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Genotypic differences in shoot silicon concentration and the impact on grain arsenic concentration in rice

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Abstract

Silicon in rice (*Oryza sativa* L.) has been demonstrated to be involved in resistance to lodging, drought, and salinity, and also enhances resistance to pests and diseases. The aim of this study was to determine the range of silicon concentration in a set of rice (*Oryza sativa* L.) accessions, and to determine if the natural variation of shoot silicon is linked to the previously identified silicon transporters (*Lsi* genes). Silicon concentration was determined in 50 field-grown accessions, representing all sub-populations of rice, with all accessions being genotyped with 700K SNPs. SNPs within 10 kb of the *Lsi* genes were examined to determine if any were significantly linked with the phenotypic variation. An XRF method of silicon determination compared favourably with digestion and colorimetric analysis. There were significant genotypic differences in shoot silicon ranging from 16.5 to 42.4 mg g^{-1} of plant dry weight, but there was no significant difference between the rice sub-populations. Plants with different alleles for SNPs representing *Lsi2* and *Lsi3* were significantly different for shoot silicon concentration. Shoot silicon correlated negatively with grain arsenic in the *tropical* and *temperate japonica* sub-population, suggesting that accessions with high shoot silicon have reduced grain arsenic. This study indicates that alleles for *Lsi* genes are excellent candidate genes for further study to explain the natural variation of shoot silicon in rice.

Key words: Arsenic, natural variation, *Oryza sativa*, silicon, XRF

1 Introduction

Global rice (*Oryza sativa* L.) production needs to increase continuously to ensure the world's food security (*Hibberd* et al., 2008). As a beneficial element, silicon alleviates biotic and abiotic stresses in rice, which helps to maintain yield (*Ma* and *Takahashi,* 2002; *Detmann* et al., 2012; *Meharg* and *Meharg,* 2015). Silicon is mainly available as monosilicic acid ranging from 0.1 to 0.6 mM in the soil solution (*Epstein,* 1994; *Ma* and *Takahashi,* 2002). Previous studies have demonstrated that monosilicic acid is taken up by rice roots as an undissociated molecule and translocated into the shoots through the transpiration stream (*Takahashi and Hino,* 1978; *Mitani-Ueno* et al., 2005). It then polymerises on the surface of cells in the shoot in the form of a silica-cellulose double layer and silica-cuticle double layer. This silica-base layer improves resistance to lodging, salinity resistance, drought tolerance, and enhances resistance to pests and diseases (*Takahashi* and *Hino*, 1978; *Mitani-Ueno* et al., 2005, *Chen* et al., 2011; *Han* et al., 2015).

Genetically rice can be classified into two major sub-species, *Japonica* and *Indica* (*Chang,* 2003) and these have been further classified into five sub-populations; *indica, aus,* (both *Indica* sub-species) *tropical japonica*, *temperate japonica,* and *aromatic* (all three *Japonica* sub-species; *Garris* et al., 2005; *Zhao* et al., 2011). Several previous studies indicate that there are differences in shoot silicon concentration between the *Indica* and *Japonica* sub-species of rice. *Deren* et al., (1992) showed that *Japonica* sub-species usually have a higher silicon concentration than *Indica* rice varieties, based on screening ten accessions in the greenhouse and 18 under field conditions. A study conducted by *Winslow* (1992) revealed that African upland *Japonica* rice accessions had 50-100% higher silicon concentration in mature flag leaves than Asian upland *Indica* accessions. In addition to the differences at the subspecies level several studies have looked at genotypic differences in silicon concentration, showing ranges of 41-60 mg g^{-1} (*Deren,* 2001) and 28 to 61 mg g^{-1} (*Norton* et al., 2010a). *Ma*

et al., (2007a) also observed that silicon uptake by the root and the concentration of silicon present in the shoot are both higher in *Japonica* than *Indica* rice accessions, which they attributed to differences in the expression of silicon transporter genes.

Two types of silicon transporters have been identified in rice to date. A gene (LOC_Os02g51110) identified for silicic acid influx in rice is classified as an aquaporin (Low silicon 1 or *Lsi1*) which is a member of the nodulin 26-like intrinsic protein (OsNIP2; 1) group of aquaporins (*Ma* et al., 2006; *Ma* et al., 2008). A homologue of *Lsi1*, known as *Lsi6* (LOC_Os06g12310; OsNIP2; 2), responsible for shoot and husk silicon distribution in rice is also classified as an aquaporin (*Yamaji* et al., 2008). The efflux of silicic acid through the plasma membrane protein known as low silicon 2 (*Lsi2;* LOC_Os03g01700) is an energy-dependent process in rice (*Ma* et al., 2007b). A homologue of *Lsi2,* known as *Lsi3* (LOC_Os10g39980), is also an energy-dependent active transporter involved in regulating shoot silicon accumulation in rice (*Yamaji* et al., 2015).

It has been shown that arsenic, classified as a class one carcinogen, can be transported through silicon transporters in rice (*Ma* et al., 2008; *Zhao* et al., 2010; *Mitani-Ueno* et al., 2011). There are two different forms of arsenic present in rice: organic arsenic and inorganic arsenic (*Williams* et al., 2005). Organic arsenic is found in rice in two main types of molecular species, dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA), as well as tetramethylarsonium (*Williams* et al., 2005; *Hansen* et al., 2011). Inorganic arsenic is found in rice as two molecular species: arsenate and arsenite (*Abedin* et al., 2002; *Williams* et al., 2005). Arsenate is an analogue of phosphate and is taken up via phosphate transporters while arsenite is taken up by silicic acid transporters in rice (*Abedin* et al., 2002; *Ma* et al., 2008). The silicon transporters *Lsi1*, *Lsi2* and *Lsi6* are also arsenic transporters (*Ma* et al.,

2008; *Zhao* et al., 2010; *Mitani-Ueno* et al., 2011). Several studies indicate that anaerobic rice cultivation leads to increased mobilisation of soil arsenic in the form of arsenite, which causes anaerobically grown rice to accumulate more arsenic through silicon transporters (*Ma* et al., 2008; *Xu* et al., 2008; *Carey* et al., 2010). Silicon fertilisation decreases shoot and grain arsenic indicating that silicon could play an important role in decreasing total arsenic uptake in rice (*Li* et al., 2009; *Seyfferth* and *Ferdorf*, 2012). Additionally, in a hydroponic system addition of silicon reduced arsenite accumulation in rice plants (*Tripathi* et al., 2013).

This study was designed to address four questions all related to the process of silicon and arsenic accumulation in rice: 1. How does the cultivation method affect silicon distribution in different organs of rice plants? 2. Are there significant genotypic differences in shoot silicon concentration across a diverse panel of rice related to the five different sub-populations of rice? 3. Is there a relationship between natural variation in shoot silicon and arsenic content in rice? 4. Can natural variation in shoot silicon be linked to known silicon transporters in rice? The results provide a deeper understanding of the natural variation in silicon concentration across rice accessions and its relationship to arsenic accumulation in rice grains.

