

UNRAVELING THE GENETICS OF SUBMERGENCE TOLERANCE THROUGH PHENOTYPING AND MOLECULAR MARKER SCORING IN RICE (*ORYZA SATIVA* L.)

Muthuvijayaragavan. R^{1*} and E. Murugan²

¹Department of Plant Biotechnology, Centre for Plant Molecular Biology and Biotechnology, Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu, India – 641 003

²Department of Plant Breeding and Genetics, Agricultural College and Research Institute, Tamil Nadu Agricultural University, Madurai, Tamil Nadu, India – 625104

*Corresponding author's: Muthuvijayaragavan. R, Email : muthu.ragavan@gmail.com, Email addresses of co-author: murugan.e@tnau.ac.in

ABSTRACT

The major rice quantitative trait locus *Submergence 1* (*Sub 1*) confers tolerance to submergence for about 2 weeks. To identify the inheritance pattern of submergence tolerance, we have conducted a polymorphic survey using allele-specific markers targeting SUB1A and SUB1C and also field evaluation for assessing the tolerance mechanism through backcross populations developed with highly susceptible genotypes ADT43, Improved White Ponni (IWP) and highly tolerant genotype FR13A. The six generations viz., P₁, P₂, F₁, F₂, BC₁ and BC₂ of the two cross combinations viz., ADT 43 x FR 13A and IWP x FR 13A were used for this investigation. The marker segregation pattern indicated that a duplicate dominant allele influence tolerance as seen from the observed F₂ ratio 15:1 for susceptible and tolerant in cross 1 and cross 2 under submergence condition. The study on inheritance pattern for submergence tolerance indicated that both the crosses the submergence tolerance was found to be controlled by duplicate dominant genes in which is confirmed through respective BC₁ and BC₂ crosses. The field study (phenotypic scoring) also confirmed the ratio in F₂ of 15: 1 (duplicate dominant epistasis).

KEYWORDS: Submergence; Segregating generations; Inheritance pattern; Markers

1. INTRODUCTION

Rice is the most important staple food crop for more than half of the global population and accounts for approximately 20% of the daily food calorie intake (Khush, 2003). Indian sub-continent and South-East Asia are the most important centers of diversity for rice (*Oryza sativa* L.), where rice is widely cultivated on the flood plains of the major river basins and deltas. Rice cultivation in these areas is not only crucial for the world food security but is also closely associated with the tradition, culture and customs of the people (Lu and Snow (2005); Sasidharan *et al.*, (2017)). Submergence tolerance is defined as the ability of a rice plant to survive and continue growing after being completely submerged in water for several days. Rice plants are less tolerant of submergence at the early growth stages (Adkin *et al.*, 1990). Submergence at vegetative stage is quite often followed by reproductive stage drought in the rain-fed lowland river plains of India and South-East Asia. The majority of rice growing areas in this region suffers from abiotic stresses such as drought, flooding and salinity (Singh *et al.* 2015). Worldwide, 15 million hectares of rice suffers from flooding stress of one kind or other causing huge economic losses. Different kinds of flooding include: (i) flooding during seed germination, (ii) complete submergence for 1–2 weeks due to flash flooding at seedling or tillering stage, (iii) stagnant water flooding of 25–50 cm in the areas with poor drainage, near the banks of overflowing rivers, (iv) saline water flooding in the coastal and tidal areas, and (v) deep water flooding lasting for a prolonged period of more than a month with water levels ranging from a 50 cm to several meters (Singh *et al.* 2015 and Septiningsih *et al.* 2013). Traditional varieties and landraces of rice are available for cultivation in each of these situations. These are low yielding but can be useful source of genes for breeding high yielding stress tolerant rice cultivars. Several QTLs and genes for different kinds of flooding stress have been identified from these germplasm (Jean *et al.*, 2016, Septiningsih and Mackill 2018). During 1970s hundreds of landraces of rice collected from the flood-prone areas of the world were screened for flash food submergence tolerance at IRRI. Consequently, FR13A, FR43B, Kurkarruppan, GodaHeenati, Thuvalu, *etc.* were recognized as flash flooding tolerant. *Aus* cultivar FR13A was found to be one of the best sources for submergence tolerance. Genetic studies using a population derived from FR13A identified a major QTL *Submergence 1* (*SUB1*) on the chromosome 9 (Xu and Mackill (1996)). This was followed by map-based cloning of three ethylene response factor-like genes, *Sub1A*, *Sub1B*, and *Sub1C*, and demonstration of the role of *Sub1A* in submergence tolerance by genetic transformation of a sensitive variety Liaogeng (Xu *et al.* 2000). Analysis of 21 rice accessions showed that all of them possessed *Sub1B* and *Sub1C* genes, but only a sub-set of 12 cultivars possessed the *Sub1A* gene, responsible for the submergence tolerance in FR13A (Xu *et al.* 2006). The *Sub1A* positive varieties possessed one of the two alleles, *Sub1A-1* or

Sub1A-2, which were associated with the tolerant or intolerant phenotype, respectively (Xu *et al.* 2006). However, there are limited studies on inheritance pattern of the *Sub1B* and *Sub1C* genes and *Sub1A*-independent mechanisms of submergence tolerance in food-tolerant rice germplasm. Submergence tolerant rice cultivars have been developed in the background of several popular mega varieties of rice using marker-assisted backcross breeding in order to transfer 0.8 to 7.8 Mbp of the donor chromosome 9 segment around the *Sub1* locus with varying degree of success (Neeraja *et al.* 2007 and Septiningsih *et al.* 2009). The inheritance and expression of traits associated with submergence stress tolerance at the seedling stage are physiologically and genetically complex. However, there is need to find novel genes and alleles for tolerance to submergence and other kinds of flooding stresses including anaerobic germination, deep water and prolonged water stagnation. Collection and characterization of rice landraces and traditional varieties adapted to flood-prone environments is important for the identification of donors with new genes and alleles. Hence, the present investigation was undertaken to reveal the inheritance pattern of submergence tolerance through phenotypic scoring and Marker Assisted Selection by utilizing two popular varieties of Tamil Nadu *viz.*, ADT 43 and IWP and one donor submergence tolerance (FR13A).

