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ESTIMATION OF mRNA ACCUMULATION AND PHYSIOLOGICAL RESPONSE TRAITS ASSOCIATED WITH SUBMERGENCE TOLERANT GENE *SUB*1A IN RICE PLANT (*Oryza sativa* L.)

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ABSTRACT

The experiment was conducted to estimate mRNA accumulation in a submergence tolerant genotype M202 (Sub1). Nineteen-day-old seedlings of two genotypes (M202 (*Sub1*) and M202) were exposed to submergence. Leaves and meristems were sampled before applying submergence treatment and at 1, 3 and 7 days of submergence for estimating *Sub*1A and *Adh*1 mRNA accumulation. The results showed that the levels of mRNA increased in abundance as plants have undergone stress, especially at 3 days of submergence. The mRNA concentration also accumulated more in the meristem than in leaf tissues.

The other experiment was conducted to compare the rate of recovery of photosynthesis and plant growth of the submergence-tolerant M202 (*Sub*1A) and the intolerant M202. Twenty three-day-old seedlings in soil-containing pots were completely submerged for up 3, 6 and 10 days (d). Photosynthetic and growth traits were measured before submergence treatment (0 day) and after 1 hour and 24 hours of recovering at 3, 6 and 10 days of submergence. When plants were prolonged under stress condition, all the photosysnthetic parameters such as photosynthetic rate, stomatal conductance, transpiration and water use efficiency decreased much more in M202 (*Sub1*) than those in M202. On the contrary, these parameters were able to recover in M202 (*Sub*1) better than in M202. Similar patterns were revealed for plant growth characters including plant height, number of leaves and tillers. The results indicated that *Sub*1A gene restricted photosynthesis and stem elongation and leaf area in the tolerant genotype M202 (*Sub*1) during submergence but increased the rate of photosynthesis and dry matter accumulation after de-submergence.

Keywords: mRNA, photosynthesis, rice plant, submergence tolerance, Sub1A.

Đánh giá khả năng tổng hợp ARNtt và các tính trạng sinh lý liên quan của gen chịu ngập Sub1A ở cây lúa (*Oryza sativa* L.)

TÓM TẮT

Thí nghiệm tiến hành đánh giá khả năng tổng hợp ARNtt trong điều kiện ngập của giống lúa M202 có chứa gen chịu ngập (*Sub*1A). Mạ 19 ngày tuổi của hai giống lúa M202 (*Sub1*A) và M202 được xử lý ngập nhân tạo. Tại thời điểm trước xử lý ngập và sau xử lý 1, 3 và 7 ngày, tiến hành lấy mẫu lá và thân của hai giống lúa để đánh giá khả năng tổng hợp ARNtt bằng chỉ thị *Sub*1 và *Adh*1. Kết quả nghiên cứu cho thấy lượng ARNtt được tổng hợp từ gen Sub1A tăng lên khi cây xử lý ngập, đặc biệt là 3 ngày sau xử lý. Lượng ARNtt được tổng hợp trong thân cao hơn so với trong lá.

Một thí nghiệm khác tiến hành đánh giá khả năng phục hồi về quang hợp và sinh trưởng của giống lúa M202 (*Sub*1) so với giống đối chứng M202. Hạt của hai giống lúa được gieo trong khay có chứa đất cho đến 20 ngày tuổi sau đó được xử lý ngập nhân tạo với thời gian là 3, 6 và 10 ngày. Một số chỉ tiêu về quang hợp và sinh trưởng được đo tại thời điểm cùng ngày trước khi xử lý ngập và tại thời điểm là 1 giờ và 24 giờ sau phục hồi khi xử lý ngập với thời gian 3, 6 và 10 ngày. Các chỉ tiêu quang hợp như cường độ quang hợp, độ dẫn khí khổng, cường độ thoát hơi nước và hiệu suất sử dụng nước đều giảm ở M202 (*Sub1*) nhiều hơn so với giống M202. Tuy nhiên, giống M202

(*Sub*1) có khả năng phục hồi các chỉ tiêu này tốt hơn so với giống M202. Kết quả tương tự với các chỉ tiêu sinh trưởng như chiều cao cây, số lá và số nhánh. Kết quả nghiên cứu đã xác định là khi bị ngập gen *Sub*1A đã kìm hãm quang hợp cũng như việc tăng chiều cao và diện tích lá. Đồng thời gen này đã kích thích tăng cường độ quang hợp và chất khô tích lũy ở giai đoạn phục hồi ở giống lúa M202 (*Sub*1) tốt hơn so với giống đối chứng M202.

