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The Presence of Silica Bodies in the Foliar Epidermis of Zoysiagrass

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ABSTRACT

The presence of silica bodies (SBs) in the foliar epidermis of zoysiagrass (*Zoysia* spp. Willd.) is a special characteristic, the function of which is not well understood. To quantify the importance of SB, we evaluated foliar SB densities and sizes of five *Zoysia* species [*Z. japonica* Steud., *Z. matrella* (L.) Merr., *Z. pacifica* (Goudsw.) M. Hotta & S. Kuroki, *Z. minima* (Colenso) Zotov, and *Z. macrostachya* Franch. & Sav.] including three ecotypes of each species using tabletop scanning electron microscopy. Our results demonstrated that abaxial SB density was significantly higher (mean: 734.3 mm^{-2}) than that of adaxial SB (mean: 317.7mm^{-2}) in all species. Abaxial SB size was also significantly larger (mean: 80.0 μm^2) than that of the adaxial SB (mean: 61.4 μm^2) in all species. *Zoysia japonica* had significantly higher SB density and size than the other tested *Zoysia* spp. Location-specific sizes of foliar abaxial SB were identified: large on the midrib, medium on other ribs, and small in the costal zone. These results support the hypothesis that foliar SBs of zoysiagrass function to protect rib bundles and Kranz anatomy from physical damages, and to assist light absorbance for C$_4$ photosynthesis through inhibiting leaves from twisting. Further work assessing SB impact on growth efficiency, survival strategy, and the utility of zoysiagrass is needed.

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Abbreviations: SB, silica body; SEM, scanning electron microscopy.

SILICON (Si) is the second most abundant element in soils, making up 23 to 40% of bulk material (Carrow et al., 2001). It is taken up by plants from the soil and accumulated in tissues. Grasses tend to assimilate more Si into their tissue than other plants. Silicon accumulation has been linked to many improvements in plant health and tolerance to stress (Carrow et al., 2001; Ma and Yamaji, 2006). The difference in Si accumulation between species has been linked to the presence of Si transporters in the roots (Ma and Yamaji, 2006). In the angiosperms, the Commelinoid monocot orders Poales and Arecales accumulate more Si in their shoots than species from other monocot clades (Ma and Takahashi, 2002). Within Poales, the families *Cyperaceae* and *Poaceae* gather notably high amounts of Si (Ma and Takahashi, 2002). This paper will focus on the silica body (SB) variability among species of the *Poaceae* genus *Zoysia* Willd. (zoysiagrass).

Silica bodies, called phytoliths or plant opals, have been observed and studied by taxonomic botanists (Chen et al., 1996; Prychid et al., 2004), environmental biologists, archeologists, and paleontologists (Sangster and Hodson, 1986; Piperno and Flannery, 2001; Prasad et al., 2011) because of their extreme durability. They are formed when silicon (Si) is accumulated in a specialized silica cells

or on a foliar SB structure from amorphous hydrated silica ($SiO_2 \cdot nH_2O$) (Chen et al., 1996). Polymerization occurs, forming solid Si-based structures whose utility is not well defined. The hypothesized functions of SBs include providing physical strength against attacks by pathogens and pests, resistance to physical wear, and leaf rigidity against twisting, allowing for better sunlight absorption for photosynthesis (Carrow et al., 2001; Prychid et al., 2004; Hartley et al., 2015; Dabney, III, et al., 2016). *Zoysia* leaves are reportedly higher in silica than many other grasses (Ruemmele and Engelke, 1990). The physiological impacts of this are described as *Zoysia* leaves being tougher, more fibrous, and difficult to cut (Beard, 1973; Duble, 1996; Loch, 2015).

