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RESEARCH PAPER

Comparative histology of abscission zones reveals the extent of convergence and divergence in seed shattering in weedy and cultivated rice

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Abstract

The modification of seed shattering has been a recurring theme in rice evolution. The wild ancestor of cultivated rice disperses its seeds, but reduced shattering was selected during multiple domestication events to facilitate harvesting. Conversely, selection for increased shattering occurred during the evolution of weedy rice, a weed invading cultivated rice fields that has originated multiple times from domesticated ancestors. Shattering requires formation of a tissue known as the abscission zone (AZ), but how the AZ has been modified throughout rice evolution is unclear. We quantitatively characterized the AZ characteristics of relative length, discontinuity, and intensity in 86 cultivated and weedy rice accessions. We reconstructed AZ evolutionary trajectories and determined the degree of convergence among different cultivated varieties and among independent weedy rice populations. AZ relative length emerged as the best feature to distinguish high and low shattering rice. Cultivated varieties differed in average AZ morphology, revealing lack of convergence in how shattering reduction was achieved during domestication. In contrast, weedy rice populations typically converged on complete AZs, irrespective of origin. By examining AZ population-level morphology, our study reveals its evolutionary plasticity, and suggests that the genetic potential to modify the ecologically and agronomically important trait of shattering is plentiful in rice lineages.

Keywords: Abscission zone, convergent evolution, de-domestication, domestication, parallel evolution, seed shattering, weedy rice.

Introduction

Seed dispersal is an important life history process with substantial impact on plant success. Because seedling survival is often greater away from the parent plant (Hyatt *et al.*, 2003), wild plants have evolved a diversity of mechanisms to disperse their seeds (Seale and Nakayama, 2020). Efficient seed dispersal is the main mechanism by which plants can colonize new habitats and expand their ranges (Clobert *et al.*, 2012). Seed dispersal is also an agriculturally important trait, as it is generally undesirable in cultivated plants, particularly in those grown for their seeds such as cereal crops (Dong and Wang, 2015; Purugganan, 2019; Maity *et al.*, 2021). In both agronomic and natural systems, seed dispersal is thus strongly shaped by selective forces.

Cultivated Asian rice (*Oryza sativa* L.), one of the world's most important food crops (Bin Rahman and Zhang, 2022),

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Abbreviations: AFT, after heading date; AZ, abscission zone; BTS, breaking tensile strength; PCA, principal component analysis; QTL: quantitative trait loci; RF, random forest.

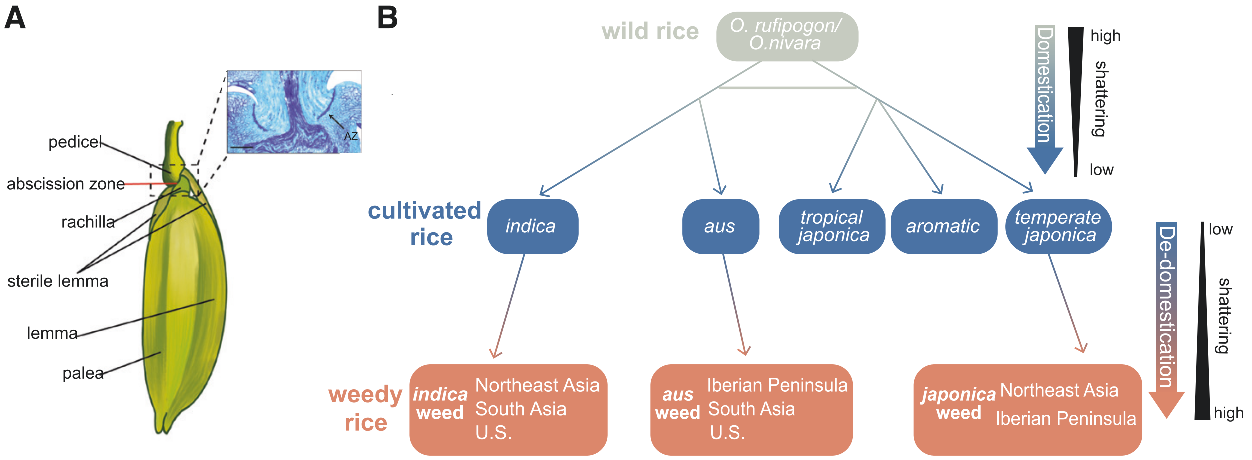

Fig. 1. Illustration of the position of the abscission zone (AZ) in a rice floret and schematic representation of some domestication and de-domestication events throughout rice evolutionary history. (A) The AZ is formed in the rachilla; in a longitudinally sectioned rachilla stained with toluidine blue, the AZ appears as a dark blue line extending from the edge of the floret to the central vascular bundle. (B) Five major cultivated groups (*Oryza sativa indica*, *aus*, *temperate japonica*, *tropical japonica*, and *aromatic*) were domesticated from wild rice (*Oryza rufipogon/Oryza nivara*), through independent, though interconnected (as represented by the gray line between the two lineages) events. Independent de-domestications of weedy rice from *indica*, *aus*, or *temperate japonica* cultivated backgrounds have subsequently occurred in different geographic locations. The degree of seed shattering decreased during domestication and increased during de-domestication events. Only cultivated and weedy rice populations used in this study are represented in the diagram; other weedy populations exist.

is an ideal example of the multiple, and sometimes opposing, selective forces shaping seed dispersal. In rice and other cereal grasses, seed dispersal is known as seed shattering, and it involves an easy release of the caryopsis (i.e. the grain; but in cereals little practical distinction is made between grain and seed) upon maturity in response to light touch or movement. The wild ancestor of cultivated rice, a species complex known as *Oryza rufipogon/Oryza nivara*, easily shatters its seeds (Ji *et al.*, 2006; Thurber *et al.*, 2010, 2011). During domestication 8000–10 000 years ago, reduced shattering was selected for in rice to facilitate harvesting and avoid yield loss (Fuller, 2007; Fuller and Allaby, 2009). Cultivated rice fields are often plagued by a weedy descendant of domesticated rice called weedy rice; like other agricultural weeds, weedy rice exploits high seed shattering to maximize its dispersal (Vigueira *et al.*, 2013; Maity *et al.*, 2021), making it a leading factor limiting cultivated rice productivity all around the world (Burgos *et al.*, 2006; Ziska *et al.*, 2015; Durand-Morat *et al.*, 2018). Modification of seed shattering levels has thus been a recurring theme throughout the evolutionary history of Asian rice, but there are gaps in our understanding of the mechanisms by which this has occurred.

Seed shattering requires the formation of one or more specialized cell layers, collectively called an abscission zone (AZ), where an organ separates from the plant (Patterson, 2001). The cells that constitute the AZ are often distinct from surrounding cells, and are generally small in size, have dense cytoplasm, lack a secondary cell wall, and have highly branched plasmodesmata (Valdovinos and Jensen, 1968; Sexton and Roberts, 1982; Yu *et al.*, 2020b). AZ location and anatomy vary among grass species (Yu *et al.*, 2020a, b). In rice, the abscission zone is formed in the rachilla, between the pedicel and floret (Fig. 1A), and consists of cells that lack lignin. The wild ancestors of rice are known to have well-developed AZs, and several studies have implicated loss or disruption of the AZ in the reduction of shattering in domesticated rice (Ji *et al.*, 2006; Konishi *et al.*, 2006; Li *et al.*, 2006). Likewise, a few studies looking at weedy rice AZs have found these to be well developed (Thurber *et al.*, 2011), suggesting that the AZ has re-evolved in weeds as a pre-requisite for high shattering.

