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Characterization of an Excellent Hybrid Rice Restorer Line R382 with Enhanced Lodging Resistance

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Abstract: Hybrid rice needs to prevent lodging due to excessive grain weight during maturity, which can lead to yield loss, grain quality deterioration and difficulty in harvesting. Breeding excellent parents (restorer and male-sterile lines) is the foundation for cultivating lodging-resistant hybrid rice. However, there is still limited information about restorers with improved lodging resistance. To identify a new restorer with significantly enhanced resistance to lodging, the restorer line Huazhan, which has been used for many years in hybrid rice breeding in China, was used as the control, to evaluate the lodging resistance of the new restorer R382. A two-year field experiment was executed to investigate the difference on lodging-related traits of the basal two internodes above-ground, such as out diameters of stems with or without leaf sheath (DWS, DWOS), culm thickness (CT), the section modulus (SM), bending stress (BS), and bending moment at break (M). The results indicated that DWS, CT, BS, M of R382 was at least 19.9%, 13.3%, 36.5% and 52.6% higher than that of Huazhan. However, the negative indicator lodging index of R382 was over 17.5% lower. The number and area of vascular bundles, and the contents of the chemical components of the cell wall including cellulose, lignin, and hemicellulose, together with the accumulation of starch granules, were significantly greater in R382. Furthermore, in the basal stems during the jointing stage, a series of genes that promote cell wall formation exhibited significantly higher expression levels in R382. Meanwhile, there was no significant difference in the yield of R382 and Huazhan. Thus, this new restorer could be a valuable donor in the future hybrid rice breeding programs for improving lodging resistance.

Keywords: rice; stem strength; vascular bundles; cell wall components



Citation: Liu, X.; Song, J.; Xiong, J.; Zhang, G.; Deng, H.; Tang, W.; Lu, X. Characterization of an Excellent Hybrid Rice Restorer Line R382 with Enhanced Lodging Resistance.

Agronomy **2024**, *14*, 1291. <https://doi.org/10.3390/agronomy14061291>

Academic Editor: Diego Rubiales

Received: 5 May 2024

Revised: 3 June 2024

Accepted: 11 June 2024

Published: 14 June 2024



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1. Introduction

Rice (*Oryza sativa* L.) is an important staple food for approximately 50% of the global population [1]. The continued growth of the world's population and the reduction in food production due to climate change will lead us to face the even more serious problem of food security [2,3]. Therefore, ensuring both the agricultural production and nutritional quality of rice is critical. Hybrid rice, successfully utilizing the heterosis of male-sterile lines and restorer lines, has been a significant breakthrough to the increase in rice yield [4]. However, lodging leads to difficulties in harvesting high-yielding rice, resulting in severe yield losses and damage to rice quality [5,6]. As a result of rising labor costs, modern rice cultivation techniques have shifted from traditional transplanting to simpler cultivation techniques such as seedling throwing and direct-seeded rice, which makes high-yielding hybrid rice more susceptible to lodging [7–9]. The breeding of high-yielding, lodging-resistant hybrid rice is conducive to ensure food security and quality in the mechanized production of modern agriculture.

There are two main types of lodging in rice: root lodging and stem lodging. Root lodging is mainly caused by shallow rooting and a small rooting area due to direct sowing and seedling throwing [1,10]. Stem lodging is defined as the bending or breaking of the lower culm internodes due to excessive bending pressure at the higher internodes, especially when experiencing strong winds and heavy rains, which is the main lodging type that leads to reductions in grain yield and quality [11]. Crop management practices, such as planting techniques, plant growth regulator use, irrigation, and fertilization, are strongly associated with rice stem lodging. Additionally, environmental conditions like soil properties, temperature, CO₂ and O₃ levels, and shade can also significantly impact the incidence of lodging in rice [12–22]. Genetic traits including plant height, gravity center height, above-ground biomass, and stem strength are important for the stem lodging resistance of rice varieties [16,19,21,22].

The stem strength of the basal internodes is identified as one of the most important traits associated with lodging resistance in rice [16,17,21,23,24], along with culm diameter, culm thickness, the thickness of mechanical tissues, and the number and area of vascular bundles [21,24,25]. In addition, the mechanical strength of basal internodes depends mainly on their chemical composition, such as silicon, and the content of non-structural and structural carbohydrates (mainly lignin, cellulose, and hemicellulose) [26–29]. In general, well-arranged internode length, a low center of gravity height, a high proportion of structural carbohydrates such as lignin and cellulose in the stalks, and short and thick basal internodes with more vascular bundles, positively contribute to the lodging resistance in rice [9,30].

