

1 Flood tolerance mediated by the rice Sub1A transcription factor

Kenong Xu¹, Abdelbagi M. Ismail², and Pamela Ronald³

¹Department of Horticultural Sciences, Cornell University, New York State Agricultural Experiment Station, Geneva, New York

²Crop and Environmental Sciences Division (CESD), International Rice Research Institute (IRRI), Manila, The Philippines

³Department of Plant Pathology and the Genome Center, University of California at Davis, Davis, California

1.1 Introduction

Over one billion people, 15% of the world's population, live in extreme poverty. Most of these people live on farms and barely produce enough food for themselves and their families. The most economic and effective method for improving farm productivity is the planting of high-yielding and more resilient varieties that thrive on these farms. Varieties that are resistant to diseases and/or tolerant of environmental stresses can have dramatic and positive impacts on the lives of the very poor worldwide.

Rice is the staple food for more than half of the world's population. Flooding is a major constraint to rice production in South and Southeast Asia, where the majority of the world's rice farmers live. Each year, 25% of the global rice croplands are inundated by flash floods, which are unpredictable and can occur several times a year. Although rice is grown in flooded soil, most rice cultivars die within a week of complete submergence, causing yield losses ranging from 10% to total destruction (Mackill et al., 2012). These losses disproportionately affect the rice farmers in the world, where 70 million people live on less than \$1 a day.

Compounding the challenges facing rice production are the predicted effects of climate change. As the sea level rises and glaciers melt, low-lying croplands will be submerged and river systems will experience shorter and more intense seasonal flows, as well as more flooding. Most of the coastal rice production areas in the tropics and subtropics are vulnerable to such conditions, especially low-lying deltas along the coastlines of South, East, and Southeast Asia. Rice production in these deltas is the major agricultural activity. These areas include the Mekong and Red River deltas of Vietnam, the Ayeyerwaddy Delta of

Myanmar, and the Ganges-Brahmaputra Delta of Bangladesh. These deltas provide between 34% and 70% of the total rice production in these countries, and any reduction in rice production due to increases in the frequency of flooding will have serious consequences on food security (Wassmann et al., 2009). This is a challenge especially in places like Bangladesh, Eastern India, Vietnam, and Myanmar, where people get about two-thirds of their total calories from rice. Large areas of Bangladesh and India already flood on an annual basis and are likely to flood even more frequently in the future, leading to a substantial loss of agricultural land. In Bangladesh and India alone, 4 million tons of rice, enough to feed 30 million people, is lost to floods each year.

Thus, an important goal for improving the rural economy and livelihood in these vulnerable countries is to develop rice varieties that can survive flooding. Because most of the world's poorest people get their food and income by farming small plots of land, the availability of rice varieties with enhanced tolerance to flooding is expected to make a major difference in food security for these farmers.

Although rice can withstand shallow flooding, most rice varieties will die if completely submerged for more than a few days. There are a few rice landraces that can survive prolonged submergences, and these are of great interest to rice breeders. For example, the ancient Indian rice landrace, FR13A, has poor grain and yield qualities but is unusual in its ability to endure complete submergence for over 14 days.

FR13A has been known to farmers in Orissa, India, since the 1950s. For over 40 years, breeders at the International Rice Research Institute (IRRI) tried to use FR13A as a donor parent to introduce the submergence tolerance trait into varieties that would be useful to rice farmers. Although submergence tolerant varieties were developed, they were not widely adopted. The main reason is that because breeding was carried out with relatively crude genetic tools based mainly on visual selection, the resulting varieties lacked many of the traits desired by farmers in the major rice-growing areas of Asia. With lack of knowledge on the exact genes needed to confer submergence tolerance, the breeders unknowingly dragged in undesirable traits along with the submergence tolerance trait, which reduced yield and grain quality.