While broadly informative, this simple sequence of proposed events does not fully encompass the complex evolutionary history of cultivated and weedy rice. Stemming from two separate but inter-related domestication events (Choi and Purugganan, 2018; Wang *et al.*, 2018; Fornasiero *et al.*, 2022), five major rice cultivar groups are recognized today: *aus*, *indica*, *temperate japonica*, *tropical japonica*, and *aromatic* (Garris *et al.*, 2005; Zhao *et al.*, 2011; Wang *et al.*, 2018) (Fig. 1B). These are all characterized by reduced seed shattering compared with ancestral wild rice, but this reduction varies in magnitude (Ji *et al.*, 2006; Thurber *et al.*, 2010). Levels of seed shattering affect ease of threshing, and have probably been selected to different degrees in cultivars based on timing and method of harvest (Ji *et al.*, 2006) and domestication origin. Weedy rice has also evolved multiple times independently, most often from a variety of cultivated rice ancestors through a process of de-domestication (Cao *et al.*, 2006; Li *et al.*, 2017; Qiu *et al.*, 2017, 2020; Hoyos *et al.*, 2020; Imaizumi *et al.*, 2021; Li *et al.*, 2022) (Fig. 1B). Each time weedy rice has evolved, it has gained a competitive advantage by re-evolving high seed shattering (Thurber *et al.*, 2010; Huang *et al.*, 2017; Li *et al.*, 2022). Both loss and gain of seed shattering have therefore occurred multiple times in rice, raising questions about the evolutionary trajectories of the AZ and the degree of convergence in their modifications.

To date, AZ histology has been characterized only in a few cultivars and wild rice accessions involved in quantitative trait locus (QTL) mapping, a few mutants, and a few weedy rice individuals (e.g. Ji *et al.*, 2006; Konishi *et al.*, 2006; Li *et al.*, 2006; Thurber *et al.*, 2011; Zhou *et al.*, 2012). This lack of population-level characterization has left us with an incomplete understanding of how the AZ morphology has changed in different rice evolutionary lineages, the degree to which similar morphological changes has occurred across populations, and how AZ morphology corresponds to the observed variability in seed shattering. Genetic studies have not provided clarity either, as alleles of genes associated with reduced seed shattering in cultivated rice, such as *SH4* (Li *et al.*, 2006), *qSH1* (Konishi *et al.*, 2006), *OsSh1* (Li *et al.*, 2020), and *OsLG1* (Ishii *et al.*, 2013), have often not been sufficient to account for the phenotype in all genetic backgrounds and can be associated with different AZ morphologies. Other shattering-related candidate genes such as *SH5* (Yoon *et al.*, 2014), *SNB* (Jiang *et al.*, 2019), *SHAT1* (Zhou *et al.*, 2012), *OsCPL1* (Ji *et al.*, 2010), *OSH15* (Yoon *et al.*, 2017), *SLR1* (Wu *et al.*, 2023), *OsGRF4* (Sun *et al.*, 2016), and *NPC1* (Cao *et al.*, 2016) have unknown roles in domestication or weed evolution. In addition, although multiple QTLs associated with increased shattering in some weedy rice groups have been found, no specific genes have been identified yet (Li *et al.*, 2023).

Here, we take a population-level approach to characterize AZ morphology in different cultivated and weedy rice groups. We develop measures to quantify aspects of the AZ and use this comparative histology to understand the morphological basis for changes in seed shattering during cultivated and weedy rice evolution. We specifically address the following questions. (i) What is the relationship between AZ morphology and seed shattering levels? (ii) What is the typical AZ morphology in cultivated rice groups and is there convergence among groups? (iii) What is the typical AZ morphology in weedy rice groups and is there convergence among groups. (iv) What has been the evolutionary trajectory of the AZ through each domestication and de-domestication event? We find that AZ length plays the largest role in determining shattering levels in rice. While there is convergence of AZ morphology among independent weedy rice groups, convergence is not prevalent among cultivated groups. Our reconstructions of the AZ evolutionary trajectories provide insights into the genetic mechanisms driving these evolutionary changes.

Materials and methods

Plant materials and histology

We included 86 weedy and cultivated rice accessions (Supplementary Table S1). Cultivated samples encompassed the five major domesticated rice variety groups. Weedy rice samples hailed from multiple locations including the Iberian Peninsula, South Asia, Northeast Asia, and the USA. The genetic background of each accession was previously determined using Faststructure (Raj *et al.*, 2014) on genotyping by sequencing data (Li *et al.*, 2022). Accessions with a >80% ancestry contribution from a single group were considered to belong to that genomic background; the remainder were considered admixed (Supplementary Table S1). One plant from each accession was grown in the greenhouse at UMass Amherst during autumn of 2019 and spring of 2020. A few accessions obtained later were grown in a Pervical PGC-105 growth chamber with a 11 h light (29 °C) and 13 h dark (24 °C) daily cycle in summer of 2020. Plants were grown to maturity and, when half of the panicle (inflorescence) had grown out of the boot (referred to as the 'heading' stage), either panicles were bagged for subsequent shattering measurements or florets were collected for microscopy.

Florets were fixed in an FAA solution (37% formaldehyde:ethanol:water:acetic acid=10:50:35:5), and dehydrated in a graduated ethanol series (50, 70, 85, 95%, and two changes of 100% ethanol), followed by clearing with an ethanol HistoClear solution (25, 50, 75%, and three changes of 100% HistoClear). The HistoClear was drained and paraplast was added and changed every 6 h six times at 60 °C. Florets were embedded in tissue cassettes with paraplast, serially sectioned into 10 µm longitudinal slices with a Leica RM2125 microtome, mounted on slides, and placed on a slide warmer at 42 °C for at least 24 h. The slides were stained with 1% toluidine blue solution for 30 min. Excess paraplast was cleared with two xylene washes. A glass cover slip was affixed to the slide with Permount. A Leica DM750 LED biological microscope with a ×4 or ×10 objective was used to image florets. Images were captured with an AmScope MU1000-HS camera and AmLite software.

Seed shattering measurements

The breaking tensile strength (BTS) required to remove individual seeds from the panicle was measured using an Imada DS2 digital force gauge, based on the protocol described by Thurber *et al.* (2010). Shattering levels were determined 30 d after heading (AFT30) when seeds reach maturity and shattering levels stabilize (Thurber *et al.*, 2011; Turra *et al.*, 2023). Ten seeds per panicle and at least one panicle for each rice accession were measured. We chose 2 gf (grams force) at AFT30 as our BTS threshold to distinguish high or low seed shattering, after considering the distribution of our shattering measurements (Supplementary Fig. S1) and the BTS values most likely to contribute to efficient (easy) seed dispersal. Four weedy accessions (rr20, arr27, arr75, and AS-08-8) flowered but only produced unfilled seeds, so we were unable to measure their shattering levels.

Image analysis

Images with a clear intact vascular bundle and symmetric flanking AZ were selected for quantification. RGB color images were first adjusted to 8-bit grayscale in ImageJ (Schneider *et al.*, 2012). Under grayscale, darkly stained regions are converted to pixels with low gray values while lightly stained regions are converted to pixels with high gray values. The AZ was identified as the darkly stained cells that span from the edge of the floret toward the vascular bundle (Supplementary Fig. S2). For each image, a square region was selected in the non-AZ region near the pedicel side (Supplementary Fig. S2) and its minimum gray value was set as a threshold. Line scans were drawn, with the plot profile tool in ImageJ, from the edge of the floret to the vascular bundle following the natural curve of the AZ (Supplementary Fig. S2). Pixels along the line scan with gray values greater than the threshold were classified as belonging to non-AZ tissue and pixels with lower values were classified as AZ tissue. The resulting profiles were used to produce three measures: relative length, discontinuity, and intensity. AZ relative length was calculated by dividing the number of pixels below the threshold by the total number of pixels in the line scan. AZ discontinuity was calculated by summing the number of

AZ pixel clusters, where these refer to a series of contiguous pixels below the threshold bounded by non-AZ tissue on one or both sides. AZ intensity was calculated by dividing the threshold value by the mean gray value of the AZ tissue, which provided a scaled measure of the staining contrast between AZ and non-AZ tissues. The three measures were performed on the left and right side of each section (Supplementary Table S2) and averaged for subsequent analyses (Supplementary Table S1).