Recent work has shown the importance of turfgrass in limiting global warming because of its high potential to fix atmospheric carbon dioxide and store energy (Agata, 2008). Turfgrass can be compared to pelagic phytoplankton, as both have very high ratios of production to biomass (Whittaker, 1970; Agata, 2008). Recently zoysiagrass has become of interest because of easy management and high tolerance to heat, drought, disease, and wear in warming climates. One special physiological characteristic of zoysiagrass is the presence of SBs on the foliar epidermis (Carrow et al., 2001). Silica bodies are not a conserved trait across the Poaceae and seem to be absent from cool-season turfgrasses such as creeping bentgrass (*Agrostis stolonifera* L.) and Kentucky bluegrass (*Poa pratensis* L.) (Ushilo, unpublished data, 2014). In contrast, SBs have been reported in warm-season grasses (e.g., buffalograss [*Buchloe dactyloides* (Nutt.) Engelm.] and switchgrass [*Panicum virgartum* L.]) (Twiss et al., 1969).

The SB of zoysiagrass has not been studied except for a brief mention in Watson et al. (1992). In this study, we observed intraspecific and interspecific variability of SB density, distribution, and size in the foliar epidermis in five species of zoysiagrass. This is the first example of using a tabletop scanning electron microscopy (SEM) to observe many specimens of turfgrass for quantitative comparisons and also the first attempt to clarify SB density and size differences among intra– and interspecific *Zoysia* spp. The results are discussed in relation to the potential for more use of zoysiagrass due to global warming.

MATERIALS AND METHODS

Seventeen ecotypes and cultivars of zoysiagrass from five species [*Z. japonica* Steud., *Z. matrella* (L.) Merr., *Z. pacifica* (Goudswaard) M. Hotta & S. Kuroki, *Z. minima* (Colenso) Zotov, and *Z. macrostachya* Franch. & Sav.] were evaluated for SB in this study (Table 1).

All ecotypes except cultivar 'Himeno' (*Z. japonica*) and an ecotype 'ZJ' (*Z. pacifica*) had been collected from indigenous lands by Tokai University (Table 1). The former *Zoysia* spp. were grown in culture soil with Akadama (red clay beads) on the pot bottoms and fertilized with Kenbyo (0.25 g N L^{-1}, 1.1 g P$_2$O$_5$ L^{-1}, 0.3 g K$_2$O L^{-1}; Hakkounougei). Plants were grown outdoors at Aso campus of Tokai University. The cultivar

Table 1. Zoysiagrass species ecotypes or cultivar evaluated for silica body density, size, and Si mapping using scanning electron microscopy and energy-dispersive X-ray spectrometry.

Zoysia sp.	Ecotype (E) or cultivar (C)	Source	Si mapping
Z. japonica	O44 (E)	Oita, Japan	
	TG1 (E)	Tanegashima, Kagoshima, Japan	
	J63 (E)	Thai	
	Himeno (C)	Zoysian Japan Company, Ltd.	x
Z. matrella	KS10 (E)	Koshikijima, Kagoshima, Japan	
	W9 (E)	Taketomijima, Okinawa, Japan	x
	T25 (E)	Taiwan	
Z. pacifica	KM15 (E)	Kumejima, Okinawa, Japan	
	Tg54 (E)	Tanegashima, Kagoshima, Japan	
	Y2 (E)	Kagoshima, Japan	
	ZJ (E)	Zoysian Japan Company, Ltd.	
Z. minima	V101 (E)	New Zealand	
	V102 (E)	New Zealand	
	V103 (E)	New Zealand	
Z. macrostachya	F11 (E)	Fukuoka, Japan	
	K27 (E)	Toyama, Japan	
	OT6 (E)	Oita, Japan	

Himeno and the ecotype ZJ had been cultured at Zoysian Japan Company, Hiroshima, Japan.