Over the last 15 years, we collaborated with Dave Mackill at the International Rice Research Institute and other researchers and breeders to carry out detailed genetic analyses of submergence tolerance in rice. Our long-term goal was to understand the underlying molecular mechanisms controlling submergence tolerance and generate tools that breeders could use to develop rice varieties with high yields and good grain quality that are tolerant to submergence. The results of this team effort led to the identification of the SUB1 locus and associated genes, development of rice "mega varieties" with submergence tolerance for farmers, and elucidation of the gene networks and physiological processes mediated by SUB1.

1.2 Isolation of the rice SUB1 locus

In early genetic studies, rice submergence tolerance derived from FR13A had been shown to have a relatively high heritability, with tolerance being partially to completely dominant (Haque et al., 1989; Mohanty and Khush, 1985; Mohanty et al., 1982; Sinha and Saran, 1988; Suprihatno and Coffman, 1981). The trait was also thought to be controlled by one or a few loci with major effects and loci with smaller, modifying effects. On the basis of these studies, we began to investigate submergence tolerance using an approach combining the power of molecular markers and quantitative trait locus (QTL) analysis. This initial study employed a population (DX18) of 169 F₂ plants and their resulting F₃ families that were derived from a cross between two breeding lines, PI613988 (japonica) and IR40931-26 (indica), the latter of which inherits strong submergence tolerance from FR13A. Kenong Xu and David Mackill demonstrated that a major QTL, SUB1, mapped between two restriction fragment length polymorphism (RFLP) markers (C1232 and RZ698) on rice chromosome 9 (Xu and Mackill, 1996). The SUB1 QTL was supported with a logarithm of odds (LOD) score of 36 and accounted for 69% of phenotypic variation in the F₂ population, concluding that SUB1 is critical for conferring submergence tolerance in rice. Simultaneously, other teams (Kamolsukyonyong et al., 2001; Nandi et al., 1997; Toojinda et al., 2003) also reported the strong phenotypic effect of the SUB1 locus, confirming its effect as the major determinant of tolerance, besides few other minor QTLs.

Previously, the Ronald laboratory had successfully used an approach of “positional cloning” to isolate a rice gene, called *Xa21*, that conferred broad-spectrum resistance to a serious bacterial disease in Asia and Africa (Song et al., 1995). This experience encouraged us to take the same approach to isolate the SUB1 QTL although it was challenging because a QTL for an important agronomic trait had never before been isolated from a staple crop species, and the rice genome had not yet been sequenced.

We first carried out fine mapping of the SUB1 QTL to characterize the SUB1 region with more markers in a large F₂ population (DX202) of 2,950 plants, which was derived from a cross between M202 (a widely grown japonica rice cultivar in California) and DX18-121 (a tolerant line from population DX18, see above). The resulting SUB1 fine map comprised ten amplified fragment length polymorphism (AFLP) markers. Two of these markers co-segregated with SUB1 and eight linked to SUB1 within 0.2 cM (Xu et al., 2000). The significance of this fine map is that it laid a foundation for physically mapping the SUB1 locus on rice chromosome 9.

We then carried out physical mapping of the SUB1 locus by identifying a set of five bacterial artificial chromosome (BAC) and 13 binary clones that overlapped each other and that entirely covered the SUB1 region (Xu et al., 2006). The five BAC clones were obtained from the two BAC libraries constructed

from rice cultivars IRBB21 and Teqing, respectively. Both BAC libraries were publically available, but IRBB21 and Teqing do not carry the submergence tolerance trait. The 13 binary clones were achieved from a genomic library constructed from the submergence tolerance parental line IR40931-26 using a binary vector that could be used to directly engineer rice plants. By developing more markers from these BAC and binary clones and analyzing the expanded F_2 population DX202 of 4,022 plants, we were able to delimit the SUB1 locus with a region of 182 kb between markers CR25K and SSR1A (Xu et al., 2006).

