Development and Characterization of Rice Lines Carrying both Sub1 and Anaerobic Germination Tolerance: SUB1A does not Inhibit AG

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Accelerated coleoptile elongation in flooded soils allowed seedling shoots to reach the water surface to maintain gas exchange and CO2 fixation. Rice genotypes with tolerance of flooding during germination (anaerobic germination, AG) exhibited fast coleoptile elongation as an escape mechanism. On the other hand, tolerance of complete submergence during the vegetative stage, conferred by SUB1A, involved growth retardation to conserve energy for maintenance metabolism, with resumption of growth upon de-submergence. Combining genes controlling flood tolerance at these two stages with contrasting mechanisms was necessary in flood-prone areas for protection during germination under direct seeding and during vegetative stage. Breeding lines combining AG + Sub1 were used to determine the timing of the expression of SUB1A and elucidate its impact when combined with tolerance of flooding during seed germination and to evaluate seedling performance under complete submergence. Time-points for SUB1A expression in IR64-Sub1 and AG + Sub1 lines germinated under hypoxia or submerged for 30 h at vegetative stage showed that during flooding treatment, SUB1A and SUB1C expression was inhibited in young seedlings (< 6 d old) in IR64-Sub1 and at 2 and 4 d of seedling growth under hypoxia in AG + Sub1 materials. SUB1A was weakly expressed in AG + Sub1 starting at seedling age of 6 d following 1 d of complete submergence, indicating that expression of SUB1A may be stage-specific. Physiological tests showed higher percentage of survival of AG + Sub1 lines under hypoxia, comparable to that of AG tolerant check and significantly different from intolerant check IR42, indicating that the presence of SUB1A does not affect tolerance of flooding during germination. When seedlings were completely submerged, Sub1 expression was not inhibited by the presence of AG and tolerant genotypes showed suppressed elongation that was significantly different from intolerant check IR42. Despite their contrasting mechanisms of tolerance, combining AG with Sub1 provides multiple flooding tolerance from early crop establishment using direct seeding through to the vegetative stages.

Keywords: Sub1, anaerobic germination, combined AG + Sub1, Khaiyan

Abbreviation: ADH—alcohol dehydrogenase, AG—anaerobic germination tolerance, ATP—adenosine triphosphate, GA—gibberellic acid/gibberellins, PDC—pyruvate decarboxylase, QTL—quantitative trait loci, RT-PCR—reverse transcriptase polymerase chain reaction, SUB1—quantitative trait loci for submergence tolerance with SUB1A as major determinant gene, Sub1—submergence tolerance, SUB1C—gene homologue of SUB1A

INTRODUCTION

In rainfed and irrigated areas, rice faces challenges of flooding during germination and seedling emergence when directly seeded and during seedling and vegetative stages. Rice genotypes with greater abilities to germinate under anaerobic conditions in flooded soils (Khaiyan, Khao Hlan On, Mazhan Red) and others with submergence tolerance during vegetative stage (FR13A, IR64-Sub1, Swarna-Sub1) have been identified in previous studies (Neeraja et al. 2007; Ismail et al. 2009; Septiningsih et al. 2009 Singh et al. 2010; Toledo et al. 2015; Asante et...
The mechanisms underlining tolerance of flooding during germination and submergence tolerance at vegetative stage are known to involve contrasting mechanisms of "escape" strategy through faster growth in the first and "quiescence" strategy through reduced elongation in the latter (Jackson and Ram 2003; Ismail et al. 2009).

