

MOLECULAR CHARACTERIZATION OF SUBMERGENCE TOLERANCE GENES AND LOCUS IN THE DEEP-WATER RICE CULTIVARS

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Received: 13.6.2018

Accepted: 30.8.2018

SUMMARY

Most of the rice cultivars exhibit suspension of growth when submerged to overcome the reduced availability of oxygen. When the situation continues, majority of the cultivars unable to recover after the flood recedes. However, there are fortunately some rice genotypes that can withstand such submerged condition for up to two weeks by adapting two totally opposite mechanisms. One type of cultivars elongates enormously at a very short span of time and the leaves come above the water level. In the second type, they remain under water without any growth. Cultivars of both types tolerate the submergence but the first category easily lodges when flood water recede. In those lines, yields are reduced drastically. In this study, we focus on characterize the genetic variation at the *Sub1* locus and to associate its relevance, if any, to submergence tolerance among the deep water landraces. As a first step, seeds of some rice cultivars collected from North-east Indian regions were initially selected for the characterization of genetic variation. The PCR based analysis involving several genes known to be associated with submergence tolerance did not reveal much difference. However, Southern hybridization revealed certain differences between submergence tolerant and susceptible cultivars. Although we did not notice major difference with regard to *Sub1* genes when tried with *EcoRI* and *BamHI*, differences were noticed with *adh1* and *RAmy3C* genes. Representative, Southern analysis showed the genetic variation among the deep-water cultivars as compared to Swarna and Sub1-Swarna. It is possible that deep-water rice cultivars may not differ in their genome at *Sub1* locus but they respond through SNORKEL genes under submergence.

Keywords: Gene, genetic variation, rice, submergence tolerance, *Sub1*

INTRODUCTION

Rice is a semi-aquatic plant. It can live, adopt and grow under water logging. Rice plants survive longer than other cereals under water. However, complete submergence is serious for the farmer as severe flooding results in reduced grain productivity and quality. Unfortunately, sudden and uncontrollable flash floods up to half a meter deep are common and last for several days. Resulted from overflowing rivers, low height earth barriers made to stop erosion, or accumulation from higher ground, and higher tides. Flooding in these areas, along with drought and salinity are main constraints affecting the yield.

Typically, deep-water rice responds by increasing in cell division and/or elongation of cells

leading to overall increase of internodes underwater stems. When plants are submerged under water, the levels of ethylene goes up triggering several mechanisms that include increased production of gibberellic acids (GA) and degradation of growth promoting abscisic acid (ABA) (Kende *et al.*, 1998; Hattori *et al.*, 2009). An interesting feature of GA mediated response is rapid elongation of stem that can reach up to 25 cm/day. Later some studies (Hattori *et al.*, 2009) have shown that such growth is regulated at least three different quantitative trait loci (QTLs). One of the most studied loci, the SNORKEL is located on chromosome 12 which encodes two ethylene responsive factor (ERF) proteins [SNORKEL1 (SK1) and SNORKEL2 (SK2)] containing DNA binding motif, one of the typical motif for any transcription factor. It is also

now well established that SK1 and SK2 are not present in the cultivars that do not elongate like deep-water rice and survive under submerged conditions for a longer duration (Hattori *et al.*, 2009). Such elongation growth response of these deep-water rice ensures that sufficient aerial tissue like leaves is in contact with the air for exchange of oxygen and to continue photosynthesis to meet the required energy for the submerged tissues (Bailey-Serres, Voeselek, 2010).

