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Silicon nutrition increases grain yield, which, in turn, exerts a feed-forward stimulation of photosynthetic rates via enhanced mesophyll conductance and alters primary metabolism in rice

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Summary

- Silicon (Si) is not considered to be an essential element for higher plants and is believed to have no effect on primary metabolism in unstressed plants. In rice (*Oryza sativa*), Si nutrition improves grain production; however, no attempt has been made to elucidate the physiological mechanisms underlying such responses.
- Here, we assessed crop yield and combined advanced gas exchange analysis with carbon isotope labelling and metabolic profiling to measure the effects of Si nutrition on rice photosynthesis, together with the associated metabolic changes, by comparing wild-type rice with the low-Si rice mutant *lsi1* under unstressed conditions.
- Si improved the harvest index, paralleling an increase in nitrogen use efficiency. Higher crop yields associated with Si nutrition exerted a feed-forward effect on photosynthesis which was fundamentally associated with increased mesophyll conductance. By contrast, Si nutrition did not affect photosynthetic gas exchange during the vegetative growth phase or in de-grained plants. In addition, Si nutrition altered primary metabolism by stimulating amino acid remobilization.
- Our results indicate a stimulation of the source capacity, coupled with increased sink demand, in Si-treated plants; therefore, we identify Si nutrition as an important target in attempts to improve the agronomic yield of rice.

Introduction

Silicon (Si) is the second most abundant element after oxygen in the Earth's crust. Because silicon dioxide comprises 50–70% of the soil mass, all plants grown in soil contain some Si in their tissues. However, Si is often assumed to be biologically unreactive and is not considered to be an essential element for higher plants. The most positive and consistent effects of Si nutrition have been found in the alleviation of both biotic (e.g. pathogens and insects) and abiotic (e.g. salt, heavy metals, light and drought) stresses in a wide variety of plant species (Epstein, 2009; Keeping & Reynolds, 2009). Indeed, a growing body of evidence suggests that the benefits of Si fertilization are minimal or even nonexistent unless the plant is under some form of imposed stress (Epstein, 2009). This has been demonstrated recently in molecular studies using Arabidopsis under unstressed conditions, where Si addition only altered the expression levels of two of the nearly 40 000 transcripts (Fauteux *et al.*, 2006). Even in high-Si-accumulating monocots, Si has limited effects on both the transcriptome (wheat; Chain *et al.*, 2009) and proteome (rice (*Oryza sativa*); Nwugo & Huerta, 2011) in the absence of stress, which lends further support to the general belief that Si has no effect on metabolism in unstressed plants, suggesting a nonessential role for this element.

Two genes encoding Si transporters (*Lsi1* and *Lsi2*) have been identified in rice roots (Ma *et al.*, 2006, 2007). Si is transported via Lsi1 and Lsi2 from the root epidermis into the root steles and then moves to the shoot by transpirational water flow via the xylem, after which it is polymerized and accumulated on the shoot tissues as silica (Ma *et al.*, 2006). In addition, *Lsi6* is involved in Si distribution in rice shoots (Yamaji & Ma, 2009). These specific Si transporters are associated with the strong ability of rice to actively take up Si in the form of monosilicic acid and may explain the high Si levels in rice, which can reach values as high as 10% of the shoot dry weight (Ma & Takahashi, 2002). Under field conditions, Si fertilization is widely used to enhance rice production. This effect of Si has been traditionally attributed to its role in alleviating abiotic and biotic stresses, as well as in improving resistance to lodging and increasing the erectness of leaves; these effects allow better light transmittance through plant canopies and thus indirectly improve whole-plant photosynthesis (Tamai & Ma,

2008). There is, however, evidence suggesting that Si addition hardly affects the net CO_2 assimilation rate (A) *per se* and also has no impact on the tiller number, root dry weight or leaf area. By sharp contrast, rice grain yield is remarkably increased by Si fertilization, as evidenced by rice mutants defective in Si uptake (Tamai & Ma, 2008). Increased production has chiefly been associated with lower transpiration of the spikelets because high moisture conditions play a key role in the normal development of the husk and the protection against pathogen attack (Tamai & Ma, 2008). The omission of Si nutrition during the vegetative growth stage, with a subsequent Si application following the beginning of the reproductive stage, results in rice grain yields similar to those found when Si is added during the entire crop cycle (Okuda & Takahashi 1961; Ma *et al.*, 1989). Given this observation, improved photosynthesis associated with enhanced leaf erectness as a result of Si fertilization can be ruled out, because this trait is defined during the vegetative growth phase.

Taking into account the observations that, in rice, Si has a significant effect on the percentage of filled spikelets and the number of spikelets per panicle, and therefore on fertility (Ma *et al.*, 1989), most carbon in the rice grain comes from photoassimilate produced in leaves (especially the flag leaf) during the grain-filling period (Yoshida, 1981; Murchie *et al.*, 1999) and Si does not affect leaf area, it can be hypothesized that Si should modify the source–sink relationships through increased sink strength. These relationships, in turn, will result in increased photosynthetic capacity of the flag leaf, with probable consequences on carbon metabolism.

Photosynthesis is a major process affecting crop growth and performance. This is not surprising, taking into account that 90–95% of plant dry mass is derived from photosynthetically fixed carbon, although a straightforward relationship between photosynthesis and crop yield is not always observed (Kruger & Volin, 2006). In addition to stomatal and biochemical limitations to photosynthesis, 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), can also remarkably limit the photosynthetic capacity of leaves (Flexas *et al.*, 2012). Early gas exchange studies assumed that g_m was large and constant and, therefore, that CO_2 concentrations in substomatal cavities (C_i) and in chloroplasts (C_c) were nearly the same (Farquhar *et al.*, 1980). However, several subsequent studies have demonstrated that g_m is sufficiently small to decrease C_c markedly (Harley *et al.*, 1992; Bernacchi *et al.*, 2002; Flexas *et al.*, 2007b; Tholen & Zhu, 2011). Indeed, the available evidence demonstrates that g_m limitations to photosynthesis are of a similar magnitude to stomatal constraints, and generally greater than biochemical limitations (Flexas *et al.*, 2012). In rice, for example, C_c is apparently not saturated and was considered to be the ultimate limiting factor for photosynthesis (Li *et al.*, 2009).