2 Material and methods

2.1 Silicon concentrations in different organs of rice (*Oryza sativa* L.) grown under flooded and non-flooded conditions

An experiment was conducted in a greenhouse at the University of Aberdeen, UK under both flooded and non-flooded conditions with four replicates for each treatment. One litre plastic pots were filled with soil (~530 g soil described in *Norton* et al., 2013). For the flooded

144 condition, a plastic liner was used to line the pots and hold the water within the pot whereas
145 the non-flooded pots were kept without a liner to allow drainage of water through the pot.
146 Five Italica Carolina (*temperate japonica*) seeds were sown in each pot, then thinned to one
147 plant in each pot after 2 weeks. To maintain the flooded condition, tap water from the
148 greenhouse was used to flood the pots to 2 cm above the soil surface when plants were 3
149 weeks old. Every 2 weeks during the first 4 weeks of growth 100 mL of half-strength
150 Yoshida's nutrient solution was added (*Yoshida* et al., 1976). The dose of Yoshida's nutrient
151 solution was increased up to 100 mL of full strength solution every week after 4 weeks and
152 continued until the filled grains had matured when samples were harvested.

153

154 At harvest, samples were collected from different parts of the mature plants: root, 3rd sheath,
155 3rd node, 3rd leaf, 2nd sheath, 2nd node, 2nd leaf, flag sheath, 1st node, flag leaf, husk and
156 unpolished grains. The sheaths, nodes, and leaves were taken from the main tiller, with the
157 most recent leaf prior to the flag leaf designated 2nd leaf. Root samples were washed
158 thoroughly with tap water followed by deionised water and confirmed to be free of soil
159 particles by examining the roots under a microscope (Leica MZ8, 10445932, 16×/14B, PLAN
160 1.0X). Samples were dried at 80°C for 5 d until a constant weight was achieved. All samples
161 were mixed and subsampled prior to being ball-milled. The silicon concentration was
162 determined by Flow Injection Analyser (FIA) after alkali digestion.

163

164 **2.2 Genotypic differences in shoot silicon concentration of rice**

165 Seeds were obtained from the Rice Diversity Panel 1 (RDP1) (http://ricediversity.org/) (*Ali* et
166 al., 2011; *Eizenga* et al., 2014). The classification of *Zhao* et al. (2011) was used for the sub-
167 population classification of rice accessions. In 2009, 312 accessions were cultivated at the

168 experimental site in Bangladesh. Seedlings were transplanted by hand in a single 2 m row of
169 10 hills, each hill (one seedling) 20 cm apart and each row 20 cm apart in a randomised
170 complete block design with four replicates of each accession. The experimental site was kept
171 flooded until the grains were filled. Plant material from the central six plants was harvested
172 and used for chemical analysis. Detailed information about the experimental site and
173 experimental methods are described in *Norton* et al., (2012). For shoot silicon analysis, fifty
174 accessions (10 accessions from each rice sub-population) were randomly selected based on
175 the initial sub-population assignment using single sequence repeat (SSR) markers (*Ali* et al.,
176 2011; Tab. 1). Subsequently, after selection and silicon analysis, these accessions have been
177 assigned to sub-populations based on the 700K SNP data (*McCouch* et al. 2016), these sub-
178 population assignments are used for classification of the accessions in this study.

179

180 ((Table 1))

181

182 **2.3 Analysis of rice shoot silicon by FIA**

183 Plant material and certified reference material (CRM) were prepared for silicon analysis as
184 described by *Carneiro* et al. (2007). A total of 1.5 g shoot material from each sample was
185 sub-sampled at random and powderised using a ball mill (Retsch, MM200, Germany). From
186 the powderised plant material, a sub-sample of 20 mg was weighed into a 50 mL
187 polyethylene centrifuge tube (CORNING®, NY). To digest the sample, 0.6 mL of hydrogen
188 peroxide (H_2O_2, > 30% W/V, Fisher Scientific) and 1.5 mL of sodium hydroxide (NaOH,
189 solutions 50%, Fluka) were added and the samples were then vortexed. The samples were
190 heated for 1 h at 90°C in a water bath, then vortexed again and left overnight. The tubes were
191 vortexed again after overnight extraction, then heated at 123°C under a pressure of 0.15 MPa

for 1 h. Samples were kept at room temperature for 2 h then vortexed, followed by addition of 18.5 mL of ddH_2O. Prior to analysis, samples were diluted 1 : 5 with Milli-Q water. Silicon concentration was measured using an FIA spectrophotometer (Tecator FIAstar 5010) at a wavelength of 410 nm (*Carneiro* et al., 2007; *Norton* et al., 2010a; *Norton* et al., 2010b).

2.4 Analysis of rice shoot silicon by P-XRF

A total of 1.5 g of dried shoot material for each rice accession was sub-sampled at random and powdered using a ball mill (Retsch, MM200, Germany). To perform the analysis of shoot silicon by P-XRF, 19 accessions were selected at random from the 50 accessions for which shoot silicon had been determined by FIA. For P-XRF analysis, 0.7 g of homogeneous powder sample was compressed into 13 mm diameter pellets using a manual hydraulic press with a 13mm die at a pressure of 10 tons (Specac, Orpington, United Kingdom). Shoot silicon contentration was measured using a commercial P-XRF instrument (Niton XL3t900 GOLDD analyzer: Thermo Scientific Winchester, UK), calibrated using Si-spiked synthetic methyl cellulose and validated using Certified Reference Materials of NCS DC73349 ‘Bush branches and leaves’ obtained from the China National Analysis Center for Iron and Steel, as described in *Reidinger* et al. (2012). The mean value of samples for each accession was used for correlation analysis between P-XRF and FIA measurements.

2.5 Relationship between silicon and arsenic concentrations in rice

The plant material used in this study was previously examined for grain arsenic concentration (*Norton* et al., 2012) which provided an opportunity to examine the relationship between shoot silicon and grain arsenic in rice. The relationship between shoot silicon (log transformed) and grain arsenic (log transformed) was investigated for the 50 rice accessions based on accession means.

2.6 Single-marker analysis

The accessions used in this study have been genotyped using a high-density SNP chip (*McCouch* et al., 2016). SNPs for the accessions were extracted using PLINK (*Purcell* et al., 2007). SNPs were extracted from 10 kb upstream of the start codon to 10 kb downstream of the stop codon of the *Lsi1, Lsi2, Lsi3,* and *Lsi*6 loci. SNPs were excluded from the analysis if they were invariant or if minor alleles were present in less than 5% of the accessions. The RDP1 population has a high degree of stratification by rice sub-population (*Zhao* et al., 2011; *McCouch* et al., 2016). To overcome this stratification, sub-population assignment was used (based on the 700 K SNP data; *McCouch* et al., 2016) as a factor in a two-way ANOVA, with SNP base call as the other factor. The two-way ANOVA was used to determine if the phenotype for the accession was significantly different for each SNP tested.

