Strategies of Transient Submergence Tolerance Cope with Translocation of Photosynthetic Products in Rice Plant

(イネの光合成産物の転流が冠水耐性に及ぼす影響)

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概要

作物栽培において、冠水は生理障害や収量低下を引き起こす環境ストレスの一つである。冠水耐性遺伝子型は冠水条件において、生存に必要な生理的プロセスを維持し、洪水ストレスの影響を軽減する特徴がある。イネは冠水耐性の有無に関係なく、完全冠水期間中の光合成速度を低下させる。光合成産物である非構造性炭水化物（NSC;デンプンと可溶性糖類）の動態は、冠水応答にとって重要であり、これは ^{13}C 標識法を用いることで完全冠水中の光合成産物の転流を評価することができる。本研究では、(1)クロロフィル蛍光 (Fv/Fm)を用いた冠水耐性遺伝子 *Sub1A* の葉身受光能力の検討、(2)冠水中の NSC 転流と冠水耐性の関係の検討、および(3)光合成産物の分配からみた冠水耐性機能の生理学的解析を行った。本研究における第一の要因は、冠水環境条件、第二の要因は、イネ遺伝子型とした。第一実験および第二実験では、Inpari30 (*Sub1A*）および IR72442(非 *Sub1A*)を供試して、播種後 14 日目の苗を水深 35cm 条件下で 6 日間冠水し、その後、株基まで水位を下げて 6 日間の回復期間を設けた。草丈、SPAD、クロロフィル蛍光、および光合成速度の測定は実験を通して同一植物サンプルから継続的に行った。その結果、冠水中の地上部長は、IR72442 が Inpari30 に比較して有意に増加した。両品種とも冠水中の光合成速度の低下が顕著であった。また葉身のクロロフィル含量とクロロフィル蛍光の低下は、Inpari30 より IR72442 で顕著であった。冠水期間中の Inpari30 は IR72442 に比較して、葉身 PSII における光合成の光エネルギー吸収機能の維持及び、回復期間の乾物生産量の増加によって、好気環境における適応性を高めた。第二実験では、冠水前、冠水解除直後および回復後の植物体のデンプン含量と可溶性糖の変化と冠水耐性の関係を比

較・検討した。個体の成長器官のデンプンと可溶性糖は、IR72442 では、冠水中にデンプンと糖類の展開中葉への転流を早め分配していたことが、急激な地上部伸長に影響を及ぼした要因と考えた。一方、冠水中に地上部伸長が抑制され、NSC の転流速度が遅かった Inpari30 は、冠水解除後も NSC 量を維持し、新たに発生した葉への転流速度が IR72442 に比較して早かったことを認めた。第三実験では、安定同位体炭素 ^{13}C を標識した IR67520（*Sub1A*）と IR72442（非 *Sub1A*）を用いて、水深 80 cm の下で 7 日間完全冠水させ、その後 7 日間の回復期間を設けた。その結果、冠水期間中の光合成産物の蓄積・転流量の変化を、標識 ^{13}C の分布から明らかにした。冠水区の IR72442 では、冠水中に酸素不足による代謝活性が抑制されたにもかかわらず、冠水前に同化された光合成産物が、冠水中に新たに発生した葉に素早く転流し、地上部が急激に伸長した。一方、IR67520 は、冠水中の冠水前の光合成同化産物の新葉への転流速度を抑制することで、地上部伸長によるエネルギーの消費を抑制しており、その結果、冠水解除後の光合成速度の回復を早め、新たな光合成同化産物を増加させることで嫌気から好気への環境変化に対応していることが明らかになった。

Summary

Submergence is an environmental challenge for crop cultivation which causes physiological perturbation and yield loss. Tolerant genotypes are characterized by the ability to maintain physiological processes to minimize the negative effects of flooding stress. Photosynthesis decrease during complete submergence, regardless of whether or not a plant tolerates submergence. The metabolic regulation of non-structural carbohydrates (NSCs; polysaccharide and soluble sugars), which control its level and distribution, are important for plants in response to submergence. For instance, the translocation of photosynthates upon complete submergence are believed to influence on plants' tolerance to submergence, which can be evaluated using ^{13}C discrimination approach. This study was aimed to evaluate: (1) the photosynthetic ability in *Sub1A* and non-*Sub1A* rice genotypes during submergence through analysis of chlorophyll fluorescence (Fv/Fm ratio) to monitor a PSII activity; (2) the relation of NSC levels with plant elongation; and (3) the relation of photosynthate distribution with plant elongation. The first factor was the environmental condition consisting of control and submergence. The second factor was rice genotypes consisting of *Sub1A* and non-*Sub1A* rice genotypes. In the first and second experiments Inpari30 (*Sub1A*) and IR72442 (non-*Sub1A*) rice genotypes were used. Measurement of plant height, SPAD, chlorophyll fluorescence and photosynthetic rate indicates that shoot length increased more significantly in IR72442 than in Inpari30 in response to submergence. The noticeable decline was observed in the photosynthetic rate of both genotypes during submergence with severe decrease in chlorophyll content and chlorophyll fluorescence in IR72442 compared to that in Inpari30. In Inpari30, PSII in chloroplasts was presumably maintained during submergence and then after flooding, leading to quick adaptation to an aerobic environment as shown by a recovery of dry weight compared with that in IR72442.

Investigation of the distribution of NSC (starch and soluble sugar) contents in plant organs suggested that elongation of a non-*Sub1A* genotype during submergence was achieved by starch and sugar consumption distributed to the newly developed organs. In contrast, a *Sub1A* genotype such as Inpari30 which did not exhibit shoot elongation and showed slower NSCs distribution during submergence, thus confirming the better growth performance on post submergence due to efficient distribution of retained NSC to the new developed organ.

The changes in photosynthate accumulation and distribution during submergence indicates that even though photosynthetic activity was inhibited during submergence, the assimilate can be translocated to the newly developed leaves of submerged IR72442 (*non Sub1A*), to address the needs for elongation. In contrast, the submergence-tolerant IR67520 (*Sub1A*) exhibited less shoot elongation and slower translocation of ^{13}C labelled substances during submergence, thus the traits presumably brought the better growth performance of this genotype on post-submergence through a quick recovery of metabolic activity.

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Table of Contents

List of Figures

List of Tables

I. Introduction

I-1. Background

Agriculture faces growing problems in terms of climate change with severe weather events that lead to significant losses of yield. In conjunction with a rising population and a higher demand for food, this poses a challenge to scientists and breeders to sustain the existing supply of food. The increase in the frequency and length of heavy rainfall due to climate change has adversely affected plant growth and development, which often causes plant death if it lasts for days. Flooding is arguably the third most significant constraint after heat and drought that decline crop production (Oladosu et al. 2020). The rise in the frequency of flood associated with climate change and the need to increase the current agricultural yield capacity 70% more remain a major challenge for the increasing human population, which is projected to hit more than nine billion by 2050 (Godfray et al. 2010).

Rice is an important crop that serves as a staple food for over 3.5 billion people worldwide and also serves as food security for many African and Asian countries (Chukwu et al. 2019). Rice is widely cultivated in varied environmental conditions, from coastal to high altitude which are significantly affected by flooding due to river discharge, heavy accumulation of rainwater, and tidal movements. These area are prone to cause transient submergence, a type of flood that last for a period of up to 2 weeks, which threaten more than 30 million hectares of rainfed lowland rice in South and South East Asia (Sarkar et al. 2004). Rice is rather tolerant to anaerobic conditions, however, severe flooding, regardless of either total or partial submergence, has often given the adverse effects on this crop.

Flooding primarily restricts gas diffusion between the plants and its surroundings due to lower diffusion rate under water than that in the air (Armstrong 1980; Voesenek and Bailey-Serres 2015). This contributes to a shortage of oxygen within the flooded plant bodies, and it

restricts the production of energy in mitochondria, and inducing fermentation, a type of anaerobic respiration. The availability of carbohydrates in the plant and the plant efficiency to use low energy production largely affect plant productivity and survival. Furthermore, low CO_2 availability in flooded leaves inhibits photosynthesis which leads to energy crisis within plant cells (Voesenek et al. 2006; Mustroph 2018).

During submergence, certain plant species including rice consume a large amounts of carbohydrates from sources such as starch, otherwise they cannot deal with oxygen deficiency. This process mediated by a specific amylase and its metabolic regulation have a critical role in this process (Mustroph 2018). With high carbohydrate availability, deepwater-rice varieties adopt a strategy called "escape strategy", which allows them rapid elongation; this leads to outgrow a flood within a short time, and thus confirming their survival (Vriezen et al. 2003; Hattori et al. 2009). On the other hand, other rice varieties used another strategy called "quiescence strategy". In this strategy, *Sub1A* gene enables the plants to survive under deep floods for up to 14 days by restricting carbohydrate usage (Septiningsih et al. 2015). So far, the underlying mechanisms of both strategies, especially causative gene functions have been intensively studied, however, only the second strategy "quiescence strategy" has been successfully adopted for rice breeding to confer flood tolerance. In contrast, the first strategy has not, because of its negative effect on crop stability observed after the flood recede (Mustroph 2018). Addressing the mechanistic difference between the quiescence strategy and the escape strategy, which are characterized by the presence and absence of *Sub1A* gene, respectively, this study evaluated the effects of submergence stress on shoot elongation, photosynthetic performance and photosynthate translocation.

Photosynthesis is a complex process involving light reactions that produce an energy in the form of adenosine triphosphate (ATP) and nicotinamide adenine dinucleotide phosphate (NADPH) as a reducing agent (Johnson 2017). In the light reaction in chloroplasts, the

photosynthetic pigments located in the photosynthesizers absorb the energy of a photon at a specific wavelength and then utilize this energy to initiate a chain of photochemical events (Hall and Rao 1999). Among photochemical reaction apparatuses, photosystem II (PSII) is known to be the most sensitive to environmental stresses, including submergence, of which damages often cause the inhibition of photosynthesis (Sayed 2003). Damage to PSII can be detected by observing the potential maximum quantum yield (proportional to the Fv/Fm ratio, a measure of chlorophyll fluorescence), which generally decreases in plants suffering environmental stress (Maxwell and Johnson 2000). An evaluation has been reported on the contribution of *Sub1A* to the inhibition and restoration of net photosynthesis and photosystem II photochemistry (Fv/Fm) along with chlorophyll content, carbohydrate and nitrogen metabolism during submergence and reoxygenation (Panda et al. 2008; Sarkar and Panda 2009; Alpuerto et al. 2016). In this study, we evaluated the photosystem II (PSII)-mediated photochemistry during submergence and recovery periods, by the indirect physiological measurement of quantum yield of chlorophyll fluorescence (Fv/Fm) and chlorophyll content using a SPAD meter.

In the Calvin-Benson cycle, ATP and NADPH are utilised to convert CO_2 into carbohydrates. Finally, glyceraldehyde 3-phosphate (GAP) is produced as an end product in the Calvin-Benson cycle, which is subsequently taken into soluble sugar, glucose (Johnson 2017). Non-structural carbohydrate (i.e. starch and sugar) concentrations in rice shoots have reportedly been associated with genotypic differences in ability of submergence tolerance (Das et al. 2009). Numerous studies using submerged rice plants describe the relations of NSC with various growth parameters (Sarkar 1997), elongation ability (Das et al. 2005), different ages of rice seedlings (Das et al. 2009), impeded metabolism (Adak et al. 2011), catabolism processes (Panda and Sarkar 2012b, 2014), and application time of nitrogen and phosphorus fertilizers (Gautam et al. 2014). In this study, we investigated the timing of NSC consumption, and the

NSC distribution in plant body during submergence using ^{13}C-labeled tracer approach, to evaluate the relations of these parameters with different elongation between *Sub1A* and non-*Sub1A* genotype. The discrimination against $^{13}CO_2$, a smaller proportion of ^{13}C being incorporated into organic material during photosynthesis can be used to assign plants to various environmental condition (Griffith 1993; Condon et al. 2006). There are limited reports on ^{13}C discrimination during photosynthesis in submerged rice; however, isotope labelling has been used to study the effect of waterlogging on several types of plants such as *Larix gmelinii* and *Salix sp.* in northeastern Siberia (Fan et al. 2018; Li and Sugimoto 2018). Using ^{13}C-labeled tracer approach, we evaluated the photosynthesis product translocation during submergence between *Sub1A* genotype of IR67520 and non-*Sub1A* genotype of IR72442.

I-2. Problem Statement

Photosynthesis is severely inhibited by slow gas diffusion rate between submerged rice plants and their surrounding environments. Some rice genotypes with *Sub1A* gene slowly grew during submergence, while other genotypes without *Sub1A* extensively elongated, which presumably contributing to an escape on from submergence. To further address different behaviours in plant growth between the two genotypes, other methods are necessary, which allow us to study photosynthesis performance during submergence (or under water). In this study we proposed the integrating measurement of chlorophyll fluorescence and chlorophyll content (SPAD) to evaluate photosynthetic ability of submerged plants. In addition, analysing photosynthate translocation using ^{13}C-labeled tracer approach are also effective to study the relation of carbohydrate distribution with elongation of submerged plants.

I-3. Novelties

This study offers the following novelties:

1. Integration of SPAD and Fv/Fm to determine photosynthetic ability of submerged rice plant
2. Analysis of the requirement of NSCs for elongation growth of submerged rice plants.
3. Use of ^{13}C-laveled tracer approach to study photosynthate translocation of *sub1A* genotype during submergence

I-4. Objectives

The current study specifically addressed the following:

1. To determine the photosynthetic ability of *Sub1A* and non-*Sub1A* genotypes by evaluating Fv/Fm performance
2. To assess the non-structural carbohydrate in relation with shoot elongation of rice under complete submergence
3. To investigate photosynthetic product translocation of rice under submergence using a ^{13}C-labeled tracer approach in response to shoot elongation Using a ^{13}C-labeled tracer approach, to investigate photosynthetic product translocation with respect to shoot elongation in submerged rice plants.

I-5. Research Scope

This research was based on three inter-linked objectives aiming at studying the photosynthetic performance in relation with shoot elongation under water: to this aim, photosynthetic capacity and translocation of photosynthates in submergence rice were characterized. These research activities were carried out in the controlled room. The three activities were:

1. Characterizing the photosynthetic ability of the submergence-tolerant rice variety, ~~of~~ Inpari30 via measuring maximum quantum yield performance during transient flooding stress and recovery
2. Analysis of non-structural carbohydrates in relation with shoot elongation of rice under complete submergence
3. Characterizing translocation of 13C-labelled photoassimilates in submerged rice plants with genetic traits of either quiescent growth or shoot elongation

II. Characterization of the photosynthetic ability of the submergence-tolerant rice variety of Inpari30 by examining maximum quantum yield performance during transient flooding stress and recovery

II-1. Introduction

Complete submergence is defined as a condition when the entire plant is fully and continuously submerged underwater for 7–14 days (Das et al. 2005; Nishiuchi et al. 2012). Submergence limits plant growth through slow diffusion rate of oxygen underwater (Jackson 1984) which likely inhibits metabolic processes (Setter et al. 1989). A transient flooding event reportedly perturbs plant metabolism twice: once during submergence and again during the re-aeration that occurs after the water recedes, both of which often bring internal stresses to reduce shoot growth and survival rates (Pradhan and Mohanty 2013). When a plant is submerged, the resultant environment limits gas diffusion between plant and the surrounding area, and light transmission to underwater, which together result in oxygen deficiency, decreased transpiration, and starvation (reduced photosynthesis ability). Upon re-aeration, the plant is suddenly exposed to an excess amount of oxygen and relatively high light intensity, which lead to oxidative stress and dehydration (Panda and Sarkar 2012a; Tamang and Fukao 2015).