2. MATERIALS AND METHODS

The six generations *viz.*, P₁, P₂, F₁, F₂, BC₁ and BC₂ of the two cross combinations (cross 1 (ADT 43 x FR 13A) and cross 2 (IWP x FR 13A)) were raised in nursery beds. Twenty-five days old seedlings were transplanted to main field at Agricultural College and Research Institute, Madurai in Randomized Block Design under submerged condition of 40 cm depth with two replications, adopting a spacing of 20cm between rows and 15cm between plants. The parents along with F₁, F₂, BC₁ and BC₂ were transplanted in the main field at the water level of 40 cm. The water level was monitored periodically and maintained at 40 cm for 14 days. After 14 days of complete submergence, the field was de-submerged and survival of the plants was scored. The scoring was done as per the standard evaluation system of rice (SES) developed by IRRI (1996) based on the per cent plant survival (PPS) as shown in Table 1. Ten plants each in P₁, P₂ and F₁, 250 plants in F₂ and 150 plants each in BC₁ and BC₂ were maintained for each cross per replication for the study. The phenotypic scoring was done at 21 days after de-submergence of water as per the standard evaluation system of rice (SES) developed by IRRI for submergence in all the six generations.

Table 1. Scale developed by HilleRis Lambers and Vergara (1982)

Score	Symptoms	Tolerance
1	Erect, little or no elongation	HT- Highly tolerant
3	Erect, green, elongated	T- Tolerant
5	Elongated and bent at middle	MT- Moderately tolerant
7	Elongated and lodged flat	S- Susceptible
9	Elongated and apparently dead	HS - Highly susceptible

The scores 1 and 3 are grouped as tolerant and scores 5, 7 and 9 are grouped under susceptible category for phenotypic scoring.

2.1. DNA Isolation

Genomic DNA was isolated from young leaves of the parents, ADT43, IWP and FL478 after transplanting to the experimental field using the modified CTAB protocol as described by Ausubel *et al.* (1994). Polymerase chain reaction- (PCR-) based amplification of the target genomic fragments by the primer pairs for each selected marker was performed in a 10 μ l reaction mix constituted by adding 25–30 ng genomic DNA, 5 pmol each of the two primers, 0.05 mM each of the four dNTPs, and PCR buffer (10x) containing 10 mM Tris (pH 8.4), 50 mM KCl, and 1.8 mM MgCl₂. To this mix, 0.5 μ l of Taq DNA polymerase was added, and the volume made up to 10 μ l using nuclease free water. The PCR was run for 35 cycles comprising of denaturation for one minute at 94°C, followed by annealing for 45 Sec at 55°C, and primer elongation for one minutes at 72°C, sandwiched between an initial denaturation for five minutes at 94°C and the final extension for seven minutes at 72°C. The amplified products were electrophoresed in 3% agarose gel, and the products were visualized using a gel documentation system.

2.2. SSR marker studies for submergence tolerance

Parental polymorphic survey was made to screen the parents (ADT43, IWP and FR13A) by using four primers *viz.*, GnS2, AEX1, ART 5 and Sub 1C 173 which are tightly linked to Sub 1 QTL. The details of the primers used for parental survey for submergence tolerance are furnished below.

Primer	Sequence (5' 3') →	Tm (°C)	Position	Type of marker	Expected amplicon size (bp)	Reference
GnS2 F	CTTCTTGCTCAACGACAACG	57	exon of SUB1A	CAPS (AluI/PvuII)	242	Septiningsih <i>et al.</i>
GnS2 R	TCGATGGGGTCTTGATCTCT	57				

AEX1F	AGGCGGAGCTACGAGTACC A	62	non-synonymous SNP for SUB1A	mismatch	231	(2013)
AEX1R	GCAGAGCGGCTGCGA	56				
ART5F	CAGGGAAAGAGATGGTGGGA	57	SUB1C promoter	15 bp insertion in NB/93-11	217	
ART5R	TTGGCCCTAGGTTGTTTCAG	57				
Sub1C173F	AACGCCAAGACCAACTTCC	57	exon of SUB1C	9 bp deletion in NB/93-11	173	
Sub1C173R	AGGAGGCTGTCCATCAGGT	59				

In each generation viz., F₁, F₂, BC₁ and BC₂ the target locus *Sub 1* was monitored by the above four gene specific markers tightly linked to target loci at *Sub 1* region on chromosome number 9 for submergence tolerance. The plants in all the generations viz., P₁, P₂, F₁, F₂, BC₁ and BC₂ in both replications were used for marker scoring. The scoring was done for the marker data by means of visualizing the band appearance. If the band of individual plant matches with the donor parent, the plant is designated as score '1' and the final scores is added to the total population in each generation. Conversely, if the band of the individual plant matches with the female parent (susceptible), the plant is designated as score '0' and the final score is added to the total population in each generation. The plant falls under the category of score '0' means it is designated as susceptible and score '1' means it is tolerant.

2.3. Data analysis

The expected values corresponding to the observed values on phenotypic scoring under field condition and marker scoring under laboratory condition for tolerance and susceptibility were calculated based on the presumed ratio. The deviations were put to Chi-square test (χ^2) (Fisher, 1936).

