LÍLIAN MARIA VINCIS PEREIRA SANGLARD

THE NEGATIVE IMPACTS OF ARSENIC ON PHOTOSYNTHESIS OF RICE LEAVES ARE ALLEVIATED BY SILICON SUPPLEMENTATION, WITHOUT UP-REGULATION OF THE ANTIOXIDANT CAPACITY

Dissertation submitted to Federal University of Viçosa, as part of the requirements for obtaining of *Magister Scientiae* degree in Plant Physiology.

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ATTESTED IN: February 26 th, 2014.

Wagner Luiz Araújo

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(Adviser)

To my mother Dayse and my father Ferdinand and my sister Letícia,
for all their love, sacrifice and constant support, always present in my heart

With all my love to my grandfather Magela and my grandmother Ada

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BIOGRAPHY

LÍLIAN MARIA VINCIS PEREIRA SANGLARD was born in Itaperuna-RJ, Brazil, on May 29th, 1990. In 2012, she graduated in Biology at Federal University of Viçosa, Viçosa-MG, Brazil. In March 2012, she began her *Master Scientiae* course in the Plant Physiology Program at the Federal University of Viçosa, Viçosa-MG, Brazil.

CONTENT

RESUMO

SANGLARD, Lílian Maria Vincis Pereira, M.Sc., Universidade Federal de Viçosa, fevereiro de 2014. **Impactos negativos do arsênico na fotossíntese em folhas de arroz são mitigados pela suplementação de silício, sem aumento da capacidade antioxidante**. Orientador: Fábio Murilo DaMatta.

O silício (Si) desempenha um papel importante em mitigar vários estresses abióticos, possivelmente por minimizar o dano oxidativo induzido por estresse. Em arroz (*Oryza sativa*), acredita-se que o arsênio (As) possa causar estresse oxidativo compartilhando com o Si a mesma via de entrada nas raízes. Apesar dos grandes avanços na compreensão dos mecanismos de absorção de As e como estes podem ser afetados por Si, os mecanismos fisiológicos pelos quais o Si pode mitigar a toxicidade do As em plantas ainda precisam ser esclarecidos. Neste trabalho, avaliaram-se os efeitos isolados e combinados de Si e arsenito [As(III)] em plantas de arroz, usando um genótipo selvagem e o mutante *lsi*1 (*low silicon rice* 1) defectivo para a absorção de Si, no que diz respeito às trocas gasosas e parâmetros de fluorescência da clorofila *a* e aos danos celulares. A presença de As(III) levou a uma diminuição da fixação de carbono, fato não relacionado com limitações estomáticas e fotoquímicas, mas associada à diminuição da condutância mesofílica. Esta redução pôde ser revertida ao longo do tempo de forma considerável pela presença do Si, nos dois genótipos. Entretanto, os efeitos benéficos do Si em plantas tratadas com As(III) não apresentaram relação direta com o aumento da regulação da capacidade antioxidante. A fertilização com Si pode ser importante tanto na tentativa de diminuir as concentrações de As(III) quanto para melhorar o desempenho fotossintético de plantas de arroz contaminadas com As, o que pode resultar em uma melhor produtividade da cultura, além de promover aumento da segurança alimentar.

ABSTRACT

SANGLARD, Lílian Maria Vincis Pereira, M.Sc., Universidade Federal de Viçosa, February, 2014. **The negative impacts of arsenic on photosynthesis of rice leaves are alleviated by silicon supplementation, without up-regulation of the antioxidant capacity**. Adviser: Fábio Murilo DaMatta.

Silicon (Si) plays important roles in alleviating various abiotic stresses, possibly by counteracting stress-induced oxidative damage. In rice (*Oryza sativa*), arsenic (As) is believed to cause oxidative stress and to share the Si transport pathway for entry into roots. Despite much progress in understanding the mechanisms underlying the uptake of As and how they can be affected by Si, the physiological mechanisms through which Si might alleviate As toxicity in plants remain to be elucidated. We combined gas exchange and chlorophyll fluorescence measurements with analysis of the activity of the antioxidant system to examine the effects of Si nutrition on photosynthetic performance and oxidative stress in rice plants (a wild-type cultivar and its *lsi1* mutant defective in Si uptake) challenged with arsenite. Arsenite treatment led to an impairment of carbon fixation that was unrelated to stomatal and photochemical limitations but, rather, was associated with decreased mesophyll conductance. This impairment could be reverted to a considerable extent by Si in a time- and genotype-dependent manner. The ameliorative effects of Si on As-treated plants were uncoupled from any noticeable up-regulation of the antioxidant capacity. We identified Si nutrition as an important target in attempts to not only decrease As concentrations but also to improve the photosynthetic performance of rice plants challenged with As, which may ultimately result in better crop yield coupled with enhanced food safety.

1. Introduction

Pollution with transition metals and metalloids is an increasing environmental problem, and arsenic (As), in particular, is highly toxic for all forms of life, including plants [1]. Plants take up arsenate [As(V)], the predominant form of As in aerated soils; however, in reducing environments such as in paddy soils, arsenite [As(III)] becomes the predominant chemical species of As [2]. The main pathway of As(V) uptake in plants is through phosphate transporters, whereas As(III) is believed to be taken up through the nodulin 26-like intrinsic aquaporin channels [3]. The accumulation of As in plants negatively impacts their morphology and physiology by inhibiting root and shoot growth, generating reactive oxygen species (ROS), decreasing photosynthetic activity and altering carbohydrate and amino acid metabolism [4,5,6]. Plants exhibit a range of As detoxification mechanisms, including metal transport, chelation and sequestration [1,7]. In addition, activation of enzymatic antioxidants, including superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR) and glutathione peroxidase (GPX), as well as antioxidant molecules, such as ascorbate (AsA) and glutathione (GSH), has been reported to neutralise As-mediated oxidative stress [8]. Failure of the antioxidant defence system may result in damage when metabolites and components of the cellular machinery react with ROS, resulting in lipid peroxidation and oxidation of proteins and nucleic acids [9], thus ultimately impairing photosynthesis and plant growth [10].

Silicon (Si) is not considered an essential element for higher plants, although it has been proven to be beneficial for improving the growth and crop yields of some plant species, such as rice [11]. Studies addressing Si nutrition have extensively demonstrated the ability of Si to alleviate biotic and abiotic stresses in a wide variety of plant species [12,13], including toxicity associated with elements such as aluminium [14], cadmium [15] and arsenic [16]. The ameliorative effect of Si on plants suffering from abiotic stresses is believed to occur, to a large extent, through counteracting oxidative stress via modulating antioxidant enzymes [17].

In rice roots, two genes encoding Si transporters (*Lsi1* and *Lsi2*) have been identified to date [18,19]. These Si transporters are associated with the great ability of rice to actively take up Si and could explain the high Si concentrations observed in this species [11]. Intriguingly, it has been observed that high levels of Lsi1 and Lsi2

expression in rice not only lead to high Si accumulation but also enhance As accumulation in rice shoots and grains [20], suggesting that As shares the Si transport pathway for entry into rice root cells. Indeed, increasing the Si concentration in a soil solution was observed to lead to decreased As accumulation in rice shoots and grains [21,22,23], most likely through a competitive inhibition effect on As(III) transport via Lsi1 and Lsi2 [7].