To test the hypothesis that Si should modify the source–sink relationships through increased sink strength, source–sink imbalances were analysed via controlled de-graining experiments, which were expected to modulate photosynthesis in unstressed rice plants. We combined advanced gas exchange analysis and chlorophyll *a* fluorescence measurements with carbon isotope labelling and metabolic profiling to measure the effects of Si nutrition on photosynthesis and the process that governs metabolism in rice, and we did this by comparing wild-type (WT) rice (cv 'Oochikara') and an *lsi1* mutant defective in Si uptake. Physiological and molecular studies using this mutant have helped to elucidate the Si uptake system, in addition to increasing our knowledge on the importance of Si to rice physiology (Ma *et al.*, 2006). Our results demonstrate that the increase in grain yield in Si-treated plants is mainly a result of a positive effect on A via a g_m-mediated effect, coupled with enhanced sink strength. Our results highlight the importance of Si nutrition in controlling the nitrogen (N)/carbon (C) balance and amino acid homeostasis. The results are discussed in the context of current models of the metabolic regulation of the sink–source relationship and photosynthetic metabolism.

Materials and Methods

Plant material, growth conditions and experimental design

The experiment was conducted in Viçosa (20°45′S, 42°54′W, 650 m altitude) in southeastern Brazil from November 2009 to March 2010. Rice (*Oryza sativa* L.) plants from cv 'Oochikara' and the low-silicon 1 (*lsi1*) mutant (Ma *et al.*, 2006) were grown in a screen house in plastic pots with 5 l of nutrient solution containing 0 or 2 mM Si under naturally fluctuating environmental conditions. Silicon 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 have been given elsewhere (Dallagnol *et al.*, 2011). The maximum photosynthetic photon flux density (PPFD) inside the screen house was *c.* 1500 μmol m^{-2} s^{-1}. The experiment had a completely randomized design, with eight treatment combinations, forming a 2^3 factorial (two genotypes, two Si levels and two grain loads, i.e. 0 and full grain burden, hereafter referred to as –G and +G plants, respectively), with six plants in individual pots per treatment combination serving as conditional replicates. De-graining treatments were performed by entirely removing the panicles just after the panicle emission. The biomass of these panicles was computed to estimate the total biomass of the –G plants. The experiments were repeated twice, yielding similar results for whole-plant biomass, *in situ* gas exchange parameters and crop yield.

Si concentration

Flag leaves were collected, and their Si concentrations were colorimetrically determined according to Dallagnol *et al.* (2011).

Biomass and crop yield

At the end of the experiment, plants were harvested and separated into culms, leaves, roots and reproductive parts. Total leaf areas were measured with an area meter. Plant tissues were then oven dried at 70°C for 72 h, after which the dry weights of the vegetative and reproductive parts were determined. The specific leaf area of flag leaves, total grain yield, panicle number, percentage of filled

spikelets, 1000-grain weight and harvest index were also determined.

Photosynthetic gas exchange measurements

The net CO_2 assimilation rate (A), stomatal conductance to water vapour (g_s), substomatal CO_2 concentrations (C_i) and instantaneous transpiration rate (E_i) were measured on attached leaves (flag leaf) with a portable open-flow gas exchange system (LI-6400XT, LI-COR, Lincoln, NE, USA). Measurements were made from 10:00 to 13:00 h (solar time), which is when A is at its maximum, under artificial PPFD, that is, 1000 μmol photons m^{-2} s^{-1} at the leaf level and 400 μmol CO_2 mol^{-1} air. During the measurements, the leaf-to-air vapour pressure deficit was *c.* 1.0 kPa.

Leaf gas exchange parameters were also determined simultaneously with measurements of chlorophyll fluorescence using the above-mentioned gas exchange system equipped with an integrated fluorescence chamber head (LI-6400-40, LI-COR). The actual photochemical efficiency of photosystem II (φ_{PSII}) was determined by measuring steady-state fluorescence and maximum fluorescence using a light-saturating pulse of *c.* 8000 μmol m^{-2} s^{-1} following the procedures of Genty *et al.* (1989). The electron transport rate (J) was then calculated from $J = \varphi_{PSII}\,\beta\,\alpha$ PPFD, where α is the leaf absorptance and β reflects the partitioning of absorbed quanta between photosystems II and I. The product $\beta\alpha$ was determined, according to Valentini *et al.* (1995), from the relationship between φ_{PSII} and φ_{CO2} obtained by varying the light intensity under nonphotorespiratory conditions. There were no differences in the product $\beta\alpha$ between –Si and +Si plants, therefore ruling out any confounding effect of different leaf optical properties as a result of Si nutrition among the treatments. Estimations of g_m were performed using the combined gas exchange/fluorescence data (Harley *et al.*, 1992) 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 taken from gas exchange and chlorophyll fluorescence measurements at saturating light, R_l is the rate of mitochondrial respiration in the light, not related to photorespiration, and Γ^* is the chloroplastic CO_2 photocompensation point in the absence of mitochondrial respiration. R_d was measured in the early morning at PPFD = 0 μmol m^{-2} s^{-1} in dark-adapted leaves and was taken as a proxy for R_l (Pinelli & Loreto, 2003; Centritto *et al.*, 2009). The conservative parameter Γ^* for rice was taken from Li *et al.* (2009). To convert A–C_i curves into A–C_c curves (Supporting Information Fig. S1), C_c was calculated according to Flexas *et al.* (2007b). The maximum rate of carboxylation (V_{cmax}) and the maximum rate of carboxylation limited by electron transport (J_{max}) were estimated by fitting the mechanistic model of CO_2 assimilation proposed by Farquhar *et al.* (1980) using the C_c-based temperature dependence of kinetic parameters of Rubisco (Bernacchi *et al.*, 2002). Fitting of the model involved the optimization of the parameter values by adjusting them to minimize the sums of residuals between the observed and modelled assimilation values over a range of C_c. This procedure was performed using the software package Solver in Microsoft Excel. Afterwards, the photosynthetic parameters V_{cmax}, J_{max} and g_m were normalized to 25°C using the temperature response equations from Sharkey *et al.* (2007). Corrections for the leakage of CO_2 into and out of the leaf chamber of the LI-6400 were applied to all gas exchange data, as described by Flexas *et al.* (2007a).

Because all the available methods to estimate g_m rely on models that have a number of assumptions, as well as technical limitations and sources of error that need to be considered to obtain reliable estimates of g_m (Pons *et al.*, 2009), g_m was also estimated using an alternative approach, that is, the A–C_i curve analysis method suggested by Ethier & Livingston (2004). Briefly, this method fits A–C_i curves with a nonrectangular hyperbola version of Farquhar's biochemical model of leaf photosynthesis. The model was fitted to the Rubisco-limited data using nonlinear regression analysis that minimized the error of the sum of squares between the observed and predicted data (Ethier & Livingston, 2004; Tholen *et al.*, 2008). The g_m data were normalized to 25°C following Sharkey *et al.* (2007).

