

ORIGINAL RESEARCH

Method-Dependent Variation in Stomatal Trait Estimation and Its Functional Implications in *Oryza* Species

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ABSTRACT

Reliable quantification of stomatal traits is essential for understanding plant gas exchange and its responses to environmental changes. However, grasses present methodological challenges due to their complex leaf surface topography and the distinctive dumbbell-shaped stomata arranged in longitudinal rows. Here, we combined a literature-based synthesis with comparative experiments to assess how measurement methods influence stomatal trait estimates and to characterize inter-specific variation and environmental plasticity across *Oryza* species. Literature-based synthesis revealed large cross-study variations in reported stomatal density, with apparent differences of up to one order of magnitude among studies using SEM and impression-based methods, although this variation may also reflect differences in genotypes, environments, developmental stages, and sampling protocols. Experimental comparisons confirmed consistent differences between SEM and imprint measurements, although the differences were smaller in magnitude. Across species, stomatal density, guard-cell length, and guard-cell width exhibited substantial diversity and conserved rankings across environments, while guard-cell length showed greater environmental sensitivity. Moreover, method-dependent differences significantly affected estimates of anatomical maximum stomatal conductance. Our results demonstrate that measurement methodology is an important source of uncertainty in stomatal trait estimation, even within the same leaf, and that *Oryza* species display broad interspecific morphological variations but comparatively constrained environmental responses. These findings highlight the need to standardize imaging protocols and to account for method-dependent differences for accurate interpretation of relationships between stomatal morphology and functions in rice.

1 | Introduction

Stomata, which are microscopic pores on leaf surfaces, play a crucial role in regulating plant carbon assimilation, transpiration, and plant adaptation to environmental stresses (Hetherington and Woodward 2003; Lawson et al. 2011; Clark et al. 2022; Xiong 2024). Stomatal density (number per unit area) and stomatal size (pore dimensions) are two key traits

that are widely used in evaluating stomatal functions (Lawson et al. 1998; Franks and Beerling 2009; Sack and Buckley 2016). The integrated effect of stomatal density and size is often quantified as the stomatal pore area index (SPI), an important proxy for leaf photosynthetic potential and maximum stomatal conductance (g_{smax} ; Franks and Beerling 2009; Lehmeier et al. 2017; Harrison et al. 2020; Nunes et al. 2022). Beyond their effects on maximum diffusive capacity, stomatal size and density are also

functionally relevant to stomatal opening and closure kinetics (Kardiman and Ræbild 2018), water-use efficiency (Petrik et al. 2024), and residual water loss after stomatal closure (Petrik et al. 2023, 2024). Moreover, variations in stomatal density and size can provide critical insights into plant adaptive strategies under different environmental conditions. For instance, species inhabiting water-limited environments frequently produce smaller stomata, which facilitate rapid opening and closure, thereby minimizing water loss (Lawson and Blatt 2014; Lawson and Vialet-Chabrand 2019; Caine et al. 2023). Accurate

estimation of these traits is therefore important not only for calculating anatomical conductance, but also for interpreting broader stomatal regulatory strategies related to water-use efficiency and drought-associated water loss (Petrik et al. 2024).

Over the past decades, several methods have been developed to measure stomatal density and size (Pathoumthong et al. 2023; Gibbs et al. 2024 and refs therein), with two methods, the impression-based method and direct microscopy method, being widely adopted approaches (see details in Figure 1).

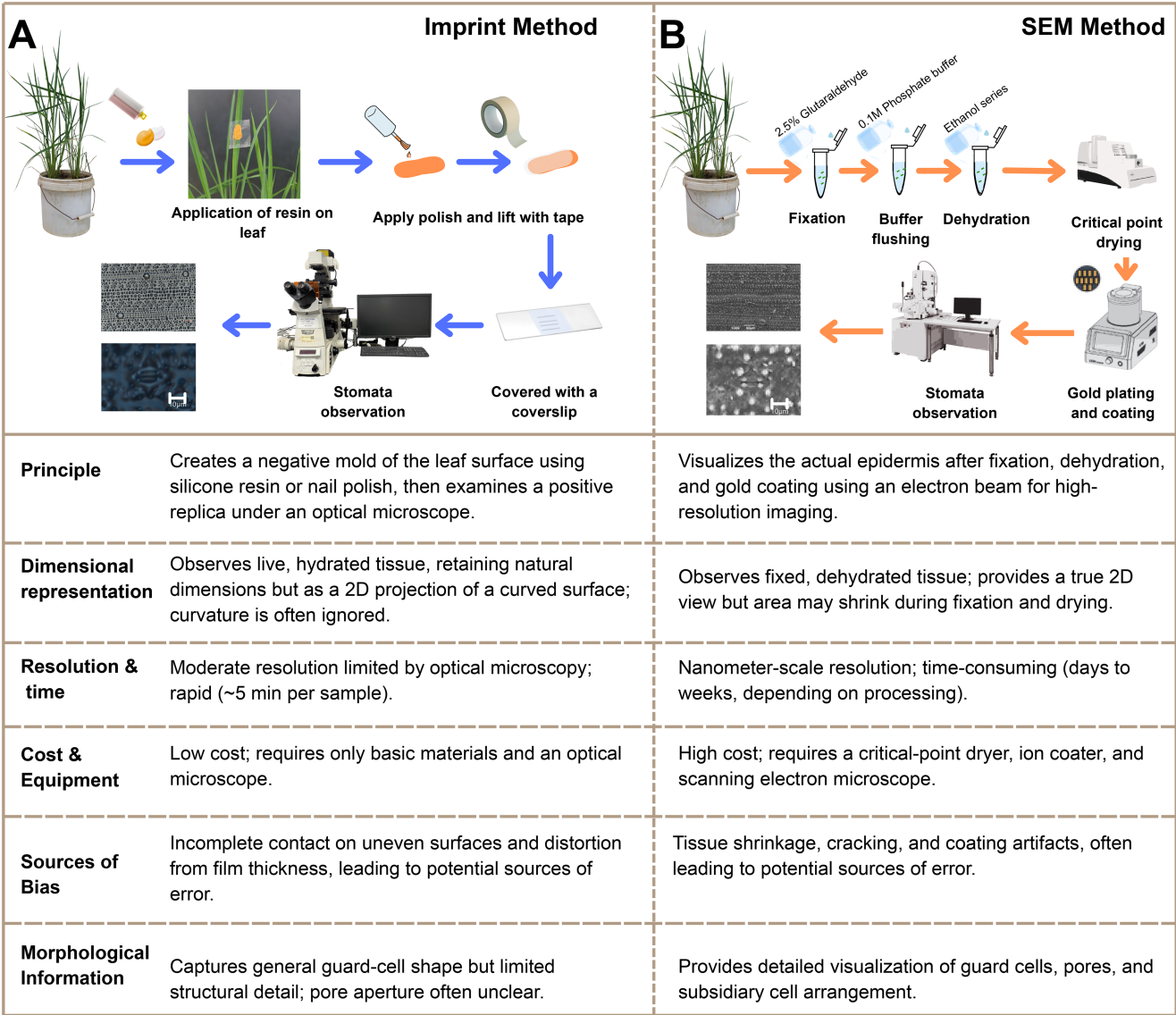


FIGURE 1 | Methodological workflow and comparative analysis of Imprint and Scanning Electron Microscopy (SEM) methods for stomatal trait quantification. The figure provides a detailed schematic of the two primary methods used for analyzing stomatal traits. (A) The imprint method used in this study involved applying a fast-curing silicone resin to the leaf surface. After curing, the silicone mold was carefully removed to obtain a negative replica of the epidermis. The negative mold was then coated with colorless nail varnish. After drying, the resulting positive replica was detached from the silicone mold, mounted on a glass slide beneath a coverslip, and examined using bright-field microscopy. (B) The SEM Method is an intensive laboratory procedure that produces high-resolution images. It requires fixing leaf tissue samples in a chemical solution, followed by buffer flushing, graded ethanol dehydration, critical point drying to remove water without causing structural collapse, and sputter coating with a conductive metal like gold to prepare the sample for observation in a high-vacuum environment. While both methods aim to quantify the same traits, they are not interchangeable. They differ fundamentally in their sample preparation, underlying assumptions, and sources of error, leading to systematic differences in the final data. A detailed comparison is provided in the Table embedded in this figure.