Any sudden decrease in gas diffusion is a major problem for plants, because of limiting the photosynthesis and respiration processes that require oxygen and carbon dioxide as substrates (Pradhan and Mohanty 2013). Among photosynthetic apparatuses, photosystem II (PSII) is known to be the most sensitive to environmental changes, including submergence before irreversible damage becomes apparent (Sayed 2003). Damage to PSII can be detected by observing the potential maximum quantum yield (proportional to the Fv/Fm ratio, a measurement of chlorophyll fluorescence), which generally decreases in plants suffering environmental stress. In general, a decline in Fv/Fm is a signature of a photoinhibition

phenomenon involving not only photodamage to the photosynthetic system, but also a photoprotective mechanism (Björkman and Demmig 1987). The chlorophyll fluorescence parameter Fv/Fm has been widely used for measurement of maximum quantum yield of PS II, as it is a non-invasive technique suitable for studying the tolerance of the photosynthetic apparatus to environmental constraints (Murchie and Lawson 2013). Fluorescence emission is an indicator of the composition of the antenna system used in photosynthesis, which is influenced by chlorophyll content per leaf area (Lichtenthaler et al. 1986). The measurement of chlorophyll fluorescence provides a lot of information on almost all aspects of PSII and also on the plant response to different environmental factors such as stress and nutrient availability (Björkman and Demmig 1987; Zhu et al. 2005). Three functions of chlorophyll are important for photosynthesis: light absorbance, light energy transport by resonance energy transfer into a specific chlorophyll pair in the reaction centre of the photosystem, and facilitation of the charge separation leading to carbon dioxide fixation (Murchie and Lawson 2013).

A rice variety, Ciherang is popular in Indonesia due to high yielding capacity, wide adaptability, and high quality grain preferred by Indonesian costumers (Ruskandar 2009). However, this variety is sensitive to unexpected inundation (Nurrahma et al. 2017). It is mandatory to provide suitable rice varieties that can maintain their intrinsic yields after unpredictable rainfall caused flooding, especially in the countries where rice stand as the main staple food. Induction of these varieties would also contribute to increased productivity of rice in the less favourable environments i.e. tidal lowland or coastal which also prone toward submergence (Rumanti et al. 2018).

A major quantitative trait locus (QTL) responsible for submergence tolerance, *Sub1A*, has been widely studied in FR13A rice (*Oryza sativa* **L.**) and regarded as conferring submergence tolerance in rice breeding (Xu and Mackill 1996; Xu et al. 2006; Panda and Sarkar 2012b). For example, introgression of *Sub1A* into the high yielding rice variety,

Ciherang in Indonesia has generated a new variety named Inpari30, which shows no significant difference from the parental variety (Ciherang) in all traits observed under normal conditions (Septiningsih et al. 2015; Nugraha et al. 2017). However, limited study reported photosynthetic activity following submergence which shall be responsible during recovery and gain assimilates for yield production to characterize newly developed submergence tolerant variety of Inpari30. However, so far limited studies report photosynthetic activity during submergence of a newly developed submergence tolerant variety of Inpari30, which is presumably responsible for plant physiology post submergence and also for recovery of sugar assimilation affecting its productivity.

The effect of submergence on shoot elongation was tested for 15 genotypes of *Oryza sativa* for 7 days under 80 cm water depth, resulted the highest ratio of submerged to control plant in IR72442 among all cultivar, and IR72442 was found to show the highest ratio of submerged to control plant among all cultivar (Sone et al. 2012). Therefore, they used this variety as a control cultivar with a typical shoot-elongating trait for a non-shoot-elongating test cultivar to evaluate the difference in chlorophyll content and chlorophyll fluorescence under submergence. Upon the obtained results, they concluded that a shoot-elongating cultivar was able to maintain the photosynthetic capacity in the new leaves developed during submergence by prompting reduction of chlorophyll and chlorophyll fluorescence in the leaf developed before submergence.

In this study, photosynthetic performance of Inpari30 during submergence and post submergence was examined to obtain a basic knowledge for explaining the physiological mechanisms underlying tolerance of Inpari30 to submergence stress. Besides, photosynthetic performance between Inpari30 with *Sub1A* gene and IR72442 without *Sub1A* was also compared by integrating measurements of gas exchange analysis, chlorophyll content (SPAD value), and chlorophyll fluorescence (Fv/Fm).

II-2. Material and Methods

1. Location

The experiment was conducted from June to July 2018, in a plant cultivation room at the Tropical Crop Science Laboratory, Kagoshima University, Japan. The daily temperature during the experiment was maintained 27°C with 350 μmol m^{-2} s^{-1} of 12 h light exposure. Temperature and light condition in the room were set at 27°C and at 350 μmol m^{-2} s^{-1} for 12 h, respectively.

2. Experimental Design

A completely randomized design was employed in this experiment. Two factors were studied. The first factor was environmental conditions: control versus submergence. The second factor was rice variety, which was fully randomized on the environmental conditions. To compare tolerance mechanisms to transient submergence, we grew two rice varieties with (Inpari30) or without (IR72442) *Sub1A*gene under complete submergence followed by several days of re-aeration. The experiment was conducted in three replications.

3. Experimental setup and conduction

Rice seeds of Inpari30 and IR72442 were soaked and incubated for three days at 30°C. Germinated seeds were then sown into commercial soil for rice seedling (N:P:K = 0.9:2.3:1.1; pH 4.5–5.2) in a growth chamber. Ten-day-old seedlings were transplanted into a sponge and inserted into a seedling tray inside experimental glass containers. The water level was maintained at 4.5 cm from the container base, the same level as the seedling tray surface also the plant stem base. The experiment was conducted in a controlled room with 12 h light exposure at 350 μmol m^{-2} s^{-1} measured 20 cm above the tray surface and at 27°C

(Day/Night). However, under submergence the light exposure was approximately 200 μmol m^{-2} s^{-1} lower. The oxygen concentration at the same water depth was ranging from 7.0-8.9 mg L^{-1} during the experiment.

At 14 days after seeding (DAS), when the third leaf was fully expanded, the water level in the glass container was raised to 35 cm above the shoot base for the test plants, while the water level for the control plants was maintained as height as the stem base. Treatment was stopped after 6 days (20 DAS), at which point the water level for treatment and control was adjusted to the stem base for maintain the same water level. The recovery stage lasted 6 days, and then all plants were removed from their containers (i.e. at 26 DAS). To evaluate the effect of stress exposure, measurement of Fv/Fm, SPAD value and photosynthesis rate were performed using the 3rd leaf that was fully expanded before treatment started.

4. Variable analysed

Variables explained below was observed before water level was increased (referred to as 'before submergence'), at 7 days of submergence when the plants were just removed from the water-filled glass container (referred to as 'desubmergence'), and at 7 days after desubmergence (referred to as 'post submergence').

4.1. PS II quantum yield (Fv/Fm)

After 2 hours of dark adaptation, an equipment to measure chlorophyll fluorescence (AquaPen-P AP-P 100, PSI, Czech Republic) was attached to the third leaf of the rice plant. The maximal quantum yield of PS II photochemistry (Fv/Fm) was obtained by actinic light emission through the quantum yield (QY) menu. Variable fluorescence (Fv) equals the fluorescence increases from minimal fluorescence (Fo) to

maximal fluorescence (Fm), Fv = Fm – Fo. Then the maximal QY of PS II Fv/Fm is the function of QY = (Fm – Fo)/Fm (Hall et al., 1993; Panda et al., 2008). Any changes in Fm or Fo would result in the change of Fv/Fm. The maximal fluorescence (Fv/Fm) value is 0.85 that means 85% efficiency of the conversion of absorbed light into photochemistry.

4.2. Leaf chlorophyll content (SPAD Value)

The amount of chlorophyll present in leaves was estimated using a leaf chlorophyll meter (SPAD-502, Konica Minolta Corporation, Japan). The chlorophyll content was represented by the average Soil Plant Analysis Development (SPAD) value of three measurements on the tip, middle, and base of the third leaf. The strong relationship between SPAD index and leaf chlorophyll concentration has been widely. This tool measures the chlorophyll content of the leave without destruction, simple, quick, and portable. Moreover, a positive correlation between SPAD reading and chlorophyll content in rice was reported (Kumagai et al. 2009). The leaf chlorophyll content is affected by Nitrogen status in the plant that commonly has a strong positive relationship with the photosynthetic rate (Sinclair and Horie 1989). Therefore, SPAD measurement can be used as an indirect countermeasure of photosynthetic capacity on the rice leaf.

4.3. Plant Length (cm)

The length of each plant was measured from the stem base to the highest shoot tip using a ruler. The data presented are the average from 12 plant samples in three replicates.

4.4. Gas exchange measurements

Measurement of net photosynthesis (P_N) was assessed using a portable photosynthesis analysis system (LI-6400; LI-COR, Lincoln, NE, USA). Before

measurements, plant samples were adapted under light exposure of 300-350 μmol m^{-2} s^{-1} same as growth environment condition. The third leaf of each sample was selected and kept inside the chamber until a stable reading was recorded. Leaf area was measured to calculate the gas exchanges rate per observed area.

During the measurement of net photosynthesis, relative humidity was ~50%, leaf temperature 25°C, ambient CO_2 concentration ~400 μmol mol^{-1}, airflow through the chamber was maintained at 500 μmol s^{-1}, and photosynthetic photon flux density (PPFD) 1100 μmol m^{-2} s^{-1}.

4.5. Total dry weight (g)

Four plant samples from each test plot were collected for total dry weight measurement. The plant samples were put in envelopes and dried in an oven for 72 h at 80°C. The ratio S/C (submerged/control) for weight change was then calculated.

5. Statistical analysis

Data were analysed from three replications using two ways of analysis of variance. Tukey test was performed at $p = 0.05$ to evaluate statistical significance of different values.

II-3. Results

1. Effect of submergence on the increase of plant length

Plant length of submerged IR72442 increased significantly more than its control (>34.3% after 6 days of submergence and >40.9% by the end of the 6-day post submergence period) and significantly more than both the submerged and control of Inpari30. Conversely, the smaller increase in plant length of submerged Inpari30 was not statistically significant compared to its control. The ratio of plant lengths (after

submergence versus control) was 1.23 for IR72442 and 1.02 for Inpari30 (Fig. 2.1). The limited elongation of Inpari30 during submergence seems to reflect the slow growth characteristic conferred by *Sub1A*.

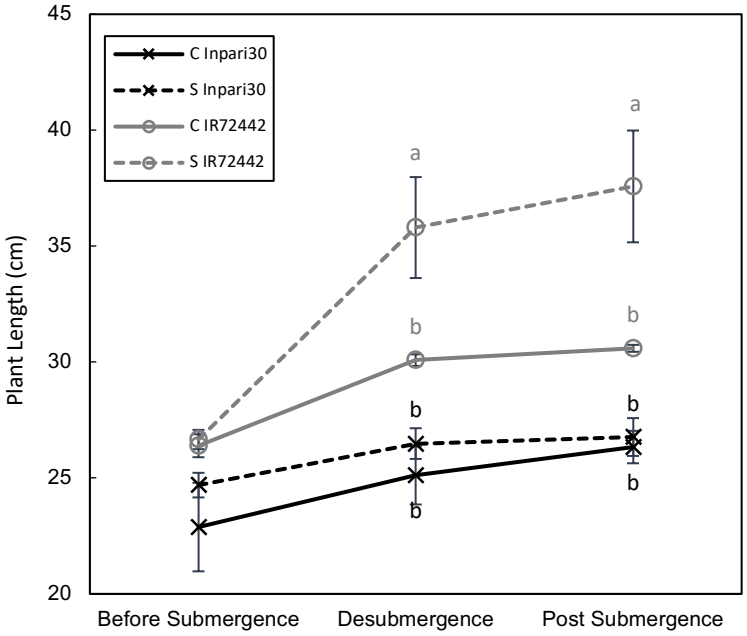


Fig. 2.1. Plant length of two rice varieties (IR72442, an elongation type; Inpari30, carrying gene *Sub1A*) before submergence, desubmergence, and post submergence (re-aeration). Different lowercase letters in the same column indicate a significant difference at $p < 0.05$ with Tukey test. Error bar represent standard deviation from three replications. C, Control; S, Submerged.

2. Submergence decreased chlorophyll content (SPAD value)

The SPAD values for submerged IR72442 decreased significantly versus control (72.4%) on desubmergence whereas those for submerged Inpari30 decreased by only 14.4% (not significant). The value of all treatment did not change much on post submergence. Inpari30 maintained higher chlorophyll levels on desubmergence and post submergence.

The chlorophyll content of IR72442 decreased on desubmergence and did not recover well on post submergence (Fig. 2.2).

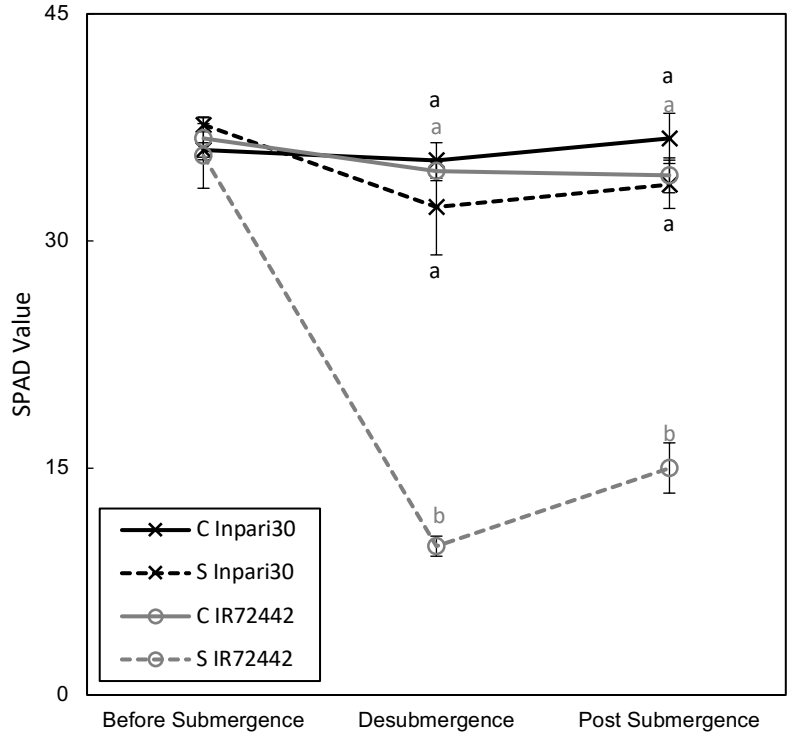


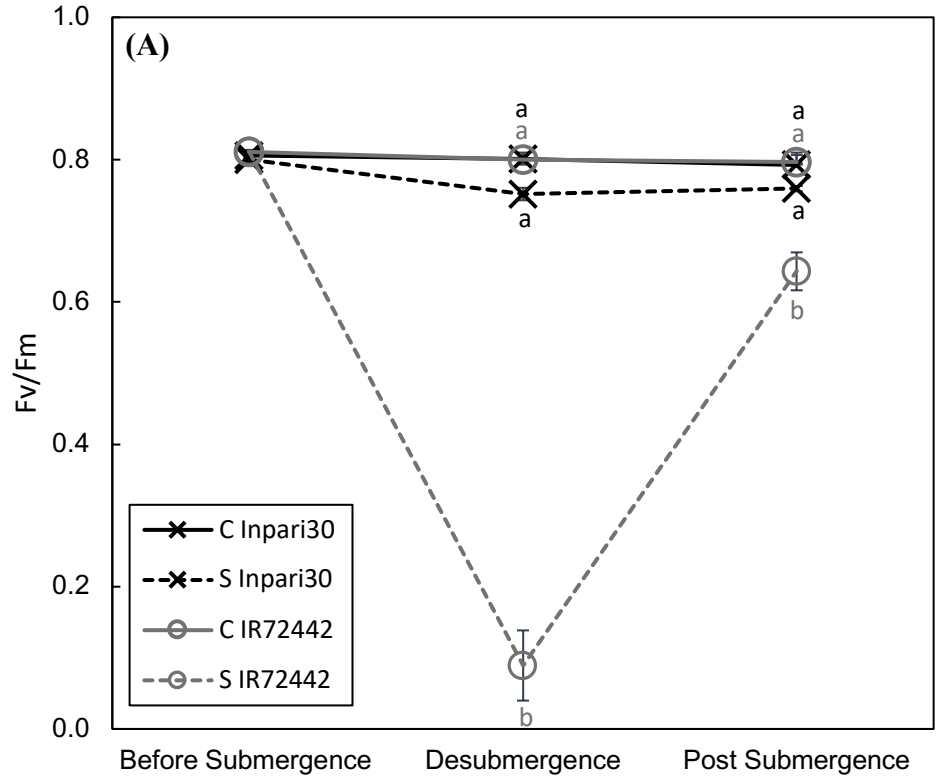
Fig. 2.2. Chlorophyll content (SPAD Value) of two contrasting rice varieties (*Sub1A* genotype of Inpari30, and non-*Sub1A* genotype of IR72442) before submergence, after desubmergence, and post submergence (re-aeration). Different lowercase letters in the same column indicate a significant difference at $p < 0.05$ with Tukey test. Error bar represents standard deviation from three replications. C, Control; S, Submerged.

