



REVIEW ARTICLE

# Adaptive mechanism of submergence tolerance by *Sub1 A*

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## Abstract

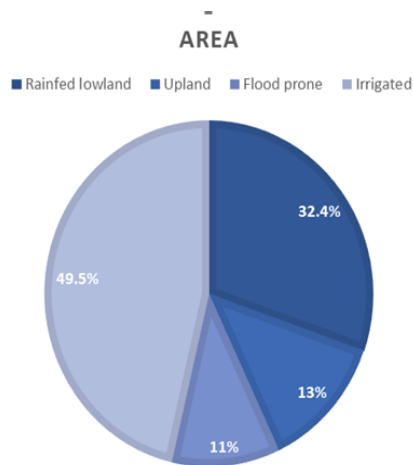
Among the various abiotic stresses affecting the growth, development and yield of rice, submergence caused by continuous flooding without adequate drainage poses a significant threat. This stress is particularly detrimental in lowland areas with poor drainage, often near coastal regions, where excessive rainfall leads to prolonged waterlogging. Continuous waterlogging during germination severely impacts the germination of directly seeded rice crops, while seedling establishment suffers post-transplantation due to seedling decay and mortality. Submergence tolerance is an adaptive physiological and biochemical mechanism that has evolved in indica rice, enabling the plant to cope with the effects of anaerobic conditions caused by prolonged submergence. The putative progenitor *Oryza rufipogon* is well adapted to marshy environments. This study discusses the mechanisms of introgression of anaerobic germination and submergence tolerance from *O. rufipogon* through molecular analysis of genomic regions. It also explains the physiological and biochemical mechanisms that influence anaerobic germination and submergence tolerance. Lowland areas characterized by flooding due to excessive rainfall and inadequate drainage, particularly near coastal regions, require anaerobic germination and submergence tolerance for rice cultivation. Identifying new sources of submergence tolerance beyond the *Sub1* gene, followed by genomic structural characterization for the development of pre-breeding genetic sources, is essential. Additionally, well-characterized quantitative trait loci (QTLs) and genes that confer submergence tolerance need to be transferred precisely.

## Keywords

adaptations; anaerobic germination; submergence; physiological mechanisms; biochemical mechanisms; *Sub1 QTL*; introgression

## Introduction

In the crop year 2022, the global area cultivated with rice was approximately 165 M ha. India was the leading producer, with an estimated harvest covering about 48 M ha. Rice holds a significant position as India's primary food crop and is cultivated across most states in the country. The habitat for rice cultivation in India can be broadly categorized into four types based on water requirements and soil conditions, as shown in Fig. 1: (1) rainfed lowland, (2) irrigated lowland, (3) rainfed highland and (4) flood-prone areas. Within the irrigated lowland category, there are three subcategories: single aeration, multiple aeration and frequently submerged, distinguished by the water



**Fig. 1.** Rice cultivation area in India according to rice's habitat: (1) rainfed lowland, (2) irrigated (3) rainfed upland and (4) flood prone (105).

levels during germination, growth and development. Rainfed areas are further divided into drought-prone and flood-prone regions. Lowland rice fields are submerged or intermittently flooded, with sporadic flooding occurring in northern India. Upland fields are never submerged, while deepwater rice varieties are cultivated in regions with low elevation and heavy precipitation (1).

Numerous significant rice-growing regions in Southeast and South Asia, including India, Myanmar, Bangladesh, Vietnam, Thailand and Indonesia, experience frequent flash floods during the monsoon season, which coincides with the early seedling growth phase (2). In Nepal, over 1.5 M ha, or 15%, of agricultural land is heavily affected by floods annually. Bangladesh, with its extensive rice fields spanning more than 2.65 M ha, is particularly vulnerable to recurring flooding. Of the 22 M ha of rainfed land, approximately 15 M ha are subjected to short flash floods within a season, resulting in an estimated economic impact of \$1 billion (3). Around 7% of the area used for rice cultivation worldwide, which constitutes 4% of the world's rice crop, is situated in submerged areas (4). Soil waterlogging, especially following heavy rainfall and inadequate levelling, is a common occurrence.

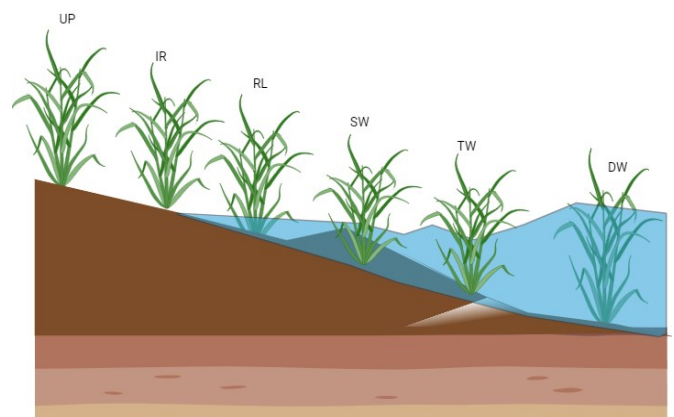
Floods can lead to anaerobic germination (AG) of seeds, where germination occurs without access to air or oxygen. Direct rice planting in flood-prone areas is challenging due to issues such as low seed germination rates, delayed emergence and significant weed infestations (5). Floods pose a significant threat to rice crops, causing damage and affecting germination rates. Anaerobic germination involves complex regulatory mechanisms, encompassing various gene families and metabolic changes such as starch breakdown, fermentation and glycolysis (6). This process is essential under flood conditions to ensure uniform germination and better seedling establishment. Flash floods, caused by heavy rain or river overflows, can last 1 to 2 weeks, inundating areas entirely (7). Floods pose a significant threat to rice crops, causing damage and affecting germination rates (8). In stagnant flood conditions, water levels typically range from 25 to 50 cm, allowing visible sections of plant shoots to remain above the water. The yield of rice grains varies depending on the depth of submergence, typically ranging from 0.5 to 1.5 t/h. A study

conducted in West Bengal, India, evaluated 577 rice genotypes under stationary flooding. Despite genetic differences in survival and yield, no flood-tolerant cultivars outperformed others, suggesting that genotypes resistant to flash floods may not be suitable (7). Deep-water floods can cause extensive water stagnation, necessitating adaptations in rice varieties. These varieties grow rapidly to avoid submersion and resist complete inundation due to increased carbohydrate intake, reaching heights of up to 5 m (3). In states such as Assam, Bihar, Orissa, West Bengal and Uttar Pradesh in India, small-scale farmers often cultivate native deep-water rice varieties.