Despite of huge efforts in screening and selection of submergence tolerant plants, not much is known until the *Sub1* locus was identified in rice. Systematic studies lead to molecular mapping of a major QTL, designated as SUBMERGENCE 1 (*sub1*) onto rice chromosome 9. The *Sub1* locus has been estimated to contribute for about 70% of submergence tolerance in rice (Xu and Mackill, 1996). Based on elaborate crosses made between tolerant and susceptible lines and segregation pattern of the F1 progeny, rice breeders postulated that the submergence trait is a quantitative trait. Xu *et al.*, (2000) further narrowed it to less than a cM region on chromosome 9 using a large number of F2 population. Besides the *Sub1* QTL, there are three more QTLs identified in deep-water-rice on chromosome 1, 3 and 12 (Nemoto *et al.*, 2004). Oxygen, a major electron acceptor drops below the optimized level so that the plant energy consumption is reduced by shutting off the metabolism involving sugars, proteins and amino acids and lipid to conserve energy (Geigenberger, 2003). This conserved energy is redirected to the translation and

transcription of stress proteins that are needed for the plant survival. These stress proteins are called anaerobic polypeptides (ANP) (Drew, 1997). Major ANPs are sucrose synthetase (SUS), glucose phosphate isomerase, alcohol dehydrogenase (ADH), pyruvate decarboxylase (PDC), and lactate dehydrogenase. They are all part of the fermentation and the glycolysis pathway.

MATERIALS AND METHODS

Plant materials

Total six rice cultivars expressed well-known submergence tolerance mechanisms were collected for this study. Rice cultivars namely IR64, Swarna, Sub1-Swarna were collected from IARI (Indian Agricultural Research Institute), New Delhi, India. These varieties response to submergence condition by non-elongation mechanism. Other cultivars named Khongan, Taothabi and Murshi response to submergence by putative elongation mechanism, which were selected from Manipur, North-east Indian region.

Experimental conditions to assess submergence tolerance

Experiments involving submergence tolerance test have been conducted using two week old seedlings by submerging completely in the water in a clear glass tanks. Duration of the submergence was 1- 2 weeks depending on the type of experiment. They were then kept 1- 2 weeks outside the tanks for recovery.

Table 1. Primers and conditions applied for PCR.

Gene	Gene Bank Accession	Forward primer (5'- 3')	Reverse primer (5'- 3')	Annealing temp (° C)	Expected fragment size (bp)
<i>Sub1A</i>	DQ011598	AGGTGAAAATGATGCAGG	CTTCCCCTGCATATGATATG	50	614
<i>Sub1B</i>	AP005705	TTTCATGTTCCCTTCTGGTG	GCTGCTAATTAACCATTCCTCAA AAC	60	567
<i>Sub1C</i>	AP006758	CGTGGTAGTGACAAGTGCCTC	CGTTTATGTTGCTCCTGAATCTGC	60	535
<i>RAmy3C</i>	AP005891	ACCCTGCATTTTCTACGACC	GTCCTGAAAGAAATATTTCCGGC	57	313
<i>Sus1</i>	AC084380	TTGTGCAGCCCGCTTTCTAC	AAGAGCAGTGCCTAGGAATGC	57	578
<i>Pdc1</i>	AC121364	CGGGTACGAGTTCCAGATG	AGAAGCTCTTTGCTGGTGTC	57	426
<i>Pdc2</i>	AC137072	CTGCGGGGATGAATTCCAAATG	GATTTGGTGGCCTTGGAGTTG	57	473
<i>Pdc4</i>	AC121364	TGACGGTAGCTTCCAGATGAC	ATACTTCAACCTCTCGGCTG	54	520
<i>Adh1</i>	AC123521	CCAGTTTCGACAGGTACTTGC	CGAGATACACAGAAGAACCG	54	484
<i>Adh2</i>	AC123515	ACAGAGGTGCTGATTGAG	CAAGATCAAGCGATGAAAGG	54	583

Genomic DNA isolation and molecular characterization

Genomic DNA isolation was carried out following the protocol of Murray and Thompson (1980). All the primers used in this study were designed using oligoanalyzer program. For primer designing, genome sequence (Phytozome database or EST sequence of rice, extracted through “blastx” were used.

(<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>).