3. Influence of anaerobic environment on Fv/Fm and photosynthesis rate

Fv/Fm for the non-submerged (=control) plants of both varieties did not differ significantly throughout the experiment. On desubmergence, Fv/Fm for submerged IR72442 decreased significantly (88.9%). Although the value largely recovered on post submergence (to 6.11 times the submerged value), Fv/Fm remained still significantly

lower than that of the control plant. In contrast, on desubmergence, Fv/Fm for submerged Inpari30 decreased marginally (not significant) and then increased very slightly on post submergence (Fig. 2.3A).

On desubmergence, net photosynthesis for both varieties decreased significantly versus their corresponding controls (>41%), and although their net photosynthesis rates recovered to some extent on post submergence (>27.4%), the values remained significantly lower than that of controls (Fig. 2.3A).



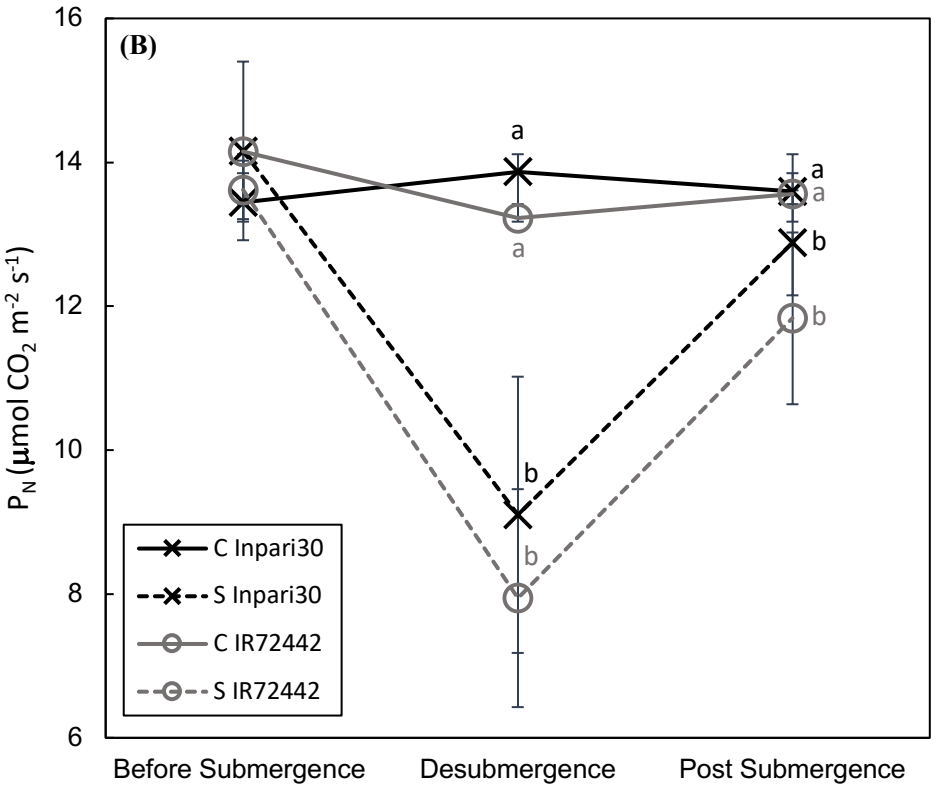


Fig. 2.3. Fv/Fm (A) and net photosynthesis rate (B) of two rice varieties (*Sub1A* genotype of Inpari30, and non-*Sub1A* genotype of IR72442) before submergence, during desubmergence, and post submergence (re-aeration). Different lowercase letters in the same column indicate a significant difference at $p < 0.05$ with Tukey test. Error bar represents standard deviation from three replications. C, Control; S, Submerged.

4. Total dry weight changes affected by submergence

Comparison of growth was made via changes in total dry weight during the submergence period and post submergence period, expressed as the ratio between submerged (S) and control (C) plants. The S/C ratio for Inpari30 was higher than that for IR72442 after submergence and after recovery (Table 2.1).

Table 2.1. Ratio of total dry weight after submergence and after recovery (re-aeration)

Treatment	Total dry weight during submergence (g plant^{-1})	S/C ratio	Total dry weight of post submergence (g plant^{-1})	S/C ratio
C Inpari30	0.0188	0.76 a	0.0152 d	2.15 a
S Inpari30	0.0144		0.0326 a	
C IR72442	0.0180	0.59 b	0.0242 bc	1.07 b
S IR72442	0.0105		0.0259 b	

Note: C, Control; S, Submerged. Total dry weight during submergence is the difference value between total dry weight desubmergence and before submergence. Total dry weight post submergence is the difference value between total dry weight post submergence and desubmergence.

5. The ratio of shoot dry weight per shoot length

The ratio of shoot dry weight and shoot length was similar between varieties and treatment before exposing to submergence. At desubmergence, submerged IR72442 decreased the ratio significantly lower than non-submerged and submerged Inpari30, though no significant difference with non-submerged IR72442. On post submergence, submerged IR72442 was significantly lower than submerged Inpari30, though there is no significant observed with non-submerged plant (Fig. 2.4).

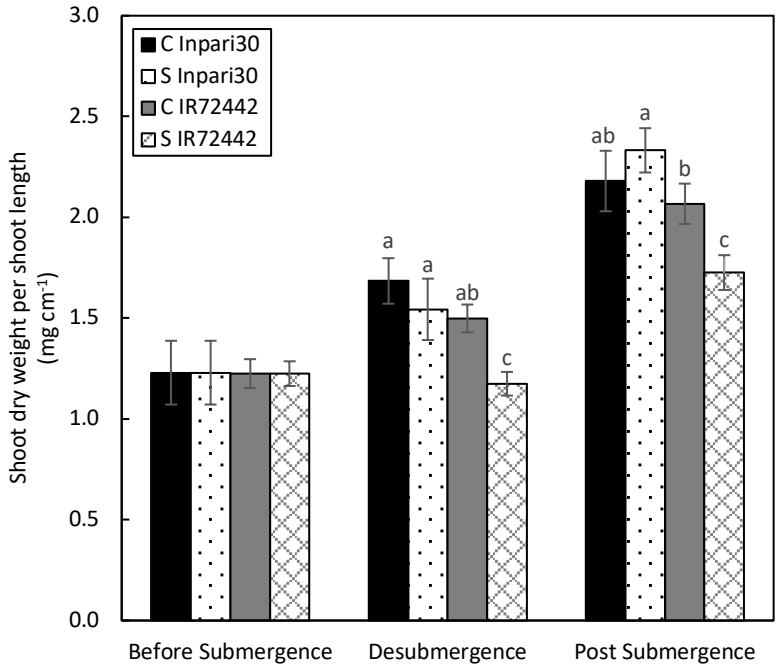


Fig. 2.4. Changes of shoot dry weight per shoot length between two contrasting rice varieties (IR72442, an elongation type without a *Sub1A* gene; Inpari30, carrying a *Sub1A* gene) before submergence, desubmergence, and post-submergence (re-aeration). Different lowercase letters indicate a significant difference at $p < 0.05$ with Tukey test. Error bar represents standard deviation from three replications. C, Control; S, Submerged.

The ratio of shoot dry weight to shoot length during the experiment is explained in Fig. 2.4. IR72442 significantly the most strongly increased plant length at desubmergence among all treatments (Fig. 2.1). However, the increase of plant length was not corelated with that of shoot dry weight. Thus, the ratio of shoot dry weight per shoot elongation was lower in submerged IR72442 compared with that of Inpari30 as shown in Fig. 2.4.

6. The changes in photosynthesis rate over SPAD value and Fv/Fm

The scatterplot figure represents the relationship between variables of submerged Inpari30 and IR72442. Given the significant changes in the photosynthetic rate observed in both varieties, the fluctuations in SPAD value and Fv/Fm value in Inpari30 were smaller than IR72442 (Fig. 2.5A, B). Plant physiological response of SPAD value and Fv/Fm to net photosynthetic rate (Pn) was lower in Inpari30 than in IR72442. Besides, this result indicates that the of SPAD value and Fv/Fm is more independent than the photosynthesis rate in explaining the effect of submergence on Inpari30, but not on IR72442.

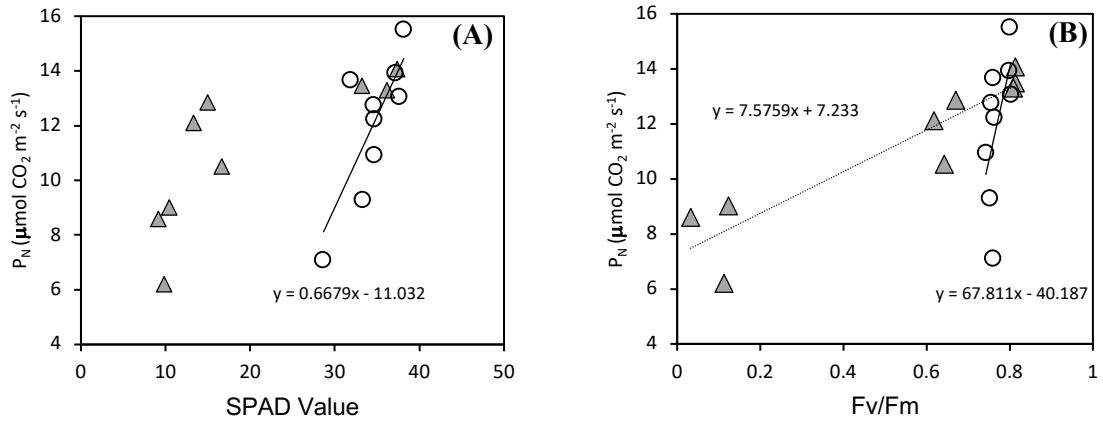


Fig. 2.5. Relationship between (A) SPAD value, (B) Fv/Fm and net photosynthesis of submerged Inpari30 and IR72442 rice plants. Triangle shape represents submerged IR72442 and circle represents submerged Inpari30.

II-4. Discussions

The adverse effect of submergence commonly does not appear immediately as ~~visual~~ visible damage, but it becomes observable soon after the water level recedes (Drew 1997). The first noticeable effect of submergence was the increased plant length of IR72442, which was greater than Inpari30 at desubmergence (Fig. 2.1). According to the mechanism of submergence tolerance proposed in previous studies (Nagai et al. 2010; (Hattori et al. 2011), the elongation exhibited by IR72442 represents an intolerance trait to submergence whereas the quiescent growth exhibited by Inpari30 represents a tolerance trait to submergence. Inpari30 carries a *Sub1A* gene (Septiningsih et al. 2015), which limits plant elongation during submergence. Thus, this variety enables a greater accumulation of shoot dry matter than IR72442 on recovery, as indicated by the S/C ratio (Table 2.1). This phenomenon can be explained by conserved energy under submergence being used for regrowth after that the water level subsides. This result contrasts with that of Sarkar et al. 1996, who reported that submergence-tolerant cultivars accumulated more carbohydrates before submergence to elongate the seedling height allowing the production of new carbohydrates as the leaves became exposed to sunlight above the water. In varieties exhibiting shoot elongation, the dry weight of leaves developed before submergence is used for leaf elongation (Kawano et al. 2008). In this study, IR72442 showed extensive shoot elongation, with low ratio of dry weight per shoot elongation (Fig. 2.4), despite a decline in photosynthetic rate observed during submergence (Fig. 2.2B).

Chlorophyll fluorescence (Fv/Fm) is an effective indicator of submergence tolerance of rice (Sone et al. 2012). In this study, Fv/Fm for the controls (i.e. non-stress conditions) did not differ significantly between Inpari30 and IR72442 (Fig. 2.2A); therefore, the decrease in Fv/Fm of IR72442 during submergence can be attributed to the submergence treatment. Conversely, net photosynthesis rapidly decreased during submergence for both Inpari30 and

IR72442 versus their corresponding non-submerged controls (Fig. 2.2B). Significant decline in Fv/Fm value in the susceptible cultivars than tolerant cultivars also reported by (Panda et al. 2008). In the study, they assumed that deterioration of PSII in tolerant cultivars was comparatively less than susceptible ones. The decline in Fv/Fm for IR72442 during submergence likely reflects a reduced ability of PSII to reduce the primary acceptor (Panda and Sarkar 2012a). This provides evidence for disorganization of the photosynthetic apparatus and is attributed to a decrease in light intensity and oxygen level in floodwater (Panda et al. 2008; Panda and Sarkar 2012a). This reduction is also indicative of photoinhibition damage in response to environmental stress resulting in a decline in the efficiency of solar energy conversion during photosynthesis in combination with an overall decline in photosynthetic capacity (Inamullah and Isoda 2005). Submerged plants of Inpari30 described to maintain absorbing energy from light, however plants declined photosynthetic rate by limiting gas exchange due to stomatal closure under anaerobic conditions.

On post submergence, Fv/Fm for the submerged IR72442 recovered but not to a level of the non-submerged controls. However, the tolerant rice variety, Inpari30, was quickly adapted to the aerobic environment, as suggested by high Fv/Fm values (Fig. 2.2A). On post submergence, the photosynthetic rate largely recovered from that on submergence in both varieties, but remained significantly lower than those of the non-submerged controls (Fig. 2.2B). Variable fluorescence (Fv) equals the fluorescence increase from minimal fluorescence (Fo) to maximal fluorescence (Fm), Fv = Fm – Fo. Then the maximal quantum yield of PS II Fv/Fm is the function of quantum yield = (Fm – Fo)/Fm (Hall et al., 1993; Panda et al., 2008). Any changes in Fm or Fo would cause the change of Fv/Fm. However, the increased Fv/Fm value in the susceptible variety does not necessarily indicate an increase of PSII efficiency, a substitute of minimal fluorescence (Fo) decreasing compared to maximal fluorescence (Fm) on

the measurement observed (Panda et al., 2008). This describes the possible cause of increasing Fv/Fm of submerged IR72442 of post submergence.

The photosynthetic rate in both varieties decreased on at desubmergence; however, Inpari30 grew effectively on post submergence as reflected by the high dry weight ratio of submerged to non-submerged control (Table 2.1). Shoot dry weight in both varieties declined at desubmergence, but not on post submergence, shoot dry weight for Inpari30 recovered to the similar level as the controls. The higher ratio of shoot dry weight per length on Inpari30 represents that by slow elongation during submergence, this variety enabled to maintain dry matter (Fig. 2.4). This observation is consistent with the results of Sarkar and Bhattacharjee (2011), who found that *Sub1A* varieties exhibit the ability to maintain higher biomass and can re-grow faster during re-aeration.

The small changes in SPAD value and Fv/Fm probably explains that both variables do not affect the large changes in photosynthesis in Inpari30, but not in IR72442 (Fig. 2.5A, B). This also means that the response of the SPAD value and Fv/Fm is independent of photosynthesis rate especially in submerged Inpari30. In contrast, the large changes in SPAD and Fv/Fm value in IR72442 as the change of photosynthesis rate perhaps related to varietal factors in response to environmental changes during submergence, such as changes of light intensity that reflected by SPAD and Fv/Fm measurements. Light intensity changed from 350 to 330 μmol m^{-2} s^{-1} during submergence which responded by Inpari30 and IR72442 differently.