Despite much progress in understanding the mechanisms underlying the uptake and distribution of As and how they can be affected by Si [7,20], the physiological mechanisms by which Si might alleviate As toxicity in plants received no attention until the recent study of Tripathi *et al.* [16]. These authors showed that Si could mediate As(III) tolerance in rice through lowering of As uptake and improved antioxidant defence system. However, how these responses may impact key physiological processes such as photosynthesis remain to be fully elucidated. Here, we hypothesised that application of Si could mitigate the toxic effects of As through improvements of the photosynthetic performance coupled with up-regulation of the antioxidant system, and this effect is expected to be further facilitated via the competitive inhibition effect of Si on As(III) uptake, as observed in rice [7,20]. To test this hypothesis, we combined detailed gas exchange and chlorophyll *a* fluorescence measurements with analysis of the activity of the antioxidant system to examine the effects of Si nutrition on photosynthetic performance and oxidative stress in rice plants challenged with As. This analysis was realised by comparing wild-type (WT) rice (cv. 'Oochikara') and its *lsi1* mutant defective in Si uptake. Our results suggest that the ameliorative effects of Si on plants challenged with As were linked to the preservation of photosynthetic activity in a time- and genotype-dependent manner, although, unexpectedly, this response was unrelated to up-regulation of the antioxidant capacity.

2. Materials and Methods

2.1. Plant material, growth conditions and experimental design

The experiment was conducted in Viçosa (20º45'S, 42º54'W, 650 m altitude) in south-eastern Brazil from January through March 2011. Rice (*Oryza sativa* L.) plants from cv. 'Oochikara' and its low-silicon 1 (*lsi1*) mutant (Ma *et al.*, 2006) were grown in

a screen house in plastic pots with 5 L of a nutrient solution containing 0 or 2 mM Si under naturally fluctuating environmental conditions. Si was supplied as monosilicic acid, which was prepared by passing potassium silicate through cation-exchange resin (Amberlite IR-120B, H^+ form; Sigma-Aldrich, São Paulo, Brazil). Further details of the applied methodology (e.g., seed germination, composition of the nutrient solutions, pH control, etc.) have been given elsewhere [24]. The maximum photosynthetically active radiation (PAR) inside the screen house was *c.* 1,500 μmol photons m^{-2} s^{-1}. Forty-five days after transplanting, As was applied in the form of $NaAsO_2$ at four concentrations (0, 15, 25 or 50 μM). The experiment had a completely randomised design, with six plants in individual pots (96 in total) per treatment combination serving as conditional replicates. Sampling and measurements were performed at 5, 9 and 13 days after the As additions (DAA). The pots were randomised periodically to minimise any variation among treatments.

2.2. Si and As concentrations

The youngest fully expanded leaves and the bulk root system were collected, and the Si concentrations in these tissues were determined colourimetrically according to Dallagnol *et al.* [24]. For As quantification, plant tissues were oven dried under hot air at 40ºC until constant weight. The tissues were then digested using a mixture of HNO_3 and H_2O_2 (3:1 by vol.), after which As was quantitatively analysed using an inductively coupled plasma emission spectrometer (Optima3300 DV, Perkin Elmer, Maryland, USA). Further details have been described elsewhere [25].

2.3. Photosynthetic measurements

The gas exchange parameters determined simultaneously via conducting measurements of chlorophyll (Chl) *a* fluorescence, were measured in youngest fully expanded leaves using two cross-calibrated infrared gas analysers (LI-6400XT, LI-COR, Lincoln, NE, USA) equipped with integrated fluorescence chamber heads (LI-6400-40, LI-COR Inc.). The net CO_2 assimilation rate (A), stomatal conductance to water vapour (g_s) and internal CO_2 concentration (C_i) were measured on attached leaves (flag leaf) from 10:00 to 13:00 hours, which is when A was at its maximum, under

artificial PAR, *i.e.*, 1,000 µmol photons m^{-2} s^{-1} at the leaf level and 400 µmol CO_2 mol^{-1} air. All measurements were performed at 25°C, and the vapour pressure deficit was maintained at *c.* 1.0 kPa, while the amount of blue light was set to 10% of PAR to maximise the stomatal aperture.

After registering the gas exchange parameters, the steady-state fluorescence yield (F_s) was measured, following which a saturating white light pulse (8,000 µmol m^{-2} s^{-1}; 0.8 s) was applied to achieve the light-adapted maximum fluorescence (F_m'). The actinic light was then turned off, and far-red illumination was applied (2 µmol m^{-2} s^{-1}) to measure the light-adapted initial fluorescence (F_0'). Using the values of these parameters, the photochemical quenching coefficient (q_P) and the capture efficiency of excitation energy by open photosystem (PS) II reaction centres (F_v'/F_m') were estimated [26]. The actual PSII photochemical efficiency (ϕ_{PSII}) was determined following the procedures of Genty *et al.* [27]. The electron transport rate (J) was then calculated from the equation $J = \phi_{PSII}\ \beta\ \alpha\ PPFD$, where α is leaf absorptance, and β reflects the partitioning of absorbed quanta between PS II and I. The product β α was determined according to Valentini *et al.* [28], from the relationship between ϕ_{PSII} and ϕ_{CO2} obtained by varying the light intensity under non-photorespiratory conditions. The conductance of CO_2 from intercellular airspaces to the sites of CO_2 fixation in the stroma of chloroplasts, termed mesophyll conductance (g_m), was estimated according to Harley *et al.* [29], as follows:

$$g_m = A/(C_i - (\Gamma^*(J + 8(A + R_l))/(J - 4(A + R_l))))$$

where A, C_i and J were obtained from gas exchange and Chl fluorescence measurements conducted under saturating light; R_l is the rate of mitochondrial respiration in light, not related to photorespiration; and Γ* is the chloroplastic CO_2 photocompensation point in the absence of mitochondrial respiration. Further details on the R_l and Γ* estimations have been described elsewhere [30].

Because all of the available methods for estimating g_m rely on models that include a number of assumptions as well as technical limitations that need to be considered to obtain reliable estimates of this parameter [31], g_m was also estimated in an additional experiment using an alternative approach, the A–C_i curve analysis method suggested by Ethier and Livingston [32]. The plants were treated with As (0 or 25 µM) for 5 days, after which photosynthetic measurements were performed. Importantly, the

overall trends of the photosynthetic parameters among treatments, including that of g_m estimated using the two methods quoted above, were essentially similar to the trends reported in this experiment (data not shown).

Additionally, previously dark-adapted (30 min) leaf tissues were illuminated with weak modulated measuring beams (0.03 μmol m^{-2} s^{-1}) to obtain the initial fluorescence (F_0). Saturating white light pulses of 8000 μmol m^{-2} s^{-1} were applied for 0.8 s to ensure for maximum fluorescence emissions (F_m), from which the variable-to-maximum Chl fluorescence ratios, $F_v/F_m = [(F_m - F_0)/F_m)]$, were calculated.

2.4. Biochemical assays

The youngest fully expanded leaves were collected between 12:00 h and 14:00 hours, immediately frozen in liquid nitrogen, and then stored at -80°C until further analysis.