Flag leaves were detached from the mother plant in the morning and immediately brought to the laboratory. The rate of $^{14}CO_2$ uptake was assessed in a leaf-disc oxygen electrode (LD2/2, Hansatech, Kings Lynn, Norfolk, UK) under saturation with CO_2 (*c.* 5 kPa) at a PPFD of 1000 μmol photons m^{-2} s^{-1} at 30°C for 30 min. Further details have been described elsewhere (DaMatta *et al.*, 2008).

Total canopy transpiration (E_c) over the course of the day was gravimetrically measured using a balance (0.1 g precision).

Metabolite levels

Leaf samples were collected at midday, immediately frozen in liquid nitrogen and then stored at −80°C until further analysis. The samples were lyophilized at −48°C and crushed in a ball mill. All other metabolites were quantified by GC-MS-based metabolic profiling, exactly as described previously (Lisec *et al.*, 2006), with the exception that the injected volumes were optimized for rice samples according to Kusano *et al.* (2011). Both chromatograms and mass spectra were evaluated using TAGFINDER (Luedemann *et al.*, 2008). Metabolites were identified in comparison with database entries of authentic standards (Kopka *et al.*, 2005; Schauer *et al.*, 2005). Identification and annotation of the detected peaks followed the recommendations for reporting metabolite data described in Fernie *et al.* (2011).

Other assays

Total N contents were estimated in oven-dried plant tissues according to DaMatta *et al.* (1999). The carbon isotope composition ratio ($\delta^{13}C$), which provides an integrated record of the balance of CO_2 supply and demand over time (Farquhar *et al.*, 1989), was assessed as described previously (DaMatta *et al.*, 2002).

Statistical analysis

The data for biomass, yield components and gas exchange were analysed using a completely randomized design following a 2 × 2 × 2 factorial (two genotypes × two Si levels × two grain

loads) with six replicates. The data were subjected to an ANOVA (three-way ANOVA with all main factors evaluated as fixed factors) which was performed using the general linear models (GLM) procedure of SAS (version 9.1, Cary, NC, USA.) adopting a confidence interval of 95%. When any interaction was found to be significant, the Slice statement of GLM was used to interpret the dependence effect between factors. Pearson's linear correlation technique was subsequently used to examine the relationships among variables.

All the variables concerning the metabolic profile were initially subjected to an ANOVA as described above. The variables that differed significantly among the treatments ($P < 0.05$) were used to perform a multivariate canonical variable analysis (CVA, based on the first and second CVs) using the CANDISC procedure of SAS (version 9.1). This analysis allows the determination of a linear combination of traits that best separate two or more groups of individuals (Johnson & Wichern, 1998).

Results

Measurements were performed in three different phenological phases: the vegetative stage (25–26 d after transplanting), during panicle emission (*c.* 50 d after transplanting) and during the milking grain stage, where the sink strength is believed to be at a maximum (*c.* 90 d after transplanting). Regardless of genotype, no noticeable effect of Si on photosynthetic gas exchange parameters was detected during both the vegetative stage and panicle emission evaluations described above. Therefore, data for these evaluations are not presented.

Si levels are increased, whereas N levels are unaltered, on Si nutrition

As expected, Si addition induced significant increases in Si concentration in leaf tissues (Tables 1, 2). On average, the Si concentration in leaves was higher (80%) in Si-treated (+Si) WT plants than in +Si *lsi1* individuals (Table 2). Regardless of treatment, N levels remained unaltered in both flag leaves and grain tissues (Table 2).

Si nutrition does not affect plant growth, but enhances crop yield

Regardless of treatment, there were no significant alterations in total biomass, total leaf area or specific leaf area (Tables 1, 2). Therefore, the Si-induced increases in crop yield (51% in WT and 34% in *lsi1* plants) resulted in an improved harvest index in both genotypes (33% on average), which was primarily associated with an increase in both the number of spikelets and the 1000-grain weight, with no effect of Si on the panicle number and the percentage of filled spikelets (Table 1, Fig. 1). Although grain yield correlated positively with leaf Si concentration ($r = 0.74$, $n = 24$, $P < 0.001$), a closer inspection of such a relationship revealed that +Si *lsi1* plants had a lower yield than Si-deprived (–Si) WT plants, in spite of the larger Si concentration in the former. One possible explanation could be an intrinsic lower grain yield capacity in the mutant than in its WT counterpart.

Photosynthetic gas exchange parameters are affected by Si nutrition

Because +Si plants had a higher grain yield, with no commensurate changes in leaf area, A per unit leaf area must increase to meet the photoassimilate demand of grains, as demonstrated by the significant enhancements in A (20% on average) in plants with full grain load (+G) relative to de-grained plants (–G) (Table 1, Fig. 2). Moreover, grain yield was correlated with A ($r = 0.64$, $n = 24$, $P < 0.001$).

To explore the causes of A variations among the treatments, we conducted a detailed gas exchange analysis and showed that g_s was unaltered (Table 1, Fig. 2), and therefore stomatal constraints are unlikely to have affected A in this study. Furthermore, no noticeable alterations in E_i and E_c were found (data not shown), suggesting that changes (if any) in cuticular conductance are unlikely to have occurred in response to Si supply. Indeed, changes in A were essentially explained by variations in g_m, which was estimated using two independent methods. Averaging combined gas exchange/fluorescence-derived g_m values (Fig. 2) resulted in a highly significant relationship ($r^2 = 0.79$) with values estimated using the curve-fitting approach (Fig. S2), as similarly found in tobacco leaves by Flexas *et al.* (2007b). Based on such a relationship, all the g_m values reported below are those obtained from the combined gas exchange/fluorescence method.