3. RESULTS AND DISCUSSION

3.1. Inheritance pattern for Submergence tolerance through Marker analysis

The chi-square test by marker analysis and phenotypic scoring for the deviations from the expected genetic ratio of the segregating generations for submergence was made in two crosses viz., cross 1 and cross 2 at Agricultural college and Research Institute, Madurai in field condition and the marker study also made at Biotechnology laboratory, Madurai and the results are presented in Tables 2 and 3. In P₁, P₂, F₁, F₂, BC₁ and BC₂ generation, the target locus *Sub 1* was monitored by markers tightly linked to target loci at *Sub 1* region for submergence tolerance in both the cross under study.

3.1.1. Cross 1 (ADT 43 x FR 13A)

The foreground selection for cross 1 (ADT43 x FR13A) in F₂ was performed on 250 plants using markers tightly linked to submergence tolerance loci *Sub 1*. Out of 250 plants, 19 plants showed fixed donor allele (tolerant allele (T)) and 231 plants showed fixed recipient allele (susceptible allele (S)) for ART 5 marker, 10 plants exhibited fixed donor allele (tolerant allele (T)) and 240 plants evidence fixed recipient allele (susceptible allele (S)) for GnS2, 12 plants showed fixed donor allele (tolerant allele (T)) and 238 plants exhibited fixed recipient allele (susceptible allele (S)) for Sub 1C 173 marker and 14 plants showed fixed donor allele (tolerant allele (T)) and 236 plants showed fixed recipient allele (susceptible allele (S)) for marker AEX1 in cross 1 under submergence. The results fit with the expected ratio of 1: 15 for the homozygous donor allele and homozygous recipient allele groups with a non-significant chi-square value of 0.780 (ART 5), 2.157 (GnS2), 0.895 (Sub 1C 173) and 0.179 (AEX1) at a probability level of 0.771. (Table 2, Fig. 2, 3 and 4)

In cross 1 of BC₁, 150 progenies were subjected to foreground selection. The total 150 plants were segregated into 4 tolerant plants with homozygous donor allele (T) and 146 susceptible plants with homozygous recipient allele (S) by molecular marker analysis using ART 5 and Sub 1C 173. Moreover, 1 tolerant plant with homozygous donor allele (T) and 149 susceptible plants with homozygous recipient allele (S) exhibited by the marker GnS2 and also AEX1 marker 2 were tolerant with homozygous donor allele (T) and 148 were susceptible with homozygous recipient allele (S).

In BC₂ generation of cross 1 (ADT43 x FR13A), 150 progenies derived from cross 1 were subjected to foreground selection using markers. The population was segregated into 39 tolerant and 111 susceptible (ART 5), 31 tolerant and 119 susceptible (GnS2), 28 tolerant and 122 susceptible (Sub 1C 173) and 32 tolerant and 118 susceptible (AEX1) by the presence of homozygous donor allele (T) or homozygous recipient allele (S) through molecular marker analysis. The result was fitted to the expected 1:3 ratio in BC₂ generation with a non-significant chi-square value of 0.080 for ART 5 marker, 1.502 for GnS2, 3.209 (Sub 1C 173) and 1.076 for AEX1 marker at probability value of 0.05.

3.1.2. Cross 2 (IWP x FR 13A)

In F₂ generation, foreground selection was made on 250 plants using markers tightly linked to the submergence tolerance for the loci *Sub 1* in cross 2 (IWP x FR13A). Out of 250 plants, 13 plants showed fixed donor allele (tolerant allele (T)) and 237 plants showed fixed recipient allele (susceptible allele (S)) for ART 5 marker, 17 tolerant and 233 susceptible were showed by GnS2. While 11 tolerant and 239 susceptible plants (Sub 1C 173) and 9 tolerant and 241 susceptible (AEX1) in cross 2 under submerged condition. The results fitted to the expected 1: 15 (T: S) ratio of this generation for the homozygous donor allele and homozygous recipient allele

groups with a non-significant chi-square value of 0.469 (ART 5), 0.130 (GnS2), 1.458 (Sub 1C 173) and 2.993 (AEX1) at a probability level of 0.05. In BC₁ generation of cross 2, the foreground selection was done for 150 plants, in which 1 plant showed fixed donor allele (tolerant allele) and 149 plants showed fixed recipient allele (susceptible allele) by ART 5 and Sub 1C 173 markers under submergence. Utilizing the marker GnS2 and AEX 1 also, 2 plants showed fixed donor allele (tolerant allele) and 148 with recipient allele (susceptible allele). (Table 3)

Regarding BC₂ generation of cross 2 (IWP x FR13A), the foreground selection was carried out over 150 plants. Out of 150 plants, 43 plants exhibited homozygous donor allele and 107 plants with homozygous recipient allele using ART5 marker, where as 29 plants showed homozygous donor allele and 121 plants with recipient allele through the GnS2 and Sub 1C 173 markers. The Sub 1 loci tightly linked marker AEX 1 exhibited 31 plants for homozygous donor allele and 119 plants for homozygous recipient allele. The results fitted to the expected ratio of 1:3 in B₂ generation with a non-significant chi-square value of 1.076 (ART 5), 2.569 (GnS2 and Sub 1C 173) and 1.502 (AEX 1).

3.2. Inheritance pattern for Submergence tolerance through phenotypic scoring

Regarding submergence in field condition through artificial screening done at Agricultural college and Research Institute, Madurai revealed that the parents, ADT43 and IWP were highly susceptible to submergence, whereas FR13A was highly tolerant to submergence. Based on the phenotypic scoring under field condition, the F₁ and BC₁ were found to be susceptible for the cross 1 and 2. In cross 1, the chi-square test for the expected ratio was 1:15 (20 tolerant: 230 susceptible) in F₂ and 1:3 (46 tolerant: 104 susceptible) in B₂. In cross 2, the chi-square test was non-significant, showing fitness for the expected ratio of 1:15 and 1:3 (tolerant: susceptible) for submergence tolerance in F₂(19 tolerant: 221 susceptible) and BC₂(31 tolerant: 119 susceptible) generations respectively (Table 4 and 5).