Phylogenetic signal

We extracted a Variant Call Format file that only included the non-admixed *Oryza* accessions examined in our study from Li *et al.* (2022). After filtering out indels and removing alleles with a proportion of missing data >0.3 using vcftools (Danecek *et al.*, 2011), and converting into a PHY format using vcf2phylip v2.0 (Ortiz, 2019), the resulting file was used to construct a maximum likelihood consensus tree using IQ-TREE (Minh *et al.*, 2020). The TVMe+R4 model was chosen as the best-fit model based on the Bayesian information criterion. The consensus phylogenetic tree was used in tests for phylogenetic signal with the R package PHYLOSIGNAL (Keck *et al.*, 2016). Local Moran's I test was chosen to detect the hotspots of positive and negative autocorrelation (Münkemüller *et al.*, 2012; Keck *et al.*, 2016).

Scanning electron microscopy

To prepare rice for SEM imaging, the pedicel was manually removed to expose the seed base. Seeds were mounted on the specimen stage with the seed base perpendicular to the field emission gun. Images were taken on a JCM-6000Plus scanning electron microscope (Joel, USA) with an acceleration voltage of 5–10 kV under high vacuum to explore the relationships among seed base surface topography, AZ morphology, and seed shattering level.

Statistical analyses and modeling

Several of our analyses involved grouping accessions by shattering level, cultivated/weedy identity, or lineage. Population structure analyses from Li *et al.* (2022) identified three accessions (AS-08-25, AS-09-70, and AS-08-08) as possibly misclassified volunteer rice or weed–crop hybrids, thus they were not included in analyses requiring weed or crop classification. Accessions without recorded shattering data at AFT30 (AS-08-08, arr75, arr27, and rr20) were removed from analyses requiring a high or low shattering classification. Finally, when grouping accessions by *Oryza* lineage, we excluded possibly misclassified volunteers and admixed accessions (Supplementary Table S1).

Statistical analysis was performed using R Studio version 4.2.2 (R Core Team, 2022). The Welch *t*-test was used to analyze differences in BTS values, relative length, discontinuity, and intensity between any two groups. For comparisons among a greater number of groups, we used the Kruskal–Wallis test from the stats package (R Core Team, 2022). Post-hoc testing was performed with the ggpubr package (Kassambara, 2022), using the Mann–Whitney test with a Benjamini and Hochberg adjustment for multiple testing. Principle component analysis (PCA) was performed using relative length, discontinuity, and intensity with the ggbiplot package (Vu, 2011) to investigate the morphological differences between high and low shattering rice.

Random forest (RF; Cutler *et al.*, 2007) was used as a classification tool to determine which of our measurements were the best predictors of shattering level and crop or weedy status. We calculated 500 RFs, each consisting of 500 decision trees with two splits at each node. Model accuracy was assessed as the percentage of successful classifications of the out of bag data (Kay and Grossenbacher, 2022). Feature importance was determined by the mean decrease in Gini index, a measure that quantifies the efficacy of a feature to classify data, over all 500 RFs (Dellinger *et al.*, 2019). Models were created using the randomForest package in R (Liaw and Wiener, 2002).

Results

Abscission zone histological variation among cultivated and weedy rice accessions

In the initial examinations of our rice samples, we observed that cells in the AZ were predominantly round and irregular in shape, smaller than surrounding cells, and lacked lignin. Cells were densely packed together in an alternating arrangement, forming a narrow polygonal AZ (Fig. 2), rather than a single or double row(s) of cells, as is often depicted in diagrams. We detected great variation in how far the cell layer extended towards the vascular bundle, how discontinuous or interrupted this cell layer seemed to be, and the extent of contrast between the AZ and its neighboring cells. To quantify this phenotypic diversity, we developed and performed measures of relative length, discontinuity, and intensity for the AZ in all rice accessions (Supplementary Tables S1, S2).

AZ relative length varied from 0.07 to 1, corresponding to a range of phenotypes from an absence of AZ (Fig. 2A) to an AZ that extended from the edge of the floret to the vascular bundle (Fig. 2B). Discontinuity is a discrete variable that ranged from 1 to 11 in our samples, spanning an AZ without any breaks (Fig. 2C) to an AZ with multiple breaks (Fig. 2D), respectively. Intensity, which ranged from 1.06 to 2.31, represents the degree of contrast between the AZ and non-AZ tissues, with higher numbers indicating a more pronounced AZ relative to the surrounding cells (Fig. 2F), and lower ones a less distinct AZ (Fig. 2E). We calculated the coefficient of variation (CV) for each feature to assess their variability while accounting for the population mean (Pélabon *et al.*, 2020). Discontinuity exhibited the highest variability, with a CV of 67.45, while relative length and intensity displayed similar CVs, 30.49 and 20.37, respectively (Supplementary Table S3).

Weedy rice often displayed a complete AZ characterized by a high relative length, low discontinuity, and high intensity (e.g. Fig. 2B, F). Cultivars frequently exhibited a partial AZ that extended from the floret edge but stopped before reaching the vascular bundle (e.g. Fig. 2C), or a disrupted AZ usually characterized by multiple breaks between the cells and less contrast with neighboring regions (e.g. Fig. 2A, D, E). All AZ measures were significantly different between cultivated and weedy rice, despite a range of variation in both groups (Supplementary Fig. S3). Cultivated rice also showed greater diversity of AZ morphology than weedy rice as evidenced by its higher CV in relative length and intensity (Supplementary Table S3).

We further examined how well these AZ measures could predict crop or weedy identity using RF modeling. More than half of the samples were accurately predicted, with a mean accuracy prediction rate of 0.64 (Supplementary Table S4). Relative length, with a mean decrease in Gini index of 22.22,

