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Physiological and Molecular Characterization of Rice Isogenic Line for *SubQTL9* under Flash Flooding

¹V. Ruanjaichon, ¹T. Toojinda, ²S. Tragoonrun and ^{1,3}A. Vanavichit

¹Rice Gene Discovery Unit, National Center for Genetic Engineering and Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakorn Pathom 73140, Thailand

²DNA Technology Laboratory,
National Center for Genetic Engineering and Biotechnology, Kasetsart University,
Kamphaeng Saen Campus, Nakorn Pathom 73140, Thailand

³Department of Agronomy, Kasetsart University,
Kamphaeng Saen Campus, Nakorn Pathom 73140, Thailand

Abstract: Discovering QTL associated with submergence tolerance facilitates the development of isogenic lines for submergence stress. One hundred and thirty two isogenic lines carry 36 kb region contained in the QTLchr9 were used to compare under controlled submerging conditions. The region contained gene encoding three ethylene responsive element binding protein as *Sub1A*, *OsEREBP1 (Sub1C)*, *OsEREBP2 (Sub1B)* genes. The Isogenic Lines (ISLs) were categorized into three groups as homozygous donors (DH206), ISLs-CC, homozygous recipients (KDML105), ISLs-cc and heterozygous, ISLs-Cc, by the three pairs of primers targeted at the target region. Each individual plant of the ISLs was submerged for 20 days to increase the physiological changes in response to flooding. In addition the increment in shoot height, plant growth and the ability to stay green of ISLs-CC and ISLs-Cc were also similar. Rapid elongation was significantly higher in ISLs-cc. All genotypes showed no significance with respect to leaf senescence. However they were shown to be significant when genotypes interacted to time-course. The accumulation of shoot elongation was affected mostly by the extension of leaf blade and leaf sheath. A prolonged flooding to reach a tolerance plant was associated with the plant adaptation of suppressing growth and the maintenance of green leaf area. However several lines within each class did not differ in plant adaptations under normal conditions. The analysis of variance for the genotypic effect (G) and time (T) duration showed that the genotype main effects (G) from CC, Cc and cc were highly significant at 1% level, meaning that differences between genotypes vary across time. Plant Elongation (PE), Total Shoot Elongation (TSE) and Relative Shoot Elongation (RSE) showed high negative significance ($p < 0.05$) correlated with percent plant survival as $r = -0.78^*$, -0.75^* and -0.34^* . In contrast, percent plant survival showed a high positive correlation ($r = 0.71^*$) with Leaf Senescence (LS). There were significant differences in plant elongation and leaf senescence among classes. This study provides a powerful tool to improve flooding tolerance in plants.

Key words: Isogenic line, submergence tolerance, *Oryza sativa* L., plant elongation, leaf senescence, plant survival

INTRODUCTION

Over the past few years, a major Quantitative Trait Locus (QTL) controlling submergence tolerance was mapped to a 6.5 cM region of chromosome 9. It was cloned, sequenced and annotated

Corresponding Author: Apichart Vanavichit, Rice Gene Discovery Unit,
National Center for Genetic Engineering and Biotechnology, Kasetsart University,
Kamphaeng Saen Campus, Nakorn Pathom 73140, Thailand
Tel: (66) 034 355194 Fax: (66) 034 355197

(Vanavichit *et al.*, 2001). Due to the physiological response mechanisms, submergence primarily reduced plant elongation and delayed leaf senescence, plants can survive and recover from submergence stress (Timothy and Eufrocino, 1996; Ito *et al.*, 1999). Genetic linkage between submergence tolerance to both suppressed elongation and delayed leaf senescence was clearly shown by the QTL mapping analysis involving several segregating recombinant inbred lines (Toojinda *et al.*, 2003). Both traits were coincidentally mapped between two marker loci, S10709 and RB0783 on chromosome 9, where their candidate genes were identified.

Submergence tolerance is characterized by complex phenotype-associated traits. The tolerant plant can be generalized as the ability to survive and continue growing after several days in submerged conditions (Adkins *et al.*, 1990). The phenotypic benefits provided the tolerant rice with the ability to survive and, also, recover, during the flooding period. The submergence-tolerant plants affected by several morphological adaptations decreased the chlorosis of tissues and reduced the shoot elongation on plant growth in order to save carbohydrates and energy for maintenance processes including protective antioxidant systems.

