 100bp ladder, Lane 4- Swarna sub1, Lane 5- Jyothi , Lane 6- KAUM 3, Lane 7 – KAUM 4, Lane 8 – KAUM5, Lane 9- KAUM6 , Lane 10- KAUM7, Lane 11- KAUM18, Lane 12- KAUM19,Lane13-KAUM20)

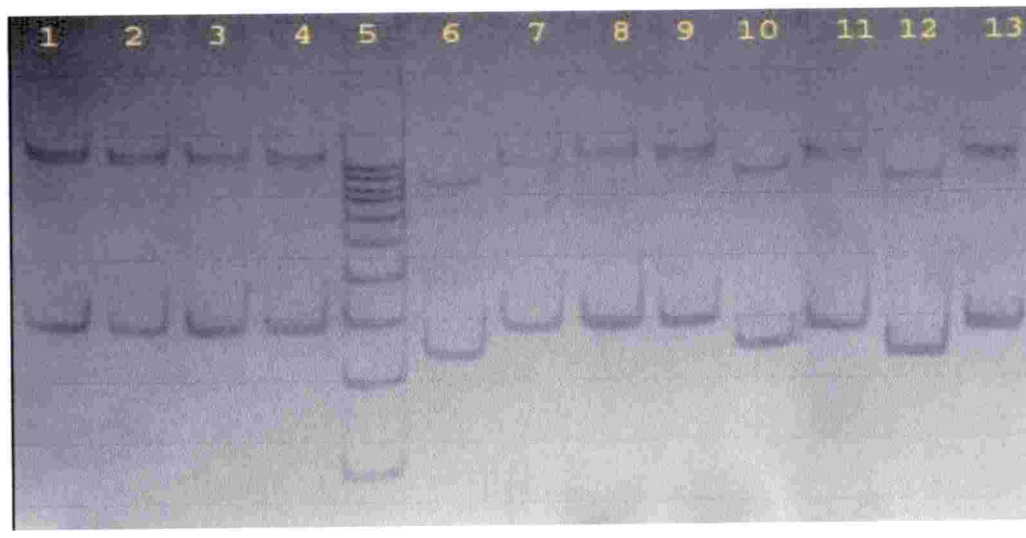

Plate 5: Amplification pattern of rice genotypes obtained by SSR marker Sub1 BC2 (Lane 1- KAUM1, Lane 2- KAUM 2, Lane 3- KAUM3, Lane 4- KAUM4, Lane 5- 100bp ladder, Lane 6- Swarna sub1, Lane 7 – Jyothi, Lane8– KAUM5, Lane 9- KAUM6 , Lane 10- KAUM7, Lane 11- KAUM18, Lane 12- KAUM 19, Lane13-KAUM20)

Ten rice genotypes were genotyped with SSR primer ART 5 and Sub 1 BC2 which are closely linked to the submergence tolerant gene *SUB1*. The product amplified at 217 bp with ART 5 and 268 bp with Sub 1 BC2 as in the tolerant check Swarna *Sub 1* was taken as the standard. Among ten rice genotypes, only two genotypes showed the presence of the *Sub 1*.

Table 10. Presence of various markers in selected genotypes

Genotypes	ART5	Sub1 BC2
KAUM1	0	0
KAUM2	0	0
KAUM3	0	0
KAUM4	0	0
KAUM5	0	0
KAUM6	0	0
KAUM7	1	1
KAUM 18	0	0
KAUM 19	1	1
KAUM 20	0	0
Swarna -*sub1*	1	1
Jyothi	0	0

DISCUSSION

5. DISCUSSION

World rice requirements are likely to increase by 1.7 per cent yearly between 1990 and 2025, which requires an additional 13 million tons of rice per year. Rice crop is affected by various abiotic and biotic stresses, thus resulting in huge yield losses wherever rice is grown. Among the abiotic stresses, submergence is a major constraint to rice production in South and Southeast Asia. Though, rice is the only cereal that can be grown in flood prone ecosystem, uncertainty of rainfall with flash flood affecting seriously the plant population and crop stand depending on stage of crop and duration of submergence is a major stress factor affecting the rice yield. Submergence is considered as the third most important constraint to rice yield in India. Traditional accessions are reservoirs of unique genes, which confer resistance to biotic and abiotic stresses. The land races and wild or weedy species together constitute 80 per cent of the rice germplasm, but they are not exploited appropriately. Even though portions of land races have been collected and maintained in the form of gene banks in several germplasm programmes, there have been very few reported studies designed to assess the various genes of resistance present in them. A few indica rice cultivars, such as FR13A, BKNFR and Kurkaruppan (Mazaredo and Vergara, 1982; Mohanty and Chaudhary, 1986) are highly tolerant and survive up to two weeks of complete submergence due to a major quantitative trait locus designated as *Submergence 1* (*Sub1*) near the centromere region of chromosome 9.