Complete sequencing of the 182 kb SUB1 region revealed that the region encodes 13 genes, including 3 that contain ethylene response-factor (ERF) domains, which were designated SUB1A, SUB1B, and SUB1C. We found that the corresponding SUB1 region in the sequenced genome of japonica rice Nipponbare (International Rice Genome Sequencing Project, 2005) spans only 142 kb and lacks SUB1A.

We next carried out an allelic variation survey of the SUB1 genes in 21 varieties (17 indica and 4 japonica). We identified two SUB1A, nine SUB1B, and seven SUB1C alleles. The SUB1A-1 and SUB1C-1 alleles are specific to all six submergence tolerant accessions studied, including FR13A, Goda Heenati, and Kurkurapan, which are of independent geographic origins. However, there was no such correlation between a specific SUB1B allele and submergence tolerance.

Using gene expression analysis, we found that SUB1A was rapidly induced upon submergence in the submergence tolerant variety. In contrast, SUB1C was upregulated only in the intolerant variety, M202. The expression of SUB1B was low and constant in both submergence tolerant and intolerant varieties. These data suggested that SUB1A controlled the SUB1-mediated submergence tolerance response.

To functionally prove SUB1A as the very gene underlying the SUB1 QTL, we created a construct containing the SUB1A-1 full-length cDNA under the control of the maize Ubiquitin1 promoter (Christensen and Quail, 1996) to overexpress SUB1A-1 in Liaogeng, a submergence intolerant japonica rice that also lacks SUB1A. Submergence screening of the resulting T_1 transgenic plants identified four independent T_1 families segregating for submergence. A detailed analysis of two of the four T_1 families showed a nearly complete correlation between high expression of the SUB1A-1 transgene and submergence tolerance. We therefore concluded that SUB1A-1 is sufficient to confer submergence tolerance to intolerant varieties, signifying the isolation of the SUB1 QTL (Xu et al., 2006).

This work was significant because it represented the first isolation of a QTL with an important agronomic effect and revealed an important genetic mechanism with which rice plants can control tolerance to submergence. Isolation of SUB1A and the 180 kb of genetic sequence surrounding the gene set the stage for advanced marker assisted breeding at the IRRI (Neeraja et al., 2007; Septiningsih et al., 2009; Mackill et al., 2012).

1.3 Sub1 rice in farmers' fields

Initially, the IRRI group monitored the SUB1 locus using markers closely linked with the gene. However, the availability of the sequences from BAC clone AP005907, which carried the sequences of the SUB1 genes, soon facilitated the development of six more markers tightly linked to the SUB1 QTL. This approach allowed for the transfer of the “donor” (SUB1) genetic region to be precisely monitored. The SUB1 donor FR13A variety carries many undesirable agronomic characters; therefore without knowledge on the precise location of SUB1A and the ability to select against other regions of the FR13A genome, these undesirable characteristics are dragged into the new variety along with SUB1 (Neerja et al., 2007). Thus, with the availability of the SUB1A sequence and other sequences in the region, the SUB1 locus could be precisely introduced into a wide range of recipient rice varieties favored by farmers, while at the same time minimizing the effects of “linkage drag” from the sub1 donor. This work resulted in the introduction of SUB1 into eight rice varieties popular in South and Southeast Asia. The first of these was the mega variety Swarna, which is grown on ca. 5 million hectares in India and on additional areas in Bangladesh and Nepal (Xu et al., 2006).

The new rice variety—called *Swarna-sub1*—was tested in farmers' fields in Bangladesh and India. In the absence of flooding both Swarna and Swarna-Sub1 yield 5–6 tons per hectare. However, in the presence of flooding, fewer plants of the Swarna rice crop survived (0–20% in most cases depending on floodwater conditions and duration; Das et al., 2009), whereas the Swarna-Sub1 rice flourished—80–95% of it survived. This enhanced survival means that farmers growing the Swarna-Sub1 variety gain a 1 to over 3 tons per hectare yield advantage following floods (Singh et al., 2009). Using this marker assisted breeding approach, the IRRI team has now generated and released several Sub1 varieties in six countries (Indonesia [4], Nepal [2], Myanmar [1], India [2], Bangladesh [2], and the Philippines [2]). In 2011, Swarna-Sub1 alone was estimated to have reached over one million farmers in South Asia (Mackill et al., 2012).

