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THESIS

FUNCTIONAL GENOMICS: DISSECTING SIGNAL TRANSDUCTION PATHWAY REGULATING SUBMERGENCE-INDUCED ELONGATION IN RICE

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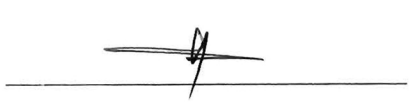
The major Quantitative Trait Loci (QTL) for submergence tolerance was mapped near the sub-centromeric region of the chromosome 9. We predicted a total of 228 coding sequence (cds) features on the submergence tolerance region with 104 related to transposable elements (TEs). Putative functions could be assigned to 21 genes, with another 51 genes annotated as expressed, leaving 71 encoded hypothetical proteins. Excluding TEs-related gene, the gene density in the 1.35 Mb submergence tolerance region is approximately 10.8 kb/gene. To understand the expression profiling of these genes in the region, 49 genes were arrayed on a filter membrane and tested against total RNAs isolated from different time-courses experiments of rice genotypes grown under submergence conditions. The expression profiling analysis of the tolerant *vs* intolerant lines revealed differential expression of putative gene encoding Ras-GTP binding protein (*OsRAS*) and ethylene-responsive element binding protein (*OsEREBP1*). Positional cloning of a submergence tolerance trait on chromosome 9 generated isogenic lines differing for submergence tolerance. We found two ethylene responsiveness genes playing significant roles in regulating shoot elongation during submergence stress. Complementation test was used to assess the effects of altered expression of specific stress-related genes, *OsRAS* and *OsEREBP*1, allowing confirmation of the importance of particular plant adaptation in response to submergence.



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Vinitchan Ruanjaichon

March 2007

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LIST OF ABBREVIATIONS

AFLP	=	Amplified Fragment Length polymorphism
BAC	=	Bacterial Artificial Chromosome
bp	=	Base pair
cDNA	=	Complementary Deoxyribonucleic Acid
cM	=	Centi Morgan
DNA	=	Deoxyribonucleic Acid
DNATEC	=	DNA Technology Laboratory
dNTP	=	Deoxynucleotide Triphosphate
EST	=	Expressed Sequence Tag
HMW DNA	=	High-Molecular-Weight Deoxyribonucleic Acid
IPTG	=	Isopropylthio-β-D-galactoside
kb	=	Kilobase
LD	=	Linkage Disequillibrium
LMP	=	Low Melting Point
LOD	=	Log of Odds
min	=	Minute
ml	=	Milliliter
mM	=	Millimolar
mRNA	=	Messenger Ribonucleic Acid
NCBI	=	National Center for Biotechnology Information
ng	=	Nanogram
PAC	=	P1-derived Artificial Chromosome
PCR	=	Polymerase Chain Reaction
PFGE	=	Pulsed field gel electrophoresis
PIC	=	Polymorphic Information Content
pmol	=	picomole
PMSF	=	Phenylmethyl sulfonyl fluoride
QTL	=	Quantitative Trait Locus

LIST OF ABBREVIATIONS (Continued)

RAPD	=	Random Amplified Polymorphic DNA
RFLP	=	Restriction Fragment Length Polymorphism
RNA	=	Ribonucleic Acid
rpm	=	Rotations per minute
RT-PCR	=	Reverse-transcribed Polymerase Chain Reaction
s	=	Second
SCAR	=	Sequence Characterized Amplified Region
SNP	=	Single Nucleotide Polymorphism
SSLP	=	Simple Sequence Length Polymorphism
STR	=	Simple Tandem Repeat
STS	=	Sequence Tagged Site
TBE	=	Tris-Borate-EDTA
TE	=	Tris-EDTA
U	=	Unit
µl	=	Microliter
µM	=	Micromolar
V/cm	=	Volt per centimeter
X-gal	=	5-Bromo-4-chloro-3-idolyl-β-galactoside
YAC	=	Yeast Artificial Chromosome
^{o}C	=	Degree celcius

FUNCTIONAL GENOMICS: DISSECTING SIGNAL TRANSDUCTION PATHWAY REGULATING SUBMERGENCE-INDUCED ELONGATION IN RICE

INTRODUCTION

Submergence tolerance is important trait for rice farming in the tropical lowland where flash flooding is unavoidable. To improve yield in flash flood area, the development of a rice variety with improved agronomic traits, such as submergence tolerance is necessary. The tolerant plant is characterized by complex phenotype-associated traits. There can be generalized as the ability to survive and continue growing after several days in submerged conditions. The phenotypic benefits provided the tolerant rice with the ability to survive and also recover during the flooding period. Consequently, the submergence-tolerant plants affected by several morphological adaptations: decreased chlorosis of tissues and reduced shoot elongation on plant growth in order to save carbohydrates and energy for maintenance processes including protective antioxidant systems.

Physiological changes of leaf elongation, senescence and the whole-plant decay are major effects that occure under submergence stress condition. These changes were highly correlated with the particular changes in the contents of central phytohormones such as gibberellins (GAs), abscisic acid (ABA), ethylene and so on. Due to physiological response mechanisms, primarily reduced plant elongation and delayed leaf senescence, plants can survive and recover from submergence stress. In metabolic change, shift to an ethanolic fermentation pathway directly increase ethylene accumulation within the shoot, which result to chlorosis of tissue and leaf elongation in response to submergence stress.

The candidate gene approach has emerged as a promising method of merging QTL analysis with the extensive data available on the cloning and characterization of genes. Over the past few years, a major quantitative trait locus (QTL) controlling

submergence tolerance was mapped to a 15.5-cM region of chromosome 9. It was cloned, sequenced and annotated. 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 (RILs). Both traits were coincidentally mapped between two marker loci, S10709 and RZ698 on chromosome 9, where their candidate genes were identified. Therefore, the large-scale genome event has open up valuable sequence for data mining and positional cloning of the responsible genes.

Discovering the transcriptions and functions of responsible genes caused the understanding of genetic mechanisms controlling submergence tolerance in rice. The profiling of gene expression also knew as transcription profiling or mRNA profiling, is one such tool. DNA array technology is an attractive and ideal tool to investigate expression profiles in developmental studies in a large-scale fashion (Tanaka *et al.*, 2000). In comparison, among the available array techniques, the use of nylon membranes and radioactively labelled probes seems to be especially reliable (Herwig *et al.,* 2001). Macroarray analysis is more economical method for efficiently exploring the function of large number of genes at the same time than the other available technique. Many types of macroarray have been proposed and applied to verify the expression profiling array that is generated by PCR-amplified DNA dots on a nylon membrane and a high-density colony grid on a membrane.

The near-isogenic lines (NILs) are a 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. According to a different mapping population, a near-isogenic line (NIL) was developed by four advanced backcrosses of Khao Dok Mali 105 (KDML105), which were 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 NIL 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 (Seaglew *et al*., 2003).

Genetic engineering is a useful tool for a study designed to find the functions of candidate genes which provide for positional cloning approach in rice. The transgenic technology is effective for utilizing information of their gene functions. Morover, the investigation of gene expression between introduced gene and native gene are determined precisionly by using TaqMan RT-PCR assay with allelic discrimination. This method may be used to calculate relative changes in gene expression with Real Time quantitative RT-PCR analysis experiment.

In the studies, we performed the computational approach to predict the candidate genes that located on the major QTL of submergence tolerance. Based on sequencing and annotated information, molecular markers in various types were developed for identification and characterization. Furthermore the predicted genes were explored for the expression profiling under submergence stress using DNA macroarray analysis. The functional roles of candidate genes which regulated plant shoots elongation and senescence are concerned by backcrossing advanced isogenic line. Consequently, transgenic technology is the most critical evidence for the successful cloning. Then the development of analysis for transgenic rice expressed is still necessary for the final proof of the precise function and a physical organization of these genes.

The objectives consist of ;

(1) Identification of genes underlying QTL determining flooding tolerance

(2) To identify the differential expression of candidate genes under submergence stress using macroarray analysis that relates to plant adaptation

(3) To obtain more information about the function of candidate genes from a tolerant allele and also to determine if the phenotypic changes observed are in NILs population and transgenic plants

LITERATURE REVIEWS

Characterization of submergence tolerance

Submergence tolerance is characterized by complex phenotype-associated traits which can be generalized as the ability to survive and continue growing after several days in submerged conditions (Adkins *et al*., 1990). The physiological machanisms provided to the tolerant rice determine plant survival and also recovery under flooding period. Submergence-tolerant plants exhibited several morphological adaptations such as decreased chlorosis of tissues and reduced elongation growth in order to save carbohydrates and energy for maintenance processes including the protective antioxidant systems.

Plant adaptation and other responses to complete submergence

Submergence stress causes the elongation rate of leaves increase and stem in some plant species. The physiological mechanisms are benefit for the survival of these plants when they are in contact with atmosphere. In rice, submergence stress causes abrupt changes in many levels such as molecular level, physiological level and morphological level. One obvious change is elongation growth, in particular on the newly emerged leaves under submergence. Several reports reviewed that susceptible rice strains are fast elongating while the tolerants ones are non-responsive. Setter and Laureles, (1996) showed that decreasing elongation growth improves the survival of the rice ability during complete submergence. Consequently, responses of submergence, the tropical Indica rice immediately suppress the leaf sheath elongation (Mazaredo and Vergara, 1982). Although the benefit of rapid elongation event is unclear in energy-demanding process under flash flooding conditions. It was thought to be a cause of susceptibility for short-term submergence (Jackson *et al*., 1987: Setter *et al*, 1997). Other evidence supported the hypothesis which is the existence of resistance to flood-induced elongation in the best-known submergence tolerant variety, FR13A (Mazaredo and Vergara, 1982). It is a basic knowledge that slow plant elongation under submergence may be more beneficial in lowland areas. Although no

genetic evidence has been reported of a relationship between elongation and susceptibility to flooding. However, Siangliw *et al*., (2003) demonstrated using BC4F2 population that there are a parallel between plant elongation and relative plant elongation in completely submerged in rice seedling. However, both traits showed negative correlation for percent plant survival, while leaf senescence did not.

Hormonal signaling pathway

Signal transduction pathway is a specific process within a cell that allows an extrinsic signal into a specific cellular response. The preliminary step before the signal is tranduced depends on high-affinity binding of the hormone to a specific protein receptor. After activated, the receptor can induce the expression of a responsivness gene by acting as a transcription factor or may pass the signal to the nucleus through a series of intermediary steps that define the length of the transduction pathway. In Arabidopsis, a variety of mutants affecting the hormone signal transduction were described upstream and downstream of cascading mechanism (McCourt, 1999). For example, *ABI3 (ABA INSENSITIVE*3) encodes a seed-specific transcription factor involved in the regulation of seed dormancy and that has multi number of different domains. The generation of a set of mutant alleles in the Columbia ecotype having mutations in different domains showed that ABI3 is a complex protein that is involved in responses to a number of different signals: *abi3-9* has a mutation in the B2 domain and is resistant to exogenous ABA application; *abi3-10* has a different mutation in the B2 domain and shows a sugar signaling response; and the *abi3-11* allele has a mutation between the B2 and B3 domains and shows a somewhat different sugar signaling response from that of *abi3-1*0. Work of this type will help determine the protein–protein interactions that underlie genetic interactions between pathways.

Phytohomones involved in Submergence-Induced plant elongation

Upon sensing the regulation in response to submergence, the action of several downstream components, including plant hormones is altered. The interplay between three phytohormones (ethylene, abscisic acid and gibberellin) have been known for a long time to be a crucial important factor in determining submergence-induced plant elongation under water. As a consequence, ethylene synthesis is promoted by submergence stress that affects to physically entrap in submerged tissues (Métraux and Kende, 1983), which results in accumulation of ethylene. In addition to ethylene synthesis, lower content of oxygen under water is also involed. The processes of ethylene biosynthesis are generated by conversion of S-adenosylmethionine to 1-aminocyclopropane-1-carboxylic acid (*ACC*) by ACC synthase (ACS, EC 4.4.14). Then, ethylene is synthesised from *ACC* by the ACC oxidase (ACO, EC 1.14.17.4) in a non rate-limiting step. Subsequent to the increase in ethylene content, the balance between levels of the growth-promoting hormone gibberellin (GA) and the growth-inhibiting hormone abscisic acid (ABA) is drastically altered. Hoffmann-Benning and Kende, (1992) found that the level of ABA decreases after 3 hours of partial submergence. Because responsiveness to GA is a function of ABA content, fall down in ABA levels increases responsiveness to GA. High content of GA including GA-responsiveness result in increased growth rates of the youngest internode. Submergence-induced petiole elongation of the dicotyledonous *Rumex palustris* involves ABA and GA as well as ethylene, which interact through a signalling cascade that resembles to the one observed in submerged deepwater rice (Voesenek *et al*., 2003).

1. Ethylene

Ethylene is one of important phytohormones that play roles in physiological and developmental processes, including the life cycle of the plant (Mattoo and Suttle, 1991: Abeles *et a*l., 1992). Effects of ethylene are regulated at multiple levels, from hormone synthesis and perception of signal transduction and transcriptional regulation. It also regulates plant response to abiotic stresses such as submergence

stress. Consequently, some parts of the ethylene signaling pathway are controlled by members of a family of transcriptional factor that are specific to plants. Many species possess mechanisms for sensing and reacting to impeded aeration that involve ethylene. When the plant is under various stresses, the increase in ethylene synthesis serves as a common step in the chain of events leading to a variety of response. Ethylene plays a central role in submergence-tolerant species (Armstrong *et al*., 1994; Voesenek and Blom, 1999). Ethylene is also the signal that induces death to epidermal cells at the node since cell death was induced by the natural ethylene precursor ACC and was inhibited by simultaneous application of norbornadien, an inhibitor of ethylene action (Bleecker *et al*., 1987: Lorbiecke and Sauter, 1999). Moreover, the induction of ACC oxidase by ethylene in shoot is involved in signal transduction in the cell (Varvara *et al*., 2001). The cell death program of cortical cells during aerenchyma formation was also shown to depend on the transduction of an ethylene signal (Drew *et al*., 1979: He *et al*., 1996). In rice, lead to a shortage of oxygen in respiring cells is resulted to strongly influence ethylene production under non-photosynthesizing tissues (Jackson, 1990).

Consequently, some parts of the ethylene signaling pathway are controlled by members of plant specific family of transcriptional factor. Recently, ethylene responsive element binding protein genes (*EREBP*) have been identified in various plants (Young *et a*l., 2004; Magnani *et a*l., 2004), which are necessary and sufficient for transcriptional control under stress responsive conditions and developmental processes by ethylene. A highly conserved DNA binding domain known as the ERF domain is the unique feature of this protein family. These proteins act as transcriptional activators or repressors of GCC-box which mediate the gene expression which may affect plant adaptation in the regulation of the responsive genes. In addition, other plant hormones such as auxin, ABA, cytokinin, gibberellins and brassinosteroids were reviewed in the complex regulations that are often mediated by ethylene (Chen *et a*l., 2005).

Molecular analysis of ethylene-insensitive mutants allowed for instance the identification of ethylene receptors. In Arabidopsis, five receptors were identified,

which show a high degree of functional overlap (Alonso and Ecker, 2001). Ethylene receptors are related to the bacterial two-component sensors. Mutations in *CTR1* result in a constitutive triple-response phenotype. (Kieber *et a*l., 1993). CTR1 is able to physically interact with the ethylene receptors ETR1 and ERS (Clark *et a*l., 1998) and was shown to be a Raf-like Ser/Thr protein kinase that is part of a MAP kinase cascade that mediates ethylene signalling (Ouaked *et a*l., 2003). Mutation in *EIN3* causes ethylene insensitivity which is epistatic to *CTR*1, implying that *EIN3* acts downstream of *CTR*1. EIN3 is a putative transcription factor that acts at the end of the ethylene transduction pathway (Chao *et a*l., 1997). It is able to *tran*s-activate expression of the *ERF1* gene (Solano *et a*l., 1998) that encodes an Ethylene-Response-Element- Binding-Protein (EREBP). Overexpression of *ERF1* in Arabidopsis resulted in the activation of ethylene response genes and subsequently in a variety of phenotypes typically obtained through ethylene treatment such as the triple-response phenotype. EIN3-like transcription factors have been characterised from other plant species. At least five tobacco *EIN3-like (NtEI*L), three tomato *EIL (LeEI*L) and two Mung Bean *EIL (VR-EI*L) genes have been identified, and some of these genes encode proteins with biological function and DNA-binding capacity identical to *EIN3*. The finding of *EIN3* orthologues from other plant species supported the idea that nuclear events induced by ethylene signal transduction rely on similar mechanisms, and that physiological responses to ethylene in plants are regulated mainly at the transcriptional level (Bleecker and Kende, 2000).

Most recent advances in research on ethylene signal transduction showed that levels of EIN3 protein are regulated by ethylene through two F-box proteins that target *EIN3* to the proteasome degradation pathway. These F-box proteins, EBF1 and EBF2, are components of the $SCF^{EBF1/EBF2}$ E3 ubiquitin-ligase complexes (Potuschak *et a*l, 2003; Guo and Ecker, 2003).

2. Gibberellins

The plant hormone family of gibberellins (GAs) has been the subject of the majority of studied that attempt to identify the key components in the regulation of

plant responses to submergence stress. GA as well as responsiveness GA, is affected a number of processes during plant growth and developments, including seed germination, shoot growth, flowering, and fruit development. They are also involved in the submergence-induced shoot elongation in *Rumex* and in Rice. However, Lorbiecke and Sauter (1998,1999), found that GA activates cells which are in the G1 phase to enter the S phase at an enhanced cell division rate which results of a growth promoting effect in rice.

To date, downstream GA signalling events are clearly understood. Most reports of molecular and genetic approaches of GA-response mutants classified their vegetative phenotypes and responses to GA in two classes. The first class is involed in GA-insensitive dwarf mutants as DELLA protein in Arabidopsis and rice (Dill *et al.*, 2001: Silverstone *et al.*, 2001: Gomi and Matsuoka, 2003: Harberd, 2003). Another one is GA-response mutation that presents GA-independent phenotypes such as slender, elongated stems and early-flowering (Jacobsen and Olszewski, 1993: Jacobsen *et al*., 1996).

3. Abscisic acid (ABA)

Abscisic acid (ABA) plays a major role in seed maturation and germination, as well as in adaptations to abiotic stress (Buchanan *et al*. 2000). Besides its important role in drought avoidance, ABA also seems to be important in the regulation of shoot elongation in submerged plants. In both deepwater rice and Scirpus micronatus, ABA inhibits submergence-induced shoot elongation. Furthermore, a fast decline in endogenous ABA concentrations in shoots is observed when these species were submerged or exposed to elevated levels of ethylene (Hoffmann-Benning and Kende 1992; Lee *et al*., 1996). It has been assumed that the sensitivity to GA of deep-water rice internodes is decreased by ABA and that an ethylene-induced down-regulation of ABA increases this sensitivity. In this aspect, ABA can be seen as a negative regulator of GA action (Hoffmann-Benning and Kende 1992, Ritchie and Gilroy 1998).

An inhibition of submergence-induced shoot elongation by ABA is also observed in the dicot *R. pal-ustri*s. The ABA concentration in this species declined 80% within 1 h of submergence or exposure to elevated ethylene levels. Submergence of *R. palustris* plants pretreated with 1-MCP, an inhibitor of ethylene perception, prevented this fast down-regulation of ABA (J. J. Ben-schop and L. A. C. J. Voesenek, *unpublished dat*a). Taken together, all these results suggest that down-regulation of ABA, mediated by ethylene, is a prerequisite for fast shoot elongation under water in both monocots and dicots.

4. Hormonal signaling between ethylene and gibberellins

Interplay between phytohormones signaling has long been recognized and ethylene is no exception in this respect. Many reports described some of the shared characteristics of plant hormone signal transduction. Especially, since gibberellin action is well known to be a key component in response to submergence stress, interaction between ethylene and gibberellins will be described in some more detail. In Rumex, semi-aquatic plant specie, the ethylene can stimulate shoot elongation by stimulating the production of and sensitivity to gibberellins (Rijnders *et al*., 1997: Voesennek *et al*., 2003). In addition, other semi-aquatic species such as deepwater rice, also enhance shoot elongation through ethylene-mediated changes in gibberellins action (Jackson and Ling, 1972). In arabidopsis, ethylene was found as a repressor of plant growth which opposes effects of GA during apical hook formation and maintenance (Vriezen *et al*., 2004). However, little is known about the signalling pathway converting the ethylene signal into increased GA levels and responsiveness which affect to submergence induced elongation in rice.

Genomics Approaches

1. Genome Mapping and QTL Analysis

The mapping of quantitative traits where there is often little knowledge of the genetic control before of the analysis, such as is usually the case with stress

tolerance, is usually carried out by ‘QTL mapping’. Recently, genome mapping and QTL analysis of submergence tolerance produced the major point of information for plant research. Several studies revealed that submergence tolerance has relatively high heritability, controlled by one or a few genes with major effect and minor modifiers (Haque *et al*., 1989: Mohanty and Khush, 1985). Five Quantitative Trait Loci (QTLs) for submergence tolerance were reported on chromosomes 6, 7, 9, 11, and 12 (Xu and Mackill, 1996: Nandi *et al*. 1997). Genetic linkage between submergence tolerance and shoot elongation was clearly shown by QTL analysis in segregating recombinant inbred line (Siangliw *et al*., 2003). Both submergence tolerance and suppression of elongation were coincidentally mapped on chromosome 9 (Toojinda *et al*., 2003). Quantitative Trait Loci (QTLs) analysis for submergence tolerance had been identified “*sub*QTL9” as a major QTL.

Developments over the past few years, allowed the discovery at a major QTL controlling submergence tolerance mapped to a 6.5-cM region of chromosome 9. It was cloned, sequenced, and annotated (Vanavichit *et al*., 2001). Due to the physiological response mechanisms, primarily reduced plant elongation and delayed leaf senescence, plants can survive and recover from submergence stress (Timothy *et al*., 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 markers loci, S10709 and RB0783 on chromosome9, where their candidate genes were identified.

2. Map-based positional cloning

The approach being taken for rice genome sequencing is the same as the one used for human, yeast, arabidopsis and C.elegans. Rice has become an attractive model for map-based positional cloning because rice has been used extensively for QTL analysis. Physical mapping based on YAC, BAC, PAC, and FPC is available for publicity. In rice, physical map (map-based positional cloning) that covers on which a major QTL for submergence tolerance locus are available using large insert libraries

based on bacterial artificial chromosomes (BACs) and yeast artificial chromosomes (YACs) (Kamolsukyunyong *et al*., 2001). The availability of the major QTL for flooding tolerance mapped onto a 6 cM subcentromeric region on chromosome 9 in rice has been one of the largest QTL ever reported (Toojinda *et al*., 2003). In addition, the major QTL was located within the 16 cM region flanked by two RFLP markers, R1164 and RZ698. Fine map was constructed near R1164 using two YAC ends, 6 BAC ends and a SSCP. Map-based analysis revealed that major QTLs for plant elongation, leaf senescence, plant vigor, and plant recovery after submergence were located on chromosome 9. The way to learn how the genes controlling the QTL regulate submergence tolerance is to sequence the whole genomic region and looking for genes of interest.

3. Genome sequencing

The advent of large-scale genomic sequencing has been conducted on a major QTL response for submergence tolerant from chromosome 9 resulting in a significant increase in gene sequence information. However, our knowledge of the function and interaction is lacking of these newly discovered genes. In recent years, the genomic sequence around the major QTL of submergence tolerance has been completely sequenced (http://www.ncbi.nlm.nih.gov/GenBank/index.html). Therefore, the investigation of structures and functions of responsible genes is essential for understanding the genetic mechanisms controlling submergence tolerance in rice.

4. Sequence-based approaches to function prediction

In the last few years, several new genomics resources and tools became available and will greatly assist quantitative trait locus (QTL) mapping and cloning of the corresponding genes. Predicted functions and gene ontologies help to guide the selection of candidate genes. The process of selecting candidate genes relies on a wealth of information gained through traditional genetics and molecular approaches. Moreover, a variety of computational programs are used to find genes and other functional sites from genomic DNA sequence. These programs seek a basic signal

based on molecular types. The most basic signal is a simple consensus sequence, or an expression regulation site that describes a consensus sequence together with allowable variation. In contrast, extended and variable – length regions, such as exons and introns are recognized by method that used. Recently, there have been some successful examples of the use of the candidate gene approach to identify QTL genes. The gene encoding the CRYPTO-CHROME2 photoreceptor was shown to be responsible for the phenotypic variation associated with a flowering-time QTL (EI-Din EI-Assal *et al*., 2001). In rice, three QTL have been identified as candidate genes (Kojima *et al*., 2002: Takahashi *et al*., 2001: Yano *et al*., 2000) whose function was known from studies of Arabidopsis.

Once a candidate gene is selected, the first follow-up experiment is usually to sequence the gene in the two parental lines and to look for variations that are predicted to have functional consequences. As only few QTL have been cloned, it is hard to make generalizations about what kind of changes will have phenotypic consequences, but certainly nonsense polymorphisms and deletion polymorphisms make the candidate gene more likely. Amino-acid changes (EI-Din EI-Assal *et al*., 2001: Maloof *et al*., 2001), as well as expression level changes, may also be important in providing functional variation (Kliebenstein *et al*., 2001: Doebley *et al*., 1997: Liu *et al*., 2003). Several functional alleles have been identified at some QTL loci (Sheldon *et al*., 1999: Michaels *et al*., 1999: Le Corre *et al*., 2002: Johanson *et al*., 2000: Gazzani *et al*., 2003). In such cases, the previous identification of high-density polymorphisms allows the interval to be screened for changes that might have functional consequences (Andersen and Lubberstedt, 2003). In this regard, genomic DNA hybri-dization to arrays can reveal changes and potential dele-tions in genes that make excellent candidates (Borevitz *et al*., 2003). A new flowering-time QTL has been identified by this approach in our group (J Werner *et al*., unpublished).

Functional Genomics Approaches

1. Monitoring gene expression

In plants, DNA arrays have been successfully utilized to examine a range of biological issues including developmental processes, environmental stress responses, identification, and genotyping of mutations (Aharoni and Vorst, 2001). The common goal of most of these experiments is to investigate the reaction of a given genotype to a specific environmental effect, to study profiling patterns of gene expression, or to compare a wild-type plant with a corresponding tolerant plant. Positional cloning approached in submergence response is a key step to clarifying the mechanisms of submergence stress and tolerance in rice. One of the most important applications for function analysis so far is the monitoring of gene expression (mRNA abundance). The collection of genes that are expressed or transcribed from genomic DNA, sometimes referred to as the expression profile or the 'transcriptome', is a major determinant of cellular phenotype and function.

Currently, fluorescent - based microarray is an efficient High-throughput methods of gene expression are available for monitoring gene expression profiles and to initiate functional analysis of the plant genome. Macroarray analysis is a useful method for efficiently exploring the function of uncharacterized genes in addition to known genes by relating the expression pattern of one gene to those of other. Many types of macroarray have been proposed and applied to expression profiling such as PCR-amplified DNA dots on slide glass or a nylon membrane and high-density colony grids on a membrane (Lenon, and Lehrach, 1991: Pietu *et al*., 1996). Moreover, the use of macroarray for quantifying measuring gene expression level is advantageous over other established techniques such as northern blot analysis and RT-PCR because it facilitates analysis of large number of genes simultaneously. In addition, macroarray technology is affordable for gene expression experiment.

2. Expression of near-isogenic lines (NILs) that differ for submergence tolerance

Analysis of near-isogenic lines that differ at QTL can be an effective approach for the detailed mapping and characterization of individual loci. The procedure utilizes molecular markers to identify heterogeneous inbred families that are isogenic at most loci in the genome from NILs that differ for markers linked to QTL of interest. The process of identifying linkage between markers and traits in a mapping population followed by test of marker effects in NILs can be powerful and useful to resolve several issues. First, marker linkage to a QTL can be confirmed by examining the phenotype on NILs that only differ for individual QTL. Initial QTL analysis indicates regions of the genome that may contain QTL but the particular phenotypic effects of these loci need to be confirmed. Second, NILs can be used for fine mapping of QTL. Evaluation of a series of NILs that contrast at a specific locus can be used to narrow the genetic interval known to contain the QTL. Third, NILs that differ at a QTL can be used to characterize the expression and function of a specific locus. 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).

Gene expression studies in which the NIL QTL is compared to the parental line (or an alternative QTL allele) for differences in gene expression can also be used to identify candidate genes. Several replicate lines are used as controls for biological variance and potential maternal effects. The conditions and tissue selected for the gene expression study must be chosen on the basis of the phenotype of the QTL. Experiments that look at differences in gene expression under several conditions will be more powerful. A set of conditions in which the QTL has no effect provides a control for changes that are unrelated to the phenotype. However, changes in constitutive gene expression may also suggest QTL candidate genes. Gene expression studies also characterize the downstream transcriptional response of the QTL. Thus, genes with expression level differences that map to the QTL are candidate genes, whereas genes that map to other locations are part of the molecular phenotype caused

by the QTL. An alternative experimental design involves the use of lines from the mapping population that have extreme phenotypes. Replicate pools of extreme lines can be profiled independently, so that differences in gene expression will be specific to the phenotype and genotype that separates the pools. This strategy was recently used to identify candidate genes for drought response QTL in rice (S Hazen, personal communication). Large-scale studies are under way to map QTL for gene expression differences (eQTL) by individually profiling lines from a mapping population. Often, the eQTL map to the gene itself, indicating that cis changes are responsible for the different levels of expression; however, the presence of groups of genes that are coordinately regulated by a single unlinked QTL suggests that trans-acting factors are controlling expression (Schadt *et al*., 2003: Brem *et al*., 2002).

