

# Physiological and transcriptomic analysis provide new insight into seed shattering mechanism in *Carex breviculmis* 'Siji'

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## Research Article

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# Abstract

*Carex breviculmis* is an excellent native grass species of the *Carex L.* of the Cyperaceae family. It has strong resistance and outstanding water-saving advantages, which is widely used in landscaping and understory ecological management. However, the strong seed shattering affects the seed yield and increases the risk of seed harvesting, which not only enhances the difficulty of seed production, but also limits the large-scale application of *C. breviculmis*. In order to explore the intrinsic mechanism of seed shattering in *C. breviculmis*, this study analyzed the histology, physiology, and transcriptomics of *C. breviculmis* seeds. Through histological observation, it was found that there are obvious abscission zones on the seeds, mainly distributed at the end of the seeds. By measuring the physiological indicators, it was found that the cellulase activity in the abscission zone gradually increased with the development of the spike at different stages, and was significantly positively correlated with the degree of seed shattering. In addition, the changes in the content of gibberellin, cytokinin, and ethylene in the abscission zone were significantly correlated with the breaking tensile strength. The second-generation transcriptome sequencing data of the abscission zone indicate that the main pathways involved in seed shattering include "plant hormone signaling transduction", "phenylalanine biosynthesis", and "starch and sucrose metabolism", and many key genes that may be involved in the seed shattering process have been identified. This study provides new insights into the seed shattering mechanism of *C. breviculmis* and has important theoretical and practical significance for breeding low seed shattering *C. breviculmis* varieties.

## Key message

During the process of seed shattering in *C. breviculmis* 'Siji', pathways related to seed shattering underwent changes. DEGs involved in extracellular cellulase activity and plant hormones, leading to seed shattering.

## 1. Introduction

The phenomenon of seed shattering is widely present in nature, which is a survival strategy formed by plants in the long-term process of species evolution to adapt to harsh environments and effectively reproduce offspring (Liquin, et al. 1996; Patterson 2001). Seed shattering refers to the phenomenon in which seeds split horizontally from the inflorescence axis or small spike axis of the parent plant, causing the seeds to fall off (Fan, et al. 2013). The seed shattering of mature plant seeds is beneficial for the reproduction and spread of species. From an evolutionary perspective, this is a beneficial process because it not only promotes the separation of aging or damaged organs from the plant, but also facilitates the release of fruits and the spread of seeds, which is conducive to the effective and sustained spread or reproduction of wild plants, thereby improving species adaptability (Di Vittori, et al. 2019; Estornell, et al. 2013; Nakano and Ito 2013). However, in agricultural activities aimed at seed production, seed shattering is a key limiting factor, which not only increases the production cost and

difficulty of harvesting seeds, but also reduces seed quality, causing huge losses to seed producers(Geitmann 2018; Pickersgill 2007; Qureshi, et al. 2022; Zhang 2011).

Related studies have shown that seed shattering may be related to factors such as structural characteristics of abscission zones, plant hormones, hydrolytic enzymes, and plant aging(Estornell, et al. 2013; Taylor and Whitelaw 2001; Zhang 2011). Among these factors that regulate seed shattering, the formation and development of abscission zone structures are the histological basis for seed shattering. Changes in the concentration of plant hormones such as ethylene, auxin, and gibberellin can produce signals that promote or inhibit seed shattering. Changes in the activity of cell wall hydrolases such as cellulase, pectinase, and polygalacturonase can trigger cell separation in the abscission zone. These processes are all regulated by gene expression(Ballester and Ferrándiz 2017; Lewis, et al. 2006; Zhang 2020). An important structure involved in the process of seed shattering is the abscission zone, where the first step in seed shattering is to form the abscission zone where the seed attaches to the plant(Maity, et al. 2021). The abscission zone is usually located at the site where the seed detaches from the parent plant. It is composed of one to several layers of cells with small morphology, dense cytoplasm, and abundant starch granules formed by lateral division of the petiole or stem base. Its formation, development, and degradation are the direct causes of seed shattering(Bleecker and Patterson 1997; Zhang 2020). Research has shown that in most plants, abscission zones are formed before cell separation and delamination activation(Han, et al. 2019; Tiwari and Bhatia 1995). The abscission zone of *Elymus sibiricus* L. is formed before the heading stage, and the abscission layer breaks around the heading stage(Xie, et al. 2017). The abscission zone structure of *Lolium perenne* L. also appears before heading and fractures occur 4 to 5 weeks after flowering stage(Elgersma, et al. 1988). Plant hormones are involved in regulating many processes of plant growth and development. They are metabolized and synthesized by fixed organs of plants, and undergo physiological and biochemical reactions by binding to fixed response protein receptors. They play a signaling role in the process of seed shattering(Xie, et al. 2021). Absciscic acid, auxin, ethylene, gibberellin, cytokinin, etc. are common endogenous hormones in plants. In the process of seed nutrition and reproductive growth, different endogenous hormones play different roles in seeds, and the effects of the same endogenous hormone are not completely the same in different time and space. The timing and content of endogenous hormones appear differently in different grass species(Bewley and Black 2012; Grabe 1956; Kozaki and Aoyanagi 2022). Absciscic acid promotes plant maturation and aging, with higher levels of absciscic acid present in aging organs or tissues(Liao, 2019). Plant hormones such as auxin and ethylene are related to the production and development of abscission zones(Tang and Chen 2017). Research suggests that the impact on seed shattering depends on the interaction between ethylene, auxin, and absciscic acid, rather than a single hormone(Roberts, et al. 2002). Absciscic acid slows down the transport of auxin, thereby promoting the abscission zone development and accelerating seed shattering(Chang, et al. 1973; Ogawa, et al. 2009). Seed shattering caused by absciscic acid is mediated by ethylene(Jackson, et al. 1973). Studying the molecular regulation mechanism of seed shattering from the perspective of molecular biology is an important research direction in recent years, and previous researchers have conducted in-depth exploration of many molecular mechanisms of seed shattering. For

instance, transcriptome analyses in rice (*Oryza sativa*) have identified key regulators such as *OsSH1* (a *YABBY* transcription factor) and *OsCCT22*, which form a regulatory module to suppress lignin biosynthesis in the abscission zone, thereby promoting seed shattering(He, et al. 2025). In *Setaria viridis*, a pan - genome association study combined with transcriptomics uncovered *SvLes1*, a gene whose mutation via CRISPR - Cas9 leads to non - shattering phenotypes, mimicking the domestication process of millet(Mamidi, et al. 2020). Similarly, in *Elymus sibiricus*, transcriptome profiling has revealed candidate genes involved in cell wall degradation and hormone signaling during abscission(Xie, et al. 2017). Through techniques such as transcriptomics, many key genes involved in regulating seed shattering have been reported, and further research has been conducted on the genetic basis that controls abscission layer development and the gene regulatory network that controls seed shattering. With the continuous improvement and update of second-generation transcriptome sequencing technology, this technology has been applied to the mining of key genes related to seed shattering. By comparing the transcriptomes of samples from different developmental stages, it is possible to quickly and effectively identify the response genes of most plant seed shattering(Sun, et al. 2019; Yang, et al. 2021). In addition, studies have found that hormones, functional genes, and transcription factors form a complex regulatory network that coordinates the process of seed shattering(Huang, et al. 2021). In this regard, weighted gene co expression network analysis can more quickly and efficiently mine functionally related genes in co expression modules, and has been widely used to mine corresponding candidate genes in various plants(Feng, et al. 2021; Jiang, et al. 2021; Zhang, et al. 2021; Zhou, et al. 2022).

*Carex L.* is a perennial herbaceous plant in the Cyperaceae family, with a wide variety of species. There are over 2000 species worldwide, widely distributed throughout the world. It is an ideal cold season native lawn grass, with advantages such as diverse species, wide adaptability, and strong stress resistance(Fan, et al. 2021; Ji, et al. 2009; Yang, et al. 2011). *C. breviculmis* belongs to the *Carex L.* and is an excellent lawn ground cover plant. Its plant type is compact and clustered, and its linear leaves are dark green. It has excellent characteristics such as cold resistance, shade resistance, and drought resistance. It has obvious water-saving advantages and is an excellent grass species for grassland planting and ecological management. It is widely used in urban landscaping, playing a role in covering the ground, maintaining soil and water, and beautifying the environment(Fan, et al. 2021; Wu, et al. 2006; Zhang, et al. 2016). However, the seeds of *C. breviculmis* are prone to falling off during the harvesting process, resulting in a significant decrease in seed yield, which to some extent limits the large-scale application. *C. breviculmis* 'Siji' was selected as the research material due to its unique biological characteristics and practical significance. As an excellent native grass species in China, it exhibits strong stress resistance, including cold, shade, and drought tolerance, making it widely used in urban landscaping and ecological management(Fan, et al. 2021). *C. breviculmis* 'Siji' was specifically chosen for its consistent evergreen phenotype and compact growth habit, which facilitate long-term observation and experimental manipulation. More importantly, this cultivar shows pronounced seed shattering during harvesting, with seed yield reduced by 30–40% due to premature shedding, significantly limiting its large-scale application(Ji, et al. 2009). Unlike most studied grasses, *C. breviculmis* belongs to the Cyperaceae family, representing a distinct phylogenetic lineage where seed shattering mechanisms remain

uncharacterized. This provides a unique opportunity to explore evolutionary divergences in abscission regulation. The selection of *C. breviculmis* 'Siji' thus combines both practical needs (addressing yield losses) and scientific value (uncovering novel molecular mechanisms in an understudied taxon), making it an ideal model for this investigation.

At present, there are no reports on the mechanism of seed shattering in *C. breviculmis*. In order to better explore the mechanism of seed shattering in *C. breviculmis*, this study investigated *C. breviculmis* seed at different developmental stages, revealing the intrinsic mechanism of seed shattering from the perspectives of histology, physiology, and molecular biology. This study provides new insights and ideas for elucidating the intrinsic mechanism of seed shattering in *C. breviculmis*, and provides guidance for cultivating high-quality low seed shattering *C. breviculmis* varieties in the future.

## 2. Materials and Methods

### 2.1 Plant Materials

This study used *C. breviculamis* 'Siji' provided by the Institute of Grassland, Flower and Landscape Ecology of Beijing Academy of Agricultural and Forestry Sciences (Beijing, China) as the plant material. All plants were vegetatively propagated within the experimental site in the Beijing Academy of Agriculture and Forestry Sciences (39°94'N, 116°29'E). And tissue samples from the time of heading to drying were collected in 2023 (from March to May). Heading stage (HS): the spike is exposed by 1/2 of the leaf sheath. Flowering stage (FS): the florets of the middle and upper spikes open, and the anthers are scattered. Grain filling stage (GS): the seed visibly swell, and the endosperm forms a milky white, semi liquid consistency. Milk ripening stage (MS): the seed coat is dark green, and the endosperm is condensed milk. Drying stage (DS): the color of the seed coat turns yellow-green, and the endosperm is waxy.