Key antioxidant enzymes, namely SOD (EC 1.15.1.1), APX (EC 1.11.1.11), CAT (EC 1.11.1.6) and GR (EC 1.6.4.2), were extracted by grinding the samples with a cold mortar and pestle with appropriate extraction buffers, as described in Pinheiro *et al.* [33].Total SOD activity was determined by measuring the ability to inhibit the photochemical reduction of *p*-nitro-blue-tetrazolium chloride at 560 nm. The activity of CAT was estimated by measuring the rate of decomposition of H_2O_2 at 240 nm, while total APX activity was determined by monitoring the decline in absorbance at 290 nm, and GR activity was assessed by measuring the rate of NADPH oxidation at 340 nm. Further details have been reported previously [33].

For determination of ascorbate and glutathione pools, flag leaf tissues (*c.* 0.05 g) were ground to a fine powder in liquid N_2, after which 0.1 mL of ice-cold 0.1 mM HCl and 0.1 mM EDTA were added. The AsA and dehydroascorbate (DHA) contents were determined according to the procedure described by Kampfenkel *et al.* [34], whereas GSH and GSSG were assayed following Griffith [35]. The redox states of these compounds were estimated as AsA/(AsA+DHA) and GSH/(GSSG+GSH).

The total antioxidant activity (TAA) was estimated via the ferric reducing antioxidant power assay of Benzie and Strain [36]. In this assay, antioxidants are used as reductants in a redox-linked colourimetric technique, employing an easily reduced oxidant system present in stoichiometric excess.

Cellular damage was analysed based on malondialdehyde (MDA) accumulation, estimated as the content of total 2-thiobarbituric acid-reactive substances, as detailed in Lima *et al.* [37].

2.5. Statistical analysis

The data obtained from the experiment were analysed using a completely randomised design following a 2x2x2 factorial scheme (two genotypes x two Si levels x two As levels) with six replicates (see further details in the 'Results' section). The data were subjected to an analysis of variance (three-way ANOVA with all main factors evaluated as fixed factors) performed using the general linear models (GLM) procedure of SAS (version 9.1.), adopting $\alpha = 0.05$. When any interaction was found to be significant, the GLM Slice statement was used to interpret the dependency effect between factors.

3. Results

Initially, we tested the effects of two levels of Si combined with four levels of As (0, 15, 25 or 50 µM) on photosynthetic performance and oxidative stress using two rice genotypes. The analyses were repeated at 5, 9 or 13 DAA. Regardless of their genotype, most plants grown under 50 µM As did not survive, and we therefore have no further data for this treatment. The majority of the results obtained under 15 µM As differed only slightly from those obtained under 25 µM As; similarly, most results obtained at 9 DAA differed minimally when compared with those obtained at 13 DAA. Therefore, we omitted the data obtained under 15 µM As as well as those gathered at 9 DAA for the sake of simplicity.

3.1. Si concentrations are increased, whereas As concentrations are decreased upon Si supplementation

Both Si and As concentrations were higher at 13 than at 5 DAA in both roots and leaves (Fig. 1 a-h). As would be expected, Si addition led to increases in the leaf Si concentration (Fig. 1a-b). Among +Si plants, the Si concentrations were significantly

higher in WT (17.5 g kg^{-1} DW on average; Fig. 1a) than in *lsi1* mutant plants (9.6 g kg^{-1} DW on average; Fig. 1b). The Si concentrations were higher (19% on average) in +As plants compared with –As individuals (significant Si x As interaction; Table 1), and this pattern was genotype dependent (significant Si x As x genotype interaction; Table 1). Similar results were found in roots (Fig. 1c-d), although the absolute values of the Si contents were lower in root than in leaf tissues, particularly in +Si individuals (Fig. 1).

We were unable to detect As in plants that were not treated with this metalloid. Regardless of the sampling date and genotype, As concentrations decreased significantly (48% in leaves and 12% in roots, on average) in +Si plants when compared with their –Si counterparts (Fig. 1e-h, Table 1).Within a given Si treatment, the As concentrations were lower in the mutant than in the WT plants (30% on average), with a significant Si x As x genotype interaction being detected. In sharp contrast to the situation observed for Si, As concentrations were remarkably higher in roots (200% on average) than in leaves (Fig. 1).

3.2. The negative effects of As on photosynthetic rates may be reversed by Si in a genotype- and time-dependent manner

Overall, As treatment led to decreases in *A*, particularly in –Si plants (significant Si x As interaction) (Table 1, Fig. 2a-b). These decreases were more pronounced at 13 than at 5 DAA, especially in WT plants, which displayed negative *A* values (-2.6 µmol CO_2 m^{-2} s^{-1}), in contrast to the positive values (3.2 µmol CO_2 m^{-2} s^{-1}) detected in *lsi1* plants (Fig. 2a-b). Changes in *A* were generally followed by changes in g_s (Fig. 2c-d). However, it is very unlikely that the impairment of *A* was caused by stomatal limitations because the parallel decreases observed in both *A* and g_s were often accompanied by significant increases in C_i (Fig. 2e-f). In contrast, the changes in *A* were mirrored by those in g_m (Fig. 2g-h), and we therefore identified diffusive limitations of the mesophyll as playing a central role in constraining *A* in As-treated plants. Most importantly, the negative effects of As on *A* were partially reversed by the addition of Si in WT plants, as noted at 13 DAA; such effects were only slight (5 DAA) or even nonexistent (13 DAA) in *lsi1* plants (significant As x genotype interaction) (Table 1, Fig. 2a-b).

3.3. Photochemical events are minimally affected by Si and As levels

The F_v/F_m ratio, which expresses the maximum PSII photochemical efficiency, decreased slightly upon As addition (Fig. 3 a-b). This effect was mostly observed in –Si plants, independent of the genotype. The q_P (Fig. 3c-d), F_v'/F_m' (Fig. 3 e-f) and J (Fig. 3 g-h) parameters were affected by both Si and As, with significant As x Si and genotype x As x Si interactions being detected, as observed at 13 DAA (Table 1). However, these traits varied minimally across the treatments, and photochemical factors are therefore unlikely to have prominent impacts on the differences observed in *A*.

3.4. The antioxidant capacity increases upon As addition, particularly in plants not treated with Si

We analysed four key antioxidant enzymes: SOD, APX, GR and CAT, whose activities were significantly affected by Si (except APX at 5 DAA), As and plant genotype (except CAT) (Table 1). The activities of all of the enzymes followed similar trends across the treatments (Fig. 4 a-h). Overall, enzyme activities increased upon As addition, especially in –Si plants. The relative As-related increases in enzyme activities were genotype dependent (significant genotype x As interaction; Table 1): greater increases were recorded for SOD (Fig. 4a-b) in *lsi1* than in WT plants (on average, 29% and 12%, respectively), and greater increases in APX (Fig. 4c-d) (on average, 82% and 42%, respectively) and GR (Fig. 4e-f) (on average, 40% and 22%, respectively) were detected in WT than in *lsi1* plants, whereas the increases in CAT (Fig. 4g-h) were independent of the genotype.