Increased g_m was found in +G plants relative to –G individuals (101% on average; Fig. 2) with significant Ge × G and Si × G interactions (Table 1); indeed, g_m was significantly higher (77%) in +G +Si WT plants than in their +G –Si counterparts, although it did not differ significantly between +G +Si *lsi1* and +G –Si *lsi1* plants. C_c tended to increase accordingly with increasing g_m (Fig. 2), resulting in a positive correlation between these traits ($r = 0.54$, $n = 48$, $P < 0.001$). Collectively, this information indicates that increases in A were largely associated with increases in g_m ($r = 0.71$, $n = 48$, $P < 0.001$), which, in turn, translated into higher CO_2 availability around the Rubisco environment. In the long term, a higher C_c mediated by a higher g_m should increase the ability of Rubisco to discriminate $^{13}CO_2$, which was reflected in significantly more negative $\delta^{13}C$ values in +G +Si plants relative to +G –Si plants with a significant Si × G interaction (Table 1, Fig. 2). Indeed, negative correlations of $\delta^{13}C$ with both g_m ($r = -0.52$, $n = 48$, $P < 0.001$) and C_c ($r = -0.35$, $n = 48$, $P = 0.025$) were found.

Both V_{cmax} and J_{max}, on a C_c basis, were unaltered in response to Si supply in both genotypes, although small, but significant, grain-related increases in V_{cmax} (11% on average) and J_{max} (13% on average) were observed (Fig. 2). The significant Si × G interaction found for V_{cmax} (Table 1) could be interpreted as a higher (20%) V_{cmax} in +G +Si plants than in their +G –Si counterparts (Fig. 2). In any case, the rate of $^{14}CO_2$ uptake, assessed under saturating CO_2 and therefore in the absence of diffusion-mediated limitations of photosynthesis, thereby reflecting the potential (biochemical) capacity for carbon fixation, was unaffected by either Si or grain load (Table 1, Fig. 2). Collectively, all the above

Table 1 Results (significance) of the ANOVA for the effects of rice (*Oryza sativa*) genotype (Ge), silicon (Si) and grain load (G), and their interactions, for the concentrations of Si and nitrogen, growth traits, yield-related traits and photosynthetic gas exchange parameters (net CO_2 assimilation rate (A), stomatal conductance (g_s), substomatal CO_2 concentration (C_i), chloroplastic CO_2 concentration (C_c), mesophyll conductance (g_m), maximum rate of carboxylation (V_{cmax}), maximum rate of carboxylation limited by electron transport (J_{max}), carbon isotope composition ratio ($\delta^{13}C$) and total ^{14}C uptake rate)

Parameter	Ge	Si	G	Ge × Si	G × Ge	Si × G	Ge × Si × G
Leaf Si	< 0.001	< 0.001	0.060	< 0.001	0.456	0.499	0.441
Leaf N	0.127	0.783	0.441	0.115	0.844	0.935	0.059
Grain N	0.371	0.295	–	0.569	–	–	–
Total biomass	0.761	0.185	0.273	0.286	0.139	0.626	0.607
Leaf area	0.590	0.132	0.482	0.201	0.105	0.462	0.797
Specific leaf area	0.582	0.600	0.289	0.106	0.935	0.570	0.673
Panicle number	0.073	0.097	–	0.418	–	–	–
Spikelet number	< 0.001	0.010	–	0.053	–	–	–
Filled spikelets	0.100	0.565	–	0.565	–	–	–
1000-grain weight	0.736	0.004	–	0.193	–	–	–
Crop yield	< 0.001	0.001	–	0.090	–	–	–
Harvest index	< 0.001	0.007	–	0.044	–	–	–
A	< 0.001	0.021	< 0.001	0.056	0.183	0.076	0.534
g_s	0.016	0.740	0.083	0.559	0.051	0.508	0.829
C_i	0.136	0.532	0.055	0.003	0.034	0.670	0.419
g_m	0.023	0.030	< 0.001	0.122	0.001	0.028	0.007
C_c	0.045	0.794	0.049	0.845	0.005	0.038	0.221
V_{cmax}	0.280	0.428	0.048	0.102	0.644	0.016	0.978
J_{max}	0.330	0.626	0.001	0.070	0.641	0.233	0.070
$\delta^{13}C$	< 0.001	0.742	0.001	0.208	0.915	0.037	0.485
^{14}C uptake	< 0.001	0.490	0.492	0.143	0.002	0.741	0.901

Table 2 The effects of silicon (Si) supply (0 or 2 mM: –Si or +Si, respectively) and grain load (0 or full grain burden: –G and +G, respectively) on the concentrations of Si (flag leaves) and nitrogen (N; flag leaves and grains) and growth parameters (total biomass, leaf area (LA), specific leaf area (SLA)) of two rice (*Oryza sativa*) genotypes (cv 'Oochikara' (WT) and the *lsi1* mutant defective for Si uptake) grown in nutrient solutions

	WT				*lsi1*			
	–Si		+Si		–Si		+Si	
Parameter	–G	+G	–G	+G	–G	+G	–G	+G
Leaf Si (g kg^{-1} DW)	11.7 ± 0.1	11.4 ± 0.1	47.3 ± 0.2	51.0 ± 0.2	6.0 ± 0.1	9.9 ± 0.4	25.5 ± 0.1	29.1 ± 0.2
Leaf N (g kg^{-1} DW)	31.3 ± 1.2	30.0 ± 0.3	26.3 ± 1.5	30.0 ± 2.6	24.4 ± 2.5	27.8 ± 2.2	28.9 ± 1.4	27.1 ± 2.2
Grain N (g kg^{-1} DW)	–	15.3 ± 0.1	–	14.0 ± 0.1	–	14.1 ± 0.1	–	13.8 ± 0.1
Biomass (g per plant)	36.1 ± 1.8	39.7 ± 1.0	39.5 ± 4.0	47.0 ± 2.6	39.9 ± 2.4	39.1 ± 2.7	40.5 ± 2.4	39.6 ± 4.2
LA (m^2)	0.14 ± 0.01	0.14 ± 0.01	0.17 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.14 ± 0.01	0.14 ± 0.01	0.14 ± 0.01
SLA (m^2 kg^{-1})	17.6 ± 1.0	19.9 ± 1.2	19.9 ± 1.1	20.6 ± 1.3	18.1 ± 1.2	20.2 ± 2.0	18.0 ± 1.2	18.2 ± 1.1

$n = 6 \pm$ SE.

information provides compelling evidence that changes in actual A were fundamentally governed by g_m.

The plant metabolite profile is affected by Si nutrition and, most particularly, by Si-mediated increases in grain load

In response to the imposed treatments, considerable changes in the levels of a wide range of organic acids, amino acids and sugars were evident. To provide an overview, the major metabolic changes observed were synthesized in a schematic summary by metabolic pathways (Fig. 3; the full dataset is available in Table S1). Interestingly, +G plants from both genotypes displayed reduced levels of several amino acids in the presence of Si, as observed for alanine, arginine, methionine, ornithine and valine. In addition, +G +Si WT plants showed reduced asparagine, aspartate, lysine, phenylalanine, proline, serine, threonine and tyrosine, which suggests a higher mobilization of these amino acids to sustain the high grain yield and demand of +G +Si WT plants. Intriguingly, sucrose, fructose and glucose were consistently lower in +G +Si *lsi1* plants than in their –Si counterparts.