### 2.2 Determination of breaking tensile strength

The digital force meter HANDPI HP-5 (Leqing, China) was used to measure the breaking tensile strength of the *C. breviculamis* 'Siji' seeds at different stages. Randomly select 5 *C. breviculamis* 'Siji' spikelets at each of the five developmental stages, and then select 10 seeds with good growth in the middle of each spikelet. That is, observe the breaking tensile strength of the seeds in the abscission zone 50 times at each stage, and take the average of the breaking tensile strength values at each stage. The specific operation process for measuring the breaking tensile strength value is as follows: fix the force gauge on a horizontal table, and fix the *C. breviculamis* 'Siji' spikelet on the top of the force gauge. Use tweezers to vertically pull down the seed and record the maximum tensile force at the moment of detachment from the stem in units of force.

### 2.3 Histological observation of abscission zone

Collect spikelet samples of the *C. breviculamis* 'Siji' at five developmental stages. Take materials from the middle 1/3 area of the spikelet. Store materials at heading stages and flowering stages in 50% FAA

fixative (anhydrous ethanol: glacial acetic acid: formaldehyde: water = 60:5:5:30, with a small amount of glycerol added) at 4 °C. Store materials from the grain filling stage, milk ripening stage, and drying stage in 70% FAA fixative at 4 °C and fix all materials for 24 hours. Dehydrate the fixed material using alcohol solutions of different concentrations, and make it transparent through a mixture of anhydrous ethanol and xylene (anhydrous ethanol: xylene = 1:1) and xylene solution (100%). Transfer the transparent material to paraffin, immerse it in wax in an oven, embed the sample in the embedding agent for 48 hours, and then use a paraffin slicer (Leica, Germany) to cut the wax block to a thickness of 6–8 µm. Finally, stain it with uranium lead double staining (2% uranium acetate saturated aqueous solution, lead citrate, each staining for 15 minutes), protect the section with a cover glass, and dry it at room temperature. Observe under a microscope (3DHISTECH, Hungary) after staining and drying. Use Hitachi SU8100 (HITACHI, Japan) scanning electron microscope to observe the detachment structure at the end of the seed.

## 2.4 Determination of hydrolytic enzyme activity in the abscission zone

Starting from the heading stage of the *C. breviculamis* 'Siji', five developmental stages of spikelets should be harvested, with three biological replicates set for each developmental stage, for a total of 15 samples. Plant materials in the middle one-third of the spikelets should be cut in a low-temperature environment, wrapped in tin foil, and stored at -80 °C. When extracting, take out the sample and place it in liquid nitrogen. Weigh 0.1 g of the sample on an electronic balance (accurate to 0.0001 g), transfer it to a mortar and grind it into powder with liquid nitrogen. Add 1000 µL of extraction buffer. Transfer the solution to a 1.5 ml centrifuge tube using a pipette, centrifuge at 12000 rpm at 4 °C for 25 minutes, and then transfer the supernatant to a new centrifuge tube for storage at 4 °C. The activity determination of cellulase and polygalacturonase was carried out using a reagent kit (Suzhou Keming Biotechnology Co., Ltd.) according to the instructions, and then measured using a full wavelength multifunctional enzyme-linked immunosorbent assay.

## 2.5 Determination of Plant Hormone Content in the Abscission Zone

Following Simura's method (Simura, et al. 2018), ultra-high performance liquid chromatography tandem mass spectrometry was used to quantify 26 plant endogenous hormones from 7 categories in the free zone samples of *C. breviculamis* 'Siji'. The preparation method of plant hormone standards refers to the study by Li et al. (Li, et al. 2016). The setting of gradient elution conditions refers to the method of Hui et al. (Hui, et al. 2018).

The instruments used in the experiment include: AB SCIEX 6500 + Qtrap mass spectrometer (AB Sciex, USA), ExionLC liquid chromatography (AB Sciex, USA), KW-100TDV ultrasonic cleaning machine (Shumei, China), H1850-R high-speed freeze centrifuge (speed above 4000r/min, Xiangyi, China), MB-96 freeze grinder (Meibi, China), and HCM100 Pro constant temperature oscillating metal bath (Dalong, China).

## 2.6 RNA Extraction, Library Construction, and Sequencing

According to the analysis results of histology and physiology, plant tissues were collected from the middle one-third of the *C. breviculamis* 'Siji' at 10am every day at five developmental stages. 100mg was collected for each biological replicate, and 3 biological replicates were performed for a total of 15 samples for second-generation transcriptome sequencing. Immediately freeze the collected samples in liquid nitrogen and store them at -80 °C for subsequent RNA-seq and qRT-PCR. Extract RNA from 15 samples using CTAB + Adlai RN40 (Adlai, Beijing) extraction kit. Use Nanodrop2000 (Thermo Fisher, USA) for RNA purity and concentration testing, and use Agint2100 Lab Chip GX (Platinum Elmer, Shanghai) for integrity testing of extracted RNA. After passing the sample testing, the total RNA sample will be sent to Baimaike Biotechnology Company (Beijing, China) for cDNA library construction and transcriptome sequencing using the PacBio Sequel II platform. When constructing the PacBio sequencing library, first use magnetic beads with Oligo (dT) to enrich eukaryotic mRNA, then add Fragmentation Buffer to randomly interrupt mRNA and use mRNA as a template to synthesize the first and second cDNA chains, purify cDNA, repair the purified double stranded cDNA, select fragment size using AMPure XP beads, and enrich the final cDNA library through PCR. Finally, transcriptome sequencing was performed on the constructed cDNA library on the Illumina NovaSeq6000 platform.

## 2.7 Data processing, gene function annotation, and differential expressed genes analysis

Obtain the original sequence using the Illumina platform, and then remove low-quality, concatenated reads to obtain high-quality and effective clean data for the next step of data analysis. To ensure the accuracy of subsequent analysis, the quality of the raw data is monitored using base quality values as indicators. Q30 (%) is selected as the evaluation standard, and Q30, GC content, and sequence repetition level are calculated for quality assessment and control. Use HISAT2-v2.2.0 to align the high-quality sequence after quality control to the reference genome. Assemble the reads on the comparison using String Tie-v2.1.5 for subsequent bioinformatics analysis such as functional annotation and expression level. In order to perform gene function annotation, all transcriptome data were retrieved into the following eight databases using BLAST-v2.14.0 software to obtain gene function annotation information: NR (non redundant protein sequence), COG (Clusters of Orthologous Groups), GO (Gene Ontology), KOG (euKaryotic Ortholog Groups), KEGG (Kyoto Encyclopedia of Genes and Genomes), EggNOG (Evolutionary genetics of genes: Non superior Orthologous Groups), Pfam (Protein family), and Swiss Prot. This experiment uses FPKM value as an indicator to measure expression level. When the expression level difference multiple FC (fold change) is  $\geq 2$  and the false discovery rate FDR is  $< 0.01$ , the gene is recognized as a differential expressed gene in this experiment. The differential expressed genes obtained from screening are used for subsequent bioinformatics analysis. In addition, expression trend analysis was generated by Short Time series Expression Miner-v1.3.7 (STEM) based on FPKM, and differential expressed gene heat maps were drawn using TBtools software. GO analysis, KEGG enrichment analysis, and WGCNA analysis were performed using the Biocloud platform ([www.Biocloud](http://www.Biocloud)).

net, accessed on June 30, 2023). Visualize the co expression network and screen key genes using Cytoscape-v3.10.1.

## 2.8 qRT-PCR validation of transcriptome sequencing

To determine the reliability of transcriptome data, differential expressed genes were randomly selected for qRT-PCR analysis and validation using the Prime Script PT Enzyme MixI quantitative PCR system. Total RNA extracted from different developmental stages of the *C. breviculamis* 'Siji' was reverse transcribed using the Prime Script kit to obtain cDNA. The Real Time PCR reaction was performed using the TB Green Premix Ex Tap II (TaKaRa, China) kit on a Quant Studio 6 Flex instrument. Primer Premier-v5.20 software was used for primer design during validation, as shown in the table. Use the  $2^{-\Delta\Delta Ct}$  method(Livak and Schmittgen 2001) to calculate the expression levels of these differential expressed genes. Each sample to be tested contains three biological replicates, and each biological replicate contains three technical replicates. Use *qCbUBQ* as an internal reference gene to correct the relative expression levels of the genes being tested.

## 2.9 Data processing and chart creation

Use Microsoft Excel 2021-v16.0.1 to organize and chart the measurement data of fracture tensile tension, and use IBM SPSS Statistics-v27.0.1 for ANOVA one-way ANOVA and Duncan method multiple comparison analysis ( $P < 0.05$ ) to analyze the significance of the data differences.

## 3. Results

### 3.1 Seed shattering degree at different developmental stages

By referring to the growth period of grasses and field observations, the spike development process of *C. breviculamis* 'Siji' was divided into five stages(Fig. 1 a). The seed shattering degree of *C. breviculamis* 'Siji' was characterized by measuring the breaking tensile strength(BTS). As shown in the figure, the breaking tensile strength of the *C. breviculamis* 'Siji' seeds gradually decreases with the development of the spike, and the value of breaking tensile strength is negatively correlated with the seed shattering degree(Fig. 1b). At the heading stage, the seed shattering is relatively low, and the breaking tensile strength is the highest, at 76.43 gf. The flowering stage is followed by 71.55 gf. The breaking tensile strength value during the grouting stage still shows a decreasing trend, reaching 61.94 gf. At the milk ripening stage and drying stage, the breaking tensile strength is less than 60 and 30 gf respectively, which is significantly lower than the previous stage. The seed shattering degree is higher at this stage.

### 3.2 Histological analysis of abscission zone

By observing the apparent structure, it was preliminarily determined that the abscission zone is located at the end of the seed(Fig. 1 c-g). Through paraffin section dissection analysis of the abscission zone

structure, it was found that the abscission zone of *C. breviculamis* 'Siji' is mainly located at the end of the seed, with small cell volume and compact arrangement. The abscission zone is already visible during the flowering stage, and as the spike continues to develop, this zone becomes increasingly clear. As the seeds mature, the color of the abscission zone gradually deepens, and the degree of lignification of the abscission cells gradually increases. During the milk ripening stage, the cell wall adjacent to the abscission zone begins to degrade, resulting in broken marks in the abscission zone. The decomposition of the abscission layer begins from a gap on the epidermis side. During the drying stage, there is a noticeable fracture in the abscission zone, which leads to the disintegration of the abscission zone and triggers seed shattering(Fig. 1h-l).

