International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(1): 369-377

Received: 01-11-2017
Accepted: 02-12-2017

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Expression profiling of anoxia-responsive genes and changes in both antioxidative enzymes and lipid peroxidation in rice seedlings under anoxia stress

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Abstract
The effect of complete submergence on growth attributes of rice via morphological screening. Four (W0, W2, W6 and W10) of twenty-seven varieties were screened and further used to test for changes in antioxidative enzymes, lipid peroxidation and the expression of anoxia-responsive genes in plants under complete submergence. Antioxidative enzymes such as SOD, CAT and POD increased significantly in W6 and W10; however, POD and CAT decreased greatly, and SOD showed no change in W0 or W2 on 6th and 8th days of submergence. In addition, malondialdehyde (MDA) content was significantly more enhanced in W0 and W2 than in W6 and W10. The expression level of *ADH1*, *CIPK15*, *SnRK1A*, *SUB1A*, *SLRL1* and *SUB1C* increased significantly in W10 but decreased in W2, except for *SUB1A* and *SLRL1* on the 4th day of submergence. The results of the current study suggest that rice varieties with better shoot and root growth sustained high survival rates. Antioxidative enzymes activities must be higher in resistant varieties. Our results further suggest that submergence induces the expression of *SUB1A,* which further promotes the expression of *SLRL1*, a central suppressor of GA signaling, and improves tolerance to submergence stress in tolerant varieties by inducing expression of enzymes responsible for the detoxification of ROS.

Keywords: anoxia tolerance; *SUB1A*; antioxidative enzyme; lipid peroxidation; ROS

Introduction
Rice (*Oryza sativa* L.), as a major staple food, millions of people are using it as a source of nutrition around the world. Abiotic stress such as flooding and the complete submergence of fields is a severe problem in South and South-East Asia, affecting more than 20 million hectares of rice each year [1]. Submergence creates hypoxic or anoxic conditions, causing poor germination, seedling establishment, and enormous yield losses in rice. Fortunately, some indica cultivars such as FR13A can survive 10 to 14 days of complete submergence and can renew growth after the floods recede [2]. The major gene conferring submergence tolerance in FR13A is an AP2/ERF transcription factor called submergence 1A (*Sub1A*) [3]. Two alleles of Sub1A (*Sub1A-1* and *Sub1A-2*) exist that differ only by a single nucleotide polymorphism (SNP) variation at position 556. Gibberillic acid repressors like Slender rice-1 (*SLR1*) and Slender rice-1 like-1 (*SLRL1*) can be enhanced by *Sub1A-1* to confer submergence tolerance [3]. *Sub1A* limits the reactivity of ethylene (GA) during submergence [4] and improves levels of alcohol dehydrogenase-1 (*ADH1*) and pyruvate decarboxylase (*PDC*) by providing adenosine triphosphate (ATP) necessary for the survival of rice during submergence stress [5]. *Sub1A* studies have led to substantial improvements in understanding the rice submergence tolerance. Agreeing to Fukao and Bailey-Serres [4] and Xu *et al.* [3], submergence tolerance is controlled by many genes because it is a multifaceted trait. The FR13A and Goda Heenati rice cultivars are examples that carry the *Sub1A*-1 gene, but identification of the *Sub1A* gene can partly resolve differences in submergence tolerance between different rice cultivars [5]. This indicates that other genes may interact with *Sub1A*-1 to provide submergence tolerance. Xiong *et al.* [2] expressed genes differentially these two rice cultivars under submergence include two novel submergence-responsive genes: *Os09 g0269900* and *Os12 g0202700*. Therefore, it is necessary to classify new genes involved in *Sub1A*-mediated tolerance in order to investigate the molecular mechanisms and further improve submergence tolerance.

Reactive oxygen species (ROS) are aggressive O_2 radicals, which can be enhanced by submergence stress in plants. These O_2 radicals are also known as active oxygen species (AOS) or reactive oxygen intermediates (ROI), which can cause oxidative injury which includes superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals [6, 7, 8, 9, 10, 11]. Via oxidative damage of chlorophyll, DNA, proteins, lipids, nucleic acids and other macromolecules, O_2 radicals can severely disrupt normal metabolism due to their cytotoxicity [12, 13, 14, 15, 16, 17]. To defend cellular membranes and organelles from injurious effects of ROS, Plants have developed a multifaceted antioxidant system to mitigate oxidative damage generated by reactive oxygen species [18, 19]. Antioxidative enzymes in combination with numerous peroxidases like ascorbate peroxidase (APX), peroxidase (POD) and glutathione reductase (GR), can ably overcome the activity of ROS [20, 21, 22, 23, 24, 25]. In the detoxifying process, the first enzyme is SOD transmutes superoxide anion radicals (O_2^-) to H_2O_2, after which H_2O_2 is converted to water and oxygen by CAT. Gill *et al.* [26] and Bonifacio *et al.* [11] concluded that, H_2O_2 detoxification is followed by the activity of an important ascorbate peroxidase (APX), which also catalyses the conversion of H_2O_2 to water by the reducing power of ascorbate. Ascorbate, glutathione and β-carotene in combination with above antioxidative enzymes plays an important role in the elimination of harmful oxygen compounds [27, 28, 29]. Other endogenous antioxidants include glutathione (GSH), and the associated glutathione metabolism enzyme is an ideal biomolecular compound and play a vital part in protecting plants from oxidative stress injury.

