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Research article

Ontogenetic changes in vitamin C in selected rice varieties

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ABSTRACT

Vitamin C (L-ascorbic acid) is a key antioxidant for both plants and animals. In plants, ascorbate is involved in several key physiological processes including photosynthesis, cell expansion and division, growth, flowering, and senescence. In addition, ascorbate is an enzyme cofactor and a regulator of gene expression. During exposure to abiotic stresses, ascorbate counteracts excessive reactive oxygen species within the cell and protects key molecules, including lipids, proteins, and nucleic acids, from irreversible damage. In this study we focus on understanding how ascorbate levels are controlled in rice (*Oryza sativa*) during plant development and in response to light intensity and photoperiod. Our results indicate that in rice ascorbate metabolism follows a different pattern compared to other species. In the rice accessions we analyzed, total foliar ascorbate content increases during development and peaks at the vegetative 2–4 and the reproductive 4 stages, whereas other research has shown that in *Arabidopsis thaliana* and other dicots, ascorbate content declines with plant age. The pattern in rice does not seem to change when plants were grown under increasing light intensity: 150, 400 or 1200–1500 μmol m^{-2} s^{-1}. We observed little diurnal variation in AsA content in rice and did not see a steady decline during the dark period as has been reported in other species such as *Arabidopsis* and tomato. The total foliar ascorbate content of twenty-three rice accessions from four major rice subgroups was compared. These genotypes differed as much as eight-fold in ascorbate content at the V2 stage indicating the potential to enhance vitamin C levels in genotypes of global interest via breeding approaches.

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1. Introduction

Plants generate reactive oxygen species (ROS) as a defense mechanism by signaling various proteins, enzymes, and pathways. ROS play a major role in the growth and development of the plant by regulating pathogen and stress defense and hormone signaling [1,2]. In response to both biotic and abiotic stresses, plants produce excessive ROS which are toxic to cells because they are capable of oxidizing cellular components causing irreparable damage [1,3]. When overproduced, ROS oxidize nucleic acids, lipids, and proteins [3]. In order to prevent such damage, plants keep ROS levels under control using enzymatic and non-enzymatic antioxidants.

Vitamin C (L-ascorbic acid, ascorbate, AsA) is the most abundant water-soluble antioxidant in eukaryotes where it is vital whether obtained by synthesis or consumption [4,5]. While humans rely on fresh produce as the main source of vitamin C, much of its metabolism and functions in plants are poorly understood [6–8]. Ascorbate also serves as a cofactor for numerous enzymatic processes and is a modulator of plant development, senescence, cell division, and growth [6–9]. In plants, AsA is involved in electron transport and is considered to be an electron donor during photosynthesis [6]. It has been reported that AsA is found in all plant tissues but is higher in photosynthetic tissues and young leaves [4,10]. No plant completely devoid of AsA has ever been reported, indicating the key function of this molecule for plant survival and health. As illustrated in Fig. 1, the biosynthesis of AsA in plants is achieved through a complex metabolic network. The known AsA biosynthetic pathways include as entry points: D-mannose, D-galacturonate, L-gulose, and *myo*-inositol (reviewed in Refs. [8,11]).

Arabidopsis thaliana (*Arabidopsis*, mouse ear cress) is the model of choice for most plant molecular biology studies and for

Abbreviations: AsA, ascorbate; ROS, reactive oxygen species.

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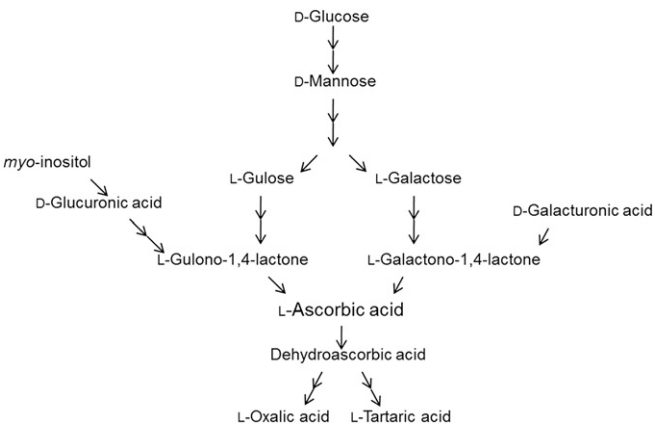