3. Transgenic approach

Successful positional cloning requires rigorous functional analysis of the candidate genes representing a responsive tolerance. The number of genes that regulate the process and how they coordinate it has yet to be unraveled. The studies designed to find the functions of candidate genes were success for positional cloning approach in rice. Currently, transformation technology is a powerful tool for utilizing information of their gene functions and may lead us to describable rice plant for abiotic stress tolerance. Transgenic approach to submergence stress – tolerant rice is an essential key for explanation role of the candidate gene from adaptive tolerance cultivars. IRRI reported developing transgenic rice with the *adh* and *pdc* genes in several cultivars (Quimio *et al*. 2000). Results on transgenic lines with the *adh* gene are inconclusive. The rice *pdc1* (reported by Hossain *et al*. 1996) gene coding for pyruvate decarboxylase (PDC) is one of the enzymes involved in alcohol fermentation. The overexpression of the *pdc* gene was used not only to assess the role of alcohol fermentation but also to produce lines with enhanced metabolic capacity and submergence tolerance. For example, overexpression of the pdc may have induced the expression of other genes that control the rate of carbon flow though the glycolytic and ethanolic fermentation pathways under anaerobic conditions. Transgenic lines with overexpression of both the *adh* and *pdc* genes may provide

more information regarding the importance or validity of the fermentation pathway for inducing flooding tolerance (Minhas and Grover 1999).

MATERIALS AND METHODS

Part I: Effect of submergence stress on physiology in rice

Plant materials

Two rice cultivars were used for this study. The first cultivar is FR13A, an Indian landrace and one of the most submergence tolerant lines. Second, susceptible cultivars included KDML105 a traditional aromatic cultivar from Thailand, which is commercially important but intolerant to submergence. Moreover, GA-related dwarf mutants (Tangin *d35*, Wild type *D35*, Waito *d18*, Remei *sd1*, T65 *d1*, *SLR* hetero were also used to monitor the phenotypic changes in submergence response which might be related to genes controlling a tolerant characteristic.

Submergence treatment

Three weeks after seedling establishment, the plants were completely submerged for 2, 4, 6, and 8 under low light condition. The plastic tank filled with treated water was used in three independent experiments. The water level was kept 60 cm above the tallest entries to prevent their leaf tips emerging into the atmosphere. Water temperature was 28-32 ^{o}C throughout the experiments. Plants were desubmerged after 10 days and the survival rate was observed after a 1 week recovery. Control plants were left unsubmerged through-out in a tank with the water maintained at a constant level as for the remaining plants prior to the experiment. The order of measurements was randomized within a day for both plants and treatments.

Determination of plant responses to submergence stress

Total Shoot Elongation under water (TSE) was used as a measure of the increment in shoot height during submergence and calculated as the average difference in shoot height (in practice, shoot length) relative to the plant height before

submerged. In randomly selected plants per cultivar, shoot height was taken as the distance from the soil surface to the tip of the longest leaf.

Relative Shoot Elongation (that they are so called as plant growth) was used to compare the impact of submergence on shoot elongation. The expanding in height of each individual line was set to be 100% under non-submerged conditions and elongation growth underwater compared with this value. The resulting value is termed RSE. If the values of RSE were less than 100% indicating that plant growth was suppressed under submergence, whereas the value was over than 100% that it meant in the induction of plant growth under water.

Leaf Senescence (LS) is characterized by dramatic yellowing resulting from chloroplast degeneration. Submergence tolerant plants are able to retain green leaves for longer than intolerant lines when under water. In this experiment, a SPAD-502 Chlorophyll Meter (Minolta Co., LTD, Japan) was used to measure the value of leaf chlorophyll concentration. The output signals are processed to calculate a “SPAD index”. This index has been successfully used to calculate a relationship exists between SPAD index and leaf chlorophyll concentration. The measurements were obtained at three random points of each leaf. In the present work, results are presented in terms of meter output.

Statistical analyses

The STATGRAPHICS 2.1 software was performed using mainly ANOVA’s for comparison of means in each of the response traits under submergence stress.

Part II: Data mining of *SubQTL* region on chromosome9

In this experiment, we performed the computational approach to predict the candidate genes, which located on the major QTL of submergence tolerance for identification and characterization. The 1.35 Mb of the finished sequences from 14 BAC/PAC clones as P0651G05, P0645D04, P0603H10, OSJNBa0009H03, OJ1190B07, P0663H05, P0453B09, B1151D08, B1106B03, B1054C11, B1043F11, OSJNBa0044K01, P0592C05, and OJ1381_H04 were subjected to analysis. The complete sequence of the critical region of submergence tolerance and its annotated information bear the accession number at National Center for Biotechnology Information, (http://www.ncbi.nlm.nih.gov/GenBank/index.html).

Sequencing Data

We downloaded an available sequence data found in GenBank (www.ncbi.nlm.nih.gov) and from the rice databases of The Institute for Genomic Research (TIGR; www.tigr.org). The complete sequences of the critical region of submergence tolerance bear the accession number as P0651G05, P0645D04, P0603H10, OSJNBa0009H03, OJ1190B07, P0663H05, P0453B09, B1151D08, B1106B03, B1054C11, B1043F11, OSJNBa0044K01, P0592C05, and OJ1381_H04 were subjected to analysis.

Data mining analysis

BLAST (Basic Local Alignment Search Tool) search and gene prediction programs were used to determine the genomic sequence of PACs for protein-coding genes. BlastX and Blastn programs were employed to search for non-redundant and EST databases at the NCBI (National Center for Biological Information) and TIGR (The International Genomic Research). BLOSUM 62 was utilized in the BLAST program as a default matrix. The homology was considered significant if an E-value was greater than –15 for at least 95% identity for 250 nucleotide tract. Potential coding regions were predicted by GenScan (Arabidopsis), GeneMark HMM (rice),

and Grail (Arabidopsis) (Burge *et al*., 1997: Lukaskin and Borodovsky, 1998: Uberbacher and Mural, 1991). Predicted protein sequences were investigated against a non-redundant amino acid database using blastp (Altschul, 1997). Repetitive sequences were masked using Repeat-masker and tRNA Scan—SE (http://ftp.genome.wash-ington.edu/RM/RepeatMasker.html (Lowe and Eddy, 1997). Annotated genes were categorized according to the homology level. Putative open reading frames (ORFs) were analyzed or scanned by several sequence motif searches including PROSITE, BLOCKs, ProDom, PRINTS, and Pfam (http://www.motife.genome.ad.jp) (Hofmann *et al*., 1999: Hofmann *et al*., 1999: Corpet *et al*., 1999: Attwood *et al*., 1999: Bateman *et al*., 1999). Proteome analysis database (http://www.ebi.ac.uk/pro-teome/) and ExPASy Molecular Biology serve (http://www.expasy.ch/) were used as a resource to identify the functional classification of proteins in this region

Part III: Transcriptional Profiling in Response to Submergence Stress

Our goal of this study is to identification of candidate genes by inferring from the transcriptional networks of the genes which associated with a major QTL regulating submergence tolerance on chromosome 9 (*sub*QTL) and also display quantitative variation.

Plant materials, Stress treatment, and RNA isolation

Submergence tolerant rice cultivar, FR13A, and susceptible cultivar, KDML105 (a traditional aromatic cultivar from Thailand, which is commercially important but intolerant to submergence), were used for the experiments. Three weeks after seedling plants were carried to submergence stress for 0, 2, 4, 6, performing in an aquarium under day light condition in two separated experiments. For control treatments, the plants were grown under normal condition in the same days. The plants were subjected to the stressed treatments and control condition for various periods and then frozen in liquid nitrogen for further analysis. Total RNA was prepared using High pure Total RNA isolation kit (Roach). Quality of the RNA preparation was determined on agarose gels. Polyadenylated mRNA was prepared from other RNA extraction using oligotexTM-dT resin (Qiagen). All traits evaluation methods were used in this study according to reported by Siangliw *et al*. (2003).

Construction of mini-scale macroarray

DNA macroarray used in the study contained 29 PCR-amplified DNA fragments are represented candidate genes responsible for a major QTL of submergence tolerance including cell wall modification, GA and ethylene signaling pathway (Table1). All DNA fragments were amplified with gene-specific primers, purified, and analyzed on agarose gels. Fifty ng concentration-adjusted PCR products were added with 1 N NaOH, 5 M NaCl and plus 0.01 mM bromophenol blue for 10 min to insure complete denaturation, and spotted in duplicate onto positively charged HybondTM – N+ (7.5 x 11.5 cm2) (Amersham Phamacia Biotech, GERMANY) by

using 96-pin Replicator System (Nalge Nunc International, GERMANY). Each sample is spotted as duplicate crossways in the pattern of 2 X 2 arrays. The final result is 384-DNA-spot array (4 X 96) standing for 96 samples of amplified-PCR fragments on each membrane. After finish spotting process, soak the membrane in denaturation solution (0.4 N NaOH, 10 mM EDTA) for 5 min, then neutralized in cool neutralization buffer (2 M ammonium acetate, pH 7.0) for 5 min and wash with 2XSSC for 2-5 min. Stabilize the membrane at 80 °C for 2 h; the membrane can be stored at room temperature until further use.

Preparation of the probe

Five microgram of each total RNA sample is combined with 1 μg oligo(dT_{15}) in a total volume of 6 μl, heated to 65°C for 5 min, cool down to 42 °C for annealing and then chilled on ice. Five microlitres of dCTP (3000 Ci/mmol), 2 μl of 0.1 M DTT, 4 μl of first strand buffer (Invitrogen), 1 μl of dNTPs (10mM each of dTTP, dGTP, dATP and 37 μM dCTP), 1 μl of 40 U /μl RNASEOUT (Gibco BRL) and 1 μl of Superscript ^{TM} II reverse transcriptase (Invitrogen) were then added. Each sample is then incubated at 37 °C for 1.5 h to generate ^{32}P-labelled cDNA.

Array hybridization and data analysis

After wetting in 2XSSC, the arrays were pre-hybridized for at least 30 min at 68°C in Rapid-hyb buffer (Amersham Phamacia Biotech, GERMANY) containing sheared salmon sperm DNA (10 μg/ml). Heat-denatured (3 min, 95°C) labeled single strand cDNAs were added together with fresh hybridization buffer and hybridized at 68°C for at least 12 h. DNA arrays were washed with washing solution I (2XSSC, 1% SDS) for 30 min at 68°C, and washing solution II (0.1XSSC, 0.5%SDS) for 30 min at 68°C, then wrapped in plastic wrap and exposed to a phosphor screen (Amersham Phamacia Biotech, GERMANY). After 2-4 hours exposure, the images of membrane were scanned using Typhoon 9240 (Amersham Phamacia Biotech, GERMANY) and resulted profiles were analyzed with commercial image processing system ImageQL program (Amersham Phamacia Biotech, GERMANY). As each sample was spotted

on the membrane with duplication, signals acquired were calculated in the average of each spot for background subtraction. The values were normalized to the average value of the housekeeping genes such as beta-actin gene. After twice hybridizations using independent treated samples, differential expressed spots with the ratio (treated/control) > 2 or < 0.5 were selected for further analyses.

Table 1 Classification of possible function based on their gene annotation from a major QTL for submergence tolerant and known genes from hormonal signaling pathway and cell wall modification

No.	Gene Name	Possible function	Target area	Chr	BAC/PAC
1.	Sub08,Gamma response I protein	Splicing factor	Internal exon	9	PAC-0645D04
2.	Sub09,Ras GTP binding protein	Signal transduction	All exon	9	PAC-0645D04
3.	Sub10, unknown protein	Unknown	Internal exon	9	PAC-0645D04
4.	Sub11,TRAP170 protein	Trasmembran protein	Terminal exon	9	PAC-0645D04
5.	Sub12, Transparent tasta 1	Transcription factor	All exon	9	PAC-0645D04
6.	Sub6,Bzip transcription factor	Transcription factor	Internal exon	9	PAC-0651G05
7.	Sub7,Anth Phosphoribosytransferase	Unknown	Internal exon	9	PAC-0651G05
8.	Sub8,Hypothetical	Unknown	Internal exon	9	PAC-0651G05
9.	Sub9,Hypothetical	Unknown	Internal exon	9	PAC-0651G05
10.	Sub10,Plastid ribosomal protein	Unknown	Initial exon	9	PAC-0651G05
11.	Sub11,ARC,LRR domain	Unknown	Single gene	9	PAC-0603H10
12.	Sub12,Hypothetical	Unknown	Terminal exon	9	PAC-0603H10
13.	Sub13,Hypothetical	Unknown	Terminal exon	9	PAC-0603H10
14.	Sub14,GTPase regulator protein	Unknown	Internal exon	9	PAC0663H05
15.	Sub15,WRKY transcription factor	Unknown	Initial exon	9	PAC0663H05
16.	Sub16,EREBP transcription factor	Unknown	All exon	9	PAC0663H05
17.	Actin biosynthesis	Cell wall synthesis	Internal exon	9	-
18.	G alpha subunit (D1)	GA signaling	Initial exon	5	-
19.	GA20 oxidase (SD1)	GA biosynthesis	Internal exon	1	-
20.	beta 3hydroxylase (*D18*)	GA biosynthesis	Internal exon	1	-
21.	GAMYB	Transcription factor	Internal exon	-	-
22.	Amylase3(AMY3)	Starch degradation	Internal exon	-	-
23.	Expansin1 (*Exp1*)	Cell wall modification	Initial exon	-	-
24.	Expansin2 (*Exp2*)	Cell wall modification	Internal exon	-	-
25.	Expansin3 (*Exp3*)	Cell wall modification	Initial exon	-	-
26.	Expansin4 (*Exp4*)	Cell wall modification	Internal exon	-	-
27.	Enolase	Anaerobic catabolism	Initial exon	-	-
28.	Pyruvate decarboxylase (*PDC*)	Anaerobic pathway	Internal exon	-	-
29.	ACC oxidase (*ACO*)	Ethylene Synthesis	Initial exon	-	-

Real time RT-PCR analysis

To confirm differential gene expression under submergence stress, LightCycler-based real-time RT-PCR using Taqman probe with allelic discrimination assay was used in this study. The mean concentration of beta actin gene was used as control for input mRNA because it is considered variable housekeeping gene and was detected as same as candidate genes expressed level.

Part IV: Characterization of a small GTP-binding protein gene

Plant materials

Three submergence tolerant rice cultivars were used for this study. The first cultivar was FR13A (an Indian landrace and one of the most submergence tolerant lines). The second cultivar was IR49830, a submergence tolerant breeding line from the International Rice Research Institute, and the third was DH206, the F1-derived doubled haploid line from the cross between FR13A and CT6241, a submergence-intolerant line from Centro International de Agriculture Tropical. Susceptible cultivars included KDML105 (a traditional aromatic cultivar from Thailand, which is commercially important but intolerant to submergence), JHN (a black rice with moderate tolerance to submergence), CT6241, and Nipponbare (a traditional cultivar from Japan).

Preparation of genomic DNA

Fresh young leaves were collected and stored at –80°C before DNA extraction using the CTAB (cetylmethyl ammonium bromide)–NaCl method (Rogers and Bendich, 1994). Fifty ng of genomic DNA were used as a template for PCR amplification.

Gene expression analysis

To study differential gene expression under submergence stress conditions, RT-PCR analysis was used to analyze the total RNA obtained from FR13A, the submergence-tolerant genotype, in flash flooding for 2 days in natural ponds. Total RNA isolated from plants under normal growing condition was used as a control

DNA gel blot analysis

Genomic DNA was completely digested with Commercial restriction enzymes and fractionated on 0.8% agarose gel. Subsequently, the fractionated DNA was transferred to a positively charged nylon membrane (Boehringer, Germany). For hybridization, a detection system with digoxygenin non-radioactive nucleic acid labeling (Boehringer) and the predicted gene probe were used.

Designed primers and amplification

Based on the Candidate genes in a genomic sequence, all gene-specific primers were designed using Primer 3 software (http://www.genome.wi.mit.edu/cgibin/primer/primer3_www_results.cgi). The parameters were set at 40–60% GC rich, 60°C annealing temperature, and 21 base pairs in length. The basic procedure of the PCR reaction was as following: 5 µl of 10x buffer, 25mM $MgCl_2$, 200 uM each of dNTPs (Promega), 0.2 µM specific primer, 80 ng genomic DNA, and 1 unit Taq polymerase (Boehringer). The amplification reactions followed the protocol: 94°C 5 min for 1 cycle, 94°C 30 s for 35 cycles, 60°C 1 min, 72°C 2 min, and then 72°C for 7 min. The amplification products were analyzed by electrophoresis in 1% agarose gels with 1x TAE, followed by ethidium bromide staining and viewed under UV light.

Development of SSCP and Linkage Mapping

The basic procedure of the SSCP analysis was as following: 5 µl of the PCR product and 5 µl of 1x denaturing buffer (980-ml/L formamide, 50 ml/L 0.2 M NaOH, 0.5 g/L bromphenol blue, 0.5 g/L xylene cyanol) were heated for 5 min at 95C and finally quenched on ice. The samples were loaded onto 3.5% non-denaturing polyacrylamide gel (acrylamide/methylenebis acrylamide 29 : 1), and 1 × TBE buffer (89 mM Tris–borate and 2 mM EDTA pH 8.0) was used as a running buffer. The DNA products were separated on a sequencing gel at constant power of 5 W at 25°C for 14 hours and then detected by a silver staining. This marker was mapped to 172

recombinant inbred lines (RILs) derived from FR13A × CT6241 cross (Toojinda *et al*., 2003; Siangliw *et al*., 2003).

DNA sequence determination

DNA sequences of PCR products from specific primers for the candidate genes were characterized using a ABI Prism TM Dye Terminator Cycle Sequencing Ready Reaction Kit with AmpliTaq DNA polymerase for fluorescent sequencing (PerkinElmer). The Basic Local Alignment Search Tool (BLAST) algorithm and a data-base for plant *cis*-acting regulatory DNA elements (PLACE) were utilized in the analysis of the DNA sequences (Altschu *et al*., 1990: Higo *et al*., 1999).

Transgenic approach

In our work, we transferred the ras-related GTP-binding protein gene (*OsRAS*) which locates on the major QTL for submergence tolerance region of rice chromosome 9 into submergence intolerance rice to confirm its functions. Embryogenic calli of submergence intolerance rice, Jao Hom Nin (JHN) were used as host plant to bombard with constructed vectors pCAMBIA 1301-*OsRAS* containing the selectable marker hygromycin phosphotransferase (*hpt*), the reporter gene β-glucuronidase (*gus*).

Gene constructs

Ras-related GTP-binding protein gene (*OsRAS*) apllified directly from tolerant FR13A driven by its 5' upstream of native promoter and 35s *CaMV* were cloned into a transformation plasmid DNA, pCAMBIA1301 (Dr. Richard A. Jefferson; CAMBIA, Australia) containing the selectable marker hygromycin phosphotransferace (*hpt*) and the reporter gene beta-glucuronidase (*gus*) each under control of CaMV35S promoter.

Microprojectile bombardment

Plasmid were absorbed to gold particles about diameter 1.5 to 3.0 mm (Aldrich) by $CaCl_2$ and spermidine precipitation as described by Hunold *et al*. (1994) and delivered to the target tissues by using a Finner-type home made Particle Gun Bombardment (Finner *et al*., 1992) Embryogenic callus were pre-incubated on an osmotic inducing medium (a basic CC medium with addition of 0.2 M each mannitol and sorbitol) by picking 2 to 3 cm around the center of the petridish for 4 hour prior to bombardment. Six to eight microlite of plasmid DNA coated particles was distributed onto micro carrier of the gun nozzle. The petridish of target tissues were placed on 11 cm below the gun nozzle and covered with a nylon mesh. The bombardment was done using 700 kPa helium pressures at -25 inch of Hg vacuum.

Growth and selection of bombard cells

Sixteen hours after bombardment, the target tissues were transferred to 75 mg/l hygromycin B selective medium (CC medium sugar free supplemented with 2 mg/l 2,4-D and 1 g/l casein hydrolysate solidified with 0.27% phytagel). and cultured in the dark at 25^0C for 2-3 weeks. Newly formed callus were separated from the bombarded cells and transferred on the same fresh medium. After the resistant callus measuring 2 to 3 mm in diameter were transferred to 75 mg/l hygromycin B pre-regeneration medium (CC medium sugar free supplemented with 1 mg/l IAA and 4 mg/l BA solidified with 0.27% phytagel). The cultures were incubated under a photoperiod of 16 hrs (Toshiba cool white fluorescent tube 38w 3100lm) at 25^0C.

Regeneration of putative transgenic plants

After the calli with differentiated shoots were transferred to shoot induction medium (CC medium sugar free supplemented with 1 mg/l IAA and 0.05 mg/l zeatin solidified with 0.27% phytagel) without hygromycin B and cultured under a photoperiod of 16 hrs at 25^0C. After 3 weeks, the transgenic plants were transferred to root induction medium ; MS (Murashige and Skoog, 1962) medium containing 30 g/l

sucrose without plant growth regulator solidified with 0.27% phytagel for 2 weeks. Next, the plantlets were grown in tap water for 7 days for acclimatization and for fast rooting, before transferring them to pots in the greenhouse conditions.

GUS assay

Histochemical GUS assay was carried out 2 days after bombardments, essentially as described by Klein *et al*. (1988). Methanol was added (final concentration 10%) in the buffer to suppress endrogenous beta-glucuronidase activity. Blue loci, indicative of transient GUS expression, were counted after the addition of the X-Glue substrate solution. Stable transformations were detected on resistant callus and resistance plantlets after selected the bombarded cells on hygromycin B - selactive medium.

DNA isolation and southern blot analysis

DNA (5μg) was digested overnight with the appropriate restriction enzymes. It was fractionated on a 0.8% agarose gel and alkali blotted onto Amersham N^+ hybond membrane according to the manufracture's instructions.Twenty five nanogram of DNA was radiolabeled with alpha-^{32}P-labeled dCTP (3,000 Ci/mmol) through the ramdom primer method. After wetting in 2XSSC, the membrane was hybridized for 6 h at 68°C in Rapid-hyb buffer (Amersham Phamacia Biotech) containing sheared salmon sperm DNA (10 μg/ml).

PCR analysis of transgenic plants

Fresh young leaves were collected and stored at -80 ^{o}C before DNA extraction using the CTAB (cetylmethylammonium bromide)--NaCl method. Fifty ng of genomic DNA of wild type and transformant plants was used as a template in PCR reactions with the following, 1) *SubEREBP1*-specific primer pairs, 2) *OsRAS*-specific primer pairs. The basic procedure of the PCR reaction was as followed: 5 μl of 10x buffer, 25mM $MgCl_2$, 200 μM each of dNTPs (Promega), 0.2 μM specific primer, 80

ng genomic DNA, and 1 unit *Taq* polymerase (Boehringer). The amplification reactions followed the protocol: 94°C 5 min for 1 cycle, 94°C 30 s for 35 cycle, 60°C 1 min, 72°C 2 min, then 72° for 7 min. The amplification products were analyzed by electrophoresis in 1% agarose gels with 1x TAE, followed by ethidium bromide staining and viewing under UV radiation to verify amplification. The expected size of each product is between 350 and 450 bp. The GFP fragment was also amplified from transformant plants.

Design of probes and primers

According to sequence variation of *OsRAS* gene between a tolerant line and an intolerant line, we designed a set of primers and probe to distinguish a specific allele in transgenic plant. The set of primers and fluorescence probes are showed in table2.

Table2 Oligonucleotide sequence of primers and probes used in the study

Oligonucleotide	Sequence (5'-3')	Position (bp)	Fluorophores	Tm^c (°C)
Forward primer	TGT TGT TGA GGT AGA AGG AGA	-42 to –22 (5'UTR)	-	60
Reverse primer	TTG AAC ACG TAG TCG ATC TTC	39 to 60 (Exon 1)	-	60
Tolerant probe	GCGAGGAGGA**T**GGAGCTACGG	6 to 24 (Exon 1)	FAM	70
Intolerant probe	GCGAGGAGGA**G**GGAGCTACGG	6 to 24 (Exon 1)	TET	71

Taqman assay with allelic discrimination

To determine the specificity of probes, three submergence tolerance cultivars as FR13A, IR49830, DH206 including two submergence intolerant cultivars, KDML105 and JHN, were used to analysis in this experiment. Fresh young leaf tissues were collected and stored at –80 ^{o}C before a DNA extraction. The DNA from individual plant can be extracted using the CTAB-NaCl method (Rogers and Bendich, 1994). Reaction were performed in 50 µl volume containing of 5 µl 10x buffer, 25mM $MgCl_2$, 200µM each of dNTPs (Promega), 1000 ng genomic DNA, 400 nM of each primer, 100 nM concentration of each fluorescence-labeled probe (tolerance and

susceptible) and 1 U Taq polymerase (Boehringer). TaqMan assay was performed using the ABI 7700-sequence detection system (PE-applied Biosystem). The cycling condition used was as follows 95°C for 10 min, followed by 45 cycles at 95°C for 15 s and 60°C for 1 min each. All reactions of PCR were done in duplicate. The correct size of the PCR product o each assay was verified by running an amplified sample of each reaction tube on agarose gels stained with ethidium bromide.

RNA isolation and Taqman RT-PCR with allelic discrimination

For control treatments, the plants were grown under normal condition in the same days. The plants were subjected to the stressed treatments and control condition for various periods and then frozen in liquid nitrogen for further analysis. Total RNA was prepared using High pure Total RNA isolation kit (Roach). Quality of the RNA preparation was determined on agarose gels. Polyadenylated mRNA was prepared from other RNA extraction using oligotex^{TM}-dT resin (Qiagen). To study the expression of transgene and endogenous gene, Taqman RT-PCR which adapted with allelic discrimination analysis was used to analyze the total RNA obtained from five of T_0 regenerated plants, in flash flooding for 2, 4, 6 and 8 days in aquarium. Total RNA isolated from plants under normal growing condition was used as a control. Taqman assay was performed using ABI 7700-sequence detection system (PE-Applied Biosystem). The basic procedure of the RT-PCR reaction was as followed: 5 µl of 10x buffer, 25mM $MgCl_2$, 200 µM each of dNTPs (Promega), 0.2 µM specific primer, 100 ng mRNA, 5 unit AMV reverse transcriptase and 1 unit Taq polymerase (Boehringer). The amplification reactions were carried out by the following profile: 50°C 45 min for 1 cycle, 94°C 10 min for 1 cycle, then 40 cycle at 94°C for 15 sec and 60°C for 1 min.

Data analysis

Data analysis of the comparative Ct method that described by Livak (1997) was applied. The average value of the triplicates (allele of a tolerant line and an intolerant line) was calculated for the control sample and the experimental sample.

The final values were determined according to the formular described by Livak where ΔCt is an average of the triplicates. That formular was detailed below.

$2^{-(\Delta Ct)}$, (ΔCt) = [ΔCt Tol (calibrator sample) - (ΔCt Intol (calibrator sample)] / [ΔCt Tol (patient sample) – (ΔCt Intol (patient sample)].

Part V: Characterization of Isogenic lines for *OsEREBP*1

In this part, we present the associated data between the phenotypic response under flooding and the genotypic alleles of the NILs containing three gene targeted markers (GTMs), which are localized on the 16.7 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.

Plant materials

A set of near isogenic lines, NIL-132, was used for this study. The NILs population had been developed from BC4F7 by backcrossing an introgression specific region of chromosome9. According to BC4F7 generation developed by the Rice Gene Discovery Unit (RGDU), we found one of 231 individuals (912-B-30-107-23-132) from the selected plant family no. 912-B-30 generated from BC4F2 to BC4F7 population carried the heterozygous allele on subQTL9 expanding 16.7 kb among three gene targeted markers (TGMs) as RB0783-Indel, H05-11SSLP, and *OSER*2-Indel. In addition stress treatment measurements were confirmed at BC4F7 generation showed 90% plant survival. The percentage similarity to the intolerance allele KDML105 background showed approximately 94%.

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 (cetylmethylammonium bromide)–NaCl method (Roger and Bendich, 1994). The DNA pellets were re-dissolved (at a concentration of 50 ng/ul) in 50 ul of water for PCR-based polymorphism analysis.

Characterization of NIL132 of BC4F8

Three pair of primers for gene targeted markers (GTMs) were used to cover the 16.7 kb region on the long arm of chromosome9, to which major subQTL had previous been mapped (Toojinda *et al*., 2003). All polymorphic primers were developed based on the genomic sequence from GenBank accession number AC90056. Genotypic screening to maintain a tolerance allele (DH206) 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 NILs-132 of BC4F8

The evaluation for several phenotypic adaptations to survival was analyzed under complete submergence using a dark plastic tank, in August 2004. Individual plants of NILs-132 population were grown completely submerged for 20 days 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 at 0, 2, 4, and 20 days of submergence were 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.

Transgenic approach to submergence stress – tolerant rice

The aim of this section is to obtain more information about the function of the candidate genes from FR13A a tolerant line and, also, to determine whether or not the phenotypic changes observed in transgenic plants might reflect a general effect of transgenes, we attempted to introduce the genes into both rice cultivars, Nipponbare and JHN, an intolerant line.

Transgenic material and gene constuction

Ethylene responsive element binding protein gene (*subEREBP1*) was cloned and prepared for transformation experiment. Embryogenic calli of submergence intolerance rice, Jao Hom Nin (JHN) were used to co-bombard with constructed recombinant vectors pCAMBIA 1302-*SubEREBP1* containing the selectable marker hygromycin phosphotransferase (*hpt*) and *mGFP*-5 (Green fluorescence protein). The transgene is strongly over-expressed by 35s *CaMV* promoter.

Microprojectile bombardment

Plasmid were absorbed to gold particles about diameter 1.5 to 3.0 mm (Aldrich) by $CaCl_2$ and spermidine precipitation as described by Hunold *et al*. (1994) and delivered to the target tissues by using a Finner-type home made Particle Gun Bombardment (Finner *et al*., 1992) Embryogenic callus were pre-incubated on an osmotic inducing medium (a basic CC medium with addition of 0.2 M each mannitol and sorbitol) by picking 2 to 3 cm around the center of the petridish for 4 hour prior to bombardment. Six to eight microlite of plasmid DNA coated particles was distributed onto micro carrier of the gun nozzle. The petridish of target tissues were placed on 11 cm below the gun nozzle and covered with a nylon mesh. The bombardment was done using 700 kPa helium pressures at -25 inch of Hg vacuum.