Over the last 5 years, our colleagues at IRRI have been working with India's National Food Security Mission, the Ministry of Agriculture, the government of India, and with state governments, non-governmental organizations (NGOs), and public and private seed producers and breeders in India, Bangladesh, and Nepal to multiply and disseminate Swarna-Sub1 seeds and seeds of other released Sub1 varieties and to strengthen the existing seed systems. The supply will aid various states in South Asia that do not have enough seeds to distribute to farmers.

The Bill and Melinda Gates Foundation is now supporting a large program, called *Stress-Tolerant Rice for Africa and South Asia* (STRASA; www.strasa.org), that is assisting with the development and dissemination of Sub1 rice varieties in three countries (<http://irri.org/news-events/irri-news/bill-and-melinda-gates-visit-strasa-and-csisa-projects-at-icar-research-farms-in-patna-india>). STRASA was conceived as a 10-year project with the vision of reaching about 20 million farmers in

South Asia and Sub-Saharan Africa by 2017. By 2014, Sub1 varieties are predicted to be grown in over 5 million hectares (Mackill et al., 2012).

We initially introduced SUB1 into a set of popular varieties including Swarna (also widely grown in Bangladesh and Nepal), Samba Mahsuri, and CR1009 (Savitri) from India; BR11 from Bangladesh; Thadkkham 1 (TDK 1) from Laos; and IR64 from IRRI-Philippines. More recently, SUB1 has been introduced into Ciherang from Indonesia and PSBRc 18 from the Philippines. These varieties were chosen because they are popular among farmers and consumers in rainfed lowland areas, each covering between 1 and over 6 million hectares. The flood-tolerant versions of these high-yielding “mega varieties” are effectively identical to their intolerant counterparts but survive better after severe floods to yield well. The grain quality of all Sub1 lines developed so far is essentially identical to the conventional varieties, with the extra advantages of fast recovery and earlier maturity (by 10–15 days) than their non-Sub1 counterparts following submergence for various durations (Singh et al., 2009). Breeders predict that the most popular Sub1 varieties like Swarna-Sub1 and BR11-Sub1 will soon entirely replace the existing non-Sub1 versions and spread to other flood-prone areas all over these countries.

Introgression of SUB1 into these varieties also facilitated the introduction of these varieties to regions where they were not known before; for example, Swarna-Sub1, which previously had only been planted in South Asia, has now been released in Indonesia, and Ciherang from Indonesia is in the final stages of release for flood-prone areas in Bangladesh and India.

We chose to introduce these popular varieties because they were well known to farmers, millers, and consumers, and therefore less time would be needed to evaluate and commercialize the new varieties. One difficulty with such success is that although there are now ample incentives for farmers to grow these mega Sub1 varieties like Swarna-Sub1 and BR11-Sub1, there is still little incentive to introduce additional rice varieties to enhance the overall genetic diversity of the rice planted in large areas as in India and Bangladesh. Breeders, geneticists, and agronomists know from past experience that monocultures can be vulnerable to other problems, such as yield stability. The issue is to balance the demand of farmers for high-yielding, high-quality, flood-tolerant rice varieties with the need to plant genetically diverse rice varieties to minimize possible future losses to pest and disease. For these reasons, IRRI decided to introduce SUB1 into all varieties being bred for rainfed lowlands, and a considerable number of breeding lines are now being evaluated at target sites in Asia and Africa. In addition, breeding lines combining SUB1 and drought tolerance as well as SUB1 and salt tolerance have been developed and are being field tested. These new breeding lines are useful for areas experiencing both flash floods and drought as in most rainfed lowlands, as well as submergence and salt stress as in tropical coastal areas of South and Southeast Asia (Ismail et al., 2008). Substantial efforts are also being undertaken by national programs to incorporate

SUB1 into additional local popular varieties as well as into new elite lines as in Vietnam, India, Bangladesh, and Thailand.