A product of lipid peroxidation called malondialdehyde (MDA) is considered an indicator of oxidative damage. Blokhina *et al.* [30] proposed that, reduction in membrane integrity under anoxia is an indication of injury and can be measured by variations in MDA content.

Submergence stress tolerance is correlated with antioxidative enzyme activity and has been proven by numerous studies [31, 32]. Madamanchi and Alscher [33] concluded that high levels of antioxidants provide greater tolerance to oxidative damage. Therefore, the observation of antioxidative enzyme activity in plants a few days following submergence could constitute an efficient technique to identify tolerant varieties [34].

The purpose of the study was to recognize alterations among 27 rice varieties in their response to submergence tolerance. The effect of complete submergence on growth attributes of rice was studied via morphological screening. Furthermore, changes in antioxidant enzyme levels and lipid peroxidation of rice seedlings were examined. Additionally, the expression of anoxia-responsive genes was studied to further understand the tolerance levels of rice varieties to submergence stress.

Materials and Methods

Plant material and phenotypic screening

The study entitled Expression Profiling of Anoxia-Responsive Genes and Changes in both Antioxidative Enzymes and Lipid Peroxidation in Rice Seedlings under Anoxia Stress was conducted under laboratory conditions in the School of Agriculture, Yangtze University, Jingzhou, Hubei, P.R. China during 2015-18. The materials were provided by the School of Agriculture, Yangtze University, P.R. China. Twenty-seven rice varieties were evaluated for submergence tolerance under laboratory conditions listed in Supplementary Table 1. Sodium hypochlorite (0.6 %) were used for seed sterilization (15 min), double-distilled water was used to rinsed the seeds three times and then all the seeds were soaked in tap water for 48 hours in the dark at 25 °C in an incubator [35, 36]. Sixty healthy seeds from each variety were sown to a 1.5 cm depth into pots filled with fine soil and then were covered by another 1 cm layer of soil, after which the seeds were either submerged in water (10 cm) or plants grew under normal conditions were used as control. Later, all the pots were placed in an incubator that had a light intensity of 500-1000 PAR $m^{-2}s^{-1}$ (16:8 photoperiod) and a temperature of 28-25°C (day/night) [35]. The germination rate (%), survival rate (%), shoot length (cm) and root length (cm) were measured in triplicate after 21 days of submergence [35].

Extraction and assays of ROS-scavenging enzymes

Leaf samples in each experiment were collected on the 4th, 6th and 8th day of submergence. Fresh leaves (0.2 g) were blended in 1.6 mL of 50 mmol L^{-1} precooled potassium phosphate buffer (pH 7.8) with the help of quartz sand. The slurry was centrifuged at 12000 × g for 20 min at 4°C, after which the supernatant was immediately used for enzyme assays. UV-VIS spectrophotometer (UV-1800, Shimadzu, Inc. Japan) were used for all spectrophotometric analyses. The total activity of SOD was evaluated according to Li [37] by calculating the inhibition of photochemical reduction of nitro tetrazolium blue (NBT) monitored at 560 nm. For this purpose, 3 mL of reaction mixture containing 0.05 mmol L^{-1} of potassium phosphate buffer L^{-1} (pH 7.8), 14.5 mmol L^{-1} methionine, 60 µmol L^{-1} riboflavin, 2.25 mmol L^{-1} NBT, 30 µmol L^{-1} EDTA and 30 µL of enzyme extract. The reaction mixtures were illuminated for 20 min under a light intensity of 4000 lux. The reduction of NBT was inversely proportional to the SOD activity. Identical solutions that were maintained under darkness served as a blank and as the control. The total POD activity was analysed in terms of one unit (U) of guaiacol oxidized per gram per min as described by Li [37]. Two hundred millilitres of 0.2 mol L^{-1} phosphate buffer (pH 6.0) containing 0.076 mL of guaiacol (dissolved by heating) and 0.112 mL of 30% H_2O_2 solution was used. Thirty microlitres of enzyme extract was added to 3 mL of the reaction mixture, after which the solution was measured at 470 nm as fast as possible. The total activity of CAT was tested according to Li [37] by measuring the rate of decomposition of hydrogen peroxide (H_2O_2) monitored at 240 nm. The reaction mixture contained 0.15 mol L^{-1} of potassium phosphate buffer (pH 7.0), 30 % hydrogen peroxide solution and 30 µL of enzyme extract in a volume of 3 mL.

Lipid peroxidation

Lipid peroxidation was assessed as described by Li [37] with slight modification by measuring the amount of malondialdehyde produced by thiobarbituric acid (TBA). Samples were ground in a mortar containing 1.6 mL of 10 % trichloroacetic acid solution (TCA). 1 mL of the supernatant was taken and mixed with 1 mL of 0.067 % TBA, after which the solution was boiled in water for 30 min. The samples were then chilled and centrifuged again at 12000 × g for 20 min at 4 °C, after which the samples were monitored at 450, 532 and 600 nm.

