INVESTIGATING THE ROLE OF *SWEETA* DURING GERMINATION AND SUBMERGENCE OF *ORYZA SATIVA*

By

Alexandra Vacaru

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APPROVED

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Dr. Julia Bailey-Serres
Department of Botany and Plant Sciences

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Richard A. Cardullo
Howard H Hays, Jr. Chair
Faculty Director of University Honors

Abstract

Rice (*Oryza sativa*) is a globally important staple crop for half the world's population. Flash flooding and its attendant reduction in plant growth and reproduction is an annual threat, affecting more than 135 million hectares of rainfed lowland fields which support approximately 19% of the world's rice production. Strategies that intensify rice resilience to partial and complete submergence are needed to address this threat. We hypothesize that movement of carbohydrates and amino acids from leaf tissue to actively growing regions is essential for plant survival and recovery from submergence stress. Shoot meristems at the base of the stem, determine the initiation and development of leaves and eventually floral organs. We observe significantly compromised leaf elongation in carbohydrate transporter gene knock-out mutants, whereas lines engineered to have increased transporter gene expression display increased root growth. Here, the role of one carbohydrate transporter was evaluated in germinating seed. Use of a reporter of gene activity defined gene activity in specific regions: the radicle, coleoptile, and scutellar epithelium. By use of non-radioactive isotope carbon-13 (^{13}C) tracing a pilot study monitored movement of sugars from leaves to the shoot meristem region and subsequent metabolic activity in responses to submergence. These findings illustrate a potential path to improvement of submergence resilience of rice and other grain crops in a changing global climate.

Acknowledgments

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Introduction

Flooding of crops leads to plant death and devastates yields; this problem is exacerbated by climate change. To address this issue, new strategies for flooding resilience are needed. Rice is unusual in its ability among crops to survive a transient flood, including waterlogging of the root system or transient partial to complete submergence. To understand survival mechanisms, my project focuses on sugar transporter proteins called SWEETs (Sugars Will Eventually Export Together) that are located in the plasma membrane of vascular cells and move sugars from where they are made (source) to where they are used (sink) in the plant (1). This family of sugar transporters is subdivided into four sub-classes (1). My focus was on the clade III transporters which specialize in sucrose transport. For example, in *Arabidopsis*, SWEET11 and SWEET12 are essential for loading the phloem (2). Other SWEETs are critical in disease susceptibility in rice and other plant hosts (1). In rice, the *SWEETs* are not as thoroughly studied.

Plant tissues can be divided into two classes; "source" tissues that produce sugars through photosynthesis (i.e., leaves) or convert sugars brought from leaves for storage (i.e., seeds), and "sink" tissues. In both the mesophyll cells of leaves and endosperm of seeds the sugars are converted to starch a polymer of glucose. This must be broken down so that sugars can be transported to sink cells that consume sugars, such as the regions of actively dividing, elongating or differentiating cells. My project considers the movement of sugars, especially sucrose, from rice leaves or the seed endosperm to the sink tissue of the shoot or germinating seed. Meristem cells are actively dividing cells of plants that give rise to differentiated cells. In the shoot, the sugars provide energy through aerobic production of ATP for cell division and elongation at the base of each leaf and in the development of new organs from the meristematic zones of the shoot apex. In germinating seed, I considered the growth of the shoot coleoptile.

During submergence of the plant or seed, starch is broken down via less efficient anaerobic fermentation for substrate-level ATP production. This may necessitate increased levels of sink sugars to keep cells alive and plants growing. Based on gene activity (mRNA transcript) data, the SWEET transporters may be an essential player in the movement of sugars from the endosperm to developing shoot and root tissues during germination. Gene activity data suggest that the level of the SWEETA transporter may increase at the base of the shoot during submergence. We hypothesize this leads to increased movement of sugars from the leaf to the base of the shoot during submergence.