Therefore, improving these factors through cultivation management could contribute to the enhancement of lodging resistance in hybrid rice. For instance, field experiments in two different ecological regions growing the super *indica* hybrid rice Y-liangyou2 showed that the resistance of high-yielding hybrids to lodging could be improved by optimizing plant height and internode length, increasing leaf sheath fullness, and increasing structural and moderate non-structural carbohydrates to improve basal culm strength [11,31]. The stem lodging resistance of hybrid *indica* rice Zhuliangyou819 could be improved by DDS (dry direct seeding), shown by shorter basal internodes, a better stem diameter and culm wall thickness and a lower lodging index under DDS compared with the other two patterns (FDS, flooded direct seeding, and WDS, wet direct seeding) [32]. An experiment aiming to evaluate the effects of nitrogen fertilizer dosage and application time on the potential for the lodging of the hybrid rice XL723 in the United States was conducted to provide suggestions for nitrogen fertilizer management [33].

As mentioned above, to date, the research on the lodging resistance of hybrid rice mainly focuses on the cultivation methods. For example, the field configuration of *indica* hybrid rice cultivars, Yixiangyou2115 and Shuyou217, under fewer hills with more seedlings, can improve the lodging resistance of whole hills while ensuring the grain yield of machine-transplanted rice [34]. Promoting the pre-heading growth of hybrid rice (Guiliangyou2 and Y-liangyou1) can improve its lodging resistance while maintaining a high yield [35]. Eight hybrid rice varieties, together with twenty-five inbred *indica* rice varieties, were used to screen rice varieties with a high yield and lodging resistance for rice–fish co-culture systems [36]. Breeding high-performance parents is the foundation for cultivating lodging-resistant hybrid combinations. However, there are still insufficient reports on the identification of restorer or sterile lines with improved lodging resistance and further investigation of the molecular mechanism underlying the lodging resistance regulation of the parents used in hybrid rice production.

In this study, to identify a lodging-resistant male parent, we carefully compared and analyzed R382, a new restorer line recently developed by our team, with Huazhan, a restorer line currently widely used in hybrid rice breeding and production, in terms of stem morphology and chemical composition, mechanical strength, and yield traits. It was found that R382 possessed stronger stem strength with comparable yield per plant to Huazhan. Furthermore, the molecular mechanism behind this was also preliminarily analyzed at the gene expression level to give clues

for the genetic analyses conducted in the future. It was suggested that R382 can be used as a good donor for breeding hybrid combinations with a stable improved resistance to lodging.

2. Materials and Methods

2.1. Plant Materials

In this study, two restorers for hybrid rice, R382 and Huazhan, were selected for the field experiments. Huazhan (*Oryza sativa* L. subsp. *indica* cv.) is a kind of super rice variety with high and stable yield, which has strong adaptability, better resistance to adversity and stress than ordinary varieties, and better resistance to diseases and pests [37]. Huazhan have been widely popularized in hybrid rice breeding in China (www.ricedata.cn (accessed on 10 June 2024)). R382 is a novel male parent for Hybrid *indica* rice created by Hunan Agricultural University and is currently being used to construct multiple hybrid combinations.

2.2. Experimental Site and Field Management

The two-year field trials were conducted at the Field Science Center of Hunan Agricultural University in Changsha, Hunan Province (28°17′2.79″ N, 113°25′9.75″ E). In 2022, due to uncertainty of the difference in growth period between the two varieties, the two restorers were planted three times in Changsha on 31 May, 7 June, and 14 June, respectively. Seedlings at the fifth-leaf stage were transplanted. The heading process was traced and recorded from the initial heading date to full heading date. The dates on which 10% and 80% of the total panicles emerged from the flag leaf sheath were defined as the initial and full heading date, respectively. The sowing dates, initial and full heading dates for R382 and Huazhan were recorded (Table S1). As shown in Table S1, the growth period of R382 and Huazhan differed by approximately 10 days. The second batch of Huazhan was able to mature on nearly the same date as the first batch of R382 and could be used for the determination of the resistance parameters on the same day (4 October 2022). Parameters related to lodging resistance were measured at a grain maturity stage, which is roughly 30–35 days after the full-heading date [21]. In 2023, both R382 and Huazhan were sowed on 25 May. The determination of the two restorers on the day of maturity of each of them for their respective traits was related to lodging resistance. The experimental design in 2022 excluded the influence of weather conditions on the exact day of sampling (e.g., rainwater) on the phenotypic differences in materials, while the experimental design in 2023 excluded the influence of early growth and field management on phenotypic determination. Although there is a slight difference in the sampling method for the two-year experiment, the results obtained from the two-year experiment were basically similar (see Section 3), implying that both sampling methods are feasible.