Impression-based methods were initially developed and optimized for the smooth and accessible surfaces of broad-leaved plants (Gloser 1967; Weyers and Johansen 1985). These methods involve pressing a malleable medium, commonly dental impression silicone, onto the leaf surface to generate a precise negative mold that captures epidermal features in detail (Meister and Bolh r Nordenkamp 2001; Gitz and Baker 2009; Matthaeus et al. 2020). Once the mold has set, it is carefully removed and filled with a transparent casting material, such as clear resin or nail polish, to produce a durable positive replicate. The advantage of this approach is that it permits the measurement of stomatal density and size without chemically treating or physically damaging the original tissue, thereby minimizing artifacts such as cell shrinkage (Matthaeus et al. 2020). Nevertheless, its reliability diminishes when applied to leaves with intricate topographies, including those covered with dense trichomes or marked by pronounced venation, as uneven contact between the impression medium and the leaf surface can result in incomplete or distorted casts (Hultine and Marshall 2001).

Direct microscopy methods, which were also initially applied for broad flat leaves, involve the direct visualization of stomata using either optical or electron microscopy (Bogner et al. 2007; Xiong and Flexas 2020; Gibbs et al. 2024). Scanning electron microscopy (SEM), in particular, affords high-resolution imagery, enabling precise analysis of stomatal morphology and the surrounding epidermis (Matthaeus et al. 2020). However, it requires meticulous sample preparation, including fixation and dehydration procedures, which may introduce structural artifacts (Pathan et al. 2008, 2010; Talbot and White 2013); additionally, features such as thick epicuticular waxes or abundant trichomes can obscure the view and compromise image quality (Kim et al. 2011). Optical microscopy, the alternative direct-observation method, typically relies on epidermal peels or decolorized leaf tissues (Wu et al. 2020; Xiong and Flexas 2020).

Unlike broadleaf plants, grass (Poaceae) leaves exhibit distinct surface morphologies, characterized by longitudinal ridges, bulliform cells, silica cells, and trichomes (Kumar et al. 2017; Hepworth et al. 2018; Chen et al. 2020; Nelson and Moore 2020; Nunes et al. 2020). This morphological complexity can significantly affect the reliability and consistency of stomatal measurements, not only across various methodological approaches but also within the same genotype subjected to differing environmental conditions under identical measurement protocols (Ouyang et al. 2017; Kumar et al. 2021). For instance, longitudinal ridges distort the relationship between projected and actual leaf surface area. Because the true epidermal area is greater than the flat projected area captured in images, stomatal density calculated on a projected-area basis may be overestimated (Wilson 1975). Additionally, stomata are unevenly distributed along ridges, and the steep slopes can create impression gaps or shadowing in microscopy, further reducing measurement reliability. The presence of silica-rich cells in the leaf epidermis can potentially complicate surface imprint and SEM observation methods, because such cells make the surface mechanically and compositionally heterogeneous, thereby hindering uniform adhesion of impression materials and potentially obscuring stomatal apertures or

producing imaging artifacts during imaging (Kim et al. 2011; Guerriero et al. 2020).

Despite the recognized importance of precise stomatal assessment in grasses, a systematic evaluation of measurement techniques is still lacking. This gap is underscored by the considerable discrepancies reported among studies using different methods (see Gibbs et al. 2024 and refs therein). For example, focusing on the widely studied rice variety IR64 under well-watered conditions, Ouyang et al. (2017) reported an adaxial stomatal density of approximately 621 stomata mm^{-2} , whereas Kumar et al. (2021) observed much higher values, typically 1000–1250 stomata mm^{-2} . Such large baseline differences highlight the challenges of comparing absolute stomatal trait values across studies and emphasize the need for standardized methodologies or, at minimum, careful consideration of method-induced variability when interpreting results.

Beyond methodological uncertainty, stomatal traits also display substantial interspecific variation and environmental plasticity (Chatterjee et al. 2020; Wu et al. 2020; Wang, Zheng, et al. 2022). Differences among species in stomatal size, density, and distribution between leaf surfaces or lateral sides influence intrinsic water-use efficiency and photosynthetic capacity (McAusland et al. 2016; Xiong et al. 2017; Bertolino et al. 2019). Furthermore, environmental factors such as growth light, humidity, and water availability can induce significant within-species variation in these traits (Casson and Gray 2008; Caine et al. 2019). Quantifying how these biological sources of variation interact with methodological effects is essential for understanding both the diversification of *Oryza* stomata and their physiological flexibility under changing environments.

In this study, we first conducted a literature-based synthesis to assess the variation in stomatal density and size reported across studies utilizing different measurement methods in rice. We then experimentally quantified how these methods affect estimates of stomatal density and size across different *Oryza* genotypes and further investigated the mechanisms underlying the observed discrepancies. Finally, we compared stomatal traits across greenhouse, outdoor-pot, and field environments to assess how environmental plasticity contributes to cross-study variation and whether interspecific trait rankings remain stable across growth conditions.

2 | Materials and Methods

2.1 | Literature-Based Synthesis

A literature search was conducted in the Web of Science database (Clarivate, London, UK) to identify methods used to estimate stomatal density and size in rice and to characterize the variations in the reported values. The search utilized the keywords of “rice * AND stomata” and was restricted to the “Agriculture and Plant Sciences” areas across available databases.

The initial search returned 2562 records. Titles and abstracts were screened to remove duplicates, reviews, and unrelated studies, resulting in 454 articles deemed relevant to rice stomatal traits. The full texts of these papers were subsequently assessed

against two inclusion criteria: (i) The study reported extractable data for stomatal density and/or guard cell length, as well as specifying the leaf surface. For consistency across studies in grasses, guard cell length was used as the measure of stomatal size, but the guard cell width values were also extracted if available. (ii) The study provided a clearly described measurement protocol for stomatal traits within its Materials and Methods section, which makes it possible to identify the methods used in measuring stomatal traits.

A total of 69 articles met these criteria and were included in the synthesis. Raw data were extracted directly from tables or digitized from figures using PlotDigitizer (<https://plotdigitizer.com>). Additional metadata were compiled for each record, including genotypes, growth conditions, measurement protocols, and developmental stages of trait assessments. Among the 69 articles, 41 provided explicit information on stomatal density and guard cell length and width for both leaf surfaces (adaxial and abaxial). These 41 articles are listed in Table S1.

To characterize potential differences in the biological and experimental contexts represented by different measurement methods, available metadata on plant materials, growth conditions, developmental stages, leaf surfaces, sampling positions, and leaf types were compiled and descriptively summarized by each measurement method for the 69 articles (Table S2). Because the number of studies was limited and metadata reporting was incomplete and uneven, these summaries were used only to contextualize literature-scale variation and were not subjected to multivariable meta-regression.

2.2 | Plant Materials and Growth Conditions

To investigate method-dependent differences in estimates of rice stomatal density and size and the mechanisms underlying these differences, stomatal traits were assessed in 10 wild rice species grown under three conditions: (i) potted plants in a greenhouse, (ii) outdoor-pot plants, and (iii) field-grown plants. The 10 species, *O. nivara*, *O. minuta*, *O. officinalis*, *O. eichingeri*, *O. glaberrima*, *O. latifolia*, *O. rufipogon*, *O. grandiglumis*, *O. punctata*, and *O. glumaepatala*, were selected from our wild rice germplasm bank.

Seedlings at the two-leaf stage were transplanted into 13L pots, each filled with 10kg of dry soil supplemented with 10g of a balanced compound fertilizer (N: P₂O₅: K₂O = 1:1:1). Three seedlings were planted in each pot, and eight pots per species were prepared. Among these, four pots per species were grown in a glasshouse under natural light, while another four pots per species were grown outdoors on the campus of Huazhong Agricultural University (30.48°N, 114.35°E). Due to an insufficient number of seedlings, *O. glaberrima* was included in the greenhouse and the outdoor potted experiment but not in the field experiment. One month after transplanting, a mixture of pesticides from Shengle brand was applied for the prevention and control of striped stem borers (*Chilo suppressalis*). This mixture included a 20% chlorfenapyr-chlorantraniliprole suspension concentrate, a 2% emamectin benzoate emulsifiable

concentrate, and a Shengle brand castor oil polyoxyethylene ether adjuvant.