When comparing the genotypes, it is notable that de-graining treatments significantly reduced ascorbate, glutamate and valine, and increased glutarate and shikimate, in –Si *lsi1* plants. Pyruvate, aconitate, isocitrate and malate were increased in –Si *lsi1* plants, in contrast with reduced 2-oxoglutarate and γ-aminobutyric acid (GABA), when compared with their WT counterparts. The –G treatment promoted significant increases only in glutamine, methionine, glucose and sucrose, whereas lactate was decreased in *lsi1* plants. In +G plants, the absence of Si strongly affected plant metabolism, with reductions in arginine, asparagine, aspartate and

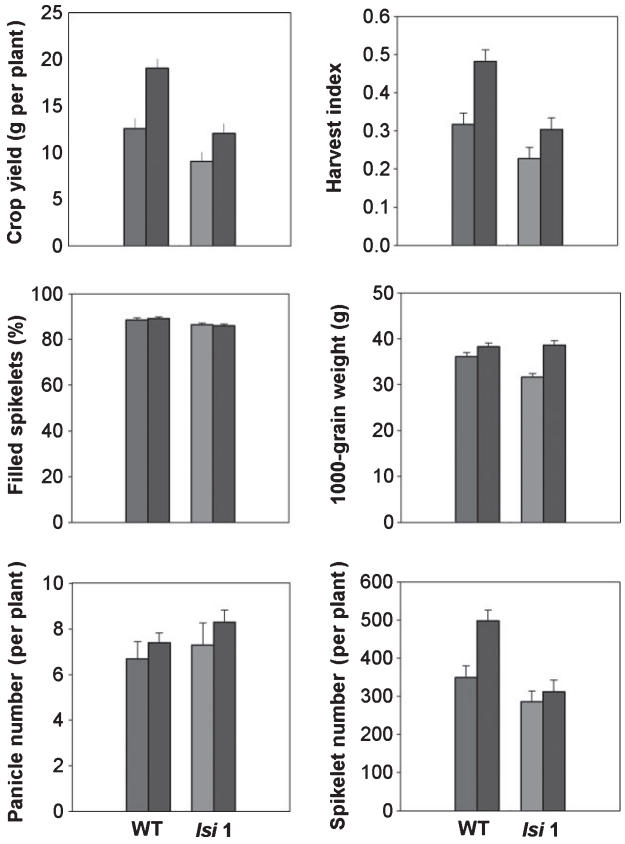

Fig. 1 The effects of silicon (Si) supply (0 or 2 mM: –Si (light grey bars) or +Si (dark grey bars), respectively) on yield components (grain yield, harvest index, panicle number, total spikelet number, percentage of filled spikelets and 1000-grain weight) of two rice (*Oryza sativa*) genotypes (cv 'Oochikara' (WT) and the *lsi1* mutant defective for Si uptake) grown in nutrient solutions. $n = 6 \pm$ SE.

pyruvate, whereas citrate, fructose, galactose, glucose, lactate and tyrosine were significantly higher in *lsi1* plants than in their WT counterparts (Fig. 3, Table S1). The +G +Si *lsi1* plants accumulated less pyruvate and more isocitrate, isoleucine, phenylalanine and tyrosine than did +G +Si WT plants.

To explore in more detail the effects of Si on plant metabolism, the metabolic dataset was analysed using CVA using the first two CVs, which cover the major variance of the dataset (Fig. 4; CV1 covers 66.5% of the total variance and CV2 17%). This fingerprinting analysis revealed that, in –G WT plants, Si did not apparently affect the primary metabolism, that is, no segregation between –Si and +Si plants was found; in sharp contrast, a clear trend of metabolic re-adjustment in response to +Si and +G conditions was observed in +G WT plants, which suggests a direct effect of Si, independent of the grain effects, in orchestrating metabolic changes in +G WT individuals (Fig. 4). Surprisingly, the results obtained by our CVA were more evident in *lsi1* plants, where a clearer separation between the effects of Si from the effects of grain load on the metabolite profile could be observed (Fig. 4). The metabolic events occurring in +G +Si conditions are best exemplified by the metabolites with the highest canonical discriminant scores and ANOVA *P* values (i.e. those metabolites with a main impact on the variance of the dataset; Table S2). A number of amino acids, such as alanine, aspartate, ornithine and threonine, as well as the sugars glucose and fructose, accounted for the main changes observed in primary metabolism (Figs 3, 4).

We next carried out a broad correlation analysis (between the relative level of each metabolite and the relative level of Si in all experimental samples) in an attempt to determine which changes were most closely associated with the change in Si concentration. When evaluating the strengths of these correlations and their significances, it became apparent that only 16 of the metabolic changes (those in alanine, arginine, glutamine, isoleucine, methionine, ornithine, valine, dehydroascorbate, 2-oxoglutarate, isocitrate, pyroglutamate, quinic acid, fructose, glucose, galactinol and glycerol) were closely associated with changes in Si (Table S3). Of these, only 2-oxoglutarate was positively correlated with Si, suggesting that increases in Si concentration negatively affected the levels of a variety of metabolites, specifically amino acids (seven of 16).

Discussion

Si nutrition improves rice production (Ishibashi, 1936; Tamai & Ma, 2008), but, surprisingly, no attempt has been made to date to elucidate the physiological mechanisms underlying the responses of plants to Si. In this study, Si concentrations in leaf tissues were manipulated by omitting Si from the culture solution (–Si plants) as well as by using the low-Si rice mutant *lsi1*. This approach revealed new insights into the links between the Si-related improvement in rice crop yield and photosynthesis, together with the associated metabolic changes. We carried out our analyses of photosynthesis and the metabolite profile using the flag leaves because most carbon in the rice grain comes from photoassimilate produced in these leaves during the grain-filling period (Yoshida, 1981; Murchie *et al.*, 1999).