The same cultivation method was used in both years. The experiment followed a randomized complete block design with three replications. Each seedling was planted singly. The row spacing of transplanted plants was 20 cm × 20 cm. All materials were transplanted in 6 rows, 8 plants per row in a block. The unhealthy seedlings were checked and made up within three days after transplanting to ensure sufficient basic seedlings. The fertilizer containing a total of about 150 kg ha^{−1} of pure nitrogen throughout the growth period was applied as follows: 375 kg ha^{−1} compound fertilizer (N/PO₅/K₂O = 15-15-15) as a base fertilizer 1 day before transplanting, 150 kg ha^{−1} urea as first topdressing 7 days after transplanting, and 150 kg ha^{−1} compound fertilizer as second topdressing at the panicle initiation stage. The irrigation method was mainly wet irrigation, and the drainage and drying was carried out 5 days before the invalid tillering. The pest and disease control method was implemented according to the normal field management. The data of the maximum and minimum temperatures of the days from sowing to maturation stage are shown in Figure S1.

2.3. Sampling and Measures

2.3.1. Morphological and Mechanical Indices of Stem Lodging Resistance

Characteristics related to the morphological and mechanical characters of culms, by using main stems of 4 independent plants from each plot that were not grown at the

edges of the plots, were measured at maturation stage [21,38]. Plant height, the length of panicles and inter-nodal length from base to top were measured [38,39]. Based on the previously reported methods with slight modifications, the first partially or completely exposed internode above the ground, usually longer than 4 cm, is defined as N1 (usually the third internode from the base in this study); while the next internode above it is defined as the N2 internode (usually the fourth internode from the base) [11,39]. The N1 and N2 internodes were used to measure the following physical and chemical parameters to meet the experimental requirements of placing internodes on supports spaced 4–10 cm apart when measuring bending stress and to elucidate the characteristic differences in the above-ground stem of the tested restorers [18,40].

- (1) Plant height (PH, cm) was measured by using a ruler to measure the length between the base to the panicle tip.
- (2) Proportion of the three basal sections to the overall plant height (PBH) is a ratio derived by adding the lengths of the three basal internodes and dividing by plant height.
- (3) Culm thickness (CT, mm) was measured using a digital Vernier caliper (EXPLOIT).
- (4) Out diameter of the stem with leaf sheath (DWS, mm) and out diameter of the stem without leaf sheath (DWOS, mm): a_1 is the outer diameter of the minor axis in an oval cross-section, b_1 is the outer diameter of the major axis in an oval cross-section, a_2 is the inner diameter of the minor axis in an oval cross-section and b_2 is the inner diameter of the major axis in an oval cross-section. DWS and DWOS refer to the average values of a_1 and b_1 of the internodes with and without a leaf sheath, respectively.
- (5) Section modulus (SM, mm^3): $SM = \pi (a_1^3 b_1 - a_2^3 b_2) / 32 a_1$. We used the outer diameters including the leaf sheath in this formula because the leaf sheath has a significant impact on the bending resistance of rice stems [41].
- (6) Bending stress (BS, mm^{-2}) is the bending load at the time of fracture of internodes placed on stands spaced 4 cm apart, using a plant stem strength tester (YDD-1A, Topu Instrument Co., Ltd., Hangzhou, China).
- (7) Bending moment of the basal internode at breaking (M, g cm), $M = SM \times BS$. M is positively correlated with the lodging resistance of rice.
- (8) Fresh weight from basal node to panicle top (FWT, g) was determined through weighing the fresh weight from the basal node to spike apex using a scale with an accuracy of 0.01 g.
- (9) Height from the basal node to panicle top (HT, cm) is the length from the basal node to the apex of the spike.
- (10) Bending moment (BM, $\text{cm} \cdot \text{g}$), $BM = HT \times FWT$
- (11) Lodging index (LI), $LI = BM / BS \times 100$. LI is negatively correlated with the lodging resistance of rice.