Rice is a globally important staple crop for half the world's population (3). In Southeast Asia, monsoon floods regularly submerge rice; events such as these affect over 30% of rice cultivation (4). Green Revolution rice varieties are semi-dwarf due to a mutation in a gene that is necessary for elongation of their stem. These varieties cannot tolerate complete submergence for more than a few days. Their response to submergence is to use the starch synthesized from the sugars produced in photosynthesis to elongate leaves, in an attempt to escape the flood and resume aerobic cellular respiration. This strategy resembles that of deep water rice varieties that are adapted to floodplains where the water table rises over the growing season. Deepwater rice has a suite of genes that promote underwater stem internodes to elongate extensively, allowing the rice to reach over 3 meters in height. In contrast, semi-dwarf Green Revolution genotypes bred to possess the *SUBMERGENCE 1A* (*SUB1A*) gene survive complete submergence by invoking growth quiescent during the stress, increasing the duration of survival for two weeks or more in farmers' fields (5,6,7). The quiescent strategy is successful because the plants use less of the available starch,and therefore consume less sugars, delaying cell death by energy starvation (8). The Bailey-Serres group and other groups have characterized SUB1A's role, as a

transcriptional regulator, in mediating changes in hormonal and growth responses during submergence and submergence recovery (5,6,7,8,10,11,12,13,14).

A recurring observation is that genotypes with *SUB1A* limit the gene activity of specific *SWEET* genes during submergence, consistent with the hypothesis that these transporters are important in movement of sugars to zones of active growth during submergence. These observations raise the possibility that reduced SWEET activity reinforces quiescence by limiting sugars that can be catabolized in growth regions. A challenge to rice farmers includes stagnant flooding, a condition where plants are partially submerged. It is feasible that the SWEETs may benefit resilience under this situation by promoting movement of sugars to the base of the plant to enhance leaf elongation. It is also possible that SWEETs may be important during germination, as starch is broken down in the seed endosperm to glucose and the resultant sugars are moved to the embryo to fuel growth of the radicle (embryonic root), coleoptile (specialized first leaf) and the shoot (15).

In my project, I surveyed *SWEET* gene activity during germination and tested the hypothesis that *SWEETA* is necessary and sufficient for aerobic germination growth. To test this hypothesis, I performed histochemical staining to monitor the tissue-specific expression of *SWEETA* and growth assays of rice differing in the presence versus absence of a functional *SWEETA*. These included wild-type (Kitaake), mutants which do not make a functional SWEETA (knock-out mutants, *sweeta*), and lines engineered to produce more SWEETA protein (overexpression lines, *SWEETA+*). I also assisted in establishing methods to monitor carbon metabolism during submergence.

Results

SWEETA transcript is consistently high during aerobic germination and in meristematic cells of the shoot during submergence.

To identify *SWEET*s that might be important during germination, I examined published (Narsai et al. (16) and unpublished (Reynoso, Pauluzzi et al., unpublished) RNA-sequence data of gene transcripts of germinating seeds and other rice tissues observed that *SWEETs* are highly regulated in regions of the young rice shoot during germination and by submergence in the shoot base region. In the shoot base study, the gene activity was examined in three cell populations using the Translating Ribosome Affinity Purification (TRAP)-RNA sequencing method. The *35S*-TRAP data cover "all" cells of the sampled tissue. The *RSS1*-TRAP data define actively dividing meristematic cells and the OSH1-TRAP data inform on the less defined shoot apical zones (Figure 1, 2). This supports the hypothesis that this transporter may play a role in supplying sucrose to different areas of the plant during germination and submergence.