Fig. 1. Major intermediates of the four biosynthetic pathways leading to vitamin C (L-ascorbic acid, AsA) formation in plants. Left to right: *myo*-inositol, L-gulose, D-mannose/L-galactose and D-galacturonic acid routes. Multiple arrows indicate reactions catalyzed by several enzymes that for simplicity were omitted in this diagram. Adapted from Ref. [8].

which the most is known about AsA metabolism. In *Arabidopsis*, the size of the ascorbate pool is regulated by light intensity [12,13], and the foliar AsA content changes diurnally, reaching a maximum at the end of the light period [5,10,12]. In this species, foliar AsA content has been reported to decline as the plant ages [14,15]. There is evidence indicating the operation of multiple AsA biosynthetic pathways in *Arabidopsis* [16]. Over-expression of genes encoding various enzymes in the vitamin C metabolic network has led to the development of *Arabidopsis* lines with enhanced AsA content (reviewed in Ref. [11]). Characterization of these high AsA *Arabidopsis* lines has shown that they are tolerant to multiple abiotic stresses including salt, cold, heat, and oxidative stress [17–20].

Although the information we have gained by studying AsA metabolism in model species is valuable, further investigations are needed to understand the biosynthesis and regulation of AsA in crops so that its potential for controlling ROS may be fully exploited to mitigate stress and improve food production. Rice (*Oryza sativa*) is considered one of the most important crops worldwide, having an enormous impact as the staple food for more than two billion people [21,22]. Various approaches including feeding studies with AsA precursors in rice have shed light on the potential for elevating AsA content as a strategy to develop cultivars with enhanced tolerance to key environmental stresses [23–25]. In support of this idea, a recent study demonstrated that a decrease by 20–30% in leaf AsA in the rice var. Nipponbare had negative effects on agronomic traits at ozone levels exceeding the current ambient concentrations [26], confirming that leaf AsA level is a trait involved in stress resistance in rice.

In order to fully harness the potential of breeding or biotechnological approaches to develop rice cultivars with improved stress tolerance, it is imperative to learn more about how steady state AsA levels are regulated in this crop. Among the basic knowledge we lack is how AsA levels vary with plant age and leaf senescence, and how light intensity and photoperiod modulate these parameters. We also need to gain insight into the variation of AsA content among rice genotypes. In this work we used multiple rice accessions to describe how foliar AsA content changes as the plant develops and as leaves age. In addition, we also studied how light intensity and photoperiod affect AsA content in varieties that represent a subsample of the diversity in the USDA mini-core collection of rice [21].

2. Results and discussion

In order to establish the steady state foliar AsA levels in rice, seven accessions were selected based on differences in properties such as tillering, origin, and intrinsic tolerance to certain stresses [27]. Table 1 presents a summary of the time (days after planting) required for each of the accessions to reach various developmental stages. Genotypes LaGrue and Nipponbare were delayed compared to others in reaching the vegetative stage 4 (V4), while STG-S and Taipei-309 took the longest time to reach the reproductive stage 8 (R8). Whole tissue was collected from each cultivar during the seed to S3 developmental stages [28] and from the newest fully expanded leaf from the V4 to R8 developmental stages [28] at the beginning of the light period (between 8 and 10 a.m.) and reduced, oxidized, and total AsA content determined (Fig. 2). Contrary to the steady decline in foliar AsA content that has been reported in *Arabidopsis* [14,15], in rice, foliar AsA peaked at vegetative stages 2 (V2) or 4 (V4), depending on the variety, and at the reproductive stage 4 (R4). It is likely that the peak in AsA content we measured at the early vegetative stages is due to the exposure of these plantlets to light, a factor that is known to control the AsA pool in rice [5]. At the R4 developmental stage, the plant has fully formed a panicle and has reached anthesis. Reproductive tissues are known to have a higher metabolic rate of cell expansion and cell division and it is plausible that the peak observed at R4 is due to higher demand for AsA in its role in both cell expansion and cell division [10,29]. Interestingly, the two weedy red rice accessions (STG-S and MS4) had the highest AsA content at the R4 stage. These two accessions along with other weedy red rice germplasm have been shown to have greater plant growth in response to elevated CO_2 levels than indica and japonica cultivars [30].