According to a different mapping population, a isogenic line (ISL) was developed by four advanced backcrosses to Khao Dok Mali 105 (KDML105), which was susceptible to submergence stress. The donor of submergence tolerance is a doubled haploid line (DH206) derived from a cross between FR13A and CT6241-7-1-2-2. This ISL of the BC4F7 generation is genetically 94% identical to KDML105 but carries the 6.5 cM of the heterozygous segment at position 102.5 to 109 cM on chromosome 9 (Siangliw *et al.*, 2003).

In this report, we present the associated data between the phenotypic response under flooding and the genotypic alleles of the ISLs containing three Gene Targeted Markers (GTMs), which are localized on 36 kb region within the 6.5 cM of *SubQTL9*. These markers were identified based on their correlation with individual trait components. In addition, the understanding of the mechanisms used by rice to tolerate submergence stress involved in GTMs compared with individual traits related to submergence stress might open new avenues to the genetic improvement of rice.

MATERIALS AND METHODS

Plant Materials

A set of near isogenic lines, ISL-132, was used for this study. The ISLs population had been developed from BC4F7 by backcrossing an introgression specific region of chromosome 9. In order to fine map the *Sub1* region, DH206, derived from a cross between FR13A and CT6241-7-1-2-2, was crossed and successively backcrossed with KDML105 (high quality and submergence intolerance) which resulted in BC4F8 of ISL-132 differing in the 36 kb of *SubQTL9* region which carried three genes encoding ethylene responsive element binding protein (ERFs) as *Sub1A*, *OsEREBP1* (*Sub1C*) and *OsEREBP2* (*Sub1B*) under the genetic background of KDML105.

DNA Extraction

Fresh young leaves were collected and ground in a 1.5 mL tube with liquid nitrogen. Genomic DNA was isolated using the CTAB (acetylmethylammonium bromide)-NaCl method (Roger and Bendich, 1994). The DNA pellets were re-dissolved (at a concentration of 50 ng μL^{-1}) in 50 μL of water for PCR-based polymorphism analysis.

Characterization of ISL132 of BC4F8

Three pair of primers (Table 1) for Gene Targeted Markers (GTMs) were used to cover the 36 kb region on the long arm of chromosome 9, to which major *Sub1*QTL had previous been mapped (Toojinda *et al.*, 2003). All polymorphic primers were developed based on the genomic sequence from

GenBank accession number AC90056 and DQ011607. Genotypic screening to maintain a tolerance allele (FR13A) at major *SubQTL9*, as well as selection against an intolerance allele (KDML105), was determined by using the GTMs.

Evaluation of Phenotypic Change under Submergence Stress in ISLs-132 of BC4F8

The evaluated for several phenotypic adaptations to survival were analyzed under complete submergence using a dark plastic tank, in August 2004. Individual plants of ISLs-132 population were grown completely submerged for 20 days at 3 weeks after germination. The water level was maintained at 60 cm above the tallest plants to prevent leaf tips from emerging into the air.

The measurements of a complex phenotype-associated trait were explained with five major traits responsible for submergence stress. The data of Plant Elongation (PE), Total Shoot Elongation (TSE), Relative Shoot Elongation (RSE) and Leaf Senescence (LS) at 0, 2, 4 and 20 days of submergence was collected. In these experiments, all trait evaluation methods were performed as reported by Siangliw *et al.* (2003) and Toojinda *et al.* (2003).

Statistical Analyses

The ANOVA and regression based software (STATGRAPHICS 2.1) was used for detecting significant correlations between the response traits and submergence stress and relationship between markers and traits.

DNA Sequencing and Promoter Analysis

DNA sequences of PCR products from specific primers for the promoter regions of *Sub1A*, *OsEREBP1* (*Sub1C*) and *OsEREBP2* (*Sub1B*) were characterized using an ABI Prism™ Dye Terminator Cycle Sequencing Ready Reaction Kit with Ampli Taq DNA polymerase for fluorescent sequencing (Perkin-Elmer, USA).