2.7 Sequence alignments

Based on the result achieved from the single-marker analysis the sequence diversity of *Lsi2* and *Lsi3* were investigated for five accessions using BAM files produced after aligning sequence reads against Nipponbare version 7 reference genome. The genome sequences of the accessions used in this study have been previously published (*Kawahara* et al., 2013; *Cardoso* et al., 2014; *Schatz* et al., 2014). The accessions were from the following sub-populations: two *indica* accessions (IR64 and Bala), one *aus* accession (DJ123), and two *tropical japonica* accessions (Azucena and Nipponbare). The genomic DNA sequence was visualised using the IGV (https://www.broadinstitute.org/igv/) to identify the difference of genomic DNA sequence within *Lsi2 and Lsi3* in vive accessions (*Thorvaldsdóttir* et al., 2013;

239 *Robinson* et al., 2011). Using Clustal Omega the DNA sequences of five accessions were
240 aligned for *Lsi2* and for *Lsi3* (*Sievers et al.*, 2011).

241 **2.8 Statistical analysis**

242 Statistical significance was set at $P < 5\%$ for all analyses, which were performed using
243 Minitab 16. The normality of distribution and homogeneity of variance of the data were
244 tested prior to one or two-way analysis of variance (ANOVA), as appropriate. Pearson
245 correlation analysis was used to investigate the relationship between measurements of shoot
246 silicon and grain arsenic.

247

248 # 3 Results

249 **3.1 Shoot silicon concentrations in different organs of rice plants**

250 Flooding increased plant silicon concentrations in the flag sheath, 1st node, flag leaf, and husk
251 compared to plants grown under non-flooded conditions (Fig. 1). The silicon concentrations
252 in grain and root tissues were significantly lower than in other organs of plants grown under
253 either condition. There was a significant difference ($P < 0.1\%$, $F = 27.40$, $R^2 = 78.20\%$) of
254 silicon concentration between different organs of the plant under non-flooded conditions: The
255 highest mean concentration was in the husks (46.8 mg g^{-1}), while the lowest was in the grain
256 (3.5 mg g^{-1}). For plants grown under flooded conditions the highest silicon concentration was
257 observed in the flag leaf (67.3 mg g^{-1}) and the lowest was in the grains (4.4 mg g^{-1}).

258

259 ((Figure 1))

260

3.2 Genotypic difference in shoot silicon content of rice

Fifty diverse rice accessions were examined by FIA to determine the difference in shoot silicon concentration of rice. There was a significant genotypic difference in shoot silicon concentrations among the 50 accessions, where genotype explained 55% of the variation ($P < 0.1\%$; $F = 5.80$; $R^2 = 55.30\%$; $df = 49$). The mean shoot silicon content of the 50 accessions was 28.1 mg g^{-1}, and the lowest mean shoot silicon was observed in Dhala Shita (16.5 mg g^{-1}) The highest mean shoot silicon was observed in Bala (42.4 mg g^{-1}; Fig. 2). There was no significant difference for shoot silicon content of the 5-major rice sub-populations (Fig. 3).

((Figure 2))

((Figure 3))

Nineteen rice accessions were selected at random from the 50 accessions analysed by FIA, for measurement of shoot silicon concentration by P-XRF. The silicon concentrations of four individual field-grown replicates of each accession were measured separately by P-XRF and FIA and the mean value of each accession was used for correlation analysis. Using both methods, genotypic differences were observed between the accessions ($P < 0.1\%$; $F = 9.90$; $df = 18$ for P-XRF; $P < 0.1\%$; $F = 7.30$; $df = 18$ for FIA). Correlation analysis indicated that there was a significant and large positive correlation between the two methods ($r = 0.95$; $P < 0.1\%$; $df = 18$; Fig 4).

((Figure 4))

3.3 Correlation between shoot silicon and grain arsenic in rice

No significant correlation was observed between mean shoot silicon and mean shoot arsenic for all of the 50 accessions, or for within each of the five sub-populations. There was a weak negative correlation (r = -0.31; P = 0.028; df = 49) (data not shown) between shoot silicon and grain arsenic concentrations for all 50 accessions. When correlation analysis was conducted separately for shoot silicon and grain arsenic on each of the sub-populations, significant negative correlations were found for the *temperate japonica* (r = -0.78; P = 0.7%; df = 9) and *tropical japonica* (r = -0.84; P = 0.2%; df = 9) accessions (Fig. 5). No significant correlations were observed for the other three major rice sub-populations (*indica, aus* and *aromatic*).

((Figure 5))

3.4 Testing accessions with different alleles of SNPs around and within *Lsi* genes for variation in shoot silicon concentration

A total of 10 SNPs from the SNP database are within 10 kb upstream and downstream of the *Lsi2* gene. Shoot silicon concentrations for accessions with the different alleles for two of these SNPs were significantly different. SNP-3.434426 is located 2551 bp before the start codon and revealed a significant difference between the C and T polymorphism (P = 0.6%), where the mean silicon concentration of accessions with the C allele was 29.3 mg g^{-1} while the mean silicon concentration of the accessions with the T allele was 23.1 mg g^{-1}. SNP-3.438416 is located 6541 bp before the start codon and revealed a significant difference between the A and C polymorphism (P = 0.8%), where the mean silicon concentration of the accessions with the A allele was 29.6 mg g^{-1} while the silicon concentration of the accessions

with the G allele had a mean of 23.1 mg g^{-1}. Both SNPs group the accessions in a similar way, the only difference was more missing SNP information for SNP-3.438416 (Fig. 6).

((Figure 6))

A total of 20 SNPs from the SNP database are within 10 kb upstream and downstream of the *Lsi3* gene. Shoot silicon concentration for accessions with the different alleles for one of these SNPs was significantly different. SNP- 10.21340470 is located 5299 bp prior to the start codon, and revealed a significant difference between the G and A polymorphism (P = 1.6%), where the mean silicon content of accessions with the G allele was 28.4 mg g^{-1} while the mean silicon content of the accessions with the A allele was 35.6 mg g^{-1} (Fig. 6). There were 20 SNPs and 19 SNPs within 10 kb of *Lsi1* and *Lsi6* respectively. However, at each of these SNPs the different alleles were not significantly different for shoot silicon concentration.