Photosynthesis, the transformation process of carbon dioxide into organic matter in the presence of plant chlorophylls under the influence of light, involves the release of oxygen from water (Gest, 2002). Chlorophyll converts the energy from captured light photons into chemical energy that is used to drive CO_2 fixation (Murchie and Lawson, 2013). Under laboratory conditions, the transport of PSII electron and the fixation of CO_2 can be very well associated. However, this tight connection between PSII function and CO_2 fixation is not necessarily

observed in field conditions. Differences can be explained by changes in the relative levels of CO_2 fixation and by competing processes such as photorespiration, nitrogen metabolism and oxygen donation (Maxwell and Johnson, 2000). The electrons released during the photochemical process are not always used for carbon fixation when CO_2 availability is perturbed by different environmental factors (e.g. anaerobic conditions, stomatal closure, etc.). In these circumstances, the excitation of electrons could be considered as a waste of energy, and then potentially producing reactive oxygen species leading to oxidative stress. The disturbance of electron transfer can be recognized from decreasing quantum yield of electron transfer in PSII (Fv/Fm), this was observed during submergence in IR72442 but not in Inpari30 despite the photosynthetic rate being in decline in both varieties (Fig. 2.2A; B) and the correlation both variables explained (Fig. 2.5B). This means that even though Fv/Fm could estimate PSII efficiency in a tolerant variety, it could not be used to predict an accurate determination of CO_2 fixation (Maxwell and Johnson, 2000).

II-5. Conclusion

Submergence decreased net photosynthesis in both *Sub1A* genotype of Inpari30 and non-*Sub1A* genotype of IR72442. However, Inpari30 supressed shoot elongation but keep Fv/Fm and SPAD levels during submergence. Plant physiological changes of SPAD value and Fv/Fm to photosynthetic rate (Pn) was lower in Inpari30 than in IR72442. Inpari30 accumulated higher dry weight per shoot elongation. In conclusion, the *Sub1A* genotype of Inpari30 confers the ability to maintain Fv/Fm under complete submergence that limits gas exchange required for photosynthesis between plants and their surrounding environments.

III. Analysis of non-structural carbohydrates in relation with shoot elongation of rice under complete submergence

III-1. Introduction

Non-structural carbohydrates (NSCs) are photosynthetic products that are often stored in various parts (sinks) of plant body, and that are used as substrates in metabolic reaction to support plant growth and development. During vegetative growth, plant grow as plants invest more of the assimilated carbohydrate into new leaves (Geiger et al. 2000). However, plants also respond the environmental changes by regulating the translocation and partitioning of assimilated carbons which also affect photosynthetic capacity (Chatterton and Silvius 1979). When plants are completely submerged in water, O_2 diffusion is restricted, photosynthesis decreases due to reduced light intensity, lowering the internal O_2 content (Das et al. 2000). Consequently, the carbohydrate levels and energy status in the shoot will drop to levels harmful for plant survival (Catling 1999; Mommer et al. 2006).

Photosynthetic rates decreased during submergence in rice cultivars regardless of tolerant or intolerant to submergence stress (Nurrahma et al. 2021). However, some rice plant elongates during submergence which require initial energy. High initial carbohydrates in the stem are essential to provide energy for rapid elongation required for plant survival during submergence (Das et al. 2000). Later, it was demonstrated that survival under submergence is dependent on the ability to store non-structural carbohydrates (NSCs) and conserve energy through reduced underwater elongation (Das et al. 2005). *Sub1A* introgression lines with submergence tolerance do not consume stored sugars and starch during submergence compared with non-*Sub1A* parent lines sensitive to submergence (Gautam et al. 2014; Singh et al. 2014). Thus, tolerance genotype is not necessarily associated with the carbohydrate status before submergence, but rather with the ability to sustain energy levels throughout submergence (Das

et al. 2000). The cultivars that maintain high NSC after submergence develop new leaves faster and accumulate greater biomass during recovery (Singh et al. 2014).

Sub1A, a major quantitative trait locus (QTL) responsible for submergence tolerance, has been widely studied in FR13A rice (*Oryza sativa* L.) and has utility values for conferring submergence tolerance in rice breeding program (Xu and Mackill 1996; Xu et al. 2006; Panda and Sarkar 2012b). *Sub1A* has already been introgressed into the high-yielding rice variety Ciherang to generate a new variety named Inpari30 that does not have any significant trait differences compared to the parental Ciherang variety when grown under normal conditions (Septiningsih et al. 2015; Nugraha et al. 2017; Nurrahma et al. 2017). Other studies reported the effect of submergence on shoot elongation in 15 genotypes of *Oryza sativa* under 80 cm water depth for 7 days. Among the 15 cultivars, the non-*Sub1A* rice type, IR72442 exhibited the highest plant growth rate during submergence, indicates that this cultivar exploit elongation mechanism (called an escape strategy) to submergence (Sone et al. 2012). Both of Inpari30 with *Sub1A* and IR72442 without *Sub1A* were used in this experiment to evaluate starch and sugar accumulation during submergence.

Rice non-structural carbohydrates (NSCs) subjected to submergence stress have been investigated in associations with various growth parameters (Sarkar 1997), elongation ability (Das et al. 2005), different ages of rice seedlings (Das et al. 2009), impeded metabolism (Adak et al. 2011), catabolism processes (Panda and Sarkar 2012b, 2014), and application time of nitrogen and phosphorus fertilizers (Gautam et al. 2014). However, the most critical period for consumption of NSCs (starch and soluble sugars), the NSC distribution throughout plant bodies, and the association of NSCs with plant growth has not been clearly described in submerged *Sub1A* (Inpari30) and non-*Sub1A* (IR72442) genotypes. In this study, we analysed the behaviours of NSCs during submergence in both Inpari30 with *Sub1A* and IR72442 without *Sub1A*. The results related to NSCs obtained in this study would (expectedly) contribute to

development of a novel and effective agronomical approach that allows rice plants to be more tolerant to severe submergence.

III-2. Material and Methods

The research was conducted from August to November 2019 at the Tropical Crop Science Laboratory, Kagoshima University, Japan. The experiment was arranged in two factors. The environmental factors consisted of a control and submergence treatment. The rice variety factor was randomized completely within the environmental factor. Two *Oryza sativa* L. genotypes, Inpari30 with *Sub1A* and IR72442 without *Sub1A* were compared during 6 days of complete submergence followed by 6 days of re-aeration.

Inpari30 and IR72442 rice seeds were soaked in an incubator at 30°C for three days. The germinated rice seeds were then sown in commercial soil (N:P:K = 0.9:2.3:1.1; pH 4.5–5.2) in a greenhouse. Ten-day-old seedlings were transplanted into hydroponic sponge (30 mm) which were then inserted into seedling trays inside experiment glasses (45 cm x 45 cm x 60 cm). Water level was maintained at 4.5 cm from the container base, which was the same height as the seedling tray surface. Seedlings were grown at 27°C for 12 hours of light per day with an intensity of 300-350 μmol m^{-2} s^{-1} that was measured at 20 cm above the tray surface.

On 14 days after seeding (DAS), the plant had totally 4 leaves among which the third leaf was the most fully expanded. The submergence treatment was applied at 14 DAS by increasing the water level of the transparent container box to 35 cm above the plant shoot base. The water level of the control was maintained at 1 cm from the glass base throughout the experiment. The submergence treatment ended after six days (20 DAS, observed as desubmergence); the water level was then maintained at 1 cm from the glass base for the six-day recovery period. After the recovery period (26 DAS), all plants were removed from the containers for post submergence observation. Variables explained below was observed before

water level was increased (referred to as 'before submergence'), at 6 days of submergence when the plants were just removed from the water-filled glass container (referred to as 'desubmergence'), and at 6 days after desubmergence (referred to as 'post submergence').

2.1. Photosystem II (PS II) Quantum Yield

Fv/Fm was measured following the method using by (Nurrahma et al. 2021). After 2 h in the dark, the fully-developed third leaf of the rice plant was clipped with chlorophyll fluorescence equipment (AquaPen-P AP-P 100, PSI, Czech Republic). The maximal quantum yield of PS II photochemistry, calculated as variable fluorescence (Fv) divided by maximum fluorescence (Fm), was obtained by emitting an actinic light through the quantum yield menu.

2.2. Gas Exchange Measurement

Net photosynthesis (P_N) was measured using a portable photosynthesis analysis system (LI-6400; LI-COR, Lincoln, NE, USA) as mentioned by (Nurrahma et al. 2021). Before the measurement, plant samples were adapted to light exposure of 100–200 μmol m^{-2} s^{-1}. The third leaf from each plant was collected and kept inside the chamber until a stable reading was recorded. The leaf area was then measured to the calculate gas exchange rate. During measurement, environmental parameters in a chamber box were set as follows; relative humidity, ~50%; leaf temperature, 27°C; ambient CO_2 concentration, ~400 μmol mol^{-1}; airflow, 500 μmol s^{-1}; photosynthetic photon flux density, 1100 μmol m^{-2} s^{-1}.

2.3. Elongation (cm)

The length of each plant was measured from the base of the stem to the highest shoot tip using a ruler. Elongation was calculated by subtracting plant length measured just before

treatment from that of after treatment. Data are presented as the average from 12 plant samples in 3 replications.

2.4. Starch and Sugar Analysis

Forty plants were harvested at each sampling time and then separated into each leaf and roots. The samples then dried at 80°C using drying oven for 72 hours. After that, the dried samples were milled using mortar and pestle, passed through 0.5 mm screen, and then 100 mg each of fine powder of the samples were collected in a glass test tube.

Next, 0.2 mL of aqueous ethanol (80% v v^{-1}) was added to the tube to wet the sample and aid dispersion. A vortex mixer was used to stir the tube contents, then 3 mL of distilled water was added. The tube was then incubated in a boiling water bath for six minutes with vigorous stirring at every 2 minutes. The solution was then transferred to another tube and the same process was repeated. The resulting sample solution was separated into two aliquots; one aliquot was used for soluble sugar analysis and another for starch analysis using total starch assays kit (K-TSTA) from Megazyme (AOAC Method 996.11, AACC Method 76-13.01) (Horwitz 2010; international 2010).

Soluble sugar was analysed using amyloglucosidase of Megazyme with some modifications. Soluble sugar was analysed by taking 1 mL of sample solution to the tube, then 3 mL of sodium acetate buffer and 0.1 mL amyloglucosidase, stirred using a vortex mixer, then incubated at 50 °C for 30 min. Then, the test tube solution was transferred to a 25 mL volumetric flask using a funnel to assist transfer and a wash bottle to rinse the contents thoroughly. The volume was adjusted to 25 mL with distilled water and mixed thoroughly. An aliquot of this solution was centrifuged at 3000 rpm for 10 min and the clear, undiluted filtrate was used for the assay.

Both of sugar and starch samples were then colorized with GOPOD reagent with following steps. A 0.1 mL sample of the diluted solution containing both the solvent and

dissolved sample was transferred to the bottom of a glass test tube and 3.0 mL of GOPOD Reagent was added and incubated at 50°C for 20 min. D-Glucose controls consisted of 0.1 mL of D-glucose standard solution (1 mg mL^{-1}) and 3.0 mL of GOPOD Reagent. Reagent Blank consist of 0.1 mL of water and 3.0 mL of GOPOD Reagent. The absorbance was measured for each sample and the D-glucose control at 510 nm against the reagent blank.

2.5. Statistical Analysis

Data were analysed from three replications using two ways of analysis of variance. Tukey test was performed at $p = 0.05$ to evaluate statistical significance of different values. T-test was performed at $p = 0.05$ to compare between submerged Inpari30 and IR72442 for Fig. 3.3 and 3.4.

III-3. Results

3.1. Photosynthesis rate and Fv/Fm of chlorophyll fluorescence affected by submergence

The photosynthesis rates was quite similar between control and submerged plant in Inpari30 and IR72442 before submergence. Then, it gradually decreased till desubmergence. In post submergence, no significant difference however was observed between the submerged and non-submerged control plants of the same genotypes (Fig. 3.1A).

At desubmergence, Fv/Fm was 3.2% and 15.6% lower in submerged Inpari30 and IR72442, respectively, compared to the non-submerged. In post submergence, Fv/Fm in submerged Inpari30 and IR72442 was 4.7% and 12.2% lower than that of the controls, respectively. The lowest Fv/Fm value was observed in submerged IR72442, which was 4 times lower than that of submerged Inpari30 (Fig. 3.1B).

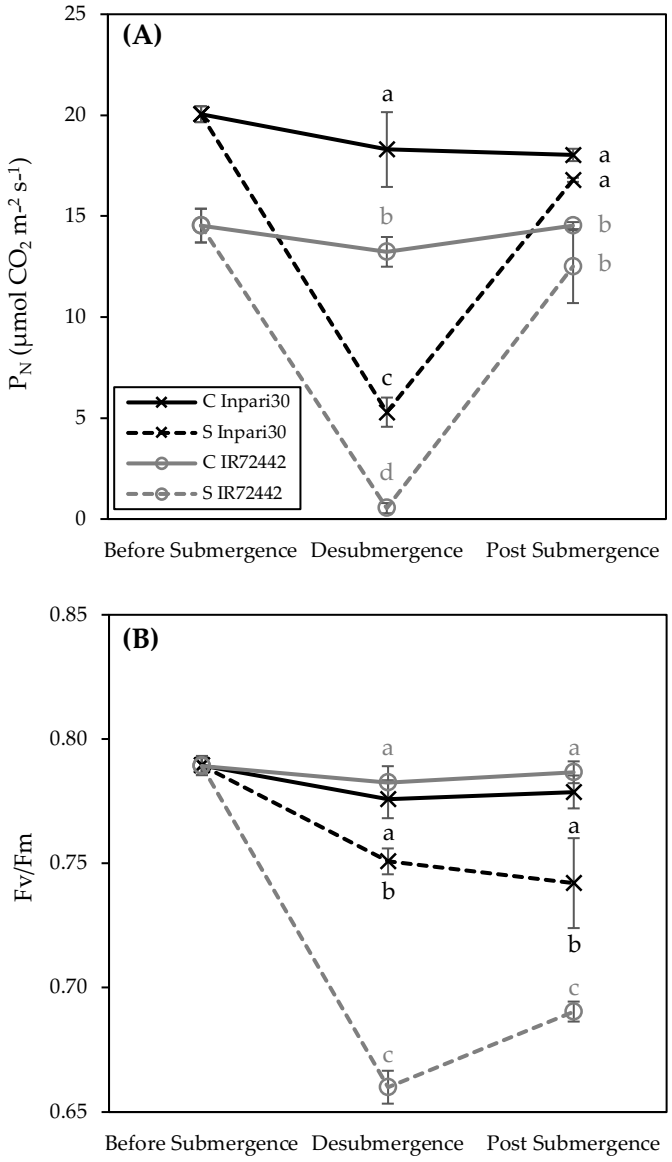


Fig. 3.1. Photosynthesis rate (A) and Fv/Fm (B) of two rice genotypes (Inpari30, a *Sub1A* genotype; IR72442, a non-*Sub1A* genotype) before submergence, desubmergence, and post submergence. Different lowercase letters in the same column indicate a significant difference at $p < 0.05$ with Tukey test. C, Control; S, Submerged.