The total pools of key antioxidant molecules (AsA and GSH) as well as the TAA were affected by Si, As and genotype on both sampling dates (Table 1, Fig. 5a-j). Overall, these traits increased significantly upon As addition, though to a greater extent in –Si plants than in their +Si relatives. Moreover, WT plants treated with As displayed larger pools of AsA (Fig. 5a-b) and GSH (Fig. 5c-d) (with minimal differences in their redox state; Fig. 5e-h) and a higher TAA (Fig. 5i-j) than the corresponding mutants (significant Si x As and genotype x As interactions; Table 1).

Regardless of the genotype and sampling date, the MDA concentration increased significantly upon As addition (Fig. 6). In As-treated plants, the highest MDA concentration was found in –Si WT individuals (87 nmol g^{-1} FW on average; Fig. 6a),

and the concentration was decreased significantly in the presence of Si (25% on average; Table 1), whereas in *lsi1* plants, Si had little impact on MDA concentrations (Fig. 6b), as noted by the similar (though significantly different) MDA pools detected in –Si and +Si plants. Notably, under As treatment, the MDA concentrations were virtually the same in +Si WT plants and *lsi1* plants, regardless of Si amendment (73±1 nmol g^{-1} FW on average at 13 DAA).

4. Discussion

To the best of our knowledge, this study is the first to demonstrate that Si can partially revert the negative impacts of As on photosynthetic performance in a time- and genotype-dependent manner. This effect was apparently uncoupled from any noticeable up-regulation of the antioxidant capacity of the rice plants, in sharp contrast with our working hypothesis.

As previously demonstrated [30], Si had no effect on *A* in rice plants during the vegetative growth stage under unstressed (–As) conditions. In contrast, we showed that As strongly impaired *A* in a time- and genotype-dependent manner: given that the concentration of As increased in plant tissues upon As addition, its toxic effects on *A* would be expected to increase accordingly over time. By decreasing the As concentrations in rice plants, Si was able to revert the toxic effects of As to a large extent, particularly in the *lsi1* mutant, which displayed the lowest As concentrations recorded. Indeed, in both WT and *lsi1* plants challenged with As, *A* tended to rise from 5 to 13 DAA in presence of Si, despite increases in As concentrations. Taken together, these data indicate that there was an acclimation of the biochemical reactions involved in CO_2 fixation to As stress, mediated by an as yet uncharacterised role of Si. We recently suggested that Si likely plays an unidentified role in rice metabolism, even under unstressful conditions [38]. It is therefore tempting to speculate that energy metabolism and signalling pathways are tightly interregulated at the whole-plant level in a manner that allows the plant to cope with stressful situations in the presence of adequate Si nutrition.

We showed that the effects of As on *A* were largely associated with diffusive limitations of the mesophyll. Indeed, the high *J* values observed (Fig. 3 g-h), even when the values of *A* were negative, not only suggest that the synthesis of both ATP and NADPH required to fuel carbon fixation reactions was largely uncompromised in As-

treated plants (in contrast to what has been proposed elsewhere – see [5]) but also strongly indicate that a large draw-down from C_i to C_c (CO_2 concentration inside the chloroplast) occurred as a result of decreased g_m [39]. Hoffmann and Schenk [4] recently suggested that symptoms of As stress in rice plants were associated with increasing As(III) binding on the outer side of the plasmalemma to proteins such as aquaporins; if so, the observed decreases in g_m might be linked to impaired aquaporin activity, as these proteins are an important component governing g_m [39,40]. In contrast, Si may lead to increases in g_m [30] coupled with increases in the activity of aquaporins [A.O. Lavinsky and F.M. DaMatta, *unpublished results*]. These assumptions might provide a mechanistic link to at least partially explain the ameliorative effects of Si on *A* via increases in g_m in rice plants challenged with As. In any case, in addition to the diffusive limitations of the mesophyll, biochemical factors may further constrain *A*, as suggested by proteomic studies conducted in As-treated rice plants, which have recorded down-regulation of the ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) large subunit and chloroplast 29 kDa ribonucleoproteins [41].

Considering that carbon fixation, which generally represents the main sink for absorbed light in chloroplasts, was found to be depressed, especially in –Si WT plants, adjustment of light capture, use and dissipation is required to provide photoprotection to the photosynthetic apparatus. Here, we showed that Chl *a* fluorescence parameters varied minimally, which suggests that light capture and use also differed minimally across the treatments. Indeed, because both F_v/F_m and F_v'/F_m' (a useful proxy for thermal dissipation [26]) were relatively unresponsive to the imposed stress, it may be suggested that modification/inactivation of the PSII reaction centre and photoprotection by xanthophylls engaged in sustained thermal energy dissipation are unlikely to have occurred in this current study. On the other hand, because the electron fluxes through PSII were more than several times greater than required for the observed *A*, particularly in –Si plants at 13 DAA (*cf.* Fig. 2a-b and Fig. 3 g-h), it may be proposed that alternative pathways for photosynthetic electron flow, such as photorespiration, cyclic flow of electrons within PSII or the Mehler-peroxidase reaction (the PS I-mediated photoreduction of O_2) [9,10], could have played a major role in dissipating the excess reducing power under elevated As. It has been suggested that an increase in the rate of O_2 photoreduction by the Mehler reaction as *A* is inhibited under stressful conditions may provide a mechanism for the photochemical dissipation of excess excitation energy within the photosynthetic apparatus [42,43]. Increases in the activities of SOD, APX

and GR, together with increased concentrations of antioxidants such as AsA and GSH, accompanied the observed increases in the ratio of *J*/*A*, suggesting an increased rate of O_2 photoreduction [43,44]. Additionally, the likely g_m-mediated reduction of C_c (which can favour Rubisco oxygenation over carboxylation), coupled with increased CAT activity, provides indirect evidence supporting a prominent role of photorespiration as a key mechanism for consuming excess energy [10,45] under conditions of As stress.

The consistent increases in the TAA due to both enzymatic (e.g., SOD, APX, GR and CAT) and non-enzymatic (e.g., ASA and GSH) antioxidant defences suggests that the plants challenged with As suffered from higher oxidative pressure, as has generally been noted in other studies under conditions of As stress [3,46]. The maintenance of two key redox pairs, AsA/(AsA+DHA) and GSH/(GSSG+GSH), under As stress is indicative that their redox potentials did not shift towards oxidising directions, which is fundamental for proper operation of the AsA-GSH cycle and, in turn, for avoiding the creation of an oxidised environment in plant tissues [47]. Nevertheless, in sharp contrast to our working hypothesis, the addition of Si did not result in up-regulation of the antioxidant system. This conclusion also contrasts with the findings of Tripathi *et al.* [16] who showed that Si could mediate As(III) tolerance in young rice seedlings via reinforcement of the antioxidant defence system. In any case, our results argue against the suggestion that Si-related enhancement of the antioxidant defence capacity is the universal mechanism for Si-enhanced tolerance to various forms of abiotic and biotic stress in plants [17,48]. The most likely explanation is that the relatively lower expression of the antioxidant system in +Si plants than in their –Si counterparts in the presence of As is simply a result of lower As concentrations in +Si individuals, which would translate into a decreased need to trigger antioxidant responses. Further support for this conclusion comes also from a recent study showing that up-regulation of the antioxidant system corresponded to greater As accumulation in rice plants [49].