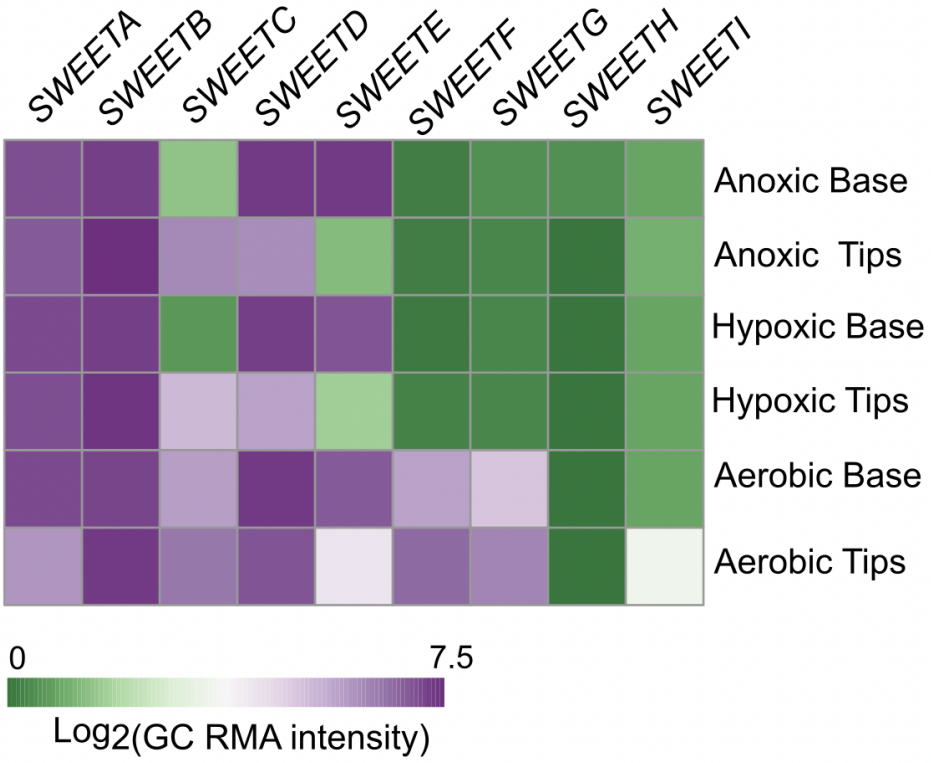

Figure 1. Evaluation of *SWEET* gene activity from tissues of rice seeds germinated in air (aerobic) or under submergence with oxygen deprivation (anoxic, hypoxic) as reported by Narsai et al. (2015) (16). The tip and base of the coleoptile and associated young shoot tissues were sampled. *SWEETA* is one of the genes shown.