In parallel, the same 10 genotypes (except *O. glaberrima*) were grown in field plots with 10 plants for each genotype at the Huazhong Agricultural University following a local established management protocol: The total fertilizer inputs over the growing season were 180 kg N ha⁻¹, 40 kg P ha⁻¹ and 100 kg K ha⁻¹. Nitrogen was applied in three splits as basal, tillering, and panicle fertilizer at 40%: 25%: 35%, respectively. Phosphorus was applied as a basal fertilizer, and potassium fertilizer was applied as basal and panicle fertilizer in a ratio of 50%:50%. Measurements were performed on the most recently expanded leaves at the tillering stage. For greenhouse-grown plants, both imprint and SEM methods were employed on the same leaves to estimate stomatal density and size. In contrast, only the imprint method was applied to outdoor-pot plants, and only the SEM method was used for field-grown plants.

2.3 | Imprint Method

For each genotype, at least four fully expanded, healthy leaves from different pots in both greenhouse and outdoor-pot plants were selected, and we marked the sampled regions in the middle of the lamina. We prepared the leaf surface impressions using a fast-curing silicone resin (HUGE). The target area was gently cleaned with a soft brush, and a thin, even layer of resin (5–30 mm in diameter) was then applied to both the adaxial and abaxial surfaces. The resin was allowed to cure completely according to the manufacturer's instructions and was then carefully peeled off to obtain a negative mold of the epidermis.

A positive replica suitable for light microscopy was made by coating the resin mold with colorless nail varnish (Gesiman). Once dried, the varnish film was detached and mounted on a glass slide beneath a coverslip. Images were captured using a bright-field optical microscope (Nikon ECLIPSE Ti) fitted with a digital camera. For each sample, more than four non-overlapping fields per surface were photographed under consistent illumination and magnification. Images exhibiting artifacts (e.g., dust, trapped air, overlapping tissue, or blur) were excluded (Figure 1).

Guard-cell length (GCL) was defined as the linear distance between the junctions of the two guard cells at each end of the pore, and guard-cell width (GCW) was measured at the apical midpoint (see Figure S1). Measurements were taken at 600× magnification, with a minimum of four stomata per leaf analyzed across the predefined regions. Stomatal density was determined at 200× magnification by counting stomata within a field of view whose area was calibrated using a stage micrometer. All measurements were performed in ImageJ (National Institutes of Health, Bethesda, MD).

2.4 | SEM Method

For the greenhouse plants, lamina pieces were excised from the same lamina regions used for resin impressions to allow a

direct comparison between the methods. Field samples were processed using the same selection and preparation criteria. For sample preparation and measurement, we followed the protocol described in Xiong et al. (2017). Approximately 10×10 mm lamina pieces were infiltrated under vacuum (DZF-6050, Hasuc Co.) with 2.5% glutaraldehyde in 0.1 M sodium-phosphate buffer (pH 7.6) until the tissues sank to the bottom of the tube. Fixation continued overnight at 4°C. Samples were rinsed three times (20 min each) in 0.1 M sodium-phosphate buffer (pH 7.6), dehydrated through a graded ethanol series (30%–100%), and transferred to iso-amyl acetate (three changes, 20 min each). After critical-point drying with liquid CO₂, specimens were mounted on aluminum stubs, sputter-coated with gold (E-1010 ion coater), and examined under a JSM-6390LV scanning electron microscope (JEOL).

For each leaf, at least four micrographs were taken from each side (narrow and wide) and both adaxial and abaxial surfaces (Figure S2) under high vacuum. Stomatal density was measured at 300× magnification, while guard-cell length and guard-cell width were determined at 1000× magnification. For guard cell size, at least four stomata were analyzed using ImageJ, following the same measurement definitions as for the impression microscopy (Figure S1).

2.5 | Anatomical g_{smax} Calculation

The anatomical maximum stomatal conductance (g_{smax}) was calculated following the method of Nunes et al. (2022), using a double end-correction of the equation originally proposed by Franks and Farquhar (2001). For each leaf, g_{smax} was estimated according to:

$$g_{smax} = \frac{d \cdot SD_{leaf} \cdot a_{max}}{v \cdot \left(l + \frac{\pi}{2}\right) \cdot \sqrt{\frac{a_{max}}{\pi}}}$$

where SD_{leaf} represents the stomatal density, calculated as the sum of adaxial and abaxial stomata per unit leaf area, d is the diffusivity of water in air ($0.0000249 \text{ m}^2 \text{ s}^{-1}$ at 25°C), v is the molar volume of air ($0.024464 \text{ m}^3 \text{ mol}^{-1}$ at 25°C), and π is the mathematical constant. The maximum pore area (a_{max}) was estimated as an ellipse according to:

$$a_{max} = 0.9 \cdot PL \cdot PW$$

where PL is the pore length (which is assumed to be $0.44 \times$ guard cell length, GCL), PW is the pore width (assumed to be $0.13 \times$ GCL), and l is the pore depth (assumed to be guard cell width, W_g). The factor 0.9 accounts for the geometrical correction for an elliptical shape.

2.6 | Data Analysis and Statistics

All data processing, statistical analyzes, and visualizations were performed in R version 4.5.1. Data manipulation and plotting were conducted using the *tidyverse* and *ggplot2* packages. Linear mixed-effects models were fitted using *lme4* and *lmerTest*; Type III ANOVA was conducted using *car*, and standardized effect sizes were calculated using *effectsize*.

For the literature-based synthesis, stomatal trait values obtained using SEM and imprint methods were compared separately for the adaxial and abaxial surfaces using Wilcoxon rank-sum tests. Because the included studies differed in genotype, growth environment, developmental stage, treatment, sampling position, and measurement protocol, these comparisons were interpreted as cross-study associations rather than independent estimates of measurement-method effects.

Experimental measurements were averaged within each biological replicate for every combination of genotype, measurement method, leaf side, and leaf surface. Effects of method, side, and surface on stomatal traits in greenhouse-grown plants were evaluated using the following linear mixed-effects model: Trait ~ Method * Side * Surface + (1 | Genotype/Replicate).

Paired SEM and imprint measurements were compared using paired *t*-tests. Method differences were calculated as SEM – Imprint and summarized using mean differences, 95% confidence intervals, Cohen's d_z and Hedges' g_z . To determine whether method differences varied with leaf side or leaf surface, the difference values were analyzed using: Method difference ~ Side * Surface + (1 | Genotype/Replicate).

In both models, biological replicate was nested within genotype. Fixed effects were evaluated using Type III ANOVA, with denominator degrees of freedom estimated using the Satterthwaite approximation. Partial η^2 was used to quantify fixed-effect magnitude, and variance components were extracted to quantify variation attributable to genotype, biological replicate within genotype, and residual variation.

Agreement between SEM and the imprint method was further evaluated using Bland–Altman analyzes of paired measurements aggregated to the biological-replicate level. Mean bias was calculated as the mean paired difference, and the 95% limits of agreement were calculated as the mean difference ± 1.96 times the standard deviation of the paired differences. Relative differences were calculated as:

$$\frac{\text{SEM} - \text{Imprint}}{(\text{SEM} + \text{Imprint})/2} \times 100.$$

Confidence intervals for mean differences were estimated using a genotype-stratified bootstrap procedure with 500 replicates. Proportional bias was evaluated by regressing paired differences against their corresponding pairwise means.

Coefficients of variation were compared between measurements obtained using SEM and the imprint method using paired Wilcoxon signed-rank tests and among growth environments using Kruskal–Wallis tests. Environmental comparisons were conducted separately within each measurement method. Outdoor-pot and greenhouse plants measured using the imprint method, and field-grown and greenhouse plants measured using SEM, were compared using Welch's two-sample *t*-tests, with 95% confidence intervals calculated using the Welch–Satterthwaite approximation. Overall environmental effects were assessed using method-specific linear mixed-effects models with environment as a fixed effect and genotype as a random intercept.