Regardless of changes in the antioxidant capacity, symptoms of oxidative stress associated with ROS triggered by As could be noted based on increased lipid peroxidation as shown by higher MDA concentrations. Nevertheless, we noted that, with the exception of the –Si WT plants, the MDA pools were quite similar in the As-treated plants, despite the complete restoration of *A* in *lsi1* plants under As stress, amended with Si. Therefore, we believe that the ameliorative effects of Si on *A* observed in rice plants challenged with As were unrelated to oxidative stress. In any

case, we identified Si as an important target in attempts to not only decrease As concentrations but also to improve the photosynthetic performance of rice plants challenged with As, which may ultimately result in better crop yield coupled with enhanced food safety.

5. References

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Table 1. The results of ANOVA for the effects of silicon, arsenic and genotype and their interactions are shown for the tested traits.

Parameters	Si	As	Ge	Si x As	Si x Ge	As x Ge	Si x As x Ge
Leaf Si	***	***	***	***	***	***/**	***/**
Root Si	***	***	***	***	***	***	***/*
Leaf As	***	***	***	***	***	***	***
Root As	***	***	***	***	***	***	***
A	ns/***	***	***/ns	**/***	ns	***	ns
g_s	***/ns	***	**/*	***/**	ns	***/ns	**/ns
C_i	**/***	**	ns/**	**/***	**/ns	ns/**	ns
g_m	*/**	***	ns	ns/**	ns/*	**/***	ns
F_v/F_m	**/*	**/***	*/ns	***/ns	ns	ns	**/***
q_P	**	***	***	ns/***	***/**	***/ns	**/***
F_v'/F_m'	ns/**	**/*	*/ns	Ns	ns/*	ns	ns/**
J	**/*	***/**	ns	ns/***	ns	ns/**	ns/***
SOD	*/**	***	***	*	ns	ns/*	ns
APX	ns/***	***	**/***	ns/***	ns/***	**/***	ns/***
GR	***/**	***	***	**/ns	***/**	***/**	***/*
CAT	**/*	***	ns	**	ns	ns	ns
AsA	***	***	***	***	**/ns	***	**/ns
GSH	***	***	***	***	**/ns	***	**/ns
AsA/(AsA+DHA)	***	***	***	***	ns/ns	***	**/ns
GSH/(GSH+GSSH)	***	***	***	***	**/ns	***	**/ns
TAA	***	***	***	**/***	ns/**	***	ns/***
MDA	***	***	***	***/**	***	ns/***	***

Significance: ^{ns} not significant, * $P< 0.05$, **$P< 0.01$, ***$P< 0.001$. The levels of significance are shown for traits tested at 5 or 13 days after arsenic addition; when a single level of significance is shown for a given trait, the same significance was found for both days.

Abbreviations: silicon (Si), arsenic (As), genotype (Ge), net CO_2 assimilation rate (A), stomatal conductance (g_s), internal CO_2 concentration (C_i), mesophyll conductance (g_m), variable-to-maximum chlorophyll fluorescence ratio (F_v/F_m), photochemical quenching coefficient (q_P), efficiency of the capture of excitation energy by open photosystem II reaction centres (F_v'/F_m'), electron transport rate (J), superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), catalase (CAT), total ascorbate (AsA), total glutathione (GSH), ascorbate redox state (AsA/(AsA + DHA)), glutathione redox state (GSH/(GSH + GSSG)), total antioxidant activity (TAA) and malondealdehyde (MDA).

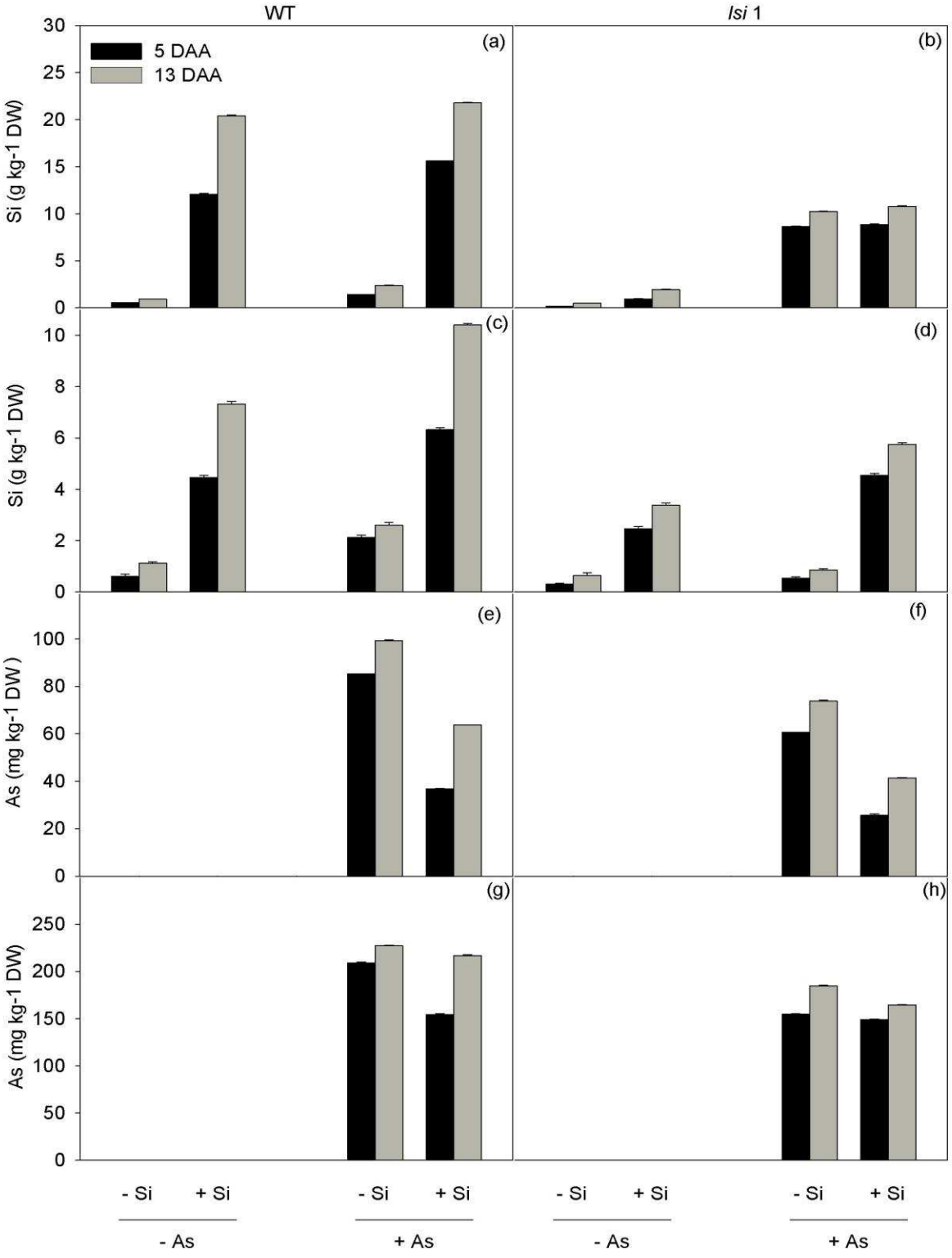

Figure 1. The effects of silicon (Si) and arsenic (As) on the concentrations of Si and As in plant tissues. Two Si (0 or 2 mM: –Si or +Si, respectively) and two As (0 or 25 µM: –As and +As, respectively) levels were supplemented to nutrient solutions. Results are shown for Si in the flag leaves (a-b) and roots (c-d) and As in the flag leaves (e-f) and roots (g-h) of the two rice genotypes [cv. 'Oochikara' (WT) and its *lsi1* mutant defective for Si uptake] grown in nutrient solutions. Measurements were performed at 5 or 13 days after As addition (DAA). $n = 6 \pm$ SE. Arsenic was not detected in –As plants.