Growth and selection of bombard cells

Sixteen hours after bombardment, the target tissues were transferred to 75 mg/l hygromycin B selective medium (CC medium sugar free supplemented with 2 mg/l 2,4-D and 1 g/l casein hydrolysate solidified with 0.27% phytagel). and cultured in the dark at 25^0C for 2-3 weeks. Newly formed callus were separated from the bombarded cells and transferred on the same fresh medium. After the resistant callus measuring 2 to 3 mm in diameter were transferred to 75 mg/l hygromycin B pre-regeneration medium (CC medium sugar free supplemented with 1 mg/l IAA and 4 mg/l BA solidified with 0.27% phytagel). The cultures were incubated under a photoperiod of 16 hrs (Toshiba cool white fluorescent tube 38w 3100lm) at 25^0C.

Regeneration of putative transgenic plants

After the calli with differentiated shoots were transferred to shoot induction medium (CC medium sugar free supplemented with 1 mg/l IAA and 0.05 mg/l zeatin solidified with 0.27% phytagel) without hygromycin B and cultured under a photoperiod of 16 hrs at 25^0C. After 3 weeks, the transgenic plants were transferred to root induction medium ; MS (Murashige and Skoog, 1962) medium containing 30 g/l sucrose without plant growth regulator solidified with 0.27% phytagel for 2 weeks. Next, the plantlets were grown in tap water for 7 days for acclimatization and for fast rooting, before transferring them to pots in the greenhouse conditions.

DNA isolation and southern blot analysis

DNA (5μg) was digested overnight with the appropriate restriction enzymes. It was fractionated on a 0.8% agarose gel and alkali blotted onto Amersham N^+ hybond membrane according to the manufracture's instructions.Twenty five nanogram of DNA was radiolabeled with alpha-^{32}P-labeled dCTP (3,000 Ci/mmol) through the ramdom primer method. After wetting in 2XSSC, the membrane was hybridized for 6 h at 68°C in Rapid-hyb buffer (Amersham Phamacia Biotech) containing sheared salmon sperm DNA (10 μg/ml).

PCR analysis of transgenic plants

Fresh young leaves were collected and stored at -80 ^{o}C before DNA extraction using the CTAB (cetylmethylammonium bromide)--NaCl method. Fifty ng of genomic DNA of wild type and transformant plants was used as a template in PCR reactions with a set of *SubEREBP1*-specific primer pairs and *GFP* primers. The basic procedure of the PCR reaction was as followed: 5 μl of 10x buffer, 25mM $MgCl_2$, 200 μM each of dNTPs (Promega), 0.2 μM specific primer, 80 ng genomic DNA, and 1 unit *Taq* polymerase (Boehringer). The amplification reactions followed the protocol: 94°C 5 min for 1 cycle, 94°C 30 s for 35 cycle, 60°C 1 min, 72°C 2 min, then 72° for 7 min. The amplification products were analyzed by electrophoresis in 1% agarose gels with 1x TAE, followed by ethidium bromide staining and viewing under UV radiation to verify amplification. The expected size of each product is between 350 and 450 bp. The GFP fragment was also amplified from transformant plants.

RNA isolation and RT-PCR analysis

For control treatments, the plants were grown under normal condition in the same days. The plants were subjected to the stressed treatments and control condition for various periods and then frozen in liquid nitrogen for further analysis. Total RNA was prepared using High pure Total RNA isolation kit (Roach). Quality of the RNA preparation was determined on agarose gels. Polyadenylated mRNA was prepared from other RNA extraction using oligotex^{TM}-dT resin (Qiagen). To study the expression of transgene and endogenous gene, Taqman RT-PCR which adapted with allelic discrimination analysis was used to analyze the total RNA obtained from five of T_0 regenerated plants, in flash flooding for 2, 4, 6 and 8 days in aquarium. Total RNA isolated from plants under normal growing condition was used as a control. Taqman assay was performed using ABI 7700-sequence detection system (PE-Applied Biosystem). The basic procedure of the RT-PCR reaction was as followed: 5 μl of 10x buffer, 25mM $MgCl_2$, 200 μM each of dNTPs (Promega), 0.2 μM specific primer, 100 ng mRNA, 5 unit AMV reverse transcriptase and 1 unit Taq polymerase

(Boehringer). The amplification reactions were carried out by the following profile: 50°C 45 min for 1 cycle, 94°C 10 min for 1 cycle, then 40 cycle at 94°C for 15 sec and 60°C for 1 min.

RESULTS

Part I: Effect of submergence stress on physiology in rice

During early developmental growth of seedling rice, flooding causes an induction in shoot elongation, accompanying plant growth as well as emergence of chlorosis tissue. Twenty days-old rice seedlings from FR13A (a tolerant line) and KDML105 (an intolerant line) were submerged for 2, 4, 6, and 8 days under low light condition compared to the controls which has grown under normal condition (air grown). As the results, flash flooding intolerant cultivar (KDML105) showed the typical symptom: rapid shoot elongation during submergence and low survival rate after de-submergence. The tolerant cultivar FR13A was not effected by these symptoms.

A mechanism of shoot elongation under water in rice is one such key to examine submergence tolerance trait. The intolerant plants increased rapidly their height underwater at 3.84 cm per day compared to 1.89 cm. per day for the tolerant plants. However, both FR13A and KDML105 showed no significant differences in plant elongation (0.58 and 0.62 cm per day) under normal growth condition. Plant growth was observed during normal condition and against submergence stress. Under submergence after 10 days, the FR13A and KDML105 had an elongation three times and six times faster compared to the air grown condition.

Complete submergence decreased seedling survival in both cultivars; the decrease is significantly less in the submergence tolerance cultivar than in KDML105. The survival rate are different between FR13A (87%) and KDML105 (12%) after de-submergence for 14 days. The survival rate of FR13A was not affected by submergence treatment during 10 days. However, the expansion of submergence time reduced the survival rate to 30% when plants were submerged for 20 days (Figure 1, Table 3).

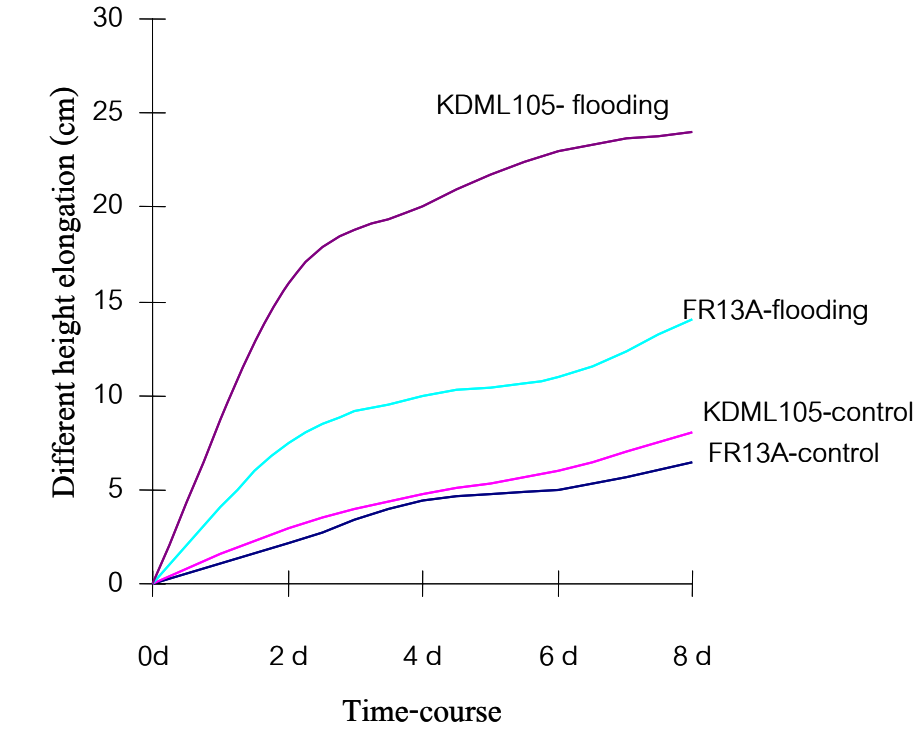


Figure 1 Effect of duration (in days) of submergence relative to none-submerged on shoot elongation of both cultivars, FR13A and KDML105. Each data represented the average of three replications. This picture showed the increment height of shoot elongation under submergence after 10 days relative to before submerged in various dwarf rice mutants including both cultivars, FR13A and KDML105.

In addition, submerged FR13A showed little changes in the chlorophyll concentration during submergence and after de-submergence, while KDML105 showed more than 75% reduction of chlorophyll concentration after 6 days submergence.

Table 3 Plant height, TSE, RSE, chlorophyll content, and survival rate. Three weeks old seedling plants of both cultivars were submerged during 10 days with the same conditions as described in Material and Methods. Control plants were grew in the normal condition.

Traits	**FR13A**		**KDML105**	
	Control	*Submergence*	*Control*	*Submergence*
Plant height (cm)	28-32.43	28-42.9	29-34.26	29-67
TSE (cm)	4.43	14.9*	5.26	38**
RSE (fold)	-	3	-	7
Chlorophyll content	32.4	15.6*	34	8**
Survival rate (%)	100	87	100	12

* *All values are statistically different from control at the 5% level (n=35)*

TSE = Total shoot elongation

RSE = Relative shoot elongation

Shoot elongation in response to submergence using dwarf rice as a model

Rice varieties with many GA-related dwarf mutants are a favorable plant to monitor the phenotypic changes in plant height. Dwarfism has clearly distinct phenotypes. Two mutation types of dwarfism were identified in rice. First, dwarfism is caused by lack of a gene function of GA biosynthesis pathway, the dwarf mutants can be overcome by exogenous GA treatment. They are also called GA biosynthesis mutants. Second dwarfism is seen in GA-response mutants that defect specifically in GA signaling. They are unresponsive to additional GA whose treatment. The ability to survive and recover from submergence is strongly related to the ability to suppress leaf elongation under water. Under submergence conditions, GA and ethylene have played important roles in leaf elongation and senescence. We have set several hypotheses that dwarf mutants and submergence tolerant lines may share some mechanisms that lead to regulating leaf elongation. To study the ability of plant elongation under submergence stress, we used two kinds of a dwarf rice such as Waito (*d18*), T65 (*d1*), Tanginbonzu (*d35*) and Remei (*Sd1*) to investigate the relationship between suppressed plant elongation and survival ability under submergence stress. Several line evidences suggested that reduced plant elongation in response to complete submergence was critical for rice plant to survive during submergence. In flash flooding, elongation in FR13A (submergence tolerant) was suppressed, in contrast to KDML105 (intolerant ones). The relative plant height determination of KDML105 was 3.5 time superior to FR13A after 10 days of submergence. For dwarf rice elongation determination, T65 (*d1*), Tanginbozu (*d35*), Nihorbate (*d1*), and Nishiki (*d18*) were classified into the same group that showed low level of plant elongation in contrast to KDML105 and Remei (*Sd1*), which showed high incremental height of elongation. Although watio (*d18*) showed the lowest elongation growth (even lower than FR13A), it was not able to survive after a 20 days submergence stress (unlike FR13A). The results suggest that lowest elongation growth and the beginning plant height affected to plant survival under submergence stress (Table 4 and Figure 2, 3).

Table 4 Plant elongation in response to submergence stress using various dwarf mutants. Twenty-one days old seedling plants were submerged for 10 days and then the total shoot elongation was measured comparatively after and before submergence. Then, plant survival rate were obtained after 14 days of submergence followed by 14 days of de-submergence

Rice Cultivars	Air grown during 10 d		Submergence during 10 d		TSE relative to air grown control (ratio)	Plant survival after 14 days de-submerged (%)
	TSE	*CC*	*TSE*	*CC*		
Tangin *d35*	3	38	15.7	0	5.2 time	0
WT *D35*	5.4	38	36	0	6.6 time	0
Waito *d18*	4.41	39	8.88	4.7	2 time	3
Remei *sd1*	3.2	38	22.8	0	7.1 time	0
T65 *d1*	5.9	37	15.9	0	2.6 time	0
SLR hetero	9.7	32	43	0	4.4 time	0
KDML105	5	34	30.75	7.8	6.1 time	12
FR13A	4.67	31	15.13	24.5	3.2 time	79

TSE = Total shoot elongation

RSE = Relative shoot elongation

CC = Chlorophyll content

n = 10

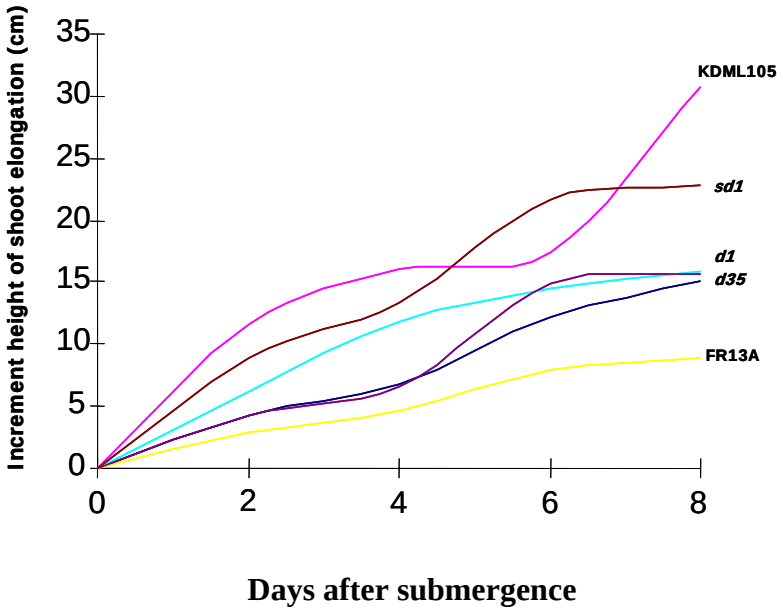


Figure 2 The picture showed that increment height of shoot elongation after 8 days of submergence in various dwarf mutants as well as FR13A and KDML105.

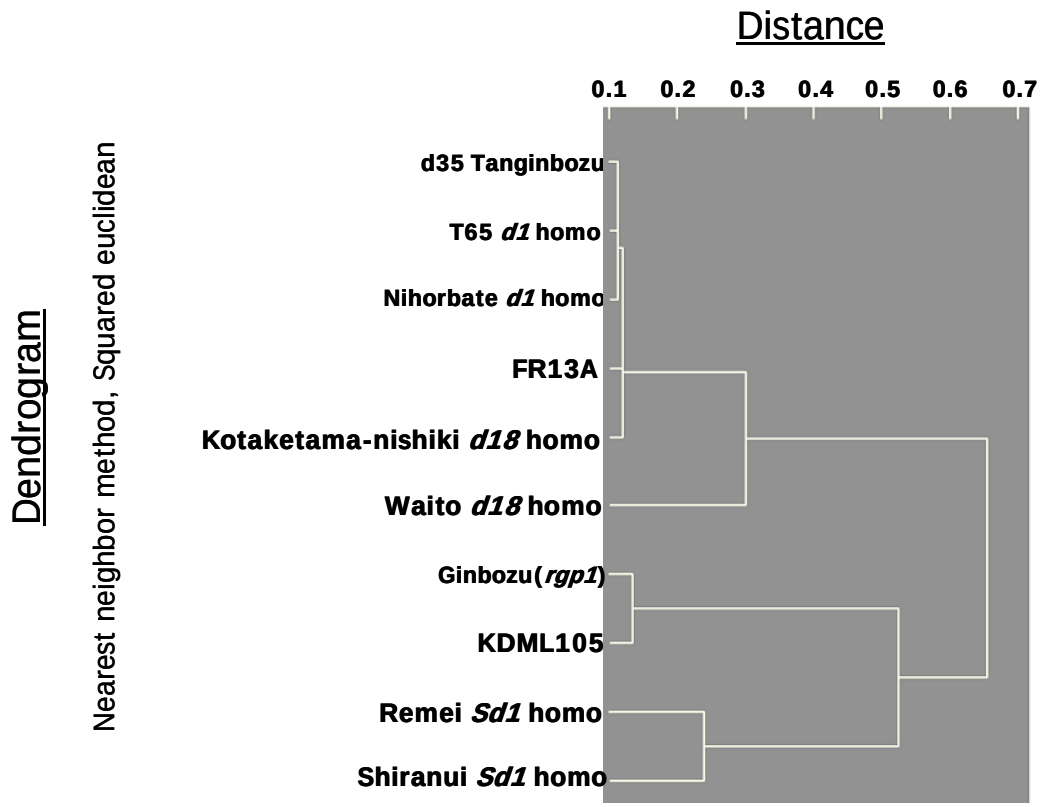


Figure 3 Cluster analysis of incremental shoot elongation in response to submergence stress for 10 days in various dwarf rice mutants.

Part II: Identification of Genes Underlying QTL

Determining Flooding Tolerance

In this part, sequencing data interval *Sub*QTL9 region was collected by anchoring sequence-based genetic markers to the sequence map. We then identified genes as candidates with respect to position, which located on the major QTL of submergence tolerance. We also presented the characterization of all the putative open reading frames and its representative sequences on the critical region of the major QTL for submergence tolerance, a contig with a sequence localized around 1.35 Mbp of the long arm of rice chromosome 9.

Structural features of *SubQTL9*

Chromosome 9, the second smallest segment in rice, has a total map distance of approximately 22 cM. The sub-centromeric region was chosen as the entry point for genome sequencing based on several interesting features for both structural and functional genomics. Nearby its centromere, the major QTL for submergence tolerance was mapped into a 15.5 cM region where two molecular markers S10709 and RZ698, were located (Figure 4). Several traits at this major QTL were characterized with plant survival, plant elongation, induced shoot elongation, visual tolerance score, and leaf senescence. The *Sub*QTL9 was detected consistently in experiments within the years and in the genetic backgrounds of all three mapping populations. The candidate sequence interval spanning the candidate QTL flanked by S10709 to RZ698 was then identified. The assembly of fourteen BAC/PAC clones in this interval yielded a contiguous sequence region of 13500 base pairs within which we identified 228 predicted gene structures. The complete sequence of the critical region of submergence tolerance bears the accession number shown in table 5 (http://www.ncbi.nlm.nih.gov/GenBank/index.html).

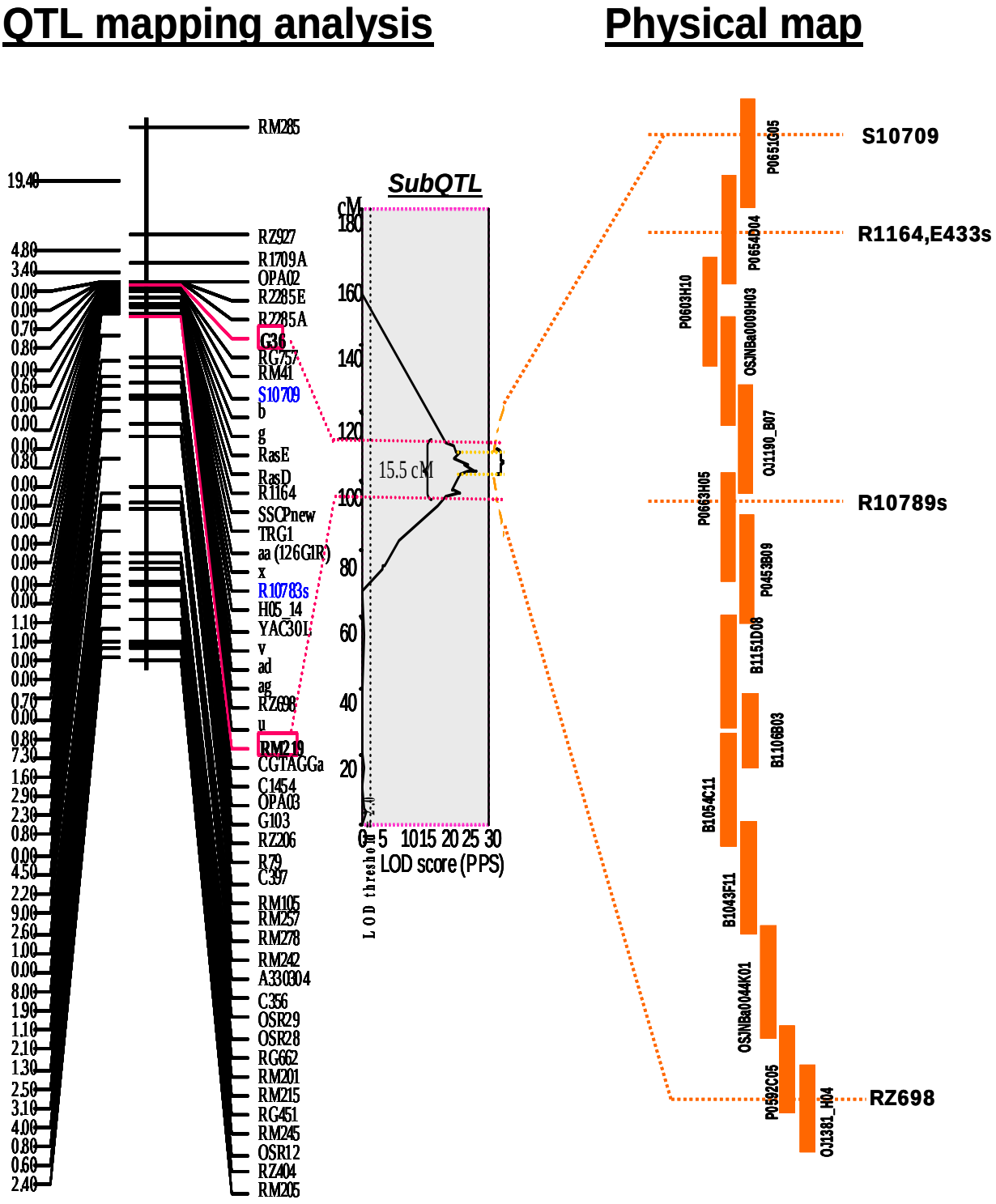


Figure 4 Relationship between a critical region 15.5 cM of QTL mapping analysis controlling submergence tolerance and physical map spaning 1.35 Mb in rice chromosome 9.

Table 5 Features of 14 BAC/PAC contigs along the major QTL of submergence tolerance region on chromosome 9.

Clone name	Accession No.	Size (kp)	CDS feature (None-TEs related genes)	Genetic markers
P0651G05	AC090055	143.1	24-**(7)**	S10709
P0645D04	AC090054	139.0	21-**(5)**	R1164
P0603H10	AC090057	127.3	19-**(1)**	-
OSJNBa0009H03	AP005818	151.7	24**(1)**	-
OJ1190B07	AP005562	140.8	21-**(5)**	-
P0663H05	AC090056	161.9	25-**(5)**	R10783s
P0453B09	AP005705	173.5	23	-
B1151D08	AP005907	152.8	17**(3)**	-
B1106B03	AP006449	155.5	16	-
B1054C11	AP006464	141.5	13**(1)**	-
B1043F11	AP006156	167.3	22**(2)**	-
OSJNBa0044K01	AP005839	160.6	18(**6**)	-
P0592C05	AP004756	150.4	26**(12)**	-
OJ1381_H04	AP004011	130.9	20**(5)**	RZ698
Total		**1.35 Mbp***	**228(53)**	**15.5cM**

**The 1.35 Mbp region was represented a total of non-overlapping contig from 14 BAC/PAC clones between the molecular marker of S10709 and RZ698.*

Novel genes prediction and classification

In general, the functional genome annotation is based on the idea that some sequence similarities detected between two proteins mean that they are homologous. They may come from the same ancestor and share the same biochemical function. In this section, we present the characterization of all the putative open reading frames and its repetitive sequences in the critical region, a contig of which sequences localized around 15.5 cM of submergence tolerant genes of rice chromosome 9 (Figure 4).

A total of 228 CDS features were found on the submergence tolerance region (Table 7). Of these, a function could be assigned to 28 (12.2%), while 25 (10.9%) were annotated as encoding an expressed protein including a full-length cDNA(s), and 175 (76.7%) were predicted as encoding a hypothetical protein without similarity to an entry in the public databases. In total, 53 potential protein-coding genes were identified, along with a 1.35 Mbp region as predicted through computational search by the packages indicated in Materials and Methods. These genes were named SUB 1-53 as a working nomenclature (Table 6). A summary of the identified genes and other features is presented in table 7. The average length of the gene model was 2,789 bp (from start to stop codon) and contains 6 exons with an average size of 487 base pair. The average predicted coding region was 1,171 bp (range 399-5127). Almost all genes contained intron (s), with an average intron (s) size of 569 nucleotides. Of the gene with introns, the average number of introns in a gene was 4 (range 1-24). The main features, localization, and similarity search results of each gene are described in table 5. Within the 1.35 megabase-region, all of genes were predicted and confirmed by homology search. The putative function was analyzed to find the presence of functional domains. The results showed that the biological function of the ORFs was related to essential processes of gene regulation and gene signaling that might be involved in transcription regulation, signal transduction pathway, ubiquitilation, and proteolysis based on their amino acid motif. According to promoter analysis, we found that many types of the DNA sequence elements were classified as a hormonal signaling in various plants. At least in the case of submergence tolerance, a gene

underlining the QTL might be driven by a mediator molecule of signal transduction pathways involved in plant hormones such as gibberellins, ethylene, ABA, and auxin (Table 8). The roles of these regulatory proteins in submergence tolerance are elucidated in the next experiments.

Analysis of intergenic region

Altogether, the 228 predicated genes, including exons and introns, account for around 48.93% of the 1.35 Mbb contig. In other words, about half of the 14 BAC/PAC of *SubQTL9* sequence are intergenic regions. The overall GC content of the contig is 43.50%, with an average content of 61.89% in exons and an average content of 39.94% in other region (introns plus intergenic region).

Benefits of molecular marker as simple sequence repeats (SSRs) have been well known not only to detect genetic variations within or between species but also to develop molecular markers tightly linked to agronomically important traits in breeding programs. Our analysis of 1.35 Mbp *SubQTL9* region revealed a total of 65 SSRs using RepeatMasker and Censor with default parameters. Most of them were classified into 64% of a di-nucleotide with lengths ranging from 6 bp to 46 bp. While other classes of simple repeats were found as tri-nucleotide (25%), tetra-nucleotide (8%) and mononucleotide (4%). The number of n ranged was showed at 5 bp to 29 bp. These are typical microsatellite motifs. They were found upstream of the 5'-UTR of several genes and sometimes in the ORF of specific genes.

Transposable elements (TEs)

One feature that might contribute to high recombination hotspot is the high number of transposon and retrotransposon flanking genes. The transposable elements (TEs) were predominated with unclassified (38%), CACTA, En/Spm sub-class (30%), Ty1-gypsy sub-class (25%), Ty1-copia sub-class (7%), and mutator sub-class (1%). Most of them were long terminal repeats (LTRs) of retrotransposon with two kinds of direct repeats and inverted repeats as Ty3-gypsy sub-class. Surprisingly, according to

the analysis by tRNAscan-SE, *SubQTL*9 region has no tRNA gene in the 1.35Mbp fragment. However, sequences with significant similarities to rice mitochondrial protein (HGWP repeat containing protein, rhodanese-like family protein, and chaperone heat shock protein) were found. The average physical genetic distance of 87 kb per cM was surprisingly high considering the sequencing area is proximal to the centromere. On the basis of these findings, we found that the gene density in the gene-rich region with none-TEs-related gene is approximately 5.92 kb/gene. High gene density with small physical to genetic distance makes this subcentromeric region particularly gene-rich and hotspot for recombination.

Table 6 The characteristics of the 53 open reading frames (ORFs) found in submergence tolerance region.