### Ecotypes of rice with adaptive characteristics of submergence tolerance

*Oryza sativa*, commonly known as Asian cultivated rice, exhibits vast variability and phenotypic diversity across different ecotypes that are well-suited to various hydrological conditions (9). A recent study established a rice diversity panel consisting of 867 rice accessions, accompanied by clear geographic distribution and cultivation data (10). This panel includes six different cultivated types: Thirty-eight deepwater rice (DW), twelve swamp rice (SW), three tidal wetland rice (TW), 316 irrigated rice (IR), 191 rainfed lowland rice (RL) and 308 upland rice accessions. The classification of these cultivation types, depicted in Fig. 2, was primarily based on the hydrological conditions of their respective regions, TW and IR correspond to briefly aerobic situations, RL refers to rainfed lowland areas and UP signifies fully aerobic habitats.

A study involving 140 wild rice samples and one cultivated rice sample identified a significant number of single nucleotide polymorphisms (SNPs) that influenced gene expression levels. Among these SNPs, 27.86% were located in the promoter region, while 493399 were missense variants that altered protein sequence. The resulting phylogenetic tree revealed distinct branches for *O. sativa xian (indica)* and *geng (japonica)* forms, both descending from wild rice groups. The cultivated varieties were categorized under the primary *xian (indica)* branch, while deep-water and swamp rice were grouped into separate branches. Within the Asian *geng (japonica)* subgroup, two distinct branches emerged: one included irrigated rice, while the other comprised upland and rainfed lowland rice. The cultivated varieties were distinctly separated from their wild rice counterparts.



**Fig. 2.** Represents the different ecotypes of rice. UP-rice under upland condition, IR-rice under irrigated condition, RL-rice under rainfed lowland condition, SW-rice under swamp wetland condition, TW-rice under tidal wetland condition and DW-rice under deep water condition.

### Effects of waterlogging in plants

Plants are sensitive to biophysical constraints such as temperature, pH, water depth, turbidity, flooding duration and cell morphology. These factors significantly influence crop growth, development and yield. Turbidity, caused by sediment, organic matter and phytoplankton, reduces sunlight penetration, which in turn affects photosynthesis, oxygen and carbon dioxide levels, respiration and can lead to anoxia, ultimately impacting plant survival (2). An experiment was conducted, which compared plant growth and survival under two conditions: complete submersion without light and submersion with some light. The result indicated that solar radiation significantly improves plant growth and survival in submerged conditions, highlighting the importance of light for aquatic plants (11). When rice plants lack light and oxygen, they generate reactive oxygen species (ROS), which can damage cellular structures and ultimately lead to plant death. However, certain rice cultivars possess mechanisms to detoxify ROS and resume growth by producing carotenoids, ascorbate and phenols, which are natural antioxidants that help reduce oxidative damage. These compounds promote plant growth, aid in the regeneration of new leaves and facilitate the restoration of chlorophyll levels. Ascorbate, one of these natural antioxidants, has been found to accumulate in rice plant root cells under hypoxic conditions. Conversely, when the plants are re-exposed to oxygen, the levels of ascorbate decrease. Additionally, research suggests that ROS continue to damage plants even after they are re-exposed to air following an anoxic or hypoxic condition (12). Moreover, during flooding or waterlogging, several harmful chemicals accumulate in anoxic or hypoxic soils, which then enter the plant and affect the tissues of both roots and shoots. For instance, ethanol that accumulates in the submerged parts of rice plants does not decrease upon re-exposure to oxygen after a period of oxygen deprivation. Instead, the trapped ethanol is converted into acetaldehyde, causing damage to cells post-anoxia (13). Researchers investigated the aeration of rice plants during complete submergence, focusing on underwater photosynthesis and the partial pressure of oxygen ( $pO_2$ ) in floodwater (14). Root  $pO_2$  levels were measured using microelectrodes over two days and the experiment involved either keeping leaf gas films on super hydrophobic leaves intact or removing them. In darkness, root  $pO_2$  levels dropped significantly, closely correlating with floodwater  $pO_2$ . However, in light, root  $pO_2$  levels were higher, primarily due to light intensity affecting underwater photosynthesis rates. Plants with intact leaf gas films exhibited higher underwater photosynthesis, contributing to their tolerance to submergence. These complex interactions illustrate the challenges plants face and the intricate mechanisms they employ to cope with submergence stress.

### Metabolic adaptability to anaerobic germination processes

Rice plants, like other crops, require oxygen for energy production through aerobic respiration. However, when submerged, they experience oxygen shortages due to the slower diffusion of oxygen in water. This triggers rapid metabolic and molecular responses, causing rice seed germination to suppress aerobic respiration and shift to anaerobic respiration. Anaerobic fermentation pathways, such

as alcoholic, lactic and alanine fermentation, become vital during this process (15). Anaerobic fermentation, where ATP synthesis is reflected towards glycolysis and ethanol production, is crucial for plant germination and seedling growth under oxygen-limited conditions. This process provides the energy needed for essential bioprocesses, such as protein synthesis in coleoptile cells, by suppressing oxidative phosphorylation and the tricarboxylic acid cycle (16). Additionally, anaerobic fermentation requires the reoxidation of NADH to NAD<sup>+</sup> to sustain glycolysis. Rice's anaerobic germination tolerance is enhanced by increased starch breakdown in endosperms, converting sugars into substrates for glycolysis and alcohol fermentation, thereby promoting germination and coleoptile outgrowth (17).

Key enzymes like aldehyde dehydrogenase, pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) play crucial roles in converting pyruvate into alcohol, which is essential for rice plant germination, growth and establishment in low-oxygen environments. These enzymes also catalyze ethanol metabolism (18). Mutation in the rice ADH1 gene, such as adh-null mutant, reduces ADH protein levels, making plants less resistant to anaerobic stress. To increase germination tolerance during submergence, a functioning ADH protein is necessary. Lowering cytoplasmic pH reduces intracellular lactate concentrations and enhances PDC activity, thereby improving anaerobic germination tolerance. The regulation of cytoplasmic pH in rice prevents acidosis and enhances survival under low-oxygen conditions by boosting enzyme activity in starch hydrolysis and ethanol production (19). These intricate metabolic pathways and enzyme activities are vital for rice plants to survive and thrive under submergence stress conditions (Fig. 3).