The scanning electron microscopy results show that the seed fracture surface presents a non planar structure, and the abscission zone is composed of 5–8 circles of elliptical or irregularly shaped cells, which are distributed radially around the periphery. As the seed gradually develops and matures, the morphology of the cells in the abscission zone changes significantly. During the heading and flowering stages, there are obvious gaps between the cells of the abscission zone and the surface of the abscission zone is rough. During the grouting stage, the surface of the abscission zone begins to become smooth and the cell contours become blurred, indicating that the cells in this zone are beginning to degrade. In the milk ripening and drying stages, it can be seen that the surface of the abscission zone becomes significantly smoother, with large areas of fracture surfaces appearing in this area. The abscission zone presents a large area of concave areas, and the cell contours become more blurred. In the figure, it can be seen that there is a vascular bundle at the center of the fracture surface, which is concave in shape. Vascular bundles can provide support, connecting seeds to plants and serving as channels for material transport between seeds and parent plants. Studies have shown that vascular bundles are an important factor affecting seed shattering. As the seeds mature, the vascular bundle structure gradually expands and invaginates, indicating that the abscission zone of *C. breviculamis* 'Siji' gradually strengthens with the complete development of the vascular bundle in the abscission zone(Fig. 1m-q).

### 3.3 Physiological analysis of abscission zone

In order to investigate the relationship between cell wall hydrolytic enzymes and seed shattering in the abscission zone of *C. breviculamis* 'Siji', the activity changes of cellulase and polygalacturonase in the abscission zone of *C. breviculamis* 'Siji' at different developmental stages were measured(Table A.1). The results showed that cellulase showed a gradually increasing trend with the development of spike and was positively correlated with the degree of seed shattering. The activity of polygalacturonase showed a trend of gradually increasing and then decreasing, and reached its maximum value at the critical stage of seed shattering(Fig. A.1).

We measured the changes in the content of endogenous plant hormones in the abscission zone during the process of seed shattering of the *C. breviculamis* 'Siji', and analyzed the 26 endogenous plant hormones measured. Use a hierarchical clustering tree to perform cluster analysis on all plant hormone samples, and obtain a hierarchical clustering tree diagram representing the similarity between samples

in each stage. The clustering tree diagram of plant hormone samples is shown in the figure. The hierarchical clustering tree diagram reveals the differences in hormone levels between the five stages. The results in the figure show that the hormones identified in the dying stage are significantly different from the other four stages, and the plant hormones in the other four stages can be further divided into two categories. The hormones identified in the heading stage and flowering stages are similar, and the hormones identified in the milk ripening stage are similar to those identified in the grouting stage. The relative values of all endogenous plant hormones measured in the abscission zone were used as the hormone levels for five developmental stages(Fig. 2a). The hierarchical clustering method was employed to obtain the clustering heatmap of each plant hormone in the abscission zone, as shown in the figure. The results of the clustering heatmap indicate that the measured plant hormones can be divided into seven categories, namely auxin, gibberellins, cytokinins, ethylene, abscisic acid, jasmonic acid, and salicylic acid. The plant hormones identified in the heading stage and flowering stage are similar, while those identified in the grouting stage and milk ripening stage are similar. In the grouting, milk ripening, and drying stages, the ICA and ICAID of auxins, GA7 and GA19 of gibberellins, cytokinin, ethylene, salicylic acid, and jasmonic acid contents all decrease, while the IAA and Me IAA of auxins and GA24 of gibberellins increase(Fig. 2b). The measurement results of endogenous plant hormone content changes in the seed abscission zone of *C. breviculamis* 'Siji' at different developmental stages are shown in Table. The results of the plant hormone content change table indicate that a total of 26 plant hormones were detected during the seed shattering process of *C. breviculamis* 'Siji', which were divided into 7 categories: auxin, gibberellin, cytokinin, ethylene, abscisic acid, jasmonic acid, and salicylic acid. Among them, the content of auxin decreased significantly during the key stage of seed shattering, namely the drying stage, while the content of IAA and MeIAA increased significantly during the grouting stage, and the content of ICA and ICAId decreased significantly. The content of most gibberellins significantly decreases during the drying stage, while the content of GA1 significantly increases during the drying stage. The overall content of cytokinins and ethylene also showed a decreasing trend. The content of abscisic acid is highest during the milk ripening stage, and significantly decreases during the drying stage, reaching its lowest value. The content of jasmonic acid and salicylic acid significantly decreased during the grouting stage, and remained at a low level during the milk ripening and drying stages thereafter(Table A.1).

We analyzed the correlation between the breaking tensile strength value and various physiological indicators at different developmental stages, and the results are shown in the figure. Through analysis, it was found that the breaking tensile strength is significantly negatively correlated with cellulase activity, with a correlation coefficient of -0.94, and significantly positively correlated with GA7 content of gibberellins, IPA, CZ content of cytokinins, ethylene content, and salicylic acid content, with correlation coefficients of 0.89, 0.88, 0.97, 0.88, and 0.87, respectively. Among them, the breaking tensile strength is highly significantly positively correlated with GA19 of gibberellins, with a correlation coefficient of 0.98. This result indicates that as the developmental stage progresses, cellulase activity increases, and at the same time, the degree of seed shattering in *C. breviculamis* 'Siji' gradually increases. It can be inferred that the seed shattering process of *C. breviculamis* 'Siji' is closely related to cellulase activity. In

addition, through the analysis of the correlation between breaking tensile strength value and plant hormones in the abscission zone, it was found that the changes in the content of gibberellin, cytokinin, ethylene, and salicylic acid during the seed shattering process were significantly correlated with the degree of seed shattering in the *C. breviculamis* 'Siji'. It can be inferred that they play a key role in the seed shattering activity of *C. breviculamis* 'Siji' seeds(Fig. A.1).

### 3.4 Transcriptome data analysis of abscission zone

The transcriptome sequencing data results are shown in Table A.2 and Table A.3. After filtering low-quality reads, adapter sequences, and ploy-N sequences from the transcriptome sequencing data of 15 samples, a total of 100.44 Gb clean data was obtained. The Q30 base percentage of each sample was 93.19% or above, and the GC content of each sample was 45.83% or above, indicating good sequencing quality(Table A.2). Perform sequence alignment between the clean reads of each sample and the designated reference genome, and the alignment results are shown in the table. From the comparison results, the number and percentage of reads with unique positions obtained by aligning clean reads with the reference genome ranged from 91.67–94.04%. The number and percentage of clean reads compared to the reference genome at multiple locations ranged from 2.31–5.55%. The number and percentage of positive chain reads compared to the reference genome in clean reads ranged from 49.43–53.09%. The number and percentage of negative chain reads compared to the reference genome in clean reads ranged from 49.55–53.12%. The alignment efficiency between reads of each sample and the reference genome ranges from 95.46–97.36%(Table A.3). The results of transcriptome data alignment with the reference genome sequence meet the requirements and can be further studied. Based on the comparison results, a total of 29463 genes were identified. Perform alternative splicing prediction analysis, gene structure optimization analysis, and discovery of new genes, discovering 6123 new genes, of which 2313 were functionally annotated.

The analysis of gene expression levels is shown in the figure. The correlation statistics of sample expression levels indicate that there is a high correlation between sample replicates, and the data has good reproducibility. The clustering results of the sample expression level correlation heatmap are consistent with the clustering results of hormones, further indicating that the seed shattering process of *C. breviculamis* 'Siji' can be divided into three distinct stages(Fig. 3a).In order to analyze the transcriptional changes of differential expressed genes during the seed shattering process, we compared the changes in the transcriptome levels of differential expressed genes between each stage with the heading stage, and counted the up-regulated and down-regulated genes of four groups of differential expressed genes. The results showed that in the "HS vs DS" group, 4417 differential expressed genes were up-regulated and 5850 differential expressed genes were down-regulated, making it the group with the largest difference, indicating that more genes were expressed in the late stage of seed maturation(Fig. 3b). In order to further reveal the relationship between differential expressed genes and seed shattering, we performed KEGG classification on co differential expressed genes. The results showed that all differential expressed genes were annotated and enriched into five major categories in the KEGG database, involving 32 metabolic pathways. Representative pathways include "Plant hormone

signal transduction" (7.81%), "Phenylpropanoid biosynthesis" (5.15%), and "Starch and sucrose metabolism" (4.82%), which are closely related to seed shattering(Fig. 3c).

To verify the reliability of transcriptome data, we randomly selected 10 genes from the differential expressed genes in the transcriptome of *C. breviculamis* 'Siji' for qRT-PCR analysis. We used an internal reference gene *qCbUBQ* to correct the relative expression levels of the tested genes. These 10 genes include *Cbr04G004490* *Cbr04G006490* *Cbr04G007090* *Cbr04G008620* *Cbr14G001330* *Cbr19G003490* *Cbr21G001050* *Cbr22G001960* *Cbr27G003590* *Cbr32G001900*. The genes and their primer sequences are shown in the Table A.4. The results showed that the expression levels of these 10 genes determined by qRT-PCR were consistent with RNA-seq data. Linear regression analysis showed a significant positive correlation between the two, with a correlation coefficient  $R^2$  of 0.9374, indicating the reliability of the transcriptome data in this experiment(Fig. A.2).

### 3.5 Analysis of Time Expression Patterns of Differentially Expressed Genes

Using STEM clustering tools to cluster all differential expressed genes, the results showed that differential expressed genes were assigned to 28 different expression pattern files. According to the significance of the expression pattern files, descending sorting was performed, and the results showed that 8 pattern files had significance(Fig. 4a). Among them, files 5 and 14 have similar expression patterns, with an overall decreasing trend in gene expression. Files 15 and 25 have similar expression patterns, with an overall upward trend in gene expression. Based on the analysis of the trend expression patterns in files 5 and 25, we conducted KEGG enrichment analysis on the differential expressed genes clustered into files 5 and 25. The results showed that most of the differential expressed genes in the two pattern files were annotated and enriched in pathways related to seed shattering. In file 5, 111 differential expressed genes were enriched in the plant hormone signal transduction pathway (8.70%), 62 differential expressed genes were enriched in the phenylalanine biosynthesis pathway (5.64%), and 72 differential expressed genes were enriched in the starch and sucrose metabolism pathway (4.86%) (Fig. 4b). In file 25, 26 differential expressed genes were enriched in the plant hormone signal transduction pathway (12.04%), 14 differential expressed genes were enriched in the phenylalanine biosynthesis pathway (4.86%) and 13 differential expressed genes were enriched in the starch and sucrose metabolism pathways (6.02%)(Fig. 4c).