1.4 The SUB1 effect

SUB1 exerts its effect by limiting gibberellic acid (GA)-activated elongation growth and ethylene-induced leaf senescence. Complete submergence restricts light intensity, slows O₂ and CO₂ exchange between shoot tissue and floodwater, and enhances the accumulation of ethylene due to increased synthesis and entrapment. Ethylene accumulation triggers chlorophyll degradation and leaf senescence (Ella et al., 2003) and causes excessive elongation of leaves and internodes of the submerged plants in an attempt to maintain contact with air. This is mediated through ethylene-induced suppression of abscisic acid (ABA) synthesis but enhanced synthesis and sensitivity to GA (Das et al., 2005). Reduced photosynthetic capacity during and following submergence, together with excessive growth during submergence, results in severe carbohydrate starvation and consequent death of the submerged plants. In collaboration with Bailey-Serres at the University of California-Riverside, we have demonstrated that SUB1A exerts its effect by limiting GA-activated elongation growth and conserving carbohydrates (Figure 1.1). The SUB1 locus enables plants to endure complete submergence for prolonged periods due to activation of a “quiescence strategy” that conserves the shoot meristem and energy reserves until the flood subsides.

1.5 The SUB1-mediated gene network

In addition to flooding, other environmental stresses such as drought, salinity, and heat stress are predicted to be increasingly problematic for farmers as the climate warms. For example, in Africa, three-quarters of the world's severe droughts have occurred over the past 10 years (African Agricultural Technology Foundation, 2010). Losses to pests and diseases are also expected to increase over the next 50 years. Much of the losses caused by these pests, diseases, and environmental stresses, which already result in 30–60% yield reductions globally each year, occur after the plants are fully grown: a point at which most or all of the land and water required to grow a crop has been invested. Thus, there is a need to identify genes that confer robust tolerance to environmental stresses and diseases and to use this information to develop new varieties.

As part of this goal, we and others are using genomic, molecular-genetic, allelic diversity, and computational approaches to identify other genes and gene networks involved in tolerance to stress and devastating diseases. For example, we recently demonstrated the usefulness of transcriptomics and interactomics

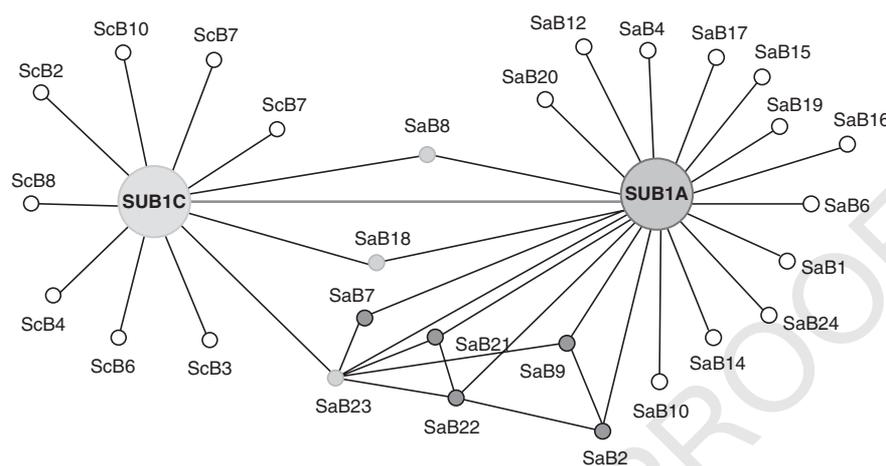


Figure 1.2 The rice Sub1A/Sub1C interactome. The interactome map represents 28 proteins identified from high-throughput Y2H screening using Sub1A and Sub1C as baits. Proteins in blue represent interactors with both Sub1A and Sub1C (Seo et al., 2011). For color details, please see color plate section.

approaches to identify genes and proteins that are part of the predicted rice SUB1A-mediated response network, and we have shown, through genetic analysis, that this approach efficiently identifies key genes regulating these biological pathways (Jung et al., 2010; Seo et al., 2011).