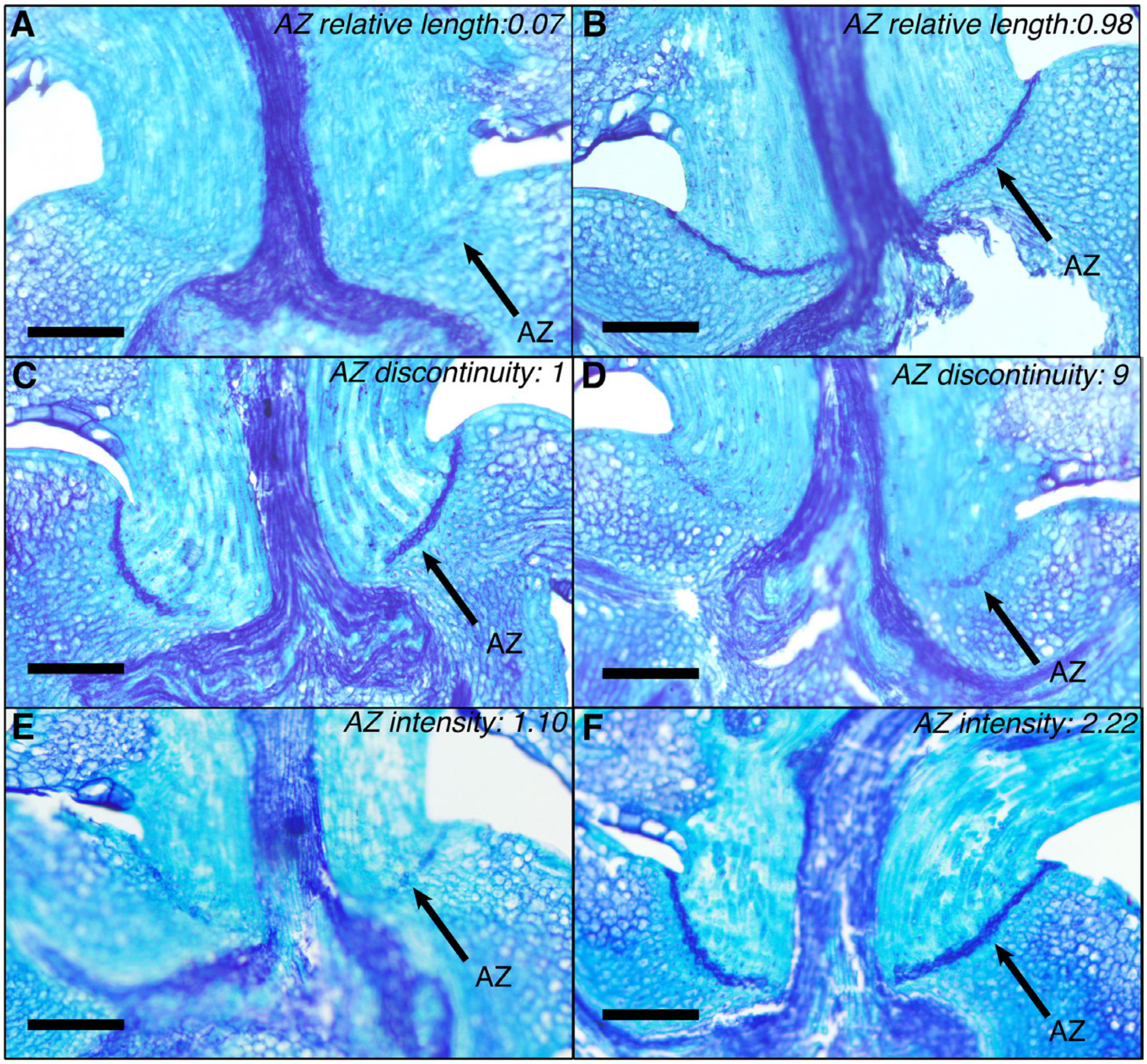

Fig. 2. The variation in abscission zone (AZ) morphologies in rice captured by measures of relative length, discontinuity, and intensity. (A–F) Toluidine blue-stained mature rice spikelets with extreme values for each measure; the AZ is stained dark blue and indicated by the black arrow. Bars: 100 µm. (A) AN-09-03, a *temperate japonica* crop with an AZ relative length of 0.07; (B) AS-09-156, a *temperate japonica* derived weed with an AZ relative length of 0.98; (C) sin11, an *indica* crop with an AZ discontinuity of 1; (D) AN-09-30, a *temperate japonica* crop with an AZ discontinuity of 9; (E) ste8, a *temperate japonica* crop with an AZ intensity of 1.10; (F) rr35, an *indica*-derived weed with an AZ intensity of 2.22.

played the most important role in predicting identity, followed by intensity and discontinuity, with mean decreases of 12.21 and 6.52, respectively (Supplementary Table S5).

Correlating abscission zone histology with seed shattering ability

To investigate how variation in AZ morphology corresponds to different levels of seed shattering, we categorized our samples into high and low shattering groups and compared the AZ morphology between them. Though weeds tend to be high shattering, a range of shattering levels exists in both groups (Supplementary Tables S1, S6). The median BTS values for the low and high seed shattering groups were 0 gf and 26.80 gf, respectively (Supplementary Table S3). Compared with the high shattering group, the low shattering group demonstrated much greater variation in BTS values (Fig. 3A).

Significant differences were observed between high and low shattering rice for all three AZ features (Fig. 3B–D). The AZ of high shattering accessions was characterized by higher relative length, lower discontinuity, and higher intensity than the AZ of low shattering accessions, a similar pattern to the weed–crop comparison (Supplementary Fig. S3). Larger variation was observed in low shattering rice (Fig. 3B–D), consistent with the higher CVs for these measures (Supplementary Table S3). A PCA indicated that principal component 1 (PC1), which explained 81.7% of the variation, provided better separation between high and low shattering groups than PC2, although

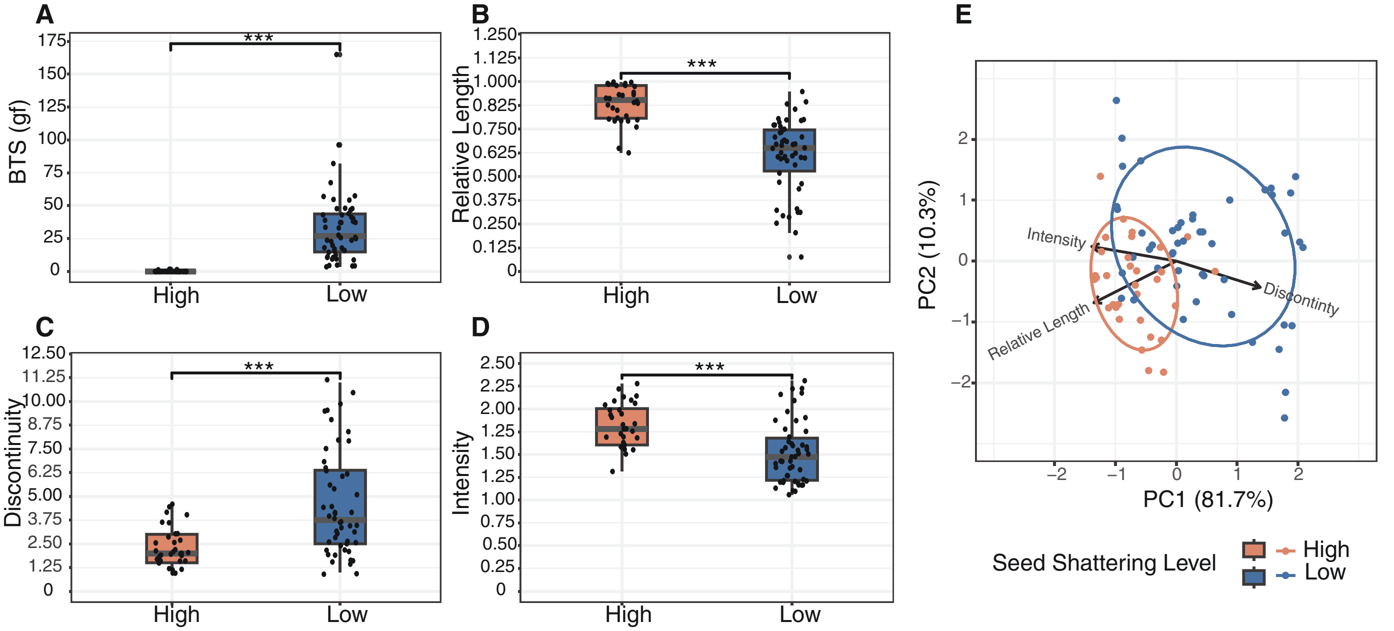

Fig. 3. Variation in abscission zone (AZ) relative length, discontinuity, and intensity in high and low shattering rice. (A–D) Boxplots of seed shattering abilities and AZ morphologies in high (32 accessions) and low (50 accessions) shattering rice groups; each dot represents measurements from a different accession, with low shattering accessions coded with the blue boxplot and high shattering accessions coded with the orange boxplot; upper, middle, and lower lines of the box represent the 75th percentile, median, and 25th percentile respectively; whiskers above and below the box extend to the upper and lower values, up to 1.5 the interquartile range. Significant differences between groups were determined through the Welch *t*-test; significance levels are denoted by $^*P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$. (A) Median breaking tensile strength (BTS) measured 30 d after heading; (B) AZ relative length; (C) AZ discontinuity; (D) AZ intensity; (E) principal components analysis (PCA) of AZ features for 82 rice accessions.

there was overlap between the groups (Fig. 3E; Supplementary Table S7). High shattering accessions were more tightly clustered, indicating that the AZ morphology of high shattering rice was more convergent (Fig. 3E).