Rice cultivars introgressed with SUB1 QTL showed less elongation and better survival under submergence (Panda and Sarkar 2013). These cultivars maintained greater activities of alcohol dehydrogenase compared to the susceptible cultivars. Chlorophyll content decreased with increased period of submergence and the rate of 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.

Based on the marker study using four tightly linked gene specific markers viz., ART 5, GnS2, Sub 1C 173 and AEX1, data were subjected to Chi-square test to determine their goodness of fit to various genetic ratios of the segregating generations under submergence for cross 1 (ADT43 x FR13A) and cross 2 (IWP x FR13A). The F₁ of the cross 1 and cross 2 were susceptible to submergence. In F₂ of the cross 1, exhibited 19 tolerance and 231 susceptible to marker ART 5, 10 tolerant and 240 susceptible (GnS2), 12 tolerant and 238 susceptible (Sub 1C 173) and 14 tolerant and 236 susceptible (AEX 1) whereas in cross 2 the F₂ had a segregation of 13:237 (ART 5), 17:233 (GnS2), 11:239 (Sub 1C 173) and 9:241 (AEX 1) under submergence for tolerance and susceptible respectively. The results of both the crosses fitted into 1:15 ratio (Tolerant: Susceptible). The 150 plants derived from BC₁ in cross 1 and cross 2 were segregated into 4 tolerant and 146 susceptible (ART 5 and Sub 1C 173), 1 tolerant and 149 susceptible for GnS2 and 2 tolerant and 148 susceptible for AEX 1 marker under submergence in cross 1. Whereas in cross 2, 1: 149 of tolerant and susceptible for ART 5 and Sub 1C 173. Regarding the markers GnS2 and AEX 1, 2:148 of tolerant and susceptible plants were exhibited through marker study. However, in BC₂ of cross 1 were segregated into 39 tolerant and 111 susceptible (ART 5), 31 tolerant and 119 susceptible (GnS2), 28 tolerant and 122 susceptible (Sub 1C 173) and 32 tolerant and 118 susceptible (AEX 1) under submergence. In cross 2 also the segregation was 43: 107 for ART 5 marker, 29: 121 (GnS2 and Sub 1C 173) and 31: 119 (AEX 1) for tolerant and susceptible. In both crosses the chi-square test was non-significant showing the fitness of the expected ratios of 1:3 (tolerant: susceptible) in back cross (BC₂) (Tables 2 and 3) under marker study for submergence tolerance.

Most rice cultivars are damaged when subjected to complete submergence for several days. Modern high-yielding rice varieties are generally sensitive to complete submergence; however, a few tolerant landraces have been identified (Sarkar and Bhattacharya, 2011). Cultivars highly tolerant to flash flooding are rare. Only 0.1% of 15,000 rice germplasm evaluated at the Central Rice Research Institute, Cuttack, India survived under 12 days of complete submergence (Sarkar and Mohanty, 2010). *Sub1* provided a substantial enhancement in the level of tolerance of all the sensitive mega varieties. *Sub1A* is confirmed as the primary contributor to tolerance, while *Sub1C* alleles do not seem important. Lack of dominance of *Sub1* suggests that the *Sub1A-1* allele should be carried by both parents for developing tolerant rice hybrids (Septiningsih *et al.*, 2009). In field condition under submergence through artificial screening revealed that the parents, ADT43 and IWP were highly susceptible to submergence, whereas FR13A was highly tolerant. Based on the phenotypic scoring under field condition, the F₁ and BC₁ were found to be susceptible for both the crosses 1 and 2. In cross 1, the chi-square test for the expected ratio was 15:1 for susceptible: tolerant in F₂. In BC₁ all are susceptible in both the crosses. Where as in BC₂, the submergence tolerance was exhibited 3:1 for susceptible and tolerant. (Table 4)

The phenotyping of rice cultivars for submergence tolerance could be done more efficiently by employing chlorophyll a fluorescence technique (Sarkar and Bhattacharjee, 2011). Subsequently, FR13A occupied the core position in the National and International rice breeding programmes from which the submergence tolerance gene SUB1A was identified and cloned (Xu and Mackill, 1996; Xu *et al.*, 2006). Based on the present study, the inheritance pattern for submergence tolerance revealed that the tolerance was recessive to susceptible in both the

crosses. The segregation pattern of F₂ revealed that the submergence was controlled by duplicate dominant epistasis by two genes as per the observed F₂ ratio of 15:1 for susceptible and tolerance in both the crosses. The above inheritance pattern for submergence tolerance was confirmed by the segregation of test cross ratio with 3:1 (susceptible: tolerant) in BC₂ generation. Thus, the gene action for submergence tolerance was observed to be governed by digenic duplicate interaction confirmed through both marker and field condition.

In contrary, the earlier findings by few workers reported dominance of tolerance over non-tolerance and involvement of both major and minor genes in the inheritance pattern (Mohanty and Khush, 1985, Mohanty *et al.* 2000). Segregation analysis of progeny from crosses between tolerant and intolerant types (Suprihatno and Coffman, 1981) gave an early indication that submergence tolerance is controlled by a small number of genes that are partially or completely dominant.

4. CONCLUSION

The present study revealed that submergence tolerance was controlled by two genes with duplicate dominant interaction. The coincidence of QTL for traits important for submergence tolerance provides significant genetic evidence linking physiological responses to submergence tolerance in rice. Hence it is easy and possible to develop and introgress the submergence tolerance gene to desired genotype through conventional breeding method to develop high yielding coupled with submergence tolerance rice varieties. Harnessing molecular biology to ameliorate the effects of environmental stress is a major challenge for biotechnologists (Grover *et al.* 1999; Ratinasabapathi, 2000) and one that is being met with respect to submergence tolerance. The identification of tightly linked DNA markers holds particular promise for transferring the submergence tolerance trait from FR13A or related lines into commercially important cultivars. Such markers are already known to be carried over when tolerance is introduced into an agronomically desirable background by conventional crossing and progeny phenotyping (Siangliw *et al.* 2003). The potential for identifying other mechanisms of tolerance derived from resilient cultivars unrelated to FR13A (e.g. GodaHeenati and Vaidehi) remains unexplored and represent a promising additional resource for plant breeders.