3.2. Changes in starch and soluble sugar contents in relation to changes of elongation

Environmental change occurred two times, from before submergence to desubmergence which refers to 'submergence condition' and from desubmergence to post submergence which refers to 'recovery condition'. Here we compared the shoot elongation, change of starch and sugar contents in shoots to evaluate the needs of starch and non soluble sugar for plant elongation. The result revealed greater differences in the first period from before submergence to desubmergence than in the second period from desubmergence to post submergence (Fig. 3.2). During submergence condition, submerged IR72442 exhibited a rate of shoot elongation 1.7 times higher than control IR72442 and more than 5.2 times higher than both control and submerged Inpari30 submerged IR72442 grew 1.7 times faster than non-submerged IR72442, and 5.2 times faster than Inpari30 with or without submergence (Fig. 3.2A). In contrast, significant differences in shoot elongation rate were not observed between treatments during recovery condition (Fig. 3.2B). The shoot starch contents revealed that submerged IR72442 exhibited smallest changes in these parameters than control IR72442 and both control and submerged Inpari30 (Fig. 3.2C). No significant differences were observed between control and submerged Inpari30 (Fig. 3.2D). Submerged IR72442 decline in shoot sugar content among all treatments from before submergence to desubmergence (Fig. 3.2E,F).

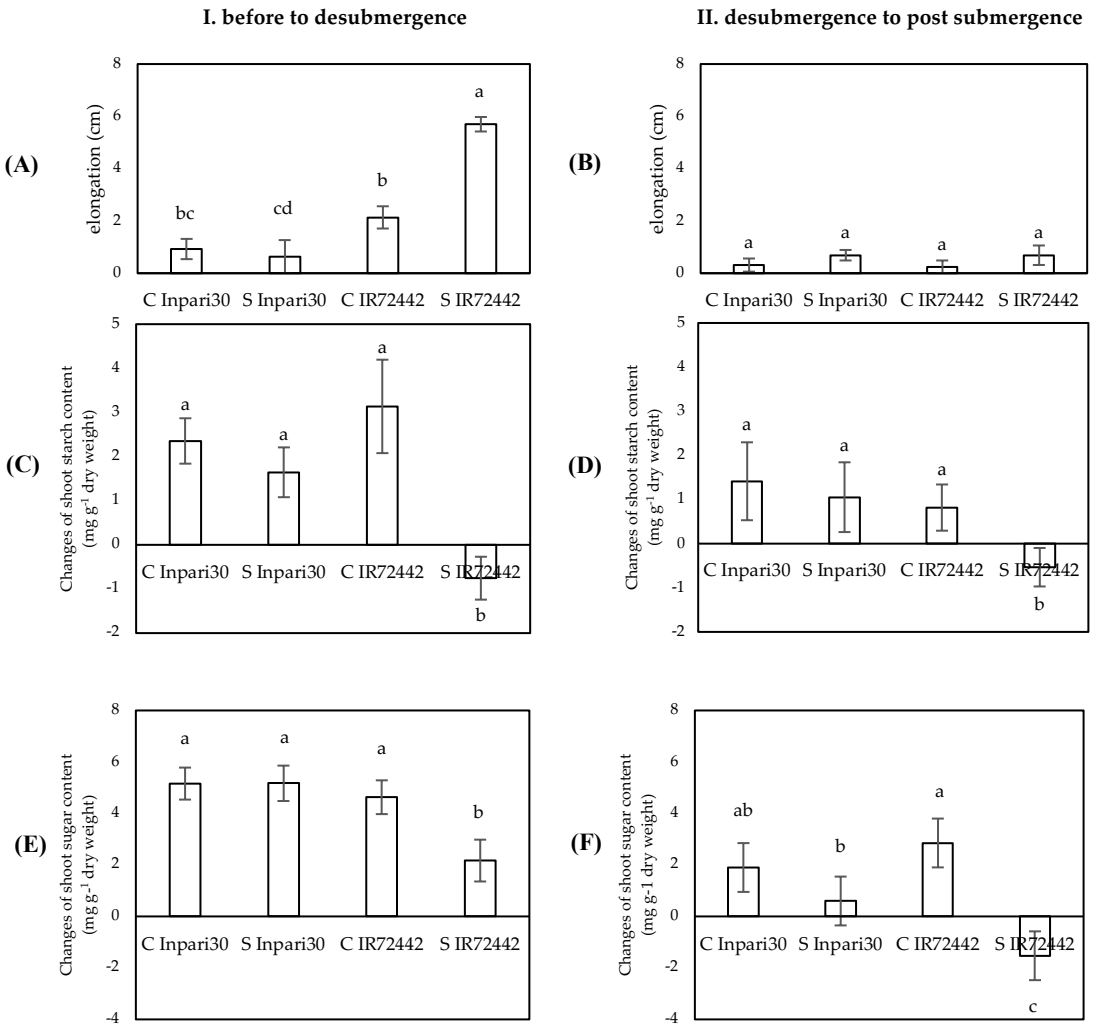
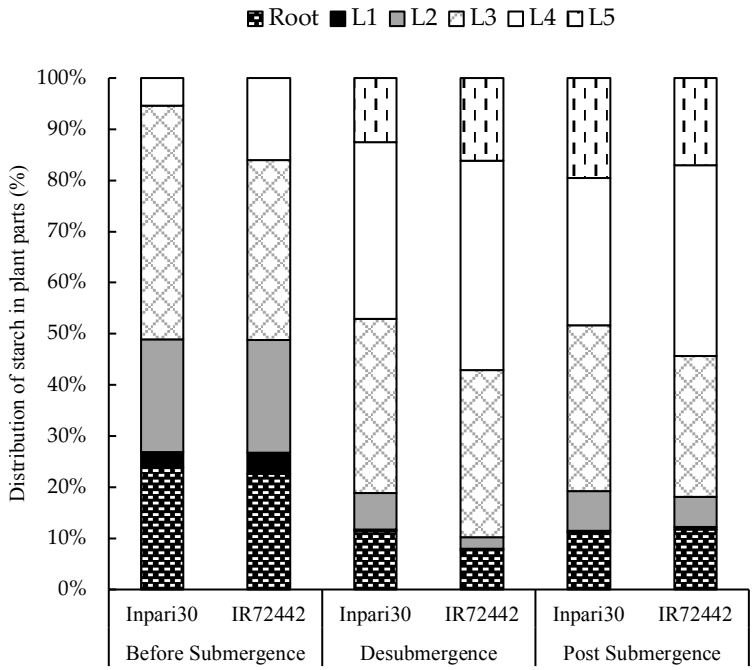


Fig. 3.2. Elongation between (A) desubmergence and before submergence, (B) post submergence and desubmergence; Changes of shoot starch content between (C) desubmergence and before submergence, (D) post submergence and desubmergence; and changes of shoot sugar content between (E) desubmergence and before submergence, (F) post submergence and desubmergence. Different lowercase letters in a single graph indicate a significant difference at $p < 0.05$ with Tukey test. Error bar represent standard deviation from three replications. C, Control; S, Submerged.

3.3. Distribution of starch and soluble sugar to the plant organs

At desubmergence, Inpari30 distributed less amounts of starch and soluble sugars to the newly emerged 5th leaf than IR72442. However, Inpari30 distributed higher amounts of starch and sugar to the 5th leaf than IR72442 on post submergence. The distribution of soluble sugars to the 5th leaf tends to be higher than starch. The distribution of starch and sugars to the 5th leaf was negatively correlated with reduced accumulation of NSCs in the roots and the 2nd, 3rd and 4th leaves (Fig. 3.3).

(A)



(B)

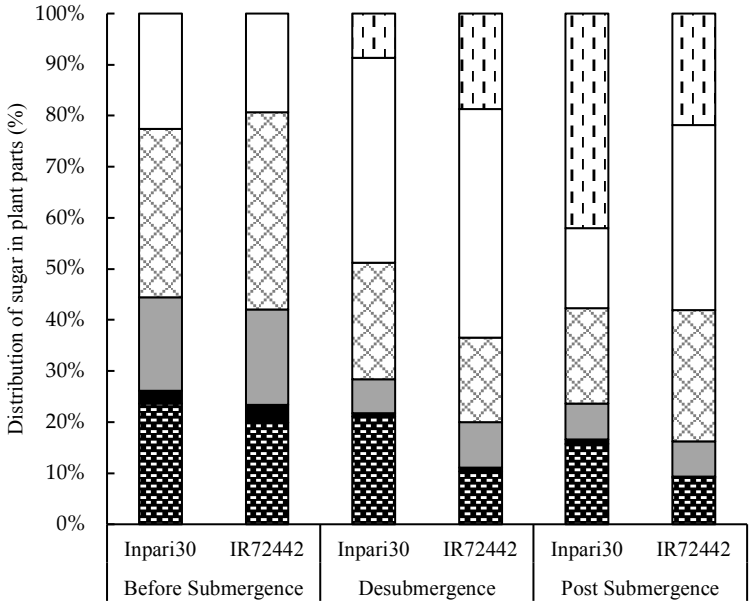


Fig. 3.3. Distribution of (A) starch and (B) soluble sugars of submerged Inpari30 (*Sub1A* genotype) and IR72442 (non-*Sub1A* genotype) in plant organs observed at before submergence, desubmergence and post-submergence.

At before submergence, the proportion of starch and sugar contents in shoots were similar in both genotypes. However, both at desubmergence and post submergence Inpari30 has highest proportion of starch content than IR72442, and proportion of starch to soluble sugars in shoots was higher in Inpari30 than IR72442 Fig. 3.4)

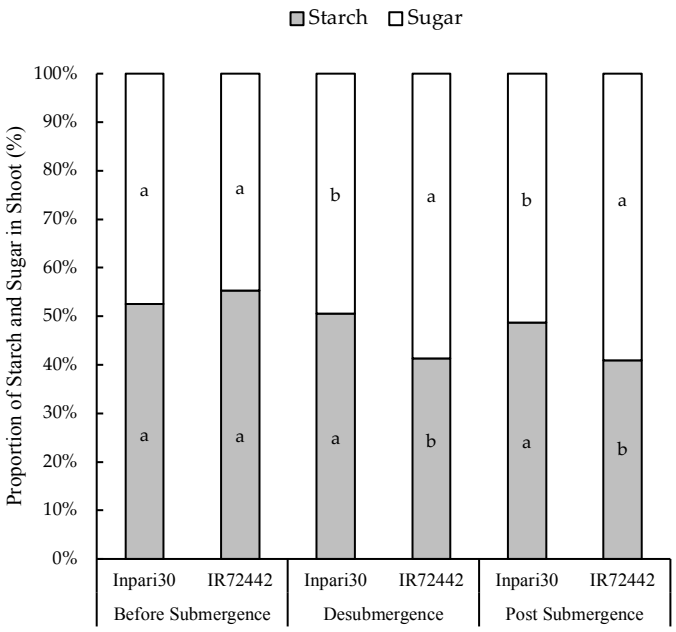


Fig. 3.4. Proportion of starch and soluble sugars in shoot of submerged Inpari30 (*Sub1A* genotype) and IR72442 (non-*Sub1A* genotype) before submergence, on desubmergence and post submergence (re-aeration). Different lowercase letters in the same parameter indicate a significant difference at $p < 0.05$ with T-test.

III-4. Discussions

In this experiment, the photosynthetic rate decreased at desubmergence in both the *Sub1A* genotype of Inpari30 and non-*Sub1A* genotype of IR72442 (Fig 3.1A). Further, chlorophyll fluorescence (Fv/Fm) at desubmergence was lowest in submerged IR72442 among all plots we set (Fig 3.1B). Chlorophyll fluorescence (Fv/Fm) as an effective indicator to know the ability of PSII has so far been used to evaluate submergence tolerance of rice (Sone et al. 2012). The

decline of Fv/Fm value likely reflects a reduced ability of PSII for electron transfer to the primary acceptor molecules that are located in this protein complex (Panda and Sarkar 2012b). The reduction of Fv/Fm can be also used as a indicator of "photoinhibition damage" caused by environmental stresses, because these hassles are well known to often result in a decline in the efficiency of solar energy conversion during photosynthesis (Maxwell and Johnson 2000; Inamullah and Isoda 2005). Therefore, the reduced Fv/Fm value suggests that photosynthetic aparatus for light-dependent reaction was disorganized in submerged IR72442, and that this defect may be attributed to reduced light intensity as well as insufficent oxygen level in floodwater (Panda et al. 2008; Panda and Sarkar 2012b).

Photosynthetic activity is a major factor determining sucrose availability for translocation (Lemoine et al. 2013). Plant carbohydrates are comprised of NSCs, such as starch and soluble sugars which play important roles in metabolic processes in plants. As photosynthesis rates decreased upon desubmergence, we measured starch and soluble sugar contents to evaluate the relation with elongation (Fig 3.2). Environmental condition was changed twice; one from before submergence to desubmergence which referred to as 'submergence condition' and the other from desubmergence to post submergence which referred to as 'recovery condition'. Submerged IR72442 exhibited highest elongation on submergence condition accompanied with decline of starch and sugar contents. Decline of starch and sugar on submerged IR72442 continued even in recovery period while elongation was ceased. However, submerged Inpari30 did not exhibit elongation on submergence condition with no significant changes of starch and sugar contents. Change of starch and sugar contents in submerged Inpari30 was also observed on recovery condition. This result suggests that the change of starch and soluble sugar in submerged IR72442 was tightly associated with the rapid shoot elongation upon submergence. Shoot elongation is one of emergence responses to escape from complete submergence (Hattori et al. 2011). Conversely, other report describes that complete submergence reduces the rate of

growth as well as carbohydrate accumulation in shoot tissues (Krishnan et al. 1999). Shoot elongation processes compete with maintenance processes for energy during submergence (Setter and Laureles 1996; Sarkar 1997; Panda and Sarkar 2012b). Moreover, shoot elongation during submergence may result in weak and droopy blades that are easily damaged by wind and water (Catling 1999).

Distribution of starch and soluble sugars in specific plant organs reflects the translocation of assimilates between organs thoughout an experimental time course (before submergence, on desubmergence, and post submergence). Submerged IR72442 distributed more amounts of starch and soluble sugars to 5th leaf than submerged Inpari30 during submergence. However, the tendency of NSC distribution was changed during post submergence; submerged Inpari30 distributed more amounts of starch and sugars to 5th leaf than submerged IR72442 (Fig 3.3). A proportion of sugars in submerged IR72442 was higher that of starch both at desubmergence and post submergence (Fig 3.4). This result suggested that in order to elongates, submerged IR72442 transported a proportion of starch and sugars to the newly-developed 5th leaf. Transport of photo-assimilates is generally believed (or considered) to depend on source supply and sink demand; roots and young leaves in healthy plants are major sinks during early developmental stages. Balanced growth and development of plants could be achieved if prioritised photo-assimilate translocation is established between sinks and sources. However, when photosynthetic rates is reduced by environmental stresses such as submergence, older leaves is responsible for an only source of photo-assimilates to younger leaves (Lemoine et al. 2013).

III-5. Conclusion

Decreasing photosynthesis during submergence was observed both in *Sub1A* and non-*Sub1A* genotype. Fv/Fm values and NSC contents decreased more in submerged IR72442 than in submerged Inpari30. Investigation of the distribution of starch and soluble sugar content in plant organs suggested that elongation of non-*Sub1A* genotype accounts for rapid starch and sugar consumption which is inferred by their rapid destribution to the new developed organs such as the 5th leaf during submergence. In contrast, *Sub1A* genotype of Inpari30 did not grow so much and exhibited slower NSCs distribution during submergence, thus leading to a better growth performance during post submergence perhaps by quick destribution to the new developed organ and efficent use of saved NSCs. This study suggests-that *Sub1A* genotype can managed both shoot elongation and NSC level during submergence more efficiently than non-*Sub1A* genotype. The knowledge obtained in this study would be applicable to develop novel agronomical cultivation approaches for improving rice tolerance to submergence stress through the efficient manage of non-structural carbohydrates (NSCs). Further studies are however necessary to determine both the detail schemes of enzymes and also the expression of genes involved in consmptions and distribution of NSCs upon submergence for assessing and distinguishing the behaviors of starch and soluble sugers in NSCs.