RNA isolation and analysis of relative gene expression by qRT-PCR

The total RNA was extracted from rice seedlings on the 4th and 6th days of submergence using TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's instruction. RNA concentrations were determined using a spectrophotometer (NanoDrop 2000, Thermo Fisher

Scientific, Wilmington, MA, USA). PrimeScript RT reagent kit with gDNA eraser (Takara, Japan) were used to synthesized first-strand cDNA. qRT-PCR was performed using SYBR Green and a StepOne Plus Real-Time PCR system in accordance with the manufacturer's instructions (QuantStudio™ 6 Flux Real-Time PCR System, Applied Biosystems by Life Technologies, California, USA). The qRT-PCR primers specific for different genes are listed in Supplementary Table 2. To stabilize the expression data, the *Actin1* gene was used as an endogenous control.

Statistical analysis

The experimental design was completely randomized design (CRD) with three replications. Analysis of variance (ANOVA) was performed using Data Processing System (DPS) software. In the figures, all data presented were the mean ± standard errors of three replicates.

Table 1: Number, names, location and types of rice varieties.

Number	Names	From	Indica/Japonica
W0	Huajing 74	(Guandong) China	*Indica*
W1	Tetep	Vietnam	*Indica*
W2	Amol 3 (Sona)	Iran	*Indica*
W3	Zhong 4188	(Zhejiang) China	*Indica*
W4	BG367	Bangladesh	*Indica*
W5	Zihui100	(Anhui) China	*Indica*
W6	Katy	USA	*Japonica*
W7	Suyunuo	(Jiangsu) China	*Japonica glutinous*
W8	IR64	Philippines	*Indica*
W9	Basmati 385	Pakistan	*Indica*
W10	Nanyangzhan	(Guangxi) China	*Japonica*
W11	Basmati 370	Pakistan	*Indica*
W12	IR58025B	Philippines	*Indica*
W13	Jiangxisimiao	(Jiangxi) China	*Indica*
W14	Lianjian33	(Zhejiang) China	*Indica black glutinous*
W15	Meiguolixiang	USA	*Indica*
W17	Ganxiangnuo	(Jiangxi) China	*Indica glutinous*
W18	IRAT261	Nigeria	*Japonica*
W19	Kyeema	Australia	*Japonica*
W20	Chenglongshuijingmi	(Zhejiang) China	*Indica*
W21	IR65598-112-2	Philippines	*Japonica*
W22	Khazar	Iran	*Japonica*
W23	Lemont	USA	*Japonica*
W24	Star bonnet99	USA	*Japonica*
W27	IAPAR9	Brazil	*Japonica*
W31	IR66897B	Philippines	*Japonica*
W32	IR66167-27-5-1-6	Philippines	*Japonica*

Table 2: List of primer sets for qRT-PCR analysis.

Gene Name	Forward primer (5'-3')	Reverse primer (5'-3')
Actin1	GGTAACATTGTGCTC AGTGGTGG	GGTGCAACGACCTT AATCTTCAT
ADH1	ACGAGTTTCAGTTCG TCACCCTCT	AACCACAACTCGAG CGCACAAATC
CIPK15	TGGAGATGAATAGC AGCAGTC	GCATACATTGTCTC AACATGA
SLRL1	GGCGGCGACAATAA CAACAACAGT	TACAAACACACGCT GCTACCATCC
SnRK1A	GAGACACCAAACTC AGCCACTG	ATGCCTCAAGCCAA ACCCAG
SUB1A	AGGTGAAAATGATG CAGG	CTTCCCCTGCATAT GATATG
SUB1C	ATACTCATCGAGTGC TGCTCCGAC	TTAGCTCCAGAAGC GCATGTC