Plant vitamin C content is regulated by environmental factors and in particular by light intensity and photoperiod (reviewed in Ref. [31]). With this in mind we studied whether light intensity would modify the pattern we found in Fig. 2. Two accessions were selected for this purpose, one with relatively low AsA content (Nipponbare) and one with relatively high AsA content (PI312777). The accessions were grown at 150, 400 and 1200–1500 μmol m^{-2} s^{-1} light intensities until the V8 stage. As illustrated in Fig. 3A light intensity had an effect on the growth rate (plants in the picture are the same age). Fig. 3B shows that light intensity had a significant effect on the size of the AsA pool in the Nipponbare and PI 312777 rice accessions; however the pattern of accumulation did not significantly differ from plants that were grown at 150 μmol m^{-2} s^{-1} (Fig. 2). Ascorbate data for Nipponbare plants growing under high light is missing at the V8 stage (Fig. 3B) because the plants entered the reproductive stage sooner than expected. Our results for the Nipponbare accession are consistent with previous findings by Fukunaga et al. [5] who reported AsA content to be ~3-fold higher when 2-week-old plantlets were grown at 236 μmol m^{-2} s^{-1} as compared to the levels measured under what they considered normal light conditions

Table 1
Age (days after planting) of plants sampled for ascorbate ontogenetic analysis shown in Fig. 2.

Variety	Seed	1-day imbibed	S3	V2	V4	V8	R4	R8
PI 312777	0	1	8	14	30	65	134	148
LaGrue	0	1	8	14	47	65	134	148
Nipponbare	0	1	8	14	47	65	106	134
MS4	0	1	8	14	30	65	134	148
STG-S	0	1	8	14	30	65	134	156
Taipei-309	0	1	8	14	30	65	134	156
Zhe-733	0	1	8	14	30	65	134	148

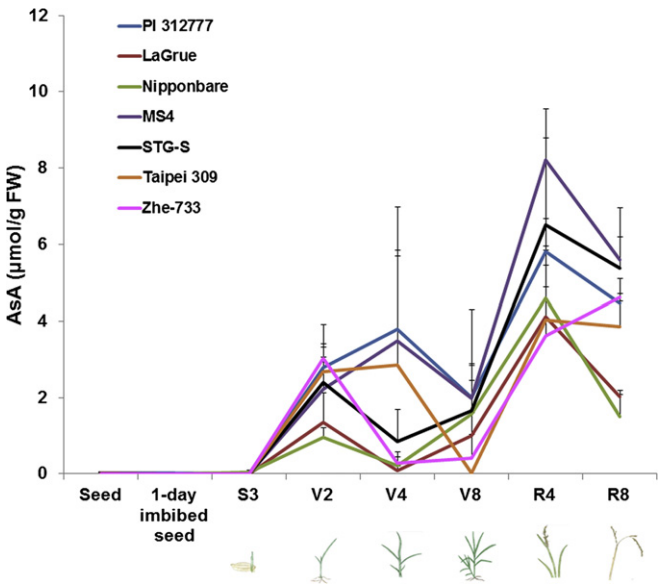

Fig. 2. Total ascorbate content of selected rice accessions throughout development. Plants were grown at 30 °C day/20 °C night temperature, 70% relative humidity, 12:12 h photoperiod, and 150 µmol m^{-2} s^{-1} light intensity. Values reported at developmental stages V2 to R4 are the AsA content of the youngest fully expanded leaf, while from seed to S3 are whole tissue AsA content. Reduced AsA was ~97% of the total for all genotypes. Data represent the mean ± standard deviation ($n = 3$). Illustrations of developmental stages were drawn according to Ref. [28].