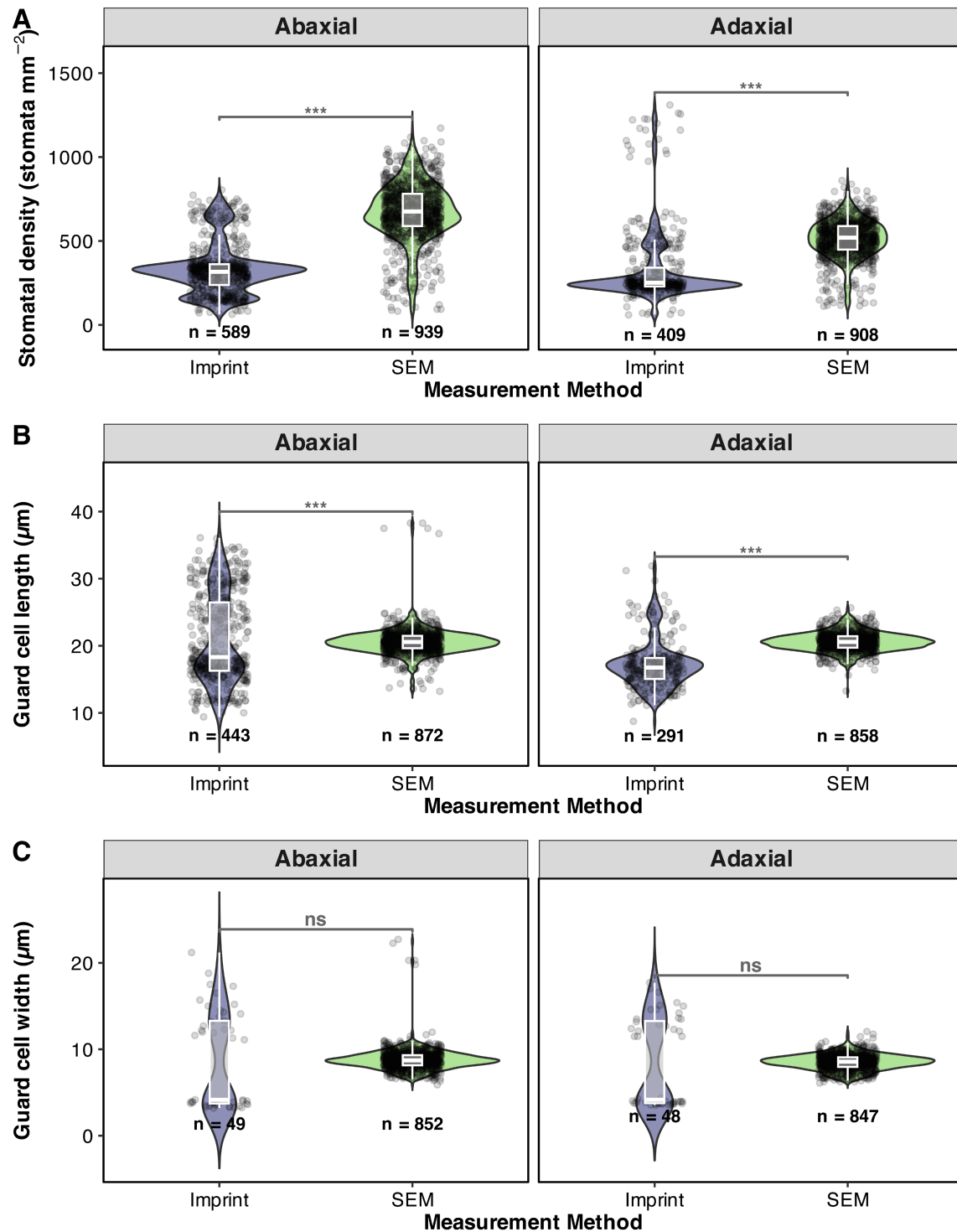


FIGURE 2 | Comparative analysis of stomatal density (A), guard-cell length (B), and guard-cell width (C) measurements obtained via leaf stomatal imprinting and Scanning Electron Microscopy (SEM) methods. Violin plots, overlaid with boxplots, illustrate the distribution of values for each method on both the abaxial and adaxial surfaces of the leaf. Individual data points are jittered for clarity, while boxplots denote the median and interquartile range (IQR); whiskers extend to 1.5 times the IQR. Statistical comparisons between the imprint and SEM methods were conducted using the Wilcoxon rank-sum test. Significance levels are indicated by brackets, *** denotes a significant difference ($p < 0.001$). Sample sizes (n) for each method are provided accordingly.

Anatomical maximum stomatal conductance (g_{smax}) was analyzed using the corresponding paired and environmental comparisons. Pearson's correlation coefficient (r) was used to quantify linear associations among g_{smax} estimates, whereas

Spearman's ρ and Kendall's τ were used to assess the consistency of genotype rankings between measurement methods and growth environments. All statistical tests were two-sided, and $p < 0.05$ was considered statistically significant.

3 | Results

3.1 | Cross-Study Variation and Controlled Comparison of Stomatal Traits Between Measurement Methods

Across the compiled literature, reported stomatal traits varied substantially among studies. Studies using scanning electron microscopy (SEM) tended to report higher stomatal density and larger guard-cell length at the adaxial surface than impression-based methods in rice (Wilcoxon rank-sum tests, $p < 0.001$; Figure 2), while guard-cell width values were generally lower in SEM-based reports. However, because the compiled studies differed in genotypes, growth environments, developmental stages, treatments, sampling positions, and measurement protocols, these literature-scale differences should be interpreted as cross-study associations rather than direct estimates of method effects. We therefore used the experimental datasets to separate, as far as possible, methodological and biological sources of variation. Indeed, the stomatal density from SEM in our dataset was generally lower than many literature values (Figure S3A), likely reflecting methodological differences. In contrast, our guard-cell length and width estimated from both methods fell to the bottom range of literature reports (Figure S3B), likely representing a genuine biological characteristic rather than a methodological bias.

In our greenhouse-grown plants, stomatal traits were quantified using both SEM and the Imprint method for 10 *Oryza* species. When data were pooled across species, surfaces, and leaf sides, all three traits—stomatal density, guard-cell length, and guard-cell width—showed consistent method offsets ($p < 0.001$ for all three traits; Figure 3). Linear mixed-effects models fitted to the trait values further confirmed significant effects of Method on stomatal density ($F = 15.47$, $p < 0.001$), guard-cell length ($F = 70.27$, $p < 0.001$), and guard-cell width ($F = 88.98$, $p < 0.001$; Table 1). SEM produced higher estimates of stomatal

density and guard-cell length but lower estimates of guard-cell width than the imprint method. For guard-cell width, Surface also had a significant effect ($F = 5.61$, $p = 0.018$), and significant Method $\times$ Side and Side $\times$ Surface interactions were detected ($F = 7.13$, $p = 0.008$ for both interactions). Variance partitioning of the paired method differences showed contrasting sources of variation among traits (Table 2). For stomatal density, genotype, biological replicate within genotype, and residual variation accounted for 1.5%, 20.6%, and 77.9% of the total variance, respectively. The corresponding proportions were 32.5%, 10.9%, and 56.6% for guard-cell length, and 12.7%, 9.9%, and 77.4% for guard-cell width. Among the fixed effects, Side significantly affected the method difference in guard-cell width (partial $\eta^2 = 6.8\%$, $p < 0.01$), and the Side $\times$ Surface interaction was also significant (partial $\eta^2 = 3.7\%$, $p = 0.037$; Table 2). No significant fixed effects were detected for stomatal density or guard-cell length. In greenhouse plants, the Imprint and SEM methods showed similar variability for all traits (Figure 4A), within-method comparisons also showed limited differences in CV across environments (Figure 4B–C).