On this basis, we selected two key pathways about seed shattering, plant hormone signal transduction and phenylalanine biosynthesis, in documents 5 and 25, and analyzed the related genes in these pathways. We selected 28 plant hormone signal transduction genes from file 5 and 24 from file 25 to draw gene expression heatmaps. These genes are mainly related to four hormones, including gibberellin, cytokinin, ethylene, and salicylic acid. These plant hormones are significantly correlated with the seed shattering of *C. breviculamis* 'Siji'(Fig. A.3 a, b). 45 genes related to phenylalanine biosynthesis were identified. Specifically, 31 genes were identified from document 5 and 14 genes were identified from document 25. These genes are involved in the regulation of lignin monomer and flavonoid synthesis,

some of which are key enzyme genes regulating seed shattering, such as *CAD*, *4CL*, *TOGT1*, *CCR*, *bglB*, *POX*, etc(Fig. A.3 c, d).

### 3.6 Weighted gene co-expression network analysis reveals specific modules of seed shattering

In order to reveal the specific modules involved in the seed shattering process of *C. breviculamis* 'Siji' and more accurately mine candidate genes related to seed shattering, we used a weighted gene co-expression network analysis(WGCNA) method to construct a weighted gene network and jointly analyze gene expression levels and physiological data to identify gene modules related to seed shattering. After testing and merging, a total of 6 gene co-expression network modules were obtained from 7683 genes. The correlation between MEturquoise and MEblue modules and traits is high, with 2950 and 2660 genes in MEturquoise and MEblue respectively. The MEturquoise and MEblue modules are highly correlated with the most intuitive indicator breaking tensile strength value, with a correlation of 0.69 between MEturquoise and BTS and 0.77 between MEblue and BTS. In addition, MEturquoise is highly correlated with CE, PG, IAA, Me IAA, ICAld, IPA, IP, CZ, TZR, GA1, GA7, GA19, GA24, ACC, JA, MeJA, DHJA, JA Ile, OPDA, SA, and SAG, with correlation coefficients  $> 0.5$  or  $< -0.5$ . MEblue is highly correlated with CE, PG, IAA, Me IAA, ICAld, IPA, IP, CZ, TZR, GA7, GA19, GA24, ACC, JA, MeJA, DHJA, JA Ile, OPDA, SA, and SAG, with correlation coefficients  $> 0.5$  or  $< -0.5$ (Fig. 5a). Many genes in the MEturquoise and MEblue modules are enriched in pathways related to seed shattering, including plant hormone signal transduction pathways, phenylalanine biosynthesis pathways, and starch and sucrose metabolism pathways(Fig. 5b, c). In the MEturquoise module, we identified 12 core genes involved in important seed shattering activities such as signal transduction of gibberellin, cellulose degradation, and biosynthesis of lignin monomers(Fig. 5d). In the MEblue module, we identified six core genes that are involved in multiple important seed shattering processes such as cellulose degradation(Fig. 5e).

### 3.7 Differential expressed genes involved in cellulose degradation pathways

In order to further explore the differential expressed genes related to grain shattering in the *C. breviculamis* 'Siji', this study conducted a more in-depth exploration of the degradation process of cellulose during seed shattering. In the degradation process of cellulose in the abscission zone, the degradation of cellulose into cellulose disaccharides requires the action of endoglucanase, while  $\beta$ -glucosidase plays a role in the further degradation of cellulose disaccharides into D-glucose. Through research, it was found that there are a total of 20 differential expressed genes involved in cellulose degradation, including 7 endoglucanases and 13  $\beta$ -glucosidase. From the grouting stage onwards, the expression levels of these genes undergo changes. Most of the differential expressed genes encoding endoglucanase and  $\beta$ -glucosidase are down-regulated during the seed shattering process, while three genes encoding these two enzymes are up-regulated during the seed shattering process(Fig. 6a). We measured the changes in cellulase activity in five stages of the *C. breviculamis* 'Siji'. The measurement

results showed a significant increase in cellulase activity in the abscission zone during the grouting stage, which was related to differential expressed genes involved in cellulose degradation(Fig. 6b).

### 3.8 Differential expressed genes involved in plant hormone signaling transduction

In order to further analyze the differential expressed genes involved in the seed shattering process of *C. breviculamis* 'Siji', we conducted a more detailed study on the synthesis pathways and signal transduction processes of three plant hormones highly correlated with the seed shattering process of *C. breviculamis* 'Siji', including gibberellin, cytokinin, and ethylene, based on hormone content indicators.

The changes in gibberellin content are significantly correlated with the breaking tensile strength values during the seed shattering process of *C. breviculamis* 'Siji', with a correlation of up to 0.98. Differential expressed genes in gibberellin synthesis and signal transduction pathways may play an important role in the seed shattering process. In the synthesis pathway of gibberellin, the precursor of gibberellin, GGPP, is cyclized to the precursor of gibberellin, ent-kaurene, under the catalysis of enzymes. Ent-kaurene generates GA12 aldehyde under the action of ent-kaurene oxidase (*KO*) and ent-kaurenoic acid hydroxylase (*KAO*), and GA12 aldehyde is converted to various gibberellins under the action of GA20 oxidase (*GA20ox*), GA3 oxidase (*GA3ox*), and GA2 oxidase (*GA2ox*). This study found that there are five enzymes involved in the synthesis of gibberellin from GGDP in the *C. breviculamis* 'Siji', and the differential expressed genes encoding these five enzymes showed a change in trend before and after grouting stages. The gene expression trends before and after grouting stage were completely opposite. The expression levels of most differential expressed genes encoding these five enzymes are lower before grouting stage and increase after grouting stage. For example, *OsCPS1*, *KAO*, *GA20ox*, *GA2ox*, and *GA3ox*. A small number of differential expressed genes encoding *GA20ox* have higher expression levels before grouting stages and lower expression levels after grouting stage. The gibberellin signaling pathway is controlled by *GID1*, *DELLA*, and *TF*. Studies have found that the differential expressed genes encoding *GID1* are up-regulated, while those encoding *DELLA* and *TF* are down-regulated. Additionally, the gene encoding *GID1* has a higher expression level in dying stage. Based on previous studies, the levels of GA3, GA7, GA19, and GA51 significantly decreased during the grouting stage, which is closely related to the differential expressed genes involved in gibberellin synthesis. Through previous measurements of changes in gibberellin content, we also found that most gibberellins had zero content during the drying stage, which may be closely related to the significant increase in differential expressed genes involved in encoding *GID1* during the drying stage(Fig. 6c).

The changes in the content of cytokinins are significantly correlated with the breaking tensile strength values during the seed shattering process of the *C. breviculamis* 'Siji'. This study indicates that the expression levels of differential expressed genes encoding key enzymes in the cytokinins synthesis pathway change during the critical stages of milk ripening stage and drying stage during seed shattering. Most of the differential expressed genes encoding key enzymes *CIZSOG*, *IPT*, and *UGT* involved in cytokinin synthesis were up-regulated in milk ripening stage and dying stage. In the cytokinins signaling

pathway, most differential expressed genes are down-regulated in drying stage, and these genes may play a role in the seed shattering process of *C. breviculamis* 'Siji'. Based on previous research findings, the content of most cytokinins significantly decreased in milk ripening stage and reached its lowest value in drying stage, which is related to changes in the expression levels of differential expressed genes involved in cytokinin synthesis and signal transduction in milk ripening stage and drying stage(Fig. 6d).

Previous studies have shown that changes in ethylene content are also significantly correlated with the breaking tensile strength values at different developmental stages of *C. breviculamis* 'Siji', and are another important plant hormone involved in the seed shattering in *C. breviculamis* 'Siji'. As shown in the figure, differential genes encoding *metK* are up-regulated during ethylene synthesis, and *metK* is a key enzyme in ethylene synthesis. In the signal transduction pathway of ethylene, differential expressed genes encoding *CTR1* are up-regulated, while differential expressed genes encoding *ETR*, *SIMKK*, and *ERF1/2* are down-regulated. Through previous measurements of changes in ethylene content at different stages, it was found that the changes in ethylene content gradually decrease with the progression of developmental stages, which is related to changes in differential gene expression levels involved in ethylene biosynthesis and signal transduction pathways(Fig. 6e).

## 4. Discussion

### 4.1 Formation and degradation process of the abscission zone structure affecting the seed shattering of *C. breviculamis* 'Siji'

There have been many studies on seed shattering, which indicate a significant correlation between the formation and degradation of seed abscission zones and seed shattering characteristics(Akasaka, et al. 2011; Burson, et al. 1978; Elgersma, et al. 1988; Simons, et al. 2006). From the processing results of paraffin sections and scanning electron microscopy, it can be seen that the abscission zone cells of *C. breviculamis* 'Siji' are mainly distributed around the vascular bundles, and the formation of abscission zone cells occurs earlier than the occurrence of seed shattering. The formation of abscission zone cells can be clearly observed around the grouting stage. The abscission zone is composed of 5 to 8 elliptical or irregularly shaped cells, which are radially distributed around the vascular bundle. The arrangement of abscission zone cells is regular and compact, and the degree of lignification of abscission zone cells is relatively high. Scanning electron microscopy revealed that during the milk ripening stage, the surface of the abscission zone became significantly smoother and the cell contours were very blurred. This is consistent with the phenomenon observed in paraffin sections during the milk ripening stage, where the cell walls adjacent to the abscission zone were degraded and fragmented, resulting in incomplete detachment layer structure. There have been many studies on the formation and degradation of plant abscission zones, and Inoue et al.'s research confirmed that the abscission zone of wild rice seeds is formed during flowering stage and begins to degrade after pollination(Inoue, et al. 2015). Elgersma et al. found that the abscission zone of *Lolium perenne* seeds had formed during the heading stage, but no significant cellular degradation process was observed during the formation of the abscission zone(Elgersma, et al. 1988). In this study, the *C. breviculamis* 'Siji' already had abscission zones during

the grain filling stage, but the seeds did not fall off until the milk ripening stage, when the dropping ability rapidly increased and the seeds began to fall off, resulting in a significant decrease in breaking tensile strength. This discovery is consistent with Zhao's research on the seed shattering characteristics of *Elymus sibiricus*, and there may be multiple reasons for the phenomenon of seed shattering characteristics not improving despite the presence of abscission zones. The main influencing factor may be the degree of abscission degradation, which occurs at a specific developmental stage(Ji, et al. 2009). The cellular histological analysis of the abscission zone of *C. breviculamis* 'Siji' proves that the differences in seed shattering at different developmental stages are closely related to the degree of degradation in the abscission zone. It is only during the milk ripening stage that significant degradation occurs in the abscission zone. At this stage, the degree of degradation suddenly increases, the seed shattering property correspondingly increases, and the breaking tensile strength value significantly decreases.