To explore further, the sequence alignments of *Lsi2* and *Lsi3* were performed using available high-quality genome sequences. The accessions used were Nipponbare, Azucena, IR64, Bala, and DJ123 which are from the *tropical japonica*, *tropical japonica*, *indica*, *indica*, and *aus* rice subgroups respectively. From the sequence analyses of *Lsi2* and *Lsi3* a number of polymorphisms within the genes were identified. For *Lsi2*, there was a synonymous SNP substitution within the first exon, where DJ123 has “C” allele while the other four accessions have “T” allele. For *Lsi3*, 4 SNPs were detected in exons and 6 SNPs in introns. There was only one non-synonymous SNP observed in the first exon of *Lsi3* where DJ123 and Bala have “T” allele and other accessions have “A” allele. The available 3000 rice genome

sequence data indicate that this polymorphism between "A" and "T" in *Lsi3* is associated with the *aus* sub-population in rice where 15 accessions have "A" allele and 184 accessions have "T" allele (*Alexandrov* et al., 2015). This non-synonymous polymorphism between "A" and "T" in *Lsi3* with the "T" allele is very rarely observed in *indica* and *japonica* subpopulations of rice in 3000 rice genome sequence data (*Alexandrov* et al., 2015).

4 Discussion

In this study, genotypic differences in shoot silicon concentration were identified from field-grown rice accessions. However, no differences in shoot silicon were observed across the five different sub-populations of rice. Additionally, SNPs detected in the accessions were significantly linked to known silicon transporter genes in rice, which indicates that these genes are potentially involved in the natural variation of silicon accumulation in rice.

Flooded conditions increased silicon concentration in the upper part of the plant (flag sheath, 1st node, flag leaf and husk) compared to the non-flooded conditions, which suggests that the uptake or translocation of shoot silicon into these plant organs might be controlled by different processes (compared to those determining silicon uptake in other tissues) which differ between aerobic and anaerobic conditions. It has been shown that silicon dissolution and bio-availability plays a significant role in the variation of silicon concentration in grasses (*Quigley* et al., 2017). Therefore, the difference in dissolved silicon in flooded and non-flooded conditions might affect the accumulation of silicon in the rice plants used in this study. It was also notable that there was no significant difference in silicon concentration in different tissues between the internodes (*e.g.* flag leaf, 2nd leaf) under non-flooded conditions but there was a significant difference between the silicon concentration of internodes under

356 flooded conditions (Fig. 1). Previous studies have shown that transpiration is one of the most
357 important factors responsible for higher *silicification* in plants and that transpirational flow is
358 higher under anaerobic conditions than under aerobic ones (*Mitani-Ueno* et al., 2005; *Kato*
359 and *Okami*, 2011; *Kumar* et al., 2017; *McLarnon* et al., 2017). Therefore, one potential
360 explanation for increased silicon accumulation in the upper organs or developing organs of
361 rice plants (e.g. flag sheath, 1st node, flag leaf and husk) grown in flooded soils is a higher
362 transpirational flow in these plants. Importantly, the data presented in Fig. 1 show that tissue
363 silicon concentration is reasonably evenly distributed across tissues with only that from
364 flooded plants in tissue associated with flowering and seed production significantly higher
365 than the rest. Since this reproductive tissue was removed from the field samples used in this
366 study we can be reassured that a mean value obtained from straw will be a good estimate of
367 the tissue concentration of the majority of rice plant.

368

369 Fifty accessions from five different sub-populations (ten accessions from each sub-
370 population) were selected at random to examine the difference of shoot silicon concentration
371 in rice, and this revealed highly significant differences of shoot silicon concentration. A
372 genotypic difference in shoot silicon concentration across a wide group of accessions has
373 been observed previously (*Deren*, 2001; *Norton* et al., 2010a). The 2.6-fold difference of
374 shoot silicon content in this study is similar to the previous 2.2 fold range detected for
375 genotypic differences of shoot silicon concentration in rice (*Norton* et al., 2010a). However,
376 the maximum value observed in our study is slightly lower than that detected previously
377 (42.4 mg g^{-1} in this study, 60 mg g^{-1} (*Deren*, 2001), 61 mg g^{-1} (*Norton* et al., 2010a).

378

379 The plant material used for determination of shoot silicon concentration in the 50 rice
380 accessions was grown under flooded, irrigated conditions (*Norton* et al., 2012). Previous

studies estimated that 27-44% of the silicon taken up by rice plants is supplied by irrigation, while the remaining percentage must be supplied by soil constituents (*Desplanques* et al., 2006; *Klotzbücher* et al., 2015). All the accessions tested in this study had a silicon concentration below 50 mg g^{-1} which, according to *Dobermann* and *Fairhurst* (2000), is below the critical level of mineral deficiency for rice production. The low shoot silicon concentration (16.5-42.4 mg g^{-1}) observed in this study may be due to removal of rice straw from the paddy field, which is common practice in Bangladesh, and has been shown to contribute to lower shoot silicon in the subsequent rice crop (*Seyfferth* et al., 2013). Future work should focus on linking the flooded and non-flooded pot-based experiment and the removal of straw at the field scale to establish the importance of water management and field management on silicon accumulation in field-grown rice.

Several studies have demonstrated that the *Japonica* sub-species of rice have higher shoot silicon than *Indicas* (*Winslow*, 1992; *Winslow* et al., 1997; *Ma* et al., 2007a). These studies may have been limited by the number of accessions that were screened. For example, *Ma* et al. (2007b) only screened two rice accessions to examine the genotypic difference in silicon uptake of rice. To improve the current understanding of silicon biology in rice, we investigated field-grown shoot samples of 50 rice accessions across five sub-populations. Within the material tested in this study the data suggest that the natural variation observed in shoot silicon is not governed by genetic differences between rice sub-populations, but rather is largely due to the genetic differences within individual sub-groups.

Data on more than 50 accessions would have opened the opportunity to conduct genome-wide association (GWA) mapping where 200 accessions is considered a lower limit. However, the FIA colorimetric method for the determination of silicon in rice shoots proved