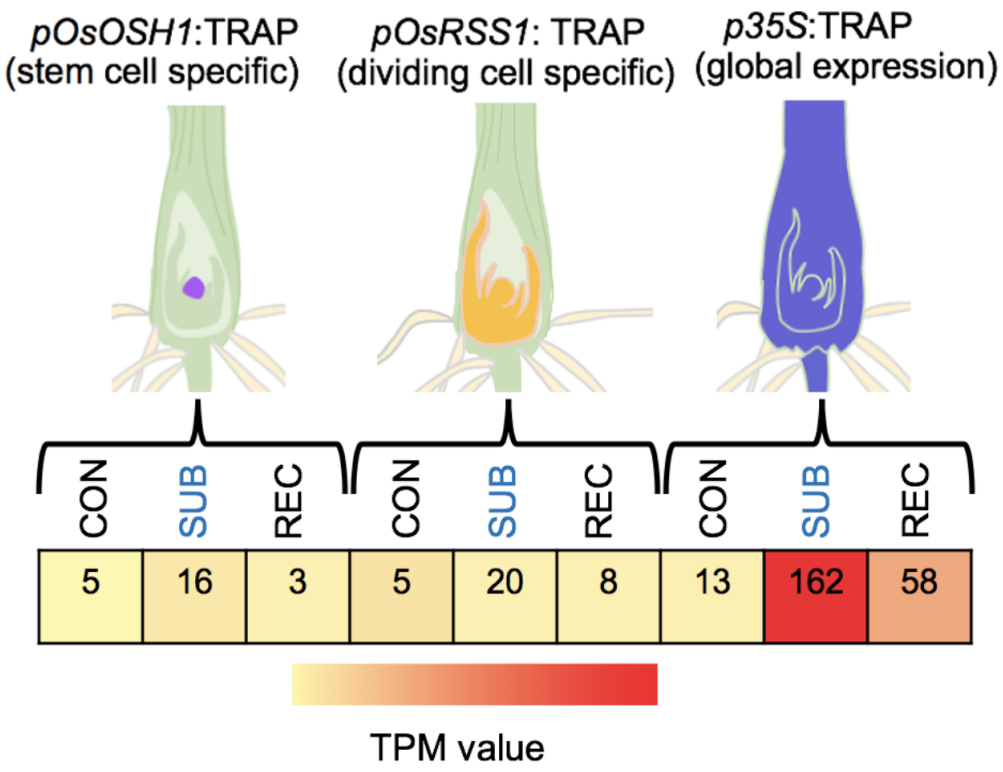

Figure 2. Summary of *SWEETA* expression in three domains of expression in the rice shoot base from unpublished data (Reynoso and Bailey-Serres, unpublished).

Histochemical GUS Localization of SWEETA-GUS

To confirm that *SWEETA* is synthesized in germinating seed, I used two independent transgenic lines of rice that express the SWEETA gene fused to a GUS reporter open-reading frame (SWEETA-GUS Line 1, Line 2). This construct is regulated by the native promoter of the gene and therefore, the pattern of expression is likely to reflect the *bona fide* site of gene activity. These plants produce a fusion protein that can be visualized by staining tissues for beta-glucuronidase activity. I observed very bright GUS staining in the scutellar epithelium, the

root (radicle) and the coleoptile (Figure 3). No staining was observed in the wild-type negative control. The pattern of expression of the SWEETA fusion protein is consistent with the conclusion that SWEETA may transport sucrose from the endosperm, through the scutellar epithelium transfer layer, to the growing radicle and coleoptile of the germinating seed. However, this experiment needs to be repeated at least two times before a final conclusion can be made. I also intend to dissect and photograph the seeds to better understand the location of SWEETA-GUS activity.

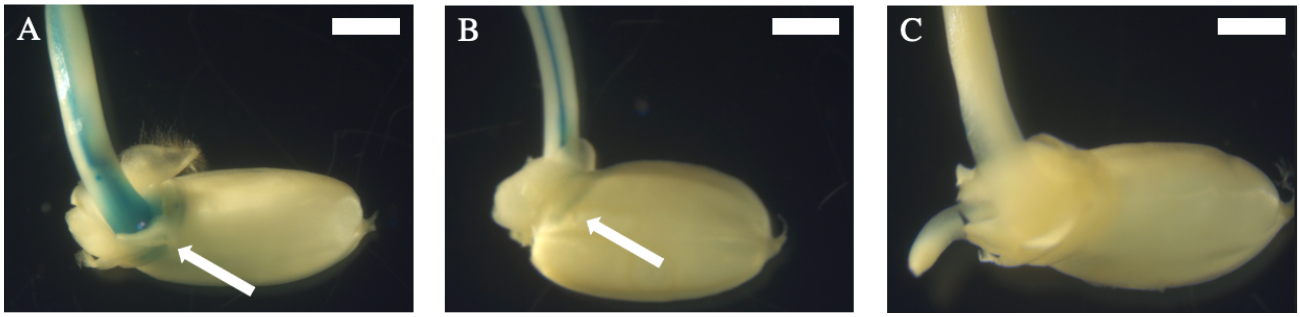

Figure 3. Visualization of SWEETA-GUS fusion protein during aerobic germination.

Line 1 SWEETA-GUS (A), Line 2 SWEETA-GUS (B), wild-type Kitaake (C). Images are from one independent experiment. The white scale bar is 1 mm. The white arrow identifies the GUS signal present in the scutellar epithelium region.

SWEETA is required for maximal primary root and coleoptile growth during aerobic germination.