## 4.2 Physiological indicators affecting the seed shattering of *C. breviculamis* 'Siji'

The degradation of cell wall components can affect the seed shattering of plants, and the main factors affecting the degradation of cell wall components include cell wall hydrolases such as cellulase, polygalacturonase, and peroxidase(Fuller, et al. 2010; Liljegren 2012; Tada, et al. 2015). Cellulase and polygalacturonase are the two main degradation enzymes of plant cell walls(Zhang 2020). Cellulase mainly degrades cellulose components in plant cell walls, while polygalacturonase mainly degrades pectin components in cell walls. Many studies have shown that these two enzymes are involved in the seed shattering process of the abscission zone cells(Bonghi, et al. 1992; Greenberg, et al. 1975; Zhang, et al. 2005). Regarding the activity changes of these two enzymes during seed shattering, studies by scholars have shown that the activities of cellulase and polygalacturonase significantly increase during seed shattering(Atkinson, et al. 2002). Previous studies have confirmed that polygalacturonase has a positive regulatory effect on cell separation, fruit ripening, seed shattering, cell growth, and pod splitting(Atkinson, et al. 2012; Sander, et al. 2001; Xiao, et al. 2014; Zhao 2017). And in some studies, it has also been demonstrated that cellulase and polygalacturonase have similar activity changes during seed development, confirming their interaction in cell wall hydrolysis(Taylor and Whitelaw 2001). These studies are consistent with the results obtained in this study. Based on the observation of cellular histology and the determination of enzyme activity, it is speculated that the mechanism of seed shattering in *C. breviculamis* 'Siji' is that the heading stage to the grouting stage is an important period for seed formation. The plant transports nutrients into the seed through the vascular bundle, and the activities of cellulase and polygalacturonase inside the seed gradually increase. Referring to Zhang's research(Zhang 2020), it is speculated that the reason why the seed does not shed is due to the support of vascular bundles and the presence of lignified cells in the seed abscission zone, which can maintain a non shedding state, and the breaking tensile strength value is correspondingly high. The milk ripening stage and drying stage are the most obvious stages for seed shattering. At this time, the activities of cellulase and polygalacturonase are both high. Under the action of these two enzymes, the detached

cells degrade, making the seeds easily shed and the breaking tensile strength value significantly reduced.

In addition to cell wall hydrolases, plant hormones also play a key role in transmitting the signal of seed shattering(Nakano, et al. 2015). Some studies suggest that gibberellins and cytokinins play important roles in the process of abscission. Gibberellin plays an important regulatory role in rice seed shattering, and increasing the content of gibberellin in rice abscission zones can promote rice seed shattering. Similar to rice, gibberellin also induces fruit drop in grapes and apples(Conesa, et al. 2015; Mahouachi, et al. 2009). However, some studies have found that exogenous application of gibberellin can inhibit the shattering of *Lonicera caerulea* fruit by suppressing cell wall degradation and delamination formation. Similarly, in *Citrus reticulata* Blanco, the application of gibberellin can delay the premature shattering of citrus fruits(Zavaleta-Mancera, et al. 1999). These studies indicate that gibberellin has different effects on abscission in different species. During the development of the *C. breviculamis* 'Siji', the content of GA7 and GA19 significantly decreased. The change in GA7 content was significantly positively correlated with the breaking tensile strength, and the change in GA19 content was extremely significantly positively correlated with the breaking tensile strength, indicating that gibberellin may have a negative regulation on the seed shattering process of the *C. breviculamis* 'Siji', inhibiting seed shattering.

Cytokinin plays a positive role in the process of cotton leaf shattering, and increasing endogenous cytokinin levels can accelerate the process of cotton leaf shattering. On the contrary, cytokinins can also delay leaf shattering, allowing leaves to stay on the plant for a longer period of time(Chauvaux, et al. 1997). In this study, the level of cytokinins decreased and showed a significant positive correlation with the breaking tensile strength, indicating that cytokinins inhibit the shattering of *C. breviculamis* 'Siji'. Meanwhile, in the analysis of the correlation between the changes in gibberellin and cytokinin content during the seed shattering process of *C. breviculamis* 'Siji', it was found that gibberellin and cytokinin content were significantly positively correlated, indicating that the two may have a synergistic effect in regulating the seed shattering process of *C. breviculamis* 'Siji'. Most studies have shown that ethylene also plays a key role in the seed shattering process. In this study, the change in ethylene content was significantly positively correlated with the breaking tensile strength, and there were significant differences in ethylene content changes at different developmental stages, indicating that ethylene also plays an indispensable role in the shattering process of *C. breviculamis* 'Siji'.

Generally, as plants develop, auxin levels gradually decrease, leading to organ abscission(Conesa, et al. 2015). Research on seed shattering in Cruciferae family suggests that auxin may play a dominant role in regulating seed shattering, and exogenous application of auxin leads to delayed pod cracking in Cruciferae family plants(Wang, et al. 2020). In this study, the content of auxin in the abscission zone of *C. breviculamis* 'Siji' gradually decreased during development, which is consistent with the research that auxin in the abscission zone can inhibit plant organ abscission. Research has shown that abscisic acid also plays a role in the seed shattering process. However, in this study, the correlation between abscisic acid and breaking tensile strength was not significant. Some scholars believe that the main function of abscisic acid is to regulate seed dormancy, and its secondary function is to regulate seed shattering.

Jasmonic acid plays an important role in plant development and is released under stress conditions(Pauwels, et al. 2008). Some studies have shown that jasmonic acid accumulates significantly during the shattering process, promoting the abscission of organs such as leaves(Singh, et al. 2022). However, some scholars have also found that the level of jasmonic acid decreases during the process of flower organ abscission(Lo'ay 2017). This viewpoint is similar to the results obtained in this study, where the content of jasmonic acid in the abscission zone of *C. breviculamis* 'Siji' significantly decreases with the developmental stage. Research suggests that an increase in salicylic acid content can inhibit shedding(Zhang, et al. 2006). In this study, the salicylic acid content showed a gradually decreasing trend, indicating that as the degree of seed shattering deepened, the salicylic acid content in the abscission zone of the *C. breviculamis* 'Siji' continued to decrease. Low concentrations of salicylic acid may have a promoting effect on seed shattering, which is consistent with previous studies.

#### **4.3 The degradation of cellulose and the interaction among plant hormones may affect the seed shattering of *C. breviculamis* 'Siji'**

The process of seed shattering is often accompanied by the degradation of the cell wall, and cellulose is an important component of plant cell walls. Cellulose in plants can be degraded into glucose through the action of cellulase. Cellulase is a collective term for a group of enzymes that degrade cellulose to produce glucose. It is a complex enzyme mainly composed of exoglucanase, endoglucanase, and  $\beta$ -glucosidase. Previous research results have shown that changes in cellulase activity are closely related to seed shattering(Xie, et al. 2017; Zhang 2020). Our research found that with the development of spikes, the cellulase activity in the abscission zone of *C. breviculamis* 'Siji' seeds significantly increased, causing seed shattering. Further research on the degradation pathways of cellulose during the process of seed shattering revealed that endoglucanase and  $\beta$ -glucosidase are key hydrolytic enzymes involved in the cellulose degradation process in the abscission zone of the *C. breviculamis* 'Siji'. Endoglucanase degrades cellulose into cellulose disaccharides, while  $\beta$ -glucosidase plays a role in the further degradation of cellulose disaccharides into glucose. During this process, some differential expressed genes encoding endoglucanase and  $\beta$ -glucosidase were significantly up-regulated, which may play an important positive regulatory role in the degradation of cellulose in the abscission zone of *C. breviculamis* 'Siji'.

The process of seed shattering is regulated by various factors such as physiology and transcriptomics, which form a complex regulatory network. Plant hormones play an irreplaceable role in transmitting seed shattering signals(Jiang, et al. 2021). In this study, we identified three main plant hormones that are highly correlated with the seed shattering activity of *C. breviculamis* 'Siji', namely gibberellin, cytokinin, and ethylene. Previous studies have shown that gibberellin has different effects in different species(Chauvaux, et al. 1997; Mahouachi, et al. 2009; Zavaleta-Mancera, et al. 1999). The results of this study showed that the content of GA1 significantly increased with the development stage, while the content of GA7 and GA19 significantly decreased. Gibberellin has an important impact on the seed shattering process of *C. breviculamis* 'Siji'. By comparing the hormone content and differential gene expression of regulatory hormones at different developmental stages, it was found that the changes in

gibberellin content were consistent with their corresponding transcriptional data. Two differential expressed genes that may affect the seed shattering of *C. breviculamis* 'Siji' during gibberellin synthesis were identified, namely *GA2ox* and *GA3ox*. The differential expressed genes encoding *GA2ox* were down-regulated, resulting in a significant decrease in GA19 content during the drying stage, while the differential expressed genes encoding *GA3ox* were significantly up-regulated during the milk ripening stage, resulting in a significant increase in GA1 content. We also identified regulatory factors in three gibberellin signaling pathways, including *GID1*, *DELLA*, and *TF*. In this study, it was found that the differential expressed genes encoding *GID1* were up-regulated. The expression level of *GID1* significantly increased during the drying stage, while the differential expressed genes encoding *DELLA* and *TF* were down-regulated. This result is consistent with previous studies, such as Ge et al.'s speculation that the expression level of gibberellin receptor gene *GID1* in grape will be up-regulated under gibberellin treatment conditions(Ge Hui, et al. 2011).