Từ khóa: Cây lúa, chịu ngập, gen *Sub*1A *, mRNA, quang hợp.*

1. INTRODUCTION

Flood is one of the most damaging among the serious problems of agriculture. It adversely affects plant growth and production which often lead to decreased crop yields. Worldwide, the flooded area, severity of flooding and the scale of damage are alarmingly increasing over the years. Moreover, under global climate changes, crops will be exposed more frequently to episodes of drought, high temperature and flood.

While many kinds of crop including soybean, wheat and maize are categorized as flooding sensitive (Komatsu et al., 2012), rice (*Oryza sativa*) is the best-characterized flooding-tolerant crop. Rice is known as a semiaquatic species with increased shoot elongation when the plant is totally or partially submerged. According to submergence habit, two main ecotypes can be distinguished: deepwater and lowland rice (Jackson et al., 1987; Kende et al*.,* 1998). Deep water rice and the widely cultivated lowland rice overcome submergence stress by antithetical strategies (Fukao and Bailey-Serres, 2004). While the deep water rice responds to submergence by promoting internode elongation to outgrow floodwaters, the submergence-tolerant lowland rice cultivars, typically East Indian accession FR13A, restrict leaf and internode elongation during inundation and can recommence the initiation of leaf development upon desubmergence (Ahmed et al., 2013; Das et al., 2005; Singh et al., 2001).

Several studies have shown that *Submergence*-1 (*Sub*1) located on Chromosome 9 is a major quantitative trait locus affecting submergence tolerance in lowland rice. The QTL accounts for 35 to 69% of phenotypic variance in tolerance in diverse backgrounds (Nandi et al., 1997; Sripongpangkul et al., 2000; Toojinda et al., 2003; Xu et al., 2000; Xu and Mackill, 1996). Detailed genetic and physical mapping of *Sub*1 revealed that this locus contains a variable cluster of two to three genes (i.e. *Sub*1A, *Sub*1B and *Sub*1C) that encode proteins with the DNA binding domain common to the ethylene response factors (ERFs)/ethylene-responsive element binding proteins/Apetala2-like proteins (Xu et al., 2006). Whille the genes *Sub*1B and *Sub*1C are present in a wide range of *indica* and *japonica* varieties, *Sub*1A is limited to a subset of *indica* varieties and absent from all studied *japonica* germplasm (Xu et al., 2006).

Submergence can lead to conditions of oxygen deprivation. Physiological responses in plants then are the increase typically requires in pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) (Drew, 1997). Fukao et al. (2006) observed an abundant increase in transcript level of *Adh* genes in a submergence-tolerant genotype M202 (*Sub*1) while this transcript was greatly limited in M202.

This study aims to investigate (i) whether there are any differences in expression of *Sub*1 and *Adh*1 genes in leaf and main stem based on estimation of mRNA accumulation and (ii) effects of *Sub*1 on physiological traits during submergence and after desubmergence.

2. MATERIALS AND METHODS

2.1. Plant materials and growth conditions

Rice (*Oryza sativa*) cv M202 and the *Sub*1 introgression line M202 (*Sub*1) were used in this study. M202 is a *japonica* inbred line that lacks *Sub*1A but possesses *Sub*1B and *Sub*1C. The near-isogenic line M202 (Sub1) was generated by introgression of the *Sub*1 region from the submergence-tolerant *indica* cultivar

FR13A (Xu et al.,2004). M202 (Sub1) possesses all three *Sub*1 genes, *Sub*1A, *Sub*1B and *Sub*1C.

Seedlings were transplanted into pots (10 cm x 10cm) 5 days after germination. Each genotype was grown in separate pots and replicated four times. Each pot contained 25 plants and was placed in a controlled glasshouse at 250C and natural light. Light gray plastic tanks (150 cm x 80 cm) were filled with 250 liter of water. Nineteen-day-old seedlings in soil-containing pots were completely submerged for up to 7 days (d). The tank water was not circulated or refreshed during the experiment. Leaves and meristems were sampled at 0, 1, 3 and 7 days after submergence (DAS).

2.2. RNA extraction and qRT-PCR

Total RNA was extracted from 100 mg of tissue (leaves and meristems) using the RNeasy plant mini kit (Qiagen). Single-stranded cDNA was synthesized from 2 mg of total RNA using SuperScript II RNase H reverse transcriptase (Invitrogen) as described in the manufacturer's protocol. Briefly, 10 µl Rnase were added to 2 µg RNA to obtain 10 µl solution which was then added with 1µl dNTP (10 mM). The mixture was pipetting and incubated at 65°C for 7 min. After cooling down, the mixture was added with 4µl 5X buffer, 2 µl DTT and 1 µl Rnasin which was then incubated at 42°C for 2 min. Finally, 1 µl Superscript II was added and mixed by pipetting. The mixture was incubated at 42°C for 50 min, followed by incubation at 70°C for 15 min. cDNA mixture was cooled down before using for qRT-PCR.