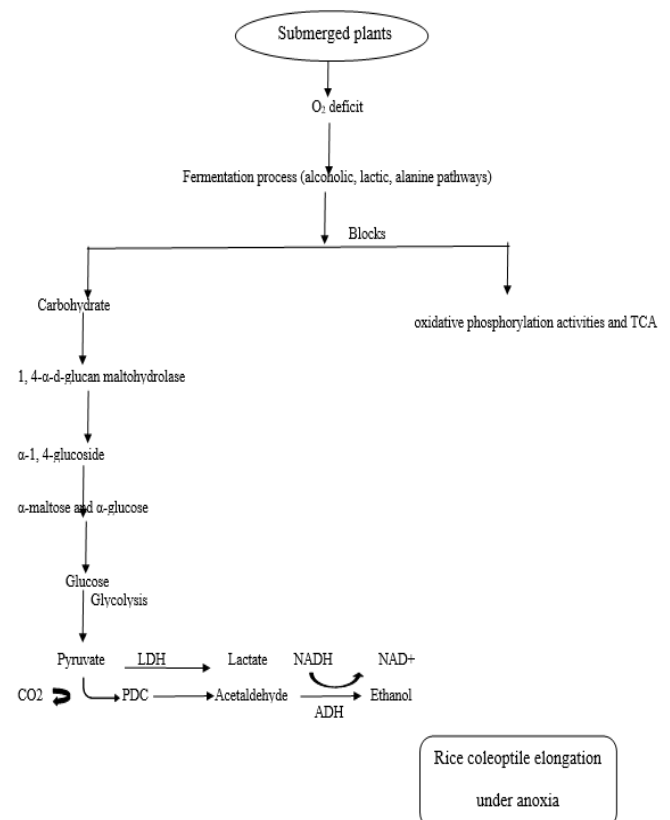


Fig. 3. Metabolic adaptations in rice (106).



### Trehalose-6-phosphate phosphatase

*OsTPP1* and *OsTPP2* are key genes in rice that convert trehalose-6-phosphate (T6P) to trehalose, a crucial molecule for development and metabolic balance. T6P partially inhibits *SnRK1*, a major regulator of energy-related signalling. *OsTPP7* lowers T6P levels in localized pools, influencing T6P-mediated sugar signalling. This mechanism may explain the increased rate of starch mobilization and growth in genotypes carrying the *qAG-9-2* (20).

### Role of $\alpha$ -amylase under anoxia

$\alpha$ -amylase, also known as 1,4- $\alpha$ -D-glucan maltohydrolase, is a crucial enzyme in rice (21) that mobilizes stored starch by hydrolyzing  $\alpha$ -1,4-glucoside bonds, producing  $\alpha$ -maltose and  $\alpha$ -glucose (22).

Under anoxic conditions, starch degradation in the rice endosperm is regulated by  $\alpha$ -amylase (23). Exogenous gibberellic acid induces  $\alpha$ -amylase activity in anoxic rice embryoless half-grains, but this response is significantly slower compared to aerobic conditions. Specific isoforms of  $\alpha$ -amylase, such as G and H, are produced under anoxia, while isoforms A and B are present under both aerobic and anaerobic conditions in seedlings (23).

The *Amy1A* and *Amy3D* genes encode different isoforms of  $\alpha$ -amylase. *Amy1A* is hormonally regulated, while *Amy3D* is influenced by sugar levels. Under aerobic conditions, the expression pattern of the  $\alpha$ -amylase genes in the scutellar epithelium are similar to those in the aleurone layer (24). The *RAmy3* subfamily, which contains most of the amylase genes, responds to sugar starvation and oxygen deficits, with a specific gene (LOC Os08g36910) playing a key role in this process. Anoxia affects gene expression by increasing mRNA levels of the *Amy3* subfamily and decreasing those of the *Amy1A* subgroup. *Amy3*  $\alpha$ -amylases aid in starch breakdown, contributing to rice grain germination and survival under anoxic condition. Unlike *Amy1A*, *Amy3* does not require GA for activity. *Amy1A* is more efficient at breaking down starch granules, while *Amy3D* selectively breaks down oligosaccharides. Together, *Amy1A* and *Amy3* coordinate starch degradation during early germination, particularly when GA sensitivity or availability is limited (24).

### Chlorophyll

Chlorophyll, a vital pigment in plants, plays a crucial role in photosynthesis. In rice, chlorophyll levels often decrease following floods and this degradation serves as an indicator of flooding tolerance in both sensitive and tolerant genotypes (2). Ethylene, a plant hormone, accelerates chlorophyll breakdown under submergence by activating the enzyme chlorophyllase. To enhance photosynthesis and maintain the necessary energy reserve, it has been suggested that chlorophyll preservation can be achieved by suppressing ethylene production and its activity. Researchers demonstrated that chlorophyll is essential for underwater photosynthesis and aids in rapid recovery of plants after de-submergence (25). Therefore, retaining chlorophyll content during and after flooding is critical for plant survival. Comparative studies between the rice varieties Swarna and Swarna-Sub1 showed that Swarna had significantly lower

chlorophyll levels after 10 days of complete submergence, whereas Swarna-Sub1 retained higher chlorophyll levels (2). Researchers further emphasized that controlling ethylene activity and maintaining chlorophyll levels are key strategies for rice plants to withstand flooding stress and recover effectively submergence (25).

### Adaptations in morphology and anatomy to water-rich environments: An exploration

Prolonged waterlogging and flooding in rice plants cause oxygen deficiency, which inhibits aerobic respiration, affecting both the tricarboxylic acid cycle and oxidative phosphorylation (26). To compensate, rice plants rely on ethanol fermentation and develop specific defences (27).

A key adaptation is the formation of aerenchyma, which is essential for efficient gas transport between the shoots and roots. There are two types of aerenchyma: lysigenous and schizogenous (28). Aerenchyma formation is triggered by the gaseous hormone ethylene, which induces programmed cell death (PCD) during flooding stress (29). Ethylene accumulation also leads to increased production of reactive oxygen species (ROS), further contributing to aerenchyma formation (30). In addition to aerenchyma, rice plants develop adventitious roots (ARs) that emerge from each node as the plant grows (31). When soil roots become dysfunctional due to hypoxia, these flooding-induced ARs are essential for gas exchange, as well as water and nutrient uptake (32). Ethylene promotes the growth of ARs, while reactive oxygen species (ROS) are produced in nodal and AR primordial epidermal cells in response to ethylene. Polar auxin transport helps coordinate AR growth and epidermal cell mortality (33). Comparative studies have shown that rice cultivars like FR13A and BRR1 dhan51 exhibit superior aerenchyma formation under flooding conditions compared to varieties such as Ukunimadhu (34).

Rice leaves, like those of other semi-aquatic species, are hydrophobic, allowing them to maintain a gas microlayer called leaf gas films on their submerged surfaces (35). This hydrophobicity, mainly due to epicuticular wax films, enhanced internal aeration and improved flood tolerance in rice (36). Leaf gas films are essential for capturing oxygen at night and carbon dioxide during the day for photosynthesis (37). Research has demonstrated that these leaf gas films play a critical role in flood tolerance (13). The leaf gas film1 (*LGF1*) gene significantly enhances flood tolerance in rice. Rice plants also develop a barrier against radial oxygen loss (ROL) during waterlogging, reducing oxygen loss in the rhizosphere (38). Under waterlogged conditions, rice roots accumulate lignin and suberin, which activate genes related to rice biosynthesis (39). In mutant rice plants lacking suberin lamellae, the ability of an apoplastic tracer to penetrate roots remains unaffected. These adaptations enable rice plants to survive and thrive in waterlogged environments (40).