RESULTS

Molecular Characterization of 36 kb *SubQTL9* region in ISLs

The use of Isogenic Lines (ISLs) is a powerful tool in genetics approach to examine the physiological processes linked to a predicted gene while other regions are similar. The region was represented on a part of the genomic sequence of a Nipponbare PAC clone (GenBank accession number AC090056) and DQ011607 from Indica rice. The genotypic screening to maintain a tolerance allele (DH206) at major *SubQTL9*, as well as selection against an intolerance allele (KDML105), was done in three groups as ISLs-132CC (12 plants), ISLs-132Cc (12 plants) and ISLs-132cc (12 plants), respectively To classify a submergence trait haplotype of ISL-132-Sub1 of BC4F8 population compared to FR13 A (a tolerant line) and KDML105 (an intolerant line), an interval segment of major *SubQTL9* region, designated as the C region, was determined by effective PCR screening markers related to submergence tolerance trait as reported by Siangliw *et al.* (2003), Ruanjaichon *et al.* (2004) and Fukao *et al.* (2006) (Table 1).

Using *Sub1A* marker, no length polymorphism was observed among rice plants. Therefore DNA sequencing analysis was carried out to detect the point mutation among these identical PCR products. The result showed that the nucleotide sequence of exon 1 contained two SNP at the nucleotide position of 556 (T to C) and 677 (A to G). Consequently, a tolerant plant, FR13A, has the bases T and A similar to ISL-132CC whereas ISL-132cc and KDML105 have the bases C and G (Fig. 1).

In addition we did not find polymorphism with *OsEREBP2* marker between ISL-132cc and KDML105. On the other hand, FR13A showed identical allele from ISL-132CC. Finally, *OsEREBP1* marker which was developed from a gene encoding ethylene responsive element binding protein



Fig. 1: Sequence analysis of *Sub1A* coding region between ISL-132CC and ISL-132 compared to FR13 A (a tolerant line) and KDML105 (an intolerant line)

Table 1: Effective DNA markers in the *Sub1QTL9* were used for haplotype screening

Rice subspecies	BAC/PAC accession	Marker name	Coordinate (5'---3')	Mutation type
Indica cv.	DQ011607	<i>Sub1A</i>	-	SNP ^A
Japonica cv.	AC090056	<i>OsEREBP2 (Sub1B)</i>	78349-78566	Indel ^B
Japonica cv.	AC090056	<i>OsEREBP1 (Sub1C)</i>	94816-95174	Indel ^B

^AIndel = Insertion-Deletion, ^B SNP = Single nucleotide polymorphism

showed a 25 nucleotides Insertion-Deletion at 3'UTR in FR13A as well as in ISL-132CC. But ISL-132cc and KDML105 were not found (Fig. 2). According to 3 molecular markers results, we found that the allele pattern of *Sub1A*, *OsEREBP1* and *OsEREBP2* from ISL-132CC is similar to FR13A (Fig. 3). These genomic patterns might be associated with the tolerant symptom when the plant is under water. Besides, the understanding of the mechanisms used by rice to tolerate submergence stress involved in 4 candidate gene markers compared with individual traits related to submergence stress might open new avenues to the genetic improvement of rice.

To survey other regions around the major QTL for submergence tolerance in ISL-132 population, many types of molecular markers reviewed by Sianglew *et al.* (2003) were used for screening. The results showed that the interval segment of major *Sub1QTL9* region was represented as a genotypic sequence of ISL-132 population as similar as KDML105 (data not shown). Furthermore, SSR genotyping with 12 chromosomes was used for quality control of isogenic line compared to KDML105 (recipient). We found that genome of ISL-132CC has 90% convergence with KDML105 using 42 SSR loci. The selected recombinant event with in the C region could potentially be used for examination of plant adaptation traits. The result could be treated as single Mendelian factors that are likely due to a single locus.

In addition, rice gene-chip expression array was applied for analysis of Single Feature Polymorphism (SFP) in whole genome (606,000 loci). Using Significant Analysis of Microarray

identified as a major QTL. The main finding in this study is the characterization of the ISL-132 of BC4F8 in response to submergence stress. These were classified into three haplotypes which exhibited differences in plant adaptation under water.

PE was calculated as the plant height (cm) for 20 days under submergence stress. The measurement was taken as the distance from the soil surface to the tip of the longest leaf. The results showed that the development of ISL-132CC and ISL-132Cc have similarity in average plant height of 38.3 and 38.9 cm for 20 days under flooding. However the development of ISL132-cc has the highest level of average plant height at 62.7 cm under flooding (Fig. 4A).

The TSE during flooding was used as an indicator of the increment in shoot height and calculated as the average difference in shoot height before and after flooding. A set of three genotypic classes were characterized under flooding for 20 days into two different types of TSE. The individual plants of the homozygous donor (DH206), ISLs132CC and the heterozygous class, ISLs-132Cc have average incremental height of 5.4 and 7.1 cm, respectively. But ISLs-132cc, the individuals carrying homozygous alleles of KDML105 has the highest level of average incremental height at 31.2 cm under flooding (Fig. 4B).