While designing primers, GC content was maintained between 40 - 60% and range of T_m was chosen between 55 - 65°C wherever possible. Primers and conditions applied for PCR were shown on table 1. Following PCR amplification program was set up: Initial denaturation at 94°C for 3 min followed by thermal cycling at 94°C for 30s; annealing at 50°C to 60°C for 30 - 45s and extension at 72°C for 1 min 30s for 35 cycles. Final extension was given at 72°C for 5 min. The obtained amplicons were analyzed by running electrophoresis on 0.8 - 1.0% agarose gel.

Radioactive labelled probe synthesis for Southern hybridization

The PCR - amplified fragment of the target genes were labeled by α -P³² dCTP using a nick

translation system (Invitrogen, Life Technologies, India) according to the manufacturer’s instruction. The probes were synthesized one day before and stored at - 20°C.

RESULTS AND DISCUSSION

Submergence tolerance mechanisms in deep - water - rice cultivars

One set of experiments involving submergence have been conducted using two week old seedlings by submerging them in the water for two weeks and kept one week for recovery. Interestingly, the

Taothabi and Khongan both elongated above the water levels all the times while Sub1-Swarna did not elongate and normal Swarna elongated slightly. The experiment clearly shows the two opposite phenotypical responses during submergence. First type is elongation mechanism to escape from flooded conditions regarding GA3 pathway and another type expressed no elongation, conserved energy and used it for new-growth (Figure 1). Two varieties namely Murshi, Niphu Thokpi remained their plant height in complete submergence, familiar to Sub1-Swarna’s response, then were dismissed next experiments (or their results, if have, do not need to be mentioned).

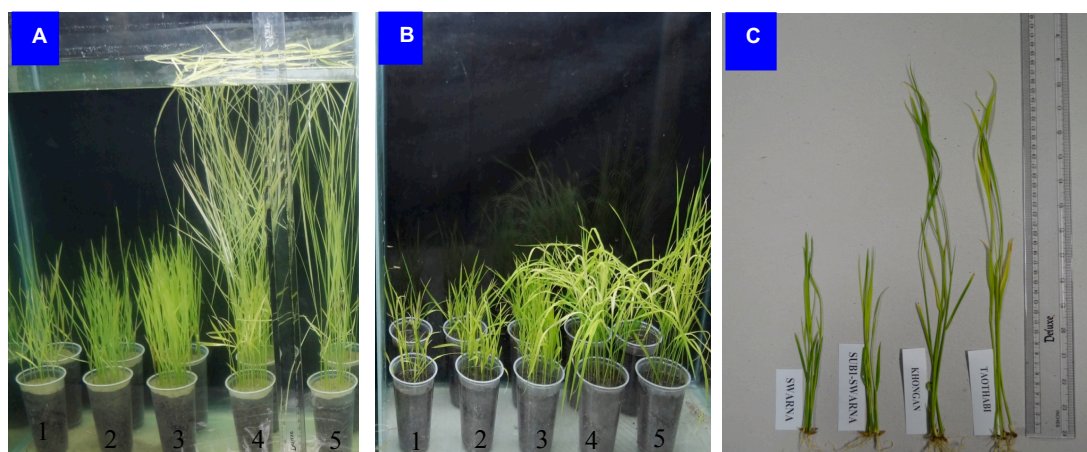


Figure 1. Comparison of growth among different rice cultivars under (A), submergence conditions and (B), under normal conditions. 1). Swarna, 2). Sub1- Swarna, 3).Murshi, 4). Khongan, 5). Taothabi (C). Plant height of 4 main rice varieties.

In order to characterize the genetic variation in the North-east Indian rice, Southern hybridization and PCR based approaches were used. Sub1-Swarna that has been developed through traditional plant

breeding methods through introgression of *Sub1* QTL was included in this study for a better comparison and was used as a positive *Sub1* gene control. Swarna variety was used as a negative *Sub1*

control. Main study focused on the effects of other unknown and novel genes, if any, on the submergence tolerance. The IR64, a popular variety susceptible to submergence conditions, is also included.