Tolerance of flooding during germination refers to the ability of seeds to germinate and the seedlings to elongate under hypoxic or low oxygen conditions, which usually happens in water logged soils. During anaerobic germination (AG), rice seeds of tolerant genotypes germinate and seedlings grow fast because of the capacity of germinating seeds to mobilize starch to produce energy using alternative pathways for carbohydrate metabolism under low oxygen stress (Ismail et al. 2012; Miro et al. 2017) and regulated by several quantitative loci (QTL) as reviewed in Ma et al. (2020). Genome-wide association study (GWAS) and 7-k single nucleotide polymorphism (SNP) array revealed 30 significant QTL regions for AG, with 14 colocalized with previous reports and 16 potentially novel (Thapa et al. 2022). One cloned AG gene, OsTPP7, is involved in trehalose-6-phosphate (T6P) metabolism (Kretzschmar et al. 2015). Under anaerobic stress, OsTPP7 activity may increase sink strength in proliferating heterotrophic tissues by indicating low sugar availability, thus enhancing starch mobilization and driving rapid coleoptile growth-enhancing AG. AG-tolerant genotypes usually show faster root growth and coleoptile elongation to emerge from flooded soils, with shoots extending above 10 cm water depth within 5 – 10 d after seeding. AG tolerance is distinguished from seedling vigor, which refers to faster seedling shoot and root growth under aerated conditions. Germinating rice seeds under low oxygen (O2) condition show 18-fold less ATP production for growth and maintenance processes (Greenway and Setter 1996), shift their carbohydrate catabolism to anaerobic pathway (alcoholic fermentation) with increased activities of both pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Ismail et al. 2009; Miro et al. 2017). They also show increased concentration of peroxidases for regulation of cell wall expansion and are able to rapidly convert stored starch into simple sugars accompanied by increased amylase activities (Lu et al. 1998; Ismail et al. 2009). Hormones such as ethylene (Ku et al. 1970) and gibberellic acid (GA) (Raskin and Kende 1984) in plant tissues are known to regulate shoot elongation. Once the coleoptile emerges from the flood water, it resumes its normal growth. Anaerobic germination tolerance is becoming more important with the expanding needs for shifting from transplanting to direct seeding with the benefits of reducing labor cost and water use, shortening the growth duration and reducing greenhouse gas emission; however, direct seeding is usually hindered by water logging or early floods, particularly in areas where water control is difficult, as in rainfed areas.

Submergence tolerance conferred by SUB1A gene is required in flash flood-prone stress where plants are usually flooded for 10 – 14 d. Younger seedlings are more sensitive to submergence stress because of their low carbohydrate contents. Tolerant rice genotypes recover and resume growth after a few days of de-submergence, producing new leaves, while intolerant genotypes usually show elongated leaves and leaf sheaths that start to turn yellow and subsequently decay, leading to the death of the seedlings. SUB1A acts by downregulating ethylene and stress responsive genes, resulting in slow metabolism and leading to growth retardation (Fukao et al. 2006; Xu et al. 2006; Jung et al. 2010). Fukao and Bailey-Serres (2008) further showed that the SUB1A-1 expression, induced by ethylene under submergence, increased accumulation of GA-signaling repressors slender rice-1 (SLR1) and SLR1-like-1 (SLRL1) and diminished GA-inducible gene expression. During complete submergence, tolerant rice plants with the SUB1A-1 allele enter into a ‘quiescent phase’ until water subsides and then fully recover, and resume growth through to harvest, producing good yield. Intolerant genotypes grow faster under flooding, depleting their carbohydrate reserves and leading to cell and tissue injuries as well as inability to recover. The SUB1A gene has been evaluated in different genetic backgrounds and widely studied for improving survival, grain yield, and resilience especially in regions where uncontrolled flooding is common (Ismail et al. 2013).

Since the mechanisms of tolerance required under these two development stages are contrasting, with AG promoting rapid shoot growth and SUB1A suppressing growth to conserve energy until floodwater recedes, it is important to assess the positive and potential negative consequences of combining the two tolerance mechanisms for breeding. Previous studies showed that the landrace FR13A which carries the SUB1A-1 allele is intolerant of anaerobic germination, while Khaiyan (KH), which is identified for AG tolerance, is susceptible to submergence (Ismail et al. 2009). Therefore, the objectives of this study are to determine the effectiveness of this combination as well as to determine the specific time points for the expression of SUB1A during germination and seedling establishment and whether it would have any inhibitory effects on AG tolerance during germination. The performance of genotypes carrying both AG and SUB1A under flooding during germination
and submergence during vegetative stage was also evaluated.