IV. Translocation of ^{13}C-labeled photo-assimilates in *Sub1A* rice subjected to submergence

IV-1. Introduction

Submergence stress is one of the most important environmental stresses that negatively affects crop growth and yield due to the limited supply of oxygen and light under water (Tamang and Fukao 2015). One of the most common plant responses to submergence is fast plant elongation to re-attain contact with the aerial environment. This is one of the prominent characteristics of deep water and floating rice (Catling 1999; Jackson 2008). This response helps to resume aerobic metabolism and photosynthetic CO_2 fixation but can also result in death if carbohydrate reserves are depleted before the leaves can re-emerge above the water surface (Hattori et al. 2011). Another response named as "quiescence strategies" which are characterized by the availability of *Sub1A* gene, involves the repression of cell elongation and carbohydrate metabolism during submergence; thus, avoiding the unnecessary consumption of energy that would be used to restart plant growth when the water recedes (Hattori et al. 2011). The quiescence survival strategy succeeds when floodwaters subside within 14–16 days and plants gain access to sufficient levels of O_2, CO_2, light, and nutrients to recommence photosynthesis, aerobic respiration, and other metabolic activities (Tamang and Fukao 2015).

Photosynthesis is one of the crucial processes for plant growth which regulates carbon fixation and metabolism (Panda and Barik 2021). The rates of these processes decrease during complete submergence, regardless of whether or not a particular plant tolerates submergence (Setter et al. 1989). Given that NSCs are the main energy source used in plants, monitoring carbon assimilation during photosynthesis is crucial to study a mechanism underlying submergence tolerance of rice (Hirano et al. 1995). Recent advances in methods determining photosynthetic rate using gas exchange analyser allow quick photosynthesis measurement,

however this equipment cannot properly estimate photosynthesis activity under certain environmental conditions leading to stomatal closure and thus reduced internal CO_2 concentration (Waring and Maricle 2012). Therefore, alternative approaches to determine photosynthetic rate in plant exposed to adverse conditions described above.

During photosynthesis, atmospheric CO_2 diffuses into the leaf interior through tiny stomatal pores in the leaf epidermis. Then CO_2 is utilized to generate the simple sugars, which is later used to synthesize a multitude of organic compounds required for plant growth and development (Condon et al. 2006). The carbon-13 isotopic abundances has become a common indicator of photosynthetic function in plants (O'leary et al. 1992). Atmospheric CO_2 contains approximately 1.1% ^{13}C and 98.9% ^{12}C (O'Leary 1988; Farquhar et al. 1989; Griffith 1993). In plant tissues, however, the overall abundance of ^{13}C relative to ^{12}C is usually less than that in atmospheric carbon dioxide due to the isotopic discrimination (Farquhar et al. 1989). Discrimination against $^{13}CO_2$ occurs because the physical and chemical properties of the heavy isotope result in the relatively less proportion of ^{13}C being incorporated into organic material during photosynthesis (Griffith 1993). Environmental stresses like drought, salinity, and flooding are usually accompanied by stomatal closure, which result in an increase in ^{13}C discrimination that can be used to assign plants photosynthetic activity (O'Leary 1988; Condon et al. 2006). Isotope labelling has been successfully used to explain the speed of translocation in leaves of rice (*Oryza sativa* **L**.) (Troughton et al. 1974). This approach was used to distinguish photoassimilates translocation between the sources and sinks in a lower-yielding traditional rice variety (Nakateshinshenbon) and a super-yielding rice variety (Takanari) (Mohapatra et al. 2004) and to evaluate the efficiency of water use in rice under drought stress (Scartazza et al. 1998; Gao et al. 2018). There are a few reports on the use of ^{13}C discrimination to study photo-assimilate translocation in submerged rice; however, isotope labelling has been

used to study the effect of waterlogging on several types of plants such as *Larix gmelinii* (Li and Sugimoto 2018) and *Salix* sp. in north eastern Siberia (Fan et al. 2018).

Cultivars with *Sub1A* gene greatly enhance submergence tolerance at vegetative stage with the ability to maintain its characteristics for limited elongation at booting and flowering stage (Ray et al. 2017). This suggested that vegetative stage is more prominent for study the physiological basis of *Sub1A* genotype as the result can be implemented in different growing stages. Previously, the effect of submergence on shoot elongation during vegetative stage was tested using 15 genotypes of *Oryza sativa*. Among them, shoot elongation of IR72442 was the most prominent; and six genotypes including IR67520 which are characterized by *Sub1A* gene (Sakagami 2012), displayed slow shoot elongation (Sone et al. 2012). A comprehensive analysis on the elongation of rice using ^{13}C-labelled has been done (Hirano et al. 1995), but a similar analysis on photosynthate translocation has rarely been conducted in submerged rice plants with *Sub1A* genotypes. These physiological criteria enable us to supply more direct and reliable information (del Amor 2013). This study allowing future crop improvement program to create plants with more efficient photosynthate utilization by limiting translocation from specific plant parts during submergence to gain more photoassimilate required to survive at post-submergence.

IV-2. Material and Methods

Location and experimental design

The experiment was conducted in a glass chamber set in an environmentally controlled room, where the daily temperature was maintained at 27°C with 350 μmol m^{-2} s^{-1} of 12-h light. A randomized complete block design using three replicates and studying two factors was employed. The first factor was environmental conditions, i.e., without submergence (referred

to as “control”) versus complete submergence. The second factor was rice genotypes, which were both treated in the same environmental conditions. We used rice genotypes IR67520-B-14-1-3-2-2 (hereinafter IR67520) as a *Sub1A* genotype and IR72442-6B-3-2-1-1 (hereinafter IR72442) as a non-*Sub1A* genotype. Both genotypes were grown in complete submergence for 7 days followed by 7 days of re-aeration to monitor physiological parameters associated with submergence tolerance.

Plant material and culture

Seeds were sprouted in a nursery cell tray measuring 30 cm × 54 cm, with 200 holes filled with soil, at a rate of one grain per hole. Sowing took 3 days in an incubator set to 30°C. After sowing, the cell tray was watered by placing it in a water bath and then grown to leaf age 3.9–4.0 in a glass chamber controlled at 27°C.

Ten days after sowing, plants were exposed to ^{13}C using two acrylic plate tanks measuring 100 cm × 80 cm × 38 cm. To generate $^{13}CO_2$, a reactant box was placed inside the tank. The box contained a reaction mixture consisting of 150 ml of 50% phosphate (H_3PO_4) and 15 g of labeled barium carbonate ($Ba^{13}CO_3$, 99atom%^{13}C. Then rice plants were incubated for 5 h under light (600μmol m^{-2} s^{-1}) to assimilate the $^{13}CO_2$ in the air-tightly sealed tank. After labelling, half of the plants were transferred to the transparent water bath, then the water level was increased to completely submerge the plants at a depth of 80 cm for 7 days (flood treatment). Another half were treated as control plants. After submergence, the plants were cultivated at the same water level as the control group for 7 days to re-aerate. Observations of variables explained below was conducted at three time points; the first time point was just before that water level was increased (referred to as ‘before submergence’), the second point was 7 days after submergence when plants were just removed from the water-filled bath

(referred to as 'desubmergence'), and the third was 7 days after desubmergence (referred to as 'post submergence').

Analysis of variables

1. **Plant length (cm)**

   Plant length was determined by measuring the length from the stem base to the highest shoot tip using a ruler. The data presented are the average measurements from 18 plant samples collected from 3 replicates.

2. **Total dry weight (g)**

   Plant samples were dried for 30 min at 105°C to stop all enzymatic reactions. Drying was then continued for 48 h at 80°C and the dry weights were measured.

3. **Gas exchange measurements**

   At specified times before and immediately after flooding, ten plants from each variety in each treatment were sampled to measure the photosynthesis rate. Net photosynthesis (P_N) was measured using a portable photosynthesis analysis system (LI-6400; LI-COR, Lincoln, NE, USA) as mentioned by (Nurrahma et al. 2021). Before the measurement, plant samples were adapted to light exposure of 100–200 μmol m^{-2} s^{-1}. The third leaf from each plant was collected and kept inside the chamber until a stable reading was recorded. The leaf area was then measured to the calculate gas exchange rate. During measurement, environmental parameters in a chamber box were set as follows; relative humidity, ~50%; leaf temperature, 27°C; ambient CO_2 concentration, ~400 μmol mol^{-1}; airflow, 500 μmol s^{-1}; photosynthetic photon flux density, 1100 μmol m^{-2} s^{-1}.

4. **Leaf chlorophyll content (SPAD value)**

   The amount of chlorophyll in leaves was estimated using a leaf chlorophyll meter (SPAD-502, Konica Minolta Corporation, Japan). The chlorophyll content was

represented by the average SPAD value of three measurements on the tip, middle, and base of the third leaf.

5. **Measurement of ^{13}C content (mg) and partitioning rate (%)**

   The total carbon and ^{13}C contents were determined using a continuous-flow mass spectrometer (Thermo Finnigan DeltaxPplus). The ^{13}C content in each organ was calculated as follows: ^{13}C content (atom %) = dry matter weight × total carbon content × ^{13}C abundance, where ^{13}C abundance (atom % excess) is the difference between the $^{13}C/(^{12}C + ^{13}C) \times 100$ ratio of plants supplied with $^{13}CO_2$ (labelled sample) and those supplied with $^{12}CO_2$ (unlabelled sample). The distribution of ^{13}C in whole plant was calculated using the following formula: ^{13}C partitioning rate (%) = (^{13}C amount in specific plant part/^{13}C amount in the whole plant) × 100%.

6. **Leaf Cross Section**

   Starch granules was microscopically observed as described below. The 3rd leaf sheath was collected from IR67520 and IR72442 plants submerged for 5 days, and then the bottom part of the sheath was cut vertically and observed under a scanning electron microscope (SM-5600 LV, Nihon Denshi Co. Ltd.) according to the supplier's instruction.

Statistical analysis

Data were analyzed using two-way analysis of variance. When significant differences were found, the Tukey test was performed at $p = 0.05$. T-test was performed to compare between submerged IR67520 and IR72442.

IV-3. Results

1. Plant growth and biomass accumulation

The changes in plant length of both genotypes after undergoing submergence are shown in Fig. 4.1 and 4.2. Submerged IR72442 was 41.1% longer than control IR72442, and submerged IR67520 was 41.3% shorter than control IR67520 at desubmergence. After submergence, submerged IR72442 grew 15.2% less than control IR72442, in terms of plant length. Submerged IR67520 grew the slowest (in terms of plant height) among all the treatments.

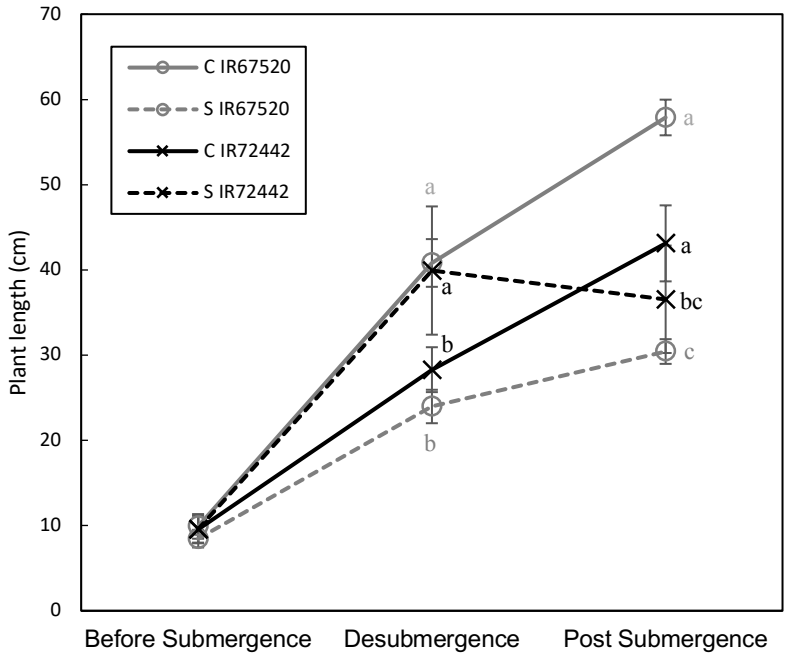


Figure 4.1. Changes in plant length during the experimental period. Different lowercase letters in the same row indicate a significant difference at $p < 0.05$ with the Tukey test. Each error bar represents the standard deviation of three replicates. C, control; S, submerged

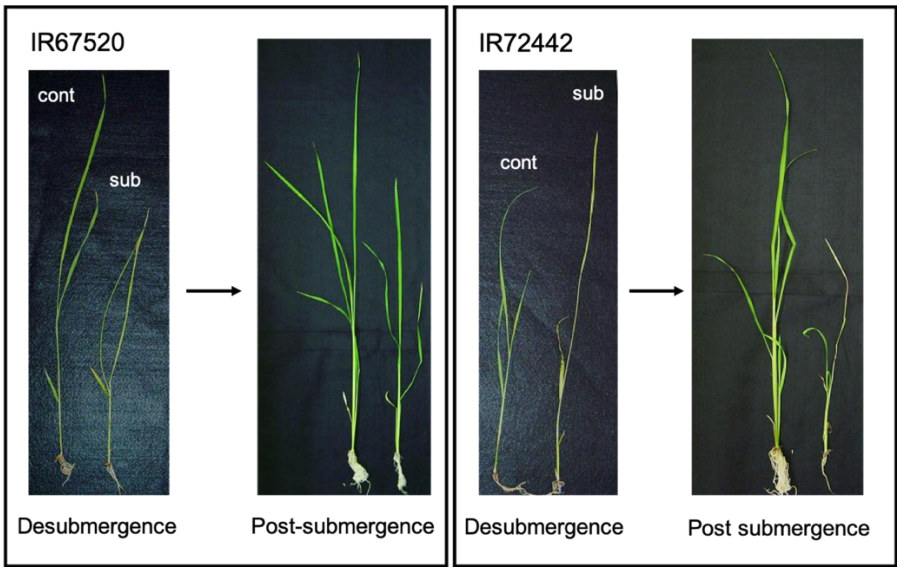


Figure 4.2. Plant length comparison between control and submergence of IR67520 and IR72442 at desubmergence and post submergence

The total plant biomass ratio is the plant biomass of submerged plants divided by the plant biomass of control plants. Figure 4.3 shows similar total plant biomass ratios for both genotypes before submergence. Upon submergence, the total plant biomass ratio of IR72442 continuously decreased thoroughout the experiment period, while that of IR67520 also decreased upon submergence but slight increased at post-submergence in. On desubmergence, the total plant biomass ratio of IR72442 was higher than that of IR67520, but post-submergence, the total plant biomass ratio of IR72442 was lower than that of IR67520.

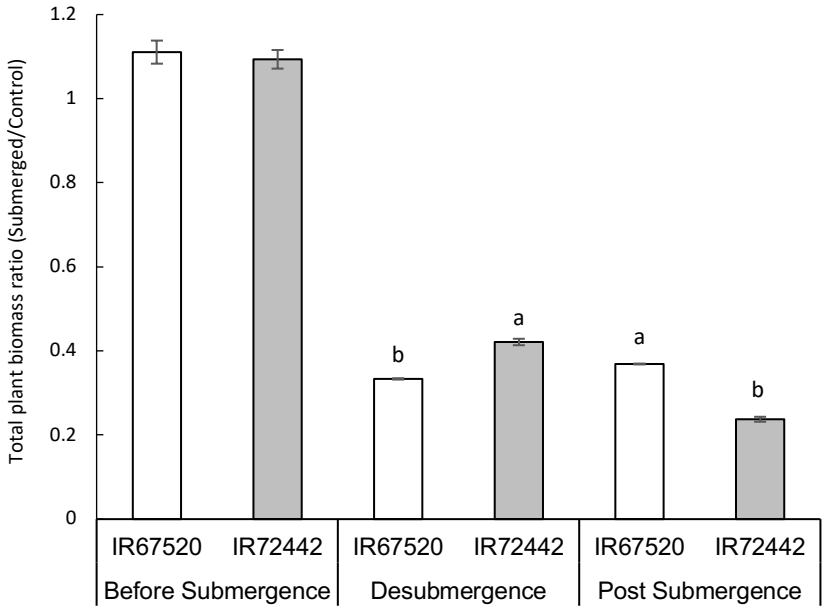


Figure 4.3. Total plant biomass ratio of submerged and control plants. Different lowercase letters in each variety before submergence, during submergence, and post-submergence indicate a significant difference at $p < 0.05$ with the t-test

Plant biomass continuously increased during the experiment for both submerged IR67520 and IR72442 (Fig. 4.4). Before submergence, the 3rd leaf (sheath and blade) was the latest developed in both genotypes, and IR67520 grew fast in terms of plant biomass

compared to IR72442, accompanied with a significant difference in weight of the 2nd leaf. During submergence, the 4th leaf was developed in both genotypes, and the total plant biomass did not significantly differ between both genotypes. However, biomass of the 2nd and 3rd leaves in IR67520 was more than that in IR72442 while biomass of the 4th leaf in IR67520 was contrarily less than that in IR72442. At post-submergence, the total plant biomass of IR67520 was much higher than that of IR72442, which was probably attribute to rapid biomass increase in the 2nd, 3rd, 4th, and 5th leaves and roots of the former.