PCR based analysis to detect genetic variation among the rice cultivars

Also, PCR based approach was followed for the amplification of important genes involved in submergence tolerance and few housekeeping genes

were utilized in the PCR experiments. PCR primers were designed for *Sub1A1*, *Sub1B* and *Sub1C* genes along with alcohol dehydrogenase

1 and 2 (*ADH1*, *ADH2*), pyruvate decarboxylase (*PDC1*, *PDC2*, *PDC4*), rice amylase (*RAmy3C*), and sucrose synthase (*SUS*) genes. All the primers were tested in these cultivars for the presence of the above mentioned genes. No significant differences were noticed in the PCR based amplification, excepted *Sub1A* (Figure 2).

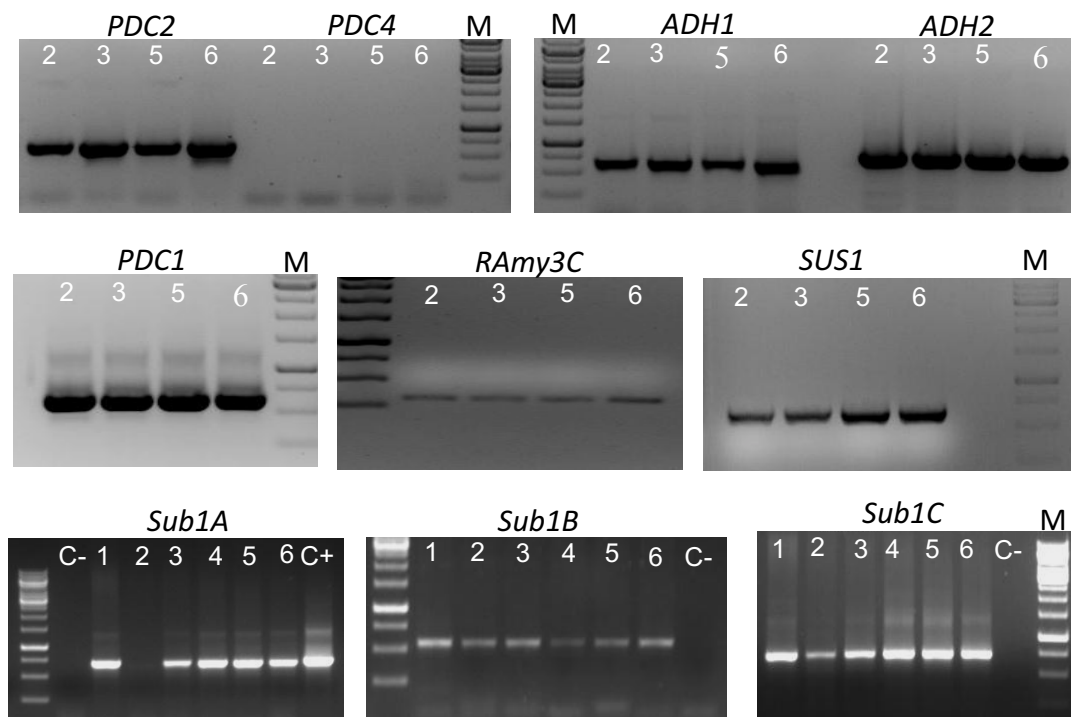


Figure 2. Agarose gels showing the PCR amplified products using certain gene specific primers. All the primers were tested in all the 6 plants 1). IR64, 2) Swarna, 3) Sub1-Swarna, 4) Murshi, 5) Khongan, 6) Taothabi/ for the presence of various genes. M. 1kb ladder.