A tabletop SEM (HITACHI-Miniscope TM3000) was used to observe and characterize SB in the leaves. For evaluating SB, five leaves of each ecotype or cultivar were randomly sampled from different shoots in several plant pots. At the middle part of each leaf, two pieces were cut for adaxial (upper) and abaxial (lower) side observation. Ecotype ZJ (*Z. pacifica*) has needle-shaped or rolled leaves, to observe its adaxial side, we had to cut and manually open them. Five leaf pieces for the adaxial side and five pieces for the abaxial side of each ecotype or cultivar were analyzed. Samples were attached to the conductive double-sided tape, set on the sample stage in the SEM, and SB density was measured at ×200 with SB size measured at ×400 magnification. Ten SEM images for adaxial or abaxial side were taken at random for each sample. Silica body numbers were counted per 0.25 mm^2. To measure the average SB length and width, three SBs were randomly selected per adaxial or abaxial image and values averaged (three subsamples per image). Only the 15 ecotypes grown at Tokai University were used for exact comparison of SB density and size, as different culture conditions, especially Si supply difference, have been reported to influence SB formation (Schaller et al., 2012).

To verify SB Si content and map Si distribution, energy-dispersive X-ray spectrometry (EDX) connected with the SEM was used. Silicon distribution mapping was performed on foliar adaxial and abaxial samples of cultivar Himeno (*Z. japonica*) and ecotype 'W9' (*Z. matrella*) with three replicates for each cultivar or ecotype.

All data were analyzed using the software OriginPro (OriginLab, 2016). Two-way parametric ANOVA with replicates were completed to determine the effects of foliar adaxial or abaxial side and species or ecotype for SB density and size. When the criteria were met for ANOVA at the 0.05 probability level, mean separation tests were performed using Tukey's test at the 0.05 probability level.

RESULTS

Silica bodies were observed in large numbers on both adaxial and abaxial sides of the leaves of all but two of the *Zoysia* spp. examined (Tables 2 and 3). Silica bodies were only found on the abaxial side of cultivar Himeno (*Z. japonica*) and ecotype ZJ (*Z. pacifica*) (Table 3).

SB Density and Size

The density of SB on the abaxial side was significantly higher than that on the adaxial side for all species and ecotypes (Tables 2 and 3). When averaging all species tested the mean density of abaxial side SB was significantly larger (734.3 mm^{-2}) than that of the adaxial side (317.7 mm^{-2}). *Zoysia. japonica* had a significantly higher abaxial SB mean density (885.6 mm^{-2}) than the five examined *Zoysia* spp. (Table 2). Notably, the cultivar Himeno (*Z.*

Table 2. Mean density and size of foliar silica body of five *Zoysia* spp.

Species	Density (*n* = 30)		Size (*n* = 90)					
			Length		Width		Area = length × width	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
	—— no. 0.25 mm^{-2} ——		——————————————— μm ———————————————				——— μm^2 ———	
Z. japonica	85.7†	221.4c	9.0a	11.0d	7.6ab	10.6c	69.1a	116.8e
Z. matrella	87.3a	154.8f	8.2bc	8.5b	6.6c	8.2de	54.8b	70.3c
Z. pacifica	81.8ab	194.4d	7.7c	8.3bc	6.4c	7.4f	49.9b	62.4d
Z. minima	80.0ab	181.4de	8.6ab	8.9b	8.0a	8.7d	69.4a	78.3b
Z. macrostachya	62.3b	165.9ef	8.6ab	8.9b	7.3b	8.0e	63.7a	72.2bc

† Means followed by same letters within a column or within a pair of "adaxial" and "abaxial" of the same species in each category are not significantly different, using Tukey's test at the *p* < 0.05.

Table 3. Mean density and size of foliar silica body of five *Zoysia* spp. with three ecotypes grown under same condition except for two of cultivar 'Himeno' (*Z. japonica*) and ecotype 'ZJ' (*Z. pacifica*) arranged under double lines.