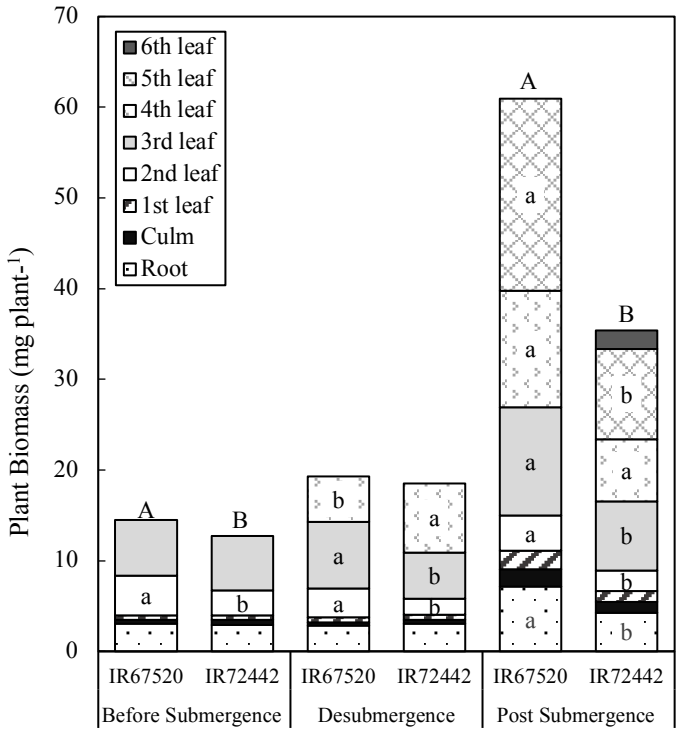


Figure 4.4. Changes in plant biomass of submerged IR67520 and IR72442. Different lowercase letters in the same plant part before submergence, during submergence, and post-submergence indicate a significant difference at $p < 0.05$ with the t-test

2. Photosynthetic rate and chlorophyll content

Photosynthesis rate did not significantly differ between non-submerged IR67520 and non-submerged IR72442 throughout the experiment time course. Photosynthesis rate of submerged plants was not obtained because of technical limitations. At post submergence, the photosynthetic rates of submerged IR67520 and submerged IR72442 were 46% and 69% lower than those of the corresponding non-submerged controls, respectively. When comparing the two genotypes, the photosynthetic rate of submerged IR72442 was 40% lower than that of submerged IR67520 at post-submergence (Table 4.1).

Table 4.1. Photosynthesis rate (μmol CO_2 m^{-1} s^{-1}) of the plant during the experimental period

Treatment	Before submergence	Desubmergence	Post-submergence
C IR67520	11.2 ± 1.9	20.8 ± 3.5	13.4 ± 1.3 a
S IR67520		-	7.2 ± 3.1 b
C IR72442	10.6 ± 2.3	19.2 ± 2.7	13.9 ± 3.8 a
S IR72442		-	4.3 ± 1.0 b

Note: C, control; S, submerged; -, data not recorded. Different lowercase letters in the same row indicate a significant difference at $p < 0.05$ with the Tukey test

The chlorophyll contents of submerged IR67520 and IR72442 significantly decreased upon submergence stress, as indicated by reduced SPAD values at desubmergence that were 41% (IR67520) and 84% (IR72442) of those in controls (Fig. 4.4). The SPAD value of submerged IR67520 recovered upon re-aeration, while that of submerged IR72442 remained 43% lower than that of the control. The SPAD values of submerged IR72442 at desubmergence and post-submergence were 71% and 39% lower, respectively, than those of submerged IR67520.

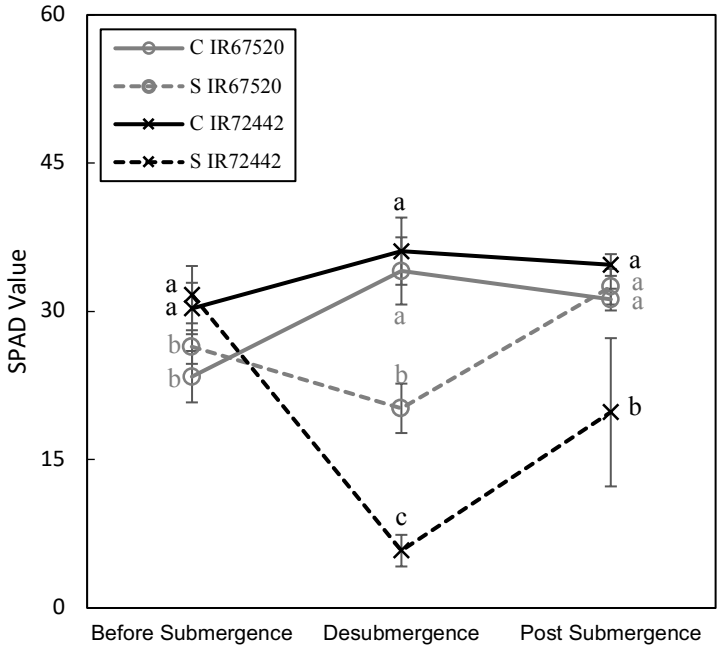


Figure 4.5. Changes of SPAD value during the experimental period. Different lowercase letters in the same row indicate a significant difference at $p < 0.05$ with the Tukey test. Each error bar represents the standard deviation of three replicates. C, control; S, submerged

3. Translocation of photosynthetic product

The ratio of ^{13}C to ^{12}C in each plant organ is expressed as excess of ^{13}C (%), and the results presented in Table 2 indicate higher levels of excess of ^{13}C in the upper leaves immediately after exposure to $^{13}CO_2$ in both submerged IR67520 and IR72442. Both loss and gain of ^{13}C abundance occurred in different plant parts.

At desubmergence, submerged IR67520 had higher ^{13}C abundance in the 3rd leaf blade and lower ^{13}C abundance in the 3rd sheath, 4th leaf blade and culm than submerged IR72442. Decreasing ^{13}C abundance occurred both genotype from before submergence to desubmergence especially in the 2nd and 3rd leaf blade and sheath, while increasing occurred in 4th leaf blade. The ^{13}C abundance in 3rd leaf blade at desubmergence was decreased from 20.2% to 13.7% in IR67520 and from 16.8% to 6.18% in IR72442. In 3rd leaf sheath and 4th leaf blade however, increased ^{13}C abundance was observed in IR67520 from 0 to 10.2% and from 0 to 6.07%. In contrast, ^{13}C abundance was decreased in the 3rd leaf sheath of IR72442 from 28.4% to 26.7% and increased in the 4th leaf blade from 0 to 10.5%.

At post-submergence, submerged IR67520 had lower ^{13}C abundance in the 3rd and 4th leaf sheath, 4th and 5th leaf blade, culm and root than submerged IR72442. The ^{13}C abundance in the 3rd leaf blade was decreased in IR67520 from 13.7% to 8.21% and increased in IR72442 from 6.2% to 7.4% although difference in the values was not statistically significant. Decreasing ^{13}C abundance occurred in both genotypes from desubmergence to post submergence especially in the 3rd leaf sheath, 4th leaf blade and culm from 10.2% to 6.2%, 6.07% to 3.1% and 7.8% to 1.7%, respectively, in IR67520 and from 24.7% to 15.4%, 10.5% to 7.2%, 9.1% to 3.8%, respectively, in IR72442. While increasing ^{13}C abundance occurred in 4th leaf sheath and 5th leaf blade from 0 to 0.33%

and 0 to 0.25%, respectively, in IR67520 and from 0 to 2.38% and 0 to 1.58%, respectively, in IR72442 (Table 4.2).

Table 4.2. ^{13}C abundance (atom % excess) between submerged IR67520 and IR72442 during submergence

Part of plant	Before submergence		Desubmergence		Post-submergence	
	IR67520	IR72442	IR67520	IR72442	IR67520	IR72442
1 LB	2.80	2.26	0.68	0.74	0.65 a	0.30 b
2 LB	11.39	10.47	1.51	1.60	0.92	1.28
2 LS	9.62	5.26	3.03	3.45	3.08	2.39
3 LB	20.18 a	16.78 b	13.73 a	6.17 b	8.21	7.44
3 LS		28.35	10.21 b	24.68 a	6.17 b	15.43 a
4 LB			6.07 b	10.50 a	3.08 b	7.18 a
4 LS					0.33 b	2.38 a
5 LB					0.25 b	1.58 a
5 LS						0.25
6 LB						0.29
Culm	8.11	8.60	7.82 b	9.06 a	1.67 b	3.76 a
Root	2.33	3.11	2.52	2.82	1.44 b	3.76 a

Note: ^{13}C atom excess (%) is calculated between submerged IR67520 and IR72442 using the formula: ^{13}C atom excess (%) = ^{13}C atomic weight/(^{12}C atomic weight + ^{13}C atomic weight) × 100. T-test was performed to compare between submerged IR67520 and IR72442.

Before submergence, the majority of ^{13}C was partitioned into the 3rd leaf in both IR67520 and IR72442 (Fig. 4.6). At desubmergence, a large part of the ^{13}C in the 3rd leaf was translocated to the 4th leaf in IR72442. As a result, the 4th leaf accounted for the highest proportion of ^{13}C in submerged IR72442. In IR67520, the 3rd leaf accounted for the highest proportion of ^{13}C in all plant parts during submergence. At post-submergence, more amounts of ^{13}C partitioned to the newly developed upper leaves increased in IR72442 than in IR67520.

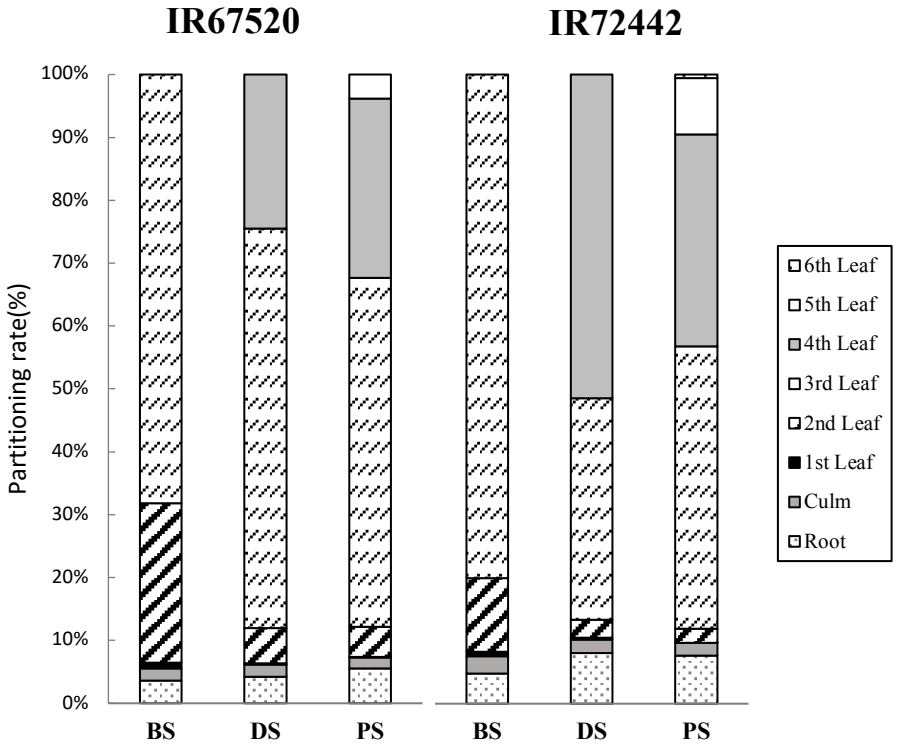


Figure 4.6. Partitioning rate of ^{13}C (%) in the whole plant part of submerged IR67520 and IR72442. The rate was calculated by dividing the amount of ^{13}C in the plant part by the amount of ^{13}C in the whole plant multiplied by 100 (%). BS: Before submergence; DS: Desubmergence; PS: Post submergence.

4. Starch granules in the leaf cross section of submerged plant

Scanning electron microscopic analysis revealed that starch granules were accumulated in the 3rd leaf sheath of submerged IR67520, while the granules were almost undetectable in the leaf sheath of IR72442 (Figure 6).

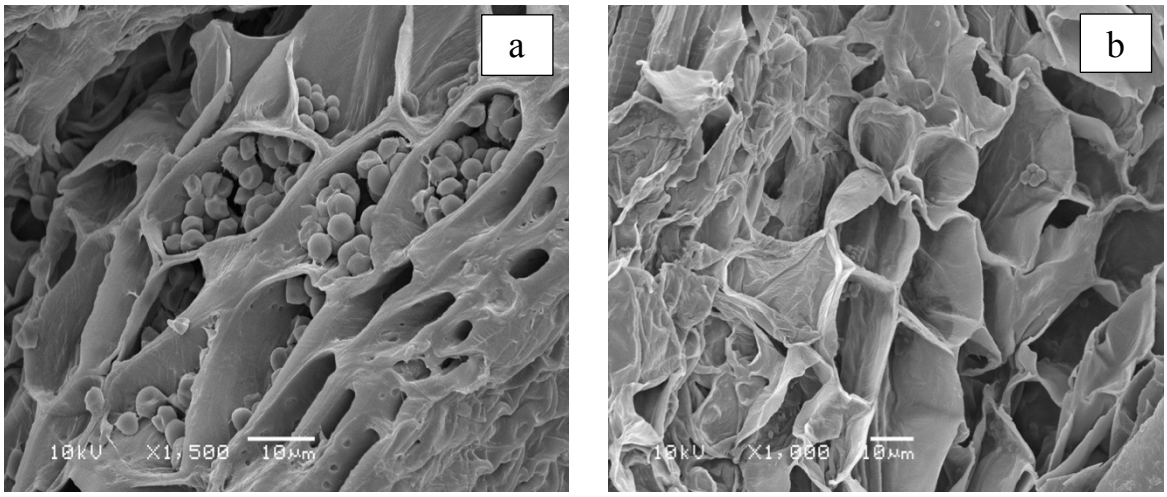


Fig. 4.7. Scanning electron microscopic observation of the 3rd leaf sheath of submerged (a) IR67520 and (b) IR72442. Sample was taken from the plant at 5 d of submergence where the bottom part of 3rd leaf sheath cut vertically then observed using SM-5600 LV, Scanning Electron Microscopy, Nihon Denshi Co. Ltd.

IV-4. Discussions

4.1. Influence of Submergence on Plant Growth and Biomass Accumulation

Submergence declines gas diffusion under water approximately 10^4 folds slower than open air (Panda et al. 2008). This reduces the supplies of CO_2 and O_2 to the plant that then leads to a decline of respiration and photosynthesis rates, respectively (Armstrong 1980; Panda and Barik 2021). To cope with such an adverse change, most of rice genotypes rapidly elongate their shoots under complete submergence (Panda and Barik 2021). In this study, IR72442 (non-*Sub1A* genotype) increases its height during submergence which contrasts with the *Sub1A* genotype of IR67520 (Fig. 1). Sone et al. (2012) reported the similar observations. Submerged IR72442 displayed faster growth than IR67520 at desubmergence, have lower submerged to control biomass ratio and accumulate lower total biomass ratio than IR6752o at post submergence. Even though submerged IR67520 grew less than submerged IR72442 at desubmergence, the biomass of submerged IR72442 was significantly higher than those of IR72442 at post submergence (Figs. 1, 2, and 3). Flooding hampers the accumulation of dry matter in sensitive genotypes (Singh et al. 2014). However, *Sub1* varieties are able to retain higher biomass and can grow faster upon re-aeration (Sarkar and Bhattacharjee 2011).