**MATERIALS AND METHODS**

**Breeding for AG + Sub 1:** Available breeding lines with AG (KH-/Mahsuri-derived) or Sub1 (Swarna/Sub1 derived from FR13A) were used as parent 1 and parent 2, respectively. Both KH and FR13A are landraces (Ismail et al. 2009). Advanced breeding lines in high-yielding background were developed by the International Rice Research Institute (IRRI), Philippines. KH-derived line and Swarna/Sub1 (IR05F101), with improved AG and Sub1, respectively, were crossed and backcrosses were made as described in Fig. 1. AG + Sub1 material from IR83770 BcF1 were advanced and used in phenotyping and genotyping. BC2 F3, (IR83770-9-3, IR83770-5-4-20, IR83770-9-2) and all other seeds used in this study were obtained from the IRRI Plant Breeding Division.

**Phenotyping for AG:** Seeds of IR42 (AG intolerant check), KH (tolerant check), and AG + Sub 1 lines were sown in trays filled with soil at about 25 cm depth. After sowing, seeds were lightly covered with fine soil, then tap water was applied and maintained for a depth of 10 cm from the soil surface until the experiment was terminated. The experiment was carried out in a greenhouse at IRRI, with daytime temperatures of 27 – 32 °C with nighttime temperatures of 20 – 24 °C. Each setup was replicated twice. Percentage survival was counted after 2 wk as the ratio of seedlings that emerge from flood water divided by the number of total seeds sown per genotype (n = 20). Data was analyzed using Statistical Analysis Software (SAS) tool (SAS Institute Inc., v.11.0) using t-test option in ANOVA for pairwise comparison of means between treatments, air and hypoxia, and between genotypes.

**Phenotyping for Sub1.** Seeds of IR42 and Khaiyan (submergence sensitive checks), varieties IR64/Sub1 and PSBRc 68 (tolerant checks), and 3 AG + Sub1 lines were sown in trays filled with soil, watered, and kept for 2 wk in the same greenhouse. After 2 wk, the trays were placed in concrete tanks and filled with tap water to a depth of 1.2 m for 10 d. When IR42 turned yellow, water was drained and trays were lifted from the tanks for seedlings to recover. Each setup was replicated twice. Percentage survival was counted after 2 wk as the ratio of surviving seedlings per genotype divided by the total number of seedlings before submergence (n = 10). Plant height and percentage survival were taken at day 21 following de-submergence. Data was analyzed using SAS using t-test option in ANOVA for pairwise comparison of means between genotypes for survival and for shoot elongation.

**Sample Preparation for RNA Extraction**

For time-point analyses of SUB1A and SUB1C during submergence at germination, IR64/Sub1 seeds were placed in several flasks, in sterile water or 0.1% agar solution as media, with or without light treatment by wrapping some of the flask in foil, and harvested 4 d later. In another setup, 6 d old IR64/Sub1 seedlings germinated in petri dishes were transferred into flasks and submerged for 30 h using sterilized water and kept either under light or dark conditions. In a third setup — anaerobic germination test of IR64/Sub1 and AG + Sub1 — seedlings (2, 4, 6 d) were transferred in flasks filled with 0.1% sterile agar solution, bubbled with nitrogen gas, and maintained under hypoxic conditions (0.03 mol O2 m−2) for 6 h prior to extraction. In another setup, seeds/seedlings of IR64/Sub1 and AG + Sub1 (2, 4, 6-d) were transferred to flasks and completely submerged for 1 d. Twenty embryos or plant tissues each were dissected under aseptic conditions. RNA extraction was performed using a modified protocol for starchy tissue (Vergara and Ismail 2007). The quantity and quality of the RNA were checked with a Nanodrop spectrophotometer (Thermo Scientific, US). Residual DNA digestion was performed using DNase (Promega, Madison US).