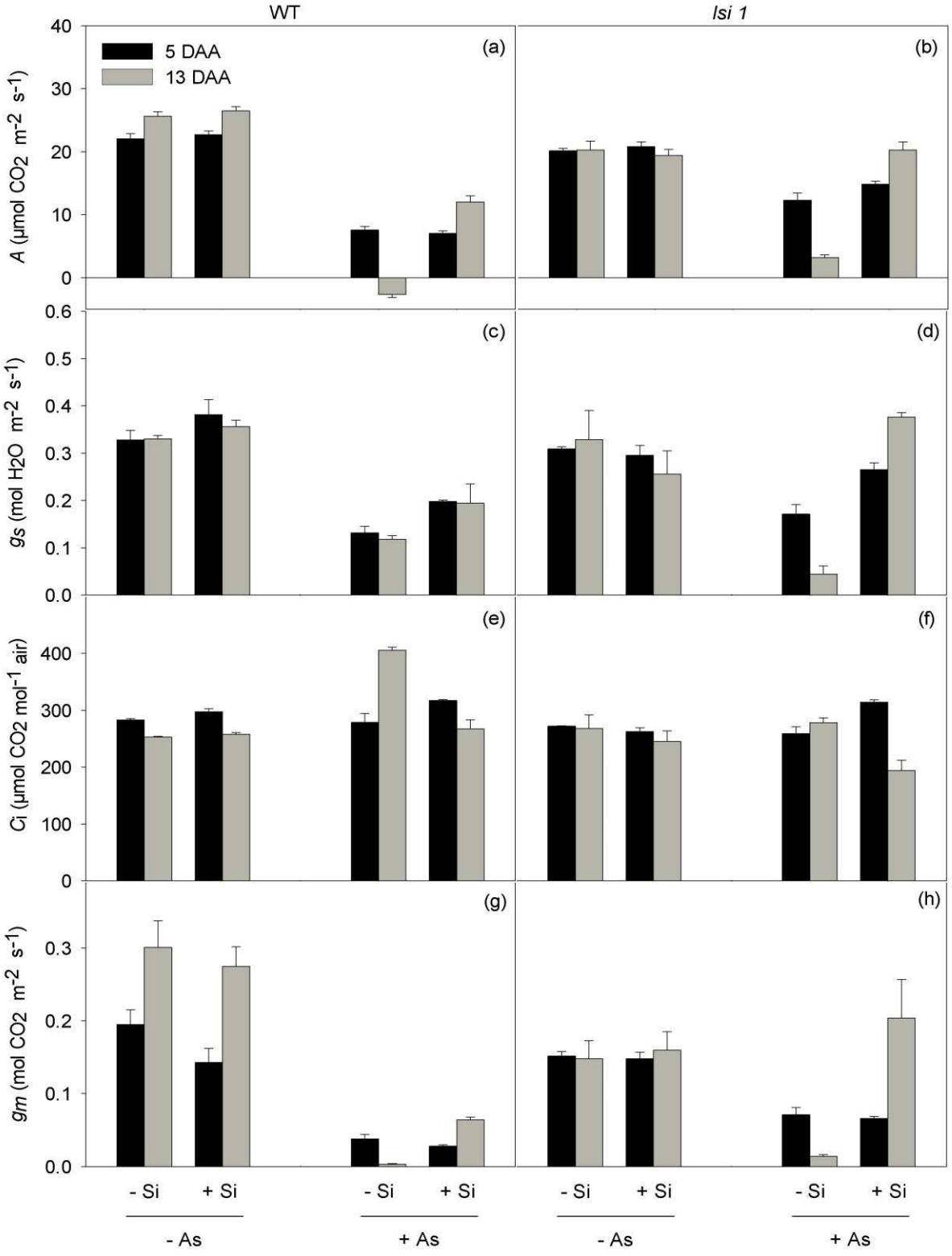

Figure 2. The effects of silicon (Si) and arsenic (As) on photosynthetic gas exchange traits. Two Si (0 or 2 mM: –Si or +Si, respectively) and two As (0 or 25 µM: –As and +As, respectively) levels were supplemented to nutrient solutions. Results are shown for the net CO_2 assimilation rate, A (a-b), stomatal conductance, g_s (c-d), internal CO_2 concentration, C_i (e-f) and mesophyll conductance, g_m (g-h), in the two rice genotypes [cv. 'Oochikara' (WT) and its *lsi1* mutant defective for Si uptake] grown in nutrient solutions. Measurements were performed at 5 or 13 days after As addition (DAA). n = 6 ± SE.