Data Availability Statement: The data supporting the findings of this study are fully presented within the article. Additional data, clarifications, or specific datasets related to the study may be obtained from the corresponding author upon reasonable request through email communication.

Acknowledgements: The authors convey their highest gratitude to the reviewers for the feedback. Many thanks to the University Grants Commission, New Delhi, India for financial support through Rajiv Gandhi National Fellowship.

Conflicts of Interest: The authors declare no conflicts of interest.

REFERENCES

1. Khush, G. S (2003). Productivity improvements in rice. *Nutr. Rev.* 61 S114–S116.
2. Lu, B. R. & Snow, A. A (2005). Gene flow from genetically modified rice and its environmental consequences. *BioSci* 55 669–678.
3. Sasidharan. R, Julia Bailey-Serres, Ashikari. M, B. J. Atwell, T. D. Colmer, K. Fagerstedt, T. Fukao, P. Geigenberger, K. H. Hebelstrup, R. D. Hill, M. J. Holdsworth, A. M. Ismail, F. Licausi, A. Mustroph, M. Nakazono, Ole Pedersen, P. Perata, M. Sauter, Ming-Che Shih, B. K. Sorrell, G. G. Striker, J.T. van Dongen, J. Whelan, Shi Xiao, Eric J. W. Visser and Laurentius A. C. J. Voesenek (2017). Community recommendations on terminology and procedures used in flooding and low oxygen stress research. *N. Phytol.* 214 1403–1407
4. AdkinSW, Shiraishi T, McComb JA (1990). Submergence tolerance of rice-A new glasshouse method for the experimental submergence of plants. *Physiologia Plantarum* 80: 642–646.
5. Singh, A., Mukul, M. Joshi, M. Ram, M. Arya and P.K. Singh (2015). Screening and evaluation of rice cultivars for submergence tolerance using SSR markers. *The Bioscan*, 9(1-2) 255-259.
6. Jean, Z.C., Gonzaga, J. Carandang, D.L. Sanchez, D.J. Mackill and E.M. Septiningsih. 2016. Mapping additional QTLs from FR13A to increase submergence tolerance in rice beyond SUB 1. *Euphytica*, 209(3): 627-636.
7. Septiningsih, E.M, B.C.Y. Collard, S. Heuer, J. Bailey-Serres, A.M. Ismail, D.J. Mackill (2013). Applying genomics tools for breeding submergence tolerance in rice. In: Varshney RK, Tuberosa R, eds. *Translational genomics for crop breeding: abiotic stress, yield and quality*, 1st edn. Chichester, UK: John Wiley and Sons, Inc., 9–30.
8. Septiningsih, E. M. and Mackill, D. J (2018). Genetics and Breeding of Flooding Tolerance in Rice. In: Sasaki T., Ashikari M. (eds.) *Rice Genomics, Genetics and Breeding*. Springer, Singapore.
9. Xu K, Mackill D.J (1996). A major locus for submergence tolerance mapped on rice chromosome 9. *Mol Breed.* 2 219–24.
10. Xu K, Xu X, Ronald PC, Mackill D.J (2000). A high-resolution linkage map in the vicinity of the rice submergence tolerance locus Sub1. *Mol Gen Genet.* 263

681–9.

11. Xu K, Xu X, Fukao T, Canalas P, Maghirang-Rodriguez R, Heuer S, Mackill D.J (2006). Sub1A is an ethylene responsive-factor-like gene that confers submergence tolerance to rice. *Nature*. 442 705–8.
12. Neeraja C, Maghirang-Rodriguez R, Pamplona A, Heuer S, Collard B, Septiningsih E (2007). A marker-assisted backcross approach for developing submergence-tolerance rice cultivars. *Theor Appl Genet*. 115 767–76.
13. Septiningsih EM, Pamplona AM, Sanchez DL, Neeraja CN, Vergara GV, Heuer S, Ismail AM, Mackill D.J (2009). Development of submergence tolerant rice cultivars: the Sub1 locus and beyond. *Ann Bot*. 103 151–160.
14. HilleRisLambers D, Vergara BS (1982). Summary results of an international collaboration on screening methods for flood tolerance. *Proceedings of the 1981 International Deepwater Rice Workshop*. International Rice Research Institute, Los Baños, Philippines. p. 347–353.
15. IRRI (1996). Standard evaluation system for rice, 4th edition. International Rice Research Institute. Manila, Philippines.
16. Ausubel, F.M., R. Brent, E. Robert, D.D. Kingston Moore and J.G. Seidman (1994). *Short protocols in molecular biology*, 2nd Edn. London: John Wiley and Sons Inc.
17. Fisher, R.A (1936). The use of multiple measurements in taxonomic problems. *Ann. Eugen*, 7: 179-189.
18. Panda, D., and R.K., Sarkar. 2011. Non-structural carbohydrate metabolism associated with submergence tolerance in rice. *Genetics and Plant Physiology*. 1: 155-162.
19. Sarkar, R. K., Bhattacharjee B (2011). Rice genotypes with Sub1 QTL differ in submergence tolerance, elongation ability during submergence, and re
20. Sarkar R. K., Mohanty P (2010). An overview on submergence tolerance in rice: farmers' wisdom and amazing science. *J. Plant Biol*. 37: 191
21. Mohanty, H.K. and G.S. Khush (1985). Diallel analysis of submergence tolerance in rice. *Oryza sativa L. TheorAppl Genet.*, 70 467–73.
22. Mohanty, H.K., S. Mallik, A (2000). Grover. Prospects of improving flooding tolerance in lowland rice varieties by conventional breeding and genetic engineering. *Curr. Sci.*, 78(2) 132-140.
23. Suprihanto. B and W.R. Coffman (1981). Inheritance of submergence tolerance of rice (*Oryza sativa L.*). *Sabroa Journal* 13 98-108.
24. Grover A, Sahi C, Sanan N and A. Grover (1999). Taming Abiotic stresses in plants through genetic engineering: current strategies and perspective. *Plant science* 143 1-11.
25. Ratinasabapathi. B (2000). Metabolic engineering for stress tolerance: installing osmoprotectant synthesis pathways. *Annals of Botany* 86 709-716.
26. Siangliw. M. Toojinda. T, Tragoonrung. S and A. Vanavichit (2003). Thai jasmine rice carrying QTLch9 (SubQTL) is submergence tolerant. *Annals of Botany* 91 255-261.