**Gene Expression Studies.** Expression analyses of SUB1A and SUB1C in checks and Sub 1 + AG lines were performed using reverse transcriptase-polymerase chain reaction (RT-PCR) as described in Ismail et al. (2009). The reaction used SuperScript one step RT-PCR with Platinum Taq system (Invitrogen, Carlsbad, CA, US) in a reaction mixture containing 50 – 75 ng RNA, 12.5 uL 2x buffer reaction mix, 0.25 mM each of forward (F) and reverse (R) primers, and 0.5 uL Taq mix (0.04 U/uL), with the final volume completed to 20 uL using diethylpyrocarbonate (DEPC) water. The reaction mixture was run under the following conditions: cDNA synthesis at 46°C for 30 min, denaturation at 94°C for 2 min, followed by 26 cycles of 94°C for denaturation for 2 min, annealing at 72°C for 30 s, 72°C extension for 2 min, and a final extension at 72°C for 10 min. The primer sequences used for SUB1A were F- 5'- GATGTGTTGGAGGAAGTGA-3’; R- 5’- TGTTTTGGTGAT CGATGGG-3’; for SUB1C were F-5'-AACCGCAAGACCACTTCC-3’; R-5’-AGGAGGCTGTCATCAGGT-3 (Singh et al. 2010); and for GAPDH were F-5’-GCGGAACCTGAGGAGATC-3’; R-5’-TTCCCTCCAGCTTTGCT-3’ (Vergara and Ismail 2007). Both SUB1A and SUB1C were used as these genes were shown to be transiently expressed after flooding in seedlings (Singh et al. 2010). Normalized transcript levels of glyceraldehyde 3-phosphate dehydrogenase (GAPDH) expression patterns were used...
Products were loaded onto Tris-Boric-EDTA (TBE) - agarose gels, run for 1.5 h, then stained with SYBR Safe dye at 75 uL concentrated stain/75 ml electrophoresis buffer (Thermo Fisher Scientific US) for 15 min.

RESULTS AND DISCUSSION

The development of AG + Sub1 lines is one of the breeding objectives at IRRI to pyramid tolerance of flooding during germination and vegetative stages into advanced breeding lines (Fig. 1). The material was then used to assess positive and potential negative effects of combining these contrasting traits. The pollen donor was Swarna-Sub1 (IR05F101), a released variety in India, and was crossed with an advanced high-yielding AG-line derived from Khaiyan/Mahsuri cross. Khaiyan is a landrace from Bangladesh (IRRI germplasm database).

Two backcrosses were subsequently made and each time leaf samples from the derived were harvested and used for genotyping for the presence of SUB1A (data not shown). IR83770 (pedigree is Khaiyan/Mahsuri//IR05F101) was also checked to contain SUB1A-1 by marker selection and was selfed. Three advanced lines (IR83770-9-3, IR83770-5-4-20, IR83770-9-2) were selected for further experiments. The development of AG + Sub1 materials was completed in three years.

To test for gene expression of SUB1A and SUB1C in young seedlings and under hypoxia, submergence-tolerant variety IR64-Sub1 was used for initial optimization of the setup and analyses, the effect of media, length of flooding, effect of light, and timing of expression. Both SUB1A and SUB1C belong to the gene cluster of SUB1 on the short arm of chromosome 9. Previous studies conducted by Xu et al. (2006) and Singh et al. (2010) using 10 to 14 d old seedlings have shown that 30 h of complete flooding is sufficient to induce the expression of SUB1A with transient expression of SUB1C in specific varieties. SUB1A was expressed in leaves, leaf sheath, nodes, and internodes and may be associated with percentage survival (Singh et al. 2010). Our results showed that for 4 d old seedlings submerged in water or in 0.1% agar in the dark, SUB1A and SUB1C were not expressed in leaves of IR64-Sub1 (Fig. 2). However, seedlings which are 6 d old and then submerged for 30 h either under dark or light conditions showed strong expression of SUB1A but not SUB1C (Fig. 2). These results are comparative to previous studies showing expression after 30 h flooding (Xu et al. 2006; Singh et al. 2010), and establishes a time-point of 6 d after sowing before SUB1A expression under controlled hypoxia.