### Physiological mechanisms of submergence tolerance in rice

Rice, being semi-aquatic, has the ability to adapt to various flooding conditions through a range of physiological and biochemical pathways. Submergence tolerance is a multifaceted trait intertwined with numerous physiological

and agronomic factors. When exposed to oxygen scarcity, such as hypoxia or anoxia during submergence, rice plants activate stage-specific tolerance and adaptation mechanisms to survive. The two primary survival strategies they employ are the low oxygen escape strategy (LOES) and low oxygen quiescence strategy (LOQS) (41). These strategies enable rice plants to cope with low oxygen levels either by escaping the submerged environment through shoot elongation (LOES) or by entering a quiescence state to conserve energy and resources (LOQS). These mechanisms are essential for survival and adaptation to rice in flooded conditions.

In deep-water lowland rice, the low oxygen escape strategy is frequently observed as an adaptation mechanism. The plant's elongated shoots protect it from complete submersion as floodwater levels steadily increase. Two key ethylene-responsive factor genes, SNORKEL1 (SK1) and SNORKEL2 (SK2), were cloned from the deep-water rice variety C9285 and are essential for shoot elongation in deep-water rice. The accumulation of biomass is essential for maintaining growth after de-submergence, a process supported by delayed coleoptile elongation and the development of cultivars tolerant to flash flooding. Deep-water rice varieties that possess the SK1 and SK2 genes exhibit rapid internode elongation in response to submergence, according to an allelic survey (42). The bioactive compound gibberellic acid (GA) accumulates in submerged plant tissues, facilitated by the action of the SNORKEL genes (43).

According to the researchers, ethylene accumulation during flooding regulates the expression of the SK1/2 genes in C9285 (44). Ethylene triggered gibberellic acid production during flooding, which, in turn, promotes internode elongation in deep-water rice (13). Moreover, another study demonstrated that a rare allele of the SD1 gibberellin biosynthesis gene aids in adapting to water depth (45). The SD1 protein boosts the production of gibberellin, GA4, enabling deep-water rice to survive submergence. Additionally, rice cultivars utilize the low oxygen quiescence to limit energy to minimize energy consumption and conserve non-structural carbohydrates (NSC), ensuring an energy reserve during submergence. This approach allows rice plants to grow above floodwaters and survive under submerged conditions (13). The quiescence response is triggered by a mutation in a transcription factor from the ethylene response factor family, located in the *Sub-1* locus on chromosome 9 (46). Rice cultivars with this mutation exhibit full tolerance to complete submergence and can survive for up to two weeks in these conditions (46, 47). The *Sub-1* locus encodes three transcription factors and mutations in cultivars lead to elevated levels of *Sub1A* transcripts during submergence. This reduces ethylene biosynthesis and inhibits internodal elongation. *Sub1A* also enhances the expression of mRNA and proteins for SLENDER RICE 1 and SLENDER RICE-LIKE 1, both of which are negative regulators of gibberellic acid signalling (48). Research has shown that the *Sub1A-1* allele of the *Sub1A* QTL increases brassinosteroid (BR) production (48). Brassinosteroids activate the GA catabolic enzyme CA2ox7. Consequently, during the quiescence stage, plants suppress shoot elongation, conserve NSCs and recover more effectively after submergence (49).

## Genetic resources of submergence

Farmers have historically cultivated traditional rice varieties that demonstrate tolerance to submergence, showcasing their adaptability to various biotic and abiotic stresses (50). The discovery of flood-tolerant landraces in rice dates back to the 1950s, when a few traditional varieties were identified as capable of withstanding flooding. Notably, two Indian accessions, FR13A from Dhalputtia and FR43B from Bhetnasia in Odisha, along with several Sri Lankan accessions, such as Kurkaruppan, Goda Heenati and Thavalu, exhibited remarkable submergence tolerance (51). Additionally, landraces like Khao Hlan On, Khaiyan, Nanhi and Ma-Zhan Red were found to be tolerant to submergence during germination (52). Varieties like Leuang Pratew 123 and Khao Tah Haeng 17, which grow during the early vegetative stage, have shown resilience to both flash floods and stagnant floods up to 80 cm (53).

## Functional analysis of genes involved in submergence tolerance

The *SUBMERGENCE 1* (*Sub1*) locus in rice provides tolerance to submergence through a quiescence strategy. *Sub1A-1*, one of three known ethylene responsive factor (*ERF*) genes (47), helps conserve energy by suppressing gibberellic acid (GA)-mediated shoot elongation and limiting ethylene production in resistant genotypes (54). *Sub1A-1* is conserved in various rice cultivars and is also present in wild rice species like *O. nivara* (55). When introduced into rice, *Sub1A-1* significantly enhances the expression of ERF-VII transcription factor genes *erf66* and *erf67*, which are critical for adaptation to submergence, by regulating downstream genes associated with submergence tolerance (33). In addition to submergence tolerance, *Sub1A-1* aids in adapting to other abiotic stresses, such as darkness, drought, oxidative stress, ethylene stress and rapid dehydration after de-submergence. During submergence, ethylene accumulation makes rice plants more responsive to GA, which promotes shoot elongation. *Sub1A-1* also influences the production and signalling of brassinosteroids (BR), which are crucial for submergence tolerance. The LEAF GAS FILM 1 (*LGF1*) gene further contributes to submergence tolerance by promoting the development of a leaf gas film that enhances hydrophobicity and gas retention during submergence. Additionally, the weedy rice gene, *OsGF14h* plays a key role in anaerobic seed germination and seedling development during submergence by increasing GA synthesis and suppressing ABA signalling, thus maintaining submergence tolerance. Rice's resistance to submersion may also derive from genetic sources beyond *qSub1*, such as the overexpression of the ethylene-producing *OsARD1* gene (33).

## *Sub1A*

Rice utilizes the ethylene responsive factor (*erf*) *SUBMERGENCE1A* (*Sub1A*) and its allelic variation *Sub1A-1* as key survival mechanisms during submergence (46). Ethylene, a gaseous plant hormone, accumulates when rice is submerged in water and activates *Sub1A-1* (56). This activation is critical for regulating the transcription of genes related to fermentative metabolism, which provides energy in low-oxygen environments. *Sub1A-1* also controls the breakdown of carbohydrate, ensuring glucose availability for recovering after

the water recedes, while simultaneously regulating shoot elongation to prevent excessive energy consumption. Following de-submergence, *Sub1A-1* helps rice adapt to dehydration by improving the detoxification of reactive oxygen species (ROS) and enhancing the response to abscisic acid (ABA) (57). While the less tolerant *Sub1A-2* allele offers some level of submergence tolerance, it lacks the positioning of *Sub1A-1* mitogen-activated protein kinase (MAPK), which is likely important for downstream gene regulation and protein activation (46).