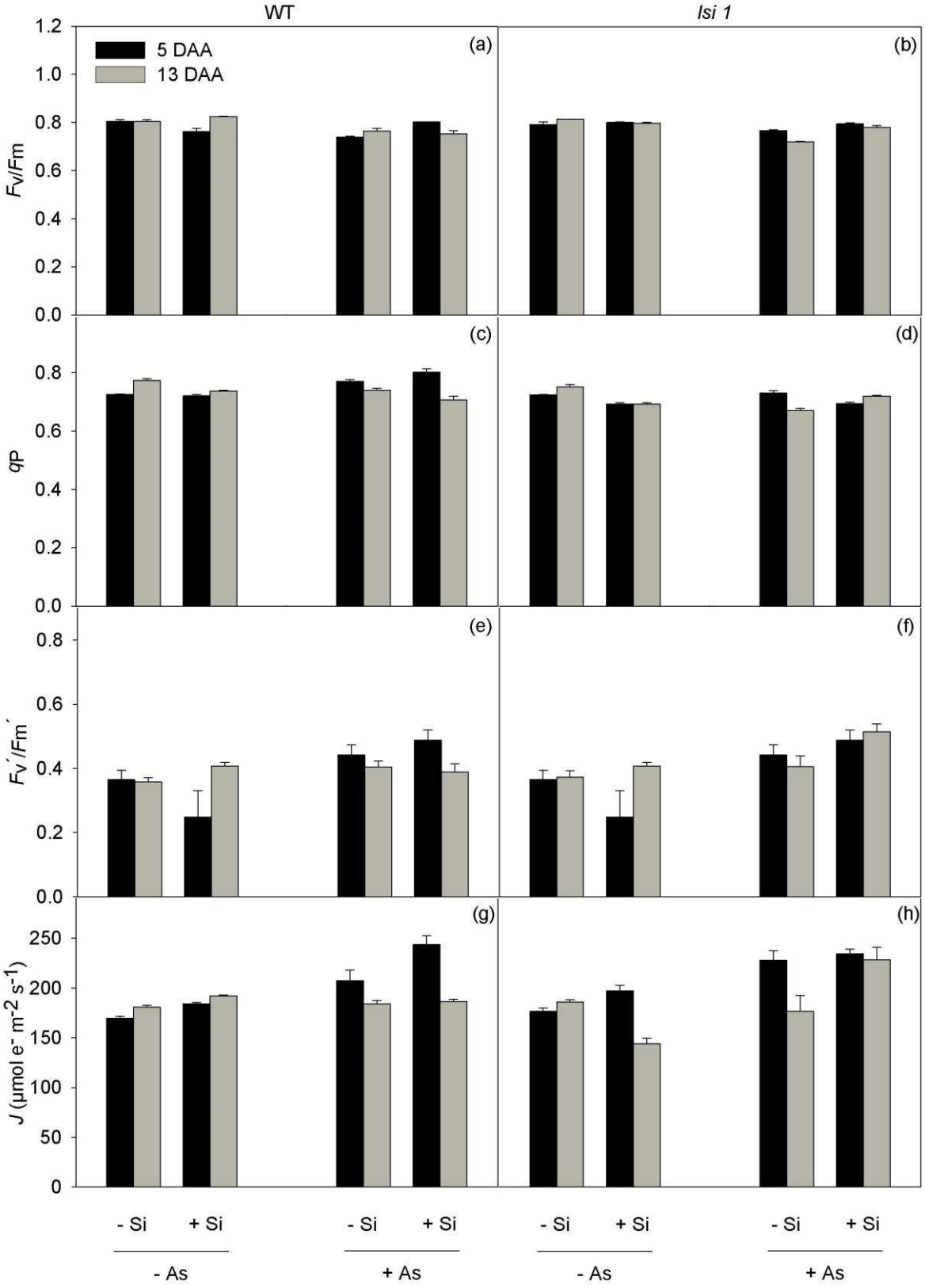

Figure 3. The effects of silicon (Si) and arsenic (As) on photochemical traits. Two Si (0 or 2 mM: –Si or +Si, respectively) and two As (0 or 25 µM: –As and +As, respectively) levels were supplemented to nutrient solutions. Results are shown for the variable-to-maximum chlorophyll fluorescence ratio, F_v/F_m(a-b), photochemical quenching coefficient, q_P (c-d), efficiency of the capture of excitation energy by open photosystem II reaction centres, F_v'/F_m' (e-f) and electron transport rate, J (g-h), in the two rice genotypes [cv. 'Oochikara' (WT) and its *lsi1* mutant defective for Si uptake] grown in nutrient solutions. Measurements were performed at 5 or 13 days after As addition (DAA). $n = 6 \pm$ SE.

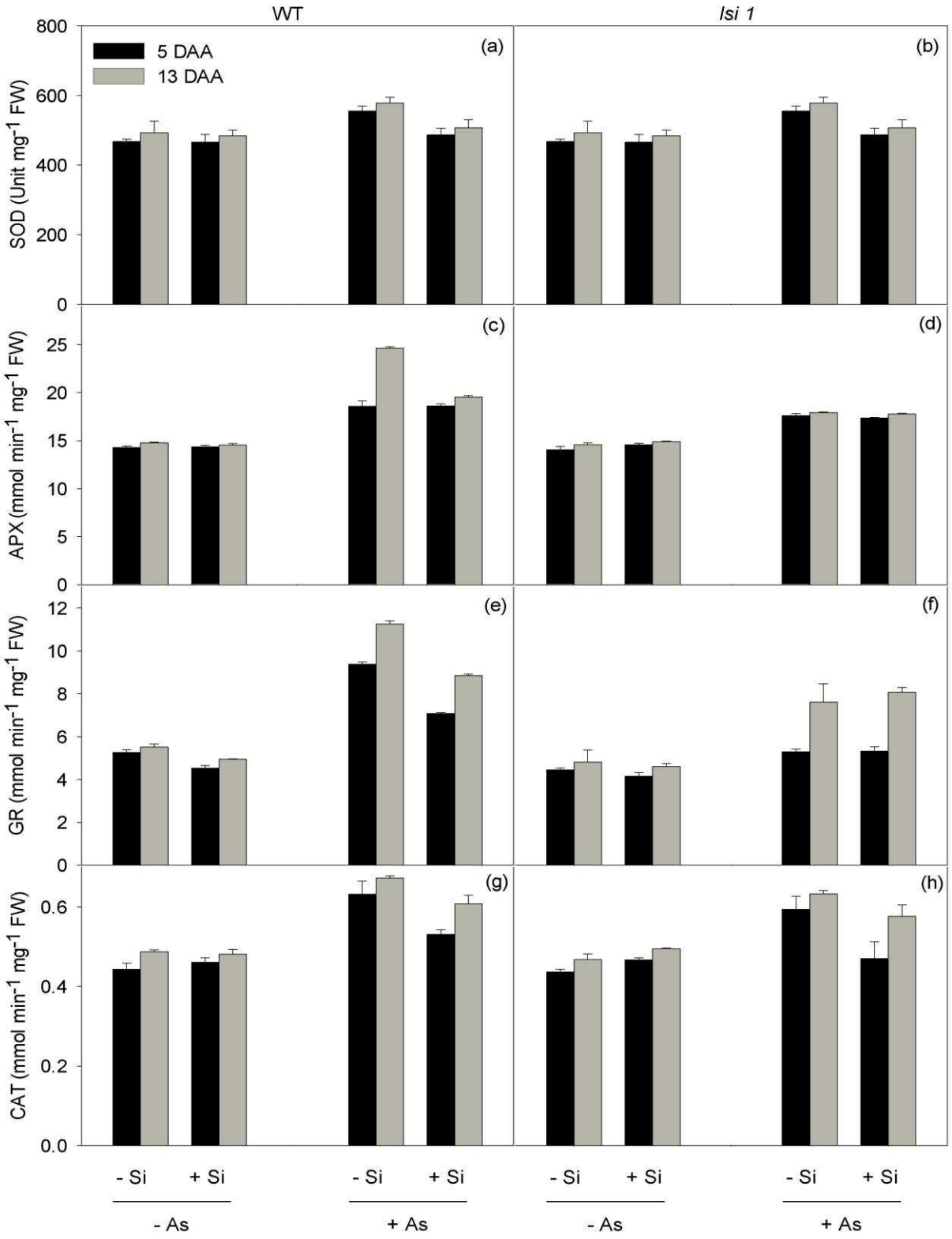

Figure 4. The effects of silicon (Si) and arsenic (As) on the activity of antioxidant enzymes. Two Si (0 or 2 mM: –Si or +Si, respectively) and two As (0 or 25 µM: –As and +As, respectively) levels were supplemented to nutrient solutions. Results are shown for the leaf activities of superoxide dismutase, SOD (a-b), ascorbate peroxidase, APX (c-d), glutathione reductase, GR (e-f) and catalase, CAT (g-h), in the two rice genotypes [cv. 'Oochikara' (WT) and its *lsi1* mutant defective for Si uptake] grown in nutrient solutions. Measurements were performed at 5 or 13 days after As addition (DAA). n = 6 ± SE.