Using an RF classifier, a more accurate prediction rate of 0.80 (Supplementary Table S8) was achieved when we used high/low shattering level as the output instead of crop/weedy identity. Among the three features, AZ relative length was found to be most important for predicting seed shattering level, with a mean decrease in Gini index of 24.33, followed by intensity at 10.7, and AZ discontinuity at 3.53 (Supplementary Table S5).

The extent of convergence in abscission zone morphology during rice domestication and weedy rice evolution

Our population-level examination of AZ morphology allows for systematic histological comparisons across different cultivar and weed groups. These comparisons allow us to understand the extent of AZ convergence underlying functional similarities, such as reduced seed shattering in different cultivars during domestication, or reacquired shattering in the de-domestication of weeds with distinct origins. Additionally, our comparisons allow us to reconstruct the trajectories of the AZ morphology throughout the evolutionary history of rice.

Comparisons among cultivated rice groups

To determine what the large variation in AZ morphology among rice cultivars implied for convergent evolution of reduced shattering during domestication, we compared morphologies across the three cultivated groups with the largest sample sizes: *aus*, *indica*, and *temperate japonica*. *Aus* crops had the highest median seed shattering levels (Fig. 4A), with the AZ typically extending partially or almost completely from the edge of the floret to the vascular bundle. *Indica* crops exhibited moderate seed shattering (Fig. 4A), and typically had a partial AZ extending from the edge of the floret but stopping halfway between the edge and the vascular bundle (Fig. 2C). No significant differences were observed between these two cultivar groups for any of the three AZ features, but a slightly lower relative length and a larger variation of intensity was evident in *indica* compared with *aus* cultivars (Fig. 4B, D). *Temperate japonica* cultivars exhibited the lowest seed shattering levels (Fig. 4A) and diverse AZ morphology. Compared with *indica* and *aus*, the AZ of *temperate japonica* crops appeared to be more disrupted or underdeveloped. The AZ in *temperate japonica* cultivars usually had multiple breaks and was less discernible from its surrounding cells (Fig. 2A, D, E). This aligned with higher discontinuity values (Fig. 4C) and lower intensity values in *temperate japonica* crops (Fig. 4D), and usually corresponded to a truncated AZ (Fig. 4D). Significant differences were observed in all three AZ measures between *temperate japonica* and the

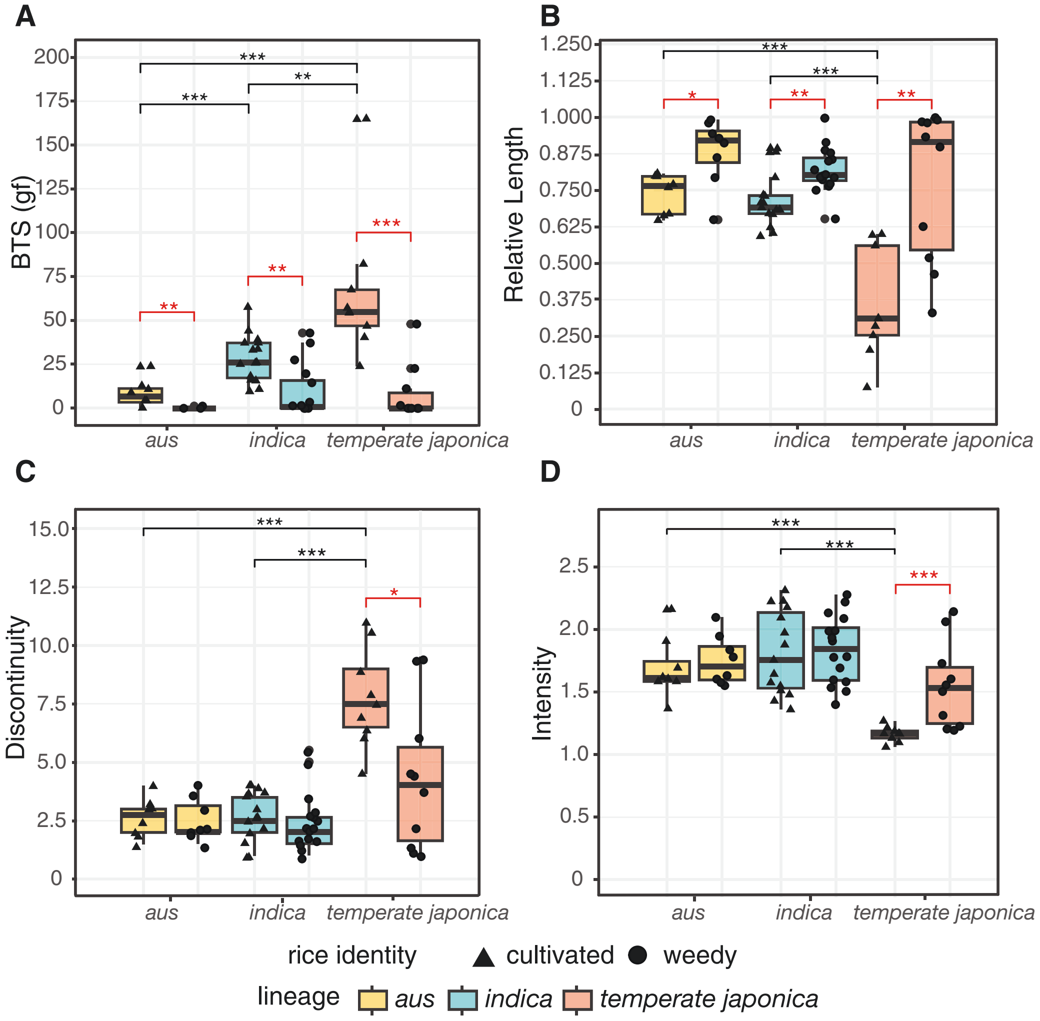

Fig. 4. Variation in seed shattering level and abscission zone (AZ) relative length, discontinuity, and intensity in cultivated and weedy rice groups. (A–D) Boxplots of shattering levels and AZ morphologies in *aus* cultivated rice (8 accessions), *aus* derived weedy rice (8 accessions), *indica* cultivated rice (15 accessions), *indica* derived weedy rice (16 accessions), *temperate japonica* cultivated rice (9 accessions), and *temperate japonica* derived weedy rice (10 accessions). Measurements from cultivated and weedy rice accessions are denoted as a dot and triangle, respectively, with each of these in the plot representing results from a different accession. Rice lineage is represented by yellow (*aus*), blue (*indica*), and orange (*temperate japonica*) boxplots; upper, middle, and lower lines of the box represent the 75th percentile, median, and 25th percentile respectively; whiskers above and below the box extend to the upper and lower values, up to 1.5 the interquartile range. Significant differences among three cultivated or weedy groups were determined using the Kruskal–Wallis test followed by post-hoc testing, and levels of significance are denoted by *P<0.05, **P<0.01, ***P<0.001 with black color; significant differences between weedy and cultivated groups from the same lineage were determined using the Welch t-test, and levels of significance are denoted as *P<0.05, **P<0.01, ***P<0.001 with red color. (A) Median breaking tensile strength (BTS) measured 30 d after heading; (B) AZ relative length; (C) AZ discontinuity; and (D) AZ intensity.

other two crop groups (Fig. 4B–D). Overall, our results suggest that reduced shattering levels in domesticated rice groups were achieved via a variety of AZ morphologies.