(52 µmol m^{-2} s^{-1}). Our AsA measurements for Nipponbare plants grown under high light (greenhouse conditions) are in agreement with those reported by Frei et al. for plants growing under similar conditions [26].

For a more detailed analysis of the effect of photoperiod on foliar AsA content, leaf tissue of the accession PI 312777 was sampled at the V2 stage every 4 hours during a 24 h period. As illustrated in Fig. 4 AsA content was relatively stable throughout the light and dark cycle and did not steadily decline during the dark period as has been reported in other species, including *Arabidopsis* and tomato (*Solanum lycopersicum*) [12,31].

To gain a better understanding of the variation of AsA among leaves of adult plants, four accessions were grown to the reproductive

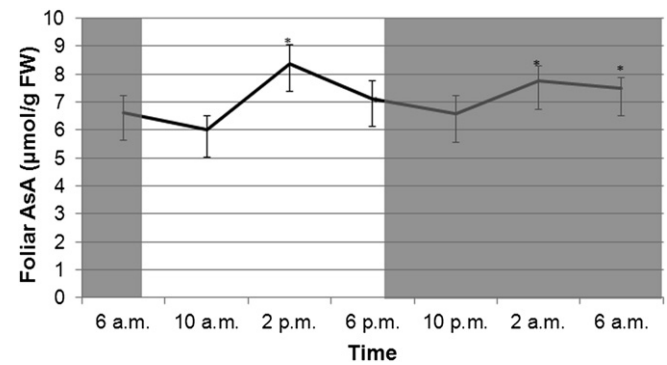

Fig. 4. Foliar ascorbate content in rice accession PI 312777 at the V2 vegetative developmental stage. Reduced AsA was above 85% for all time points. Values represent the mean AsA content ± standard deviation ($n = 3$). The grey area represents the dark hours of the photoperiod. FW = fresh weight. *Indicates significant differences between data collected at 10 am compared to data collected at other times (*t*-test, $P = 0.05$).

stage (R4), and all non-senesced leaves were sampled. Fig. 5 illustrates the average foliar AsA content for four rice accessions at the R4 developmental stage and the architectural differences among cultivars, including leaf number and position, and number of tillers. Except for Nipponbare, the average AsA content was higher in the younger leaves than the lower, older leaves. This pattern has been observed in *Arabidopsis*, tomato, and multiple species of *Nicotiana* and *Lupinus* sp. [15,32–35]. For those leaves that were large enough, we analyzed the AsA content in the proximal and distal parts of each blade. Interestingly, we were able to detect a gradient of AsA within each leaf, higher in the distal region, a pattern opposite of what we have measured in tomato and tobacco (data not shown).

To explore the genetic variability for AsA content, we expanded our study to twenty-three globally diverse rice accessions [21]. Fig. 6 shows the foliar AsA content of these accessions at the V2 stage. As illustrated there is a wide distribution of AsA contents in each of the indica and japonica subgroups.

Our results have implications for the possible development of varieties with enhanced AsA content that, based on previous studies conducted under greenhouse conditions, have the potential to lead to cultivars that are tolerant to various abiotic stresses [22,25,36–39].