SUB1A has been identified as the major determinant of submergence tolerance in rice (Septiningsih et al. 2009), but the role of SUB1C and its transient expression, even in tolerant varieties (Singh et al. 2010), is not well-understood. Septiningsih et al. (2009) showed that an intolerant SUB1C allele (SUB1C-2) combined with the tolerant SUB1A-1 allele did not significantly reduce the level of tolerance, and the SUB1C-1 expression appeared.
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then submerged for 1 d.

Sub1 and AG + Sub1 were germinated under non hypoxia maintained at 0.03 ppm O$_2$ were sown in 0.1% agar solution bubbled with N$_2$ gas.

Seedlings (4 d and 6 d for IR64

Fig. 3.  Timing of gene expression of SUB1A in IR64-Sub1 (labeled IR64-S1) and in IR64-AG + Sub1 (labeled AG+S1) in seedlings. Treatments L-R: (A) Seedlings (2, 4, 6 d) were germinated under non-hypoxia then submerged for 6 h; (B) Seedlings (4 d and 6 d for IR64-Sub1 and 4 d for AG + Sub1) were sown in 0.1% agar solution bubbled with N$_2$ gas maintained at 0.03 ppm O$_2$; (C) Seedlings (2, 4, 6 d) of IR64-Sub1 and AG + Sub1 were germinated under non hypoxia then submerged for 1 d.

Fig. 4.  Survival (%) of AG + Sub1 (IR83770) sown under 10 cm depth flooded (hypoxia) or non-flooded (aerated control) conditions, IR42 (anaerobic germination intolerant check); Khaiyan (tolerant check), n = 20. Error bars indicate standard deviation. * significantly different at $P < 0.05$.

coleoptile elongation. The appearance of abundant SUB1A transcript in IR64-Sub1 only at 6 – 7 d after sowing following 30 h under hypoxia indicates that SUB1A probably functions only at this specific stage, with no effects during the first 4 – 5 d after sowing. A weaker SUB1A expression initiated in AG + Sub1 at 6 d also indicates the functionality of SUB1A and that it is not hindered by AG tolerance mechanism at early growth.

assessment of seed germination and emergence under water-logged conditions in combined AG + Sub1 lines is important for validation for AG tolerance. IR83770 lines containing AG + Sub1 were tolerant during germination and early coleoptile growth, with no apparent SUB1A effect. Phenotyping procedures showed that seeds of the AG + Sub1 lines germinated and extended their coleoptiles beyond the 10 cm water depth with 98% survival comparable to the tolerant check, while the AG intolerant check showed poor germination and survival (19%, Fig. 4) and was found significantly different from tolerant check and AG + Sub1. This data again confirmed that SUB1A in the AG + Sub1 materials does not affect germination and seedling establishment at early stages (Alam et al. 2020). Their study also showed rapid coleoptile elongation of IR64 (Sub1, AG) with shorter coleoptiles for IR64-Sub1 under submerged germination. This indicates the potential for combining these two traits for tolerance during germination and vegetative stage, despite their contrasting mechanisms for conferring tolerance at these two stages.

Under complete submergence, SUB1A was expressed and seedlings with AG + Sub1 were shown to express the tolerance phenotype of restricted growth and improved
survival in a manner comparable to the tolerant check IR64-Sub1 (Fig. 5. A, B, and C). T-test option in ANOVA shows that intolerant variety IR42 showed a significant difference in survival in contrast to tolerant check variety Khaiyan and AG + Sub1 lines. For plant shoot elongation, intolerant variety IR42 showed the highest shoot elongation and was significantly different from tolerant check variety Khaiyan and 3 AG + Sub1 lines. Two of the 3 AG + Sub1 tolerant materials showed no significant differences in reduction of shoot elongation but grouped separately from the 3rd AG + Sub1 which showed the least shoot elongation. R-squared for data was 0.96 and coefficient of variation was low at 4.06, allowing proper ANOVA analyses. This data showed that combining AG with Sub1 does not negatively impact tolerance of complete submergence during vegetative stage. Similar findings were shown by Alam et al. (2020) in their physiological evaluation of isogenic lines carrying both specifically qAG1 (TPP7) (Khao Hlan On-derived) and SUB1A — that submergence tolerance was not compromised by the presence of qAG1 but survival was reduced in AG SUB1 plants submerged for 16 d, and that pyramiding of AG and Sub1 in their materials also resulted in reduced plant elongation growth. AG tolerance is controlled by many QTLs (Thapa et al. 2022) and any combination of these AG QTLs and interacting effects with SUB1A may result in variation in responses in combined AG + Sub1 materials, implying importance of validation in many studies.