406 not to be high throughput. However, in addition to the FIA method, a sub-set of samples
407 was also analysed by P-XRF. The two different methods were strongly correlated, but not
408 perfectly, and indicated that values for silicon concentration in samples measured by FIA
409 were slightly higher than those measured by P-XRF. The observation that both methods
410 provide comparable results highlights the conclusion that P-XRF can be used for silicon
411 analysis to detect and measure genotypic differences across populations, instead of the more
412 laborious and time-consuming alkali digestion method. Furthermore, a second advantage of
413 P-XRF is that it is a non-destructive method. This would make it much more suitable for
414 future GWA mapping studies.
415
416 The plant material used in this study was previously used to examine the variation of shoot
417 and grain arsenic (*Norton* et al., 2012). The comparison of shoot silicon and grain arsenic in
418 this study is in agreement with previous studies where, in general, plants that had high shoot
419 silicon also had lower grain arsenic (*Seyfferth and Ferdorf*, 2012; *Norton* et al., 2012; *Norton*
420 et al., 2013). However, this study also adds more insight by taking into consideration the sub-
421 population structure of rice accessions. The correlation between shoot silicon and grain
422 arsenic was sub-population specific. A strong relationship between shoot silicon and grain
423 arsenic was observed in *temperate japonica* and a weaker one in *tropical japonica*, but was
424 not observed in *indica*, *aus* or *aromatic*. This important observation suggests that the genetic
425 control of arsenic concentration in rice grain is different in *temperate* and *tropical japonicas*
426 compared to the other rice sub-populations, implying that the silicon-transport-linked
427 pathway implicated for arsenic accumulation (*Ma* et al., 2007b; *Norton* et al., 2012) may be
428 less relevant in the *other* sub-populations. Genotypic variation in rice for arsenic
429 accumulation maybe due to the impacts that silicon has on improving the antioxidant defence
430 system. *Tripathi* et al. (2013) observed that silicon addition in hydroponics, significantly

ameliorated arsenic induced oxidative stress in an arsenic-resistant accession, by lowering arsenic accumulation and improving antioxidant and thiolic systems compared to an arsenic-sensitive accessions.

The accessions used in the study have been genotyped using a 700K SNP chip (*McCouch* et al., 2016). Single-marker analysis was used to test the candidacy of the known transporters of silica in rice. The study indicates that two SNPs within 10 kb of *Lsi2* and one within 10 kb of *Lsi3* were involved in contributing to the natural variation of shoot silicon accumulation in rice (Fig. 6). The *Lsi2* gene has been shown to be pivotal for transport of silicon and inorganic arsenic in studies conducted with mutants and transgenic plants (*Ma* et al., 2006; *Ma* et al., 2007b; *Yamaji* et al., 2008; *Mitani-Ueno* et al., 2011; *Yamaji* et al., 2015). The identification of differences in shoot silicon and the link with three SNPs close to the genes further suggest that *Lsi2* and *Lsi3* are excellent candidate genes to explain the natural variation observed in shoot silicon concentration of rice. When looking at the sequencing variation of a number of diverse accessions (which have been sequenced to a high depth) it is evident that there is only a small number of polymorphisms within the genes. The highly conserved sequence for *Lsi2* may be due to its important function for silicon accumulation in rice. However, the accessions screened in this study are likely to have greater sequence variation than the accessions for which high-quality sequence is available, and therefore there may be greater sequence variation for *Lsi2* (and the other *Lsi* genes) than that is represented in the five accessions reported here. A focus for future study will be to expand sequence information to more accessions to more fully explore sequence variation associated with the polymorphic SNPs presented in Fig. 6.

5 Conclusions

This study has demonstrated strong genotypic differences in shoot silicon in a diverse collection of rice accessions, showing that there is potential to breed rice with increased silicon concentration that could improve resistance to both biotic and abiotic stresses in rice, which would help to maintain crop yields. The identification of significant SNPs linked with the shoot silicon phenotype within 10 kb of known silicon transporters warrants further study to investigate the impact of different alleles of these genes on silicon and arsenic accumulation in rice. Furthermore, the XRF method of silicon determination could be applied to GWA-mapping studies that might reveal further candidate genes for silicon concentration in rice.

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643 **Table 1:** Selected genotypes from RDP1 for shoot silicon analysis**.**

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RDP1 passport id	Genotype	Origin	Rice sub-population
221	SADRI BELYI	Azerbaijan	*aromatic*
16	BICO BRANCO	Brazil	*aromatic*
14	BASMATI 217	India	*aromatic*
112	N12	India	*aromatic*
5	ARC 10352	India	*aromatic*
160	TCHAMPA	Iran	*aromatic*
53	FIROOZ	Iran	*aromatic*
191	DOM ZARD	Iran	*aromatic*
55	GERDEH	Iran	*aromatic*
93	KITRANA 508	Madagascar	*aromatic*
58	GHATI KAMMA NANGARHAR	Afghanistan	*aus*
44	DHALA SHAITTA	Bangladesh	*aus*
50	DZ78	Bangladesh	*aus*
228	CA 902 B 2 1	Chad	*aus*
4	ARC 10177	India	*aus*
19	BLACK GORA	India	*aus*
336	PAUNG MALAUNG	Myanmar	*aus*
200	P 737	Pakistan	*aus*
378	KALUBALA VEE	Sri Lanka	*aus*
88	KHAO GAEW	Thailand	*aus*
21	BYAKKOKU Y 5006 SELN	Australia	*indica*
106	MING HUI	China	*indica*
252	DJIMORON	Guinea	*indica*
354	BALA	India	*indica*
57	GHARIB	Iran	*indica*
315	DAWEBYAN	Myanmar	*indica*
71	IR 36	Philippines	*indica*
298	LD 24	Sri Lanka	*indica*
156	TAICHUNG NATIVE 1	Taiwan	*indica*
385	NIRA	United States	*indica*
220	AZERBAIDJANICA	Azerbaijan	*temperate japonica*
155	TA MAO TSAO	China	*temperate japonica*
245	SAB INI	Egypt	*temperate japonica*
204	RAZZA 77	Italy	*temperate japonica*
263	MARATELLI	Italy	*temperate japonica*
94	KOSHIHIKARI	Japan	*temperate japonica*
113	NORIN 20	Japan	*temperate japonica*
279	KON SUITO	Mongolia	*temperate japonica*
289	LUSITANO	Portugal	*temperate japonica*
291	TOPLOEA 70 76	Romania	*temperate japonica*
107	MIRITI	Bangladesh	*tropical japonica*
46	DOURADO AGULHA	Brazil	*tropical japonica*

108	MOROBEREKAN	Guinea	*tropical japonica*
48	DULAR	India	*tropical japonica*
122	PADI KASALLE	Indonesia	*tropical japonica*
116	NPE 844	Pakistan	*tropical japonica*
174	AZUCENA	Philippines	*tropical japonica*
40	DAM	Thailand	*tropical japonica*
96	KU115	Thailand	*tropical japonica*
101	LEMONT	United States	*tropical japonica*

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Legends of figures:

Figure 1: Silicon concentrationsin different organs of rice (columns are the means of four replicates and error bars represent standard errors of the means). Letters above the columns (upper-case = anaerobic and lower case = aerobic) indicate statistically significant differences in silicon concentration of different plant organs using Tukey's test in two conditions. *denotes a significant difference between the two treatments for that plant organ.