3.2 | Variation in Stomatal Traits Among *Oryza* Species Estimated by Different Methods and Environmental Plasticity of Stomatal Traits

Across the 10 *Oryza* species studied, stomatal traits exhibited broad interspecific variation under both measurement methods (Tables 3 and S3). When averaged across environments and surfaces, stomatal density ranged from approximately 106.39 to 631.93 mm^{-2} in while guard-cell length varied from 8.87 to 25.01 μm in. Guard-cell width showed smaller variations, between 1.77 and 5.34 μm (Table 3). The interspecific differences of stomatal density and guard-cell width were highly consistent between SEM and Imprint methods (Table S4). Specifically, stomatal density showed the highest concordance between methods (Spearman's $\rho = 0.95$, $p < 0.001$), followed by guard-cell

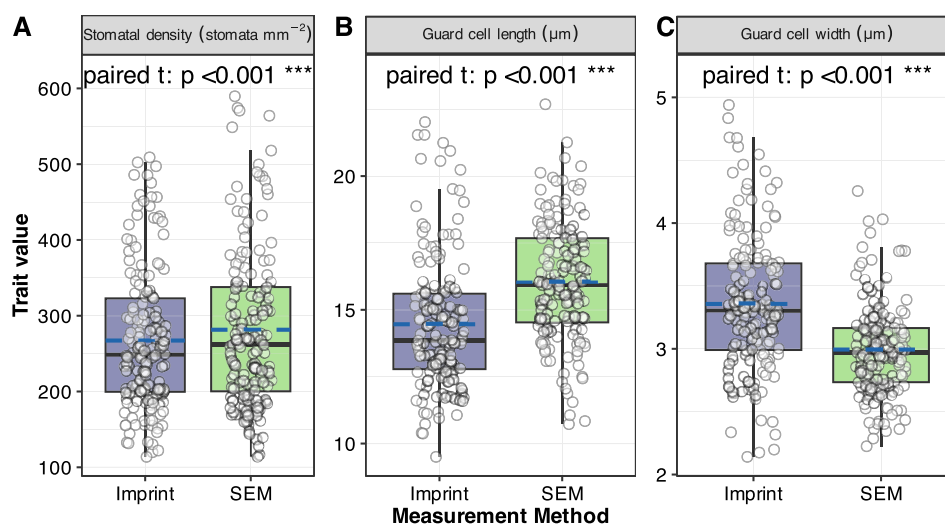


FIGURE 3 | Comparison of measurement methods (SEM vs. Imprint) for stomatal traits in wild *Oryza* species. (A) Stomatal density (stomata mm^{-2}), (B) guard cell length (μm), and (C) guard cell width (μm) measured using scanning electron microscopy (SEM) and the imprint method. In each box, the solid horizontal line represents the median, while the dashed blue line represents the mean. White space within the box shows the interquartile range, whiskers extend to $1.5 \times \text{IQR}$, and gray dots represent individual replicate values. Results of paired t -tests are shown above each panel; *** $p < 0.001$. Data are pooled across leaf surfaces (abaxial vs. adaxial) and sides (narrow vs. wide) for each species.

TABLE 1 | Results of linear mixed-effects model for stomatal density, guard cell length and guard cell width in greenhouse-grown plants.

Traits	Factor	F-value	p
Stomatal density	Method	15.47	<0.001 ***
	Side	9.70	0.002**
	Surface	507.98	<0.001***
	Method × side	2.58	0.110
	Method × surface	0.16	0.692
	Side × surface	1.77	0.185
	Method × side × surface	0.01	0.925
Guard cell length	Method	70.27	<0.001 ***
	Side	2.17	0.141
	Surface	0.97	0.327
	Method × side	0.70	0.403
	Method × surface	0.61	0.437
	Side × surface	1.08	0.299
	Method × side × surface	0.02	0.888
Guard cell width	Method	88.98	<0.001 ***
	Side	0.07	0.793
	Surface	5.61	0.018*
	Method × side	7.13	0.008**
	Method × surface	0.53	0.467
	Side × surface	7.13	0.008**
	Method × side × surface	3.74	0.054

Note: The model included Method (SEM vs. Imprint), Side (Narrow vs. Wide), Surface (abaxial vs. adaxial) and their interactions as fixed effects, with genotype and replicate nested within genotype included as random intercepts. Fixed effects were evaluated using Type III ANOVA, with denominator degrees of freedom estimated using the Satterthwaite approximation implemented in “lmerTest.” *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

width ($\rho = 0.88$, $p < 0.001$). However, the two methods were not significantly correlated for guard-cell length ($\rho = 0.61$, $p = 0.06$). Method differences (SEM – Imprint) in stomatal density, guard-cell length and guard-cell width for each species grown in the greenhouse are shown in Figure S4. For stomatal density, the significant method differences were detected only in *O. latifolia* (abaxial surface, narrow side), *O. rufipogon* (adaxial, narrow side), and *O. punctata* (adaxial, narrow side). Although several species showed detectable method differences in both guard-cell length and width, the direction of the method effect was largely consistent across traits. For guard-cell length, most species showed SEM > Imprint, but *O. glaberrima* exhibited the significantly opposite pattern (SEM < Imprint) in two side-surface combinations. In contrast, guard-cell width generally

showed SEM < Imprint, whereas *O. rufipogon* exhibited the opposite pattern (SEM > Imprint).

Environmental effects on stomatal traits were also investigated by comparing species means between greenhouse and outdoor (Imprint) or field (SEM) conditions (Table S4). Species rankings of stomatal traits by the same method were conserved across environments. For Imprint-based measurements, rank correlations between greenhouse and outdoor-pot plants were significant for stomatal density ($\rho = 0.87$, $p = 0.001$) and guard-cell width ($\rho = 0.84$, $p = 0.002$), but weaker for guard-cell length ($\rho = 0.58$, $p = 0.08$). For SEM-based measurements, correlations between greenhouse and field plants were significant for stomatal density ($\rho = 0.82$, $p = 0.007$) and guard-cell width ($\rho = 0.75$, $p = 0.020$), whereas guard-cell length showed no consistent relationship ($\rho = 0.18$, $p = 0.64$; Table S4).

Environmental responses of stomatal traits of each species were investigated by comparing greenhouse plants with those grown in outdoor pots (Imprint method) or in the field (SEM method; Figure S5). The direction of environmental effects on stomatal density and guard-cell width was not uniform, with the environmental difference of most species distributed around zero. For guard-cell width, however, outdoor-greenhouse comparisons on the abaxial surface consistently showed higher values under outdoor conditions, with significant increases detected in *O. glaberrima* and *O. grandiglumis* (Figure S5A). By contrast, guard-cell length exhibited a clear pattern between environments, that is field-grown plants generally had smaller guard cells than greenhouse plants, whereas outdoor-pot plants showed larger cells (Figure S5). In the Field–Greenhouse comparison, significant reductions in guard-cell length were observed for *O. eichingeri* and *O. latifolia* on the abaxial surface, while on the adaxial surface, all but *O. grandiglumis* and *O. punctata* showed significant decreases (Figure S5B).

3.3 | Method-Dependent Differences Influence Anatomical Estimates of Maximum Stomatal Conductance

Anatomical maximum stomatal conductance (g_{smax}) was calculated from stomatal traits obtained using each method to determine whether method-dependent differences propagated into functional estimates (Figures 5 and S6). Bland–Altman analyzes showed that, relative to the imprint method, SEM yielded stomatal density and guard-cell length values that were on average 14.26 stomata mm^{-2} (4.65%) and 1.56 μm (10.60%) higher, respectively, whereas guard-cell width was 0.36 μm lower (–10.76%; Table S5; Figure S7). The corresponding 95% limits of agreement were –61.02 to 89.53 stomata mm^{-2} , –1.98 to 5.09 μm , and –1.27 to 0.55 μm , indicating considerable variation in agreement among individual observations.

These trait-level differences propagated into the g_{smax} estimates. Specifically, SEM-derived values generally exceeded imprint-derived estimates. Nevertheless, estimates from the two methods remained strongly correlated ($r = 0.85$, $p < 0.001$; Figure S6), indicating that relative variation was more conserved than

TABLE 2 | Variance components and fixed effects explaining paired differences between SEM and imprint measurements of stomatal traits in greenhouse-grown plants.