Next, I examined the hypothesis that the SWEETA protein is required for mobilization of sucrose to the growing embryo. I grew similar sized seeds of two independent knock-out lines (*sweetA-1, sweetA-2*), wild-type (WT) Kitaake as control, and two gene addition (*SWEETA+ Line 16 and Line 18*) in sterile glass flasks with 10 mL of sterile water and measured the length of the radicle

and coleoptile after 4 d of aerobic germination in the dark (Figure 4). The growth of the coleoptile and primary root were significantly reduced in the knock-outs lines by comparison to the other two lines based on ANOVA (coleoptile p value = 2e-16; root p value = 2e-16). In contrast, there was some evidence of enhanced total root length after 4 days of growth in gene addition *SWEETA+ Line 16* (p value = 0.01). We interpret these data to indicate that SWEETA is necessary and sufficient for full radicle and coleoptile growth during aerobic germination.

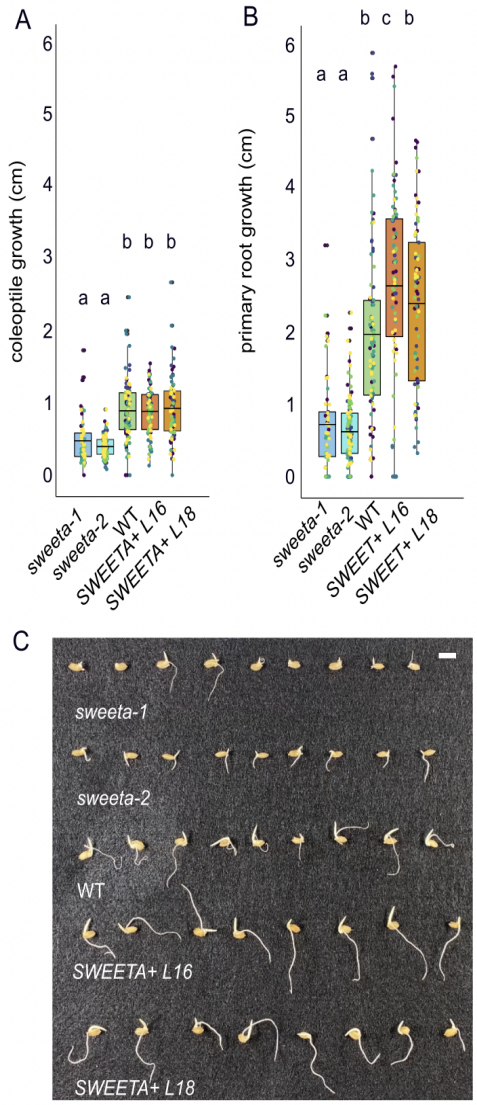

Figure 4. Aerobic growth of s*weetA* knock-out, wild-type, and *SWEETA+* measured by coleoptile length and primary root length. (A) Dot and box plot for coleoptile length of the five genotypes after four days growth for six independent biological replicates. The middle black line is the average. The top and bottom of the black boxes represent the top 25th and bottom 25th percentile, respectively. Each dot represents a single seedling's coleoptile length. An ANOVA was used to evaluate the statistical significance of each knock-out or *SWEET+* line genotype against the wild-type. (B) Dot and box plot for primary root length of the various genotypes measured four days after the start of the experiment. (C) Image of representative seedlings. Scale bar is 1 cm.

Isotopically ^{13}C labeled amino acids are detected in the rice shoot base region during submergence.

Finally, to understand the movements of sugars amongst the various *SWEETA* genotypes, I helped set-up set up a pulse-chase experiment using non-radioactive isotope ^{13}C to track the movement of sugars to the shoot meristem region before and after three days of submergence. This method uses $NaH^{13}CO_3$ (sodium bicarbonate) and lactic acid $C_3H_6O_3$ to produce $^{13}CO_2$ in a closed chamber, which in daylight is taken up by the plant and converted into ^{13}C-sugars produced by photosynthesis. Tissues are processed by liquid chromatography-mass spectrometry (LC-MS) to quantify ^{13}C present in sugars and amino acids. This allows for confirmation of sugar movement from the sink to source tissues and insight into the type of metabolism, i.e., aerobic or anaerobic. We undertook a pilot with wild-type Kitaake plants at the four-leaf (V4 stage). After 7 hours in the chamber with $^{13}CO_2$, the plants were submerged for 1 day and then leaf, shoot base and root tissue were harvested separately and flash frozen. Subsequent LC-MS was performed by our collaborators in Gregory Barding's lab at Cal Poly Pomona University. Their data show that the pulse chase was successful. In particular, submergence increased the level of labeled alanine in leaves, shoot base, and roots (Figure 5), consistent with reports of steady-state metabolite accumulation in rice leaves in response to submergence (9,10).