qRT-PCR was performed in a 50 µl-reaction mixture containing 2 µl of cDNA, 5 µl of 103 PCR buffer, 0.2 µM primers, 0.2 µM deoxynucleotide triphosphates, and 1.25 units of Taq DNA polymerase (Qiagen). Primers used for amplification of *Sub*1 genes were *Sub*1A, *Adh*1 and *Actin*1 (Table 1). The number of cycles for qRT-PCR using different primer pairs was adjusted to be in the linear range. After denaturing the genomic DNA template at 95°C for 3 min, PCR for *Sub*1A was performed with 28 cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s, extension at 72°C for 60 s, and final extension incubation at 72°C for 15 min. The number cycles and annealing temperature were 23, 25 and 54°C, 62°C for *Adh*1 and *Actin*1 respectively. The number of cycles and annealing temperature for each primer are presented in Table 1. RT-PCR products were confirmed by DNA sequence analysis.

2.3. Photosynthesis and agronomic characters after desubmergence

Seedlings of M202 and M202 (Sub1) were transplanted into pots 5 days after germination. Each genotype was grown in 7 pots (10 x 10 cm) and each pot included 4 plants. Light gray plastic tanks were filled with 250 liter of water. Twenty three-day-old seedlings in soil-containing pots were completely submerged for up 3, 6 and 10 days (d). The tank water was not circulated or refreshed during the treatment. Photosynthetic measurements were collected on a fully expanded leaf of two plants in a pot at a day before submergence treatment (0 day) and after 1 hour and 24 hours of recovering at 3, 6 and 10 days of submergence, using gas analyze Licor 6400 (temperature at 30°C, CO_2 concentration at 370 ppm, relative humidity of 60%, light intensity at 1200 $mmolm^{-2}s^{-1}$). Agronomic characters included plant height, number of leaves per main stem and number of tillers. Leaf area was measured by Leaf Area Metter Licor-3100. Individual plant was harvested and oven-dried at 80°C for 48 hours for dry matter determination.

3. RESULTS

3.1. mRNA accumulation

The *Sub*1 region on rice chromosome 9 contains a cluster of two or three *Sub*1 genes (*Sub*1A, *Sub1*B, and *Sub1*C), and genotypic variation at this complex locus confers distinctions in submergence tolerance (Xu et al., 2006). The near-isogenic line M202-*Sub*1 containes all three *Sub*1 genes. qRT-PCR analysis confirmed the presence of the *Sub*1A

transcript in both leaves and meristem of M202-*Sub*1. The level of *Sub*1A mRNA increased rapidly in abundance after 3 days of submergence in leaves (Fig. 1a), whereas it appeared after 1 day of submergence in meristems (Fig. 1a). However, the level of *Sub*1A transcript at 3 d of submergence was higher than that at 1 and 7 d of submergence. Especially, the level of *Sub*1A mRNA accumulation was higher in meristem than that in leaf tissues.

Submergence leads to conditions of oxygen deprivation, which in turn requires increased level 0f pyruvate decarboxylase (PDC) and alcohol dehydrogenase (ADH) for ethanolic fermentation (Drew, 1997). Transcript levels of the *Adh*1 genes in leaves and meristem of the two genotypes were evaluated to examine the role of *Sub*1 in ethanolic fermentation during submergence (Fig. 2). In meristem of the tolerant line M202 (Sub1), *Adh*1 mRNA gradually accumulated until day 3 and remained constant until day 7. In contrast, mRNA increase was limited in leaves. Dramatic increases in *Adh*1 transcripts occurred within 1 and 3 d of submergence in both leaf and meristem.

3.2. Recovery of photosynthesis and plant growth after desubmergence

As the days of submergence increased (0 - 6 d), photosynthetic and stomatal conductance responses in plants decreased. After stress was relieved, both M202 and M202 (Sub1) were be able to recover. In general, recovery of photosynthesis and stomatal conductance rate in M202 (Sub1) under stress condition were higher than that in M202 (Fig. 2), except for 1 hour after desubmergence at 3 and 6 days of submergence (3d+1hr and 6d+1hr respectively). The recovery in photosynthesis and stomatal conductance at 24 hours was nearly twice those at 1 hour after desubmergence from 3 and 6 d of submergence. In addition, there were significant differences in recovery with different periods of submergence. For examples, plants were able to recovery quicker and back to nearly normal photosynthesis (at 0 d of submergence) when they were submerged for 3 and 6 d. However, the recovery was much less when the plants were stressed for longer, i.e. 10 d (Fig. 2a).