Table 2. Inheritance for submerge tolerance by GnS2, ART 5, Sub 1C 173 and AEX1 markers data Cross 1 (ADT43 x FR13A)

Generations	Marker	Marker Score			Susceptible: Tolerant (Ratio)		Chi - square value	Probability between
		Total	1(T)	0 (S)	Observed	Expected		
P ₁		20	0	20	All susceptible	All susceptible	-	-
P ₂		20	20	0	All tolerant	All tolerant	-	-
F ₁		20	0	20	All susceptible	All susceptible	-	-
F ₂	ART5	250	19	231	14.78:1.21	15:1	0.780	0.50-0.30
	GnS2	250	10	240	15.36:0.64	15:1	2.157	0.20-0.10
	Sub 1C 173	250	12	238	15.23:0.77	15:1	0.895	0.50-0.30
	AEX1	250	14	236	15.10:0.90	15:1	0.179	0.95-0.50
BC ₁	ART5	150	4	146	4 tolerant	All susceptible	-	-

	GnS2	150	1	149	1 tolerant	All susceptible	-	-
	Sub 1C 173	150	4	146	4 tolerant	All susceptible	-	-
	AEX1	150	2	148	2 tolerant	All susceptible	-	-
BC₂	ART5	150	39	111	2.96:1.04	3:1	0.080	0.50-0.30
	GnS2	150	31	119	3.17:0.83	3:1	1.502	0.30-0.20
	Sub 1C 173	150	28	122	3.25:0.75	3:1	3.209	0.10-0.05
	AEX1	150	32	118	3.14:0.85	3:1	1.076	0.50-0.30

Table 3. Inheritance for submerge tolerance by GnS2, ART 5, Sub 1C 173 and AEX1 markers data Cross 2 (IWP x FR13A)

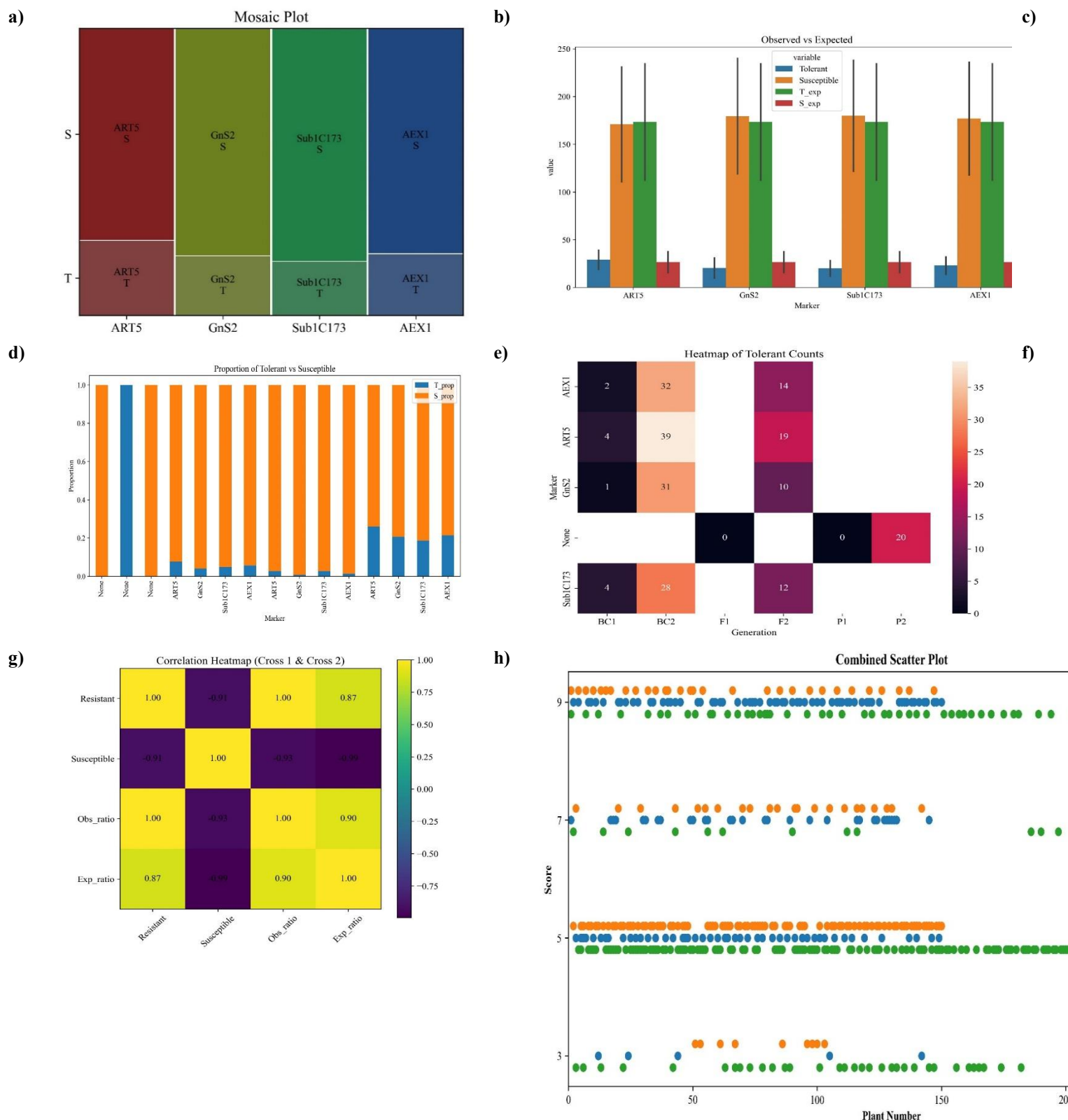
Generations	Marker	Marker Score			Susceptible: Tolerant (Ratio)		Chi - square value	Probability between
		Total	1(T)	0 (S)	Observed	Expected		
P₁		20	0	20	All susceptible	All susceptible	-	-
P₂		20	20	0	All tolerant	All tolerant	-	-
F₁		20	0	20	All susceptible	All susceptible	-	-
F₂	ART5	250	13	237	15.17:0.83	15:1	0.469	0.50-0.30
	GnS2	250	17	233	14.91:1.09	15:1	0.130	0.95-0.50
	Sub 1C 173	250	11	239	15.30:0.70	15:1	1.458	0.30-0.20
	AEX1	250	9	241	15.42:0.58	15:1	2.993	0.10-0.05
BC₁	ART5	150	1	149	1 tolerant	All susceptible	-	-
	GnS2	150	2	148	2 tolerant	All susceptible	-	-
	Sub 1C 173	150	1	149	1 tolerant	All susceptible	-	-
	AEX1	150	2	148	2 tolerant	All susceptible	-	-
BC₂	ART5	150	43	107	2.85:1.14	3:1	1.076	0.30-0.20
	GnS2	150	29	121	3.23:0.77	3:1	2.569	0.20-0.10
	Sub 1C 173	150	29	121	3.23:0.77	3:1	2.569	0.20-0.10
	AEX1	150	31	119	3.17:0.82	3:1	1.502	0.30-0.20