2.3.2. Measurement of Contents of Cell Wall Components

After drying to constant weight in a 75 °C oven, the N1 and N2 internodes with leaf sheath were used to determine the contents of main cell wall components. The determination of cellulose, hemicelluloses, and lignin in culms was performed as described previously with minor modifications according to the instructions manual of the reagent kits (Cat. No. AKSU010U, AKSU007M, AKSU008M, Beijing Box Biotechnology Co., Ltd., Beijing, China) [42–44].

As cellulose can be hydrolyzed to β -D-glucose under acidic conditions and β -D-glucose can be dehydrated to β -furaldehyde compounds in a strong acid environment, they can then be dehydrated and condensed with anthrone to produce blue-green furfural derivatives; the products have characteristic absorption peaks at 620 nm. The cellulose content was detected and calculated according to the change in the absorbance value.

The stalk samples were successively acetylated with reagents such as perchloric acid to generate acetyl lignin from the phenolic hydroxyl groups in lignin, and then mixed with reagents such as glacial acetic acid; the samples could be detected using the enzyme marker

at 280 nm, and the lignin content could be detected and calculated according to the change in absorbance value.

Since hemicellulose was converted to reducing sugar after acid treatment and further reacted with 3,5-dinitrosalicylic acid to form brown-red amine compounds, which had a characteristic absorption peak at 540 nm, the hemicellulose content was detected and calculated according to the change in absorbance value by the use of an enzyme marker at 540 nm after treatment with the acidic reagent.

2.3.3. Morphological Observations and Microscopic Analysis of Stem Cross-Sections

The N1 and N2 internodes without leaf sheaths were fixed in FAA solution (formaldehyde: acetic acid: 50% ethanol at 5:5:90). Thin transverse sections that were prepared from the internodes with a razor were stained in 0.05% (*w/v*) toluidine blue solution and observed under a stereo microscope (ECLIPSE CI, NIKON, Tokyo, Japan) with imaging systems (DS-US, NIKON, Japan). The number and area of the vascular bundles were measured using Image J (1.53 q) software.

The transverse section surface was also coated with gold for 90 s using a vaporizer and observed under a scanning electron microscope (SU8010, Hitachi, Tokyo, Japan). Observation conditions were as follows: acceleration voltage, 2000 Volt; magnification, 400.

2.3.4. RNA Extraction and Real Time-qPCR

At 0, 3, 6, 9, 12, 15, 20, 25, and 40 days after the jointing stage, RNA was extracted from basal internodes above-ground, respectively. At the early jointing stage of R382 and Huazhan, due to the short basal internodes, 9 basal internodes of main stems from independent plants of each rice variety were sampled for one biological replicate. From 20 days after the jointing, 3 basal internodes of each rice varieties were sampled for 1 biological replicate. The samples were quickly frozen in liquid nitrogen, ground into powder in liquid nitrogen, and used for the total RNA extraction. Each RNA sample for each biological replicate was reverse transcribed using primeScript™ reagent kit with gDNA eraser (TAKARA, Tokyo, Japan). RNA extraction and Real Time-qPCR (RT-qPCR) were performed as described previously [45]. To preliminarily investigate the molecular mechanism by which the chemical composition of the stems of R382 are superior to those of Huazhan, twelve genes including lignin synthesis-related genes (*OsCAD2*, *OsCOMT*, *CCoAOMT*, *Os4CL5*, *Os4CL3*, *OsPAL6* [46–50]), cellulose synthesis-related genes (*OsCesA4*, *OsBC1L4* [51,52]), a phloem sucrose transporter protein synthesis gene (*OsSUT1* [53]), and secondary cell wall synthesis-related genes (*OsMYB103L*, *OsMYB46*, *OsSWN1* [54,55]), were selected to analyze the gene expression at the basal internodes of R382 and Huazhan. The reference gene *OsActin* was used as an internal control. Primers were listed in Supplementary Table S2. The RT-qPCR reactions were performed on Roche Light Cycler 480 (Forretrasse, Rotkreuz, Switzerland) with SYBR Premix Ex TaqII (TAKARA, Tokyo, Japan). The PCR thermal cycle conditions: 95 °C for 5 min, 40 cycles at 95 °C for 10 s, 60 °C for 30 s. Three independent experiments were conducted. All the experiments showed similar results and one of them is shown in Results.