The *Sub1A*-mediated response, linked to GA signalling, restricts shoot growth during submergence to conserve energy until floodwaters recede, with this response being influenced by light, temperature and hormone levels (58). BR treatment has been found to enhance the expression of the GA catabolic gene (*GA2ox7*), which is induced in a *Sub1A*-dependent manner during submergence. BR treatment also significantly increases the transcript levels of another GA catabolic gene, *GA2ox3* (59). This suggests that BR can lower bioactive GA levels by inducing *GA2ox7* early in the submergence response, which is known to prevent the degradation of the rice SLR1 protein (60). Overall, *Sub1A*-mediated submergence tolerance likely involves interaction between the BR and GA pathways. New high-yielding rice varieties with enhanced submergence tolerance have been developed by introducing the *Sub1A-1* allele into previously well-performing varieties (61).

#### Presence of *Sub1A* QTL in wild and cultivated rice

*Oryza rufipogon*, *Oryza nivara*, *Oryza barthii*, *Oryza longistaminata*, *Oryza meridionalis* and *Oryza glumaepatula* are wild rice species that share the *aus* landrace flooding resistant 13A (FR13A) allele (46). These species are capable of exchanging genetic material when they flower simultaneously in the same location (61). *O. rufipogon* thrives in perennially wet habitats, while *O. nivara* is found in seasonally dry areas. Both species, along with wild rice, have adapted to water regimes and coexist in rice cultivation zones due to their cross-compatibility.

*O. nivara* is often considered a subspecies or ecotype of *O. rufipogon* because of their similar physical traits and low genetic divergence. The transfer of the *Sub1A* gene has proven effective in regions where flash flooding occurs when rice seedlings are still young (62). Domesticated rice genes from wild rice species with an AA genome type may have been introduced into nearby *O. sativa*, facilitating gene flow between the species.

Research on the genetic diversity of *O. rufipogon* has revealed that wild rice populations growing close to cultivated rice fields exhibited greater genetic variety than those farther away. Interestingly, Sri Lanka lacks wild rice containing the *Sub1A* gene, suggesting that these landraces were either recently introduced or that *Sub1A* was incorporated during domestication in the Indian Basin and subsequently spread to Sri Lanka (63).

#### Transfer of *Sub1* QTL from landraces to cultivars

Landraces exhibiting remarkable resilience to flooding and submersion were first identified in the early 1950s and systematic screening of these varieties began in the 1970s.

Notable accessions capable of tolerating complete submersion included FR13A and FR43B from Orissa, India, as well as Kurkaruppan, Goda Heenati and Thavalu from Sri Lanka. Researchers reported that FR13A was unique in that all of its 10-day-old seedlings survived seven days of complete submersion (64). However, this variety also has disadvantages, including its tall height, low-quality grain yield and sensitivity to photoperiod. Despite these drawbacks, the discovery of landraces with significant submergence tolerance generated optimism that this desirable trait could be incorporated into advanced breeding lines to mitigate yield losses from unexpected floods. The breeding line IR49830 7-1-2-2, developed from FR13A, did not effectively integrate submergence tolerance into high-yielding lines of short to intermediate height until the mid-1990s. Progress in this area has been noted since the 1980s (65). The International Rice Research Institute selected plants from breeding populations that were 50 days old and demonstrated extended resistance to submersion, leading to the development of semi-dwarf cultivars tolerant to flooding. However, the low grain quality and suboptimal yield of the parent landraces limited the widespread adoption of these varieties (66).

#### QTLs/genes mapped and identified

##### Anaerobic germination tolerance in rice

The anaerobic germination capability of rice BC2F2 populations, resulting from the cross between the highly flooding-tolerant rice variety Khao Hlan On from Myanmar and a flooding-susceptible variety, was studied by few researchers. Their analysis revealed the presence of five different quantitative trait loci (QTLs) on various chromosomes. These QTLs account for phenotypic variations ranging from 17.9% to 33.5%, located on the first (*qAG1.2*), third (*qAG3.1*), seventh (*qAG7.2*) and ninth (*qAG9.1*) chromosomes. The greatest QTL (*qAG9.2*) was found on the long arm of chromosome 9 and exhibited the greatest variance in phenotypic expression (67). The trehalose-6-phosphate gene linked to QTL (*qAG9.2*) plays a crucial role in starch mobilization, promoting coleoptiles elongation during anaerobic germination and enhancing germination resistance to flooding stress in the Ciherang *Sub1* (68).

Recent studies have targeted analysis of QTLs associated with rice's anaerobic germination (AG) capacity. For instance, three pairs of epistatic loci were identified on chromosomes 2, 3, 5 and 11, with variations ranging from 0.5% to 19.6% (69, 70). Significant QTLs were also discovered on chromosome 7, derived from the Chinese variety Ma-Zhan Red, which is noted for its flood resistance (71). Genome-wide association studies (GWAS) conducted using 5291 SNP markers across 432 Indica rice cultivars identified 15 loci linked to AG potential. The *HXX6* gene, which codes for a hexokinase involved in coleoptile elongation in anaerobic rice plants, has also been highlighted by researchers.

Moreover, in anaerobic conditions, a population of chromosome-segment substitution lines (CSSLS) with the IR64 genetic background was found to harbour a new QTL called *qACE3.1*, which provides resistance to anaerobic germination through coleoptile elongation (72). Researchers identified three QTLs (*qAG1*, *qAG3* and *qAG11*) and pinpointed the *AG1* gene as a refined variant of the anaerobic germination



QTL (*qAG9.2*) (Fig. 4A) (73). Further molecular research is necessary to fully understand the role of the *HXK6* gene in anaerobic germination tolerance (74).

#### Flash flood tolerance in rice

Molecular QTL mapping investigations have identified significant QTLs from the donor parents FR13A. For example, *Submergence 1* or *Sub1* locus on chromosome 9 gives rice plants the ability to endure complete submergence for up to two weeks (75). This important QTL exhibited a substantial LOD score of around 36% and accounted for 69% of the variance in submergence tolerance. Subsequent research has indicated that other minor QTLs contribute less than 30% of the phenotypic diversity in flooding tolerance, underscoring the essential role of the *Sub1* gene in conferring submergence tolerance (76). Using a population of 3000 F2 individual plants, the *Sub1* QTL was painstakingly mapped to a 0.16 cM area on chromosome 9 (77). The *Sub1* locus contains three genes-*Sub1A*, *Sub1B* and *Sub1C*-each encoding a putative ethylene-responsive factor (ERF). Among these, *Sub1A-1* has been identified as the primary predictor of rice submersion tolerance, with researchers predominantly using the FR13A landrace as the preferred donor for submergence tolerance.