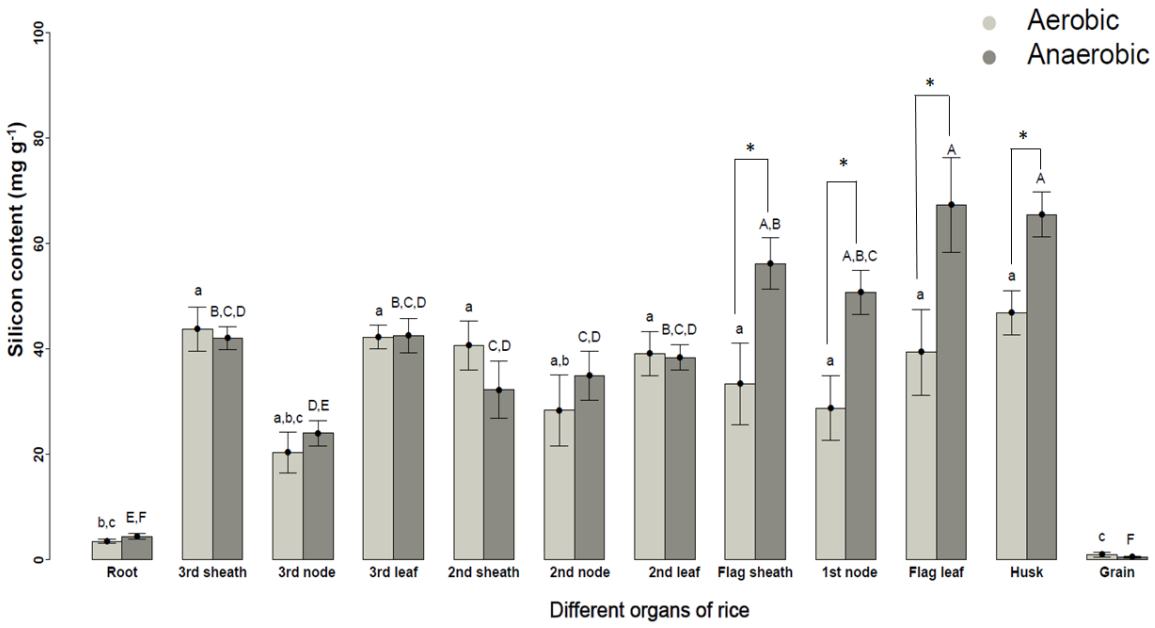
Figure 2: Mean shoot silicon concentrations of 50 rice accessions determined by FIA. Different symbols refer to the accessions belonging to the different sub-populations; circle = *aus*, square = *indica*, cross = *aromatic*, triangle = *tropical japonica*, upside down triangle = *temperate japonica*. Error bars indicate the standard errors of the means (n = 4).

Figure 3: Shoot silicon concentrations of 50 accessions in five different sub-populations of rice. ARO = *aromatic*, AUS = *aus*, IND = *indica*, TEJ = *temperate japonica* and TRJ = *tropical japonica*. The edges of each box show the upper and lower quantile and the bold line in the box shows the median value and the dotted line the mean value. The whiskers are the 10^{th} and 90^{th} percentiles.

Figure 4: Correlation of mean shoot silicon concentrations in 19 rice accessions determined by FIA and P-XRF. Error bars indicate the standard errors of the means (n = 4). Dotted line is the 1 : 1 line.

Figure 5: Correlation between shoot silicon concentrations and grain arsenic concentrations in ARO= *aromatic*, AUS = *aus*, IND = *indica*, TEJ = *temperate japonica* and TRJ = *tropical japonica* subpopulations.

Figure 6: Variation in shoot silicon concentration between different SNPs within 10 kb of *Lsi2* and *Lsi3*



696

697 **Figure 1**

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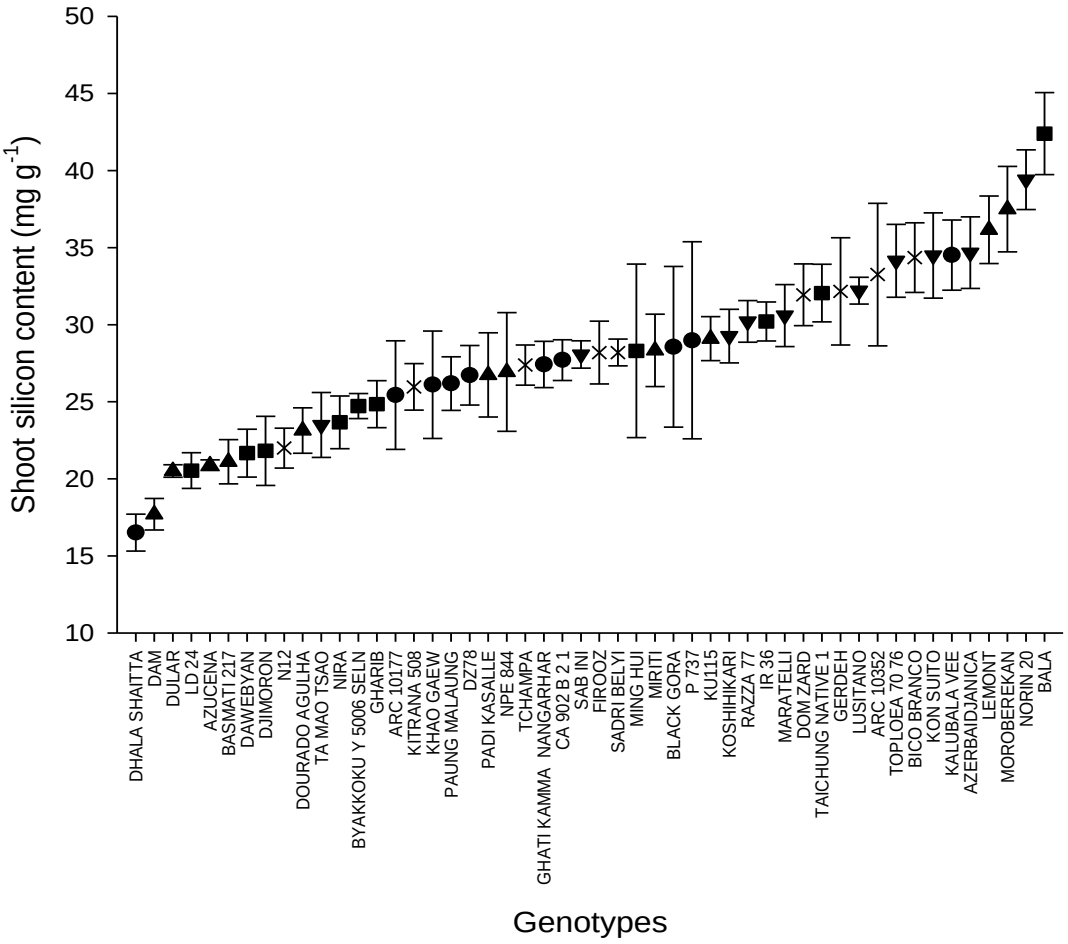