Results and Discussion

Screening of rice varieties at the seedling stage for submergence tolerance

The relative germination rate (%) revealed no significant differences among all varieties under submergence compared to their respective controls. The average relative germination rate was 95%. All the tested varieties germinated well under submergence stress compared to their respective controls (Fig 1A). The above results indicate that tolerance to flooding during germination is a complex process involving numerous mechanisms. Though, tolerance to complete submergence might be not associated with tolerance to flooding, as demonstrated by FR13A, a landrace that is highly tolerant to complete submergence during the vegetative stage [38, 39]. The relative survival rate of rice seedlings decreased significantly under submerged conditions in all varieties except W6 and W10. The highest relative survival rate was noted for W10 (95.1%) followed by W6 (93.9%), while the lowest relative survival rate (%) was recorded for W2 (26.22%) and W0 (30.4%) (Fig 1B). The above results indicated that the growth of both the shoots and roots might have started earlier in the tolerant varieties and proceeded faster than the growth in sensitive varieties. The ability of resistant varieties to break starch into soluble sugars during hypoxia stress is likely to play a key role in survival and faster growth in submergence conditions. Our results agreement with those of Angaji *et al.* [40] and Ismail *et al.* [41]. The shoot lengths of W6 and W10 significantly increased (10 cm); however, the shoot lengths of W0 and W2 significantly decreased (14 cm) and were lower than those of all other varieties under submergence stress as well as those of the controls (Fig 1C). Shoot lengths increased in W6 and W10 but decreased in W0 and W2 under submergence conditions compared to those of the controls. This finding indicates that a lack of oxygen might cause a reduction in the shoot length of susceptible varieties. Submergence stress depresses shoot growth and causes injury and oxygen depletion in susceptible varieties compared to tolerant varieties [40, 41]. Similarly, root length significantly decreased by submergence. The root lengths of W0 and W2 were highly lower (2.73-4.38 cm) than those of all other genotypes, while W6 and W10 showed no significant change in their root lengths under submergence stress compared to those of the control (Fig 1D). Roots are susceptible to oxygen shortage; since the soil was anaerobic (flooded), the roots were entirely dependent on internal oxygen transport from the shoots. Similar results were reported by Jackson and Ram [39]. Thus, noticeable damage to shoots by submergence could be the result of anoxic injury to the roots. Hence, varieties that have good root growth with higher shoot length due to submergence exhibit good survival percentages [42]. This increased activity could be due to the transport of observed oxygen from the environs over shoots to the roots under submergence. The results of the current study are in agreement with those of Sarkar and Bera [42]. In the present study, the rice varieties W6 and W10 showed higher values of relative germination rate, relative survival rate, and shoot length; therefore, these varieties are categorized as submergence tolerant. On the other hand, the varieties W0 and W2 had minimum values for relative survival rate, shoot length and root length; therefore, these varieties can be grouped as sensitive varieties.

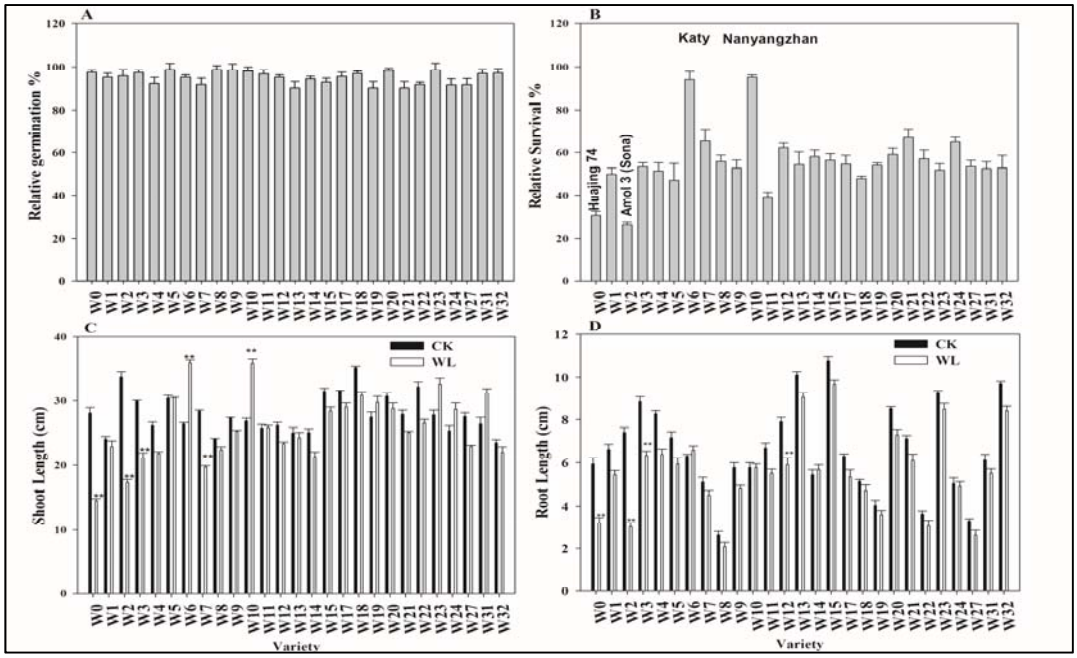

Fig 1: Phenotypic variation among 27 rice varieties

(A) Relative germination rate (%) of rice varieties after 21-days of submergence treatment at day/night temperature of 28 °C-25 °C. Values are the means ± SEs of three replicates.

(B) Relative survival rate (%) of rice varieties after 21-days of submergence treatment at day/night temperature of 28 °C-25 °C. Values are the means ± SEs of three replicates.

(C) Shoot length (cm) of rice varieties after 21-days of submergence treatment (WL) as compare to control (CK) at day/night temperature of 28 °C-25 °C. Values are the means ± SEs of three replicates. Bars with ** are significantly different at P<0.01.

(D) Root length (cm) of rice varieties after 21-days of submergence treatment (WL) as compare to control (CK) at day/night temperature of 28 °C-25 °C. Values are the means ± SEs of three replicates. Bars with ** are significantly different at P<0.01.

From the above results, based on their performance for germination, survival, shoot length and root length, W6 and W10 showed tolerance to submergence stress, while W0 and W2 were susceptible (Fig 2). Therefore, these varieties are worth further studying to assess their contributions of the induced responses of antioxidative enzymes to tolerate submergence stress.