In a cross between two moderately submergence-tolerant rice cultivars, Madabaru and IR72, four novel quantitative trait loci (QTLs) were discovered on chromosomes 1, 2, 9 and 12 in F2:3 populations. Understanding surface hydrophobicity and the maintenance of a gas film on flooded rice leaves has been advanced by the discovery of the leaf gas film 1 (*LGF1/OsHDS1*) gene, as illustrated in Fig. 4B (13). This finding opens avenues for investigating differences in rice varieties' abilities to retain a gas film, as well as for identifying alleles of the *LGF1/OsHDS1* gene that exhibit enhanced expression (78).

#### Stagnant flooding tolerance in rice

Deep-water rice has garnered increasing attention from breeders due to its unique ability to extend shoots. Research on the genetic inheritance patterns of stem elongation in deep-water rice has revealed the critical roles of the double genes *ef1*

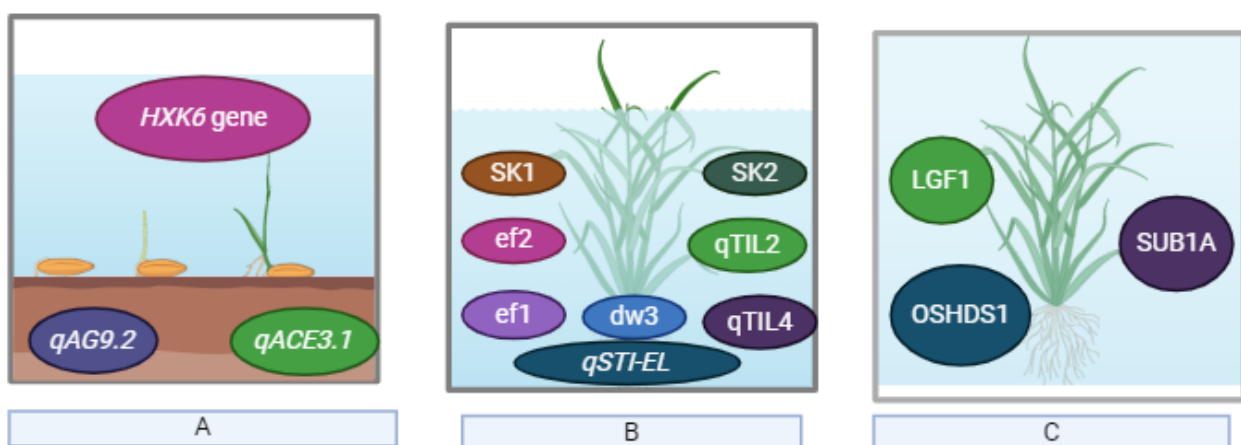
and *ef2* in regulating this process. A study conducted by (79) found that five to six genes are involved in controlling the floating capacity of rice species, exhibiting partial dominance. Di-allele crosses were employed and the progeny were examined to assess their level of submergence tolerance. A single recessive gene known as *dw3* is said to govern internode elongation in deep-water rice genotypes. According to (80), a total of 26 QTLs that control plant elongation and submergence tolerance in deep-water rice were identified in their study. The researchers carefully pinpointed two genetic areas (quantitative trait loci, or QTLs) on chromosomes 3 and 12 that affect the minimum elongation of internodes (81). Using the rate of internode elongation and the shortest elongated internode as measurements, (82) performed a QTL analysis and discovered two QTLs associated with each measurement on chromosome 3 and 12, or 1 and 12, respectively.

The analysis of QTL mapping employed three metrics: the count of elongated internodes, internode length and the shortest internode elongation. The regulation of deep-water rice expansion genes is influenced by developmental, hormonal and environmental signals, which are linked to cell elongation. During internode elongation, rice genes promote cell wall softening and modifications in cellulose microfibril orientation. Two novel QTLs, *qTIL2* and *qTIL4*, regulating early internode elongation in deep-water rice, were identified and are illustrated in Fig. 4C. These QTLs are associated with the plant's adaptation to deep-water conditions.

Previous study examined 148 inbred lines derived from a cross between IR10F365 and Ciherang-*Sub1* to investigate QTLs related to tolerance of stagnant flooding conditions (3). They identified seventeen putative QTLs for various genes under stress and non-stress situations, including PH, EL, PN, GW, PGW and PW, with one QTL for shoot elongation located on chromosome 12 (*qSTI-EL-12.1*) (83).

#### Gene editing

CRISPR-Cas9 gene editing has been utilized to improve crop traits, including the development of Ciherang-*Sub1*, an enhanced indica rice variety derived from the widely



**Fig. 4.** Represents the QTLs responsible for tolerance under different flooding. A) Seeds are under anaerobic condition-*HXK6* gene, which codes for a hexokinase implicated in coleoptile elongation in anaerobic rice plants (72), *qAG9.2* was shown to be involved in trehalose-6-phosphate metabolism, *qACE3.1*-which provides anaerobic germination resistance through coleoptile elongation (73). B) Plants under stagnant flooding- *dw3* is said to control the elongation of internodes in deep-water rice genotypes (79), *qTIL2* and *qTIL4*, that regulate early internode elongation in deep-water rice, elongation capacity (*Snorkel 1* and 2) in deep water, shoot elongation-(*qSTI-EL-12.1*) (83). C) Plants under flash flood condition-*Sub1A-1* has been found to be the main predictor of rice submersion tolerance, *LGF1/OsHDS1* gene capturing oxygen at night time and carbon dioxide during the day for photosynthesis (78).

cultivated Ciherang strain in Indonesia. This cultivar is notable for its resistance to genome alterations and the introduction of transgenes during shoot regeneration. In this study, two guide RNAs (gRNAs (gRNA1:5'CCGGCGAGGAGGCTGTCCATCAC3' and (gRNA2:5'ACGGCCGCTGCCGGATGCGTGG-3'), were designed to target and disrupt the second exon of the *Sub1A* gene, using CRISPR direct and Cas-OF Finder tools. The gRNAs were inserted into Golden Gate entry vectors, pYPQ131C and pYPQ132C, which link the OsU6 promoter to a gRNAs. These were then assembled into the Golden Gate recipient vector pYPQ142.