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701 **Figure 2**

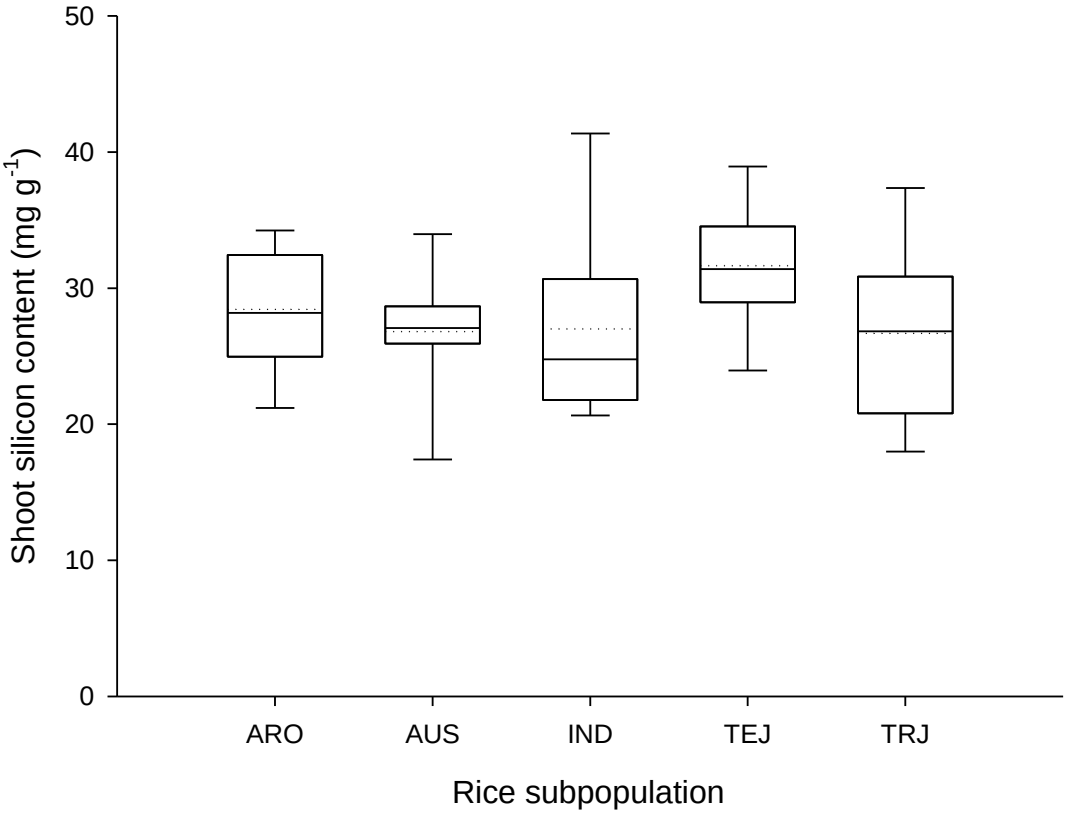
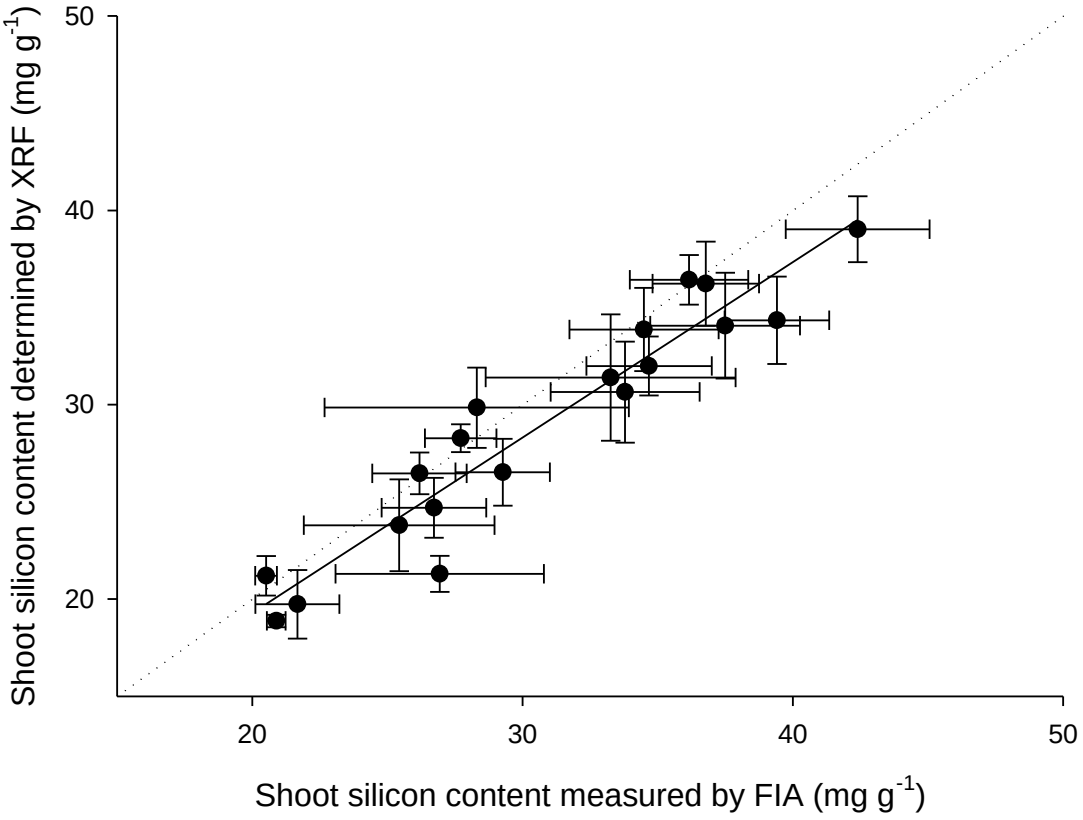

50
40
30
20
10
0
Shoot silicon content (mg g-1)
ARO
AUS
IND
TEJ
TRJ
Rice subpopulation