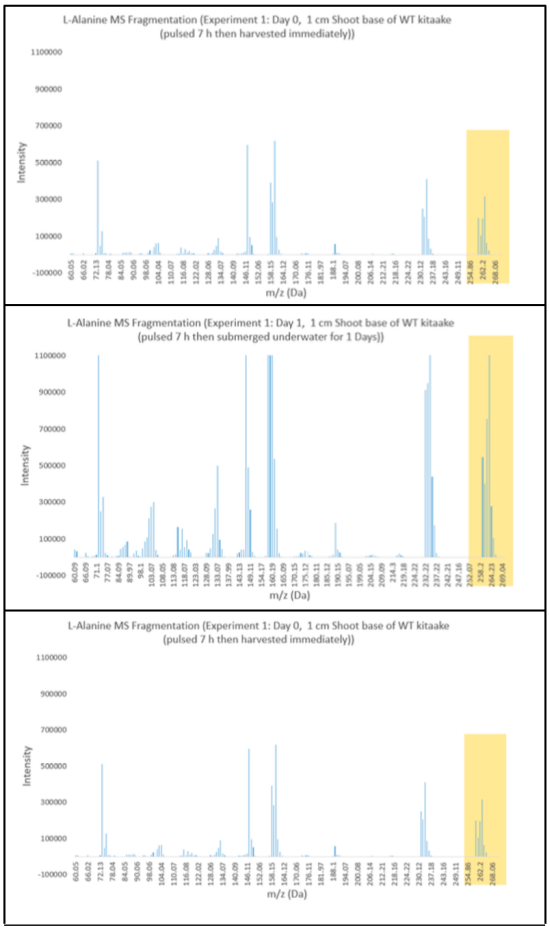

Figure 5. Pilot data of pulse chase and LC/MS analysis for ^{13}C labeled alanine accumulation in three tissues in response to submergence.

Alanine, one of many metabolites monitored, was followed as a marker of anaerobic metabolism. Its increase under submergence confirms that the assay was successful. Alanine containing the isotopes is highlighted in yellow. Analysis of sugars and additional replicates are underway.

Discussion

This study provides evidence that the *SWEETA* gene may play a beneficial role in seed germination. This observation is relevant to establishment of rice planted by direct seeding into soil or shallow paddies, the latter a method used to reduce the use of herbicides by subsistence farmers and organic grain producers. Potentially, *SWEETA* gene copy addition may be leveraged to provide increased germination to better help feed the world through the use of bioengineering or breeding. CRISPR/Cas9-based strategies that modify control sequences, such as the gene promoter, may be used to investigate the potential of this new strategy for improved aerobic germination. My next step would be to test the effect of this gene on germination of seeds underwater (anaerobic germination). The pulse chase experiment I helped to execute provides evidence that sugars produced in leaves in air are transported to the shoot base and roots during submergence. Thus, we can test the effect of *SWEETA* knockout and gene addition on this fundamental process.

Prospectus

This project leverages different aspects of submergence and sugar movement to develop a new strategy for submergence resilient rice. By using synthetic biology to engineer rice plants, this project has begun to vigorously test ways to potentially increase yield. If viable in the field, this strategy may be possible throughout the world to increase global rice yields despite increased submergence events in a changing climate. The knowledge of how meristematic and actively dividing cells survive submergence may allow for effective new strategies for other crop plants by adding additional copies or raising expression of critical sugar transporter genes. Similarly, understanding how the plant alters the movement of sugars during abiotic stress is of great value

for further insight into how the organism uses its limited resources to tolerate severe stress. Taken together, this project has increased our understanding of rice's ability to move sugars during aerobic germination and submergence.