Cytokinins can inhibit abscission, and during seed shattering, genes encoding cytokinins are up-regulated(Singh, et al. 2022; Wang, et al. 2020). In this study, the genes *CIZSOG*, *IPT*, and *UGT* encoding cytokinin synthase were up-regulated, negatively regulating the synthesis of cytokinins. The cytokinin content decreased and accelerated the seed shattering, which is consistent with previous studies, indicating that cytokinins play an important role in the seed shattering process of *C. breviculamis* 'Siji'. The shedding of plant leaves, flowers, and fruits is closely related to ethylene. Related studies have shown that *CTR1* negatively regulates ethylene, inhibits ethylene signal transduction, and up-regulates expression during tomato stem abscission, which is consistent with our research results(Meir, et al. 2010). Our research found that the differential expressed genes encoding the key enzyme *metK* for ethylene synthesis were up-regulated, and the kinase protein *CTR1* in the ethylene signal transduction process was also significantly up-regulated. These differential expressed genes negatively regulated ethylene during the seed shattering process, resulting in a decrease in ethylene content and affecting the seed shattering of *C. breviculamis* 'Siji'.

## **4.4 Convergent evolution of seed shattering in *C. breviculamis* 'Siji' and rice**

Despite belonging to distinct families (Cyperaceae for *C. breviculmis* and Poaceae for rice), both species exhibit remarkable functional convergence in seed shattering mechanisms. In rice, repeated evolution of high shattering during de-domestication of wild rice to weedy rice is mediated by the *OsSH-OsCCT22* module, which suppresses lignin biosynthesis in the abscission zone(Akasaka, et al. 2011). Similarly, *C. breviculmis* shows enhanced cellulase activity and lignin degradation in the abscission zone (Fig. 6b), with enrichment of the "phenylpropanoid biosynthesis" pathway in DEGs (Fig. 3c)—paralleling rice's lignin metabolic network for seed shattering(Xie, et al. 2017). Notably,  $\beta$ -glucosidase genes in *C. breviculmis* and *OsCAD2* in rice share functional equivalence in cell wall hydrolysis(Zhang, et al. 2024).

Hormonal regulatory networks display convergent dynamics in both species. In rice, ABA promotes abscission by inhibiting auxin transport, with ethylene mediating ABA-induced shattering(Chang, et al.

1973). While ABA showed no significant correlation with breaking tensile strength in *C. breviculmis*, gibberellins (GA7, GA19) and cytokinins (IPA, CZ) exhibited strong positive correlations ( $r = 0.89\text{--}0.98$ , Fig. A.1), mirroring rice's GA-DELLA pathway for abscission zone cell separation (Nakano, et al. 2015). Both species downregulate ethylene signaling: rice suppresses *ETR* expression, whereas *C. breviculmis* upregulates *metK* (ethylene synthesis) and activates *CTR1* (Fig. 6e), potentially co-regulating abscission zone programmed cell death (Meir, et al. 2010).

From an evolutionary perspective, convergent shattering in *C. breviculmis* and rice reflects adaptive responses to distinct ecological strategies. Wild rice enhances seed dispersal via shattering, while *C. breviculmis*—as a perennial herb—likely co-evolves shattering with clonal growth and stress tolerance. The abscission zone vascular bundles in *C. breviculmis* (Fig. 1m-q) share mechanical support functions with rice's *OsSh1*-regulated vascular development (Inoue, et al. 2015). Tandem duplication of phenylpropanoid metabolism genes, such as *4CL*, *CAD*, in both genomes underscores conserved selection for cell wall remodeling in seed shattering evolution (Liquin, et al. 1996; Xie, et al. 2017).

Notably, molecular bases of seed shattering differ between taxa. Rice shattering is controlled by the major locus *OsSh1*, whereas *C. breviculmis* exhibits polygenic regulation (Fig. 5d), possibly linked to its allogamous nature. Anatomical differences—terminal abscission zones in *C. breviculmis* vs. basal spikelet abscission in rice—highlight "functional equivalence without structural homology" during convergent evolution (Tang and Chen 2017).

## 4.5 Interplay between seed shattering, drought resistance, and environmental regulation in *C. breviculmis*

As mentioned previously, *C. breviculmis* is a high-quality grass species known for its good drought and salt tolerance. We conducted a preliminary study to understand the varying degrees of seed shattering at different stages of its growth. The question of whether this seed shattering phenomenon is related to the species' drought resistance is of great significance.

By comparing the genomes of drought-tolerant forages, the study identified several genes associated with drought resistance mechanisms, such as those involved in osmotic adjustment and antioxidant defense systems (Zhang, et al. 2024). In *C. breviculmis*, similar gene-regulatory networks might be at play. If the plant perceives drought stress, it could potentially trigger a series of molecular events that might also influence seed shattering. For example, genes related to cell wall remodeling, which are also involved in the formation of the abscission zone, could be co-regulated with drought-responsive genes. This co-regulation could be an adaptive strategy, allowing the plant to shed seeds at an appropriate time under drought conditions, perhaps to ensure the survival of the next generation in more favorable micro-habitats.

Environmental changes, particularly drought, are known to have a profound impact on plant hormones. As elucidated in the study (Mi, et al. 2022), under drought stress, *C. breviculmis* experiences significant changes in hormone levels. ABA, a well-known stress-responsive hormone, accumulates in higher

amounts. This increase in ABA can have multiple effects. It can regulate stomatal closure to reduce water loss, but it can also interact with other hormones such as ethylene. Ethylene, on the other hand, is closely associated with the seed shattering. An increase in ABA during drought might trigger a cascade of events that ultimately leads to an increase in ethylene production, which in turn could promote seed shattering.

Mild drought stress may initiate a set of compensatory mechanisms in the plant. The plant might upregulate genes involved in maintaining cell turgor and reducing oxidative stress. At the same time, it could also start to prepare for potential seed dispersal by priming the seed shattering related genes, but the actual shattering might be delayed or occur at a lower rate compared to severe drought conditions. In contrast, severe drought can disrupt normal plant physiological processes more severely. The excessive accumulation of stress-related hormones, along with the activation of genes involved in cell wall degradation in the abscission zone, could lead to a more rapid and extensive seed shattering. This is in line with findings in other plant species, such as some cereals, where severe drought has been shown to increase seed shattering as a means of ensuring at least some seeds are dispersed before the plant succumbs to extreme water stress(Ahmed, et al. 2023).

Previous excellent studies have investigated similar relationships in different plant systems. By comparing these studies with our findings on *C. breviculmis*, we can gain a more comprehensive understanding. In some plants, drought-induced changes in plant hormones have been directly linked to the activation of the seed shattering process. However, *C. breviculmis* has its own unique genetic and physiological characteristics. The genes identified in the comparative genomics study and the metabolic changes observed during drought suggest that the mechanisms in *C. breviculmis* are likely a combination of general plant stress-response pathways and species-specific adaptations.

In conclusion, the seed shattering phenomenon in *C. breviculmis* at different stages is likely related to its drought resistance. Environmental changes, especially drought, can significantly affect plant hormones, which in turn impact seed shattering. Different drought treatments lead to different patterns of seed shattering, and this complex relationship is influenced by a combination of genetic and hormonal factors. Future research should focus on further elucidating the molecular mechanisms underlying these relationships, perhaps through more detailed gene-expression studies and hormone-quantification experiments under controlled drought conditions.

## 5. Conclusion

In summary, as shown in Fig. 7, our study is the first to explore the seed shattering mechanism of *C. breviculamis* 'Siji' through histological observation, physiological indicators, and transcriptome analysis. According to our research results, the seed shattering of *C. breviculamis* 'Siji' is mainly caused by the abscission zone structure located at the end of the seed. Combining histological and physiological analysis, it is suggested that the rupture of the abscission zone may be caused by the degradation of cellulase, and gibberellin, cytokinin, and ethylene regulate the abscission signal by changing the hormone

levels in the abscission zone and regulating signaling factors, thereby affecting seed shattering. During the process of seed shattering in *C. breviculamis* 'Siji', pathways related to seed shattering such as 'plant hormone signal transduction', 'phenylalanine biosynthesis', and 'starch and sucrose metabolism' underwent changes. Further analysis revealed differential expression of genes involved in extracellular cellulase activity and plant hormones, leading to seed shattering in *C. breviculamis* 'Siji'. This study provides valuable insights into the seed shattering mechanism of *C. breviculamis* in the future.

## Declarations

### Declaration of Competing Interest

The materials used in this study were collected by ourselves. And we complied with all relevant institutional, national and international guidelines and specify the appropriate permissions obtained. We also have acquired a permission to collect all of the plant materials. The authors declare that they have no competing interests.

### CRediT authorship contribution statement

**Jiaming:** Writing-original draft, Validation, Investigation, Formal analysis, Data curation. **Liu Lingyun:** Writing-review&editing, Validation, Software. **Dong Shuanghui:** Validation, Formal analysis. **Teng Ke:** Investigation. **Guo Yidi:** Data curation, Conceptualization. **Zhang Hui:** Investigation. **Wen Haifeng:** Validation, Investigation. **Fan Xifeng:** Project administration, Writing-review&editing. **Yue Yuesen:** Project administration, Writing-original draft, Validation.

### Data availability

The Illumina sequencing data used in this study has been submitted to the BioProject database of National Center for Biotechnology Information (PRJNA1230404, <https://dataview.ncbi.nlm.nih.gov/object/PRJNA1230404?reviewer=eglu6e61h2unth2mcig3tnrh43>)