SUB	CDS (bp)	Exon	a.a	Putative characteristics	Predicted localization	remarks
1	720	6	239	rhodanese-like family protein	mitochondrial	-
2	600	6	245	Nucleic acid binding	chloroplast	RNA recognition motif (RRM)
3	687	7	696	isomerase activity	-	ATP binding protein
4	1080	12	359	oxidoreductase activity	membrane	Electron transport, metabolism
5	1404	11	467	transcription factor	Nuclei	HBP-1b(c1)
6	624	10	207	Mnd1	Nuclei	
7	573	3	190	unknown	Plasma membrane	4TM
8	870	7	289	Splicing factor4	Nuclei	Spliciosome
9	681	2	226	GTP-binding protein Rab11d	cytoplasm	
10	1323	2	440	unknown	membrane	-
11	5223	8	1740	Receptor like-kinase	membrane	TRAP170
12	921	2	306	Transparent1	chloroplast	WIP5 protein
13	747	2	248	ankyrin-like protein	chloroplast	ANK4
14	1218	5	405	ubiquitin conjugating enzyme7	Nuclei	UbE3
15	924	8	307	Serine/threonine phosphatase	Cytoplasm	Metallophos
16	468	2	155	unknown	Cytoplasm	-
17	909	6	302	chaperone GrpE type 2	mitochondrial	GrpE2
18	1401	12	466	enhancer of polycomb-like protein	Nuclei	CKS
19	2559	2	852	unknown	Nuclei	-
20	270	0	89	unknown	-	-
21	1608	23	535	NHE-8	Plasma membrane	7TM
22	699	0	232	Transcription factor	Nuclei	C-repeat/DRE-binding factor
23	751	1	250	Transcription factor	Nuclei	EREBP
24	591	2	196	antigen receptor-like protein	Membrane	3TM

Table 6 The characteristics of the 53 open reading frames (ORFs) found in submergence tolerance region. **(Continuoused)**

SUB	CDS (bp)	Exon	a.a	Putative characteristics	Predict localization	remarks
25	2022	12	673	Epstein-Barr virus EBNA-1-like protein	nuclei	ULP_PROTEASE
26	2067	1	688	far-red impaired response protein	Nuclei	FAR1 family, SWIM, WRKY
27	819	5	272	unknown	Plasma membrane	F-box protein
28	372	2	123	unknown	nuclei	unknown
29	270	3	89	unknown	Cytoplasm	unknown
30	1185	1	394	unknown	cytoplasm	kelch repeat, F-box
31	243	2	80	unknown	microbody	2TM
32	447	2	148	Ubc2 enzyme	cytoplasm	ubiquitilation
33	3645	1	1214	LRR receptor kinase	Plasma memebrane	Phytosulfokine
34	1278	10	425	unknown	nuclei	cis-trans isomerase
35	2748	19	915	aspartate kinase-homoserine dehydrogenase	mitochondrial	Bifunctional enzyme
36	525	2	174	serine/threonine protein kinase	chloroplast	-
37	1302	8	433	UQ_con, Ubc2	nuclei	Ubc2
38	1488	1	495	Unknown	Nuclei	OsNAC protein
39	327	1	108	Unknowm (Xs domain)	Cytoplasm	Xs protein
40	1698	10	565	unknown	mitochondrial	NTPase domain
41	588	1	195	unknown	1TM-outside	-
42	1401	5	466	unknown	cytoplasm	protease
43	1464	1	487	unknown	Plasma membrane	Enzyme activity
44	966	5	321	chlorophyll a/b-binding	Plasma membrane	2TM
45	2391	13	796	WD40, G beta repeat	chloroplast stroma	-
46	1449	9	482	Ferrochelatase	Plasma membrane	1TM
47	447	4	148	ubiquitin-conjugating enzyme	mitochondrail	Ubi-con
48	1554	1	517	monosaccharide transporter 6	Plasma Membrane	10TM
49	1227	9	408	phosphoenolpyruvate/phosphate translocator	Plasma Membrane	7TM
50	525	2	174	GPI-anchored protein	Plasma Membrane	2TM
51	618	1	205	Unknown	Plasma Membrane	1TM
52	1857	19	618	Phosphofructokinase	Cytoplasm	PFK
53	1503	9	300	ADP-glucose pyrophosphorylase small subunit	mitochondrial	-

Table 7 Statistics of rice chromosome 9 at a critical region of mapping QTL controlling submergence tolerance

Feature	Statistic
Total number of BACs/PACs	14
Total BAC length	1.35 (Mbp)
GC content	43.50%
Average length of gene model	2,789 (bp)
Total number of SSRs	85
Total number of genes	228
Known/putative genes	28 (12.2%)
Expressed genes	25 (10.9%)
Hypothetical genes	175 (76.7%)
Transposable elements	61
Gene density	5.92 (kb)
Average CDS length	1,171 (bp)
Average exon size	487 (bp)
Average number of exon/gene	6
Average intron size	1,981 (bp)
Average number of intron/gene	5
Integrated genetic markers	24

Table 8 Analysis of promoter region of candidate genes associated with the major QTL was identified on rice chromosome 9.

Domain type	Gene family function	Cis-acting elements	Genetically characterized factor
MYB	Secondary metabolism, Cellular morphogenesis, signal transduction in plant growth, abiotic and biotic stress responses, circadian rhythm and dorsoventrality	MRE-like sequences	*AtMYB2,ATR1, CCA1, CPC, GL1, LHY,WER*
AP2/EREBP	Flower development, cell proliferation, secondary metabolism, abiotic and biotic stress responses, ABA response, and ethylene response	GCC box	*ABI4, ANT, AP2, CBF1-3/DREB1A-C, DREB2A, ERF1*
bZIP	Seed-storage gene expression, photomorphogenesis, leaf development, flower development, defense response, ABA response and GA biosynthesis	G-box, Dof, OCS	*ABI5, HY5, PAN*
Z-C_2H_2	Flower development, flowering time, seed development, and root nodule development	GARE	*FIS2, SUP,HRT*
WRKY	Defense response	W Boxes	*TDBA12*

Part III: Transcriptional Profiling of Genes Expression in Response to Submergence Stress

The functional analysis approached on gene expression profiling is one such tool that may lead for the understanding of genetic mechanisms controlling submergence tolerance in rice. In this study, we performed mini-scale DNA macroarray analysis to investigate gene expression in response to submergence stress using FR13A, a tolerant line, and KDML105, an intolerant line which associated with plant shoot elongation under submergence. Mini-scale arrays prepared from PCR-amplified DNA fragments of candidate genes located on a major QTL for submergence tolerance at chromosome 9. The arrays, however, are contained of some known genes that known function in response to submergence.

Reproducibility of the array data

At first step in the analysis, spot-to-spot variability is crucial to the accuracy of the results. Each DNA samples was arrayed for duplicated spot on membrane. We have performed the experiments to check the reproducibility by check the signal reproducibility of the duplicated spot data and also used the same target RNA labeled at the same time for hybridization with different membranes. The PCR-amplified DNA macroarrays only 0.9% of the spots showed over two-fold variation between duplicate macroarrays. PCR-amplified DNA macroarrays showed less than 1.2-fold variation for 96% of the total spots, showing relatively high quality reproducibility (Figure 5). In addition, Light Cycle-based real-time RT-PCR using SYBR Green dye shows detection of the beta actin transcript at the same level in both FR13A and KDML105 under submergence for 2, 4 and 6 days. This type of analysis with a housekeeping gene can be used to control for the amount of cDNA in the probe labeling reaction. The no-RT control is negative, indicating the absence of genomic DNA in the probe labeling reaction of both FR13A and KDML105 (Figure 6).

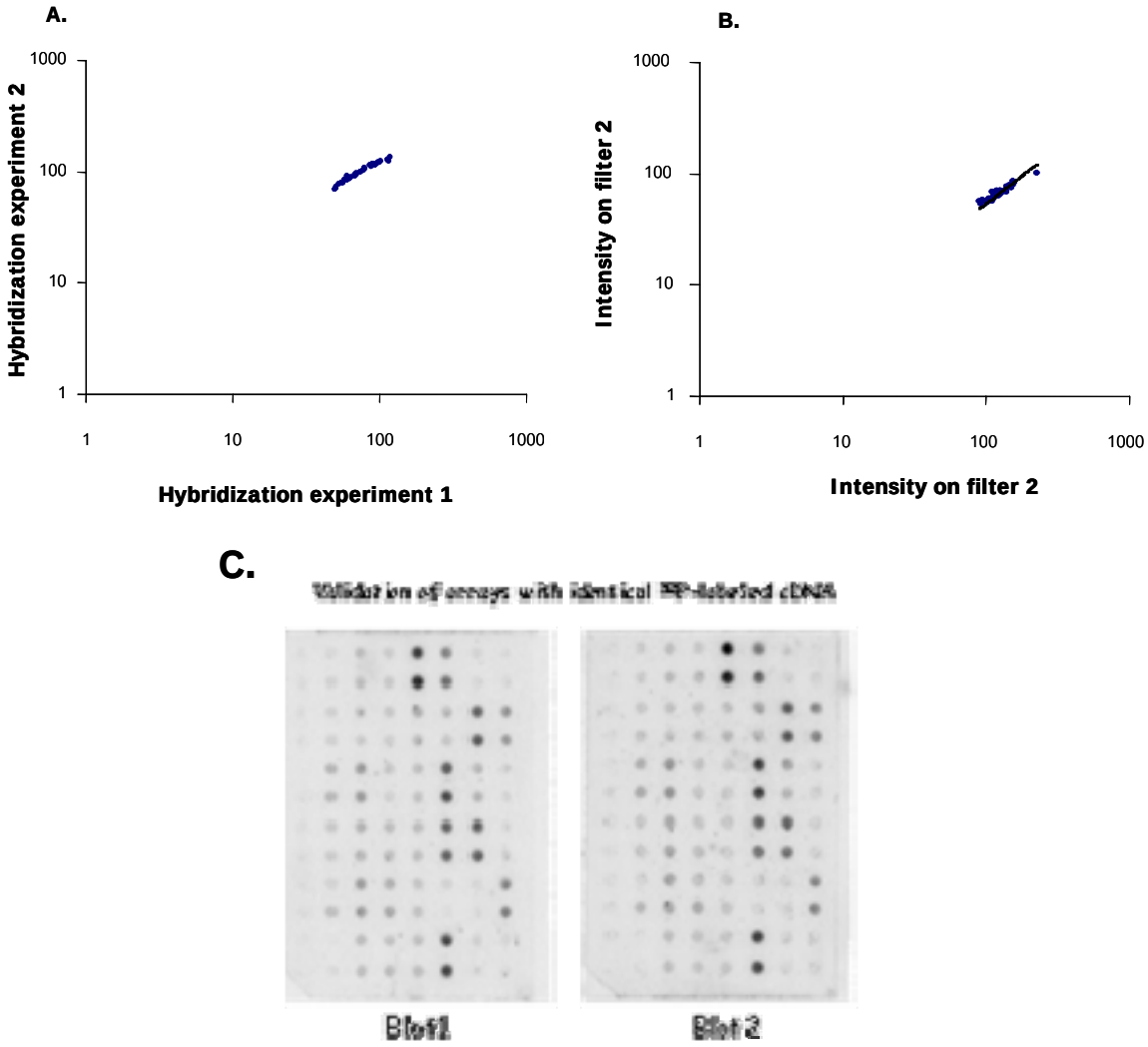


Figure 5 Macroarrays for the analysis of gene expression

A. Comparison of the normalized signal intensities obtained from two independently spotted arrays hybridized with the same labelled cDNA.

B. The independently RNA labeled at the same stress was hybridized with the same spotted array. The signal intensity of each spot on the filter was plotted.

C. Sample digital phosphor images of the results of hybridization signals on the independently spotted arrays with the same labelled cDNA.

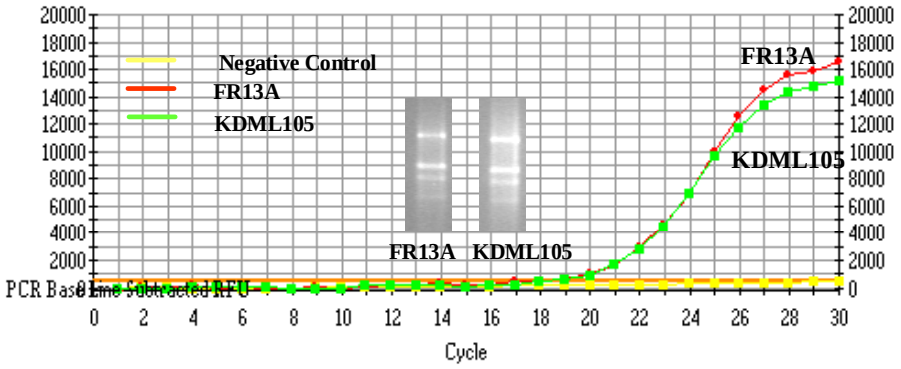


Figure 6 Based on real-time RT-PCR, SYBR Green dye was used to quantify the initial mRNA template for the amount of cDNA in the labeling reaction. The no-RT control is negative, indicating the absence of genomic DNA in the probe labeling reaction of both FR13A and KDML105.

Identification of differential candidate genes expression

To examine the effect of submergence stress on the transcription of 29 genes, a time-course experiment was performed for the plants treated with flooding stress for 0,2,4,6, and 8 days. The data from expressed intensity from FR13A and KDML105 were observed. The analysis of variance (ANOVA) showed that the population of expressed genes during two days and four days after treated with submergence was the significant source of variation ($P<0.05$). Scattered plots of spotted genes, shown in Figure6, indicate that plant resposnse to submergence stress affected the mRNA levels mainly occur after 2 and 4 days of treatment.

The estimated signal intensity relative to control within time-course experiments from FR13A and KDML105 are listed in Table8. Only three candidate genes (10%) were found to have an estimated fold changes in excess of two between 2d submerged and control conditions in FR13A samples wherase only eight (26.4%) had a twofold change at the same conditions from KDML105 samples (Figure 7, Table 9). In addition, we found that only one gene was up-regulated ratio at twofold change for 4d submerged against control condition from KDML105. A twofold

expression change was set to indicate that a gene was likely to be differentially expressed between these times of submergence. None of these genes had greater than twofold differences between conditions analysis.

In total of 24 ORFs, array analyses revealed that ras-related GTP-binding protein gene (SUB09; *OsRAS*) ethylene responsive element binding protein gene (SUB22; *SubEREBP1*) exhibited twofold for 2 days in FR13A. Due to four days under water, transcription level of SUB22 in FR13A was repressed while it was still constanted in expression from KDML105. However, differential expression was not found after 4 days tremented with them.

Consequenctly, the expressed ratio of four known-function genes, 1-aminocyclopropane-1-carboxilic acid (*ACC*), amylase (*Amy3*), G-alpha subunit (*D1*), and Enolase was induced significant in KDML105 for 2 days (Figure 8). These genes are representd for hormonal signaling pathways (Ethylene and GA) and including anaerobic pathway.

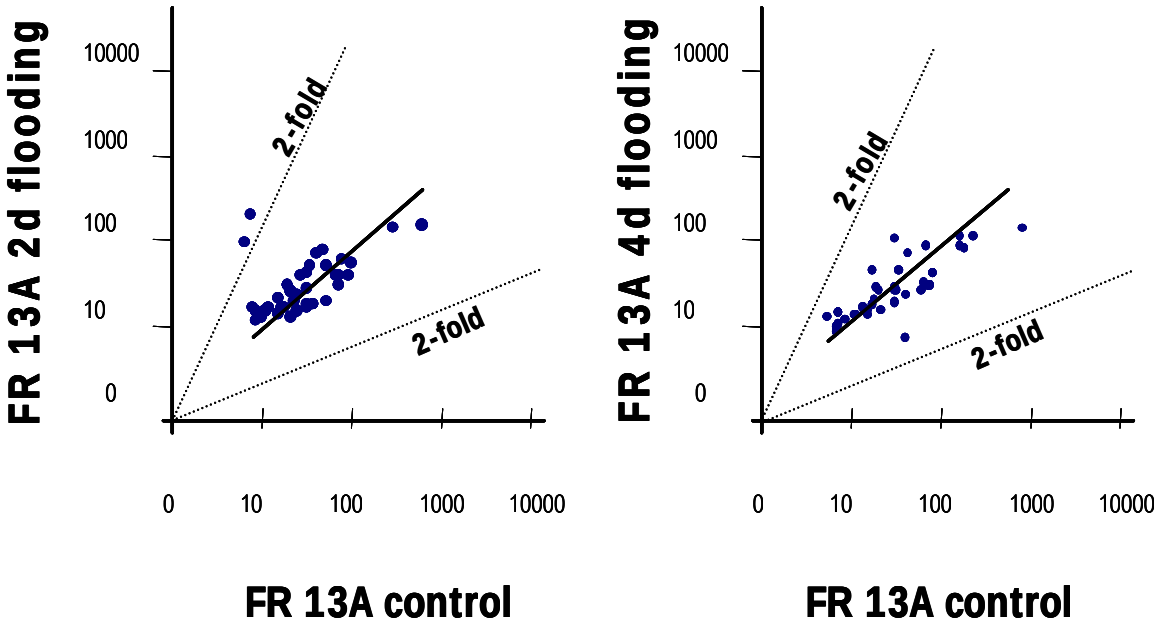


Figure 7 Standard scatter plots in gene expression analysis. The mean of the normorlized raw intensities of each gene was plotted between stress and control.

Table 9 Log_2 ratio of genes with differential expression between submergence and control for 2 and 4 days obtained from FR13A and KDML105.

ORF	Posible function	Normalized log_2 ratio (2dRelative to control)		Normalized log_2 ratio (4dRelative to control)	
		FR13A	KDML105	FR13A	KDML105
SUB05	Transcription factor	0.85	**1.02**	0.41	0.47
SUB04	Isomerase activity	0.72	**1.16**	0.33	0.56
H1	TIM17	**1.10**	**1.10**	0.49	0.61
SUB01	Rhodonase	0.67	0.84	0.63	0.32
SUB02	RNA recognition motif	0.39	0.31	0.23	-0.05
SUB08	Splicing factor	0.38	0.20	0.20	0.21
SUB10	Unknown protein	0.91	0.89	0.47	0.60
SUB11	Receptor like kinase 4TM	0.51	0.65	0.11	0.28
SUB12	Transparent tasta1	0.78	0.93	0.25	0.58
H2	unknown	0.49	0.76	-0.16	0.38
H3	unknown	0.60	0.35	0.13	-0.04
H4	unknown	0.50	0.40	0.19	0.21
SUB20	unknown	0.48	0.43	0.23	0.11
SUB21	7TM	0.64	0.56	0.11	0.32
SUB22	Transcription factor	**1.18**	**1.03**	0.20	**1.11**
PDC	Anaerobic pathway	0.80	0.88	0.21	0.55
ACO	Ethylene biosynthesis	0.87	**1.22**	0.44	0.70
Enolase	Anaerobic catabolism	0.96	**1.29**	0.43	0.39
D1	GA signaling	0.93	**1.07**	0.17	0.46
Sd1	GA biosynthesis	0.52	0.38	0.14	0.12
D18	GA biosynthesis	0.66	0.28	0.16	0.01
GAMYB	Transcription factor	0.58	0.61	0.13	0.29
Amy3	Starch degradation	0.71	**1.02**	0.30	0.61
EXP1	Cell wall modification	0.59	0.72	0.09	0.40
EXP2	Cell wall modification	0.63	0.86	0.09	0.32
EXP3	Cell wall modification	0.14	0.68	-0.51	0.37
EXP4	Cell wall modification	0.91	0.78	0.10	0.28
Unknown	unknown	0.90	0.43	-0.16	0.18
Actin	Housekeeping gene	0.69	0.59	0.24	0.36
SUB09	Signal transduction	**1.25**	0.54	0.20	0.16

If the log_2 ratio > 1 that is meant more than 2 fold change in up regulation of gene expression.

If the log_2 ratio > -1 that is meant more than 2 fold change in down regulation of gene expression.

If the log_2 ratio = 1 that is meant constancy in gene expression.

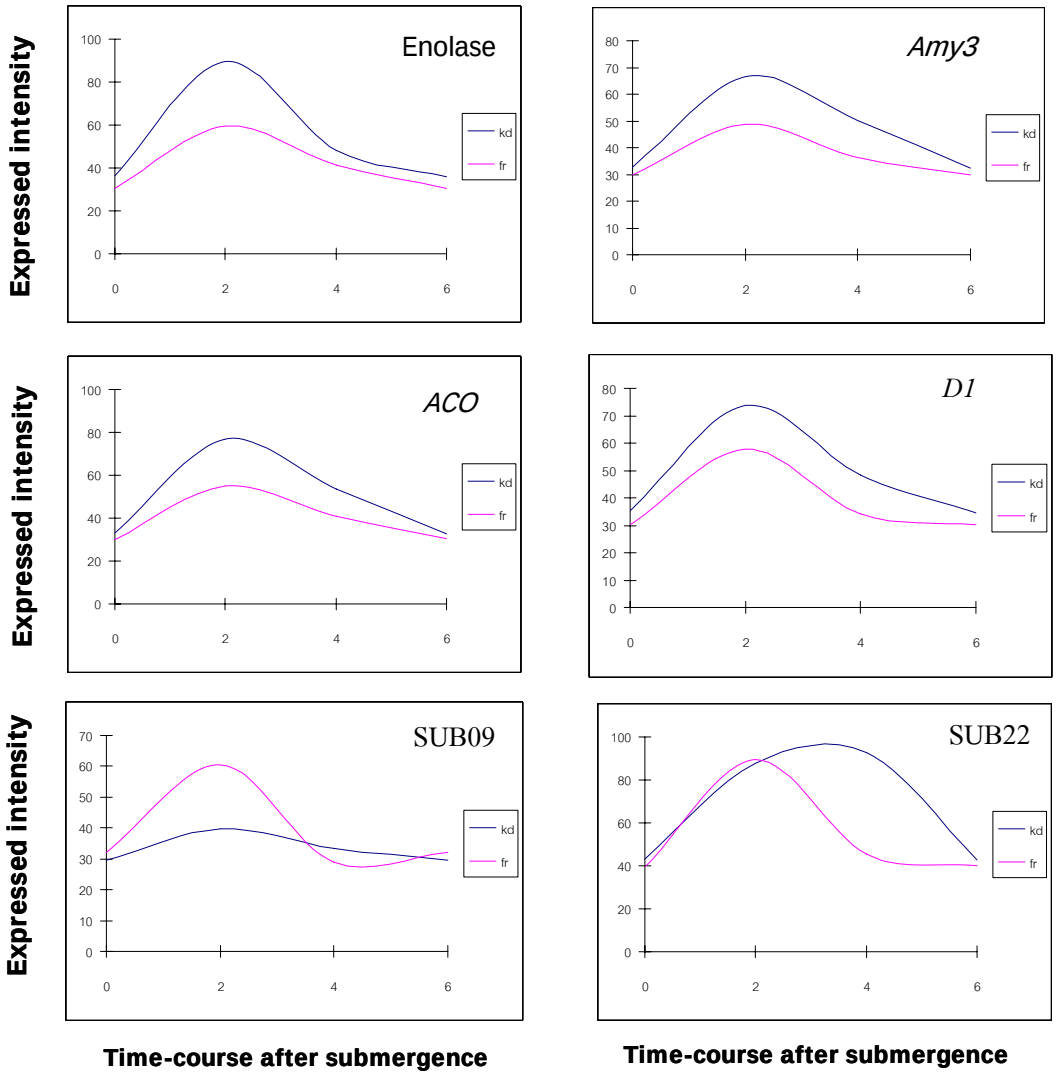


Figure 8 Comparison of mRNA accumulation after various times of submergence stress between FR13A and KDML105. The amounts of mRNA are represented in normorlized intensity value on the ordinate.

To confirm the sensitivity of macroarray analysis, the expression profiling of ras gene under submergence stress for 2 days was studied by Real-time RT-PCR using SYBR Green dye. The relative change in expression for *ras* gene was up regulated in FR13A for 2 days under submergence (Figure 9). We used the actin gene as housekeeping gene to control the initials mRNA. The real-time RT-PCR with the gene-specific primers showed a similar result to those obtained by macroarray analysis.

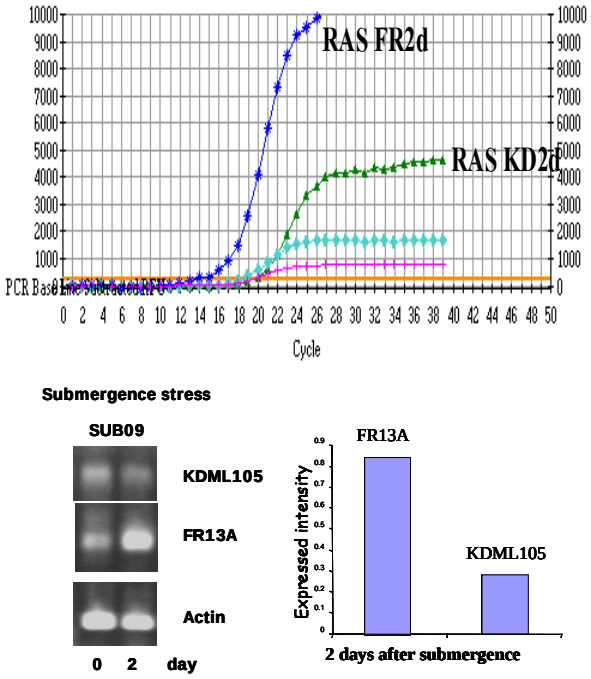


Figure 9 Confirmatory Real-time RT-PCR assay based on SYBR Green I dye detection of the effects of submergence on the expression of Ras-related GTP-binding protein gene (SUB09) in rice cutivars between FR13A and KDML105. Total RNA isolated from whole plant tissues that had been submerged for 2 days was subjected to Real-time RT-PCR with primer-specific for *OsRAS* genes. Both rice cultivars were evaluated for relative levels of *OsRAS* gene transcript in the amplification plot**.**

Functional annotation revealed by macroarray analysis

By applying various computatinal analysis programs, the genomic sequence of *OSRAS* gene encoding ras-related GTP-binding protein and ethylene responsive binding protein (*SubEREBP*) were analyzed and classified. In constantly, the *OSRAS* gene is a 5.2-kb in length and locates on *Nipponbare* PAC0663D04 (accession number AC090054). An open reading frame is consisted of two exons including a promoter. The isoelectic point (p*I*) and molecular weight (MW) were predicted using the comput pI/MW program (Bjellqvist *et al*., 1993). The predicted molecular weight for OSRAS protein is a 24.8 kiloDatons of 226 amino acids. The BLAST analysis on the homology of the deduced amino acid sequence to other RAS family showed that the percentage is hightest (98%) with identity to *rgp1*, a small GTP binding protein involved in a signal pathway responding to hormone, such as cytokinin and ethylene. According to the amino acid sequence, the small G-protein was classified as a small-Ras-related GTP-binding protein which consisted of an ATP/GTP-binding motif A (P-loop), prenylation site (CAAX box), and a domain characteristic for Ras family protein with PFAM database. A SwissProt database was searched by using the Blastp program. It revealed that the predicted amino acid sequence was similar to Ras proteins (60--98% identity) from various organisms. Moreover, the predicted amino acid sequence of *ras* gene was 98% identical to *rgp1*, Ras-related GTP-binding protein from rice with a significant E-value of *e-122*. In addition promoter sequences or *Cis*-element of hormonal-inducible gene was predicted. We found many reguratory elements which might be stimulated with plant hormones such as cytokinin (ARR1-binding element, GA (CAREs) regulatory elements and so on.

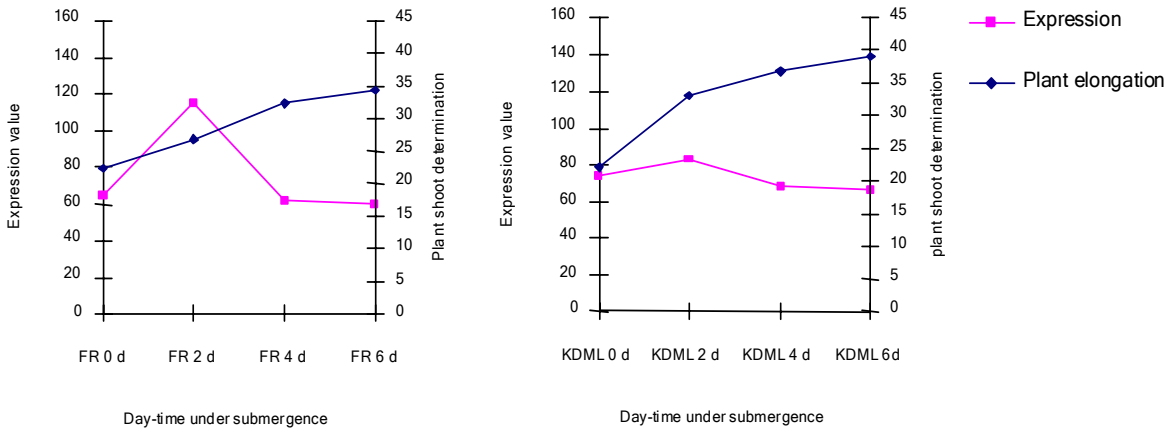
In addition, we identified the putative gene encoding ethylene responsive binding protein (*EREBP*) from *Nipponbare* PAC0663H05 (accession number AC090056). The result showed that gene sequence contains ERF domain, DNA-binding domain of ethylene-responsive element-binding factor. The deduced amino acid contains a putative polypeptide that is highly similar to a DNA binding domain of AP2/EREBP type transcription factor. The putative *EREBP* gene is contained a single open reading frame coding for a polypeptide of 225 amino acids with a

predicted molecular mass of 25 Kda. Database searching results at high similarity level revealed that the deduced amino acid sequence of the putative *EREBP* contained a conserved DNA binding domain of 64 amino acids that is presented in the large family of plant DNA binding protein. Moreover, the 2000-bp of the promoter region contained several hormone signaling elements such as ABA responsive element (ABRE), MYB binding site for ATMYB2, ANAERO consensus and DNA elements of abscisic acid induced transcription were indentified.

The correlation of the expression on *OsRAS* and *SubEREBP* genes to plant shoot elongation

Under submergence stress, high significant correlation between the expression of *OSRAS* and *SubEREBP* genes with plant shoot elongation was observed from three independent experiments. The result showed that the expression of two genes was correlated with induction and suppression of plant elongation, which were responsible to submergence stress. The *OsRAS* gene from FR13A was highly expressed for 2 day under submergence whereas the level of shoot elongation was increased slowly at the same time. In contrast to KDML105, the *OsRAS* gene was down expressed therefore plant elongation was increased rapidly. *OsEREBP1* gene from both FR13A and KDML105 were highly expressed at the second day under submergence. The expression of *OsEREBP1* gene from KDML105 was remaining increased until the fourth day and the level of plant shoot elongation was induced rapidly. The plant shoot of FR13A showed lower elongating when the expression of *OsEREBP1* was down regulated from the second day to the sixth day under submergence (Figure 10).

Ras-related GTP-binding protein gene



EREBP1

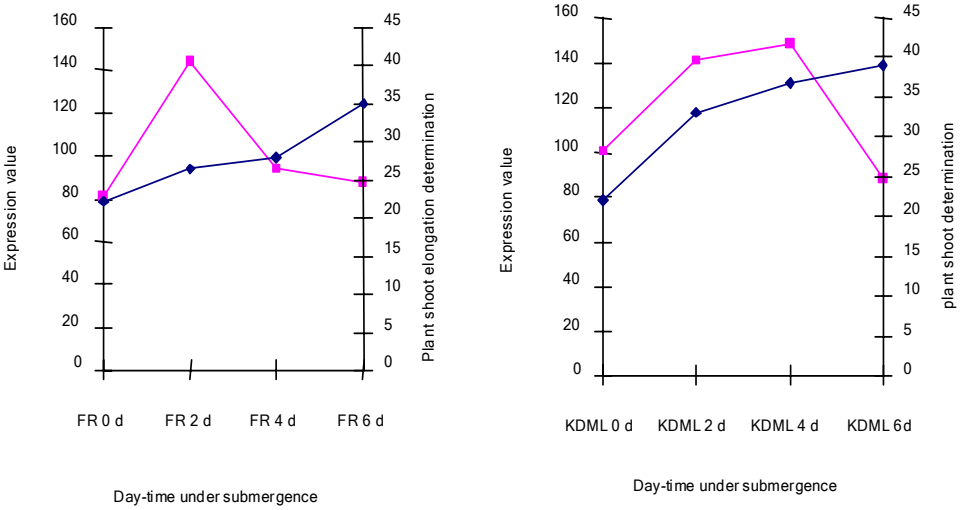


Figure 10 The correlation of the expression based on *OsRAS* and *SubEREBP* genes to plant shoot elongation.