2.3.5. Yield-Related Characters

Seeds harvested at maturity were dried to a constant weight. The number of panicles per plant (only the panicles with more than 5 grains were counted), 1000-grain weight, the seed-setting rate and yield per plant were determined [32].

2.4. Statistical Analysis

GraphPad prism 8.0.2. was used for data processing. All data were assessed for normality using the D'Agostino–Pearson test. Students' *t*-test was performed and the probability (*p*) values were calculated. The bar chart and line chart in this article are also drawn using the software. In addition, the R 'corrplot' (version 4.2.0) package was used to

draw the correlation coefficient matrix graph, while SPSS (version 2.5) was used to draw the principal component analysis graph.

3. Results

3.1. Main Stem Morphology

The lodging of rice stems was affected by the above-ground parts of the plant. The longitudinal morphological parameters of the stem, including total plant height (PH), intermodal lengths and proportion of the three basal internodes to the plant height (PBH), were measured. The height of R382 was 15.8% and 12.3% higher than that of Huazhan in 2022 and 2023, respectively (Figure 1a). However, the total length of the three basal internodes of R382 was not different from that of Huazhan in 2022, and the length of basal internodes of R382 was even shorter than that of Huazhan in 2023 (Figure 1b). Eventually, the PBH of R382 was significantly smaller than that of Huazhan (Figure 1c).

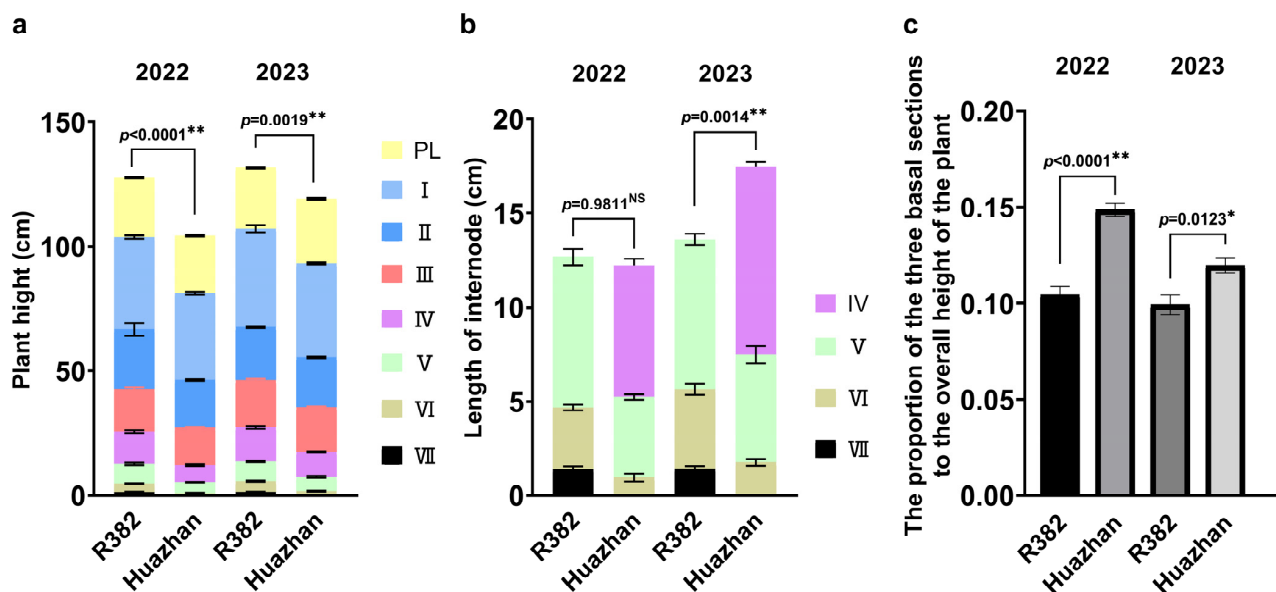


Figure 1. Comparison of R382 and Huazhan in (a) plant height and inter-nodal length, (b) the length of the three basal internodes, and (c) the proportion of the three basal sections to the overall plant height. PL, I, II, III, IV, V, VI, and VII represent the panicle length and the first to the seventh internodes of the plant, respectively; 2022 and 2023 indicates the data collected in the field experiments conducted in 2022 and 2023, respectively. Values are shown as means $\pm$ SE of 12 replicates. NS, no significant, $* p < 0.05$, $** p < 0.01$ (compared with Huazhan; Student's *t*-test).