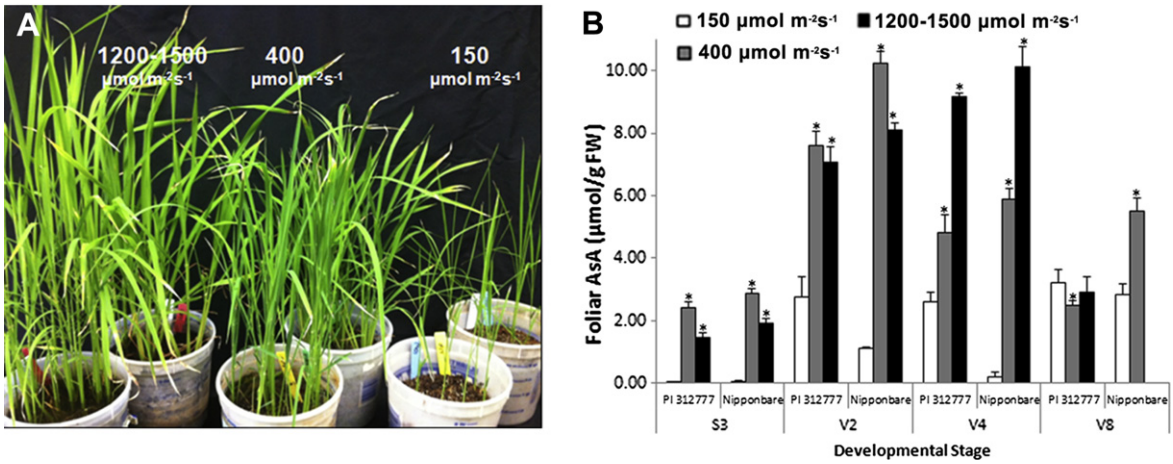

Fig. 3. Effect of light intensity in the phenotype and ascorbate content of rice accessions Nipponbare and PI 312777. (A) Phenotype of plants of the same age growing under 150, 400, and 1200–1500 µmol m^{-2} s^{-1} light intensities (Nipponbare back row, PI 312777 front row). (B) Foliar ascorbate content of Nipponbare and PI 312777 plants growing under various light intensities. Reduced AsA in samples at the S3 stage was 30%, and in all other samples was above 80% of the total. Data represent the mean ± standard deviation ($n = 6$). Ascorbate data for plants growing under high light is missing at the V8 stage because plants entered the reproductive stage sooner than expected. *Indicates significant differences between data collected for plants at the lowest light intensity compared to data collected at higher intensities (*t*-test, $P = 0.05$).

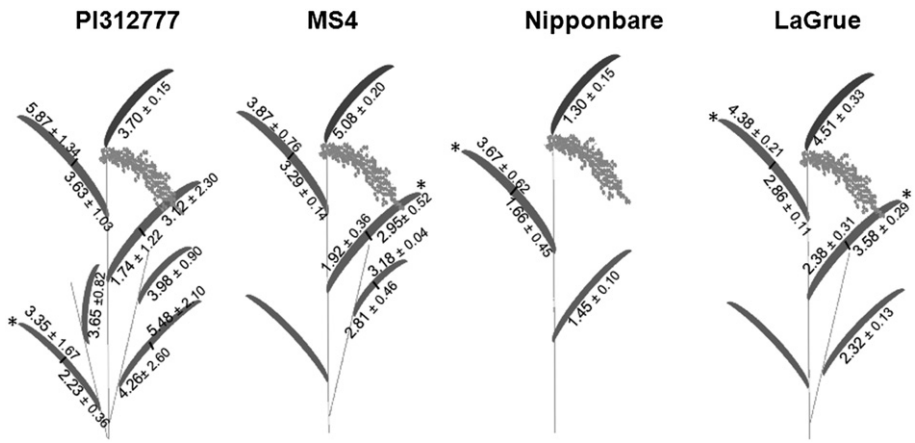

Fig. 5. Total ascorbate content in non-senescent leaves of selected rice accessions at the R4 developmental stage. Reduced AsA was above 90% of the total for all genotypes. For leaves that were large enough, we measured AsA content at both the distal and proximal regions of the blade. Values represent the mean ± standard deviation ($n = 3$), and are reported as μmol g^{-1} fresh weight. *Indicates significant differences between data collected for proximal and distal part of the leaves (*t*-test, $P = 0.05$).