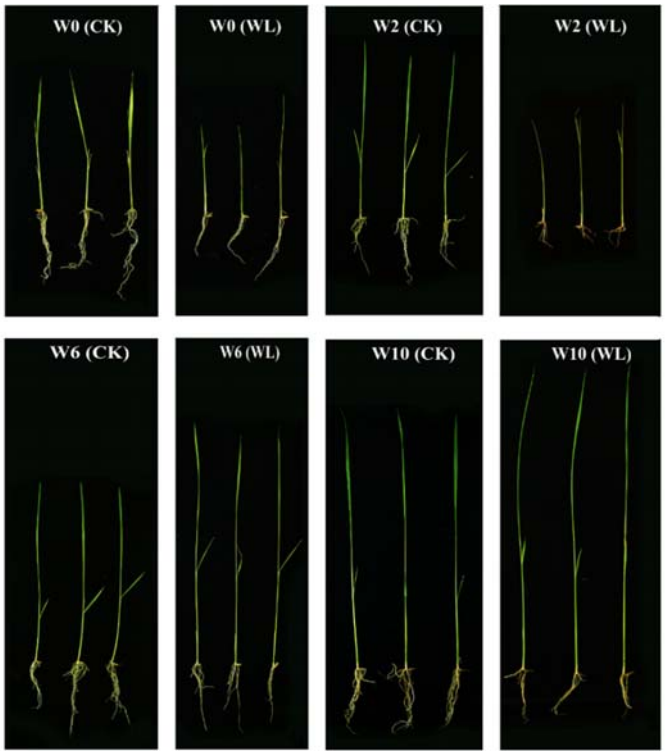

Fig 2: Images of rice plants treated with 21-days of submergence stress (WL) as compare to control (CK). Effects of submerged conditions on antioxidative enzymes activities

Antioxidative enzyme activity reflects the role of reactive O_2-scavenging enzymes in providing tolerance to submergence stress. There was a significant difference in SOD activities in W6 and W10, while W0 and W2 showed no significant differences, as shown in Fig 3A. The SOD activity in W6 and W10 were significantly higher than that of the control on the 6th and 8th days of submergence, while W0 and W2 showed no change throughout the submergence period. In addition, W6 and W10 proved to have better tolerance by increasing SOD activity on 6th and 8th days of complete submergence (Fig 3A). These observations are in line with those of Chugh *et al.* [43]. In the antioxidative systems of plants, SOD can affect the status of other activated species, such as H_2O_2 and OH [44]. Higher levels of O_2 enhance the activities SOD and other protective enzymes under submergence [45].

POD and CAT activities significantly increased in W6 and W10 on the 6th and 8th days, while no significant changes were observed on the 4th day of submergence (Fig 3B and Fig 3C). W0 and W2 showed no changes in POD or CAT activities until the 6th day, after which the activities significantly decreased on 8th day of submergence compared to those of the control. The activities of POD and CAT increased in W6 and W10 but decreased in W0 and W2 under submergence conditions. Previous studies reported higher inductions of POD and CAT activity in anoxia-tolerant barley cultivars than in susceptible ones under water-logged conditions [11]. Ushimaru *et al.* [46] testified that the potential absorption of molecular oxygen from water was the cause for the increased activity of these enzymes under submergence. We observed a similar trend in the present study: W0 and W6 displayed a higher level of SOD, POD and CAT than did W0 and W2.