Silicon nutrition increases both rice grain yield and N use efficiency

Previous analyses of the rice yield components have shown that Si supply improves crop yield by enhancing both the number of spikelets per panicle and, most particularly, the percentage of filled spikelets, with no significant effect on the panicle number or the 1000-grain weight (Ma *et al.*, 1989; Tamai & Ma, 2008). Despite the fact that the total number of spikelets was increased significantly in this study, especially in WT plants, we found increased grain weight with no significant effect of Si on the percentage of filled spikelets. Taken together, these results clearly indicate a stimulation of the source capacity, coupled with increased sink demand. A decreased percentage of filled spikelets in Si-deprived rice plants has been attributed to higher pathogen infection and increased spikelet transpiration, which is especially important if the rice crop encounters typhoon conditions during the spikelet-filling period (Tamai & Ma, 2008). Although we cannot rule out such a transpiration effect, we contend that it had only negligible importance in determining grain yield under our experimental conditions. Therefore, Si-related increases in rice production under unstressed conditions should be more directly associated with differentiation and development of reproductive

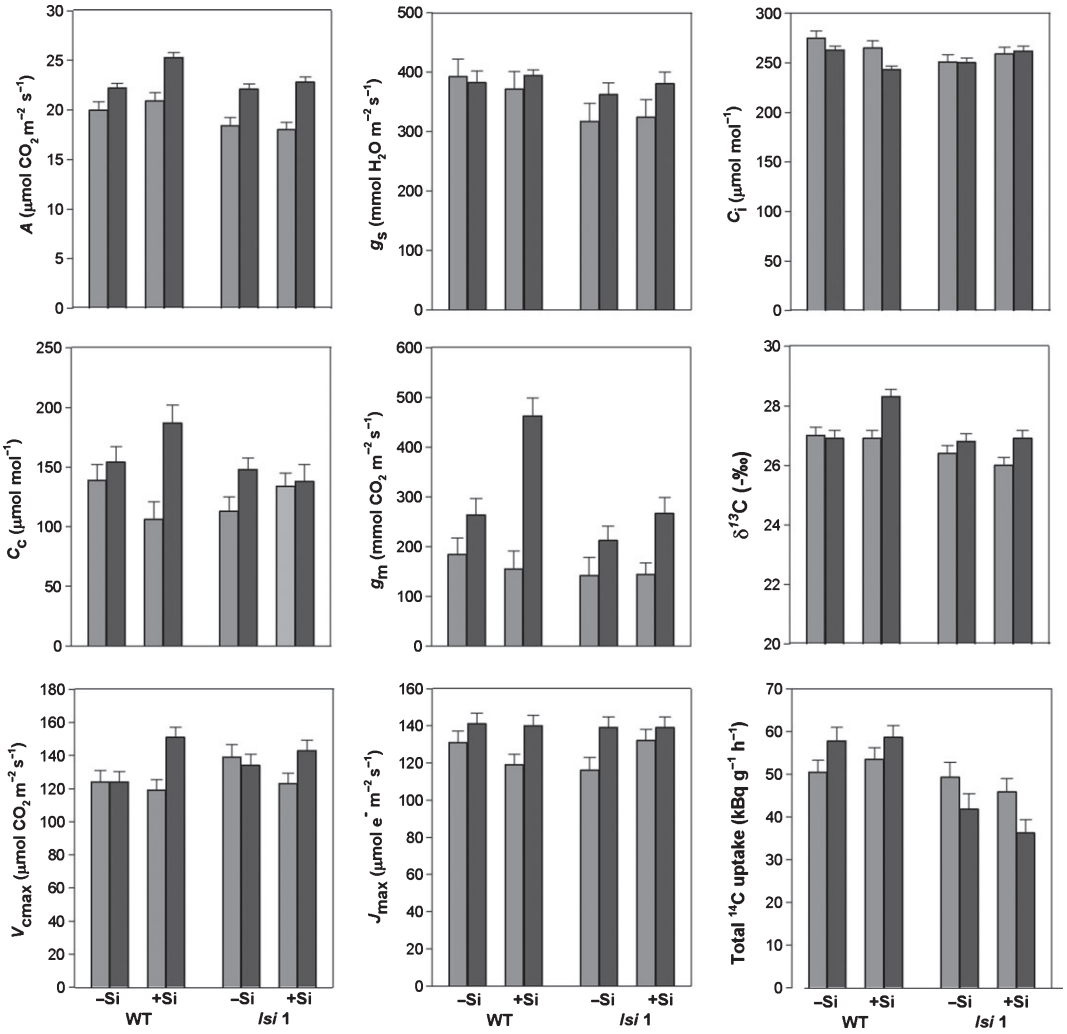

Fig. 2 The effects of silicon (Si) supply (0 or 2 mM: –Si or +Si, respectively) and grain load (0 or full grain burden: –G (light grey bars) and +G (dark grey bars), respectively) on photosynthetic gas exchange parameters (net CO_2 assimilation rate (A), stomatal conductance (g_s), substomatal CO_2 concentration (C_i), chloroplastic CO_2 concentration (C_c), mesophyll conductance (g_m), carbon isotope composition ratio ($\delta^{13}C$), maximum rate of carboxylation (V_{cmax}), maximum rate of carboxylation limited by electron transport (J_{max}) and the rate of $^{14}CO_2$ uptake) of two rice (*Oryza sativa*) genotypes (cv 'Oochikara' (WT) and the *lsi1* mutant defective for Si uptake) grown in nutrient solutions. $n = 6 \pm$ SE.

structures. Although Ma *et al.* (1989) have suggested that Si may ameliorate the low pollen viability, virtually nothing is known about the physiological basis of how Si affects rice production.

Taking into account that neither total biomass nor leaf N level varied across treatments (and assuming similar total plant N contents), N remobilization to grains must have increased with grain load in +Si plants to maintain an unaltered N content spread over a higher grain biomass. The implication of this is that both the crop yield and harvest index are effectively increased without impairing grain quality (in terms of protein content) in addition to improving N use efficiency.

Increased grain yield improves the source capacity through a feed-forward stimulation on photosynthetic rates via enhanced mesophyll conductance

We showed, for the first time, that Si leads to increases in crop yield, which brings about an increased sink strength which, in turn, exerts a feed-forward effect on A. In particular, because the higher crop yield was accompanied by increases in grain weight, our results clearly indicated that the source capacity (flag leaves) increased to a relatively greater magnitude than the sink strength. Notably, enhanced A took place with unaltered total plant biomass. This may be explained by taking into consideration the fact that additional energy is required to support increased remobilization rates of photoassimilates from vegetative parts to the grains in Si-treated plants, and to construct new biomass in the (heavier) grains, which have a greater energetic content than their vegetative counterparts.