Expression of SUB1A-1 has been shown to affect the expression of over 898 transcripts associated with: 1) anaerobic respiration and cytokinin-mediated delay in senescence caused by ethylene accumulation during submergence; 2) ethylene-dependent gene expression of 5 transcription factors (ERFs); and 3) negative regulation of GA-mediated elongation (4 ERFs). These indicate that SUB1 expression impacts multiple pathways in responses to submergence (Jung et al. 2010). This study is important for elucidating whether the presence of SUB1A could have an impact on germination in flooded soils in lines containing both AG + Sub1. Anaerobic condition during germination was previously shown to increase ethylene synthesis at 3 d in tolerant genotypes (Ismail et al. 2012) and could potentially be negatively regulated by the presence of SUB1A. There are a number of quantitative trait loci (30 loci) involved in low O2 and phytohormone sensing (auxin receptor protein), sugar-mediated signalling, and modulation of elongation and survival (Ma et al. 2020; Thapa et al. 2022). Another study showed that UDP-glucosyltransferase OsUGT75A found in japonica promotes tolerance during rice seed germination (He et al. 2023) by promoting coleoptile length and suggested that transferring this to indica can enhance coleoptile growth in submergence conditions. However, it is still not clear how these genes and processes are linked with the metabolic regulatory and signalling mechanisms associated with SUB1. Further research is needed to develop a better understanding of how the expression of SUB1A and genes/QTLs associated with AG are controlled and synchronized to allow this stage-specific functioning. Alam et al. (2020) provided detailed information in the possible mechanism of one
AG gene, TPP7, in TPP7-Sub1 combined genotypes contrasted with SUB1/SUB1A-1, using mRNA-sequencing at day 2 to 14 under flooded conditions, and inferred that there could be time-dependent and genotype-specific regulation of various mRNAs associated with DNA repair, cell cycle, chromatin modification, plastid biogenesis, carbohydrate catabolism and transport, elongation growth. In their study, TPP7 mRNA was low at day 2 and rose significantly by day 8 in IR64 (AG1) and IR64 (AG1, SUB1) but was undetectable due to a deletion in IR64 and IR64 (SUB1), validating the absence of this gene. As SUB1A-1 and TPP7 are co-expressed after 6 d, they hypothesized that SUB1A-1 would have a limited impact on the transcriptome before day 4 of development underwater. The current study’s results show that Sub1A was clearly not expressed at 4 − 5 d old and only at 6 d old seedlings with 30 h flooding (seedling age at 7 d) in IR64-Sub1 and Khaiyan-derived AG + Sub1 lines by mRNA-RTPCR; this supports their finding that SUB1A does not affect AG. In their materials, Khao Hlan On-derived IR64 (AG, SUB1) SUB1A was expressed only after 6 d (at 8 d timepoint). This shows a clear validation of time-specific expression with slight differences in genotype-specific regulation.

This study has important breeding implications, suggesting the possibility of developing rice genotypes from Khaiyan and combining contrasting tolerance mechanisms at different stages without compromising survival.

CONCLUSION

This study established that AG from Khaiyan can be combined with SUB1A to develop varieties tolerant of flooding during both seed germination and early growth as well as submergence during vegetative stage. SUB1A is not expressed in genotypes carrying both AG and Sub1 during the first 4 − 5 d after seeding under water or in agar solution devoid of oxygen, while AG has no effect on tolerance conferred by SUB1A during vegetative stage. Breeding varieties combining these two traits would provide assurance to farmers in flood-prone areas with the recent erratic patterns of flood incidences caused by climate change.

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