4.2. Influence of Submergence on Photosynthetic Activity

The negative effect of flooding on rice is most likely attribute to the exchange of water-poor gas by impeding photosynthesis (Colmer and Voesenek 2009). Although photosynthetic rate during submergence of both submerged IR67520 and IR72442 could not be determined in this study (Table 1), a previous report described that only FR13A maintained high photosynthesis rate at desubmergence among the subjected rice varieties (FR13A, IR42, Swarna, and Swarna-Sub1) (Winkel et al. 2014). SPAD values are an indirect measurement of

chlorophyll content (Kumagai et al. 2009). SPAD value was also decreased upon submergence in IR67520 and IR72442, and the reduction of SPAD value was more evident in the latter than in the former (Fig. 3). Submerged plants likely require a certain time to recover their photosynthetic ability after reaeration; this assumption was brought by our result determining that photosynthetic rates in both submerged IR67520 and IR72442 was much lower than those of the non-submerged controls (Table 1). Based on the difference in SPAD value at post-submergence, we thought that submerged IR67520 with high SPAD value can quickly recover photosynthetic rate, compared to submerged IR72442 with relatively low SPAD value. Chlorophyll is a main light-harvesting pigment for photosynthesis (Murchie and Lawson 2013). Reduction in chlorophyll contents during submergence is often observed in numerous rice varieties (Panda and Sarkar 2012b). Therefore, chlorophyll degradation in submerged rice conceivably causes reduction of photosynthesis rate. In addition, the quick recovery of chlorophyll contents at post-submergence indicates that IR67520 have more potential in the restoration of photosynthesis than IR72442. This assumption was also confirmed by the higher biomass accumulation than IR72442 at post-submergence, compared with IR72442 (Table 1 and Fig. 3). Although stomatal conductance was not investigated in this study, the proper behavior of stomata, in addition to the intrinsic function of photosynthetic apparatus including chlorophyll pigments could be associated with the maintenance and quick recovery of higher photosynthesis in genotypes tolerant to flooding (Panda and Sarkar 2013). Therefore, we will investigate stomatal behavior to evaluate its contributions to submergence tolerance of rice in the next study.

4.3. Influence of Submergence on Translocation of Photosynthetic Products

At vegetative stage of rice, photosynthetic products are mainly stored in the leaf, which are consumed through respiration in the same leaf, or translocated to other organs to be stored,

consumed or further re-located (Lian and Tanaka 1967). Under submerged conditions, rice plants are placed in hypoxia; thus, assimilation of photosynthetic products are largely suppressed (Panda and Barik 2021). In this experiment, low photosynthetic rates were observed at desubmergence of submerged IR67520 and IR72442. Total ^{13}C abundance levels in plants were mostly maintained throughout the experiment period, and distribution of ^{13}C abundance largely fluctuated between plant organs (Table 2). For example, in both genotypes, the ^{13}C abundance levels in the 3rd leaf blade was highest before submergence. These levels more largely decreased in IR72442 than IR67520 at desubmergence. This suggests that, in IR72442, the ^{13}C assimilated in the 3rd leaf blade before submergence was actively translocated to other organs, particularly to the 4th leaf blade and 3rd leaf sheath during submergence. This assumption was supported by our finding that more starch granules were detected in the 3rd leaf sheath of IR67520 exposed to submergence for 5 days than IR72442 treated in the same way (Fig. 6). This indicates that even under submergence conditions during which photosynthesis rates were low, sufficient amounts of photosynthate were transported in IR72442 (non-*Sub1A* genotype) to meet the carbon demand for generating new leaves. Similarly, maize seedlings under shaded conditions are able to transfer photosynthates from the apical regions to other plant parts to support vigorous axis extension (Tatsumi et al. 1992). In rice, ^{13}C abundance in the labeled leaf rapidly decreases and was transferred to newly generated organs containing non-labeled carbon (^{12}C) assimilates (Okano et al. 1983).

During post-submergence, photosynthetic activity in the expanded leaves was reestablished due to aerobic conditions (Table 1). The ^{13}C abundances in the 3rd leaf blade increased from desubmergence to post submergence in IR72442 and decreased in IR67520. While in the 3rd leaf sheath and the 4th leaf blade, ^{13}C abundances both genotypes declined from desubmergence to post submergence. The ^{13}C abundance in these organs of ~~in~~ IR67520 was lower than that of IR72442 at post submergence (Table 2). The difference in ^{13}C abundance

reduction from desubmergence to post-submergence is likely due to the high ability of IR67520 to assimilate non-labeled (^{12}C) carbons for regrowth after under aerobic conditions, compared with IR72442 of which photosynthesis ability seemed to be less recovered. Changes of ^{13}C abundance are reportedly affected by metabolic process such as photosynthesis and respiration (Farquhar et al. 1989). For example, in an experiment conduced in closed environment, the ^{13}C-labels accumulated in the shoots of *Camellia sasanqua* are progressively transferred to the leaves perhaps by re-absorbing $^{13}CO_2$ generated through respiration (Oitate et al. 2011). In our study, more ^{13}C abundance was found to be lost in submerged IR67520 than in IR72442 (Table 4.2), which may reflect to high respiration and photosynthesis activities of IR67520 at post-submergence.

IV-5. Conclusion

Present study examined the effect of transient submergence on photosynthesis product translocation of IR67520 (*Sub1A* genotype) and IR72442 (non-*Sub1A* genotype) using ^{13}C-labelled tracer. Our results found out that submergence enhanced plant length of submerged IR72442 at desubmergence along with decreased biomass production at post submergence. Although both genotypes had low photosynthetic rates during submergence, the SPAD value of submerged IR72442 was much lower than that of IR67520. Our study using ^{13}C abundance indicates that even though photosynthetic activity was inhibited during submergence, photosynthetic products of submerged IR72442 were translocated to the newly developed leaves to address the needs for elongation. In contrast, the *Sub1A* genotype of IR67520 exhibited less shoot elongation and had slower translocation of ^{13}C at desubmergence. Therefore, *Sub1A* genotype seemed to manage to increase plant length and accumulate high biomass at post submergence using photo-assimilates not used during submergence. Taken together, our study using 13C-labeling technique implies that slow growth of IR67520 (*Sub1A*

type) during submergence may be the optimum response to transient submergence because this genotype exhibited a good recovery of growth after re-aeration. This finding would give us an idea to breed novel rice varieties with more efficient utilization of carbon sources, which suppresses unnecessary consumption of photo-assimilates during submergence and thus attaining a better survival rate after submergence. Although we could not determine how photo-assimilates accumulated before submergence were utilized after release from flooding stress, this is a challenging question to be solved. A tracer analysis using a combination of two different carbon isotopes would help to more preciously disclose the partitioning dynamics in plant bodies of carbon sources that are assimilated before and after submergence.

V. General Discussion

Flooding is a widespread phenomenon that greatly affects the development and longevity of plants. The drastic drop in the diffusion of gas in water relative to air is a big problem for plants because it restricts the intake of CO_2 for photosynthesis and O_2 for respiration (Voesenek et al. 2006). The importance of gas diffusion during submergence is clearly demonstrated. When submerged rice is flushed with air with the high partial pressure of CO_2 (1-2 kPa), plants can survive up to 3 months during complete submergence. In contrast, plants die within 1-2 weeks in floodwater when the partial pressure of CO_2 is reduced to 0.03 kPa (Setter et al. 1997). Physiological responses to avoid the adverse effects of submergence between *Sub1A* and non-*Sub1A* genotype are investigated in this study, which include the maintenance of photosynthetic properties, non-structural carbohydrates (NSCs) consumption, photosynthate translocation and plant growth on temporary submergence.

Carbon assimilation during submergence is affected by several factors including CO_2 supply, light irradiance and a photosynthetic capacity of plants (Setter et al. 1997). A decrease of in net photosynthesis rate during complete submergence was observed regardless *Sub1A* or non-*Sub1A* genotypes. The similar observation was also reported between M202 and M202Sub1 (Alpuerto et al. 2016). A previous study demonstrates that photosynthesis rate underwater is maintained only in FR13A among the four tested races (FR13A, IR42, Swarna, and Swarna-Sub1) (Winkel et al. 2014). FR13A is a landrace from eastern India with exceptionally strong submergence tolerance and is the donor of *Sub1A*, a major QTL associated with submergence tolerance on chromosome 9 (Mackill et al. 2012). The study indicates that the ability to maintain photosynthesis rate underwater is not necessarily linked to a submergence tolerant QTL, *Sub1A* (FR13A), thereby not inherited to many of genotypes introgressed with *Sub1A*. In this study, Inpari30 (*Sub1A* genotype) was found to maintain a

higher value of Chlorophyll fluorescence (Fv/Fm) than IR72442 (non-*Sub1A* genotype) during submergence. Further, SPAD values during submergence were maintained at a higher level both in Inpari30 and IR67520 (*Sub1A* genotype), than that in IR72442. Chlorophyll fluorescence (Fv/Fm) is an effective indicator of submergence tolerance of rice (Sone et al. 2012). Therefore, the decline in Fv/Fm for IR72442 during submergence likely reflects a reduced ability of PSII that caused the reduce level of primary acceptors (Panda and Sarkar 2012a). Chlorophyll fluorescence (Fv/Fm) is also an indicator of "photoinhibition damage" caused by various environmental stressors, which leads to a decline in the efficiency of solar energy conversion in light-dependent reaction in photosynthesis process, and thus affecting an overall reduction of carbon assimilation (Inamullah and Isoda 2005).

SPAD values are commonly used as an indirect measurement of chlorophyll content (Kumagai et al. 2009). Chlorophyll is a major light-harvesting pigment of photosynthetic process that converts the energy from captured sun light photons into chemical energy (ATP) and reducing power (NADPH) that are used to drive CO_2 fixation (Murchie and Lawson 2013). Reduction in chlorophyll contents during submergence is often observed in rice (Panda and Sarkar 2012b). Therefore, chlorophyll degradation causes severe reduction of photosynthesis rate (Panda et al. 2008). Although submerged plants of Inpari30 and IR67520 reportedly maintain a capacity to absorb sun-light energy, however the photosynthetic rate in both genotypes were heavily influenced (or reduced), perhaps by limited gas exchange as well as weak light under anaerobic conditions. Swarna-Sub1 is also reported to reduce photosynthetic rate under submergence stress, even though chlorophyll contents was maintained, implying that other components of the photosynthetic machinery are compromised (Winkel et al. 2014). Measurement of SPAD value and Fv/Fm allows us to predict photosynthetic capacity of submerged rice plants.

Some of rice genotypes elongate their shoot under complete submergence (Panda and Barik 2021). In this study, IR72442 can outgrow the water by increased shoot elongation. Response of shoot elongation improved aeration and restored rates of photosynthesis when the leaves reach into the air (Voesenek et al. 2006). However, shoot elongation underwater consumes energy and carbohydrate nutrients for rapid cell elongation, which are spent as the expense of survival. -Enhanced elongation rates during submergence increase plant survival only if contact between leaf blades and the atmosphere is restored (Setter and Laureles 1996; Setter et al. 1997). The high cost of shoot elongation on IR72442 can be estimated by a low ratio of shoot dry weight per shoot elongation compared with Inpari30 (Fig. 4.4) and a lower total plant biomass ratio of submerged and control plants than IR67520 on post submergence. Application of paclobutrazol, an inhibitor of gibberellin synthesis suppressed shoot elongation in submerged rice plants and then resulted in a significant increase of plant survival, suggesting that carbon source kept by refraining shoot elongation during submergence might contribute to the survival after re-aeration (Setter and Laureles 1996). So far, *Sub1A*-introgressed varieties are reported to grow more slowly during submergence than other varieties without *Sub1A* (Sarkar and Bhattacharjee 2011).

Under the restricted photosynthesis reaction during submergence, the continuous consumption of carbohydrate source, perhaps used in anaerobic respiration, will cause the deficiency of the nutrients and energy in the shoot, and thus finally leading to plant death (Catling 1999; Mommer et al. 2006). Rapid shoot elongation during submergence of non-*Sub1A* genotypes often causes the severe depletion of starch and soluble sugars. In contrast, tolerant genotypes with *Sub1A* commonly limit shoot elongation underwater, and perhaps it guarantees the high levels of non-structural carbohydrates which can be used for recovery after re-aeration (Das et al. 2005). The result suggests that elongation and leaf development during submergence of non-*Sub1A* genotypes are achieved by starch and sugar consumption, and their

rapid distribution to the new organs. In contrast, *Sub1A* genotypes such as Inpari30 did exhibit slow shoot elongation and slow NSCs distribution during submergence, and then expressed the better growth performance during post submergence, perhaps by using the maintained NSCs for shoot growth as well as development of new organs. The result implies that carbohydrates available after re-aeration is critical for plant survival because they can be used to produce the energy for quick recovery of growth (Das et al. 2005).

A tracer analysis using 13C-labelled isotopes disclosed the dynamics of both translocation and distribution of assimilated carbons. Carbons assimilated into NSCs by photosynthesis were monitored to link their behaviour to shoot elongation during submergence. The result suggests that vigorous shoot elongation during submergence of IR72442 is associated with the rapid translocation of ^{13}C from the 3rd leaf to the newly developed leaves. This result indicates that even though cellular metabolisms including photosynthesis were inhibited by submergence, photo-assimilates in non-*Sub1A* genotypes such as IR72442 were translocated to the newly developed leaves, perhaps to address the needs for shoot elongation. In contrast, the *Sub1A* genotypes such as IR67520 exhibited slow shoot elongation and slow translocation of ^{13}C during submergence, which is likely the hallmark of growth quiescence.

VI. Conclusion

Flooding is a serious stressor that greatly affects the development and longevity of plants. The drastic drop in gas diffusion in water relative to the air is a big problem for plants because it restricts the intake of CO_2 for photosynthesis and O_2 for respiration. To determine physiological responses to avoid the adverse effects of submergence in both *Sub1A* and non-*Sub1A* genotype is a central theme of this study. These include the maintenance of photosynthetic properties, non-structural carbohydrates (NSCs) consumption, photosynthate distribution and cahnges in shoot elongation and biomass in response to temporary submergence. Slow gas diffusion under water affected a decrease of net photosynthesis rate regardless of *Sub1A* or non-*Sub1A* genotypes. However, *Sub1A* genotypes maintain higher value of Fv/Fm and SPAD under temporary submergence, compared with non-*Sub1A* genotypes. This result indicates that *Sub1A* gene has an ability to maintain PSII function which is crucial for solar energy conversion required for photosynthetic carbon assimilation. At the same time, non-*Sub1A* genotype can outgrow the floodling water by promoting shoot elongation. Rapid shoot elongation upon temporary submergence is a typical characteristc common to non-*Sub1A* genotypes, which is accompany with severe depletion of NSCs later. On the other hand, under submegence, *Sub1A* genotypes do not elongate so much and save NSCs. The distribution of ^{13}C-labelled assimilates into different plant organs suggests that shoot elongation of non-*Sub1A* genotypes upon temporary submergence is tightly associated with rapid taranslocation of photo-assimilates to the newly generating organs. In contrast, *Sub1A* genotypes with slow shoot elongation phenotype, showed slow photo-assimilate translocation during temporary submergence. Altogether, *Sub1A* genotypes are thought to exhibit the better growth perfomance after re-aeration by the maintained intrinsic function of

PSII in light-dependent reactions, the growth recovory by using the storaged NSCs and the rapid photo-assimilate translocation to the newly developing organs.

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