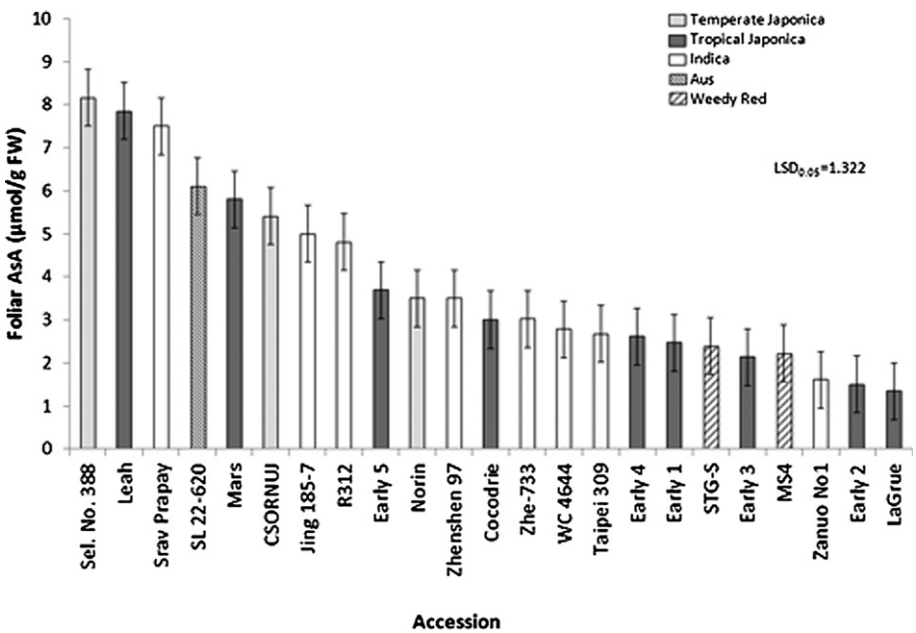

Fig. 6. Rice accessions ranked according to total foliar ascorbate content of the newest fully expanded leaf at developmental stage V2. Reduced AsA was above 90% of the total for all genotypes ($n = 3$). Data represent the mean ± Fisher least significant difference ($LSD_{0.05}$).

3. Conclusions

Previous research has demonstrated that AsA is a non-enzymatic antioxidant that plays a key role in plant growth and response to physiological stress. In this study we established the baseline AsA content in diverse rice germplasm accessions at multiple developmental stages. Foliar AsA content varied with the age of the leaf and site of sampling on the leaf blade, but was not as sensitive to the diurnal cycle as other species including *Arabidopsis* and tomato. In contrast to what has been observed in *Arabidopsis*, AsA leaf content fluctuated during the development of the rice plant with peaks at early vegetative stages (V2–V4) and at anthesis (R4). Light intensity did not seem to modify the pattern of AsA accumulation observed during vegetative growth. Significant genetic variability was observed in leaf AsA levels among germplasm accessions from both the indica and japonica subgroups of rice. These results indicate that there is sufficient genetic variability to enable altering AsA vegetative content through breeding if this trait can be linked with specific stress or developmental responses in rice. Further studies are currently in progress to elucidate the operational pathways of the AsA network during development of the rice plant. This information will lead us to a better understanding of when this molecule is most vital for the plant's growth and development and response to stress.

4. Methods

4.1. Genetic materials

Initial studies were conducted using six rice accessions including: LaGrue, a tropical japonica from the USA; Nipponbare, a temperate japonica from Japan; Taipei-309, an indica from Taiwan; PI312777, an indica from the Philippines, and two weedy red rice accessions collected from fields in Arkansas (STG-S) and Mississippi

(MS4). A wider diversity of world germplasm was later evaluated using eighteen accessions that are a subset of the USDA mini-core collection [21], and include genetic resources from the indica, aus, tropical japonica, and temperate japonica *O. sativa* sub-populations.