Molecular markers are nucleotide sequences and can be investigated through the polymorphism present between the nucleotide sequences. An ideal DNA marker should be co-dominant, evenly distributed throughout the genome, highly reproducible and having ability to detect higher level of polymorphism. (Mondini *et al.,* 2009).The present study was carried out to identify the rice genotypes having submergence tolerance through *in vitro* phenotyping and molecular screening by microsatellite. The results of the present study are discussed here:

5.1 *IN VITRO* PHENOTYPING

Screening of 10 rice cultivars was performed by pot culture experiment. 14 days old rice seedlings were subjected to submergence of 7, 14 and 21 days. Survival percentages were recorded after 10 days after de submergence. Pre submerged plant height, leaf count and post submerged recovery percentages were the main biometric observation to be made. Observations were interpreted by statistical analysis.

Statistical analysis of pre submerged plant height suggest that no genotypes have height less than tolerant check Swarna- *sub1*. KAUM7 and KAUM 19 are having on par height or similar least height after 'Swarna- *sub1*'. The increase in plant height of the tolerant line was the same under submergence and normal conditions, while other 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 SUB1A-1 that is responsible for submergence tolerance. This was also in accordance with the findings of Das and Sarkar, 2001; Singh *et al.,* 2001 and Bhattacharjee *et al.,* 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, there by leaving carbohydrate reserve for survival after maintenance after submergence when flood water recedes.

Leaf count of 10 different genotypes at 14 days old plants showed no significant difference between genotypes in leaf count. All genotypes were similar and variations are due to environment. In the case of post submerged recovery percentages, significant difference were recorded for of 7 days and 14 days of submergence.

The check variety Swarna-*sub1* has significantly higher recovery percentage of 94% after 14 days of complete submergence. Among the listed genotypes

KAUM 7 and KAUM19 had partial submergence tolerance of 66% and 58% respectively. After 14 days submergence which was statistically on par but significantly less than compared to tolerant check Swarna- *sub1*. KAUM 7 had 66% recovery which is significantly less than compared to KAUM 19.

Hence it is inferred that none of the list genotypes have 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 is significantly higher than KAUM 19. None of the other genotypes survive under 14 days of complete submergence.

In both 7 and 14 days of submergence Swarna- *Sub1* showed highest survival percentages 94 and 84 respectively.

5.2 MOLECULAR SCREENING FOR THE IDENTIFICATION OF *SUB1* QTL

In the present study, 2 SSR markers were used to identify the *Sub1* marker across 10 rice genotypes. Genotyping was done using an *InDel* marker ART5 and Sub 1 BC 2 which are tightly linked to *Sub1* locus (Septiningsih *et al.* 2009). ART5 and Sub1 BC2 were found to be polymorphic with the test genotypes and checks used. Ten rice cultivars were genotypically screened out to determine the tolerance and susceptibility status of *sub-1* gene using tightly linked Sub1BC2 indel marker. Result indicates the presence of an approximately 268 bp fragment specific for *Sub-1* mediated submergence tolerance in the differential line Swarna *Sub-1* and approximately 230 bp fragments corresponding to the susceptible cultivar Jyothi. Then the rice genotypes were screened out by ART5 marker. Swarna *Sub1* shows approximately 217 bp fragment in 8% poly acrylamide gel. Out of 10 rice genotypes, 2 genotypes, KAUM7 and KAUM 19 shown approximately similar band pattern of Swarna-*Sub1* for both the markers indicating the presence of *Sub1* gene, remaining 8 genotypes namely KAUM1, KAUM2, KAUM3, KAUM4, KAUM5, KAUM6, KAUM18, KAUM19, KAUM20 showed the absence of *Sub1* marker.

SUMMARY

6. SUMMARY

The study entitled "Molecular screening of rice genotypes for submergence tolerance" was conducted in Rice Research Station, Vyttila. The main objective of this study was *in vitro* phenotyping for the field tolerant rice genotypes under submergence and molecular screening for *sub 1* QTL using tightly linked SSR markers. Statistical analysis of biometric observation showed that both KAUM 7 and KAUM 19 showed better survival rate than other test genotypes KAUM1, KAUM2, KAUM3, KAUM4, KAUM5, KAUM6, KAUM18 and KAUM 20, but significantly different from the tolerant check Swarna *sub1*. Post submerged recovery percentage of KAUM7 and KAUM 19 were significant in both 7 and 14 days of submergence compared to other genotypes tested, but significantly less performance compared to the tolerant check Swarna- *Sub1*. In *in vitro* phenotyping KAUM 7 and KAUM 19 survive 14 days of submergence and no test genotypes survived on 21 days submergence. Presence of *Sub1* QTL in these genotypes were confirmed through molecular screening. Molecular markers are genetic loci that can be easily tracked and quantified in a population and may be associated with a particular trait. They are used to detect the presence of valuable traits such as stress tolerance. In the present study, a set of 2 SSR primers were used to identify the presence of *Sub1* QTL among the 10 genotypes. Ten rice cultivars were genotypically screened to determine the tolerance and susceptibility status using tightly linked indel markers Sub1BC2 and ART5. Swarna *Sub1* used as tolerant check and Jyothi as susceptible check. Position of Sub1 BC2 marker is in between *Sub1B* and *Sub1C*. Expected size of Sub1 BC2 forward primer is 231 bp and Sub1 BC2 reverse primer is 239 bp. Amplification pattern of rice genotypes obtained by SSR marker Sub1 BC2 in 8% poly acryl amide gel is an approximately 230 bp fragment specific for *sub-1* mediated submergence tolerance. Position of ART5 marker is in *sub1C* promoter. Expected size of ART5 forward primer is 217 bp and ART5 reverse primer is 202 bp. Amplification pattern of rice genotypes obtained by SSR marker ART5 is approximately 217 bp fragment specific for *sub-1* mediated submergence tolerance. Out of 10 different