Table 4. Inheritance of submergence tolerance by phenotypic scoring - Cross 1 (ADT43 x FR13A)

Generation	Observed values		Expected ratio	χ^2 values	Probability between
	Resistant	Susceptible			
P₁	-	20	-	-	-
P₂	20	-	-	-	-
F₁	-	Susceptible	-	-	-
F₂	20	230	1 : 15	1.301	0.20-0.30
BC₁	-	146	-		
BC₂	46	104	1 : 3	2.568	0.20-0.10

Table 5. Inheritance of submergence tolerance by phenotypic scoring - Cross 2 (IWP x FR13A)

Generation	Observed values		Expected ratio	χ^2 values	Probability between
	Resistant	Susceptible			
P₁	-	20	-	-	-
P₂	20	-	-	-	-
F₁	-	Susceptible	-	-	-
F₂	19	221	1 : 15	1.492	0.30-0.20
BC₁	-	142	-		
BC₂	31	119	1 : 3	1.502	0.30-0.20



Figures displaying the tolerant vs susceptible level in all the generations. **a)** Marker wise tolerance and susceptible proportion level. **b)** Proportion of tolerant and susceptible level for four markers. **c)** Individual marker wise tolerant segregation in all the generations. **d)** Generation wise tolerant proportion for submergence markers. **e)** Distribution of markers linked to submergence tolerance in parents and backcross progenies. **f)** Comparison of tolerance and susceptible level in both the crosses in F_2 and BC_2 generations. **g)** Correlation heat-map exhibiting the distribution of markers in cross 1 and cross 2. **h)** Individual plant score wise combined scatter plot in all the generations.