4.2. Plant growth conditions

Rice plants were grown in 6.35 cm square plastic pots (seedling to V4 stage) or 1.4 L cylindrical plastic containers (V4 to R8 stage), depending on the developmental stage used for collection. Soil used in the 6.35 cm square pots was a Sun Gro Seed Germination Mix (Sun Gro Horticulture Bellevue, WA). These plants received no fertilizer and soil was kept moist but not flooded. Soil in the 1.4 L containers was a 1:1 combination of Sun Gro Seed Germination Mix mixed with topsoil. The following supplements were added to the soil mixture: 1.8 g of High Yield dusting sulphur (Voluntary Purchasing Group, Bonham, TX), 7.5 g Scotts Micro-max fertilizer (Scotts, Maryville, OH), and 8.4 g of Ironite (Ironite Product Company, Scottsdale, AZ). Soil was fertilized at the V6 stage with Peter's Peat Lite Special (Peters, Maryville, OH). Marathon systemic insecticide (Ornamental Horticultural Products, Mainland, PA) was applied just before flooding. Plants grown in these pots were flooded at the V4 developmental stage. All plants were grown in environmentally controlled chambers (Conviron, Pembina, ND) under the following conditions: 30 °C day/20 °C night temperature, 70% relative humidity, 12:12 h photoperiod, and 150 μmol m^{-2} s^{-1} light intensity.

Plants exposed to medium light conditions were grown in an environmentally controlled chamber (Adaptis, Conviron) under the above conditions except that light intensity was set to 400 μmol m^{-2} s^{-1}. Plants exposed to high light were grown in a glass greenhouse between March and May 2012 in Jonesboro, AR, U.S.A. The greenhouse uses an Envirostep (Wadsworth Control Systems, Inc) computer system to control the temperature and humidity levels. The light intensity was measured daily between 1200 and 1500 μmol m^{-2} s^{-1}. Temperature was maintained between 20 and 30 °C and relative humidity between 60 and 70% day and night. No supplemental lamps were used.

4.3. Sampling for AsA measurements

Samples from the seed to the S3 developmental stage consisted of 50 mg of dehulled rice seeds per replicate, and the values reported represent three biological replicates. For measurements at all subsequent developmental stages, rice accessions were grown under the above conditions at the same time in a growth chamber. For accessions sampled between the S3 and V2 stages, three plants were grown per pot. Plants sampled between the V4 and R8 stages were grown individually in 1.4 L cylindrical containers as described in Section 4.2. A minimum of 50 mg of tissue was collected for AsA measurements per sample. At the S3 and V2 stage, tissue from 6 to 8 individuals was pooled from plants growing in multiple pots to obtain this mass, so the AsA values reported represent the average of 18–24 individuals sampled from across the three replicates. The values reported for accessions at the V4, V8, R4, and R8 stages represent the average of at least three individual plants sampled at a similar developmental stage per replicate. At the R4 stage, samples from the distal and proximal areas of the blade were taken from leaves of plants at the same developmental stage.

4.4. Ascorbate measurements

Total, reduced, and oxidized ascorbate contents were determined by the ascorbate oxidase assay adapted to a 96-well plate format and setup essentially as described [40]. Tissue samples were collected during the first hours of the light period, snap frozen in liquid nitrogen, and kept at −80 °C until measured. Frozen tissues were ground in fresh 6% (w/v) *meta*-phosphoric acid at a ratio of 50 mg tissue per 750 μL buffer and centrifuged at 15,000× g for 5 min. Reduced AsA was determined by measuring the decline in A_{265} after addition of 0.5 U of ascorbate oxidase (Sigma, St. Louis, MO) to 300 μL of the reaction mix including tissue extract and 100 mM potassium phosphate pH 6.9. Oxidized AsA was determined in a 300 μL reaction mixture which included 40 μM dithiothreitol and incubated at room temperature in the dark for 30 min. Total AsA was the sum of reduced and oxidized AsA and is reported as micromoles per gram of fresh weight (μmol g^{-1} FW). Calculations were based on a standard curve with pure L-ascorbic acid run in parallel. A minimum of three biological replicates were analyzed in triplicate per sample.

4.5. Statistics

Statistical analyses were carried out with Minitab 15 (Minitab, State College, PA) using the Student's *t*-test at 95% confidence limit, and with SAS (SAS Institute, Cary, NC) to calculate the Fisher least significant difference ($LSD_{0.05}$).

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