Comparison among weedy rice groups

Our observation of AZ morphologies in weedy rice suggested a high degree of morphological convergence underlying the evolution of high seed shattering weeds. To assess the extent of variation, we focused on weedy rice populations that originated from *aus*, *indica*, and *temperate japonica* crops. Regardless of the cultivars from which each weed group evolved, the AZ of weeds was usually continuous, distinct from its surrounding cells, and extended substantially toward the vascular bundle (Fig. 2B, F); this was consistent with high relative length, low discontinuity, and high intensity measures (Fig. 4B–D). No significant differences were observed between weed groups in their shattering level or any of the AZ features (Fig. 4A–D). However, a higher CV for each of the AZ measures was detected in weeds originating from *temperate japonica* crops (Supplementary Table S3), suggesting somewhat less convergence within this group.

Comparison among the transitions from cultivated to weedy rice

To gain insight into how the restoration of high shattering during de-domestication varied depending on the genetic background, we explored the evolutionary changes in the three AZ features in weeds originating from *aus*, *indica*, and *temperate japonica* crops. Overall, the derived weed groups exhibited significantly higher shattering levels than their ancestral cultivars (Fig. 4A). However, only AZ relative length differed significantly in weedy groups originating from *aus* and *indica* crops relative to their ancestors (Fig. 4B), although there was a tendency for weed AZs to also be more continuous and more intense than in their cultivated counterparts in both lineages. This weed–crop difference was consistent even though the relative length of the AZ in *aus* cultivars was generally longer than that in *indica* cultivars (Fig. 4B). In contrast, we detected significant differences in all three AZ features between *temperate japonica* crops and their derived weeds (Fig. 4B–D). This suggests a more complex process of regaining high seed shattering, such as re-evolving a new AZ if the weeds are derived from a *temperate japonica* cultivar without any AZ present (Fig. 2A) or closing the breaks between the AZ cells if the ancestral cultivar had a disrupted AZ (Fig. 2D or F).

To evaluate the impact of the three AZ features on the transitions to weediness, we performed separate RF analyses for each pair of ancestral crops and their derived weeds (Supplementary Table S9). For most models, AZ relative length was the most effective predictor, followed by intensity and discontinuity (Supplementary Table S5). The exception was the *temperate japonica* lineage, where intensity had a slightly higher mean decrease in Gini index than relative length. This indicates that intensity plays an important role in predicting crop or weedy identity in the *temperate japonica* lineage (Supplementary Table S5). Our findings further demonstrate that different AZ features were modified to evolve high shattering abilities during independent de-domestication events.

Phylogenetic signal of abscission zone morphology

We constructed an unrooted phylogenetic tree using the non-admixed samples. As expected, each ancestral crop–derived weed pair formed a distinct lineage, and the phylogeny recapitulated the known evolutionary history of cultivated and weedy rice (Supplementary Fig. S4). We mapped normalized AZ features onto the phylogenetic tree (Supplementary Fig. S4) and found that variation appeared to align more closely with shattering level rather than crop/weedy classification, although this varied by group.

Phylogenetic signal is a measure of the statistical dependence among trait values resulting from their underlying phylogenetic relationships (Revell *et al.*, 2008; Münkemüller *et al.*, 2012; Kamilar and Cooper, 2013; Vőfély *et al.*, 2019). Although often applied to species phylogenies, we assessed within-species phylogenetic signal for our weedy and crop samples. A local Moran's I test (Keck *et al.*, 2016) was computed for each tip of the phylogeny and for each of the AZ features, to detect the hotspots of positive and negative dependence. A total of 32, 54, and 46% of the rice samples showed evidence of phylogenetic signal for relative length, discontinuity, and intensity, respectively (Supplementary Table S10). In general, levels of phylogenetic signal were relatively low for all three AZ traits, consistent with the independent convergent evolution of high shattering weedy rice groups and of low shattering cultivated rice groups. Compared with discontinuity and intensity, fewer samples demonstrated evidence for phylogenetic signal for relative length, indicating that this is a trait relatively easily modified by evolution, while the others are more constrained by phylogeny. These findings align with our earlier comparisons between crops and weeds of various genetic backgrounds, where the evolution of significantly higher relative length was consistently observed in weeds. In contrast, the other AZ traits seem to have only been significantly modified in the evolution of *temperate japonica* crops and their derived weeds (Fig. 4B–D).

Examining the phylogenetic signal in each lineage can provide further insight into differences in the phylogenetic constraint of AZ morphologies. The *aus* and *indica* lineages both showed higher phylogenetic signal for discontinuity (Supplementary Table S10), suggesting that discontinuity is the least modified feature in these crops and weeds. The *japonica* lineage demonstrated a consistently stronger phylogenetic signal for all three AZ features, particularly in cultivars (Supplementary Fig. S4; Supplementary Table S10). Such a strong phylogenetic signal is likely be attributed to the stark differences in AZ morphology between *japonica* crops and other crops or weeds. Additionally, in the *japonica* lineage, samples with similar shattering levels tended to cluster together more frequently, particularly compared with the *aus* lineage where low shattering accessions were interspersed among high shattering accessions (Supplementary Fig. S4).

Seed base topography in different rice accessions

The smoothness level of the spikelet or seed base of rice samples has been used as a proxy for characterizing levels of shattering in both archeological rice remains and modern samples (Fuller *et al.*, 2009; Qiu *et al.*, 2017; Ishikawa *et al.*, 2022). We employed the same approach to compare the morphologies of seed bases for various crops and weeds that exhibited diverse AZ morphologies (Fig. 5). We found a very rough correlation between seed base morphology and seed shattering level. For instance, weeds with a complete and clear AZ and high seed shattering levels often had a seed base with a straight profile, a smooth and round abscission scar, and a relatively small and distinct vascular pore (Fig. 5A, B), which is consistent with the morphology observed in wild rice (Fuller *et al.*, 2009). Cultivars with low shattering ability and an underdeveloped AZ tended to have an uneven profile, dimpled appearance, and a larger and more irregular vascular pore (Fig. 5G, H). This difference