Part IV: Small GTP-Binding Protein Gene Is Associated with QTL for Submergence Tolerance in Rice

The objective of this study is to identify and characterize of the submergence gene, *OsRAS*, and to establish the molecular markers of submergence tolerance for breeding new rice cultivars. Using the gene encoding the Ras-related GTP-binding protein from Nipponbare rice (PAC0645D04, GenBank accession no. AC090054), specific primers were designed for PCR-SSCP analysis. These primers were able to amplify genomic DNA from several rice cultivars. Then, a promoter region of the genomic sequence of this gene was used as a specific marker for rice submergence tolerance. As a result, a small monomeric GTP-binding protein and molecular markers were identified and linked to the submergence tolerance gene. This gene was named *OsRAS*.

Identification and Analysis of the RGP1 Homologue

A total of 139-kb PAC0645D04 genomic clones located near the peak of a submergence tolerance region were used for predicting five putative genes (data not shown). The main finding of this study was a small monomeric G-protein gene region, which was located between 75391 and 78415 kb. The relationship of a small GTP-binding protein to rice submergence tolerance could be evaluated as a predicted *OsRA*S, or Ras gene. Based on several computational tools, this gene located on a major QTL controlling resistance to submergence encoded the Ras-related GTP-binding protein. The sequence based on the *OsRAS* gene was utilized to predict its function, and a GenScan and a GeneMark–HMM were used to predict two exons for the GTP-binding protein. The result showed that DNA sequence of the *OSRAS* gene had two exons with 52% GC content. In addition, an open reading frame encoded a polypeptide of 226 amino acids, with a calculated molecular mass of 24.8 kD and an isoelectric point of 6.85 (Figure 11A). The predicted genomic sequence was identical to *RGP*1, the gene for rice small G-protein, with an E value of 0.0 and a bit score of 1.223. The sequence identity was perfect across 1300 bp of the *RGP1* coding sequence. The derived amino acid sequence showed a significant homology between

small GTP-binding proteins of rice and small GTP binding proteins of other species such as arabidopsis, tobacco, garden pea, soybean, bird's-foot trefoil, maize, and so on. In addition, this rice GTP-binding protein also matched a cDNA clone expressed at the flowering stage (rice, accession no. C98582).

The PROSITE database was used to identify the functional classification of proteins in the *OSRAS* gene region. The *OSRAS* gene consists of an ATP/GTP-binding motif A (P-loop), prenylation site (CAAX box), and a domain characteristic for Ras family proteins in the PFAM database. The SwissProt database was searched by using the blastp program. The search showed that the predicted amino acid sequence was similar to the Ras proteins (60–98% identity) from various organisms. Moreover, the predicted amino acid sequence of *OSRAS* gene was 98% identical to *RGP*1, the Ras-related GTP-binding protein from rice with a significant E-value of e-122. The comparison of amino acid sequences of *OSRAS* and *RGP1* proteins is shown in figure 11C. Importantly, this gene contains the conserved residue or motif unique to members of the Ras superfamily. These sites are Rab, Ras, and Rho including ADP-ribosylation factor family and Arf-like small GTPase (Figure. 11B).

Plant Shoot Elongation under Submergence Stress

The ability to survive and recuperate after submergence is strongly related to the ability to limit leaf elongation under water. Under submergence conditions, GA and ethylene have played important roles in leaf elongation and senescence. To study the ability for plant elongation under submergence stress, we used FR13A, a tolerant line, and KDML105, an intolerant line, to investigate the relationship between suppressed plant elongation and survival ability under submergence gence stress. The plant elongation was evaluated after the 20-day-old rice seedlings were submerged for 2, 4, 6, and 8 days. The intolerant plants elongated rapidly underwater, at a rate of 3.84 cm per day, as compared to 1.89 cm per day in the tolerant plants. However, both FR13A and KDML105 showed plant elongation rate of 0.58 and 0.62 cm per day under normal growth condition (Figures 12B, 12C).

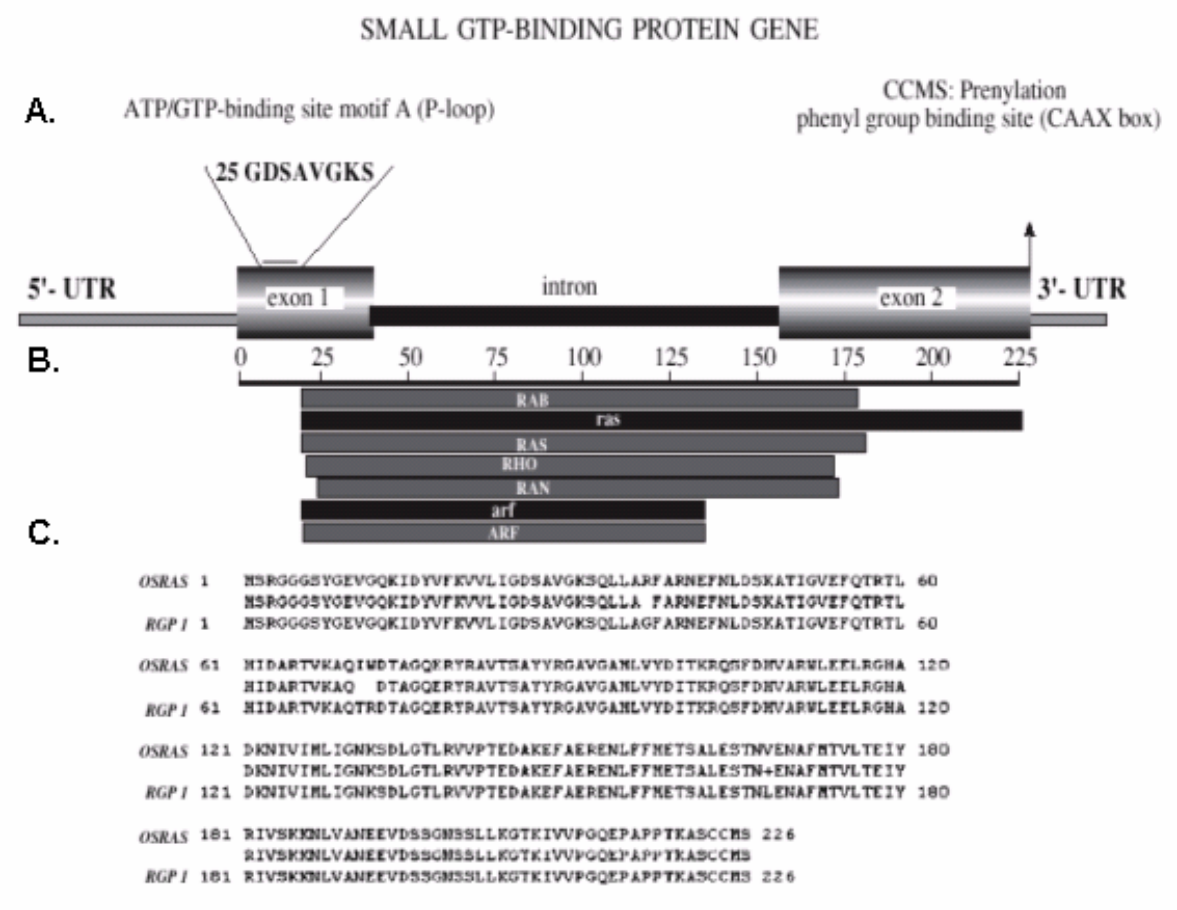


Figure 11 Sequence analysis of the *OSRAS* gene encoding a Ras-related GTP-binding protein.

A. Scheme of intron/exon organization showing that the *OSRAS* gene consists of two domains characteristic of the Ras superfamily.

B. The result from PHI-BLAST analysis showed that the conserved domain belonged to the members of Ras superfamily.

C. High similarity (98% identity) of amino acid sequences of the rice *OSRAS* and *RGP1* gene products.

Gene Expression Analysis

To investigate the expression of *OSRAS* gene, we performed RT–PCR analysis of plants grown under two contrasting flooding conditions. The specific primer generated from exon2 of *OSRAS* was used in RT–PCR analysis. In the plants experiencing flash-flooding, slow-flooding, and control treatment, expression of *OSRAS* from FR13A, the tolerant line, was upregulated up to 8 days. The gene from KDML105, the intolerant line, was also upregulated up to 8 days in the case of slow-flooding and control treatments. In contrast to the flash-flooding conditions, the *OSRAS* gene from KDML105 was suppressed after the second day (Figure 12A).

DNA Gel Blot Analysis

The DNA gel blot analysis was utilized as a tool to determine the copy number of *OSRAS* in the rice genome. Total DNA was isolated from FR13A and KDML105 cvs., digested with appropriate endonu-clease *(Eco*RI and *Hin*dIII), and was subjected to hybridization. DNA gel blot analysis was performed with B–E probes containing 5'-UTR, coding region and 3'-UTR, respectively. The result showed that one or two specific bands were obtained using most of the restric-tion endonucleases; this evidence indicates that *OSRAS* was introduced as a single-copy gene into the genomic DNA of both rice lines (Figure 13).

Specific-Primer Amplification for *OSRAS* Gene

The putative Ras-related GTP-binding protein for the *OsRAS* gene was annotated from PAC0645D04 (GenBank accession no. AC090054). This putative gene was responsible for a major QTL controlling submergence resistance. BLAST program was employed to search for a sequence similarity. The result demonstrated that the predicted genomic sequence was identical to *RGP*1, with the E-value of 0.0 and bit score of 1.223. This gene consisted of a promoter region, 5'-UTR, two exons, one intron, 3'-UTR and encoded a polypeptide of 226 amino acids. The amplification of 221 bp of 5'-UTR and the entire 5'-UTR including exon 1 and 635 bp of intron 1,

respectively. The primer C and D combination covered the entire intron 1 and the entire end of intron 1 and 250 bp of exon 2, respectively. In addition to the components of the primer sets A, B, C and D, the primer E combination product contained the entire exon 2 covering 3'-UTR region. All specific primer sets showed no length polymorphism among rice cultivars. These results indicated that there was no variation in the putative gene size among the tolerant and intolerant rice cultivars. Therefore, SSCP analysis was carried out to detect the point mutation among these identical PCR products.

SSCP Polymorphism Analysis

According to the sequence data, the primer sets B and C amplified the entire 5' UTR, exon 1 and intron 1 regions. By using SSCP analysis, a polymorphism was detected differentiating between the products amplified with the primer sets B and C. The results indicated that the primer set B did not show any single strand DNA (ssDNA) polymorphisms (data not shown), while the primer set C showed a low level of polymorphism (Figure 14B). Therefore, we conclude that the exon 1 region and a part of intron 1 of the putative gene were conserved among the rice cultivars.

Based on the sequences amplified by the primer sets D and E, the 659-bp end of intron 1 and exon 2 with 3'- UTR region were recovered. The SSCP analysis of rice cultivars showed different ssDNA patterns on polyacrylamide gel (Figures 14C, 14D). The SSCP patterns displayed one or two bands characteristic of each rice cultivar. Among seven rice cultivars, the *japonica* rice (CT6241 and Nipponbare) showed the polymorphism distinct from other cultivars. Moreover, the primer set A showed three ssDNA banding patterns, which can be used to separate rice cultivars into three groups of submergence-tolerant and intolerant cultivars. Figure 14 illustrates the product amplified with primer sets A, D, and E and the possibility of a DNA variation in their regions (Figure 14E). Therefore, a highly variable ssDNA banding in the amplified product regions related to primer sets A, D, and E indicated that the polymorphism was detected. Additional sequence analysis was conducted using PCR

products amplified from the primer sets A, D, and E in order to further confirm this polymorphism.

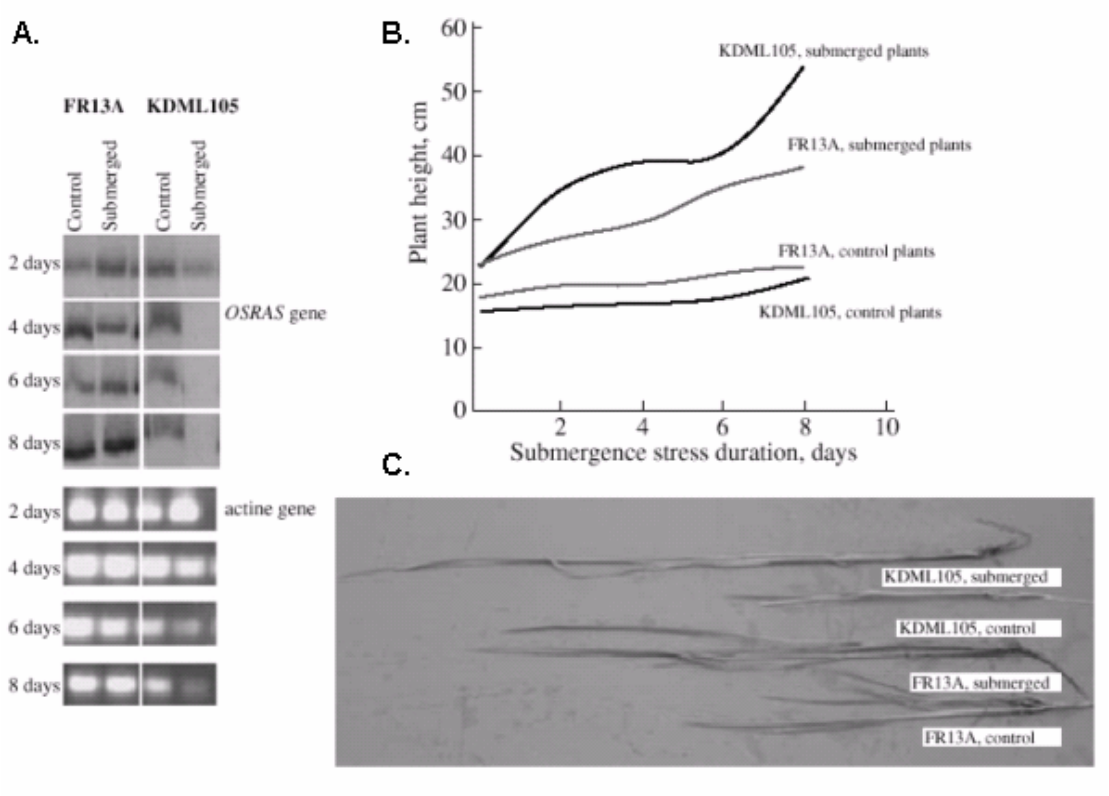


Figure 12 **A.** Expression of the *OSRAS* gene in FR13A, tolerant line, and KDML105, intolerant line, when submerged for 2, 4, 6, and 8 days. RT-PCR amplification was performed, and the products were stained with ethidium bromide. Actin was used as a control.

B. Submergence response of plant shoot elongation in FR13A, tolerant line, and KDML105, intolerant line, as compared with the control. 20-day-old seedlings were submerged for 8 days in aquarium pond, and then plant elongation was measured.

C. Phenotypic changes in FR13A and KDML105 plants submerged for 8 days and in the control plants.

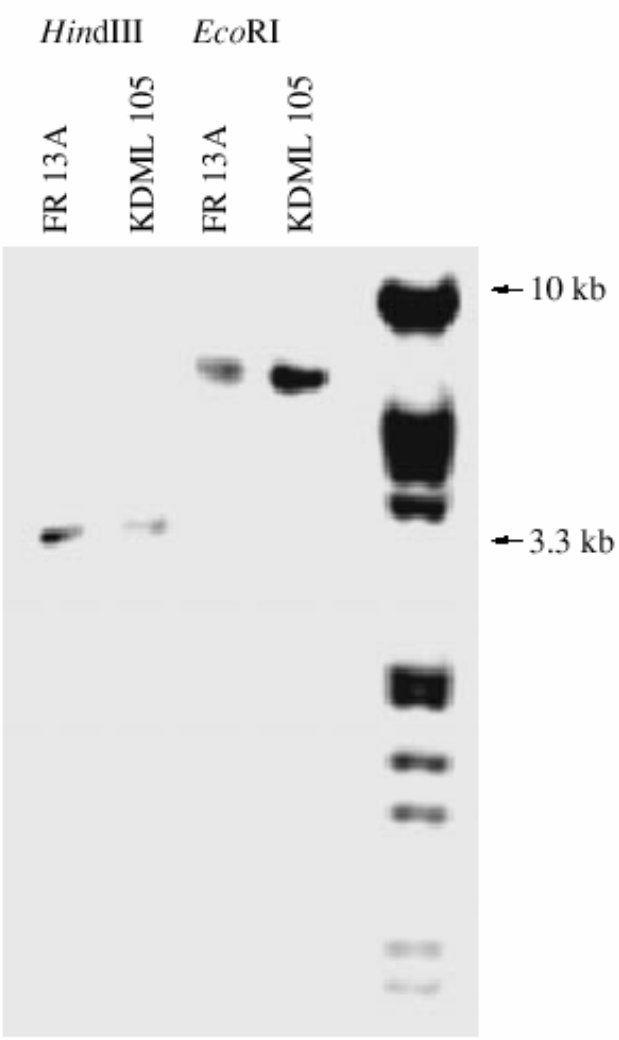


Figure 13 Southern blot hybridization of genomic DNA extracted from the tolerant cv. FR13A and the intolerant cv. KDML105. DNA was digested with appropriate endonucleases *(Eco*RI and *Hin*dIII) and subjected to hybridization. DNA gel blot analysis was performed with the B–E probes containing the 5'-UTR, coding region, and 3'-UTR.

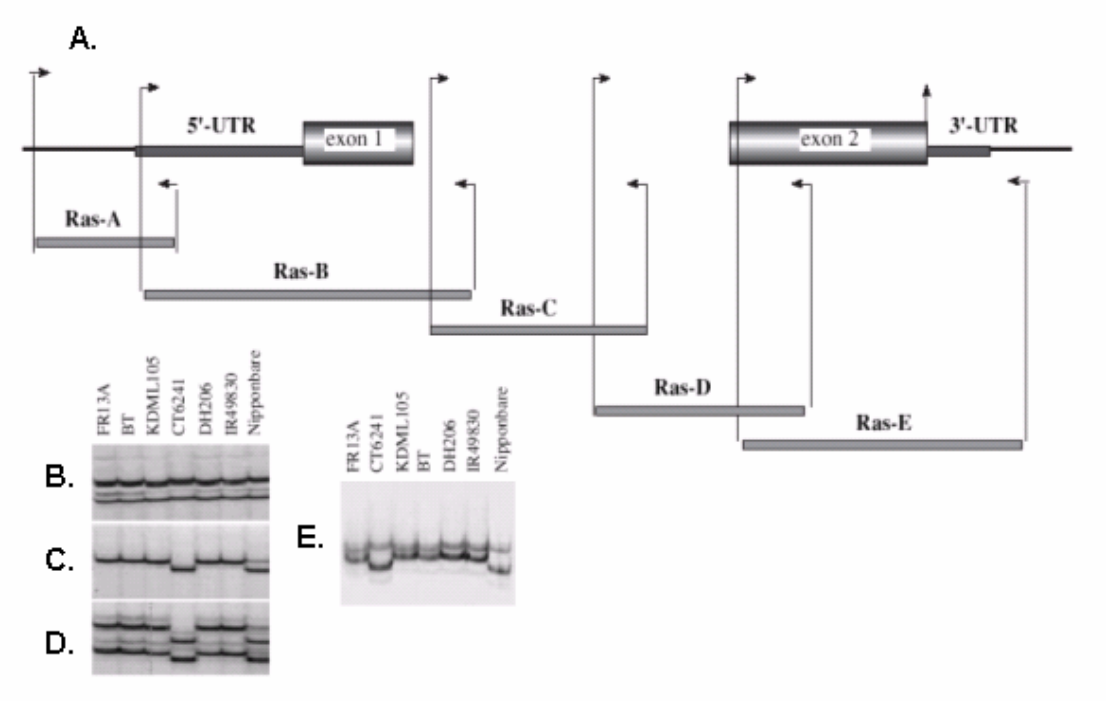


Figure 14 **A.** Scheme of the *OSRAS* gene coding for a Ras-related GTP-binding protein from rice PAC0645D04, Nipponbare (GenBank accession no. AC090054). The sequence of *OSRAS* gene was used to design the PCR primers recognizing the exon/intron domains.(b–e) SSCP analysis of the *OSRAS* gene from seven rice cultivars.

B. The products amplified with the primer set C comprise the intron region.

C. The products amplified with the primer set D consisted of intron and 250 bp of exon 2.

D. The products amplified with the primer set E contained the entire exon 2 covering 3'-UTR region.

E. The products amplified with the primer set A contained the entire promoter and 211 bp of 5'-UTR.

DNA Sequence and Sequence Homology Comparison

Sequence analysis using PCR products amplified by primer sets A–E showed that the nucleotide sequence of exon 2 was conserved among the seven rice cultivars. The analysis showed that the sequence was highly homologous to the *RGP1* gene from Ginbozu rice. In *japonica* rice, the 3'-UTR related to the primer set E showed a different 6-bp insertion/deletion, and the end of intron 1 region amplified with the primer set D showed the position of a 32-bp insertion/deletion. These results suggested that the ssDNA pattern of amplified product with the primer set D in *japonica* rice is associated with the mutation. Moreover, a base-sub-stitution mutation was identified in the region covering the TATA box and extended to the upstream 5'-UTR. Seven rice cultivars were analyzed by a sequence comparison analysis. A random point mutation with some consensus sequences was detected in the leader sequence from primer set A-amplified PCR products. It indicated that the sequence variations between the tol-erant and intolerant cultivars were point mutations located on the promoter region and were specifically distributed as five points at –100 to –300 bp as shown in figure 15. Importantly, the nucleotides changed at the position of –180 and –210 bp were present on both TATA-box regions. An unrooted gene tree was con-structed using CLUSTAL X (1.8) program to trace the relationship of the sequences amplified with the primer set A. Based on this analysis, the submergence-tolerant cvs FR13A, IR49830, and DH206 were clearly distinct from the intolerant cvs CT6241, Nipponbare, KDML105, and BT (Figure 16).

Development of SSCP Markers and Mapping

Two pairs of PCR primers were generated from the genomic sequence of the *OSRAS* gene predicted from PAC0645D04. These primers were used to amplify the fragment of the gene containing exon 2 and 3'-UTR. The SSCP markers were used for differentiation between FR13A (tolerant) and CT6241 (intolerant) cul-tivars. A linkage map was constructed using the available RFLP, SSLP, and BAC end markers for submergence- tolerance trait. One hundred and seventy two RILs from FR13A × CT6241 cross were used for mapping the SSCP markers. The SSCP-*OSRAS* marker

was mapped to the submergence resistance QTL located in a confidence interval spanning 0.3 cM from RFLP marker R1164.

Cluster Analysis of a Small GTP-Binding Protein Family

Almost all known genes encoding small GTP-bind-ing proteins were collected from several databases including Swiss, TrEMBL, Trenew, Trest, Trgen, and Trome. After analysis with Hit home database of pro-tein domains (http://hits.isb-sib.ch/), the *OSRAS* gene was closely related to the *RGP1* and clearly diverged from other eighty two proteins of the Ras superfamily.

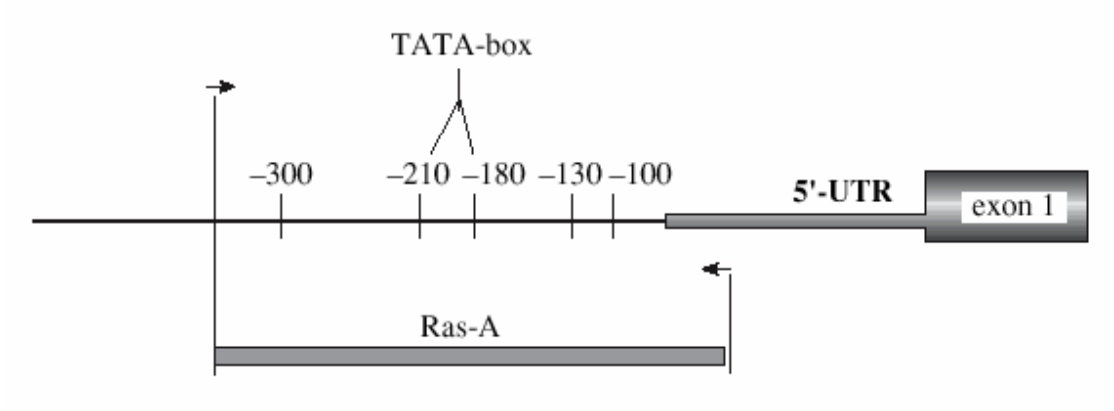


Figure 15 Scheme of the location of five SNP points in the promoter region; different patterns were found in the tolerant and intolerant cultivars.

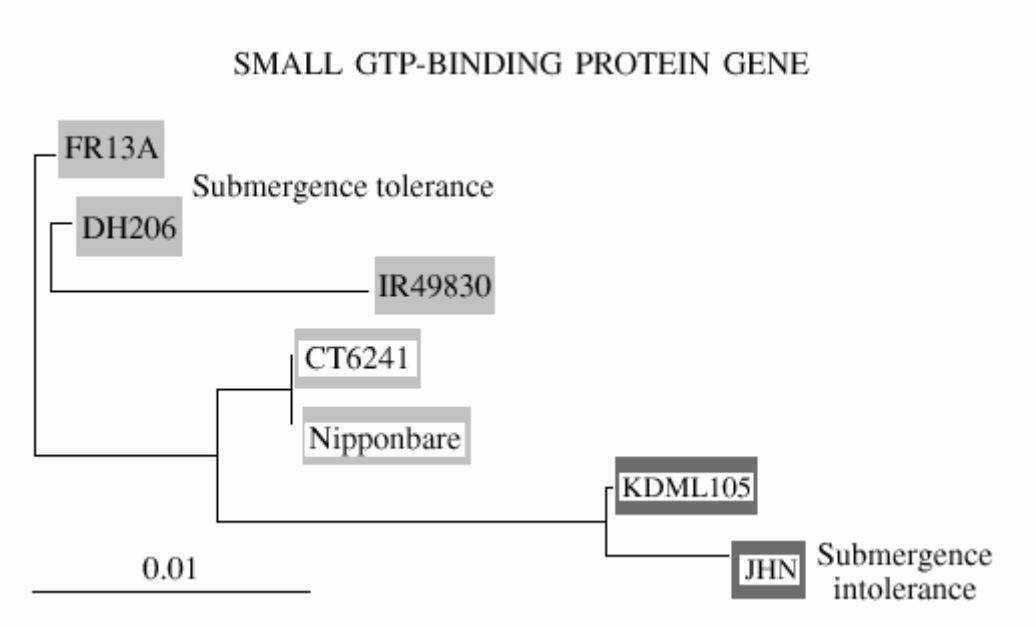


Figure 16 An unrooted dendrogram of the promoter region of the Ras-related GTP-binding protein gene constructed using CLUSTAL X (1.8). Based on this analysis, the submergence-tolerant cvs. FR13A, IR49830, and DH206 clearly diverge from the intolerant cvs. CT6241, Nipponbare, KDML105, and JHN.

Development and characterization of overexpression *OsRAS* gene in transgenic rice

In our work, we transferred the ras-related GTP-binding protein gene (*OsRAS*) which located on the major QTL for submergence tolerance chromosome 9 into Jao Hom Nin (JHN) to confirm its functions. Embryogenic calli of JHN were used as host plant to be bombarded with constructed vectors pCAMBIA 1301-*OsRAS* containing the selectable marker hygromycin phosphotransferase (*hpt*), the reporter gene and β-glucuronidase (*gus*) as shown in figure 17.

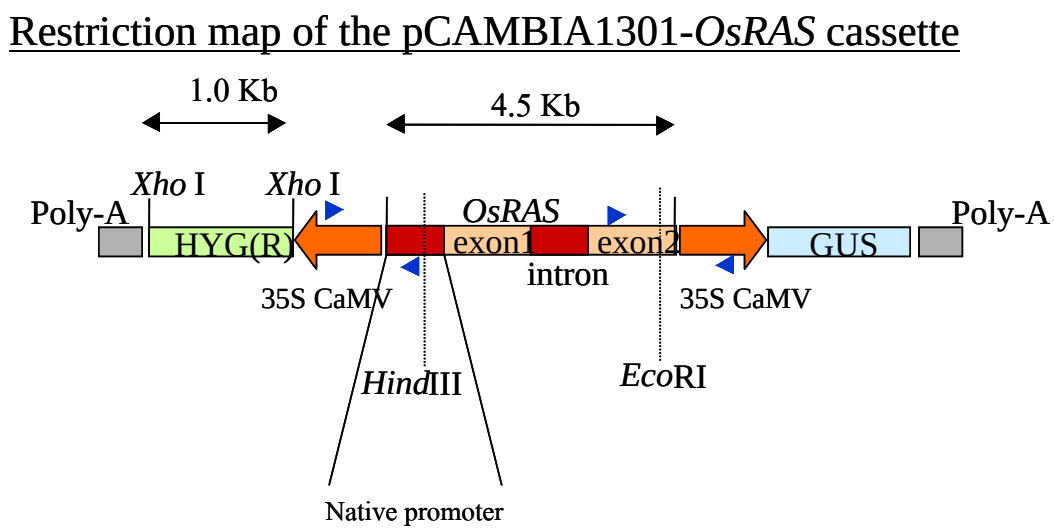


Figure 17 Construction of plamid pCAMBIA 1301. *HYG* gene for hygromycin phosphotransferase, NOS-terminator of a gene for nopaline synthase, *35S-PRO* CaMV 35S promoter and OsRAS rice genomics DNA encoding a GTP-binding protein including its native promoter.