	Density (*n* = 10)		Size (*n* = 30)					
			Length		Width		Area = length × width	
	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial	Adaxial	Abaxial
	—— no. 0.25 mm^{-2} ——		——————————————— μm ———————————————				——— μm^2 ———	
Z. japonica								
O44	101.0 a†	221.1 b	9.7a	12.6b	7.6a	10.8b	73.8 a	136.6 b
TG1	78.4 a	194.0 c	8.6b	10.0c	7.6a	11.3b	65.3 a	113.1 c
J63	77.6 a	249.1 d	8.8b	10.3c	7.7a	9.7c	68.3 a	100.7 d
Z. matrella								
KS10	72.7 a	157.4 c	8.6a	8.9a	6.9a	8.6c	60.5 a	77.9 c
W9	94.1 ab	152.9 c	8.3ab	7.9bc	6.6ab	7.8d	55.6 ab	62.6 a
T25	95.2 b	154.2 c	7.7b	8.5ac	6.3b	8.2cd	48.4 b	70.5 ac
Z. pacifica								
KM15	74.1a	190.3bc	7.2a	7.5a	6.2a	6.9b	45.4a	52.0c
Tg54	93.4a	230.8b	7.7a	9.9b	6.9b	8.3a	53.7b	83.2d
Y2	77.9a	162.0c	8.3b	7.5a	6.1a	6.9b	50.7ab	52.0ac
Z. minima								
V101	68.9a	152.3b	8.6a	8.9a	8.3a	10.0b	71.7a	90.0c
V102	88.0a	206.0c	7.5b	7.8b	7.7a	7.7a	58.0b	60.4bd
V103	83.2a	186.0c	9.7c	9.9c	8.0a	8.4a	78.3a	84.3ac
Z. macrostachya								
F11	66.2a	177.1b	8.2a	8.5ac	7.3a	8.0c	61.7a	68.7a
K27	66.7a	148.4c	7.8a	8.6c	6.5b	6.7b	51.1b	58.1b
OT6	54.0a	172.2b	9.6b	9.6bd	8.0c	9.3a	78.2c	89.8d
Z. japonica								
Himeno	0.3	134.6	0	9.1	0	8.2	0	74.2
Z. pacifica								
ZJ	10.6	110.4	7.6	7.5	6.3	5.9	48.1	45.5

† Means followed by same letters within a column or within a pair of "adaxial" and "abaxial" of the same species in each category are not significantly different, using Tukey's test at the *p* < 0.05.

japonica) and the ecotype ZJ (*Z. pacifica*) had remarkably low SB mean density on the abaxial side (Table 3). As mentioned before, these cultivars and ecotypes were not statistically compared with the other ecotypes due to different growing conditions. The overall mean SB area of adaxial and abaxial sides of the five tested *Zoysia* spp. were 61.4 and 80.0 μm^2, respectively.

Size Difference of SB on Abaxial Side

Silica body sizes were different in the abaxial epidermis more clearly than in the adaxial, which is highlighted by *Z. japonica* (Fig. 1C). Silica body size on the abaxial side was linked to specific location on the leaf. Size also varied especially in the abaxial side depending on the SB location on the leaf. The largest SBs were arrayed in midribs in large numbers, the middle size SBs in other ribs, and the smallest SB in the costal zone (space between ribs).

Si Mapping

Silicon mapping images of the foliar adaxial and abaxial sides of cultivar Himeno (*Z. japonica*) and ecotype W9 (*Z. matrella*) showed that Si was distinctly accumulated in the white parts of SBs with high amplitude contrast of SEM

(Fig. 2C and 2D). Silicon was also slightly concentrated on stomatal guard cells on both leaf sides (Fig. 2B and 2D).