Moreover, the morphological parameters on the cross-sections of the two restorer lines' stems including the outer diameters of N1 and N2 with the leaf sheath (DWS1 and DWS2), the outer diameters without leaf sheath (DWOS1 and DWOS2), and culm thickness (CT1 and CT2) were carefully compared (Figure 2). The DWS1 and DWS2 of R382 were significantly larger than that of Huazhan, respectively (2022, DWS1: 8.67 mm vs. 6.99 mm, DWS2: 7.54 mm vs. 6.29 mm; 2023, DWS1: 8.51 mm vs. 7.12 mm, DWS2: 7.64 mm vs. 5.90 mm) (Figure 2a). Moreover, the DWOS1 and DWOS2 of R382 were generally significantly larger than that of Huazhan, respectively, except for that the DWOS2 of R382 and Huazhan were similar in the 2022 field experiment (Figure 2b). It was demonstrated that the stems close to the ground of R382 are thicker than those of Huazhan. Meanwhile, the culm thickness of N1 internodes (CT1) of R382 was 13.3% (2022) and 36.7% (2023) larger than Huazhan, while the culm thickness of the N2 internodes (CT2) of R382 was 21.4% (2022) and 23.1% (2023) larger than Huazhan (Figure 2c). It was indicated that the basal internodes of R382 possessed thicker culms.

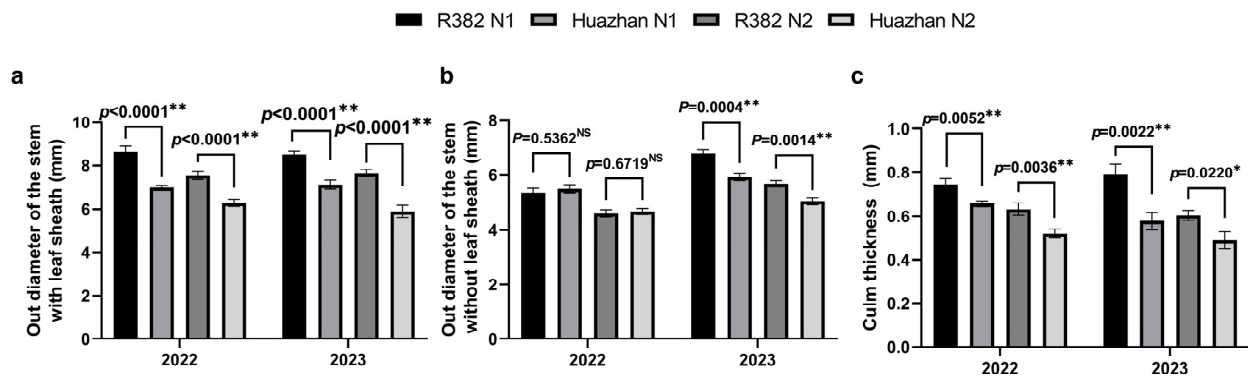


Figure 2. Comparison of (a) DWS, diameter of the stem with leaf sheath, (b) DWOS, diameter of the stem without leaf sheath, and (c) CT, culm thickness at the N1 and N2 internodes of R382 and Huazhan. Values are shown as means $\pm$ SE of 12 replicates. NS, no significant, * $p < 0.05$, ** $p < 0.01$ (compared with Huazhan; Student's *t*-test).