So far *Sub1* genes (*Sub1B* and *Sub1C*) have been amplified in all the varieties tested and cloned while *Sub1A* has been amplified in Sub1-Swarna, Khongan and Taothabi but not in Swarna. PCR based amplification of three genes (*Sub1A*, *Sub1B* and *Sub1C*) showed the conserved nature of the present in *Sub1* loci. However, the data also showed certain variations among the genotypes studied. It is likely that *Sub1A* gene plays a key role in submergence tolerance while *Sub1B* and *Sub1C* do not play an important role. Indeed, a trait of non- elongation in

submergence tolerance mechanism is regulated by *Sub1A* gene (Aaron, Schmitz *et al.*, 2013; Fukao, Bailey-Serres *et al.*, 2008). However, this gene also presents in the escape mechanism (Khongan and Taothabi vars.) which responded with submergence by fast elongation (stem or leaves). So that the interesting question is why *Sub1A* gene does not play its function in these deep-water rice varieties (stop stem elongation in submergence condition). Is a flash flooded responded mechanism independent or dependent with escape (stem elongation)

mechanism? If it is independent, is there any third mechanism in submergence tolerance?

Southern hybridization to detect genetic variation among rice cultivars

For this purpose, genomic DNAs isolated from six cultivars (Murshi, Taothabi, Khongan, Swarna, Sub1-Swarna and IR64) were digested with *EcoRI* or *BamHI* restriction enzymes and hybridized with the coding regions of a number of genes associated with submergence tolerance in rice.

Representative, Southern hybridization blots showing the genetic variation among the North-east cultivars as compared to Swarna and Sub1-Swarna are shown in Figure 3. Arrows indicate the differences in the size of the bands. Although we did not notice major difference with regard to *Sub1A* and *Sub1B* when tried with *EcoRI* and *BamHI*. Differences were noticed with *adh1* and *RAmy3C* genes.

Note the difference in Khongan as compared to other cultivars when genomic DNA was digested with *EcoRI* and probed with *adh1*. Similarly, no difference was observed in IR64 as compared to other cultivars when genomic DNA was digested with *BamHI* and probed with *RAmy3C*. Several genotypes having *Sub1* QTL have been studied to understand and identify the genetic diversity (Sarkar *et al.*, 2011). These studies revealed that greater genotypic variability in terms of various parameters such as plant height, survival rate, etc., associated with submergence tolerance. More recently, Goswami *et al.*, (2015) also showed genetic diversity at *Sub1* loci among the genotypes that are known to exhibit submergence tolerance in India. On the whole, these studies have indicated the potential genetic resources in the deep-water rice that can be deployed in lowland-flood prone areas through marker assisted breeding or through genetic transformation approaches to increase yield.