DISCUSSION

Silica body mean density (734.3 mm^{-2}) on the abaxial side was significantly higher than that on the adaxial side (317.7 mm^{-2}) for all species and ecotypes (Tables 2 and 3). This is the reverse of the stomatal density of cool–season grasses and warm–season grasses, which typically have a higher density of stomata on the adaxial side than on the abaxial side (Casnoff et al., 1989; Green et al., 1990; Ushilo and Ohshima, 2011). Moreover, SB mean size area (80.0 μm^2) on the abaxial side was significantly larger than those on the adaxial side (61.4 μm^2) for all species. It is worth noting that the abaxial mean size of *Z. japonica* of 116.8 μm^2 was significantly larger than *Zoysia* spp. tested. From mean densities and areas of SB, the ratio of SB area to foliar surface unit can be calculated. Silica body area ratios in the adaxial and abaxial epidermis were calculated to be 1.95 and 5.88%, respectively. Higher SB area ratio in the abaxial epidermis is the inverse of stomatal incidence and g_{cmax} (the maximum stomatal conductance to CO_2), which are found to be higher on the adaxial epidermis (Ushilo and Ohshima, 2011).
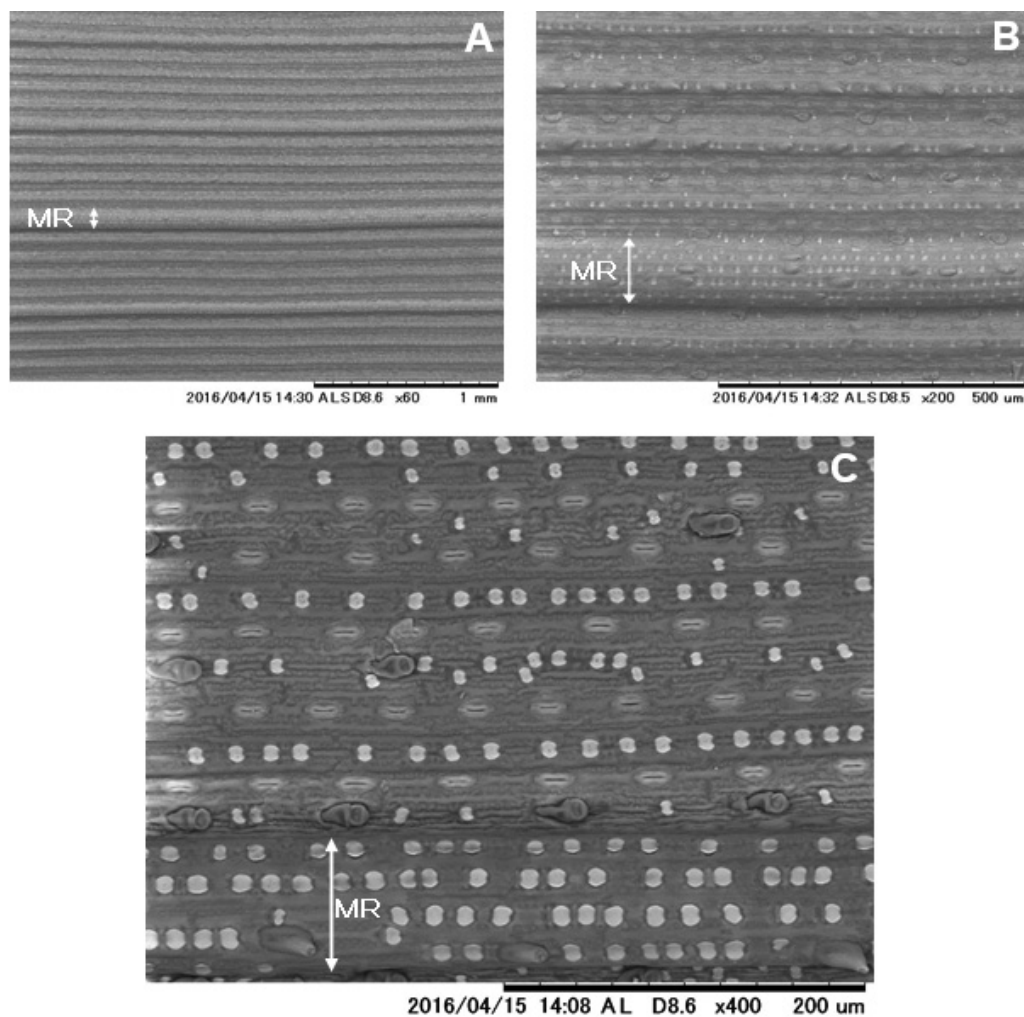