Tranformation of calli

After co-bombardmented with two types of a vector, the level of *GUS* transient expression was determined from the number of blue spot in callus. The results showed that the average number of blue spots per callus was 11.67 with recombinant plasmid contained *OsRAS* gene while the transformed with only pCAMBIA1301 showed higher rate in JHN calli. After two days, the positive targeted calli were collected in a sective media containing 75 mg per liter of hygromycin B. We found that only three calli resisted to hygromycin B from 3 independent events. These calli were then stimulated to induce shoot and root for regenerated plants. More than 60 independent T_0 plants were grown to maturity in greenhouse.

Characterization of the T_0 plant

Histochemical straining of T_0 plants was used to confirm the possibility of transgenic plants. GUS activity was observed in some parts of root and in young plantlet including the mature leaves (Figure 18). However, the original transgenic plants had no different phenotypic characteristics with the JHN wild type. On the next step, all of T_0 plants were grown to maturity until seed production to obtain T_1 generation plants. Surprisingly, No seed fertilization was observed in all the T_0 transgenic plants.

Molecular Characterization of Transgenic Rice Plants

Transgenic events are usually identified by the expression of selectable marker genes, conferring a growth advantage under selective conditions. These selectable marker genes are usually co-transformed with the gene of interest. Desired transgenic plants need to carry both: the selectable marker gene as well as the gene of interest. All regenerated plants were screened by PCR (see"Materials and Methods") to amplify a 124-bp fragment of the CaMV::*OsRAS*. All of 5 T_0 transgenic plants were amplified with *CaMV*-specific marker. Then, we tested our T_0 transgenic plants by PCR with primer specifics to the exon 1 region of *OsRAS*, which separated endogenous allele from introduced gene (Figure 19).

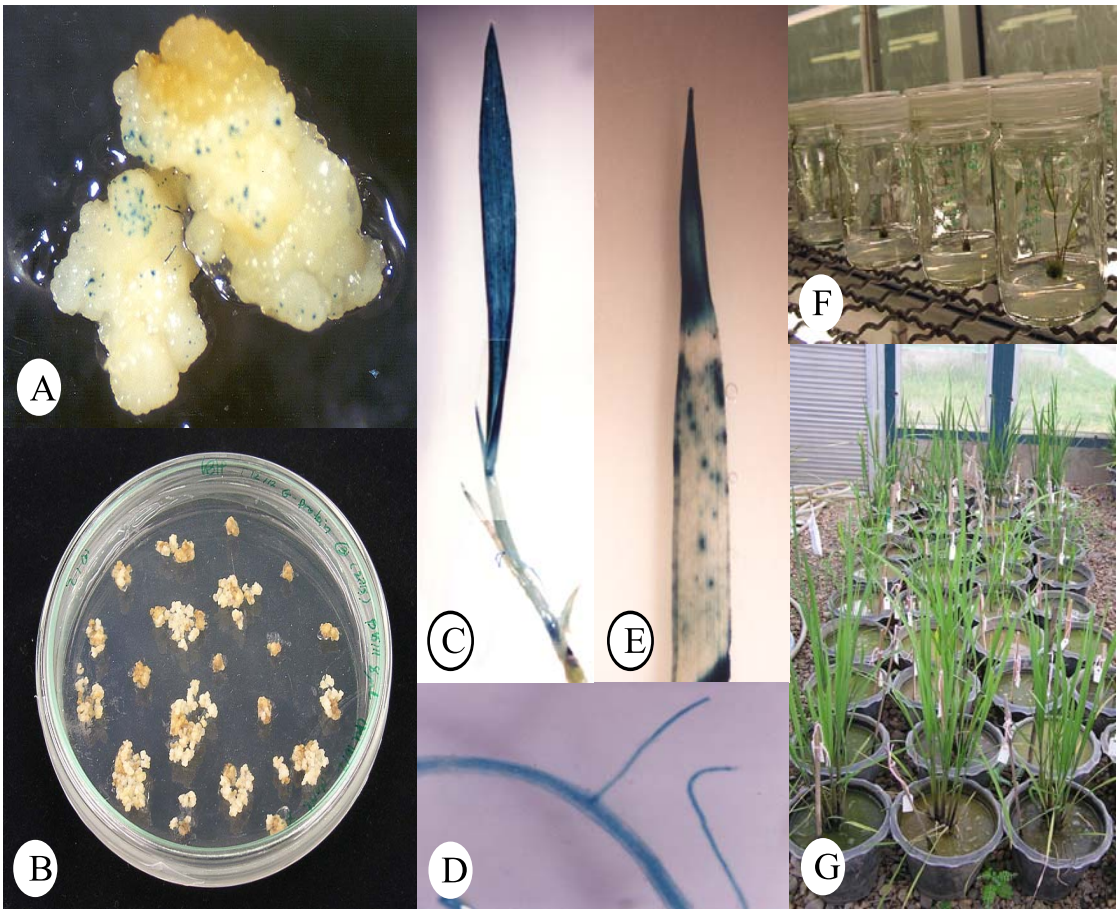


Figure18 Regeneration of transgenic Ras plants of Jao Hom Nin rice from bombarded embryogenic calli.

A. Transient expression of bombarded embryogenic calli with plasmid pCAMBIA1301-Ras.

B. Transformed callus lines formed from *hyg*-resistant cell clusters after selection on medium containing 75 mg/l hygromycinB

C. *GUS* expression in the resistant plantlet.

D. *GUS* expression in the root.

E. *GUS* expression in the maturity leaf.

F. Regeneration of transgenic plants. G. T_0 transgenic plants were grown in the soil.

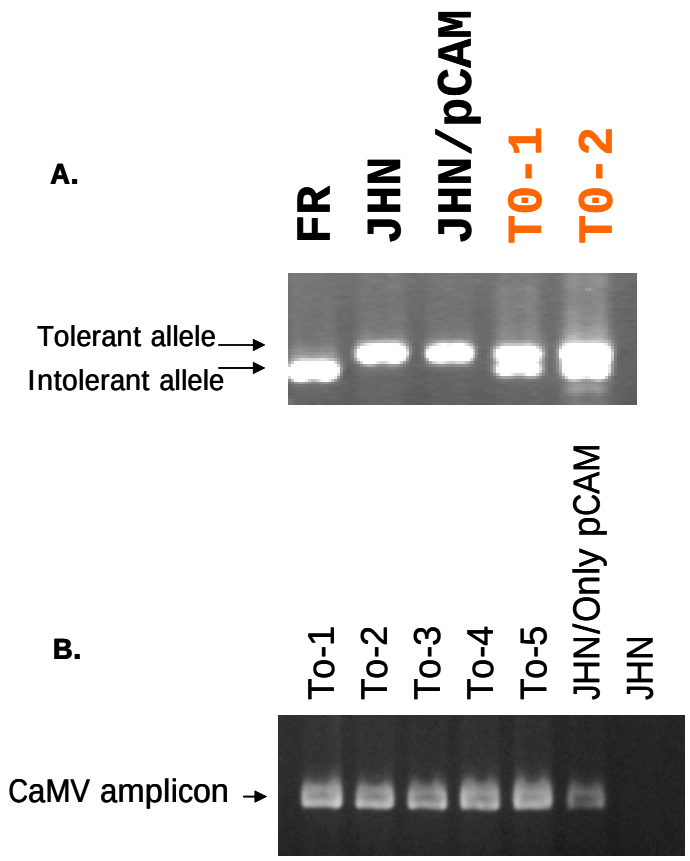


Figure 19 PCR based selection of individual transgenic plants using specific primers to detect the different allele of *OsRAS* transgene and CaMV primer. Agarose gel electrophoresis of PCR reactions of selected plants showed amplification products of the transgene **(A)** and the *CaMV* gene **(B)**.

Based on sequence comparison, we found that the exon1 of *OsRAS* gene showed single nucleic acid substitution from **T** (representing the tolerant allele, FR13A) to **G** (representing the intolerant allele, JHN) (Figure 20). A set of primers with dual TaqMan probe specific for amplicon of a 102-bp region of the *OsRAS* gene was developed to investigate the expression level of both introduced genes and endogenous genes under submergence compared to control conditions (Table 2 in materials and methods). All amplification reactions are carried out using ABI7700.

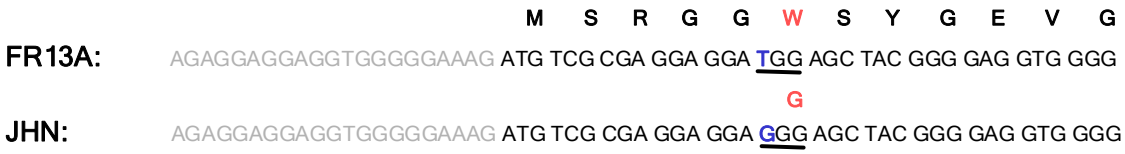
To demonstrate specificity in both tolerant and intolerant probes, four types from genomic DNA (FR13A, JHN, equal of FR13A plus JHN and T_0-1) were used in

this experiment. As shown in figure 21, fluorescence response from tolerant probe was only obtained in the dual probe reaction containing the tolerant DNA templates, FR13A, whereas there is no fluorescence response from intolerant probe in the dual probe reaction containing the tolerant DNA templates, JHN. However, genomic DNA from T_0-1 and equal tolerant and intolerant genomic DNA template was detected with fluorescence responses from tolerant and intolerant probe. The results show that the combination probe has reliable detection specificity.

Determination of copy number of transgene using Real time PCR

To quantify the number of transgene copies in five transgenic lines, two allele of ras gene were considered with quantitative real time PCR. We used samples of known concentration to construct a standard curve. This standard curve was obtained from the genomic DNA of rice. The concentration of these DNA samples can be measured spectrophotometrically at 260 nm and converted to the number of the copies according to Applied Biosystems manufactory. The concentrations of standard curve of ras gene are at 300,000 copies, 30,000 copies, 3,000 copies, 300 copies, 30 copies, and 3 copies. Consequently, the *OsRAS* gene exists as a single copy per haploid genome (or 2 copies per rice cell).

A.



B.

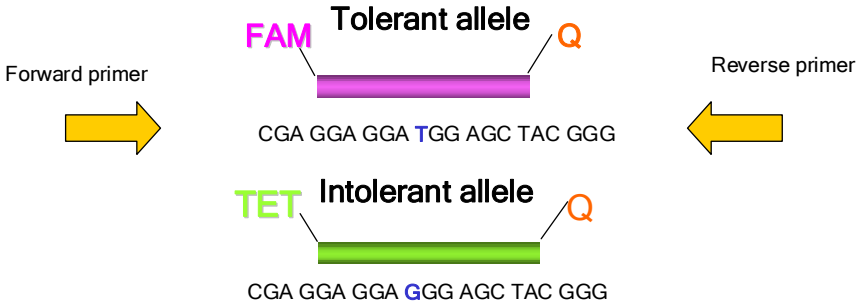


Figure20 **A.** Comparison of the 5' UTR and exon one in OsRAS sequence between FR13A and JHN rice.

B. RAS amplicon, with position of primers and dual TaqMan probe sequences used to define the differential expression of *OsRAS* gene between transgene and endogenous gene in transgenic rice

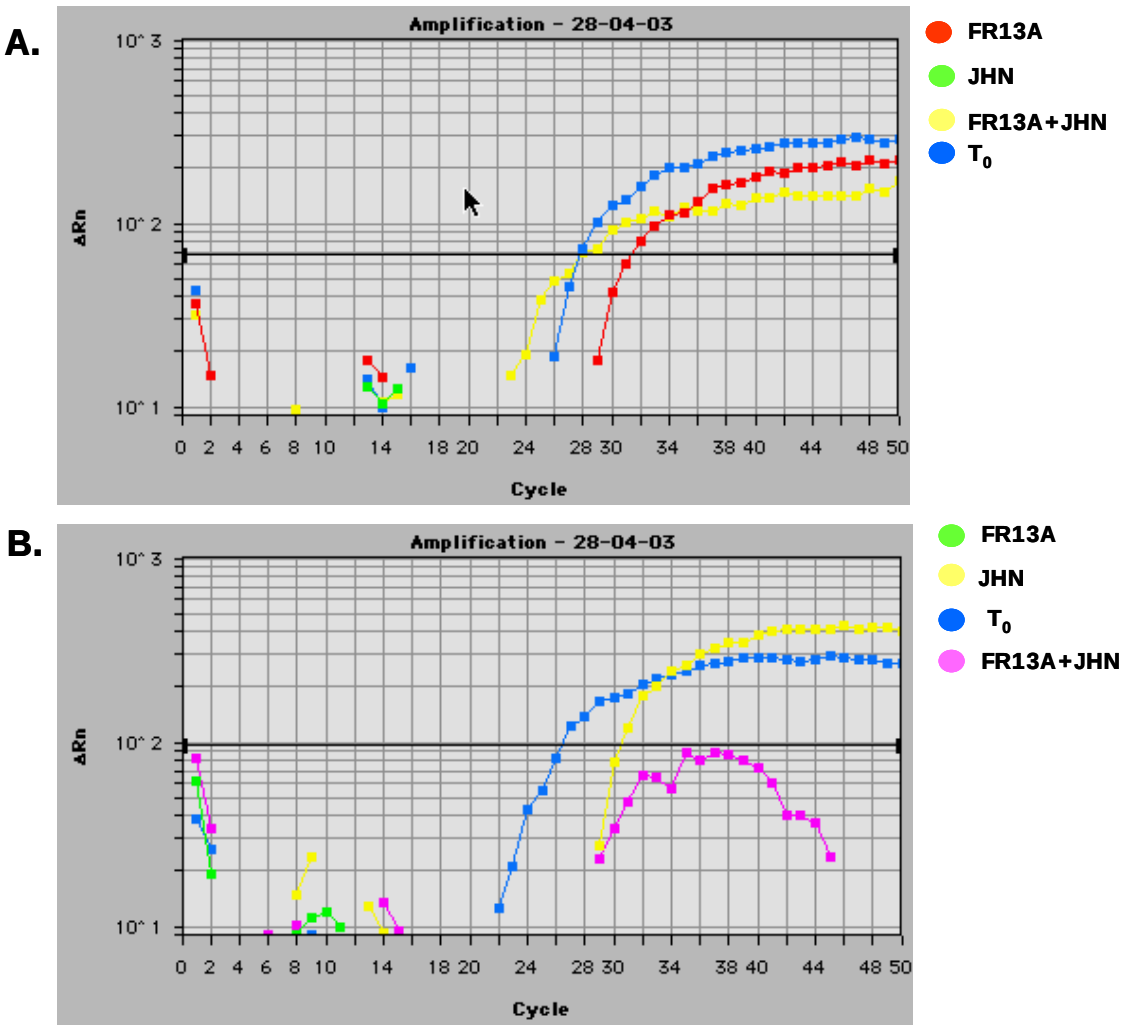


Figure21 Real time analysis with TaqMan dual probe for allele specific of *OsRAS* gene was used to detect a transcription level in rice plants. The genomic DNA was isolated from the FR13A, JHN, and T_0 transgenic plants.

A. The signal response of FAM associated with a tolerant allele.

B. The signal response of FAM associated with a tolerant allele.

Transgene expression detection

In this experiment, T_0 transgenic plants, FR13 (homozygous dominant), and JHN (homozygous recessive) were comparatively investigated for the allelic expression of the *OsRAS* gene between transgene and endogenous gene. We performed real time reverse transcriptase polymerase chain reaction (RT-PCR) using dual-fluorogenic probes with total RNA that extracted from T_0 transgenic rice and two

rice plants under normal conditions. The result of the T_0 transgenic plant showed two allelic signals with dual-fluorogenic probes whereas JHN control have only one signal with susceptible allelic probe in a single reaction. The transgene and endogenous gene were expressed as thought the same level (Figure 22). It suggests that the alleles of *OsRAS* gene from tolerant and intolerant lines were expressed under normal growth condition.

Under submergence stress, the expression of *OsRAS* gene from five T_0 plants was observed with 2, 4, 6 and 8 including control conditions. As a result, the tolerant allele was induced for 2 day under submergence when compared to the control conditions. Howevevr, the expression of tolerant allele from a tolerant line was up regulated after 2 days and decreased until 4 days while submerged. The intolerant allele, however, showed lower transcript in T_0 plants, less than the tolerant allele expressed.

Finally, real-time RT-PCR assay combines allelic discrimination sensitivity with specificity of probe hybridization, which improves the detection of introduced gene expression. This method can distinguish specifically the detection of expressed transgene and endogenous gene in transgenic plants. This is a powerful new approach for studies of gene expression in plant system. In addition, the dual probe real-time RT-PCR technique can be further developed for detection of allelic gene expression on quantitative analysis under stresses in trangenic plants.

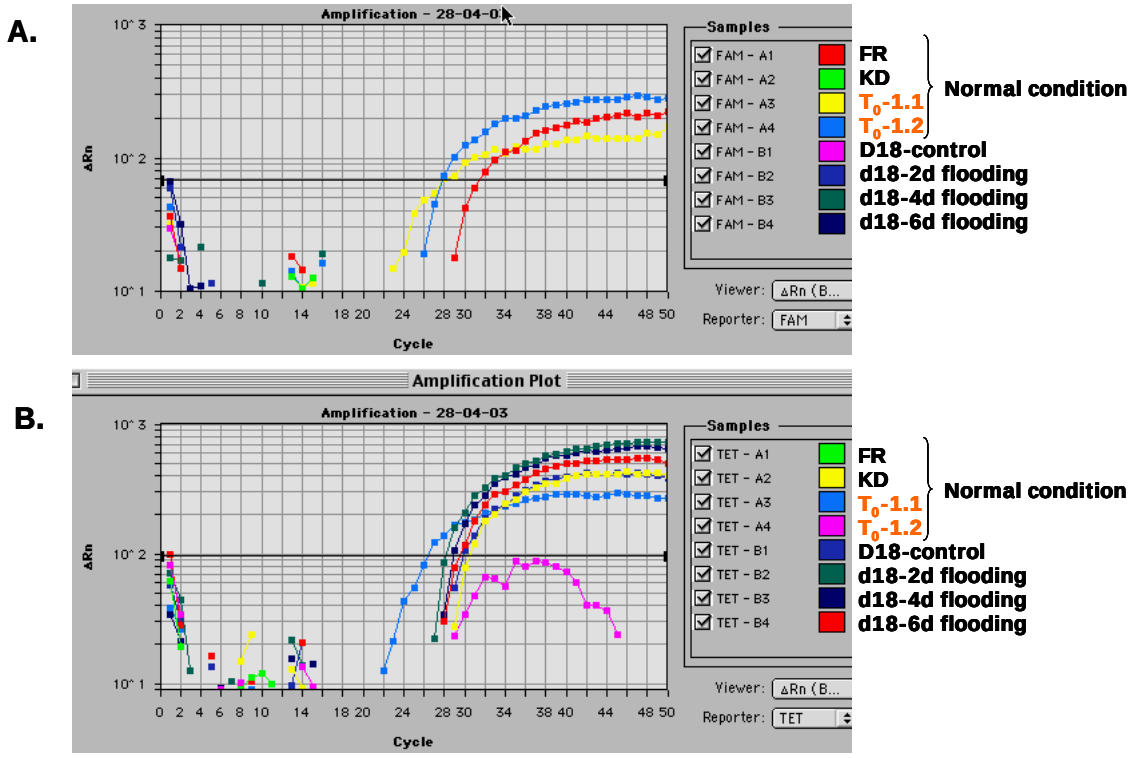


Figure 22 Real time RT-PCR analysis with TaqMan dual probe for specific allele of *OsRAS* gene was used to detect a transcription level in rice plants under flooding and control condition. The total RNA was isolated from the FR13A, KDML105, T_0 transgenic plants and dwarf rice (*d18*).

Part V: Near Isogenic Liness for *OsEREBP* are Responsible For Submergence Tolerance in Rice

We present the associated data between the phenotypic response under flooding and the genotypic alleles of the NILs containing three gene targeted markers (GTMs), which are localized on the 16.7 Kb region 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.

Characterization of near isogenic line of BC4F8

A set of near isogenic lines, NIL-132, was used for this study. The NILs population had been developed from BC4F7 by backcrossing the introgression specific region of chromosome9. The BC4F8 population consisted of a set of 87 near-isogenic lines (NILs) derived from a cross between a submergence tolerant line, DH206 and the cultivated susceptible line, KDML105 was used in the experiment. We first genotyped the 16.7 Kb segment on the long arm of chromosome9, designated as the C region, with three molecular markers known as RB0783-indel, H05-11, and *OS*ER2. The region was represented on a part of the genomic sequence of a Nipponbare PAC clone (GenBank accession number AC090056). 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 NILs-132CC (12 plants), NILs-132Cc (12 plants), and NILs-132 cc (12 plants) respectively (Figure 24). In addition the regions around the 16.7 kb were confirmed with many types of molecular markers such as single strand length polymorphism (SSLP) markers, single strand conformation polymorphism (SSCP) markers, and so on. Both sides of the flanking regions on subQTL9 were represented as a genotypic sequence of an intolerance line, (KDML105). The genetic background similar to KDML105 on average of 94% using SSLP markers per chromosome was shown (data not shown). 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.

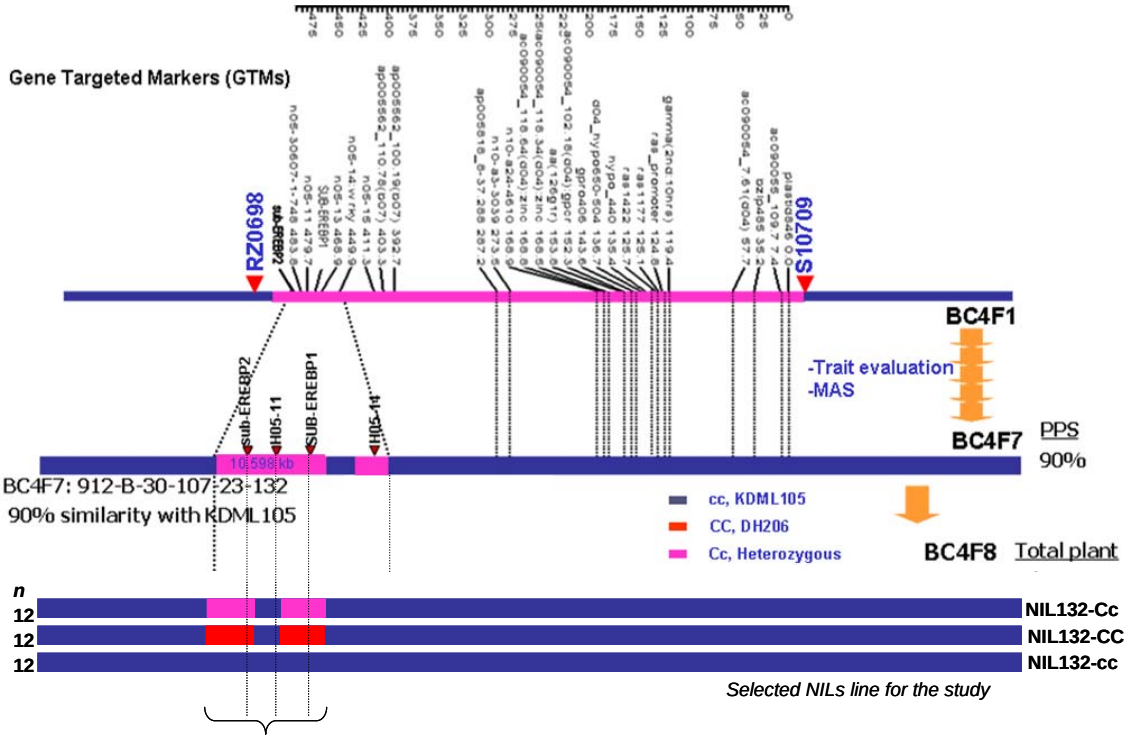


Figure 23 Graphical genotypes for the segregating segment of BC4F8 on subQTL9 expanding 16.7 kb (C region) were shown in three groups as NILs-132CC (12 plants), NILs-132Cc (12 plants), and NILs-132cc (12 plants) respectively. The genotypic screening with in the C region was done with three gene targeted markers (GTMs): RB0783, H05-11, and *O*SER2.

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 NIL132-CC, NIL132-Cc, and NIL132-cc were

significantly (*P<0.01*) different among genotypes varying across time (Table 10). The NIL132-CC and NIL132-Cc interaction showed no differences in traits such as PE, TSE, RSE, and LS at 1% level. The interaction between NIL132-cc and NIL132-CC: NIL132-Cc was highly significant (*P<0.01*) in PE, TSE and RSE and not significant in LS.

The results of figure 24 strongly indicate that the development of homozygous (NIL132-CC) and heterozygous (NIL132-Cc) are a similar genotyping effect of plant adaptation under submergence stress. The plant adaptation traits under submergence conditions such as PE, TSE, RSE, and LS are not significantly (*P<0.01*) different between NIL132CC and NIL132Cc. However, the development of homozygous (NIL132-cc) showed high significance (*P<0.01*) of responding traits (PE, TSE, and RSE) with the NIL132-CC and the NIL132-Cc during submergence. Therefore a comparison between NIL132-CC: NIL132-Cc and NIL132-cc was concerned with plant adaptation traits of PE, TSE, and RSE in response to submergence stress.

Figure 25 shows the relationship between time-course (0, 2, 4, and 20 days) and plant adaptation traits of NILs-132 population under submergence stress. The results suggest that time-course during submergence stress for 20 days affects plant adaptation traits of plant elongation, total shoot elongation, relative shoot elongation, and leaf senescence in the development of NIL132. During 2 days and 4 days under submergence conditions, all plant adaptation traits are affected less than the time-course at 20 days, especially leaf senescence.

Table 10 Shows 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 Near isogenic lines submerged for 20 days. The data shows mean square value and the significance record of main and interaction effects of time treatment and genotypes.

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

***= significant at 1% level; * = significant at 5% level*

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.

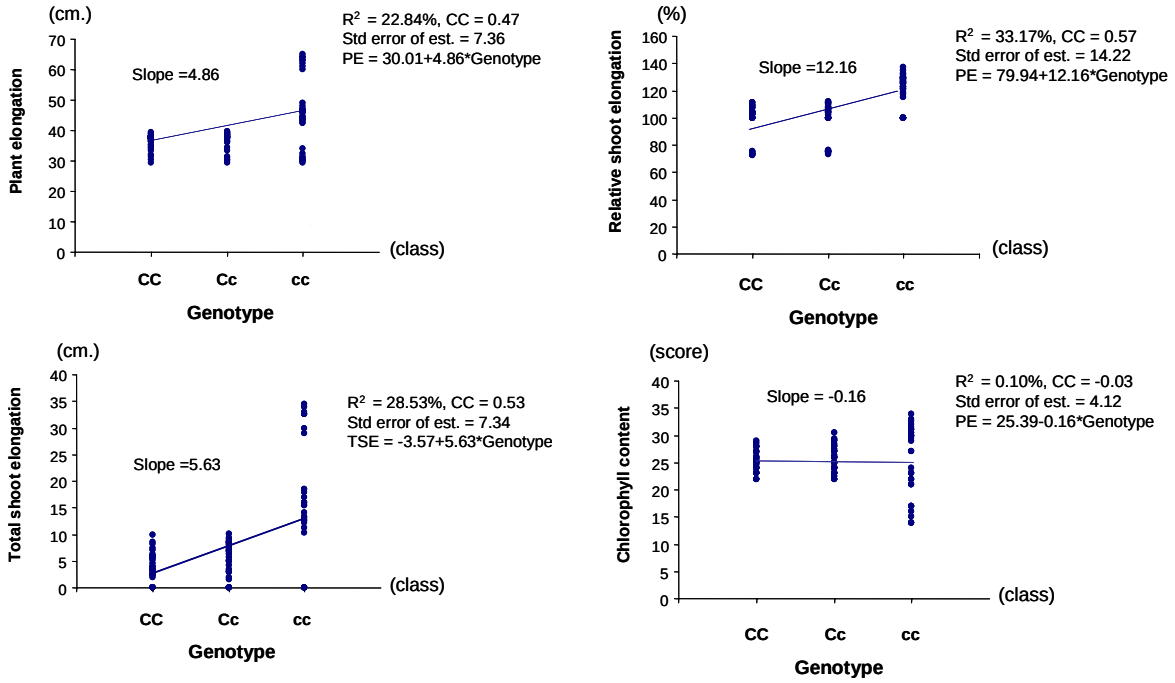


Figure 24 Graphs show the relationship among genotypes in three classes (CC, Cc, and cc) and plant adaptation traits of NILs-132 population under submergence stress for 20 days.