Plate 1. Field view of submergence evaluation



Plate 2. Field view during complete submergence



Plate 3. Field view during De-submergence

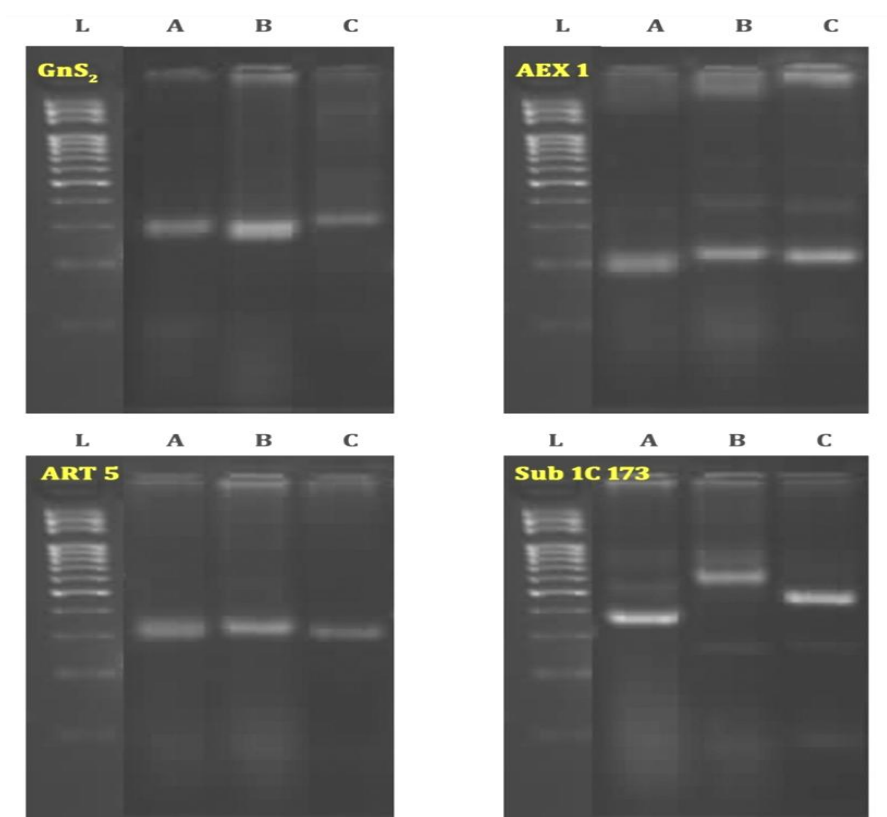


Fig. 2 SSR profile showing polymorphism between the parents by using primer GnS2, AEX 1, ART 5 and Sub 1C 173 (L- 100bp ladder, A-ADT43, B-FR13A and C-IWP)

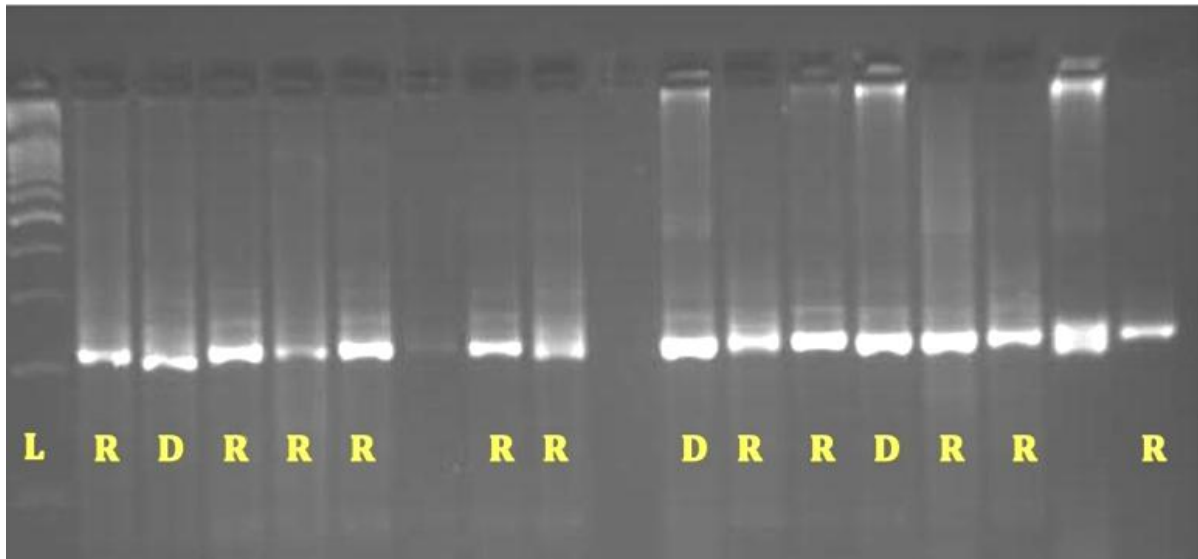


Fig. 3 Foreground selection of F2 population by using tightly linked Sub 1 marker ART 5 in Cross 2 (L-100bp ladder, R-Recipient allele and D-Donor allele)

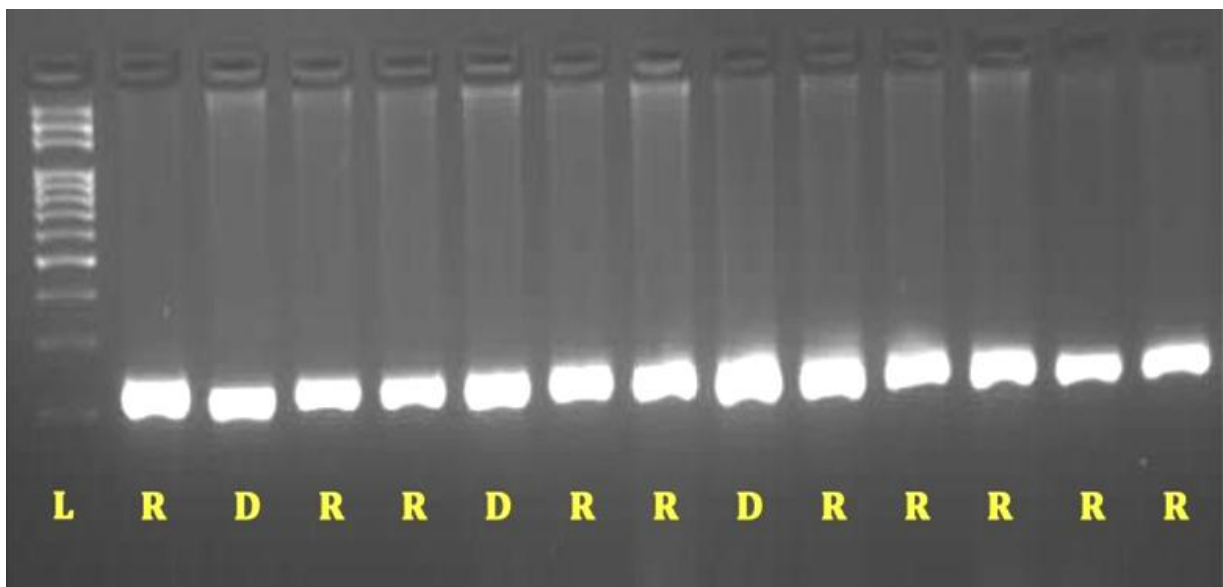


Fig. 4 Foreground selection of BC2 population by using tightly linked Sub 1 marker ART 5 in Cross 2 (L-100bp ladder, R-Recipient allele and D-Donor allele)