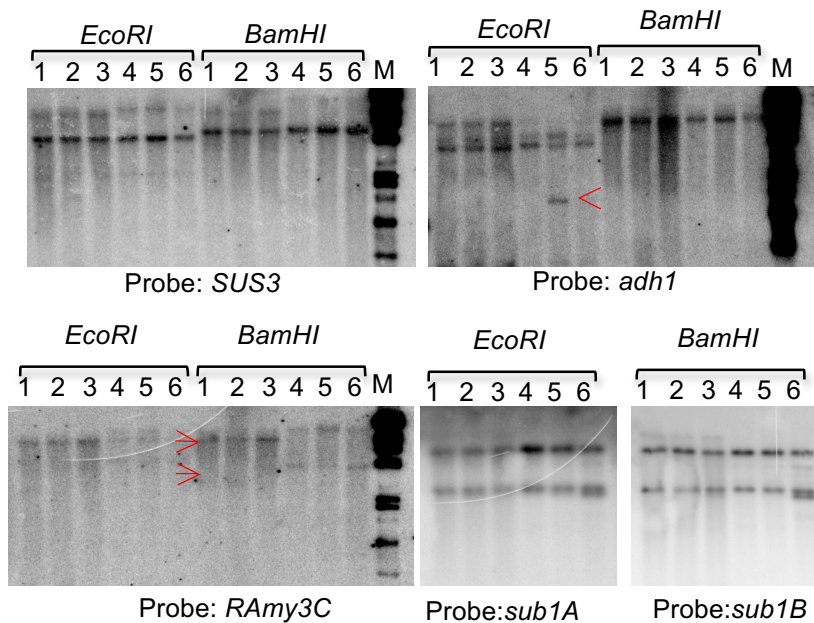


Figure 3. Southern hybridization analysis showing variations at the *Sub1* locus among rice cultivars where some are tolerant and others susceptible to submergence. 1). IR64, 2) Swarna, 3) Sub1-Swarna, 4) Murshi, 5) Khongan, 6) Taothabi/ M: λ DNA Marker.

CONCLUSIONS

The two rice cultivars of North-eastern region of India, Taothabi and Khongan showed typical phenotypic behavior of elongation under submerged conditions which is commonly observed in deep

water rice. Plants elongated rapidly when submerged and grown more than two times of normal growth. Comparatively, Swarna and Sub1-Swarna did not elongate. The PCR based analysis involving several genes known to be associated with submergence tolerance did not reveal much difference. However,

Southern hybridization using the coding regions of various genes revealed certain differences between submergence tolerant and susceptible cultivars. It is possible that deep-water rice like Taothabi and Khongan may not differ in their genome at *Sub1* locus but they respond through SK genes under submergence. It is to be proposed that over expression of *Sub1A* or *Sub1B* genes in these cultivars alter the phenotype and prevent their elongation under submergence conditions without dying, similar to Sub1-Swarna variety.

Acknowledgements: *We would like to thank International Centre for Genetic Engineering & Biotechnology (ICGEB) for providing fellowship funds, necessary infrastructure and resources to accomplish our research.*

REFERENCES

- Aaron J Schmitz, Jing J Folsom, Yusuke J, Pamela R, Harkamal W (2013) SUB1A-mediated submergence tolerance response in rice involves differential regulation of the brassinosteroid pathway. *New Phytol.* 198(4): 1060 - 1070.
- Bailey-Serres J, Fukao F, Ronald P, Ismail A, Heuer S, Mackill D (2010) Submergence tolerant rice: SUB1's journey from landrace to modern cultivar. *Rice* 3: 138 - 147.
- Drew MC (1997) Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. *Annu Rev Plant Physiol Plant Mol Biol* 48: 223 - 250.
- Fukao T, Bailey-Serres J (2008) Submergence tolerance conferred by Sub1A is mediated by SLR1 and SLR1L1 restriction of gibberellin responses in rice. *Proc Natl Acad Sci USA* 105: 16814 - 16819.
- Fukao T, Bailey-Serres J (2008) Ethylene - A key regulator of submergence responses in rice. *Plant Science* Volume 175: 43 - 51.
- Geigenberger P (2003) Response of plant metabolism to too little oxygen. *Curr Opin Plant Biol* 6/30: 247 - 256.
- Goswami S, Labar R, Paul A, Adak MK, Dey N (2015) Physio-biochemical and genetic exploration for submergence tolerance in rice (*Oryza sativa L.*) landraces with special references to Sub1 loci. *Am J Plant Sci* 6: 1893 - 1904.
- Hattori Y, Nagai K, Furukawa S, Song XJ, Kawano R, Sakakibara H, Wu J, Matsumoto T, Yoshimura A, Kitano H, Matsuoka M, Mori H, Ashikari M (2009) The ethylene response factors SNORKEL1 and SNORKEL2 allow rice to adapt to deep water. *Nature* 460: 1026 -1030.
- Kende H, Knaap EVD, Hyung-Taeg Cho (1998) Deepwater rice: A model plant to study stem elongation. *Plant Physiol.* 118(4): 1105 - 1110.
- Murray MG and Thompson WF (1980) Rapid isolation of high molecular weight plant DNA. *Nucl. Acid Res* 8: 4321 - 4325.
- Nemoto T, Cho EM, Okada A, Okada A, Okada K, Otomo K, Kanno Y, Toyomasu T, Mitsushashi W, Sassa T, Minami E, Shibuya N, Nishiyama M, Nojiri H, Yamane H (2004) Stemar-13-ene synthase, a diterpene cyclase involved in the biosynthesis of the phytoalexin oryzalexin S in rice. *FEBS Lett.* 571: 182 - 186.
- Sarkar RK, Bhattacharjee B (2011) Rice genotypes with *SUB1* QTL differ in submergence tolerance, elongation ability during submergence and re-generation growth at re-emergence. *Rice* 5: 7.
- Xu K, Mackill DJ (1996) A major locus for submergence tolerance mapped on rice chromosome 9. *Mol Breed* 2: 219 - 224.
- Xu K, Xu X, Ronald PC, Mackill DJ (2000) A high-resolution linkage map in the vicinity of the rice submergence tolerance locus Sub1. *Mol Gen Genet*, 263: 681 - 689.