Materials and Methods

Genetic material

Wild-type *Oryza sativa ssp. japonica* cultivar Kitaake was used. The *SWEETA*-GUS (gene addition line) and CRISPR-Cas9 knock-out lines were generated by *Agrobacterium tumefaciens* mediated transformation using hygromycin selection by the group of Bing Yang, University of Missouri. The *SWEET*+ construct was comprised of 1782 bp upstream of the annotated start codon (ATG), the entire *SWEETA* gene with all introns and exons but no 3'UTR, followed by a 2A self-cleavage site, His and Flag tag, then GFP CDS and the OsRPL18 CDS, ending with a NOS terminator.The SWEETA-GUS construct was comprised of 1925 bp upstream of the annotated start codon (ATG), through the native 5'UTR, all exons and introns until the codon before the annotated stop codon. This was fused to the GUS transgene and NOS terminator sequence. Homozygous seeds were confirmed by testing a minimum of 16 seeds through Hygromycin selection at 50 ug/mL on 0.5x Murashige Skoog media plates. The knock-out lines were created by CRISPR/Cas9 mutagenesis or TALEN mutagenesis targeting the first exon. Validation of the knock-out allele was performed by PCR analysis. Single copy insertion verification for the *SWEETA*+ lines was verified by quantitative PCR on genomic DNA from each independent line.

Aerobic germination assay

Seeds produced in the same season were sterilized in 50% (v/v) bleach solution while rocking for 20 minutes (14). Seeds were washed five times in sterilized water then placed in sterilized 250 mL Erlenmeyer glass beaker (Pyrex) with 10 mL of sterilized water. The top was wrapped three times with surgical tape and covered in aluminum foil for full darkness. Flasks were incubated at 37 ºC in an incubator for 4 days. Seedlings were carefully measured and photographed with a ruler. ImageJ software was used for the measurements based on the images.

GUS histochemical staining

Seeds were sterilized and grown as described in (Joon's paper, I will cite) for 4 days, harvested into 80% (v/v) acetone on ice in 50 mL Falcon tubes, and vacuum infiltrated at ambient temperature for 20 minutes in a sealed chamber. Parafilm was wrapped over the tubes to prevent spillover, which were placed at -20 ºC for 45 minutes. Samples were then washed five times with water and vacuum infiltrated with GUS staining solution for 25 min. Samples were incubated in a 37 ºC incubator until staining was visible (16 hours), washed 5 times with 80% (v/v) ethanol, and then imaged with a light microscope.

Tissue Collection for Analysis for pulse chase

The first fully extended leaf of the first tiller, of four separate plants for each genotype, were harvested and flash frozen in liquid nitrogen right before submergence. As detaching the leaf physically hurts the plants, the plants that were chosen for leaf collection were not used for the submergence experiment. Plants that were harvested for a leaf were also harvested for the 1 cm shoot apical meristem (SAM) and roots as for future sugar analysis by LC/MS if desired.

Pulse Chase Experiment

A 0.5 inch thick clear cast acrylic chamber was constructed and sealed with an inlet / outlet. A flask connected by rubber tubing was connected to the chamber. At 5.5 hours before solar noon, V4 stage rice (Kitaake) plants were transferred into the chamber and the pulsing of the $^{13}CO_2$ was saturated in the chamber to a level of ~450 ppm for 7 hours as measured by a Carbon Dioxide Detector Precise CO2 Meter Air Quality Tester, (AR8200). To evolve the $^{13}CO_2$ 4 g of ^{13}C-sodium carbonate (Sigma Aldrich) was dissolved by adding lactic acid (Sigma Aldrich) until the powder was consumed. Plants were removed from the chamber and fully submerged 1.5 hours after noon (5) or maintained on the bench in the greenhouse.

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