3.2. Histological Analyses of Basal Internodes

To determine the structural factors that contribute to the differences in culm thickness, cytological observations of the transverse sections of N1 were performed. It was revealed that the size and number of small vascular bundles in the N1 internodes of R382 were 27.35% and 21.99% larger than that of Huazhan, respectively, which ultimately resulted in a 55.31% larger total area of small vascular bundles (Figure 3a,c). Because the area of a single large vascular bundle in the N1 internode of R382 was smaller than that of Huazhan, the total area of the large vascular bundles was not significantly different, although the number of large vascular bundles was significantly greater than that of Huazhan (Figure 3a,c). The microscopic examination using scanning electron microscopy (SEM) further revealed that there were obviously more starch granules in the parenchyma cells of R382 than Huazhan (Figure 3b). Based on the positive correlation of vascular bundle and starch granule accumulation with culm strength reported previously [40,55], it was suggested that the increased number of vascular bundles in the basal internodes of R382 gives it a greater culm thickness than Huazhan, which, together with the accumulation of starch granules in the parenchyma cells, may contribute to the rigidity of the basal culms of R382.

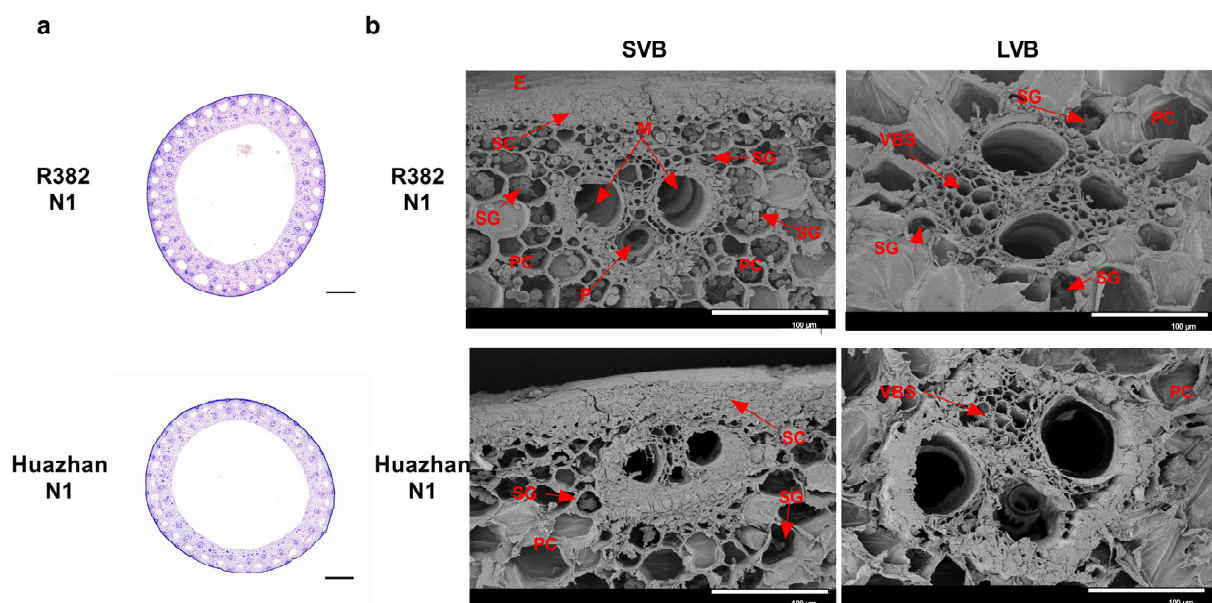


Figure 3. Cont.