Transcriptional regulation plays a key role in development and response to abiotic stresses (e.g., Sub1A, CBFs, OsNAC5, dehydration-responsive element-binding proteins [DREBs], AP2/ERFs, WRKYs). In plants, regulation of gene expression is complex, with the majority of genes differentially expressed in different tissues and cell types. We have shown that a suite of 898 genes is differentially regulated during the SUB1A-mediated rice tolerance response (Jung et al., 2010). Notably, there are 16 genes encoding transcription factors (TFs) that are differentially regulated by SUB1A (Figure 1.2). Of these, ten AP2/ERF genes belong to the ERF subfamily and six to DREB.

Our results suggest that the SUB1 locus regulates the ethylene response using AP2/ERF genes in the ERF subfamily and stress tolerance response with AP2/ERF genes in the DREB subfamily.

To further elucidate the rice submergence stress response pathway, we used an efficient and reliable yeast two-hybrid (Y2H) screening strategy (Jung et al., 2008) to identify proteins that interact with Sub1A and Sub1C (a negative regulator of submergence tolerance at the same locus as Sub1A; Xu et al., 2006). Several million transformants were screened using Sub1A and Sub1C as baits (Seo et al., 2011). Five binding proteins were chosen for further screening as baits in the Y2H to identify additional proteins in the stress response network (Figure 1.2).

We determined that Sub1A and Sub1C interact and identified 28 Sub1A and Sub1C binding proteins (SaBs and ScBs, respectively). These interactions were reconfirmed through secondary screenings. Seventy-five percent of the interactions were validated *in vivo*, using a bimolecular fluorescence complementation approach in rice protoplasts (Seo et al., 2011). The interactome includes several candidate TFs that interact with both Sub1A and Sub1C. One such protein is SAB18, a putative trihelix protein. We analyzed insertion alleles of SAB18 obtained from the POSTECH collection (Jung et al., 2008). Homozygous insertion mutants in SAB18 displayed enhanced tolerance to submergence. Lines overexpressing SAB18 did not show a submergence tolerance phenotype (Seo et al., 2011). These results suggest that Sub1A (a transcription factor) may serve as a component of large and/or changing complexes *in vivo* (Seo et al., 2011). It remains to be determined how the Sub1A-associated complex(s) is reorganized in response to submergence and drought stress.

The flood of data from functional genomics, comparative genomics, and proteomics approaches now allows diverse aspects of gene function to be assayed on a genome-wide scale. Because datasets from each technique are incomplete, error-prone, and limited in sensitivity, none of them are sufficient to fully describe the biological role for a particular gene. However, such datasets can be integrated to generate a more accurate and comprehensive view of gene function than is contained in any single dataset. Probabilistic integrated gene networks—graphical models in which linkages between genes indicate their likelihood to belong to the same biological process—provide such an approach. Such a network can guide phenotypic predictions in varied tissues and developmental contexts.

Integrated networks have proven successful for unicellular and multicellular organisms, accurately predicting gene functions and gene loss-of-function phenotypes, and are thus powerful tools for generating testable biological hypotheses. Using probabilistic gene networks, the Marcotte (Lee et al., 2004, 2008, 2010) group has successfully demonstrated proof-of-concept for yeast, worm (*C. elegans*), and mouse. Each network model is highly predictive of gene function and for organismal phenotype following gene perturbation. These results indicate that researchers can efficiently gain new functional knowledge by prioritizing genes for a given biological role based upon gene networks, then testing these candidates using available reverse genetics resources.