The incremental height of plant shoot elongation in ISL-132 was separated into two parts of leaf tissue under submergence conditions. The acceleration of leaf extension combined the elongation of both leaf sheath and leaf blade. The development of ISL132-cc showed the highest level of leaf sheath and leaf blade growth in shoot elongation when compared to ISL132-CC and ISL132-Cc (Fig. 5).

The impact of submergence stress on plant growth was compared to growth under normal conditions. RSE was used to investigate the relationship between the reduction and induction of plant growth during 20 days submergence stress. The results showed that both ISLs-132CC and ISLs-132CC showed 73.6 and 74.8% of shoot elongation compared to 100% under normal growth conditions. However, the ISLs-132cc shoots elongated 120.5% more than plant growth under normal conditions (Fig. 4C).

LS is characterized by the presence of yellowing in leaves which resulted from chloroplast damage. Submergence stress affected the ability of rice to retain its green leaf coloring area. Submergence tolerant rice is able to stay green longer than intolerant lines when flooded. After 20 days under water, each individual line was defined at the base, middle and tip of one leaf. Using LS-SPAD, the results showed that ISLs-132CC, ISLs-132Cc and ISLs-132cc were defined for average chlorophyll content of 23.8, 23.9 and 15.4 score unit at 20 day flooding, respectively (Fig. 4D). The interaction between ISL132-CC: ISL132-Cc and ISL132-cc across time is highly significant ($p < 0.01$) with chlorophyll content. The results suggest that the long-term flooding has major effects for senescence among ISL132 classes.

In all experiments, the interaction between ISLs-132CC and ISLs-132Cc is not significant ($p < 0.01$) across time. But the development of ISL132-cc interacts to NILs-132CC: ISLs-132Cc across time showed high significance ($p < 0.01$) with all obtained data in responding traits of PE, TSE and RSE under submergence stress. The result suggested that the region of 36 kb consists of a tolerant donor allele which affected submergence tolerance traits as a dominant allele. Moreover, the responding traits under flooding were controlled by a smaller region in ISLs of BC4F8. A highly correlated regression analysis was observed with Sub1A, *OsEREBP1* and *OsEREBP2* markers that contributed a high percentage of phenotypic changes in TSE and LS.

Relationship Between Genotypic Allele and Time-Course

The data from phenotypic changes among traits responded to submergence stress were observed. The analysis of variance (ANOVA) showed that the genotypic effect (G) and time-course (T) was the significant source of variation. Genotype main effects (G) from the development of ISL132-CC, ISL132-Cc and ISL132-cc were significantly ($p < 0.01$) different among genotypes varying across time