Subsequently, both pYPQ142 and the Cas9 expression vector were combined into the binary vector pMDC32 through the LR Clonase II recombination reaction. The final vector, pMDC32, was transformed into competent *E. coli* cells using a heat shock method at 42°C for 30 sec. Transformants were selected on kanamycin and confirmed through DNA sequencing. Plasmid DNA was prepared for bombardment using the QIAprep Spin Miniprep Kit (QIAGEN, Germantown, MD, USA) and stored in a -20°C freezer. Using various media, including callus induction, osmotic, selection and regeneration media, the mature seed protocol enabled the production of transgenic plants within 80 days. The optimized shoot induction medium (SIM) resulted in a high regeneration rate of 95% in Ciherang-*Sub1 indica* rice cultivars, suggesting that this medium could also be applied to other indica varieties. This regeneration medium is versatile and can be used with calli derived from both mature seeds and immature embryos, adaptable for both *Agrobacterium*-mediated and biolistic bombardment transformation methods. This study opens new avenues for exploring additional submergence tolerance genes in the Ciherang-*Sub1* background, especially after removing the dominant effect of the *Sub1A-1 QTL*'s. It also provides opportunities for future enhancements of Ciherang-*Sub1* and other indica rice varieties through gene editing and genetic engineering (84).

### Marker assisted breeding

The International Rice Research Institute (IRRI) launched a marker-assisted backcrossing (MABC) program to introduce the *Sub1* gene into major rice varieties, known as "mega-varieties", which are renowned for their high yield and excellent grain quality. The goal of the MABC program was to maintain desirable traits such as harvest timing, grain quality and agronomic characteristics while incorporating the submergence tolerance conferred by the *Sub1 QTL* (85). Microsatellite markers were utilized to enhance the integration of the recurrent parent genome with the *Sub1* region on chromosome 9 from the FR13A variety. All lines containing the "*Sub1*" *QTL* showed significantly improved tolerance to complete submergence compared to their original parent lines. This improvement was consistently observed across multiple greenhouse and field evaluations assessing submergence tolerance (61, 85, 86). The marker-assisted backcrossing technique has been employed in *Sub1* cultivars, which retain the favorable traits of their recurrent parent, such as high yield and grain quality. This process uses DNA markers in background selection to accelerate the recovery of the recurrent parent genome. Recombinant selection minimizes the donor chromosomal region

containing the *Sub1 QTL* by using closely linked markers. *Sub1* rice has demonstrated three to six times greater grain weight than non-*Sub1* varieties and as of 2012, eight *Sub1*-containing varieties have been developed. The discovery of the *Sub1 QTL* has facilitated its incorporation into widely cultivated varieties (87). Non-tolerant cultivars that experienced complete submergence during the vegetative stage showed delays in heading and maturity, leading to a reduction in grain yield (59, 83). In contrast, these enhanced mega-varieties gained popularity among farmers for their resilience. Marker-assisted backcrossing has successfully introduced the *Sub1* region from FR13A into various genetic backgrounds, demonstrating that it does not negatively affect other desirable traits and performs optimally in non-flooded conditions. This highlights the importance of preserving the intrinsic qualities of the varieties (88). Introgressed lines, such as Samba Mahsuri-*Sub1*, Swarna-*Sub1* and IR64-*Sub1*, significantly improve yields in marginal fields by replacing traditional landraces with poor yields in submergence-prone areas (89). Continued research on these lines and their submergence tolerance is vital to understanding their effects on grain production, quality and maturity (90).

### Breeding approaches for flooding tolerance during germination

The discovery of the Khao Hlan On-derived *AG1 QTL* has spurred interest in rice molecular breeding for anaerobic germination (AG) potential (20). Mega rice varieties or their *Sub1* lines, such as IR64, PSB Rc18-*Sub1* and PSB Rc82, IR64-*Sub1*, have successfully incorporated the *qAG9.1 QTL* (42). Another locus, *AG2*, derived from the Ma-Zhan Red variety, has also attracted considerable attention (68). In one study, IR64-*AG1*, a closely related donor, was used to explore the integration of the *AG1* locus into the Ciherang-*Sub1* variety through marker-assisted backcrossing (MABC). Additionally, by crossing the Korean *japonica* rice variety "Dongan" with "Khao Hlan On," an anaerobic germination-resistant donor from Myanmar, four *japonica*-type breeding lines were developed (91).

### Breeding approaches for flash flooding tolerance

The *Sub1* gene, known for its submergence resistance, has been successfully incorporated into several major rice cultivars through marker-assisted backcrossing (MABC) techniques. This has led to the development of various mega rice varieties, including IR64-*Sub1*, Swarna-*Sub1*, Thadokkam1-*Sub1*, BR11-*Sub1*, Samba and Africa (89). Additionally, two more genotypes, "PSB-RC18" from the Philippines and "Ciherang" from Indonesia, have been integrated with the *Sub1 QTL* Top of Form(92). In India, ten regionally adapted rice genotypes have been upgraded with the *Sub1 QTL* and the International Rice Research Institute (IRRI) along with Asian National Breeding Programs are using the *Sub1 QTL* to improve several popular varieties. These enhanced cultivars have shown a greater ability to endure complete submergence and have gained popularity among farmers. Yield improvements range from 1 to 3.5 tons per ha and *Sub1* rice is now cultivated by nearly four million farmers across Asia (93).



## Breeding approaches for stagnant flooding tolerance

The Philippine Rice Research Institute (IRRI) is working to enhance rice genotypes' resistance to stagnant flooding by developing a recombinant inbred line (RIL) population using the "IRRI 154" genotype for QTL mapping. In collaboration with India, Bangladesh, Nepal and other nations, IRRI has identified and evaluated new breeding techniques. However, a study revealed that varieties with the introgressed *Sub1* gene showed reduced yield and survival during stagnant water flooding(94). This is due to the gene's inability to support development under prolonged submergence stress. Similarly, another study compared high-yielding rice cultivars containing the *Sub1* QTL with non-*Sub1* parental lines, such as 'Swarna' and 'Savitri', under stagnant flooding conditions. It found that the *Sub1* QTL increased their susceptibility to floods (94).

## Approaches for developing flooding tolerance in rice using marker-assisted breeding

Marker-assisted backcrossing (MABC) is one of the most commonly used breeding methods today, primarily aimed at developing stress-resistant plant varieties and incorporating target genes from rare donor plants into popular varieties. This technique is more accurate and efficient than traditional breeding methods. The discovery of the *Sub1* QTL established a foundation for marker-assisted selection, particularly for breeding rice varieties with improved flooding tolerance. Table 1 lists popular rice varieties developed using MABC to withstand submergence, while Table 2 outlines the associated genes and their donor sources.