Fig. 1. Micrographs of SBs on the abaxial leaf epidermis of *Z. japonica* J63 by tabletop scanning electron microscopy. (A) A midrib (MR) and small ribs are shown at ×60 by the shadow mode emphasizing topographic details by producing shadowed images, which highlight the sample from the particular direction. (B) Midrib (MR) is shown below by shadow mode at ×200. (C) Three or more different silica body (SB) sizes are recognized at ×400. The SBs on the midrib (MR) are larger than at other sites in the abaxial epidermis. Scale bar is shown under each image.
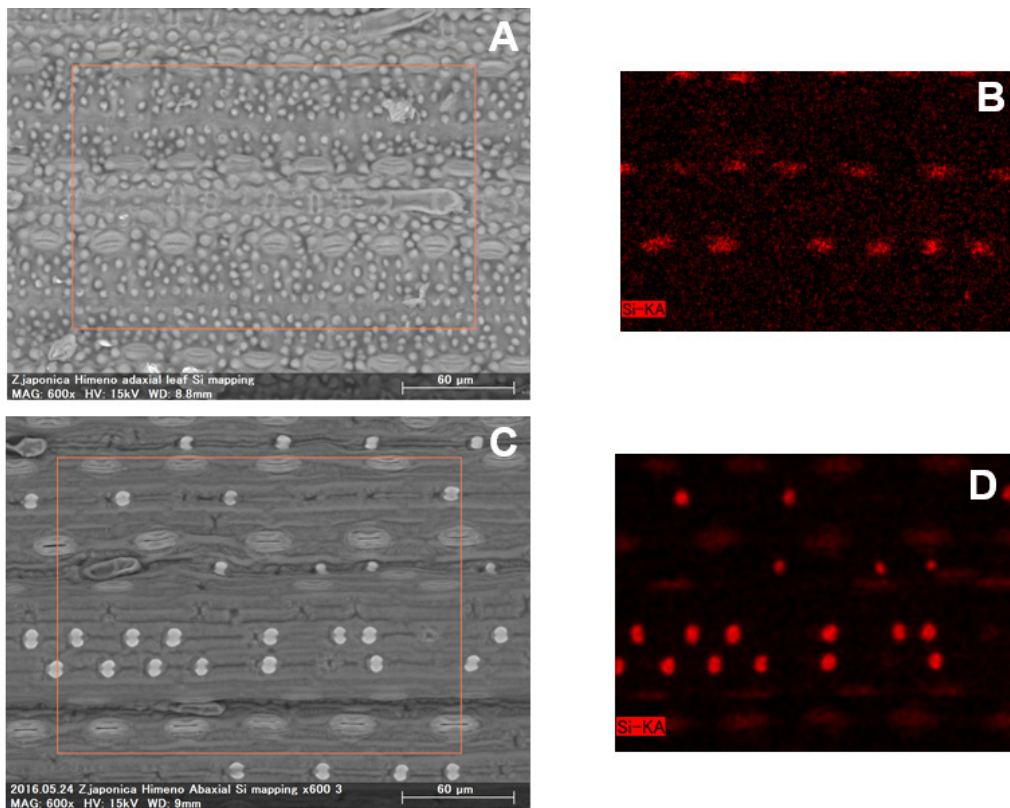
Fig. 2. Si mapping of the adaxial and abaxial sides of cultivar 'Himeno' (*Z. japonica*) at ×600 by tabletop scanning electron microscopy. (A) Image of adaxial side without silica bodies (SBs). (B) Image with Si mapping of the enclosed area of (A), showing Si condensed slightly on stomata, however, no Si on papillae and other trichomes. (C) Image of abaxial side with SB. (D) Image with Si mapping of the enclosed area of (C), showing Si condensed greatly on SB and slightly on stomata like (B) of the adaxial side.