We have used these approaches to develop an experimentally validated genome-scale functional gene network of rice genes, named RiceNet, covering the majority of encoded rice proteins. We have leveraged RiceNet to identify networks of genes governing the XA21-mediated response (Lee et al., 2011) and demonstrated that RiceNet successfully predicts gene function in rice and maize. Thus, RiceNet is broadly useful for dissecting complex immune response pathways and is particularly useful for identifying gene function in other monocot species such as wheat and barley, for which species-specific networks have not yet been constructed.

We are now using RiceNet to generate subnetworks of genes that are predicted to control Sub1A-mediated pathways, which are important for tolerance to submergence and possibly to drought. The advantage of this approach is that it facilitates non-biased identification of key networks predicted to control a particular biological response. Instead of working on a single gene predicted to have a function, researchers can study entire networks, including genes that would not have been predicted to function in the network using standard genetic and proteomics approaches.

1.6 Conclusion

From the start, the Sub1 project was guided by the needs of small-holder farmers, adapted to local circumstances, and sustainable for the economy and the environment.

The Sub1 project revealed that it was possible to identify important agronomic traits, which were thought to be quite complex, identify genes underlying these traits, and use this knowledge of gene sequences and function to develop new crop varieties that can immensely benefit farmers. It is clear from our work and that of others that this type of approach will greatly accelerate the pace with which plant geneticists and breeders can develop new crop varieties and/or improve the resilience of existing popular varieties. This approach is now being extended to other abiotic stresses such as drought and salinity, where major QTLs were identified (Thomson et al., 2010; Mackill et al., 2012), and is also being used to combine tolerances of multiple abiotic stresses.

One of the important aspects of the Sub1 project was its highly collaborative nature. Specifically, the combination of molecular geneticists, physiologists, and breeders working together and freely sharing material greatly enhanced success of this project. For example, early on, prior to publication, our laboratories shared the SUB1 genes and our Sub1 rice lines with other laboratories. This “open science” approach facilitated the breeding collaborations and also advanced our understanding of Sub1 function.

The discovery of SUB1 enabled the conversion of eight popular varieties into submergence tolerant types using marker assisted backcrossing; five of these have been commercialized in several countries in Asia and the rest are in advance stages of release. SUB1 effectively works in all environments and genetic backgrounds, from crop establishment until flowering. SUB1 has no observable effects in the absence of submergence but substantially improves survival and yield following transient complete flooding for 4–18 days in farmers’ fields. The positive impact of Sub1 varieties has been recognized in several countries, triggering enormous interest and additional resources by national programs to produce and distribute sufficient seeds of these varieties to all farmers in areas affected by flash floods.

The identification of genes that modulate SUB1-mediated tolerance to flooding will be useful for the development of “Sub1^{plus}” varieties that have a higher and wider range of tolerance. Such enhanced tolerance will be needed as farmers face a future predicted climate of unusually heavy rainfall. Additional QTLs/genes need to be pyramided with SUB1 for higher tolerance for areas encountering longer duration of flash floods. Several studies already identified few minor QTLs affecting submergence tolerance independent of SUB1 (Nandi et al., 1997; Toojindda et al., 2003; Septiningsih et al., 2012); however, exploring these QTLs is challenging because of their relatively smaller effects compared with SUB1. Furthermore, SUB1 needs to be introgressed into varieties that tolerate partial stagnant floods common in most rainfed lowlands, where floodwater of 20–50 cm depth stagnates in the field for several months (Singh et al., 2011). None of the modern rice varieties developed to date, including Sub1 types, can withstand this type of flooding; however, recent efforts at IRRI have established the possibility of combining both SUB1-mediated tolerance to transient, complete flooding and tolerance to stagnant flooding.

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