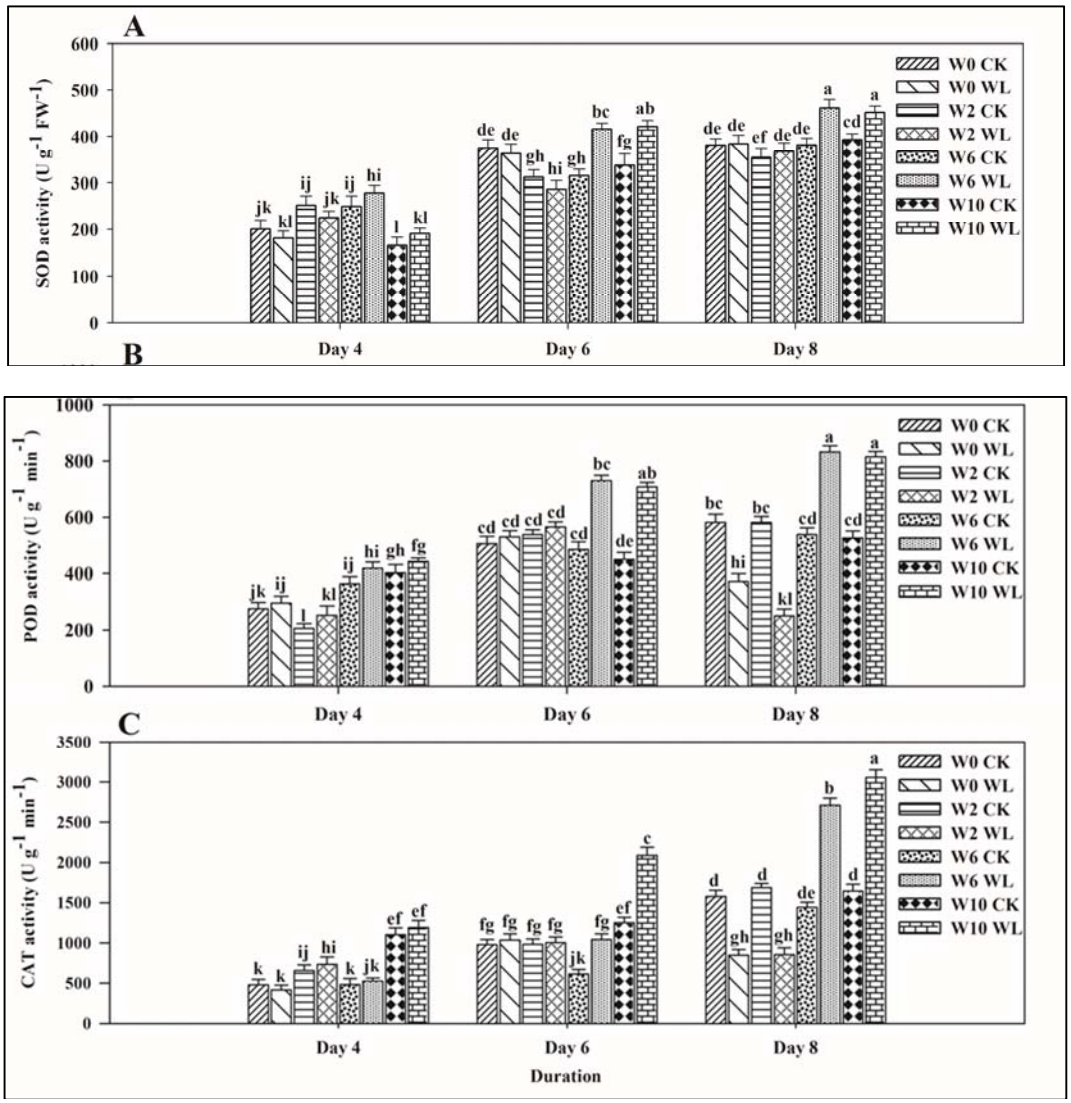

Fig 3: Variation in antioxidative enzyme activities among four rice varieties due to submergence stress.

(A) SOD activity of rice varieties at different days of submergence treatment (WL) (4th, 6th and 8th) as compare of control (CK). Values are the means ± SEs based on three independent assays for each determination. Bars with different lowercase letters indicate significant difference at $P<0.05$.

(B) POD activity of rice varieties at different days of submergence treatment (WL) (4th, 6th and 8th) as compare of control (CK). Values are the means ± SEs based on three independent assays for each determination. Bars with different lowercase letters indicate significant difference at $P<0.05$.

(C) CAT activity of rice varieties at different days of submergence treatment (WL) (4th, 6th and 8th) as compare of control (CK). Values are the means ± SEs based on three independent assays for each determination. Bars with different lowercase letters indicate significant difference at $P<0.05$.

Effects of submergence stress on lipid peroxidation

The lipid peroxidation levels, measured as the content of MDA, of the four rice varieties are shown in Fig 4. There were no significant differences in MDA contents on the 4th day of submergence treatment across all varieties (Fig 4A). The MDA contents in W0 and W2 were significantly higher than those of the control on the 6th day of the submergence treatment, while no significant changes were noted for W6 and W10 (Fig 4B). The MDA contents were significantly higher in all varieties than in their respective controls on the 8th day of submergence, but these values were much higher in W0 and W2 than in W6 and W10 (Fig 4C). Jain *et al.* [47] determined that free radical-induced peroxidation of lipid membranes is a consideration of stress-induced damage at the cellular level. A reduction in the integrity of the membrane is a sign of anoxia damage and can be measured as variations in lipid content and composition [30]. Therefore, the MDA content level during peroxidation of membrane lipids is often used as a sign of oxidative injury. In our study, the lower MDA contents in W6 and W10 than in W0 and W2 indicate less oxidative damage in W6 and W10 than in W0 and W2. Alike results were stated by Albrecht and Wiedenroth [48] and by Pfister-Sieber and Braendle [49].