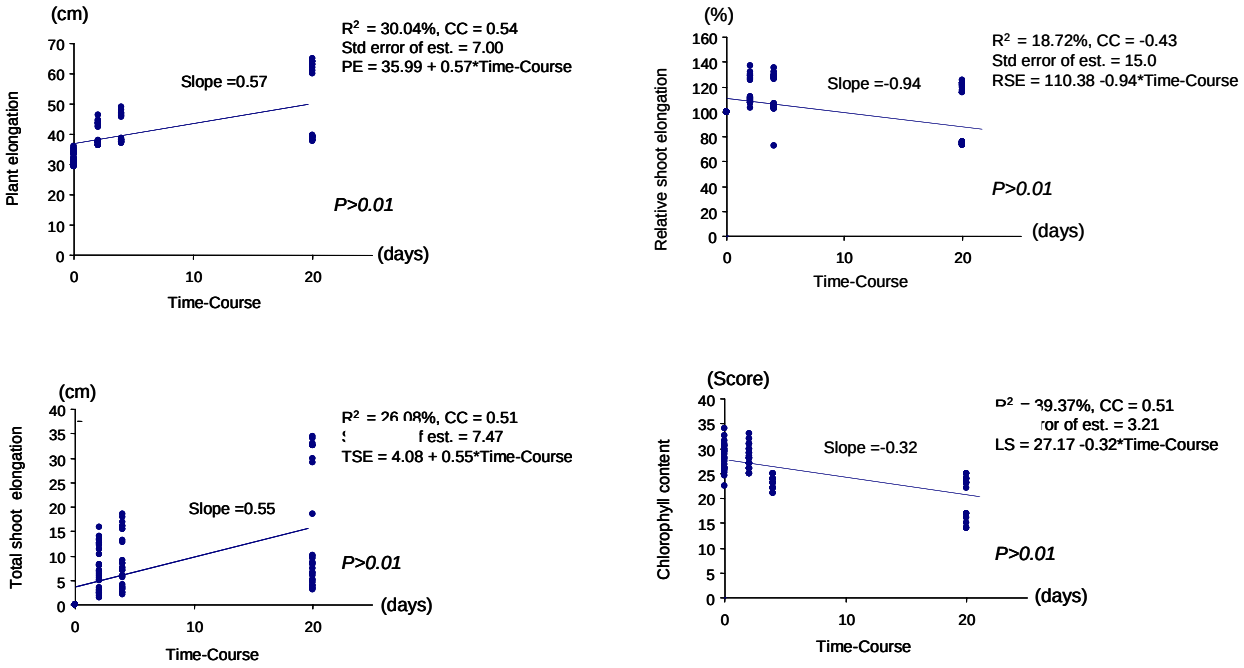


Figure 25 Graphs show the relationship between time-course (0, 2, 4, and 20 days) and plant adaptation traits of NILs-132 population under submergence stress

Expression of plant elongation at 20 day under submergence

Under submerged conditions, plant adaptation is directly a response to the ability to survive after de-submergence. To compare the impact of flooding on plant elongation, the thirty six individuals of the NILs-132 population that differ in their genotypic classes were used for investigation. A set of three classes of NIL-132CC, NIL-132Cc, and NIL-132cc, where each class contained 12 individual plants, were used in the experiment under submergence stress for 20 days.

Plant elongation (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 NIL-132CC and NIL-132Cc have similarity in average plant height of 38.3 and 38.9 cm

for 20 days under flooding. However the development of NIL132-cc has the highest level of average plant height at 62.7 cm under flooding.

The total shoot elongation (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), NILs132CC, and the heterozygous class, NILs-132Cc have average incremental height of 5.4 cm and 7.1 cm, respectively. But NILs-132cc, the individuals carrying homozygous alleles of KDML105 has the highest level of average incremental height at 31.2 cm under flooding.

The incremental height of plant shoot elongation in NILs132 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 NIL132-cc showed the highest level of leaf sheath and leaf blade growth in shoot elongation when compared to NIL132-CC and NIL132-Cc (Figure 26).

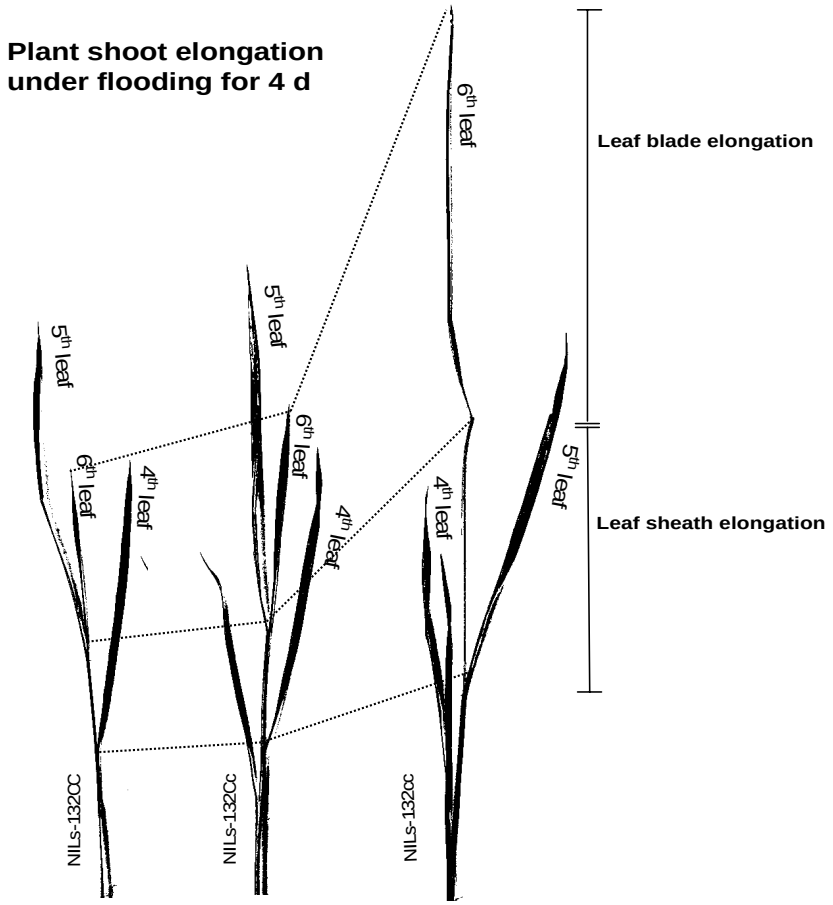


Figure 26 Submergence-induced leaf sheath elongation and leaf blade elongation were determined with three genotypic classes of NIL132. At the start of the submergence, the treatment plants used were 21 day seedlings and were submerged for 20 days.

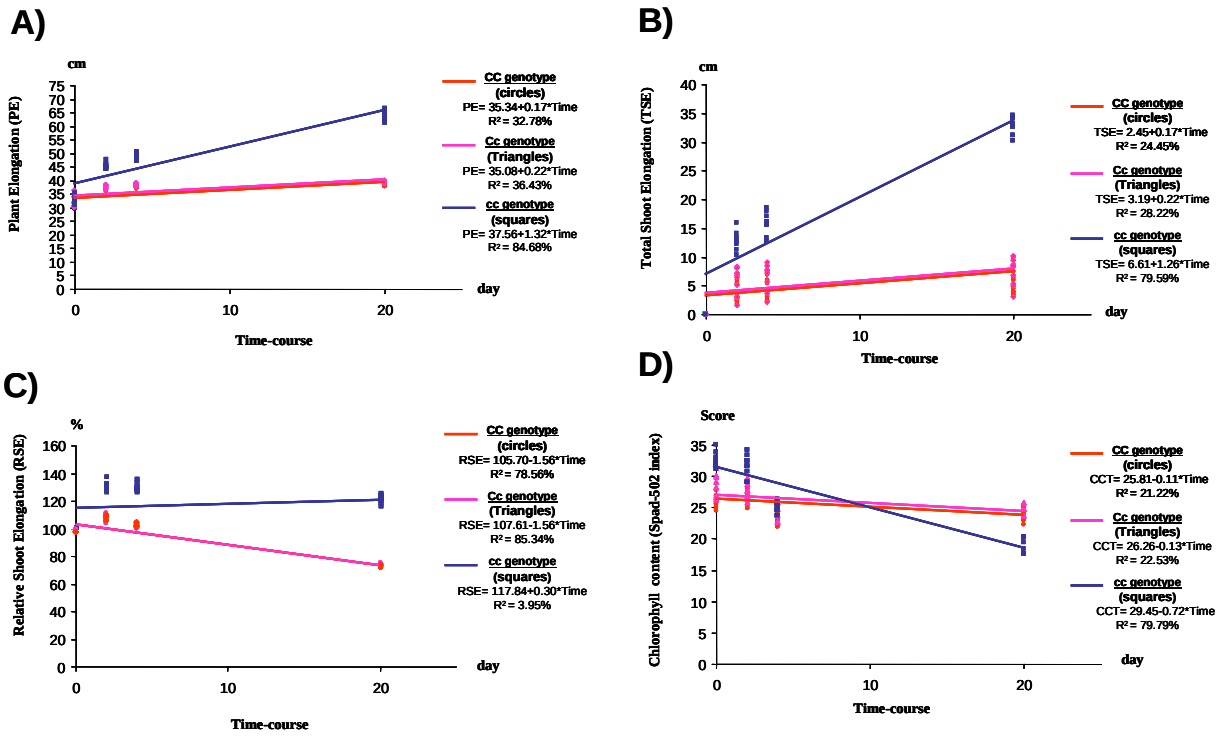


Figure 27 Effect of submergence on the physiological changes in three types of NILs; CC, Cc and cc. At the start of the submergence, the treatment plants used were 21 day seedlings and were submerged for 20 days.

A. Plant elongation

B. Total shoot elongation

C. Relative shoot elongation

D. Chlorophyll content

The impact of submergence stress on plant growth was compared to growth under normal conditions. Relative shoot elongation (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 NILs-132CC and NILs-132Cc showed 73.6 % and 74.8 % of shoot elongation compared to 100 % under normal growth conditions. However, the NILs-132cc shoots elongated 120.5 % more than plant growth under normal conditions.

In all experiments, the interaction between NILs-132CC and NILs-132Cc is not significant ($P<0.01$) across time. But the development of NIL132-cc interacts to

NILs-132CC: NILs-132Cc across time showed high significance (*P<0.01*) with all obtained data in responding traits of PE, TSE, and RSE under submergence stress (Figure 27A, 27B, and 27C).

Expression of leaf senescence at 20 day under submergence

Leaf senescence (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 NILs-132CC, NILs-132Cc, and NILs-132cc were defined for average chlorophyll content of 23.8, 23.9, and 15.4 score unit at 20 day flooding, respectively (Figure 27D). The interaction between NIL132-CC: NIL132-Cc and NIL132-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 NIL132 classes.

Table 11 Correlation matrix of submergence response traits in rice obtained in 36 individuals NILs-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 NILs-132 population (Table 11). 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, NILs-132CC and NILs-132Cc, having less TSE under flooding, mostly exhibited a higher level of %PS and LS. However, the NILs-132cc showing high TSE and low LS affected the %PS and scored as 0 % after being de-submerged for 7 days.

Identification of candidate genes at the interval region of 16.7 kb

Ethylene responsive element binding proteins (EREBPs) have been identified from various plants as a transcription factor that is necessary and sufficient for transcriptional control under stresses responsive conditions and developmental processes by ethylene. The result showed that the region of 16.7 Kb consists of three predicted genes as a hypothetical protein gene and two unknown protein genes. The unknown protein coding genes were classified into ethylene responsive element binding protein (EREBP) gene. These unknown gene sequences contain a DNA-binding domain of ethylene-responsive element-binding factor (ERF domain). The deduced amino acid sequence contains a putative polypeptide that is highly similar to the DNA binding domain of EREBP/AP2-type transcription factor. Database searching results at high similarity level revealed that the deduced amino acid sequence of the putative EREBP contained a conserved DNA binding domain of 64 amino acids which is present in the large family of plant DNA binding proteins. Moreover, the 1000-bp promoter region of two unknown protein genes contained many Cis-acting elements such as an ethylene responsive element (ERF) for senescence, a WRKY repressor recognition site GA signaling, an ABA responsive element (ARBE), a MYB binding site for ATMB2 protein and a DNA element which is a regulator of abscisic acid induced transcription etc. Several reports have shown that transcription factors are important in regulating plant responses to environmental stress.

Effect of flooding on the expression of two EREBP genes at *subQTL* region

According to the plant adaptation during submergence stress, the NILs-CC and NILs-Cc showed the same trend on physiological changes. Therefore, we used total RNA of CC and Cc to compare with cc in gene expression analysis. To investigate whether expression of two *EREBP* genes in 6.52 kb region of subQTL9 among types of NSL are influenced by submergence stress, total RNA was isolated from 21 day-seedling of different types (CC, Cc, and cc) under flooding for 2 days and control subjected to mini-scale array analysis and RT-PCR was also used for

confirmatory. The result showed that the mRNA accumulation of *OsEREBP1* gene increased up to flooding for 2 days in pooled CC and Cc type. On the other hand the transcription of *OsEREBP2* and *ACO1* (ACC oxidase) showed relatively less accumulation. However, cc type showed a high level of the transcription of *OsEREBP2* and *ACO1* under flooding for 2 days while *OsEREBP1* gene has no significant differences between stress and control for mRNA accumulation (Figure 28).

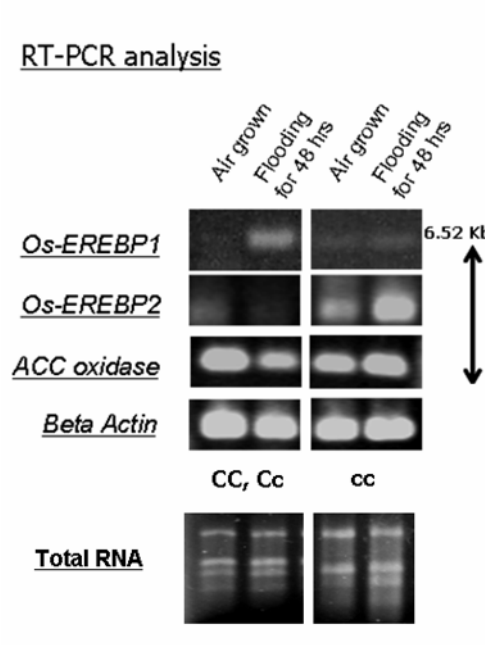


Figure 28 RT-PCR analysis of *OsEREBP1* and *OsEREBP2* mRNA from total RNA extracted from different NIL types. The *ACO* (ACC oxidase) was used to be an indicator of ethylene biosynthesis pathway.

Characterization of *OsEREBP1* gene in transgenic rice

The AP2/EREBP transcription factors play important roles in plant development and in the responses of plants to biotic and abiotic stresses. All members of the EREBP subfamily described to date are from dicotyledonous plants. To explore the understanding of mechanisms developed (reduced plant elongation and stay green) by rice to survive submergence, the transformation vector containing a single exon

gene encoding ethylene responsive element binding protein (*OsEREBP1*) was constructed as described in "Materials and Methods." JHN callus were co-bombarded with plasmid pCAMBIA-1302 (Figure 29) containing the *OsEREBP1* driven by the 35s*CaMV* promoter and a plasmid containing the hygromycin phosphotrans-ferase (*hpt*) gene as a selectable marker and *GFP* reporter gene (Valdez *et al*., 1998; Sudhakar *et al*., 1998). We analyzed 12 independently derived transgenic rice plants. The results showed that two of them were collected by using PCR-based with a *GFP* marker and introduced gene (data not shown). However, the mature T_0 plants were not different in general phenotypic characteristics from JHN wild type. But the number of seed per ear in T_0 plants was lower than of the control plant and ability of seed germination appeared lately also.

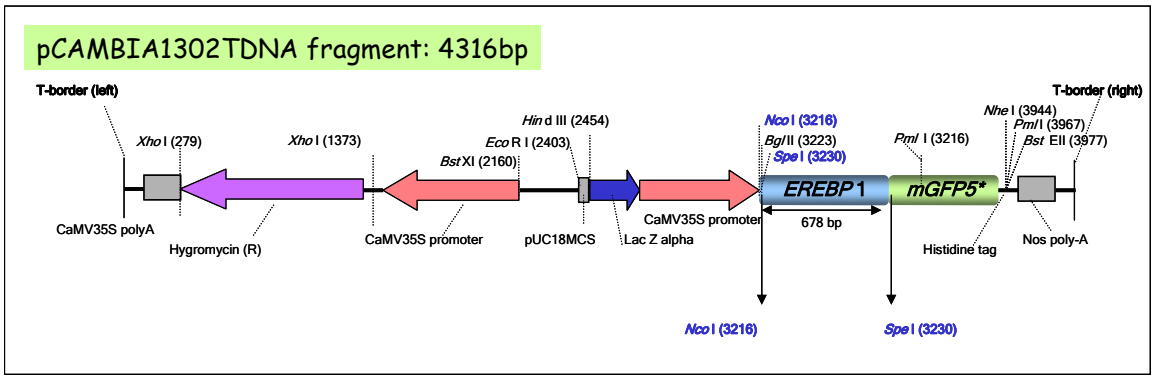


Figure 29 Construction of the pCAMBIA1302 vector. A sense-oriented 0.678-kb single exon gene derived from *OsEREBP*1 had originally been amplified from a tolerance line, FR13A, was inserted into the pCAMBIA1302 vector at the *Nco*I and *Spe*I sites.

Characterization of the T_1 transgenic plant

Transgenic events are usually identified by screening selectable marker genes using PCR analysis. These selectable marker genes (*GFP* and *35sCaMV*) are usually co-transformed with the gene of interest. Desired transgenic plants need to carry both, the selectable marker gene as well as the gene of interest. In a first selection step leaves of regenerated plant (T_1) were analyzed. Out of 344 single plants from event3

and event5, 36 PCR positive plants could be identified as potentially transgenic (Figure 30A). In addition the real time PCR with TaqMan probe with *CaMV* was used for confirmation (Figure 30B).

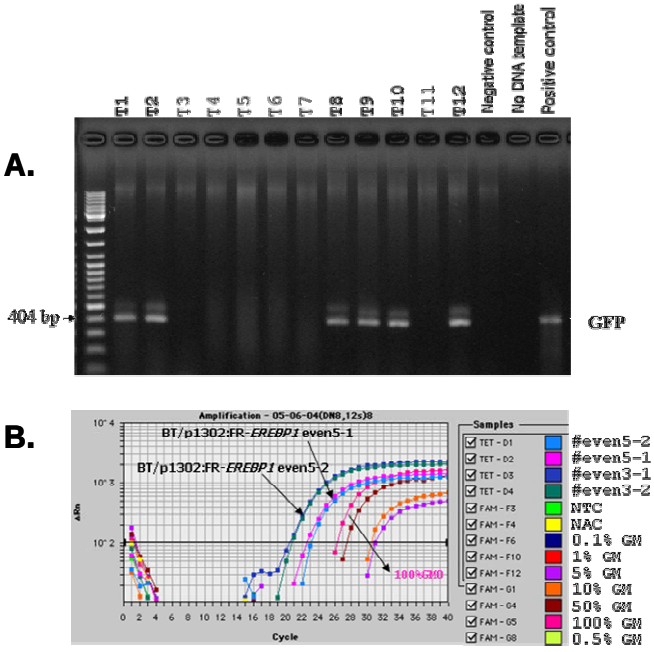


Figure 30 **A.** Amplified products were generated from T_1 plants of event 5 transformation using PCR-based with specific primers of a reporter gene (*GFP*). The 404 bp amplified fragments were positively found as potentially transgenic.

B. Real-Time with TaqMan *CaMV* probe was used to confirm a transgenic plants.

Growth of T_1 plants

We examined the effect of *OsEREBP*1 on plant growth by monitoring the height of T_1 plants after germination. During three weeks after germination, the heights of T_1 and control plants were different. (From 7 and 30 days after germination of T_1 plants) The transgenic plants grew more slowly than control plants. The plant height and the number of leaves during 21 days after germination was also effected

(Figure 31). However, the heights of T_1 and the control plants were similar for the next 2 weeks. Finally, the height of mature T_1 plants was also similar of that of control plants. Flowering date occurred 79 days after germination in T_1 plants, whereas it happened in wild type plants 68 days after germination. The mature T_1 plants had again a lower number of seed per ear compared to the controls.

Under flooding stress, the T_1 plants showed no significant difference of incremental shoot elongation with JHN wild type (*P>0.05*, n= 5). But they are different of 1.82 cm in plant heights. However, this trait is significantly (*P<0.01*) in T_1 plants and KDML105.

Table 12 Characterization of the transgenic rice plants (T_1)

Plant	**Seed fertility (%)**	**Plant height (cm)**		**Number of seeds per ear**
		3 weeks	5 weeks	
JHN (n=15)	94	8.8 ***(+/-1.2)***	13.48 ***(+/-1.4)***	137
Transgenic rice T_1 (n=15)	18	5.5 ***(+/-0.8)***	16.46 ***(+/-1.4)***	38

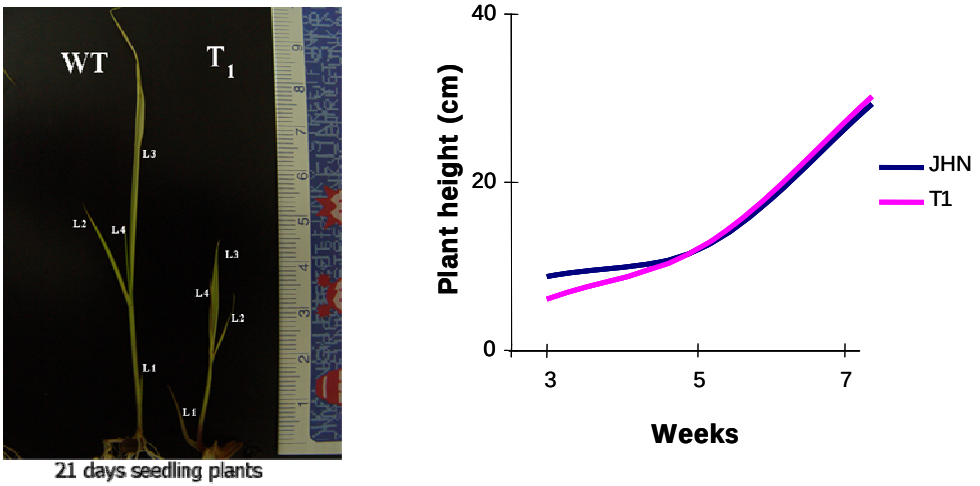


Figure 31 Increases in plant height during the growth of transgenic and control seedlings. Results are means from 15 plants in each case.

Southern hybridization analysis

The number of copies of the *OsEREBP*1 transgene was estimated by Southern hybridization. To avoid cross-hybridization of the probe for the transgene with endogenous *OsEREBP*1, we used the amplified product of *GFP* gene as the probe. Total DNA was isolated from seedlings of a mature T_1 plants, digested with appropriate endonucleases (*Eco*RV, *Hind*III, and *Eco*RI) and subjected to hybridization. One or two specific bands were obtained with three of the restriction endonucleases, indicating that *OsEREBP*1 had been introduced as a single-locus gene into their genomes of most T_1 plants (Figure 32).

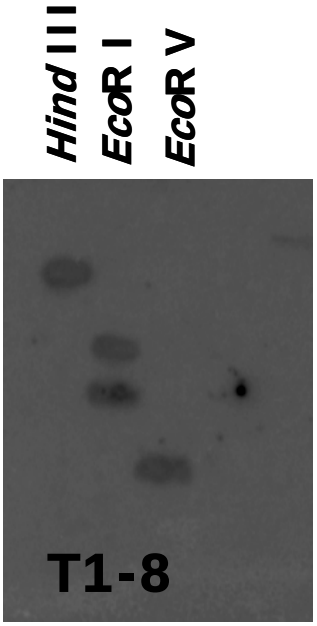


Figure 32 Results of Southern hybridization of genomic DNA. Aliquots of 15 μg of total DNA were digested with *Hind*III *(lane* 1), EcoRI *(lane* 2), *Eco*RV *(lane* 3), and digests were fractionated by electrophoresis on a 0.8% agarose gel. Bands of DNA were transferred to a nylon membrane and allowed to hybridize with radioactive-P^{32} labeled the 404 bp of *GFP* gene as probe.

Overexpression of *OsEREBP1* gene in transgenic rice

To investigate the expression of the *OsEREBP*1 transgene, we performed mini-scale array hybridization with total RNA that had been extracted from entries young plants 3 and 7 weeks after germination. All PCR-amplified genomic DNA of genes underlining *subQTL* were spotted on the nylon membrane for hybridization. The expressed intensity of these genes was confirmed by RT-PCR. The result showed that T_1 transgenic plants contained an exogenous *OsEREBP*1 gene, as confirmed by southern blot and high expressed intensity of *GFP* gene, *OsEREBP*1 appeared to be expressed constitutively in normal condition. In JHN wild type, the level of transcripts of the endogenous *OsEREBP*1 gene was generally low and the mRNA

accumulation of *GFP* gene was not found in the normal condition. In addition RNA slot blot revealed that the expression of the *OsEREBP*1 transgene increased more than two time than the endogenous gene in wild type under submergence stress for two days (Figure 33).

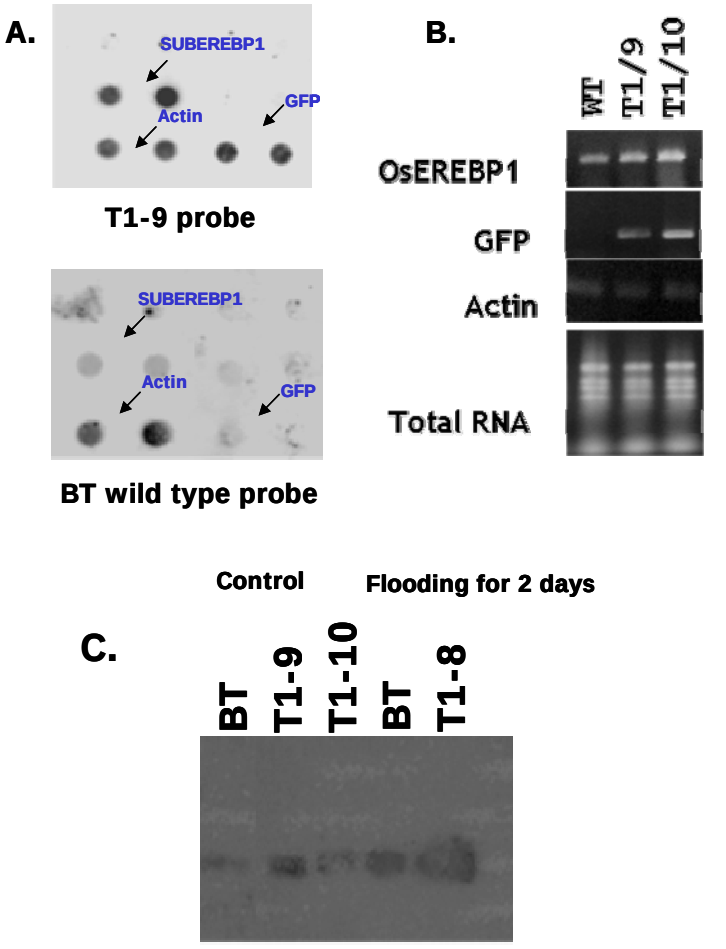


Figure 33 **A.** Mini-scale array analysis of gene expression in T1 plant contained overexpression of *OsEREBP1* comparing to JHN wild type.

B. Transcription level of introduced *OsEREBP1* gene, *GFP*, and actin gene were obtained in wild type and T1 plants using RT-PCR analysis.

C. RNA slot blot analysis of introduced gene *OsEREBP1* in submergence and control

Flooding tolerance of transgenic plants

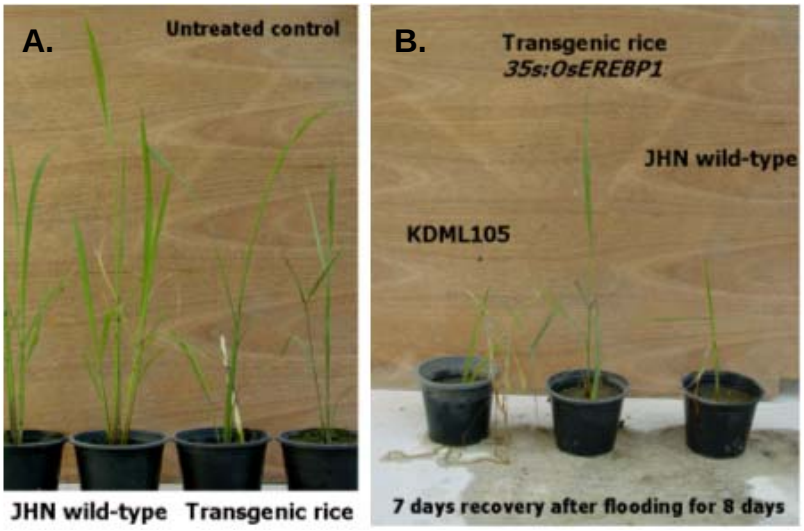


Figure 34 **A.** Phenotype of rice plants (JHN wild-type, transgenic rice) were observed under untreated control condition.

B. After submergence stress for 8 days, plants (JHN wild-type, transgenic rice and KDML105) were returned to untreated control condition.

The tolerance to flooding of transgenic plant was analyzed using *35S:OsEREBP1* plants grown in pot at air grown condition for three weeks. Under untreated control condition, we found visible characteristics of retardation in plant growth, low number of tiller and dark green leave in the transgenic plants In contrast to flooding stress, plant adaptations showed no significant correlation between JHN and transgenic rice in plant shoot elongation and leaf senescence under submergence for 8 days. However transgenic plants have a better recovery than JHN and KDML105 after de-submerged for 7 days (Figure 34).

DISCUSSION

Gene Identification and Density

In fact, abiotic stress tolerance and particularly submergence is the priority target trait in Southeast Asia countries dealing with rice plants. Among cereals, rice has the smallest genome with a size of only 430 Mbp. Consequently, the complete set of genes and their genomic locations could be identified *via* genomic sequencing with an acceptable investment. Chromosome 9 is the second smallest chromosome among the total of 12 chromosomes. At the short arm of chromosome 9 is located the only nucleolar organizer region (NOR) of the rice genome and this size is 22 Mb or 6.3% of the rice genome. However, a major QTL controling submergence tolerance was mapped at the long arm. Several evidences suggested that reduced plant elongation was a critical morphological change for rice to survive during submergence (Siangliw *et al*., 2003: Toojinda *et al*., 2003). This critical region might be associated with the regulation of the plant adaptation under flooding.