Source	Stomatal density	Guard cell length	Guard cell width
Random effects			
Genotype	27.74 (1.5%)	1.88 (32.5%)	0.03 (12.7%)
Replicate within genotype	387.14 (20.6%)	0.63 (10.9%)	0.03 (9.9%)
Residual	1463.5 (77.9%)	3.27 (56.6%)	0.2 (77.4%)
Fixed effects			
Side	F(1, 117) = 3.70 (3.1), 0.057	F(1, 117) = 1.19 (1.0), 0.278	F(1, 117) = 8.51 (6.8), 0.004**
Surface	F(1, 117) = 0.23 (0.2), 0.635	F(1, 117) = 1.02 (0.9), 0.314	F(1, 117) = 0.63 (0.5), 0.428
Side × surface	F(1, 117) = 0.01 (0.0), 0.910	F(1, 117) = 0.03 (0.0), 0.855	F(1, 117) = 4.46 (3.7), 0.037*

Note: Paired method differences were calculated as SEM – Imprint. Separate linear mixed-effects models were fitted for each trait, with Side, Surface, and Side × Surface as fixed effects and genotype and biological replicate nested within genotype as random intercepts, specified in R as (1 | Genotype/Replicate). For random effects, values are variance estimates, with the percentage of total variance shown in parentheses. For fixed effects (Side, Surface, and Side × Surface), values reported as *F*-value (partial eta squared, %) in parentheses, followed by the *p*-value. Denominator degrees of freedom were estimated using the Satterthwaite approximation. ***p* < 0.01; **p* < 0.05.

absolute magnitude. The g_{smax} was also strongly correlated between narrow and wide leaf sides ($r=0.88$, $p<0.001$). Across environments, the relationship was moderate for SEM-based greenhouse and field estimates ($r=0.66$, $p=0.051$) but strong for imprint-based greenhouse and outdoor-pot plants estimates ($r=0.85$, $p=0.002$; upper-left panel of Figure S6). Thus, absolute trait and g_{smax} values derived from the two methods should not be treated as directly interchangeable, although their relative variation was comparatively robust.

4 | Discussion

Stomata regulate both the uptake of CO₂ and the transpirational loss of water, thereby determining the balance between photosynthetic carbon assimilation and water-use efficiency (Buckley 2005; Berry et al. 2010; Wang, Wang, et al. 2022; Lawson and Leakey 2024). Precise quantification of stomatal traits is therefore critical for elucidating the relationship between anatomical structure and physiological function, as well as for interpreting interspecific and environmental variation (Matthews and Lawson 2019; Lawson and Matthews 2020; Karavolias et al. 2023). A comprehensive understanding of stomatal form and function in rice holds substantial agronomic importance (Xiong et al. 2015; Xiong 2024). Unlike dicotyledonous species, *Oryza* plants possess a distinctive stomatal architecture characterized by highly ordered stomatal files arranged longitudinally along the leaf veins (Raissig et al. 2017; McKown and Bergmann 2018, 2020; Durney et al. 2023). Although a range of techniques, including leaf impressions and various microscopy-based approaches, has been employed to quantify stomatal traits, methodological inconsistencies remain a major challenge (Gitz and Baker 2009; Sun et al. 2021; Gibbs et al. 2024). Variations in leaf surface morphology, sample preparation procedures, and imaging resolution often contribute to discrepancies in reported measurements, as evidenced by the wide variability in stomatal characteristics reported for rice across different studies (Ouyang et al. 2017; Kumar et al. 2021).

4.1 | Method-Dependent Differences in Stomatal Traits

Our literature-based synthesis revealed large inconsistencies among studies quantifying rice stomatal traits, particularly among reports using SEM and impression-based microscopy (see Gibbs et al. 2024 and refs therein). However, these literature-scale differences should be interpreted cautiously because the compiled studies differed in genotype, growth environment, developmental stage, treatment, leaf surface, sampling position, and measurement protocol, and our synthesis did not include a formal meta-regression to control for these factors. Thus, the nearly order-of-magnitude variation observed in the literature cannot be attributed solely to measurement method, but likely reflects combined methodological, biological, and sampling-related heterogeneity (Casson and Gray 2008; Casson and Hetherington 2010; Pillitteri and Torii 2012; Chua and Lau 2024).

This limitation motivated our experimental comparisons. The greenhouse experiment, in which SEM and imprint measurements were conducted on matched regions of the same leaves across 10 *Oryza* species, allowed us to evaluate relative method-dependent differences under standardized biological and sampling conditions. By using matched leaf regions, this design minimized variations associated with genotypes, growth environments, developmental stages, and sampling positions. Because no universally accepted gold-standard method is currently available for measuring stomatal morphology in intact grass leaves, the paired comparison was intended to quantify relative method-dependent differences rather than absolute measurement error. We still detected systematic differences between methods, although their magnitude was smaller than the cross-study variation inferred from the literature (Figures 3 and S3; Table 1). This indicates that measurement method contributes to stomatal trait variation, but also that much of the broader variability among published studies likely arises from differences in genotype, environment, developmental stage, sampling