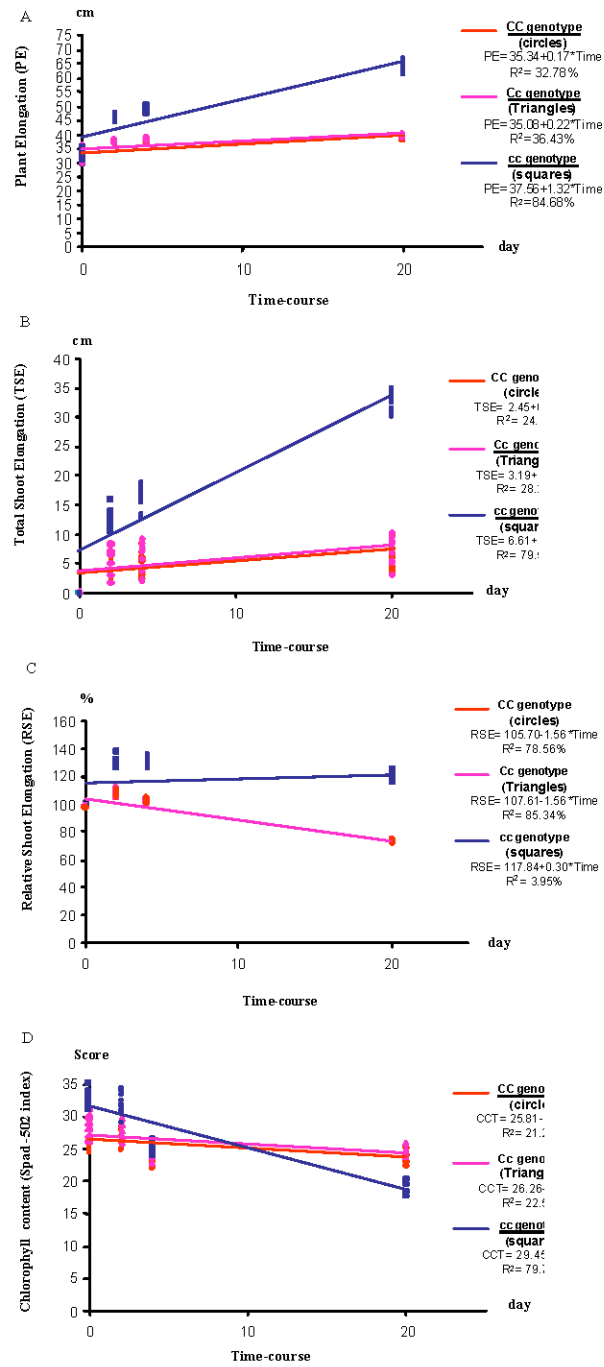


Fig. 4: Shows the relationships between genotype and plant adaptation traits, A) plant elongation (PE); B) total shoot elongation (TSE); C) relative shoot elongation (RSE); D) Chlorophyll content, at 2, 4 and 20 days under submergence condition

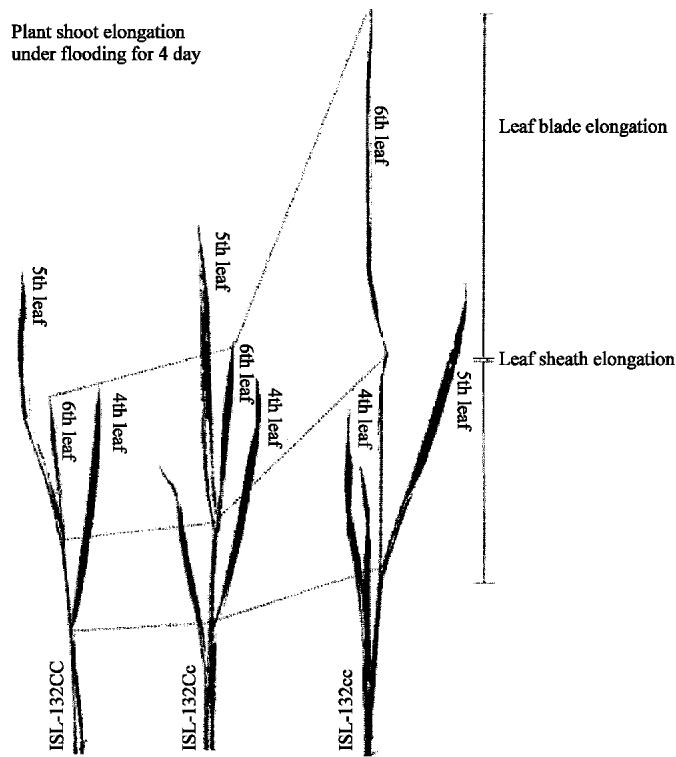


Fig. 5: Submergence-induced leaf sheath elongation and leaf blade elongation were determined with three genotypic classes of ISL-132. At the start of the submergence, the treatment plants used were 21 day seedlings and were submerged for 20 days

Table 2: Analysis of variance for Total Shoot Elongation (TSE), Plant Elongation (PE), Relative Shoot Elongation (RSE) and Leaf senescence (LS) of the three genotyping classes of isogenic lines submerged for 20 days

Traits	df	TSE	PE	RSE	LS
Treatments	11	683**	664**	2809**	146**
Genotype (G)	2	1389**	1120**	6581**	3 ^{ns}
-CcxCc	1	22*	0(<1)	67*	1(<1)
-ccxCC:Cc	1	2755**	2241**	13096**	5 ^{ns}
Time course (T)	3	957**	1002**	3689**	343**
Contrast					
TxG	6	311**	343**	1111**	95**
-TxCC:Cc	3	3(<1)	3 ^{ns}	17 ^{ns}	0(<1)
-Tx ccxCC:Cc	3	619**	682**	2206**	190**
Error (MS)	96	5	2	12	2
CV		29.6%	3.1%	3.4%	5.7