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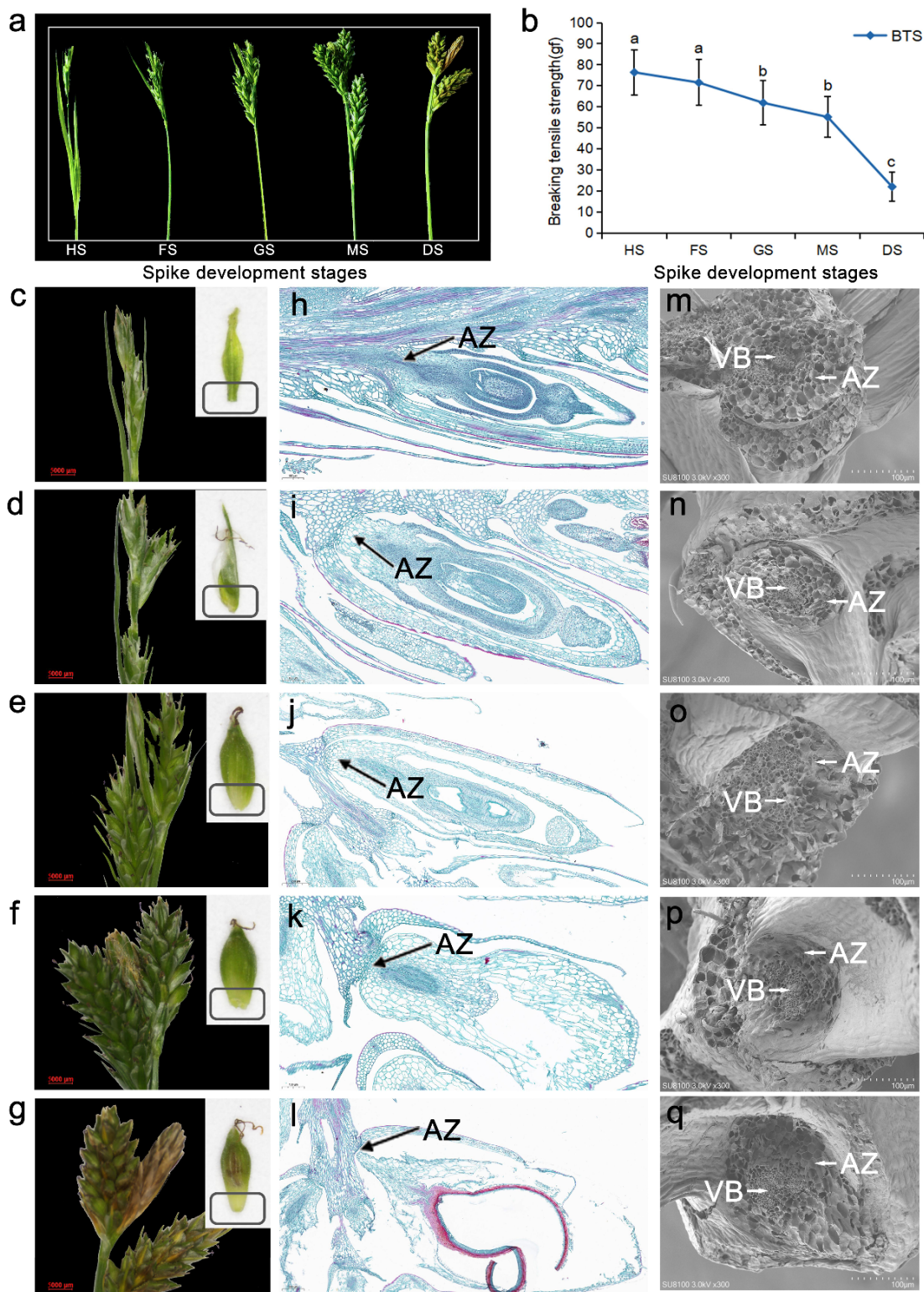
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## Figures

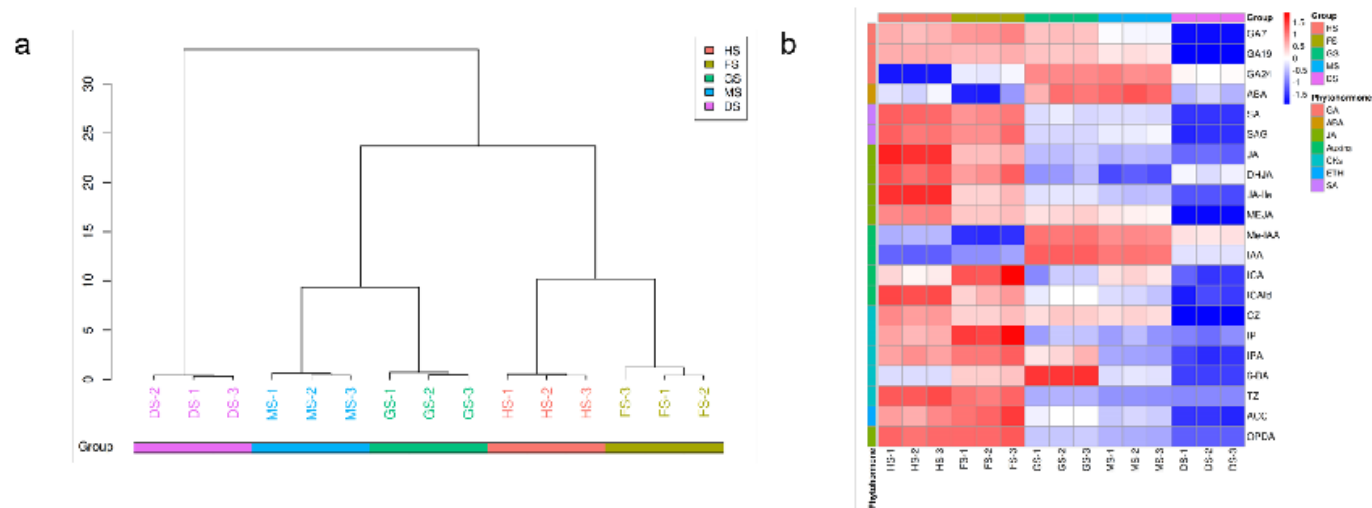


**Figure 1**

Characterization of seed shattering and abscission zone histology in *C. breviculamis* 'Siji'

**(a)** Five stages of *C. breviculamis* 'Siji' spike development. **(b)** The breaking tensile strength of the *C. breviculamis* 'Siji'. Error bars,  $\pm$ SD. **(c-g)** Morphological characteristics of the spikes. The white box showed the area where the abscission zone is located. Bars = 5000 $\mu$ m. The five rows from top to bottom

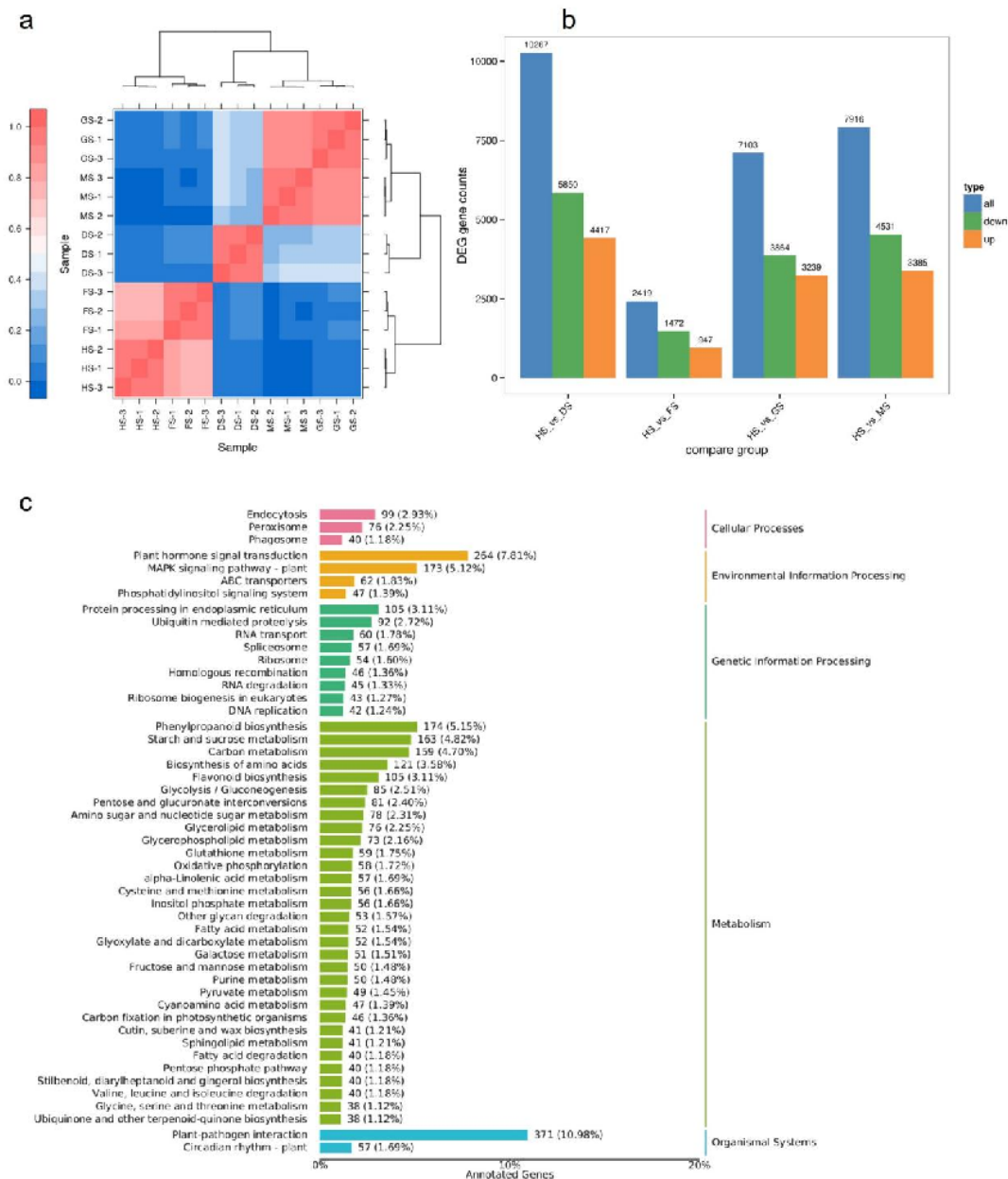
represent morphological analysis of the HS, FS, GS, MS and DS. **(h-l)** represent to longitudinal paraffin sections in the abscission zone to where the arrow points. AZ = abscission zone. **(m-q)** represent the electron microscopy scans of the seed abscission zone. AZ = abscission zone, VB = vascular bundle. The arrow points to the area where the abscission zone and the vascular bundle are located.



**Figure 2**

Sample cluster map and differential metabolite heat map of plant hormones in abscission zone of *C. breviculmis* 'Siji'

**(a)** Sample level clustering of hormone data from five different periods of *C. breviculmis* 'Siji'. **(b)** Different metabolite heat map of the five developments stages. The size of the relative content of hormones in the figure is shown by the difference in color. The columns represent the sample, while the rows represent the hormone names.



regulated genes in each group. The abscissa represents different differential gene sets, including HS vs FS, HS vs GS, HS vs MS and HS vs DS. Blue represents all differential genes, orange represents up-regulated genes, green represents down-regulated genes, and the ordinate represents the number of differential expressed genes. (c) Enriched KEGG pathways of differential expressed genes in different groups.