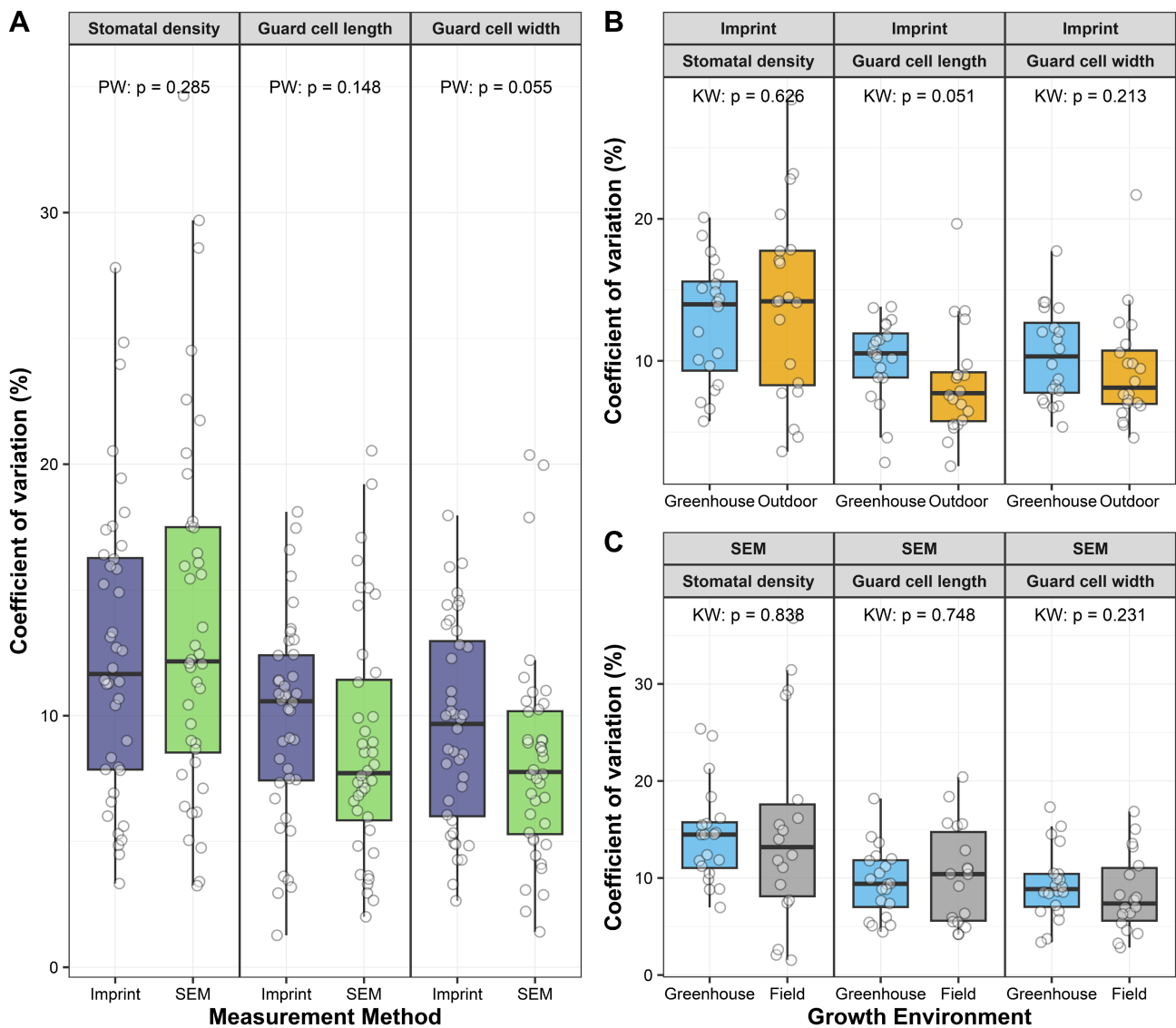


FIGURE 4 | Coefficient of variation (CV) of stomatal traits by measurement method and environment. (A) CVs of stomatal density, guard cell length, and guard cell width compared between the Imprint and SEM methods in greenhouse plants. Paired Wilcoxon signed-rank tests (PW) were performed per trait, and p -values are shown above each facet. (B) CVs obtained by the Imprint method in greenhouse versus outdoor-pot plants. (C) CVs obtained by the SEM method in greenhouse versus field plants. Kruskal–Wallis tests (KW) were applied per trait, with p -values reported above panels.

location, and protocol details (Giuliani et al. 2013; Kondamudi et al. 2016; Xiong et al. 2017). In addition, variation between the narrow and wide sides of the leaf blade, which is often unreported in the literature, may introduce further inconsistency, particularly for guard-cell dimensions (Yuan et al. 2015). Our paired comparison should therefore be interpreted as a controlled estimate of relative method-dependent offsets, rather than as evidence that either method provides an absolute reference value (Figure S7; Table S5).

The high residual variance observed for stomatal density further highlights the importance of fine-scale sampling effects (Table 2). Image quality also varied among species, leaf surfaces, and individual samples, potentially affecting the identification of guard-cell landmarks. The closer agreement between methods for stomatal density than for guard-cell dimensions suggests that gross scaling errors or severe tissue deformation were unlikely to be the sole causes of the discrepancies. Instead, variation in image clarity,

landmark placement, and sample preparation may have contributed to the observed differences in guard-cell length and width, particularly in dumbbell-shaped grass stomata, whose boundaries may be represented differently in SEM and imprint images.

Even under standardized biological and sampling conditions (Franks and Farquhar 2001; Franks and Beerling 2009; Sack and Buckley 2016), the observed method-dependent differences in stomatal density and guard-cell dimensions propagated into substantial differences in anatomical maximum stomatal conductance (g_{max} ; Figures 5 and S6). Bland–Altman analyzes revealed systematic offsets and wide limits of agreement, indicating that SEM- and imprint-derived values are not directly interchangeable (Table S5; Figure S7). However, the strong cross-method correlation in g_{max} suggests that relative rankings were more robust than absolute values. Because neither method is a gold standard, cross-study comparisons should use consistent protocols or explicit cross-method calibration. This finding

TABLE 3 | Summary of stomatal trait variation among environments, leaf surfaces, and measurement methods for the wide leaf side of wild *Oryza* species.

Trait	Environment	Surface	Method	Mean $\pm$ SE	Min	Median	Max	CV (%)
Stomatal density (stomata mm ⁻²)	Field	Abaxial	SEM	325.62 $\pm$ 15.80	164.40	317.59	541.77	25.22
	Field	Adaxial	SEM	226.12 $\pm$ 13.11	134.51	205.50	336.27	30.12
	Greenhouse	Abaxial	Imprint	302.08 $\pm$ 7.74	131.26	281.12	554.50	32.42
	Greenhouse	Abaxial	SEM	312.30 $\pm$ 8.05	139.35	291.66	631.93	32.59
	Greenhouse	Adaxial	Imprint	226.98 $\pm$ 6.79	106.39	202.25	472.60	37.85
	Greenhouse	Adaxial	SEM	233.64 $\pm$ 6.89	114.31	209.56	503.30	37.29
	Outdoor	Abaxial	Imprint	311.37 $\pm$ 14.97	135.74	310.76	545.59	30.03
	Outdoor	Adaxial	Imprint	247.78 $\pm$ 13.46	131.92	245.87	405.20	33.91
Guard cell length (μ m)	Field	Abaxial	SEM	13.01 $\pm$ 0.36	9.89	12.79	17.64	14.23
	Field	Adaxial	SEM	12.61 $\pm$ 0.19	10.27	12.65	14.19	7.64
	Greenhouse	Abaxial	Imprint	14.73 $\pm$ 0.18**	8.87	14.51	21.35	15.59
	Greenhouse	Abaxial	SEM	16.01 $\pm$ 0.19**	11.29	15.70	23.58	15.40
	Greenhouse	Adaxial	Imprint	14.62 $\pm$ 0.24**	9.35	14.18	24.66	20.77
	Greenhouse	Adaxial	SEM	16.14 $\pm$ 0.23**	9.75	15.93	24.00	18.20
	Outdoor	Abaxial	Imprint	17.17 $\pm$ 0.33	13.39	16.89	21.96	12.02
	Outdoor	Adaxial	Imprint	16.54 $\pm$ 0.40	12.36	16.04	25.01	15.22
Guard cell width (μ m)	Field	Abaxial	SEM	2.92 $\pm$ 0.10	2.22	2.78	4.30	17.70
	Field	Adaxial	SEM	2.98 $\pm$ 0.07	2.50	2.89	3.84	11.39
	Greenhouse	Abaxial	Imprint	3.15 $\pm$ 0.04*	1.93	3.11	4.85	17.35
	Greenhouse	Abaxial	SEM	2.99 $\pm$ 0.04*	1.97	2.98	4.78	17.46
	Greenhouse	Adaxial	Imprint	3.45 $\pm$ 0.04 ***	1.98	3.35	5.34	15.95
	Greenhouse	Adaxial	SEM	3.09 $\pm$ 0.04 ***	1.77	3.03	4.41	15.83
	Outdoor	Abaxial	Imprint	3.68 $\pm$ 0.11	2.51	3.60	5.24	17.94
	Outdoor	Adaxial	Imprint	3.33 $\pm$ 0.12	2.05	3.18	4.91	21.72

Note: Values represent the mean $\pm$ standard error (SE), with accompanying minimum, median, maximum, and coefficient of variation (CV). Data are shown separately for the three stomatal traits—guard cell length, guard cell width, and stomatal density—measured using either scanning electron microscopy (SEM) or the imprint method under field, greenhouse, and outdoor conditions. Asterisks indicate significant differences between SEM and the imprint method within the greenhouse, where *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$.

emphasizes that method-dependent differences can generate physiologically meaningful variation in derived functional parameters. Recent advances in automated image analysis and machine learning have greatly accelerated stomatal phenotyping (Fetter et al. 2019; Li et al. 2019; Meeus et al. 2020); however, as highlighted by Gibbs et al. (2024), the reliability and comparability of such approaches ultimately depend on the quality and consistency of image acquisition. Standardized imaging and measurement protocols therefore remain essential for biologically interpretable and cross-study comparable stomatal data.