The cultivar Himeno (*Z. japonica*) and ecotype ZJ (*Z. pacifica*) were included in this study to highlight the plasticity of SB formation. The cultivar Himeno (*Z. japonica*) is a single-plant selection found on the Kagoshima Satsunan Islands as a naturally occurring mutant of *Z. japonica*. DNA fingerprinting indicates that there have been no crosses with *Z. pacifica*, *Z. matrella*, or others (Naganuma et al., 2001). Its leaves appear comparatively tenderer than those of the other three strains of *Z. japonica* analyzed in this study. The adaxial side of cultivar Himeno (*Z. japonica*) had almost no SB (Table 3, Fig. 2A and 2B) and the abaxial SB size was smaller than that of the other *Z. japonica* ecotypes observed in this experiment (Table 3). The ecotype ZJ (*Z. pacifica*) also had very low SB density (41.2 mm^{-2}) on the adaxial side compared with (441.6 mm^{-2}) on the abaxial side. In this case, the difference is speculated to be due to *Z. pacifica* cylindrical leaf, which makes the inner adaxial side enclosed by the abaxial side. This may have led to a reduced need for SB to protect the inside from damage.

High–amplitude contrast SEM Si mapping results show SBs distinctly containing Si (Fig. 2). Moreover, since SBs were only detected on the abaxial side of leaves of Himeno (*Z. japonica*) (Fig. 2), it may be speculated that smaller–sized SiO_2 particles are possibly present in an amorphous state throughout the leaf tissue, with SB only being formed after higher accumulation of amorphous SiO_2 (Cutler et al., 2008).

The differences of SB size by location suggest that SBs may function to protect vascular bundles in the ribs. Silica bodies may also function to strengthen bundles to inhibit flat leaves from twisting to assist the leaf to use sunlight more efficiently for photosynthesis. Alternatively, SB size differences by location could be passively determined according to the amount of silicic acid [$Si(OH)_4$] moving to the leaf tissues through the rib vascular bundles. Due to low mobility, deposition could be disproportionately occurring close to the ribs. However, it may be acceptable to adopt the former suggestion from the cost-benefit theory. Silicic acid is primarily absorbed by energy–dependent Si transporter of root epidermis (Ma and Yamaji, 2006) and formed in foliar epidermis at negative energy cost (Currie and Perry, 2007). Moreover, SBs are not the pure deposit of silicic acid but a complex of Si–containing polymers combined with carbon compounds thought to be regulated by genes (Currie and Perry, 2007; Alexandre et al., 2015; Gallagher et al., 2015).

On the abaxial side, SBs were clearly arrayed in large numbers and with the largest size on the midribs. Under the ribs, the Kranz anatomy of C$_4$–plant is observed, which supports highly effective photosynthesis at high light intensity and temperature, as well as translocation (Turgeon, 2007; Carmo-Silva et al., 2009). The largest Kranz anatomy exists under the midrib of *Z. japonica* (Carmo-Silva et al., 2009). We propose that SB protect these Kranz anatomies from biological and physical damages and increase leaf rigidity for effective photosynthetically active radiation absorption. Moreover, SB may protect plant from ultraviolet damages due to the low ultraviolet transmittance through the amorphous SBs (Cutler et al.,

2008, Ushilo and Uchida, unpublished data, 2016). Zoysiagrass appears to develop a highly effective strategy to create SBs to protect itself from many kinds of stresses and damages, improving canopy photosynthesis.

CONCLUSION

Our results demonstrate that SBs were significantly more abundant and larger on the abaxial than on the adaxial side of the leaves in *Z. japonica*, *Z. matrella*, *Z. pacifica*, *Z. minima*, and *Z. macrostachya*. Moreover, the abaxial SB density and size in the foliar epidermis of *Z. Japonica* was significantly higher and larger than of the other five species of *Zoysia*. Other research has linked the presence of SB on zoysiagrass leaves to increased shoot strength and protection of essential tissues. This can lead to more efficient C_4 photosynthesis and translocation, as well as resistance to physical and ultraviolet damage (Schaller et al., 2013). We conclude that the prevalence of SBs on *Zoysia* spp. leaves may be one of the important factors contributing to their superior growth efficiency and/or adaptability to climate change in comparison with turfgrasses that lack SB. In future studies, SB and the epidermal micro- and nanostructure of zoysiagrass and others will be examined and discussed in a wider ecological and functional context.

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