The candidate genes approach is one such tool as a promising method of merging QTL analysis with the extensive data available on the cloning and characterization of genes. Of the 53 genes identified by the prediction programs in the 1.35 Mbp contig, only two lacks intron while the others contain from 1 to 53. The gene density with non-TEs related protein coding sequence is about one gene per 6.89 kb, different to those obtained from other rice BAC/PAC clones sequenced, resulting one gene per 9.9 kb (International Rice Genome Sequencing Project). However, our result is consistent with the gene density reported by Yu *et al*. (2005) for the entire *japonica* genome. This report excluded that the TE-related genes were found every 7.2 in the genome.

Retrotransposons in the *subQTL*

Retrotransposons, a group of mobile elements that transpose via the RNA intermediate, are important components of the eukaryotic genomes (Boeke and Corces, 1989). The structure of retrotransposons resembles to the one of integrated retroviruses, with long terminal repeats (LTRs), internal domain encoding a group-specific antigen and a polyprotein (Pol). The Pol region has conserved domains characteristics of protease, reverse transcriptase, integrase and RNase H genes. This region is present in both retrotransposons of 0.65 Mbp-region. All clones containing retrotransposons are currently in the annotated rice BAC/PAC database. About half of the retrotransposon present in this region are LTR retrotransposons. Many of the rice retrotransposons including hypothical protein genes, such as the two described here, are pseudogenes because they are interrupted by stop codons, no termination codon in CDS, frameshifts or no initiation codon in CDS. Thus, the rice genome indeed contains many retrotransposons, although many are inactive.

Functional gene analysis

Under flooding, plant limits oxygen supply and gass diffusion which directly cause anaerobiosis of submerged plant parts or tissues. Low oxygen supply impedes mitochondrial respiration, since dioxygen is the final electron acceptor in the respiration chain. As a consequence, submergence-induced elongation is mediated by the interplay between three phytohormones: ethylene, abscisic acid and gibberellin. Most of plant responses to abiotic stress are controlled by a regulator of transcription or a sensing molecule in hormonal signaling pathway. In total of 24 predicted genes in the *sub*QTL region, eight of them are a nuclear locarization protein, five of them are a functional protein in mitochondria and chloroplast, while three of them are cytoplasm protein. The remainders are predicted to be a plasma membrane or transmembrane proteins that act as a protein receptor in signaling pathway. Based on biological functions and similarity search using computational tools, these predicted genes might control a part of plant adaptation under submergence stress.

In the last decade, several monomeric and heterotrimeric guanine nucleotide binding proteins have been identified to associate with secretory vesicles and to be implicated in exocytosis. According to SUB09 (*OsRAS*), the role of the small GTP binding protein in submergence tolerance is intriguing. So far GTP binding proteins play roles as signal transducer for gibberillin sensitivity in several dwarf mutants in arabidopsis (Peng *et al*., 1997), maize (Winkler *et al*, 1994) and wheat (Peng *et al*, 1999). Recently, an alpha subunit of GTP binding protein was identified in *d1*, a rice gibberillin unsensitive dwarf mutant (Ashikari *et al*., 1999). Damage caused by submergence stresses and enhanced by exogenous application of gibberillic acid was related to GA-promoted elongation underwater (Setter and Laureles, 1996; Setter *et al*., 1997). On the other hand, GA-deficient mutants were submergence tolerant just as susceptible lines when fed with the GA-biosynthesis inhibitor. In a study comparing submergence tolerant and intolerant doubled haploid lines, exogenous GA promoted elongation under water of both group and rendered the progenies tolerant (M. Jackson, personal communication).

Transcriptional Profiling of Genes Expression in Response to Submergence Stress

Among 29 analyzed genes, two genes showed differential expression variation in FR13A, a tolerant line, in submergence response. Macroarray analysis revealed the putative gene encoding Ras-related GTP-binding protein and Ethylene responsive binding protein were differentially up-regulated in the submergence tolerant strain. The validated result by Real-time RT-PCR analysis confirmed these differential expressions. These results suggested that two genes had their expression affected by submergence stimulation of FR13A.

Submergence stress causes abrupt changes at molecular, physiological, and morphological levels in rice plants. One obvious change is the internode elongation, in particular on the newly emerged leaves under submergence. Susceptible rice strains are fast elongating while the tolerants ones are non-responsive (Toojinda *et al*., 2003).

In flash flooding, the plant elongation of FR13A was suppressed, whereas KDML105 were 2 time longer than FR13A during submergence for 8 days. However, there was no difference of plant elongation under normal growth condition between FR13A and KDML105. This finding suggests that lowest elongation growth is benefit to rice plants subjected to complete flooding. Setter and Laureles, (1996) demonstrated that rapid elongation during flash flooding might reduce plant survival because of the maintenance processes for energy.

The small GTP-binding protein function in submergence tolerance is intriguing. Several evidences suggested that reduced plant elongation was a critical morphological change for rice to survive during submergence (Siangliw *et al*., 2003). Results from macrarray analysis showed that the *Ras* gene from FR13A expressed underwater at 2 days. However the *Ras* gene from KDML105 was suppressed after 2 days under flash flooding condition. Therefore, the expression of *Ras* gene was correlated to the tolerant phenotype under flash flooding. Flooding affects several morphological changes including a dwarfism. However, this is a distinct phenotype which related to the response of rice to survive under submergence stress. Kameda *et al.* and Sano *et al*. (1992, 1994) studied the *rgp1* function in transgenic rice and tobacco. The result revealed that the transgenic *rgp*1 showed phenotypic association of a apical dominance reduction, many tillers, dwarfism, abnormal flower structure and viral and bacterial infection resistance enhancement. Based on genomic sequence similarity and a cluster of identical proteins between *Ras* and *rgp*1, this seems to indicate that the *Ras* might have the same function as *rgp*1, related to the cytokinin biosynthesis and/or related to metabolic pathways. However no clear evidence was presented under flash flooding.

Several reports have shown that transcription factors are important in regulating plant responses to environmental stress. Ethylene responsive element binding proteins (*EREBPs*) have been identified from various plants as a transcription factor that is necessary and sufficient for transcriptional control under stresses responsive conditions and developmental processes by ethylene. Our macroarray

analysis result showed that the putative *EREBP* gene from FR13A was suppressed after 2 days under submergence whereas *EREBP* from KDML105 was up regulated under submergence after 4 days. The result suggests that reduced expression of *EREBP* gene in FR13A is correlated to the dwarf phenotype under flash flooding. However, the constitutive expression of the putative *EREBP* in KDML105 is correlated to the submergence-stimulated elongation growth. Kende *et al*., (1998) reported that rapid elongation under submergence stress was controlled by ethylene in the oxygen deficiency condition.

Other genes of which we demonstrated their altered expressions also seem to be involved in submergence response of intolerant line. Expression of three known-function genes, 1-aminocyclopropane-1-carboxylic acid (*ACC*), amylase (Amy3), and G-alpha subunit (D1), was induced as a submergence response as in the previously reported case (as reviewed by Ito et al., 1999). ACC synthase is one of the enzymes involved in ethylene biosynthesis. Jackson et al, (1997) found that the accumulation of ethylene in completed submergence affects the chlorosis and rapid elongation of rice that is observed in susceptible line. Adaptation to submergence is complex process involving several hormones. Gibberellin is one of the phytohormones, which play an important role in regulating elongation growth. Two genes encoding for amylase and G-subunit (*D1*) were used to explore the GA response and GA biosynthesis in plant shoot elongation. Under submergence stress, high levels of GA were observed from plant tissues. The expression of two genes in KDML105 was correlated for the rapid elongation under flash flooding. This finding suggests that this phenotypic change might be caused by the GA accumulation in submergence response. Anaerobic response of rice plant observed in the adaptive metabolic mechanism showed increasing of the alcoholic fermentation rate under submergence stress. Moreover, pyruvat decoboxylase (PDC), a key enzyme in alcoholic fermentation, has its expression involved in glycolysis and alcoholic fermentation. Uddin et al., (1998) demonstrated that increased expression of PDC was associated with tolerant rice varieties. According to our result, the transcription of PDC under submergence in normal light condition was up regulated in KDML105, the intolerant

line. However, the lower transcript of PDC was observed in FR13A, the tolerant line. Yamada (1959) reported that rice plants photosynthesize in the light even when submerged. It seems likely that rice plants under submergence in the light are not in an anaerobic state because of the presence of oxygen generated by photosynthesis.

Small GTP-binding protein gene for submergence tolerance

Numerous small GTP-binding proteins have been described in various higher plants (Anai *et al*., 1991: Palme *et al*., 1992: Yang *et al*., 1993: Youssefain *et al*., 1993: Merkle *et al*., 1994). The main finding of this study is the identification and character-ization of the predicted *OSRAS* gene encoding a member of the Ras family related to GTP-binding proteins from rice. The predicted sequence analysis of the *OSRAS* protein confirmed that this GTP-binding pro-tein was highly homologous to the *RGP1* protein. The amino acid sequence of *RGP1* was similar to the Rab/Ypt subgroup of a small GTP-binding proteins involved in intracellular transport (Sano and Ohashi, 1995). Therefore, the sequence domain and expression pattern data from the *RGP1* would help explore the function of *OSRAS* related to the phenotypic changes of rice under submergence.

An amino acid sequence analysis revealed a strong similarity of *OSRAS* to other Ras-related GTP-binding proteins. Among the previous studies, small GTP-biding proteins of six species (rice, Arabidopsis, garden pea, soybean, tobacco, and bird's-foot trefoil) showed significant (75–98%) similarity to *OSRAS* (Sano and Youssefinan, 1991: Dombrowski and Raikhel, 1995: Nagano *et al*., 1993: Schiene *et al*., 2000: Haizel *et al*., 1995: Borg *et al*., 1997). This result showed that *OSRAS* is a member of small GTP-binding proteins belonging to the Ras family. Bourne *et al.* (1990) used a sequence-based analysis to identify five alpha helices in *RGP*1, and they also found the G-domain, which serves as an attachment point for the cycle between a presumably inactive GDP-bound and an active GTP-bound state. Moreover, Geyer *et al.* (1997) reported that the C-terminus of this predicted protein has a prenylation site of cysteine-containing motif CAAX, which is known to be

involved in post-translation modifications. This evidence suggests that this gene has a common topology structure with the Ras super-family of small GTP-binding protein.

In this study, the sequence variations of the putative Ras-related GTP-binding protein gene, including the promoter region and 3'-UTR, were investigated to locate the most variable regions. Similarly to other mutations in the coding regions, all variations found in the putative gene within different rice cultivars resulted from point mutations instead of insertion/deletion. The exons of the gene were highly conserved, whereas the introns manifested many random nucleotide variations. This result was consistent with the studies involving other plant species (Weining *et al*., 1995). When the putative rice sequence was compared with those from other plant species, such as *Ara-bidopsi*s, maize, tobacco, garden pea and soybean, the homology was ranging between 60 and 79% (Dombrowski et al., 1995: Nagano *et al*., 1993: Schiene *et al*., 2000: Haizel *et al*., 1995: Borg *et al*., 1997).

The 5' leader sequence comprising the promoter may be involved in gene regulation at the transcrip-tional or translational levels. At the transcription step, high nucleotide variation in this region may be involved in gene expression control through the initial binding of the RNA polymerase and the starting point selection (Greenfield, *et al*., 1992). The study of the regulation of the *RAB16* gene expression in rice revealed that the *ci*s-acting element and ABA-responsive elements are conserved in the pro-moter region and these elements interact with different transcription factors *in vivo* (Ono *et al*., 1996). Hsieh and Huang, (1998) reported that the 5'-flanking region of the rice metallothionein like gene homologous to regulatory protein was identified. Such regions included two TATA box, one CAAT box and several short sequences. The losses of putative TATA-boxes on the promoter were related to the stress response. Mistsunaga *et al.* (1994) reported that the sugar-regulated gene and the *RAmy3D* gene were specifically up regulated in the presence of the protein-binding sequence motif of the promoters. The SSCP analysis showed the correlation between the different ssDNA patterns of the product amplified with the primer set A on the promoter region and the sequencing results. This analysis indicated that there was a

nucleotide change between the submergence-tolerant and intolerant-rice cultivars. According to the SSCP results, the alteration of a submergence-tolerant response could result from the effect of a putative small GTP-binding protein gene expression and the variation in the promoter region including TATA-box. In this study, the identification of submergence-tolerant from among seven different rice cultivars was demonstrated by using SSCP markers and QTL analysis. The results suggest that plant survival after submergence correlated with the sequence divergence level on the promoter region. The SSCP markers linked with submergence tolerance provide a superior selective screen to assist the breeding of submergence-tolerant genotypes.

Genetic linkage between submergence tolerance and shoot elongation was clearly shown by QTL analysis in segregating recombinant inbred lines (Sano *et al*., 1991). Both submergence tolerance and suppression of elongation were coincidentally mapped on the chromosome 9 region where the GTP binding protein gene was identified. By using the SSCP marker developed from the *OSRAS* gene, the gene position located in the major QTL of submergence tolerance was identified. The *OSRAS* gene was mapped around 0.3 cM from RFLP marker R1164, with a physical map distance approximately 4.0 kb.

In conclusion, we were able to identify the *OSRAS* gene coding a Ras-related GTP binding protein, which was highly homologous to *RGP1* of Ginbozu rice cultivars. The *OSRAS* gene was differentially expressed under a submergence stress and was mapped on chromosome 9 near the R1164 marker. Although it is reasonable to conclude that there was a differential expression of a gene encoding a Ras-related GTP-binding regulatory protein from the tolerant line under flash-flooding conditions, a complementation experiment is needed to explore the function of the gene. The GTP-binding protein function related to a signal transduction associated with the submergence tolerance was not found. By screening a FR13A BAC library, we identified several clones of the small GTP-binding protein gene which will help to finally elucidate the precise functional and a physical organization of this gene. Finally, it can be expected that a small GTP-binding protein gene associated with

submergence tolerance in rice should be very useful for promoting breeding process in submergence stress-tolerant rice.

Characterization of the NILs for *OsEREBP*

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*., (2004) reported that the complex phenotype-associated traits such as leaf senescence, percent plant survival, suppression elongation, and some others were coincidently mapped at subQTL9 for submergence tolerance identified as a major QTL. The main finding in this study is the characterization of the NILs-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 a 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 NIL-132 of BC4F8 in comparisons between three genotypic classes. Each plant contained a segment in the same region which is different in each class (DH206 segment for NILs-CC, KDML105 segment for NILs-cc, and heterozygous for NILs-Cc). This study showed that the NILs-CC and NIL-Cc obtained a high percentage of plant survival with Gene Targeted Markers (GTMs). The result of this study suggested that the region of 16.7 kb 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 NILs of BC4F8. A highly significant statistic of regression analysis was observed with Gene Targeted Markers (GTMs), RB0783 and OSER2, which contributed at a high percentage of phenotypic

changes in total shoot elongation (TSE) and Chlorophyll content. Since BC4F8 is only 94% similar to KDML105 with 6% of its alleles being from DH206, the effects of plant adaptation under flooding are mostly caused by different alleles at the interval region of 16.7 kb containing three predicted genes. The new developing ISLs for one gene at one locus and their function can be inferred by gene cloning and several computer analysis tools.

Expression of plant elongation under submergence

The results from physiological adaptation revealed a strong pattern of shoot elongation among three genotypic classes explained as validation data of TSE and RSE during flooding. The higher level of TSE of the NILs-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 NILs132-CC and NILs132-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, 1981) and leaf blade (Jackson *et al*., 1987). Slow leaf sheath elongation or suppressing elongation at leaf sheath of individuals NILs132-CC and NILs132-Cc might be an effective role of the genotypic classes.

Finding a link in plant adaptation between fast elongation underwater and susceptibility to flooding was not positive in the past experiments (Yamada, 1959; Mazaredo and Vergara, 1982). We were able to obtain a rapid plant elongation, inresponse to submergence stress, for the three genotypic classes for few days. The susceptible homozygous NILs-132cc showed a prolongation of plant growth on shoot elongation whereas the plant growth was suppressed in both NILs-132CC and NILs-132Cc. However, the plant growth did not differ in control plants of each class. The relationship between susceptibility and resistance to fast elongation underwater was recently reported by Toojinda et al., 2001 and Siangliw *et al*., 2001. Plant growth results compared between flooded and normal conditions were revealed that the

ability of plant growth of NILs-132CC and NILs-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 Laurels, (1996). Vatapetain and Jackson (1997) revealed that the plant actively growing during submergence has much more susceptibility than the slowly growing plant in submergence response. The advantage for the plants of suppressed elongation might be regulated by their genotypic allele to maintain the energy source necessary for the plant to recover after de-submergence.

Expression of plant senescence under 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 promotion of chlorophyll content under flooding stress (Jackson and Ram, 2003). The ability to stay green (or leaf senescence) underwater is similar to responsiveness to survival and the NILs population were screened for their reaction of survival. The senescent leaves were obtained for intolerant homozygous of NILs-cc but the tolerant homozygous NILs-CC and heterozygous NILs-Cc stayed green during flooding for 20 days. ANOVA analysis of chlorophyll content data showed high significance among genotypes which vary across time. However, no significant chlorophyll content 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 rate was shown to be highly positive (Toojinda *et al*., 2003). Moreover, a strong negative correlation between elongation and survival was found among four different cultivars (Singh *et al*., 2001). The leaf senescence and tolerance score were indeed genetically

linked to elongation during submergence of the rice plant. The rapid plant elongation and leaf senescence negatively correlated to plant survival in rice was a parallel to the responses given by exogenous ethylene (Jackson *et al*., 1987). All these data suggested that plant elongation and leaf senescence were directly linked to the physiological adaptation and plant survival after submergence.

The results can classify the NILs-132 into 2 classes. First, (NILs-CC and NILs-Cc) represent a 16.7 kb tolerance segment strongly related to the ability to limit leaf elongation, to stay green and to survive including recuperatation after submergence. While NILs-CC is homozygous for the tolerance segment and NILs-Cc is heterozygous. The second class compared by NILs-cc dose not has the tolerance segment and have different phenotypic expression under water. The physiological adaptations related directly to the survival ability. Although it is reasonable to conclude that NILs-classes consist of a genotypic allele of a donor, DH206, the 16.7 kb range was related to the tolerance trait under submergence on plant shoot elongation and chlorophyll content. Finally, the NILs-132 containing a tolerance segment of 16.7 kb associated with submergence tolerance in rice should be very useful to promote the breeding process in submergence stress-tolerant rice.

Effect of flooding on the expression of two EREBP genes at *subQTL* region

The physiological adaptations to submergence stress are controlled by the plant hormone (ethylene) and involved specific activation of target genes. They protect cells against anaerobic conditions or participate in the regulation of the flooding response. In anaerobic conditions under flooding stress, an ethanolic fermentation pathway directly increased an accumulation of ethylene within shoots which are effected in tissue chlorosis and rapid shoot elongation. Consequenctly to the ethylene signal transduction steps, the ethylene responsive element binding protein genes (*EREBP*) have been identified in various plants (Young *et a*l., 2004; Magnani *et a*l., 2004), which are necessary and sufficient for transcriptional control under stress responsive conditions and developmental processes by ethylene.

In the study, mini-scale array and RT-PCR using total RNA extracted from NILs-CC, NILs-Cc, and NILs-cc plants of isogenic lines showed that *OsEREBP1* associated with tolerance symptoms was expressed at a high level under flooding for 2 days in NILs-CC and NILs-cc plants, while *OsEREBP2* was unresponsive in gene expression. However, the *ACO*1 gene was reduced at the same condition. For the NILs-cc type, the transcription level of *OsEREBP*2 gene as well as *ACO1* gene are submergence stress for 2 days. *OsEREBP*1 gene did not responed to submergence stress. As results, we suggest that the reduction of ethylene biosynthesis might occur in these of CC and Cc NILs while the process of ethylene synthesis could be activate in response to submergence in cc near isogenic line. Under water, the lower content of oxygen in plant part increases the ethylene accumulation. According to the expression of the ACC synthase gene, a rate limiting step in ethylene biosynthesis was up-regulated under submergence within 1 hour (Zhoul *et al*., 2001). Then, ethylene is synthesised from *ACC* by the ACC oxidase (ACO, EC 1.14.17.4) in a non rate-limiting step. That expression of *OsEREBP1* gene might have an effective role in the capacity to convert ACC to ethylene, which is a benefit for a tolerant plant in flooding response. In Arabidosis, *ERF*1 gene encoding an Ethylene-Response-Element-Binding-Protein (EREBP) showed that an overexpression of *ERF1* gene results in the activation of ethylene response genes (Solano *et a*l., 1998). The mechanisms in nuclear events by ethylene signaling pathway are similar in various plants and physiological responses to ethylene in plants are regulated mainly at the transcriptional level (Bleecker and Kende, 2000).

Promoter analysis of the candidate genes at the interval region of 16.7 kb

Ethylene is one of the important phytohormones that plays multiple roles in physiological and developmental processe including the life cycle of the plant (Mattoo and Suttle, 1991; Abeles *et a*l., 1992). It also regulates plant response to abiotic stresses such as submergence stress. Consequently, some parts of the ethylene signaling pathway are controlled by members of a family of transcriptional factor that are specific to plants. In recent times, ethylene responsive element binding protein

genes (EREBP) have been identified in various plants (Young *et a*l., 2004; Magnani *et a*l., 2004), which are necessary and sufficient for transcriptional control under stress responsive conditions and developmental processes by ethylene. A highly conserved DNA binding domain known as the ERF domain is the unique feature of this protein family. These proteins act as transcriptional activators or repressors of GCC-box mediated gene expression which may affect plant adaptation in the regulation of the responsive genes. In addition, other plant hormones such as auxin, ABA, cytokinin, gibberellins and brassinosteroids were reviewed in the complex regulations that are often mediated by ethylene (Chen *et a*l., 2005).

Cis-acting elements play important roles in gene regulation under stress conditions. There are also involved in cross-talk among plant hormones which have been a topic in plant responses to abiotic stresses for decades. As a results two genomic sequence of ethylene responsive element binding protein (EREBP) gene were determined by computational tools. Both genes contained a conserved domain of EREBP/AP2-type that was classified into a subfamily of transcriptional factors (Riechmann and Meyerowitz, 1998). ERF-related motifs have been reported in the promoter region of ethylene responsive gene. This DNA-binding domain, highly conserved, interacts specifically with sequences containing AGCCGCC motifs (GCC box) (Hao *et a*l., 1998). Several GCC-binding proteins have been shown in participating in signal transduction events in plant response to abiotic stress. For example, DREB1A and DREB1B showed significant tolerance under cold and drought stress condition in Arabidopsis (Liu et al., 1998). The EREBP protein has a DNA binding motif which is involved in ethylene responsive gene expression in tobacco (Ohme-Takagi and Shinshi, 1995). In addition, the promoter region of two *OsEREBP* genes contained many *ci*s-acting elements associate with many phytohomones such as: 4 copies of MYC recognition site for ABA signaling, WRKY repressor protein for GA signaling on *OsEREBP1* and many repeats of ERE element relate with senescence for ethylene biosynthesis. Combinatorial control among phytohomones was reviewed in many ways on gene regulation. Lorenzo et al., (2003) found that the ERF1 transcription factor plays on an intersection point for both JA and

ethylene signaling pathway. The microarray studies in cell expansion revealed that 40% of all BR-induced genes are up-regulated by auxin (Goda et al. 2004; Nemhauser et al. 2004). Therefore, the regulation of gene expression in response to submergence stress might be controlled by complex interactions among hormones such as GA, ethylene and ABA. Plant adaptations under submergence condition may be provided by the competition of phytohormones at different levels.

Overexpression of *OsEREBP1* in transgenic rice

Current molecular technology as a complementation experiment is needed to explore the understanding of mechanisms developed by rice to survive submergence. To address the role of *OsEREBP1* in rice under flooding, a sense-oriented 0.678-kb single exon gene derived from *OsEREBP*1, which had originally been amplified from the tolerant line, FR13A, was inserted into the pCAMBIA1302 vector at the *NcoI* and *SpeI* sites. No obvious phenotypic change was found in the T_0 and T_1 transgenic lines during their vegetative phase, but the transgenic lines showed late germination during three weeks after seedling. The transgenic T_0 plant grew to maturity and had no distinct morphological characteristics. It was short in stature, with early bowering and tillering. All these features were stably inherited by the T_1 and T_2 progeny. In the T_2 progeny, although the number of seeds per ear was slightly lower than in wild-type plants, the number of tillers was higher and consequently the total number of seeds was 2.6-fold superior than in wild-type plants.

CONCLUSION

Genomics approache allows large-scale analysis of gene characteristics (structural genomics) and expression (functional genomics). The 0.73 Mb finished sequences from 6 PAC and 2 BAC clones encompassing the flooding tolerance on rice chromosome 9 was recently reported (http://www.ncbi.nlm.nih.gov/GenBank/index.html). The average physical to genetic distance of 112.3 kb per cM was surprisingly high considering the sequencing area is proximal to the centromere. On the basis of these findings, the gene density in the gene-rich region with none-TEs gene is around 6.89 kb/gene. High gene density with small physical to genetic distance makes this subcentromeric region particularly gene-rich and hotspot for recombination. Within the subQTL 0.65 Mb-region, all of 105 genes were predicted and confirmed by homology search. One feature that might contribute to high recombination hotspot is the high number of tranposible elements (TEs) and pseudogenes. Neither genes encoding enzymes involved in fermentation pathway such as ADH and PDC nor genes in glycolytic pathway were present in the QTL region. These genes were clustered into two distinct subgenomic regions surrounded by large number of retrotransposons forming two gene-dense islands. These two gene islands were essential for maintaining submergence tolerance. Genes located on the first island were involved in signal transduction such as the TRAP170-receptor like-kinase, and a monomeric GTP-binding protein. On the second island, two *OsEREBP*, ethylene response element binding proteins were located. At least in the case of submergence tolerance, genes underlining the *sub*QTL may play important roles in signal transduction pathways (specificity and cross-talk) which are involved in plant hormones.

In addition, we performed DNA macroarray analysis to investigate gene expression in response to submergence between FR13A, a tolerant line, and KDML105, an intolerant line which are associated with plant shoot elongation and general symtoms under submergence. Our findings suggest that some of the major functions in submergence tolerance of FR13A are regulated by ras-related GTP-

binding protein gene and ethylene responsive binding protein gene when their expressions are stimulated. Therefore, increased expression of these genes may play important roles in response to submergence tolerance. Moreover, the expression of two candidate genes was correlated to the tolerant phenotype under flash flooding. Flooding affects several morphological changes including a dwarfism. This is a distinct phenotype related to the response of rice to survive under submergence stress. By screening a FR13A BAC library, several clones of the small GTP-binding protein gene and Ethylene responsive element binding protein gene were identified. This will help to finally elucidate the precise functional and a physical organization of these genes.

Small GTP-binding proteins play critical roles in signal transduction in mammalian and plant systems. We were able to identify the *OSRAS* gene coding a Ras-related GTP binding protein, which was highly homologous to *RGP1* of Ginbozu rice cultivars. The *OSRAS* gene was differentially expressed under a submergence stress and was mapped on chromosome9 near the R1164 marker. Although it is reasonable to conclude that there was a differential expression of a gene encoding a Ras-related GTP-binding regulatory protein from the tolerant line under flash-flooding conditions, a complementation experiment is needed to explore the function of the gene. Morover, sequence variation of a small GTP-binding protein identified in the subgenomic region was analyzed. The major quantitative trait locus (QTL) controlling submergence tolerance on the 6.5-cM region of chromosome 9 was previously mapped, sequenced and annotated. One of the most interesting candidate genes located in this QTL was a 5.2-kb sequence, which included a coding sequence consisting of two exons and a promoter. The deduced amino acid sequence corresponded to a 24.8 KD protein consisting of 226 amino acids with 98% identity to *RGP*1, a small GTP-binding protein involved in a signal pathway responding to hormones, such as cytokinin and ethylene. According to the amino acid sequence, the putative small G-protein was classified as a small Ras-related GTP-binding protein. DNA gel blot analysis showed that the putative gene encoding the Ras-related GTP-binding protein was present as a single copy in the rice genome. Comparison of

genomic sequences from several rice cultivars tolerant to submergence identified single nucleotide polymorphisms located in the TATA box of the Ras promoter region. Linkage analysis showed that the putative gene for GTP-binding protein was tightly linked to the peak of the QTL previously mapped on the long arm of chromosome 9. The single strand conformation polymorphism of the putative GTP-binding protein gene can be used for allele discrimination and marker assisted selection for tolerance to flash flooding.

Understanding the genetics of submergence tolerance has been limited by the availability of Near Isogenic Lines (NILs). Discovering QTL associated with submergence tolerance facilitates the development of NILs for submergence stress. One hundred and thirty two NILs carry 16.7 kb region contained in the QTLchr9 were used to compare under controlled submerging conditions. The NILs were categorized into three groups as homozygous donors (DH206), NILs-CC, homozygous recipients (KDML105), NILs-cc, and heterozygous, NILs-Cc, by the three pairs of primers targeted at the target region. Each individual plant of the NILs 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 NILs-CC and NILs-Cc were similar. Rapid elongation was significantly higher in NILs-cc. All genotypes showed no significance with the leaf senescence. However they were shown to be significant when genotypes interacted to time-course. The accumulation of shoot elongation was mostly affected by the extension of leaf blade and leaf sheath. A prolonged flooding to reach the tolerance phenotype 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 Chlorophyl content (CT). There were significant differences in plant elongation and leaf senescence among classes. This study provides a powerful tool to improve flooding tolerance in plants.

To address the role of *OsEREBP1* in rice under flooding, a sense-oriented 0.678-kb single exon gene derived from *OsEREBP*1, which had originally been amplified from a tolerance line, FR13A, was inserted into the pCAMBIA1302 vector at the *NcoI* and *SpeI* sites. No obvious phenotypic change was found in the T_0 and T_1 of transgenic lines during their vegetative phase, but the transgenic lines showed late germination for three weeks after seedling. To extra study on the submergence experiment, the T2 transgenic lines will be investigated to explore the role of *OsEREBP1* under flooding condition.

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