Similar patterns were observed in transpirational rate and water use efficiency (WUE) (Fig. 3). As plants were kept under the stress for longer period (0 - 10 d), the transpiration and WUE decreased. Recovery of transpiration in M202 (Sub1) under submergence condition was higher than that in M202, except for at 3d+1hr and 6d+1hr. Especially, M202 (Sub1) could recover tranpirational rate back to normal (at 0 d) after all periods of submergence (3 - 10 d) (Fig. 3a). In contrast, WUE in M202 (Sub1) was only higher than that in M202 at 3d+1h and 6d+24h (Fig. 3b). Although WUE recovery occurred in both genotypes, the plants were not able to reach back the level of WUE at 0 d. There were also significant differences in recovery of transpiration and WUE with different periods that plants were under submergence.

Under submergence condition from 0 - 10 d, plants still increased plant height and number of leaves but not number of tillers (Fig. 4-6). While the M202 were taller than M202 (Sub1), M202 (Sub1) had more leaves and the same number of tillers at the beginning of submergence (0 d) (Fig. 4a-6a). After desubmergence, this pattern was still maintained, except that M202 (Sub1) had more tillers. In addition, the increasing rates in number of leaves and tillers were higher in M202 (Sub1) than that in M202 (Fig. 5b-6b).

Different from the above agronomic characters, leaf area and dry matter accumulation only increased up to 3 days of submergence and but decreased if plants were prolonged under stress condition (6 - 10 d) in both genotypes (Fig. 7-8). Although the tolerant M202 (Sub1) possessed more leaves and leaf area was higher in M202 at 0 d (Fig. 7). Initial dry matter accumulation was also higher in M202 (Fig. 8). However, M202 (Sub1) recovered better with larger leaf area, higher dry matter and growth rate after desubmergence. The

recovery rate at 3d+24h was noticed to be higher than that at 6d+24h and 10d+24h (Fig. 8b). This implicates that the long period plants were exposed to stress (i.e. submergence in this case) will result in weak recovery ability or even plant death (growth rate was nearly 0 at 10d+24 - Fig. 8b).

4. DISCUSSION AND CONCLUSIONS

The results presented here demonstrate that the *Sub*1 regulates diverse acclimative responses to submergence, including the induction of *Sub*1 and *Adh*1 mRNA accumulation as well as photosysnthesis, stomatal conductance, transpiration, water use efficiency, leaf, leaf area, internode elongation, tillers and dry matter accumulation. Levels of mRNA transcripts increased rapidly in abundance once M202 (Sub1) was subjected to stress and reached highest at day 3 (Fig. 1). This finding was in line with previous studies such as Fukao et al. (2006, 2011 and 2012). The levels of *Sub*1A and *Adh*1 mRNA could remain increased for longer, up to 14 d of stress (Fukao et al., 2006) instead of 7 d as in this study. Presence of *Adh* gene was also reported to be associated with waterlogging tolerance in wheat and barley (Ahmaed et al., 2013). In addition, mRNA accumulated more in meristem than that in leaf tissues (Fig. 1).

Generally, physiological responses such as photosynthesis, stomatal conductance, transpiration and water use efficiency decreased in both genotypes as plants were exposed to submergence (Fig. 2-3). The longer period plants were subjected to stress, the lower values in those traits and the harder plants were able to recover. While both genotypes still increased in height and number of leaves up to 6 d of submergence, their growth in terms of tiller number, leaf area and dry matter accumulation decreased under prolonged stress (> 6 d of submergence) (Fig. 4-8). Especially, *Sub*1A restricted plant elongation and leaf area in the tolerant M202 (Sub1) genotype, i.e these traits were expressed less than M202 at the beginning of submergence (Fig. 4, 7). This is in consistence with previous studies showing that shoot elongation in the lowland rice varieties is restricted under submergence to conserve energy reserves and reduce carbohydrate consumption to enable development restarting upon eventual de-submergence (Fukao et al., 2006; Ismail et al., 2009; Kawano et al., 2009). *Sub*1A was also reported to delay leaf senescence (Fukao et al., 2012), inhibit floral initiation and delay flowering (Pen˜a-Castro et al., 2011), which are components of the quiescence survival strategy in rice. As a consequence, recovery rates in the tolerant genotype were higher in all measured traits. However, long-term submersion may still cause extensive carbohydrate consumption leading to energy starvation (Jackson & Ram, 2003) and plant death as indicated in nearly zero growth rate after recovery at 10 d (Fig. 8b). Besides, *Sub*1 locus including *Sub*1A, *Sub*1B and *Sub*1C also conditions various metabolisms such as restrained accumulation of reactive oxygen species (Fukao et al., 2011), lower carbohydrate consumption, activation of ethanolic fermentation (Fukao et al., 2006) and possible negative interplay between the Sub1A-1 and CIPK15 (Calcineurin B-like interacting protein kinase 15) pathways (Kudahettige et al., 2011).

In conclusion, these data confirm that the introgression of *Sub*1A region into M202 is sufficient to dramatically enhance the viability and to confer the ability to resume plant growth upon desubmergence.

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