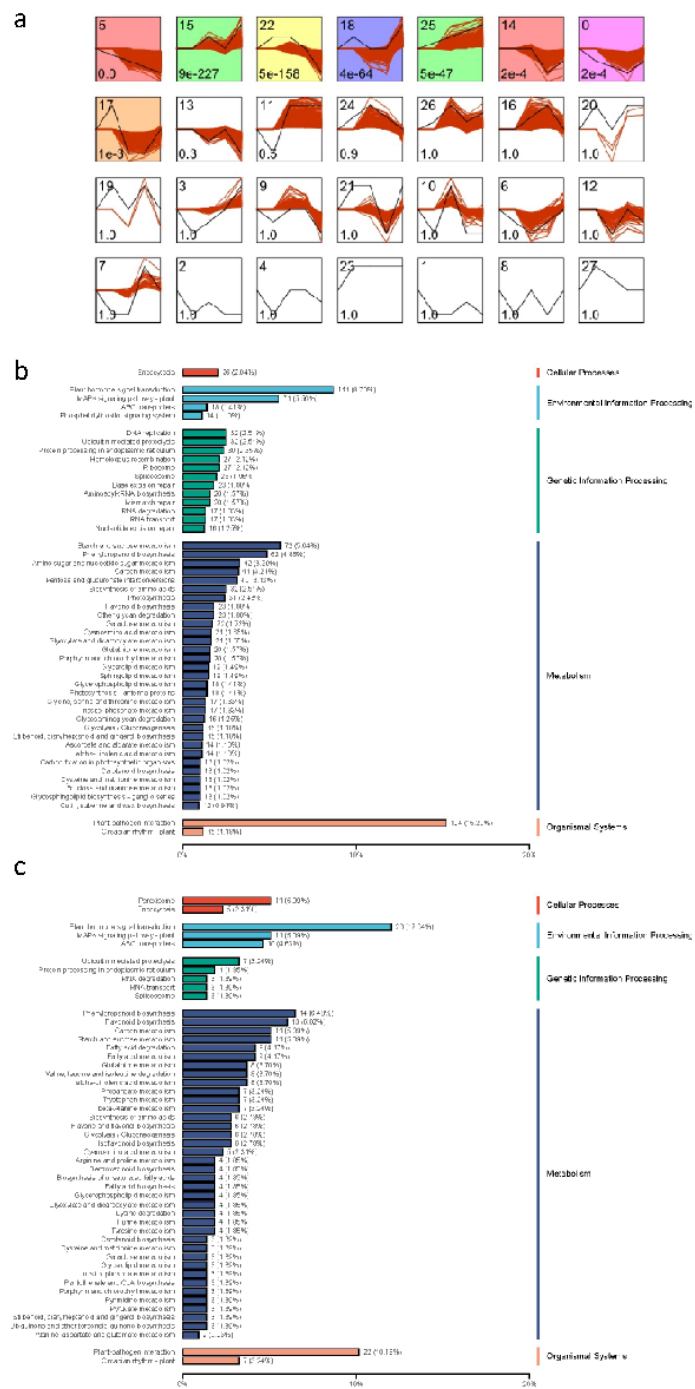


Figure 4

**(a)** The differential expressed genes were assigned to 28 different expression pattern files. **(b)** KEGG classification of differential expressed genes of file 5. **(c)** KEGG classification of differential expressed genes of file 25.



Weighted gene co-expression network analysis reveals specific modules of seed shattering of *C. breviculmis* ‘Siji’

**(a)** Trait-module relationship and gene expression patterns of Meturquoise and Meblue modules identified by WGCNA in *C. breviculmis* ‘Siji’. Each row in the figure represents a gene co-expression network module; each column represents a trait related to seed shattering. Each cell shows the corresponding correlation and p-value. **(b)** KEGG classification of Meturquoise. c KEGG classification of Meblue. d and e represent the MEturquoise and Meblue module gene network.

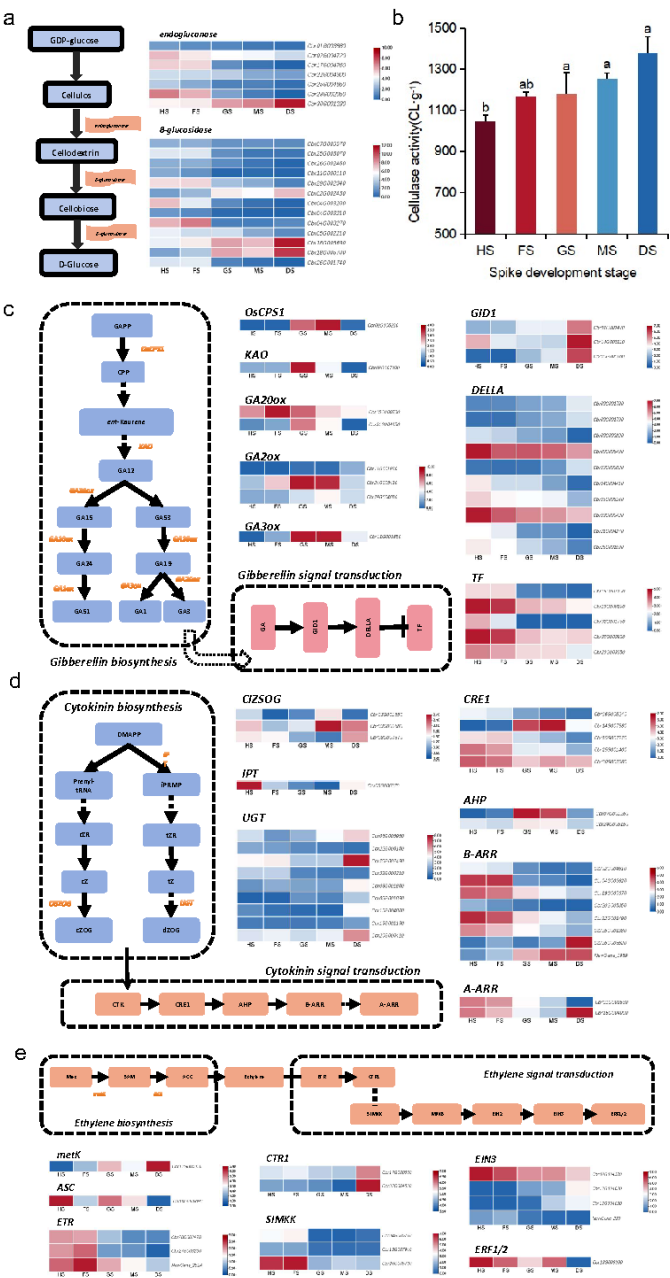


Figure 6

DEGs related to cellulase degradation pathway and plant hormone biosynthesis and signal transduction pathway of *C. breviculmis*'Siji'

(a) Cellulase degradation pathway related DEG expression in *C. breviculmis* 'Siji'. (b) The measured results for cellulase activity. Error bars,  $\pm$  SD. (c) Expression patterns of DEGs involved in gibberellin biosynthesis and signaling pathways. (d) Expression patterns of DEGs involved in cytokinin biosynthesis and signaling pathways. (e) Expression patterns of DEGs involved in ethylene biosynthesis and signaling pathways.

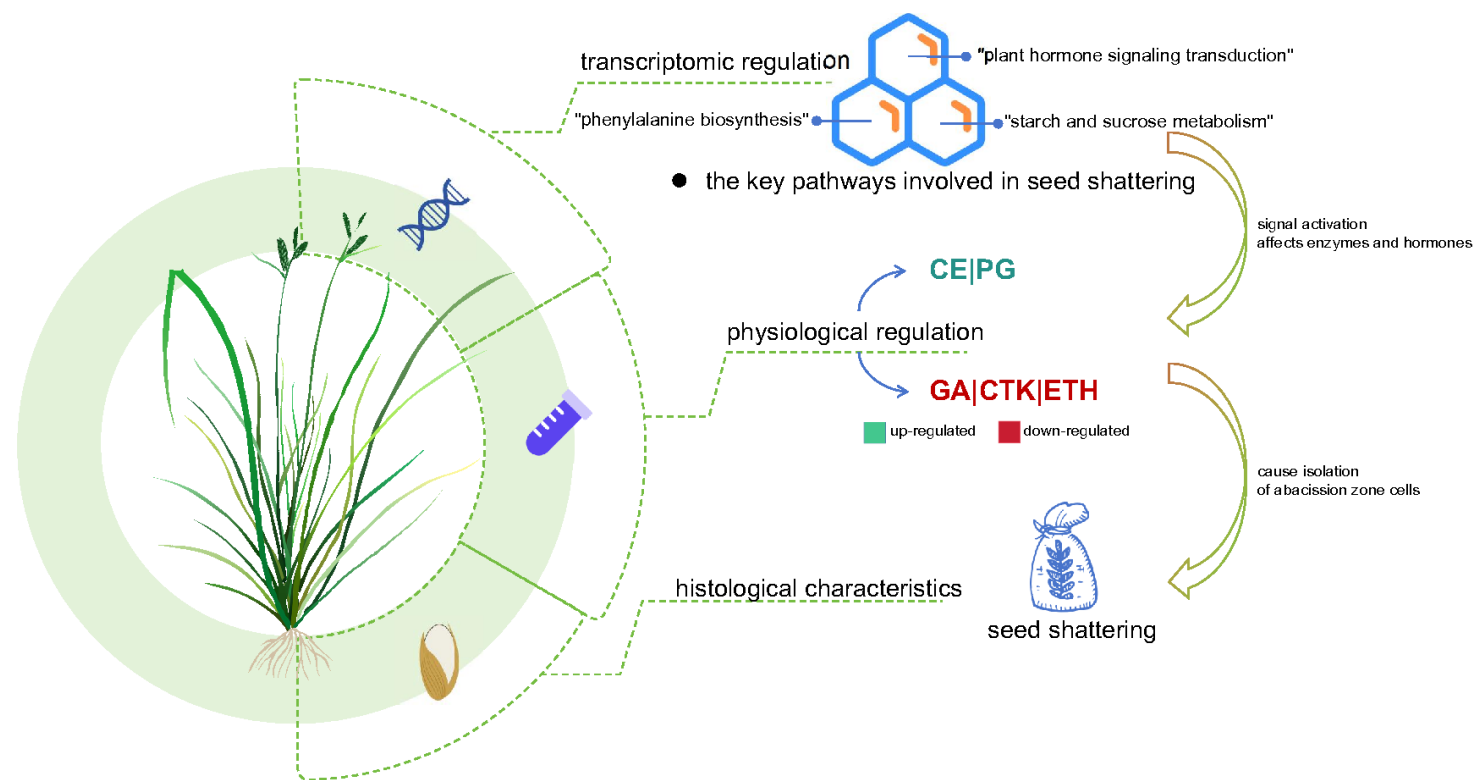


Figure 7

The model of seed shattering mechanism of *C. breviculmis* 'Siji'

## Supplementary Files

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