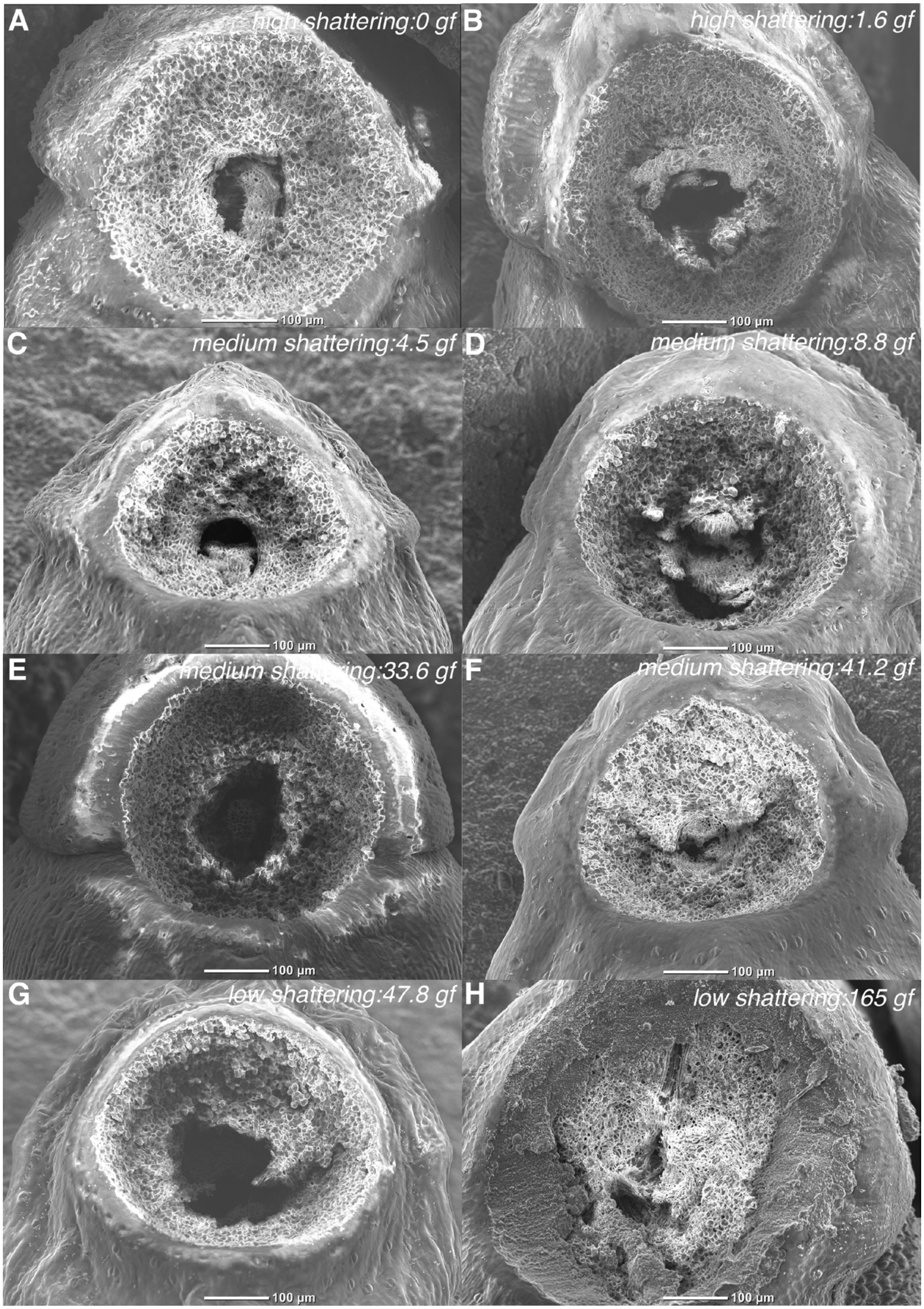

Fig. 5. SEM images of seed base topography from a sample of rice accessions with different shattering abilities. (A) rr42, an *indica* derived weed with a median BTS of 0 gf; (B) rr51, an *indica* derived weed with a median BTS of 1.6 gf; (C) 71481, a *tropical japonica* crop with a median BTS of 4.5 gf; (D) sau80, an *aus* crop with a median BTS of 8.8 gf; (E) sin11, an *indica* crop with a median BTS of 33.6 gf; (F) AN-09-46, a *temperate japonica* crop with a median BTS of 41.2 gf; (G) sus4, a *tropical japonica* crop with a median BTS of 47.8 gf; (H) AN-09-30 a *temperate japonica* crop with a median BTS of 165 gf. Bars: 100 μm.

in seed base morphology was clear when comparing rice with substantially distinct AZ morphologies, such as a complete AZ (Fig. 5A) versus a disrupted AZ (Fig. 5H). However, differences were difficult to categorize in the majority of our samples, which showed a range of intermediate variation in seed base topography (Fig. 5B–G). Additionally, the topography of the seed base surface is influenced not only by the completeness of the AZ, but also by the separation mechanism, including the forces and angle of detachment. Variation in seed base morphology from the same rice accession (Supplementary Fig. S5) highlights limitations of using this method to infer shattering levels. Distinguishing smooth from rough or uneven regions can be another challenge, since the AZ is dome-shaped, not a flat surface. Despite its popularity, relying solely on this method to assess seed shattering is risky; we thus recommend caution in qualitatively classifying seed shattering level based solely on perceived topography or in comparing seed shattering level through quantitative measurements of the smooth area of the seed base.

Discussion

The modification of seed shattering levels has been a recurrent theme throughout the evolutionary history of rice. Our examination of AZ morphology in typically low shattering cultivated rice and high shattering weedy rice provides a comprehensive view of the evolutionary modifications to the process of abscission from a histological perspective. Specifically, we show how variation in three quantitative measures to describe AZ morphology contributes to different seed shattering abilities. By examining lineage-specific variation in the AZ, we uncover the typical morphologies in independently evolved rice populations, assess the extent of morphological convergence underlying functional similarities in shattering, and offer insight into the potential genetic mechanisms involved. These discoveries deepen our understanding of the processes of both rice domestication and de-domestication.

Abscission zone morphology and its relationship to seed shattering in rice

The cellular characteristics of the AZ are consistent in both cultivated and weedy rice (Fig. 2). They appear as small, cytoplasmatically dense cells that are easily distinguishable from surrounding cells, aligning with previous research (Yu and Kellogg, 2018; Yu *et al.*, 2020b). In our examined rice samples, the complete absence of any AZ cells at the rachilla is uncommon and observed only in several *japonica* cultivars. This suggests that the molecular mechanisms determining AZ cell identity and location are probably conserved and functional in most types of cultivated and weedy rice. Alterations in AZ cell number seem to be important determinants of shattering ability, and two of our AZ measures—relative length and discontinuity—unambiguously capture these alterations (Fig. 2A–D). In contrast, variation in AZ intensity does not necessarily reflect a cell number alteration (Fig. 2E, F), but may be attributed to either the density at which cells were packed into the AZ layer or differences in cellular components that affected staining, such as cellulose or hemi-cellulose, which are typically stained dark blue by toluidine blue. However, our results suggest that cell number changes that affect the length of the AZ are the most frequently observed throughout rice evolutionary history and more strongly account for differences in shattering ability.

A popular indirect assessment of AZ functionality and shattering ability in archaeological and modern rice samples has been examination of the seed base under a light or scanning electron microscope (e.g. Fuller *et al.*, 2009; Qiu *et al.*, 2017; Ishikawa *et al.*, 2022). The topography of the seed base surface after detachment is thought to reflect the ease of cell separation between the pedicel and floral side of the AZ. Our results suggest that this approach to assess shattering is risky. Weedy (as does wild) rice usually has a complete and functional AZ, often resulting in a smooth seed base surface with small sealed vascular bundle pores after shattering (Fig. 5A, B). However, deviations of this morphology were also seen in samples that we knew to have high shattering (Supplementary Fig. S5G–I), and large variation of seed surfaces was observed in samples with medium or low shattering and with partial or disrupted AZs (Fig. 5C–G; Supplementary Fig. S5A–F). These intermediate scan morphologies are regularly observed and cannot clearly be assigned to a shattering or non-shattering functionality.

Implications for the reduction of shattering during domestication of cultivated rice

Reduction of seed shattering has long been considered a crucial step in the domestication of rice, as a way to facilitate harvesting and avoid yield loss (Purugganan, 2019). Based on studies of a few lines used in crosses, mutagenesis, or transformations (e.g. Ji *et al.*, 2006; Li *et al.*, 2006; Thurber *et al.*, 2011; Zhou *et al.*, 2012), a simplified paradigm of how shattering was reduced during rice domestication has emerged in the last couple of decades. This paradigm proposes that reduced shattering was achieved through transition of a complete AZ in wild rice to loss of the AZ in cultivated rice (Purugganan and Fuller, 2009). By characterizing AZ morphology in multiple accessions of the main cultivated rice lineages, however, we have shown the extent of morphological variation in AZs among different rice cultivars, which belie a simple or single ‘loss of shattering’ mechanism for cultivated Asian rice.