The Si effects on photosynthesis were fundamentally associated with increased g_m, and were particularly pronounced in +G WT plants. In these plants, increases in C_c mediated by higher g_m apparently led to increased V_{cmax}, in addition to allowing Rubisco to increase discrimination against $^{13}CO_2$, an observation further supported by the negative correlation between $\delta^{13}C$ and C_c (and also g_m), which ultimately resulted in lower $\delta^{13}C$ in +G +Si WT

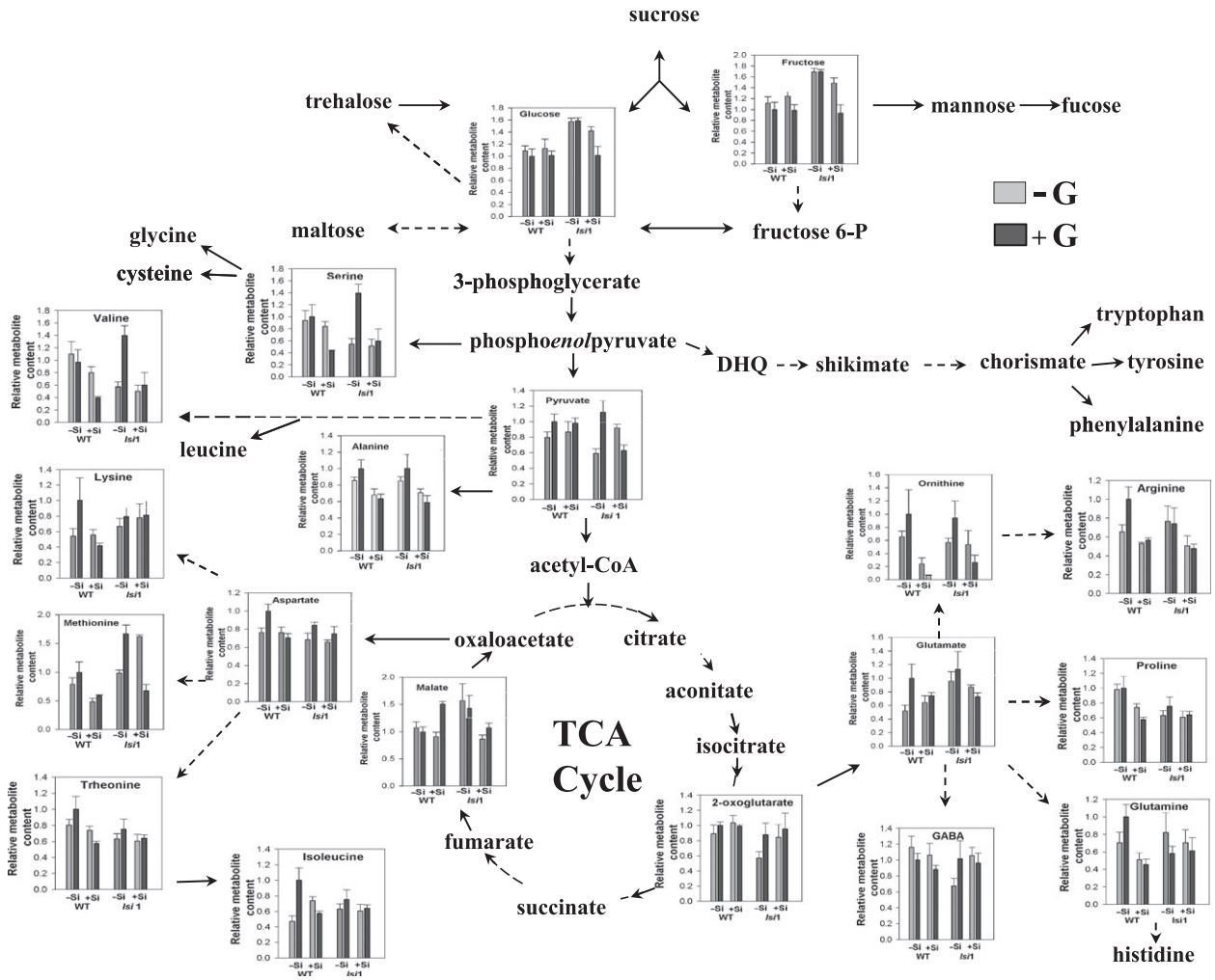

Fig. 3 Changes in metabolite contents in flag leaves of two rice (*Oryza sativa*) genotypes (cv 'Oochikara' (WT) and the *Isi1* mutant defective for Si uptake) under the effects of grain load (0 or full grain burden: –G and +G, respectively) and Si supply (0 or 2 mM: –Si and +Si, respectively). The schematic summary presents metabolites (relative levels after normalization against the +G –Si WT plants) with significant differences between treatments. For an easy overview, metabolites without a significant difference between treatments are only written in the figure. Values are presented as the means of six replicates ± SE. The full dataset, including all metabolites measured by GC-MS, can be found in Supporting Information Table S1. DHQ, 3-dehydroquinate; GABA, γ-aminobutyric acid; TCA, tricarboxylic acid.

individuals. In any case, alterations in actual A were not accompanied by significant changes in the rate of $^{14}CO_2$ uptake in +G WT plants, regardless of Si supply, which suggests that, when limitations to CO_2 diffusion are fully overcome by the supersaturated CO_2 supply, g_m-related differences in A are abolished. Earlier attempts to demonstrate an effect of Si nutrition on rice photosynthesis (e.g. Nwugo & Huerta, 2008, 2011; Chen *et al.*, 2011) most probably failed because these investigations examined plants during their vegetative growth phase, when sink strength is relatively low. This was also noted in this study, and was further corroborated by the similar A values among plants from the –G treatment.

Recently, Centritto *et al.* (2009) have posited that, under drought conditions, g_m also plays an important role in determining photosynthesis because rice genotypes with inherently higher g_m are capable of maintaining a higher A. To the best of our knowledge, the current study is the first to report a direct effect of sink strength on g_m. The mechanisms underlying this relationship are not immediately evident. Although several investigators have attempted to explain the mechanistic bases of g_m variations, which may depend on leaf thickness, surface area of chloroplasts exposed to intercellular airspace, mesophyll cell wall thickness, membrane permeability to CO_2 and carbon anhydrase activity (Evans *et al.*, 2009; Tholen & Zhu, 2011), our understanding of this subject remains far from clear. Accordingly, the limited progress in elucidating the mechanisms that govern g_m could be linked to the lack of an appropriate method to evaluate the contributions of both anatomical and biochemical components of g_m (Tholen & Zhu, 2011). In any case, in rice, greater g_m has chiefly been associated with thinner mesophyll cell walls (Scafaro *et al.*, 2011), and some evidence suggests that Si application in rice might result in decreased thickness of cell walls (Hossain *et al.*, 2002). Taken together, this information could explain, at least partially, the increases in g_m observed in +G plants, particularly when supplied with Si.