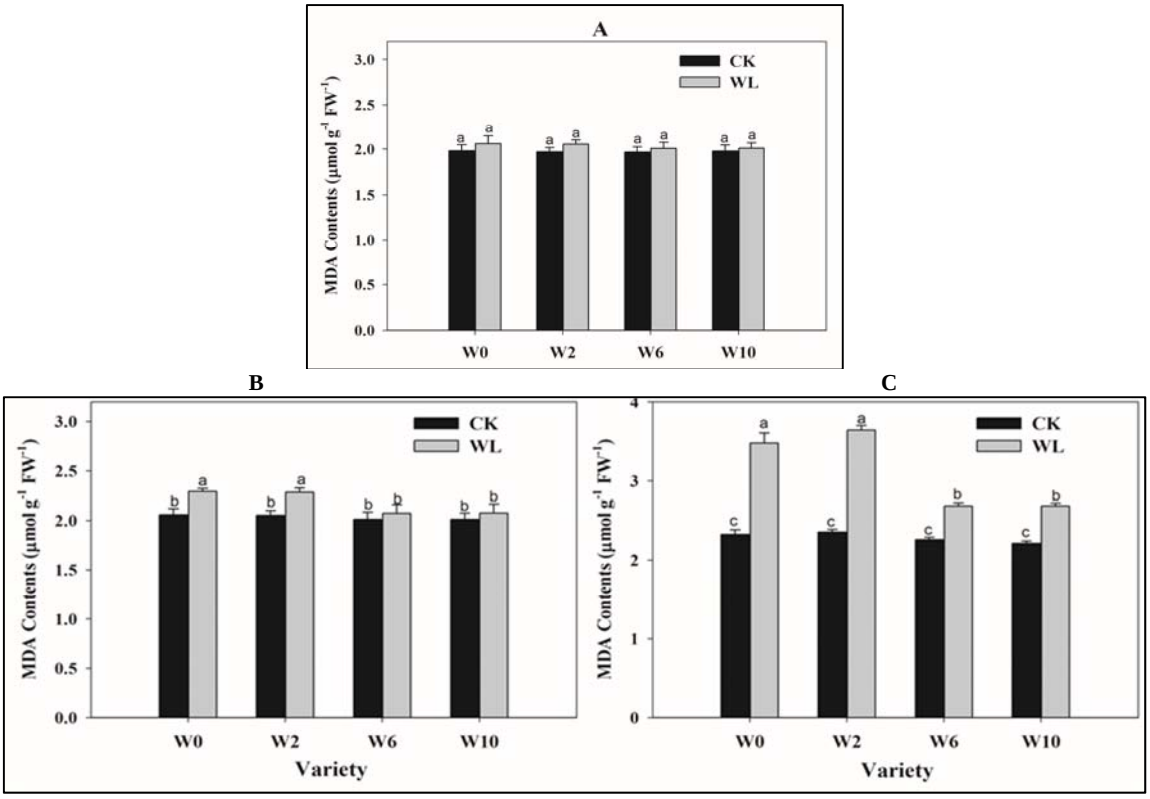

Fig 4: Changes in malondialdehyde (MDA) contents among four rice varieties under ssubmergence stress

(A) MDA contents of four rice varieties at 4th day of submergence treatment (WL) as compare of control (CK). Values are the means ± SEs based on three independent assays for each determination. Bars with different lowercase letters indicate significant difference at P<0.05.

(B) MDA contents of four rice varieties at 6th day of submergence treatment (WL) as compare of control (CK). Values are the means ± SEs based on three independent assays for each determination. Bars with different lowercase letters indicate significant difference at P<0.05.

(C) MDA contents of four rice varieties at 8th day of submergence treatment (WL) as compare of control (CK). Values are the means ± SEs based on three independent assays for each determination. Bars with different lowercase letters indicate significant difference at P<0.05.

Analysis of anoxia-related genes expression

On the basis of the above results, W2 (susceptible) and W10 (tolerant) were selected to further study and compare the expression levels of 6 genes. The studied genes include *SUB1A*, *SLRL1*, and alcohol dehydrogenase 1 (*ADH1*), each of which plays an important role in redirecting the switch from aerobic to anaerobic fermentation in low oxygen environments [50]; interaction of calcineurin B-like protein kinase 15 (*CIPK15*), which is a component of the signal transduction pathway that stimulates the elongation of rice coleoptile under hypoxia and participates in the detection of sugar during anaerobic germination [22]; and Snf1-related protein kinase 1 (*SnRK1A*), which is a plant global energy and stress sensor regulated by *CIPK15* [22], together with an allele of *SUB1C* ethylene responsive factor (ERF). Though, it is not acknowledged whether the *SUB1C* allele has any effect on the level of tolerance, as stated by Xu *et al.* [3].

The expression level of all genes was significantly higher in W10, while the expression level of *ADH1*, *CIPK15*, *SnRK1A* and *SUB1C* was significantly lower than that of the control in W2 on the 4th day of submergence (Fig 5). The expression level of *SLRL1* and *SUB1A* enhanced significantly in both varieties on the 4th day of submergence, but the increase was much higher in W10 than in W2. The expression level of the genes on the 6th day of submergence treatment for both varieties was inconclusive. The expression level of *SUB1A*, *SLRL1*, *ADH1*, *CIPK15*, *SnRK1* and *SUB1C* was much higher in W10 than in W2 under submergence conditions. The expression of *SUB1A* increased the growth of the GA signalling repressor *SLRL1* and reduced GA-inducible gene expression under submergence stress. An increase in the expression level of *SLRL1* was stimulated by ethylene, which in turn encouraged *SUB1A* expression in W10; these results are in agreement with those of Fukao *et al.* [51]. Fukao and