We found that an absent or severely reduced AZ morphology is, in fact, only typical of cultivated *temperate japonica*, with AZs either highly discontinuous or barely detectable (Fig. 2A). *Indica* and *aus* cultivars, in contrast, most often display clearly defined partial AZs (Fig. 2C), a morphology not often associated with domestication. The similarity between *indica* and *aus* is consistent with the closer phylogenetic relatedness between these groups and suggests that their reduction in shattering could have a shared evolutionary basis. However, differences in shattering levels between *aus* and *indica* (Fig. 4A), and in the degree of variation for features such as AZ intensity and relative length (Fig. 4B–D; Supplementary Table S3), indicate that even among closely related rice lineages, domestication has shaped loss of shattering in different ways.

Our results of AZ morphology add nuance to a growing understanding of the genetic basis and evolutionary history of domestication traits in lineages of cultivated rice. For many years it has been known that a non-synonymous mutation in the *SH4* locus, which codes for an *Myb3* transcription factor, is fixed in cultivated rice and was a step in the reduction of shattering in all cultivated rice lineages (Li *et al.*, 2006; Zhang *et al.*, 2009). Expressing the wild rice *SH4* allele in a *japonica* cultivar with a highly disrupted AZ resulted in a more continuous and extended AZ toward the vascular bundle and increased shattering (Li *et al.*, 2006). However, that the *SH4* domestication allele is not sufficient for loss of shattering and is likely to be affected by genomic background has also been evident from reports of weedy and wild rice that shatter and yet carry this allele (Thurber *et al.*, 2010; Ishikawa *et al.*, 2022). Other loci contributing to loss of shattering have been identified, such as *qSH1* (Konishi *et al.*, 2006) and *SHAT1* (Zhou *et al.*, 2012), and may modulate the effect of *SH4* in different lineages. Interactions between lineage-specific shattering loci may thus possibly explain the diversity of AZ morphologies we observed.

Determining how these interacting genes shape AZ morphology will take further work; however, in light of recent literature, our findings suggest some possible lineage-specific causal loci. For example, the slightly longer AZ in *aus* crops compared with *indica* crops could possibly be due to the *OsSh1* gene within the *qSH3* QTL (Lin *et al.*, 2012; Ishikawa *et al.*, 2022). Cultivated alleles of *qSH3* are associated with a very slight shortening of the AZ, as observed between inbred lines carrying the *qSH3* cultivar allele and those without it (Ishikawa *et al.*, 2022). Moreover, population analyses indicate that the *qSH3* cultivated allele is present in *indica* cultivars but not in *aus* (Ishikawa *et al.*, 2022). *qSH1* is another major seed shattering locus proposed to have been involved in loss of seed shattering during domestication, although the cultivated allele has only been observed in some *japonica* cultivars (Konishi *et al.*, 2006; Zhang *et al.*, 2009). A single nucleotide polymorphism (SNP) 12 kb upstream of a homeobox gene results in the absence of an AZ in 'Nipponbare' (Konishi *et al.*, 2006). One of our *temperate japonica* accessions (ste1) carrying this Nipponbare allele shows a partial AZ, suggesting that this mutation itself is not enough to attribute to the absence of the AZ. The presence of other morphologies in the *japonica* population suggests the potential involvement of yet other loci in the modification of AZs in these cultivars. Overall, distinct AZ morphologies observed in different rice lineages support a diversity of genetic mechanisms responsible for reduced seed shattering during rice domestication.

Implications for the evolution of shattering during the de-domestication of weedy rice

Compared with rice domestication, the independent de-domestications of different weedy rice groups around the world constitute relatively recent events, <10 000 years old and in some cases just a few hundred years (Reagon *et al.*, 2010; Li *et al.*, 2017; Qiu *et al.*, 2017). The rapid reacquisition of high seed shattering in each of these groups suggests that this trait is crucial to success as a weed and that it is not a difficult trait to re-evolve. Regardless of their distinct cultivated ancestries, most weedy rice consistently exhibit a complete or almost complete, highly continuous, and intense AZ (Figs 2, 4B–D), indicating that these features are essential for high shattering ability.

This convergence in AZ morphology in weeds stands in contrast to the diversity of morphologies that underlie reduction of shattering during domestication. The convergence is all the more striking given that different weedy rice populations would seem to have faced varying challenges in re-evolving high shattering, depending on their ancestral background. For example, *aus*-derived weeds would seem to require only slight modifications to AZ length to make a functional tissue with capacity for high shattering; in contrast, weeds derived from *temperate japonica* had to almost re-evolve an AZ *de novo*.

That re-evolution of all these AZ features has occurred more than once in all rice lineages studied here suggests that the genetic potential necessary is plentiful in weed ancestral groups, through either standing genetic variation or the existence of a plethora of mutational targets that can rescue AZ morphology. Our current knowledge of the genetic mechanisms underlying high seed shattering and AZ morphology in weedy rice remains limited. The few QTL mapping studies carried out so far have found little overlap in the seed shattering QTLs among different weed×crop mapping populations and have generally not implicated known candidate shattering genes (Thurber *et al.*, 2010, 2013; Subudhi *et al.*, 2014; Qi *et al.*, 2015; Li *et al.*, 2023). This finding is perhaps not surprising, given the variety of evolutionary trajectories for convergence of AZ morphologies in independently evolved weedy rice lineages.

The evolutionary plasticity of the AZ in rice, and the diversity of genetic mechanisms that can be involved would seem to imply that the recurrent evolution of weedy rice is, to some degree, largely unavoidable, and that management efforts are best directed at early detection of contaminating weeds and removal from fields. Conversely, the malleability of the AZ also suggests that there is a diversity of mechanisms by which ease of threshing in cultivated rice can be fine-tuned by breeders. Coupling our morphological data with mapping and population genetic studies will increase our understanding of the genetic mechanisms and their morphological consequences impacting the ecologically and agriculturally important trait of seed shattering.

Supplementary data

The following supplementary data are available at *JXB* online.

Table S1. Rice samples used in this study.

Table S2. Measurements for the three AZ features for both sides of the AZ in each rice accession.

Table S3. Statistical summary of different groupings for different measurements (BTS values at AFT30, the three AZ features).

Table S4. The probability of the accurate prediction for crop/weedy identity based on random forest modeling.

Table S5. The mean decrease in Gini index of the three AZ measures in different models.

Table S6. Raw BTS values at 30 d after heading date (AFT30).

Table S7. Principal component analysis of AZ features.

Table S8. The probability of the accurate prediction for high/low shattering level based on random forest modeling.

Table S9. The probability of the accurate prediction for crop/weedy identity in different clades based on random forest modeling.

Table S10. The phylogenetic signal (local Moran's I) for different AZ measures.

Fig. S1. Frequency histogram of the median breaking tensile strength (BTS) measured 30 d after heading (AFT30) across all the examined rice accessions.

Fig. S2. Illustration of the measures carried out for each AZ image.

Fig. S3. Variation in AZ relative length, discontinuity, and intensity in cultivated (40 accessions) and weedy rice (42 accessions).

Fig. S4. Phylogenetic tree of cultivated and weedy rice with mapping of the normalized AZ features and shattering levels.

Fig. S5. SEM images of seed base topography from the same rice accession.

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Author contributions

XL and ALC: conceptualization supervision, and funding acquisition; XL, DL, and JL: methodology and investigation; XL and DL: formal analysis and visualization; XL, DL, and ALC: writing—original draft preparation and review and editing.

Conflict of interest

No conflict of interest declared.

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Data availability

All primary data to support the findings of this study are openly available in Dryad Digital repository (doi:10.5061/dryad.dv41ns265; Li *et al.*, 2024), including the raw images of AZs for all the examined rice accessions, the gray-scaled images of AZs with the AZ line scans, the square regions that were selected as controls for non-AZ tissues, and the gray values along the line scans of AZs.

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