genotypes only 2 genotypes, KAUM 7 and KAUM19 showed approximately similar band pattern of tolerant check 'Swarna-*Sub1*'. Other 8 genotypes exhibits similar band pattern that of susceptible check 'Jyothi'. Hence from biometric observation and molecular screening analysis, it is clear that KAUM7 and KAUM19 have sub1 QTL partially in their chromosome.

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APPENDICES

APPENDICES

APPENDIX I

CTAB Extraction Buffer (pH-8.0)

CTAB	2%	
Tris HCl (pH)	100 mM	
EDTA	20 mM	
NaCl	1.4M	
β – mercaptoethanol	0.2% (V/V)	Freshly added prior to DNA extraction
PVP	4% (W/V)	

APPENDIX II

TE Buffer (pH-8.0)

Tris HCl (pH-8.0)	10 mM
EDTA	1mM

APPENDIX III

TBE Buffer (50X) for 100 ml solution (pH- 8.2-8.5)

Tris base	53.5 g
Boric acid	27.5 g
EDTA	4.9g

APPENDIX IV

8% Polyacryl amide gel

30% Acrylamide-bisacryl amide

10X TBE buffer

Ammonium persulphate

Deionized water

TEMED

APPENDIX V

Fixer

Absolute ethanol	50 ml
Glacial acetic acid	2.5 ml

Make up the volume to 500 ml with Millipore water.

APPENDIX VI

Stainer

Silver nitrate	0.75g
37% formaldehyde	750µl

Make up the volume to 500 ml with Millipore water.

APPENDIX VII

Developer

Sodium hydroxide	7.5 g
37% formaldehyde	750 µl

Make up the volume to 500 ml with Millipore water.

APPENDIX VIII

Gel loading dye (6X) for 120 ml solution

Bromophenol blue	200 mg
Xylene cyanol	50 mg
Glycerol	90 ml
EDTA (0.5 M, pH -8.0)	20 ml
Water	10 ml

MOLECULAR SCREENING OF RICE GENOTYPES FOR SUBMERGENCE TOLERANCE

Submitted by

ARYALEKSHMI A. S.

(2013-09-118)

Abstract of Thesis

Submitted in partial fulfilment of the

requirement for the degree of

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Kerala Agricultural University, Thrissur

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ABSTRACT

Rice (*Oryza sativa L.*) is the most important staple food crop that providing food to about half of the population. One of the major constraints in rice cultivation worldwide is submergence stress. This mainly affects flood prone areas like kuttanadu Pokkali as well as low lying paddy fields in Kerala. *Sub1* is a major quatitative trait locus (QTL) representing a cluster of three ethylene responsive factor (ERF) genes: *Sub1A, Sub1B* and *Sub1C* that confers submergence tolerance to rice genotype. Submergence tolerant rice variety is credited with maintaining energy and alcoholic fermentation which require high levels of carbohydrates. Sub1 locus contain *Sub1A, Sub1B* and *Sub1C*, all of which are encode ethylene responsive factor and are up regulated under submergence, but only *Sub1A* is responsible for the flash flood tolerance. The near isogenic lines generated by the introgression of the *Sub1* from FR13A into to susceptible cultivar shows restricted shoot elongation similar to FR13A. Submergence tolerant plants suppress the expression of gene encoding α- amylase and sucrose synthase, which are involved in sucrose metabolism. *Sub1 A* enhance the expression of genes encoding *SLENDER RICE -1 (SLR1)* and *SLR1 like 1(SLRL1),* which are key repressor of gibberellins (GA) signalling in rice; it also negatively regulate s the GA response in order to restrict shoot elongation under submergence. Rice varieties with the *Sub1* gene were shown higher survival rate with better yield even after 2 weeks of submergence stress. The advancement of marker assisted selection (MAS) have striking impacts on developing improved *Sub1* varieties that can meet the increasing demands of consumers along with population hike. In the present study, two rice genotypes were observed with *Sub1* QTL using SSR markers through molecular characterization in order to find out the best submergence tolerant lines. Moreover this finding lays the foundation for further researches with *sub 1* QTL for crop improvement.

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

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