The data shows mean square value and the significance record of main and interaction effects of time, **Significant at 1% level; *Significant at 5% level, ^{ns}: Non-significant Plants were treated under water for 0, 2, 4 and 20 days in a plastic aquarium tank, PE, Plant Elongation; TSE, Total Shoot Elongation; RSE, Relative Shoot Elongation; LS, Leaf Senescence

(Table 2). The ISL132-CC and ISL132-Cc interaction showed no differences in traits such as PE, TSE, RSE and LS at 1% level. The interaction between ISL132-cc and ISL132-CC: ISL132-Cc was highly significant ($p < 0.01$) in PE, TSE and RSE and not significant in LS. The results suggested that the time-course was the main effect for leaf damage by development of leaf senescence.

Table 3: Correlation matrix of submergence response traits in rice obtained in 36 individuals ISLs-132 of BC4F8 population from the submergence-tolerant (DH206) and submergence-intolerant (KDML105)

Traits	TSE	RSE	LS	PS%
PE	0.96*	0.47*	-0.71*	-0.78*
TSE	-	0.50*	-0.67*	-0.75*
RSE		-	-0.20*	-0.34*
LS			-	0.71*

Plant were treated under water for 20 days in a plastic aquarium tank, %PS, percent plant survival; TSE, total shoot elongation; RSE, relative shoot elongation; LS, leaf senescence. p-values below 0.05 indicate statistically significant non-zero correlations at the 95% confidence level

Traits Response to Flooding Correlation

Strong phenotypic correlations among traits responsive to submergence stress were observed in the ISLs-132 population (Table 3). A high correlation of the PS% was found among PE, TSE, RSE and LS traits. The PE, TSE and RSE showed high correlation (-0.78*, -0.75* and -0.34*) and have a negative correlation with the PS%. The LS measured by the SPAD-502 chlorophyll meter presented high positive (0.71*) correlation with percentage plant survival, whereas leaf senescence had negative correlation with PE, TSE and RSE.

In all experiments two classes, ISLs-132CC and ISLs-132Cc, having less TSE under flooding, mostly exhibited a higher level of PS% and LS. However, the ISLs-132cc showing high TSE and low LS affected the PS% and scored as 0% after being de-submerged for 7 days.

DISCUSSION

The efficiency of survival and recovery after submergence is strongly related to the ability to limit leaf elongation and to stay green under water. Under submergence conditions mechanisms of plant adaptation have played important roles in leaf elongation and senescence. Toojinda *et al.* (2003) reported that the complex phenotype-associated traits such as leaf senescence, percent plant survival, suppression elongation and so on were coincidentally mapped at *SubQTL9* for submergence tolerance identified as a major QTL. The main finding in this study is the characterization of the ISLs-132 of BC4F8 in response to submergence stress. These were classified into three genotypic classes which exhibited differences in plant adaptation under water.

The isogenic lines are the powerful tool in the genetic approach and the examination of the physiological processes linked to a predicted gene because of the unrelated variations which reached out from the target trait. This successful approach has been used in various plants such as maize (Dorweiler *et al.*, 1993), tomato (Alpert and Tanksley, 1996 and rice (Yamamoto *et al.*, 1998). In the study, we have used ISL-132 of BC4F8 in comparisons between three genotypic classes. Each plant contained a segment in the same region which different in each class (DH206 segment for ISLs-CC, KDML105 segment for ISLs-cc and heterozygous for ISLs-Cc). This study showed that the ISLs-CC and ISL-CC obtained a high percentage plant survival with gene targeted markers (GTMs). The result of this study suggested that the region of 36kb consists of a tolerance donor allele which affected submergence tolerance traits as a dominant allele. Moreover the responding traits under flooding were controlled by a smaller region in ISLs of BC4F8. A highly significant statistic of regression analysis was observed with gene targeted markers (GTMs), Sub1A, OsEREBP1 and OsEREBP2, which contributed a high percentage of phenotypic changes in TSE and LS. Using SSR genotyping, BC4F8 is only 94% similar to KDML105 with 6% of its alleles being from DH206, the effects of plant adaptation under flooding are major caused by different allelic at the interval region of 36 kb containing three predicted gene. The new developing ISLs for one gene at one locus and their function can be inferred by gene cloning and several computer analysis tools.