Bailey-Serres [4] further concluded that *SUB1A* stimulates the expression of the gene encoding alcohol dehydrogenase (*ADH1*), an enzyme necessary for fermentative metabolism, and represses the GA-mediated induction of the genes that are involved in starch degradation and cell elongation in leaves, thereby preserving energy until floodwaters recede. This conclusion is in agreement with the results of the current study. *CIPK15* promoted anaerobic starch degradation during seed germination and during early seedling growth under submergence stress by improving the growth of the sucrose non-fermenting-1 (SNF1)-related kinase 1 (*SnRK1*) protein and triggering the level SnRK1A-dependent signalling cascades by directly interacting with the kinase, as suggested by Tamang and Fukao [52]. The increase in the expression level of *SnRK1A* in W10 might be due to sugar starvation caused by the rapid consumption of soluble carbohydrates during the germination and seedling stages, as reported by Lu *et al.* [53]. The expression level of *SUB1C* increased under submergence stress in W10; these results are in agreement with those of Xu *et al.* [3], who reported that the transcription of *SUB1C* is strongly upregulated by submergence in tolerant varieties. The above results indicated that increased expression levels of *SUB1A* in W10 promote plants submergence stress tolerance by regulating genes involved in anaerobic metabolism [4, 51]. However, W10 again showed higher expression levels for all studied genes under submerged conditions than did W2, indicating the tolerance level of W10 was higher than that of W2.

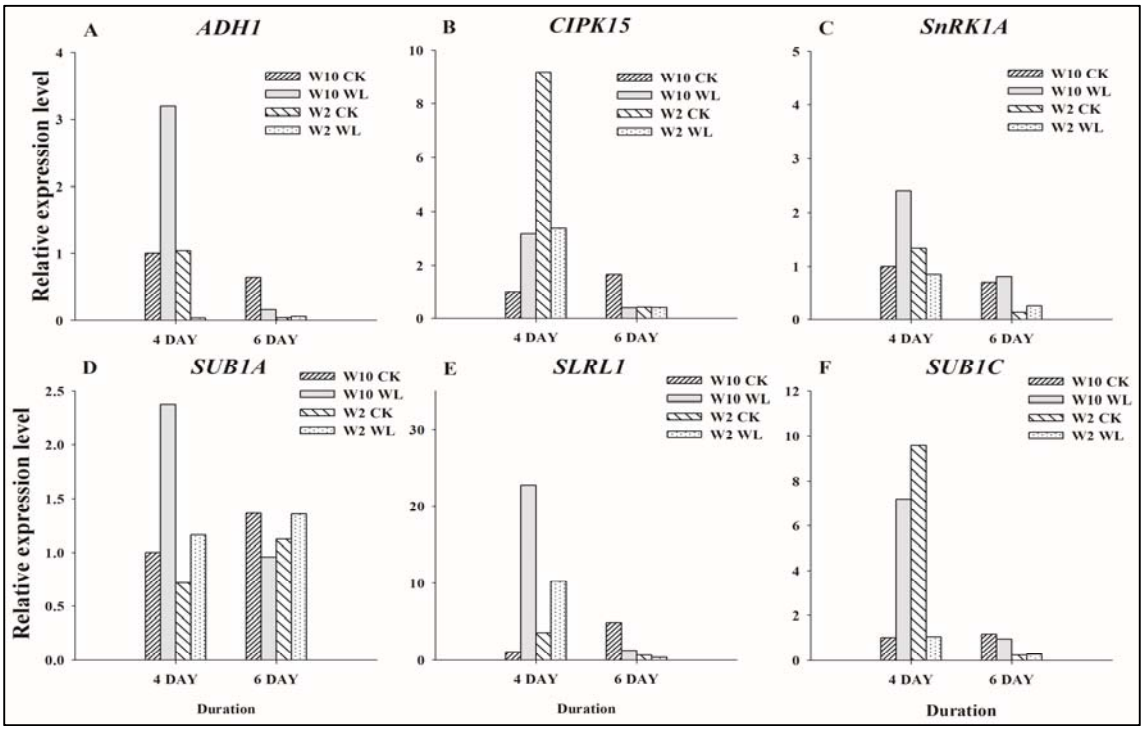

Fig 5: Expression profiling of anoxia-responsive genes

(A) Expression level of *ADH1* at 4th and 6th day of submergence treatment (WL) as compare to control.
(B) Expression level of *CIPK15* at 4th and 6th day of submergence treatment (WL) as compare to control.
(C) Expression level of *SnRK1A* at 4th and 6th day of submergence treatment (WL) as compare to control.
(D) Expression level of *SUB1A* at 4th and 6th day of submergence treatment (WL) as compare to control.
(E) Expression level of *SLRL1* at 4th and 6th day of submergence treatment (WL) as compare to control.
(F) Expression level of *SUB1C* at 4th and 6th day of submergence treatment (WL) as compare to control.

Conclusion

Antioxidative enzymes are very important for submergence tolerance in rice. There are genetic differences between germination, survival, shoot elongation, root growth and antioxidative enzyme synthesis levels of tolerant and sensitive rice varieties. In this study, W6 and W10 showed tolerance to submergence stress by exhibiting better shoot growth, root growth, and survival; higher levels of ROS-detoxifying enzyme (SOD, POD and CAT) activities; low levels of MDA; and high expression levels of all studied anoxia-responsive genes than did W0 and W2 under submerged conditions. Therefore, this study demonstrated that plants that have better shoot and root growth sustain higher survival rates under submerged conditions than do plants with poorer shoot and root growth. Submergence-tolerant varieties must have the capacity to accumulate higher antioxidative enzyme activities. Our results further propose that increased expression levels of *SUB1A* encourage plants submergence stress tolerance by regulating genes involved in anaerobic metabolism.

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