4.2 | Interspecific Variation in Stomatal Traits Among *Oryza* Species

Substantial interspecific variation was observed among the 10 *Oryza* species examined, encompassing stomatal density,

guard-cell dimensions, and their distribution across leaf surfaces and sides. Stomatal density varied nearly fivefold among species, whereas variation in guard-cell length and width was more constrained, suggesting that size and density are not strictly coupled but may represent partially independent axes of structural diversification (Table S3). This pattern is consistent with earlier findings in cultivated rice and other grasses, where evolutionary adjustments in stomatal size and density combinations optimize the balance between gas-exchange capacity and hydraulic control (Ohsumi et al. 2007; Xu and Zhou 2008; Laza et al. 2010; Xiong et al. 2017; Wu et al. 2020; Nunes et al. 2022). Species such as *O. rufipogon* and *O. glumaepatala* exhibited relatively high densities with smaller guard cells, implying a strategy favoring fast response and greater potential g_{smax} , whereas *O. latifolia* and *O. eichingeri* showed fewer but larger stomata, potentially reflecting adaptation to more mesic habitats or reduced evaporative demand (Lawson and Blatt 2014;

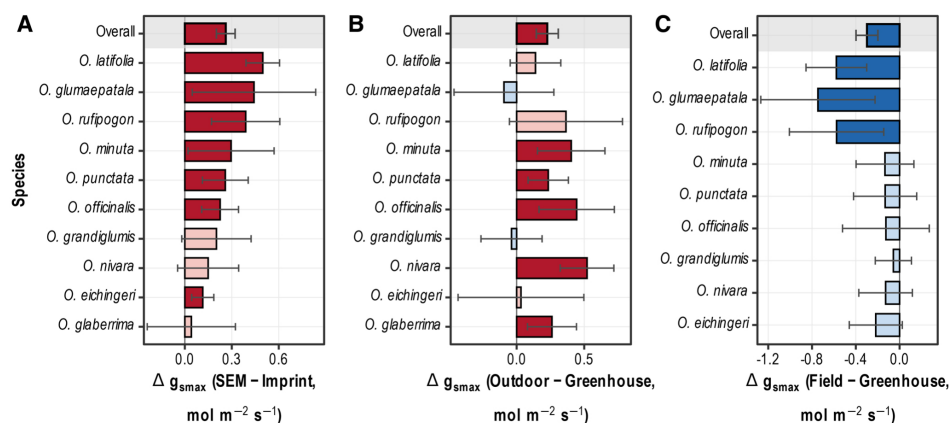


FIGURE 5 | Anatomical maximum stomatal conductance differences (Δg_{smax}) across measurement methods and growth environments. (A) Comparison of SEM- and imprint-based estimates on the same leaves. (B) Comparison of imprint-based estimates between outdoor-pot plants and greenhouse plants. (C) Comparison of SEM-based estimates between field-grown and greenhouse plants. (A), genotype-level contrasts were tested with paired t-tests, and the overall effect summarizes all paired differences. In (B) and (C), genotype-level contrasts were tested with Welch two-sample t-tests, while the overall effect was estimated using a mixed model with genotype as a random intercept. Dark red or dark blue bars indicates statistically significant differences ($p < 0.05$), light red or light blue bars indicates non-significant contrasts, and error bars represent 95% confidence intervals. The shaded grey band highlights the overall effect across species. For (A), leaf-level g_{smax} was calculated by averaging narrow- and wide-side measurements for each surface (abaxial and adaxial) and then summing both surfaces. For panels (B) and (C), only Wide-side measurements (abaxial + adaxial) were used, to match across environments.

Xiong et al. 2018; Lawson and Vialet-Chabrand 2019; Xiong, Dun, et al. 2022). Distinct spatial patterns were also evident, as most species showed consistently higher stomatal density on the abaxial surface, in line with the amphistomatic tendency of grasses but with species-specific magnitude of asymmetry (Mott et al. 1982; Xiong and Flexas 2020; Hörak 2025; Samantara et al. 2025).

4.3 | Environmental Responses of Stomatal Traits

Environmental conditions exerted measurable but trait-specific effects on stomatal characteristics across *Oryza* species (Rowland-Bamford et al. 1990). Our comparisons between greenhouse, outdoor, and field environments revealed that stomatal density and guard-cell width were relatively stable, whereas guard-cell length exhibited consistent environmental sensitivity (Figure S5). Field-grown plants generally produced smaller guard cells than greenhouse plants, while outdoor-pot plants showed the opposite trend. These responses indicate that stomatal size is more plastic than density and may respond to microclimatic cues such as vapor pressure deficit and light intensity. Similar reductions in guard-cell length under field or drought conditions have been reported in cultivated rice and other grasses (Caine et al. 2019; Chen et al. 2021; Ferguson et al. 2024).

Despite the moderate magnitude of environmental shifts observed here, species rankings for most traits were conserved across environments, implying that the direction and strength of phenotypic plasticity are constrained by inherent anatomical and developmental limits (Lawson et al. 1998; Raissig et al. 2017; McKown and Bergmann 2018, 2020). The tight coupling of epidermal patterning with longitudinal cell files and vascular organization in grasses (Xiong et al. 2017; Nunes et al. 2020) may restrict the extent to which stomatal density and allocation can

adjust post-developmentally. Instead, functional acclimation likely occurs through adjustments in stomatal aperture behavior rather than through large-scale changes in stomatal number (Taylor et al. 2012).

The combination of controlled environment and field measurements in this study underscores that stomatal traits derived from greenhouse-grown plants may not fully represent their field counterparts. Recent studies have similarly emphasized that findings obtained under controlled conditions often fail to replicate under field environments, reflecting the growing recognition of a disconnect between discovery research and real-world crop performance (Xiong, Flexas, et al. 2022; Khaipho-Burch et al. 2023; Flavell et al. 2025). This divergence has important implications for phenotyping and modeling, as many studies rely on controlled-environment data to infer plant function and productivity under natural conditions. Incorporating environmental variability into stomatal trait assessment is therefore essential for accurate interpretation of trait-function relationships and for improving predictions of plant performance under changing climates.

5 | Conclusions

This study demonstrates that methodological choices substantially influence the quantification of stomatal traits in rice. By integrating a literature synthesis with cross-method and multi-environment comparisons among *Oryza* species, we show that even under standardized sampling, scanning electron microscopy and impression techniques yield systematically different estimates of stomatal density and guard-cell dimensions. These discrepancies, although smaller than literature-wide variation, propagate into key functional parameters such as anatomical maximum stomatal conductance, highlighting the need for methodological transparency and cross-calibration. Beyond

technical considerations, the combined evidence from greenhouse, outdoor, and field measurements reveals that stomatal traits are shaped by both intrinsic genetic constraints and environmental plasticity. The finding that guard-cell length responds most strongly to environmental context indicates that trait-function relationships established under controlled conditions cannot be directly extrapolated to the field, a limitation increasingly recognized across plant science research. Our findings emphasize that accurate assessment of stomatal form and function in grass requires standardized imaging protocols, explicit reporting of methodological details, and validation across environmental gradients.

Author Contributions

D.X. planned and designed the research; Q.S. performed the experiments; Q.S. and D.X. conducted data analyzes and wrote the paper with inputs from X.Y., X.L., K.C., J.H., and S.P.

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Conflicts of Interest

The authors declare no conflicts of interest.

Data Availability Statement

All data are given in main text and in the Tables S1–S3.

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Supporting Information

Additional supporting information can be found online in the Supporting Information section. **Table S1:** Literature-based synthesis of stomatal trait measurements in rice. **Table S2:** Descriptive distribution of biological and experimental metadata among the 69 literature studies included in the synthesis, by measurement method. **Table S4:** Rank consistency of stomatal traits between measurement methods and environments. **Table S5:** Method comparison between SEM and Imprint for stomatal traits: summary statistics and proportional effect tests. **Figure S1:** Demonstrated methods for annotating guard cell length (GCL) and guard cell width (GCW) using the imprint and SEM method. **Figure S2:** Representative morpho-anatomical sections showing the diversity in leaf size, shape, and stomatal appearance estimated using both Imprint and SEM methods across the 10 *Oryza* species grown in greenhouse. **Figure S3:** Comparison of stomatal traits from experimental measurements and a literature collection. **Figure S4:** Method difference (SEM – Imprint) in stomatal density, guard cell length and guard cell width. **Figure S5:** Plant growth environment affects stomatal density, guard cell length, and guard cell width. **Figure S6:** Correlations of anatomical maximum stomatal conductance (g_{smax}) across measurement methods, leaf sides, and environments. **Figure S7:** Bland–Altman summary statistics comparing SEM and Imprint methods for (A) stomatal density, (B) guard-cell length, and (C) guard-cell width. **Table S3:** Mean values of stomatal density, guard cell length, and guard cell width in wild *Oryza* species.