The results from physiological adaptation revealed a strong pattern of shoot elongation among three genotypic classes explained as a validation data of TSE and RSE during flooding. The higher level of TSE of the ISLs-132cc class affected the accumulated elongation in at least two parts of the leaf extension. Most parts of plant elongation were observed at leaf sheath and leaf blade. However, only ISLs132-CC and ISLs132-Cc showed significant physiological adaptation in leaf blade elongation. Recent finding reports revealed that the acceleration of elongation under flooding conditions combines the elongation of both leaf sheath (Mazaredo and Vergara, 1982) and leaf blade (Jackson *et al.*, 1987). Slow leaf sheath elongation or suppressing elongation at leaf sheath of individuals ISLs132-CC and ISLs132-Cc might be an effective role with genotypic classes.

The plant adaptation linking fast elongation underwater and susceptibility to flooding was not positive in the past experiments (Yamada, 1959; Mazaredo and Vergara, 1982). Plant growth on rapid elongation of the three genotypic classes in response to submergence stress was obtained for a few days. The susceptible homozygous ISLs-132cc showed the prolongation of plant growth on shoot elongation whereas the plant growth was suppressed in both ISLs-132CC and ISLs-132Cc. However the plant growth did not differ in control plants of each class. The relationship between susceptible and resistance to fast elongation underwater was recently reported by Toojinda *et al.* (2003) and Siangliw *et al.* (2003). Plant growth compared results between flooded and normal conditions revealed that the ability of plant growth of ISLs-132CC and ISLs-132Cc was inhibited when compared to control plants. The effect of plant growth on rapid shoot elongation is highly negatively correlated ($r = -0.34^*$) with the survival data after flooding for 20 days. The relationship between plant growth on shoot elongation and survival using five rice cultivars, which has been confirmed using the IRRI gene Bank database on 903 cultivars, was reported by Setter and Laureles (1996). Vartapetian and Jackson (1997) revealed that the plant actively growing during submergence is much more susceptibility than the slowly growing plant in submergence response. The advantage of plants in suppression elongation might be regulated by their genotypic allele to maintain the energy source for recovering plants after de-submergence.

Evidence of leaf damage by the development of leaf senescence in underwater conditions was revealed in Arabidopsis and maize (Zhang *et al.*, 2000; Subbaiah and Sachs, 2003). In rice, one of the best indicators to show recovery from submergence is leaf senescence promoted under flooding stress (Jackson and Ram, 2003). The ability to stay green or leaf senescence underwater is similar to responsiveness to survival and the ISLs population were screened for their reaction to survival. The senescence leaf was obtained for intolerance homozygous of ISLs-cc but the tolerance homozygous, ISLs-CC and heterozygous, ISLs-Cc stayed green during flooding for 20 days. ANOVA analysis of LS data showed high significance among genotypes which vary across time. However, no significant LS data was shown among the three genotypic classes. The results suggested that the time-course was the main effect for leaf damage by development of leaf senescence. The ability to stay green in the leaf area showed positive correlation with the percent plant survival but negative correlation with plant shoot elongation. The phenotypic association between the low level of leaf senescence and high plant survival were shown to be highly positive (Toojinda *et al.*, 2003). Moreover a strong negative correlation between elongation and survival was found amongst four difference cultivars (Singh *et al.*, 2001). The leaf senescence and tolerance score were indeed linked genetically to plant elongation during submergence of the rice plant. The rapid plant elongation and leaf senescence being negatively correlated to plant survival in rice was parallel to the responses given by exogenous ethylene (Jackson *et al.*, 1987). All of these data suggested plant elongation and leaf senescence was directly linked to the physiological adaptation and plant survival after submergence.

The results can classify the ISLs-132 classes, ISLs-CC and ISLs-Cc representing a tolerance segment in 16.7 kb strongly related to the ability to limit leaf elongation, to stay green and to survive including recuperation after submergence. The ISLs-132 classes having homozygous segment of

tolerance donor of ISLs-CC or heterozygous of ISLs-Cc was differentially expressed on phenotypic changes under water with ISLs-cc which contained the intolerant segment. The physiological adaptations related directly to the survival ability. Although it is reasonable to conclude that ISL-classes consist of a genotypic allele of a donor, DH206, the range 36 kb was related to the tolerance trait under submergence on plant shoot elongation and leaf senescence. In addition, cloning of genes related to submergence tolerance might open new avenues for genetic improvement of rice crops. Finally, the ISLs-132 containing a tolerance segment of 36 kb associated with submergence tolerance in rice should be very useful to promote the breeding process in submergence stress-tolerant rice.

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