XÁC ĐỊNH ĐẶC TÍNH PHÂN TỬ CỦA CÁC GEN VÀ LOCUS CHỐNG CHỊU NGẬP Ở CÁC GIỐNG LÚA NỘI

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TÓM TẮT

Hầu hết các giống lúa giảm hoặc ngừng sinh trưởng khi ngập úng để đối phó với tình trạng thiếu hụt đột ngột ô xi hòa tan trong môi trường. Khi tình trạng ngập tiếp diễn trong thời gian dài, đa số các giống mất khả năng hồi phục sau khi nước rút đi. Tuy nhiên, may mắn là có một số giống có thể chịu đựng điều kiện ngập lên đến hai tuần bởi hai cơ chế hoàn toàn trái ngược nhau. Cơ chế thứ nhất, chúng kéo dài lá và thân để trốn thoát

sự ngập trong thời gian ngắn. Cơ chế thứ hai, chúng chịu đựng sự ngập nhờ ngừng sinh trưởng, không vươn lóng thân và lá. Khi nước rút đi, các giống trốn ngập dễ dàng bị gãy đổ. Dẫn đến năng suất và chất lượng hạt của giống bị giảm mạnh. Trong nghiên cứu này, chúng tôi tập trung vào xác định sự đa dạng di truyền của locus *Sub1* và các gen có liên quan đến tính trạng chống chịu ngập trên các giống lúa nổi. Bước đầu tiên, hạt của các giống lúa bản địa được thu thập từ vùng Đông Bắc Ấn Độ để làm vật liệu nghiên cứu. Sử dụng phương pháp phân tích PCR, lai Southern. Kết quả phân tích PCR cho thấy, nhóm gen *Sub1*, nhóm gen đã được biết đến quyết định khả năng chống chịu ngập, biểu hiện không có sự khác biệt lớn. Tuy nhiên, lai Southern cho thấy có sự khác biệt ở mức phân tử giữa các giống chống chịu và giống mẫn cảm ngập. Trong nghiên cứu này, chúng tôi không thấy sự khác biệt ở các gen được cho là quyết định chính khả năng chịu ngập, nhóm *Sub1*. Sự khác biệt lớn chỉ xảy ra với các gen *adh1* (phân giải rượu) và *RAmy3C* (phân giải tinh bột) khi dùng enzyme cắt hạn chế *EcoRI* và *BamHI*. Phân tích lai Southern cho thấy có sự khác biệt về nền di truyền giữa các giống lúa nổi so với giống Swarna (mẫn cảm ngập) và Sub1-Swarna (giống chịu ngập). Điều này chỉ ra là các giống lúa nổi không khác biệt về di truyền tại locus *Sub1*, chúng phản ứng thông qua các gen *SNORKEL* dưới điều kiện ngập úng.

Từ khóa: biến thể di truyền, chịu ngập, gen, lúa, *Sub1*