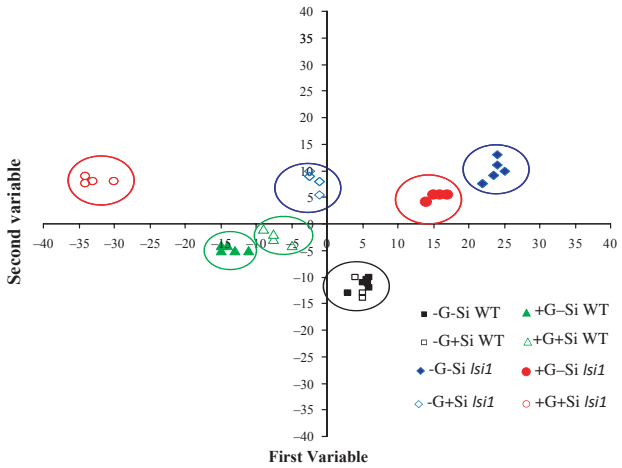

Fig. 4 Canonical variable analysis (CVA) of the metabolite data obtained in the flag leaves of two rice (*Oryza sativa*) genotypes (cv 'Oochikara' (WT) and the *lsi1* mutant defective for Si uptake) under the effects of grain load (0 or full grain burden: –G and +G, respectively) and Si supply (0 or 2 mM: –Si and +Si, respectively). CVA was performed on the variables that differ significantly among the treatments ($P < 0.05$). Supporting Information Table S1 shows the analysis of the CVA loadings. Metabolites were determined as described in the Materials and Methods section.

Si nutrition affects source–sink strength relationships and stimulates amino acid remobilization

Our data indicated that Si might act as a signal to promote amino acid remobilization (as is believed to occur with diseased rice plants, in which Si nutrition may trigger mechanisms of host resistance via alterations in plant metabolism; Dallagnol *et al.*, 2011). Support for this assumption also comes from a recent study demonstrating that Si nutrition can modulate the expression of a leucine-rich repeat (LRR) family protein and can play a central role in the perception of an as yet uncharacterized Si signal (Fleck *et al.*, 2011).

Our analysis of the metabolite profiles of +G +Si *lsi1* plants indicated that leaves were characterized by reduced sucrose, fructose and glucose, in accord with the relatively small changes in *A*. Indeed, the reduced levels of several amino acids in +G +Si WT plants were associated with an increased sink strength mediated by Si nutrition, with little, if any, impact on biomass accumulation. Indeed, it has been suggested that amino acid export can be regulated by sucrose transport or metabolism (Barneix, 2007), and Winter *et al.* (1992) have postulated that both sucrose and amino acid export to the sieve tube are dependent on photosynthetic metabolism in the source cell. In addition, our evaluation of the metabolites that correspond to the Si concentration revealed three sets of compounds: those intimately involved in respiration (isocitrate and 2-oxoglutarate), a handful of amino acids (alanine, arginine, glutamine, ornithine, isoleucine, methionine and valine) and four sugars/sugar derivatives (glucose, fructose, galactinol and glycerol). When taken together, these data clearly demonstrate that, at least under the conditions explored here, Si nutrition plays an important role in modulating the rate of flux from 2-oxoglutarate into amino acid metabolism, supporting the emergent view that amino acid metabolism is a tightly and intricately controlled network (Sweetlove & Fernie, 2005; Less & Galili, 2008). Together, these data also support our view of a role for Si nutrition in orchestrating amino acid remobilization (although such remobilization should be just beginning, because no detectable changes in leaf N concentration were found). Similar to the metabolic situation observed here, treatment of a sensitive soybean (*Glycine max*) genotype with glyphosate had a rapid impact on photosynthesis and respiration, triggering the appearance of an N-rich amino acid profile (Vivancos *et al.*, 2011). When considered together, these and our results suggest that alterations in the levels of some amino acids are most probably associated with a higher flux to proteins and other N-containing compounds to support increased N demand by the grains. Naturally, the metabolites measured represent only a small part of the whole rice metabolome, and it is yet to be determined in future studies whether similar observations can be made across a broader spectrum of metabolites. In any case, Si *per se* may directly impact the metabolite profile of rice, as noted for *lsi1* plants, where a clear separation of the effects of Si from the effects of grain load on the metabolite profile could be demonstrated. Therefore, Si could have some as yet unknown function in rice metabolism, even under unstressed conditions.

Conclusions

In this article, we demonstrate that Si nutrition leads to an improved crop yield, even under unstressed conditions, paralleling an increase in N use efficiency in rice. In addition, we demonstrate that Si nutrition results in altered primary metabolism, with Si clearly stimulating amino acid remobilization. However, it is important to note that the exact mechanism by which this is achieved is, as yet, unknown. Overall, higher crop yields bring about an increased sink strength, which, in turn, exerts a feed-forward, mesophyll conductance-associated effect on photosynthesis. Therefore, our report identifies Si nutrition as an important target in attempts to improve the agronomic yield of rice.

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Supporting Information

Additional Supporting Information may be found in the online version of this article.

Fig. S1 The effects of silicon (Si) supply and grain load on the response of the net CO_2 assimilation rate (A) to chloroplast CO_2 concentration (C_c) of two rice genotypes (cv 'Oochikara' (WT) and the *lsi1* mutant defective for Si uptake) grown in nutrient solutions.

Fig. S2 The effects of silicon (Si) supply and grain load on the mesophyll conductance (g_m) estimated using the curve-fitting approach (Ethier method) of two rice genotypes (cv 'Oochikara' (WT) and the *lsi1* mutant defective for Si uptake) grown in nutrient solutions, and the relationship between estimates of g_m (Harley and Ethier approaches).

Table S1 Relative metabolite contents in flag leaves of two rice genotypes

Table S2 Over-representation analysis of the canonical variable loadings of metabolites with a main impact on the variance of the dataset

Table S3. Pairwise correlation coefficients, with their corresponding P values, calculated between the contents of Si and all other metabolites

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