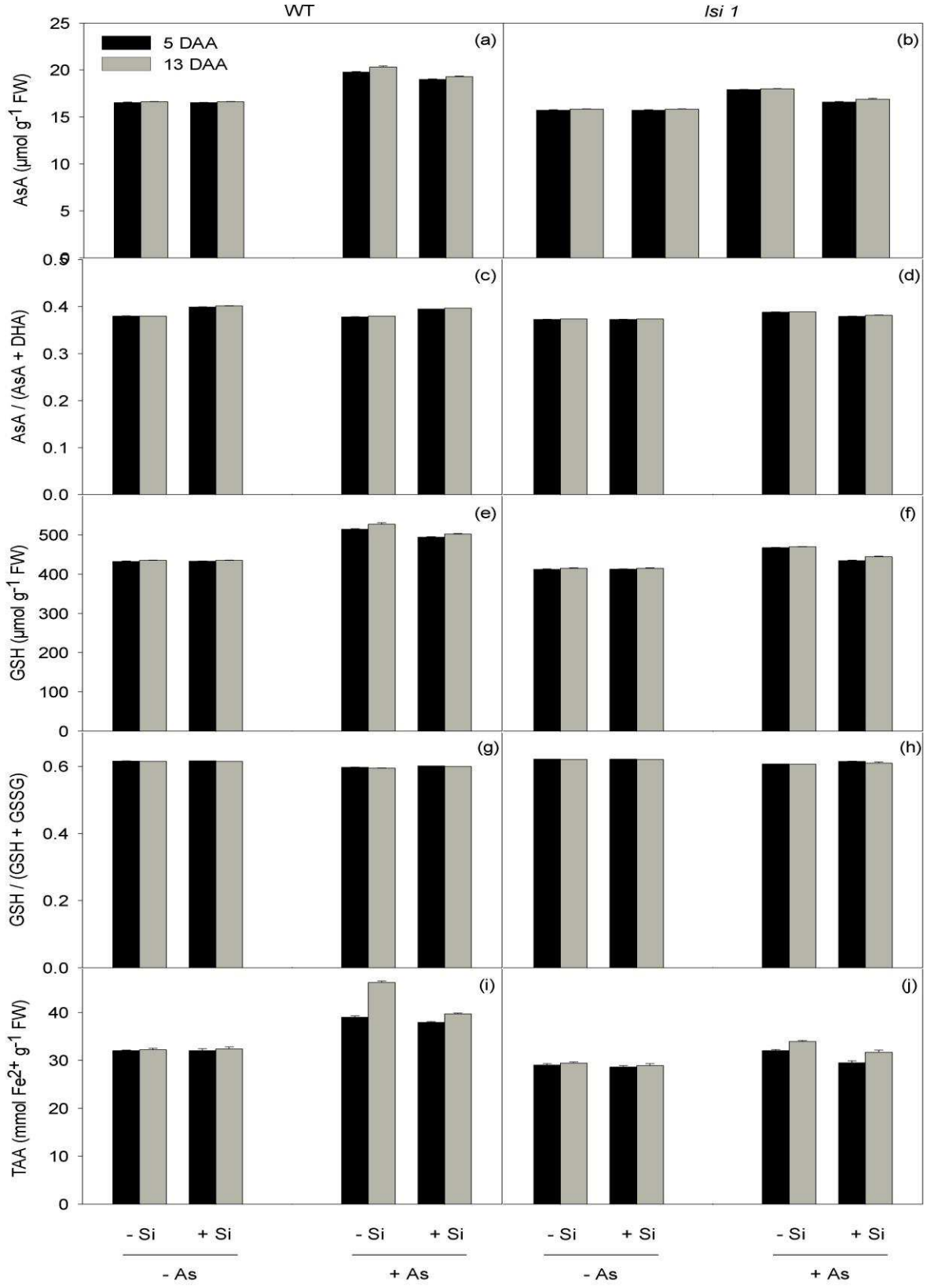

Figure 5. The effects of silicon (Si) and arsenic (As) on antioxidants and total antioxidant capacity. Two Si (0 or 2 mM: –Si or +Si, respectively) and two As (0 or 25 µM: –As and +As, respectively) levels were supplemented to nutrient solutions. Results are shown for the leaf concentrations of total ascorbate, AsA (a-b), and total glutathione, GSH (c-d), the ascorbate redox state, AsA/(AsA + DHA) (e-f), glutathione redox state, GSH/(GSH + GSSG) (g-h), and total antioxidant activity, TAA (i-j), in the two rice genotypes [cv. 'Oochikara' (WT) and its *lsi1* mutant defective for Si uptake] grown in nutrient solutions. Measurements were performed at 5 or 13 days after As addition (DAA). $n = 6 \pm$ SE.

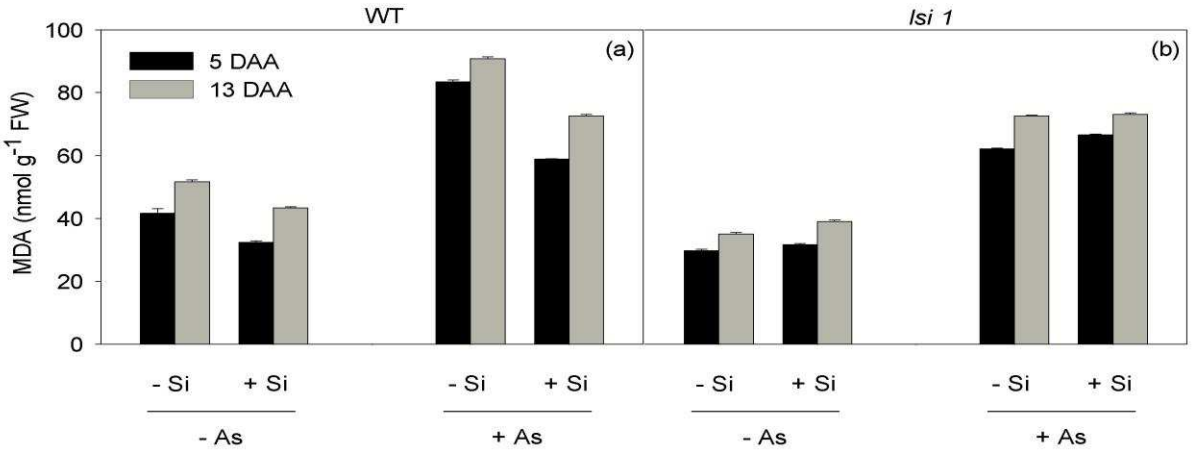

Figure 6. The effects of silicon (Si) and arsenic (As) on the leaf concentrations of malondealdehyde (MDA). Two Si (0 or 2 mM: –Si or +Si, respectively) and two As (0 or 25 µM: –As and +As, respectively) levels were supplemented to nutrient solutions. Results are shown for the two rice genotypes [cv. 'Oochikara' (WT) and its *lsi1* mutant defective for Si uptake] grown in nutrient solutions. Measurements were performed at 5 or 13 days after As addition (DAA). $n = 6 \pm$ SE.