## Year of *Sub1* mega variety development (Fig. 5)

### Screening of materials for submergence

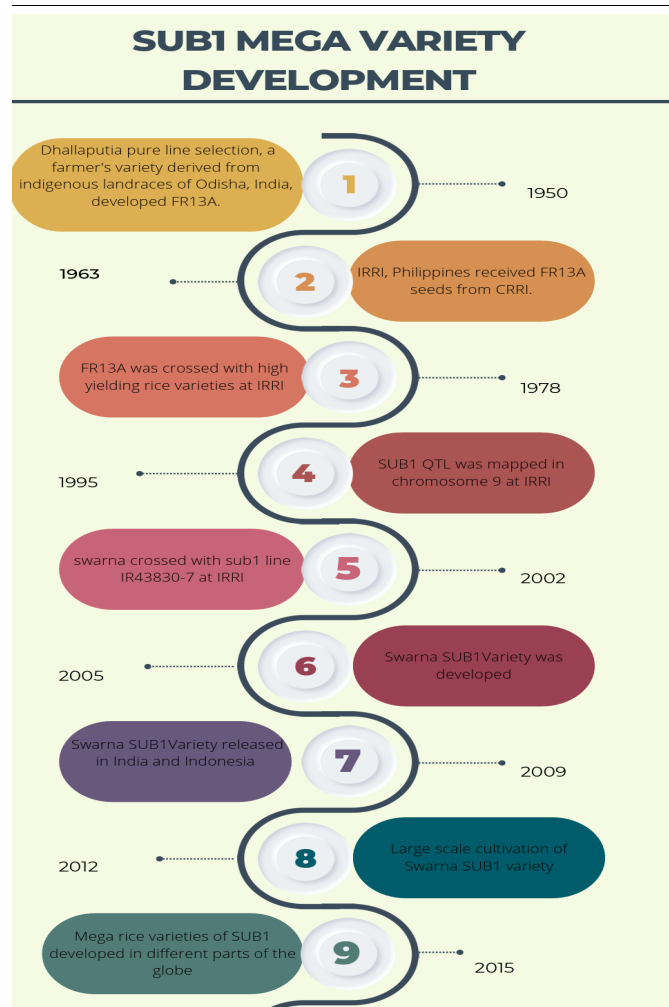
The IRRI phenotyping protocols for biological stress tolerance in rice outline specific procedures for testing tolerance to anaerobic germination, complete submergence and long-term stagnant flooding. For anaerobic germination, seed boxes are filled with sieved garden soil and dry seeds are sown. The water level is maintained at a depth of 3-5 cm to ensure normal growth. In flooded setups, a concrete table with a 10 cm water depth is used and the water level is adjusted as necessary.

For complete submergence, soil is placed in a concrete bed and seedlings are sown 0.5 cm below the soil surface. The plants are then covered with sieved garden soil and regularly weeded. Fertilization is done at the time of

sowing with 0.5 g of solophos and 0.5 g of muriate of potash per L of soil. Submergence treatment is initiated between 11:00 and 14:00 to allow for photosynthesis, ensuring that plants are fully submerged within 3-5 h by adjusting water flow.

**Table 2.** Genes and its donor.

	Genes	Donor	References
Anaerobic	AG1	Kho Hlan On	(6)
	AG2	Ma-Zhan Red	(102)
	qSUR	Kalarata	(103)
Complete submergence	<i>Sub1</i>	FR13A, Goda Heenati Kurkaruppan, Thavalu, Goda Heenati and FR13B	(49) (51)
	SK1,SK2	C9285	(104)



**Fig. 5.** Development of *Sub1* variety (107).

**Table 1.** Global survey: Leading *SUB1*-enhanced mega varieties and tolerant lines cultivated through marker-assisted back crossing (MABC).

Introgression of <i>Sub1</i>	Donor parent	Recurrent parent	origin	Year of release	References
Pratikshya <i>Sub1</i>	IR49830	Pratikshya		2017	
CR1009 <i>Sub1</i>	IR40931	CR1009/Savitri		2017	
Pooja <i>Sub1</i>	IR49830	Pooja	India		(96)
Gayatri <i>Sub1</i>	IR49830	Gayatri		2016	
Sarala <i>Sub1</i>	IR49830	Sarala			
Varshadhan <i>sub1</i>	IR49830	Varshadhan			
KDML105 <i>Sub1</i>	IR40931	KDML105	Thailand	2017	(97)
Bachthom 7- <i>Sub1</i>	IR40931	Bachthom 7	Vietnam	2016	(42)
TDK1 <i>Sub1</i>	IR40931	TDK1	Laos	2017	(98)
PSB Rc 18 <i>Sub1</i>	IR64 <i>Sub1</i>	PSR Rc 18	Philippines	2017	(99)
Jaya lines	Swarna <i>Sub1</i>	Jaya	India	-	(100)
Jyothi lines	Swarna <i>Sub1</i>	Jyothi	India	-	(101)

For long-term stagnant flooding, healthy pre-germinated seeds are sown in unfertilized soil, molluscicide is applied and nitrogen, phosphorus, potash and zinc are used as basal fertilizers. Seedlings are transplanted in the field at a spacing of 20×20 cm to maintain a population of 100 plants before submergence. Water levels in ponds are gradually increased and maintained and floodwater conditions are monitored. Hand weeding, snail control and other plant protection measures are employed throughout the process. The number of surviving plants is recorded at maturity.

In an anaerobic stress test, 115 landraces and four control varieties were subjected to 15 days of stress at a water depth of 10 cm. Several landraces-Manvilayan, Mattaikar, Karuthakar, Poovan Samba, Edakkal and Varappu Kudainchan -were identified as tolerant to early water submergence. These tolerant landraces can serve as potential donors in future hybridization programs (95).

### Future prospects

In India, long-duration rice cultivars like CR1009-*Sub1* and Swarna-*Sub1*, which have been effectively introgressed with the *Sub1* gene, have shown durable tolerance to submergence and are successfully grown in flood-prone areas. However, due to climate change, torrential rains during the first growing season often result in flooding. Therefore, it is crucial to introgress submergence tolerance into short- and medium-duration rice varieties, as this is a pressing demand from the farming community. Expanding the benefits of *Sub1* to rice cultivars grown in different geographic regions would allow more farmers to successfully cultivate rice under varying climatic and flood conditions. Future breeding programs could focus on combining *Sub1* with other stress-tolerant traits, such as salinity tolerance, to develop rice ecotypes that can withstand multiple environmental stresses. Advanced gene-editing technologies like CRISPR-Cas9 can facilitate the precise modification of key genes in the *Sub1* QTL region, speeding up the development of new submergence-tolerant varieties. Additionally, new sources of submergence tolerance should be identified and their component traits can be introgressed into various rice genetic backgrounds using genomic selection.

### Conclusion

Rice, a resilient crop, has been enhanced with the *Sub1* QTL, representing a significant breakthrough in addressing aeration and growth restrictions. This innovation reduces crop losses and ensures food security in flood-prone areas. Advances in genome editing tools, molecular genetics and breeding methods have made this achievement possible. The success of the *Sub1A* gene demonstrates the potential of genetic interventions in developing crops that are resilient to climate change.

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### Authors' contributions

SS and RP have conceptualized the draft. SS reviewed the literature, drafted manuscript. RP finalized the manuscript. All authors read and approved the final manuscript.

### Compliance with ethical standards

**Conflict of interest:** Authors do not have any conflict of interest to declare.

**Ethical issues:** None.

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