Figure 3



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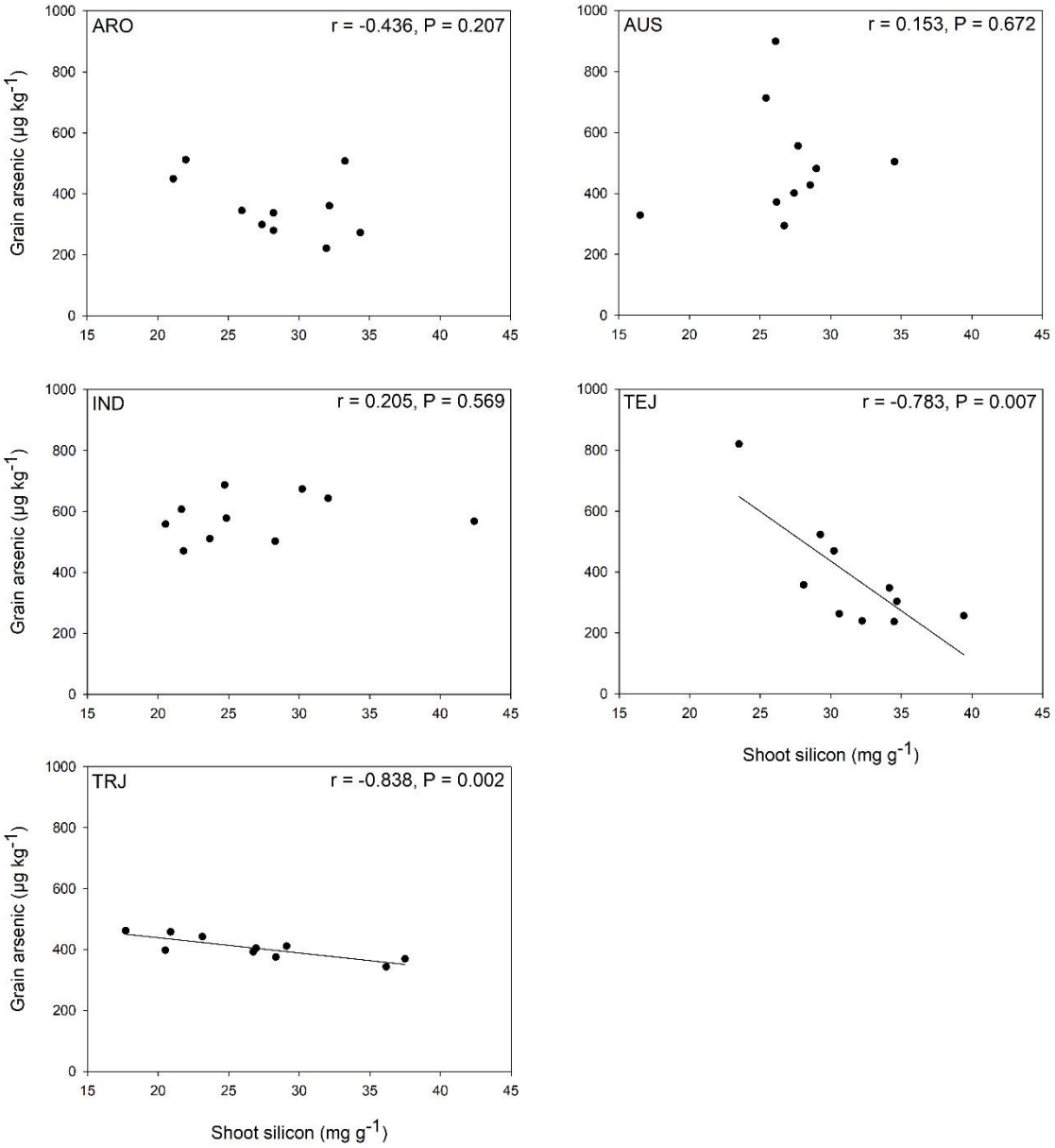
707 **Figure 4**



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709 **Figure 5**

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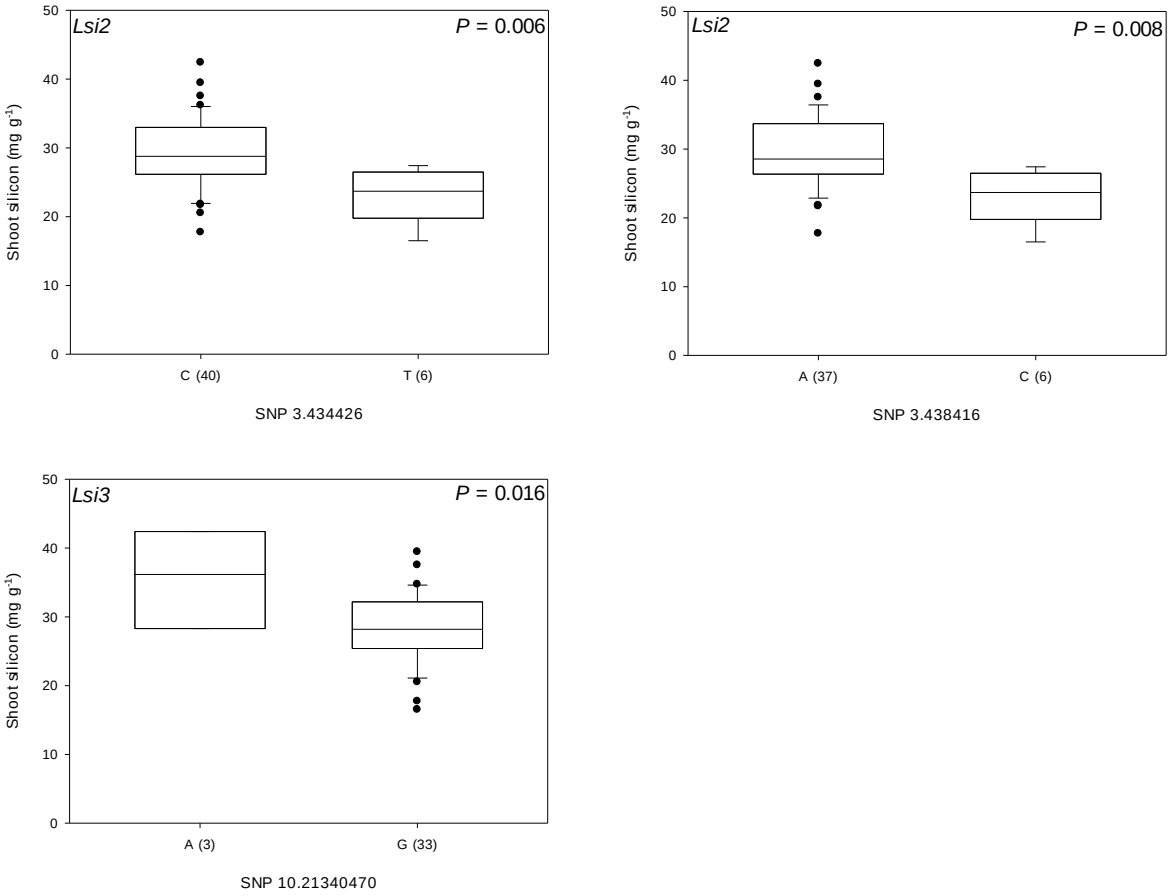
Lsi2
P = 0.006
Shoot silicon (mg g-1)
C (40)
T (6)
SNP 3.434426
Lsi2
P = 0.008
A (37)
C (6)
SNP 3.438416
Lsi3
P = 0.016
A (3)
G (33)
SNP 10.21340470

Figure 6