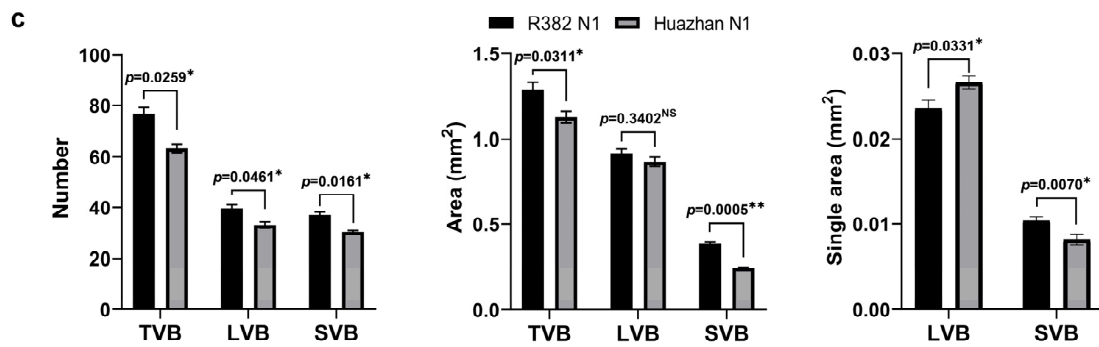


Figure 3. Comparison of cross-sectional anatomy of basal internodes of stems from two restorers. (a) Paraffin sections, (b) scanning electron microscopy images of R382 N1 and Huazhan N1, (c) quantitative analysis of the area and number of vascular bundles. E, epidermis; SC, sclerenchyma cell; SG, starch granules; M, metaxylem; PC, parenchyma cell; P, primary xylem; VBS, vascular bundles; TVB, total vascular bundles; LVB, large vascular bundles; SVB, single small vascular bundles. Values are shown as means $\pm$ SE of 5 replicates. NS, no significant, * $p < 0.05$, ** $p < 0.01$ (compared with Huazhan; Student's *t*-test).

3.3. Mechanical Properties of Stems

Compared with that of Huazhan, the bending stress (BS) of N1 and N2 was 36.5% and 46.9% higher in R382, respectively, in 2022 (Figure 4a). The difference between the BS of the two materials demonstrated by the 2023 experiment was even greater, with the N1 and N2 BS of R382 being 58.8%, and 81.1% larger, respectively, than that of Huazhan (Figure 4a). Although the SM values of the two materials were not significantly different in 2022, the SM value of R382 was significantly greater than that of Huazhan in 2023 (Figure 4b). M shows that R382 was stronger than Huazhan in both years (2022: M1, 850.6 g cm vs. 557.5 g cm, M2, 428.0 g cm vs. 247.9 g cm; 2023: M1, 926.4 g cm vs. 340.1 g cm, M2, 364.3 g cm vs. 157.6 g cm) (Figure 4c).

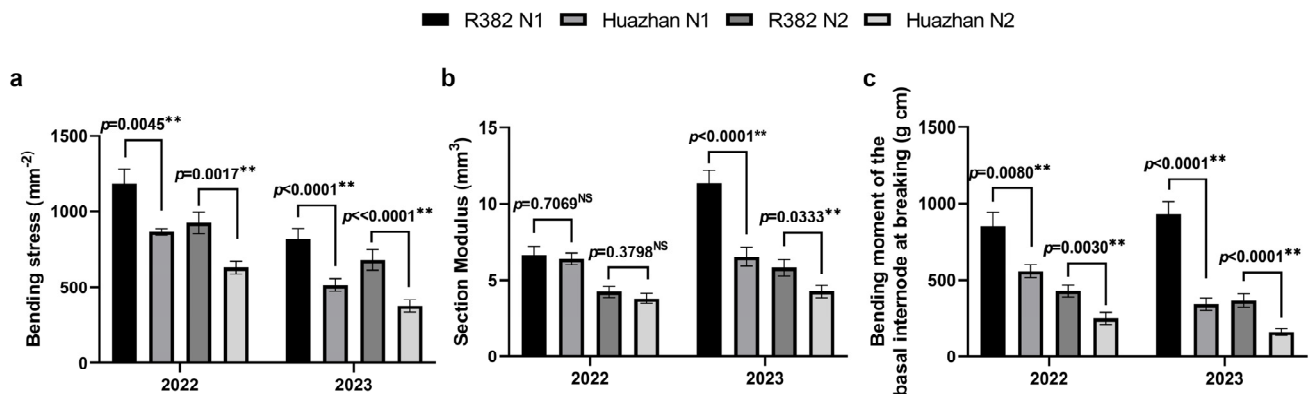


Figure 4. Evaluation of R382 and Huazhan for lodging resistance based on (a) bending stress, (b) section modulus, (c) bending moment at breaking in 2022 and 2023. Values are shown as means $\pm$ SE of 12 replicates. NS, no significant, ** $p < 0.01$ (compared with Huazhan; Student's *t*-test).

Studies in rice cultivation often use the lodging index (LI) as an important parameter for evaluating rice lodging resistance [25,36]. LI, which is negatively correlated with lodging resistance, is the ratio of bending moment (BM) to BS breaking stress (BS), where BM is obtained by multiplying the fresh weight (FWT) and height (HT) from the basal node to the apex of the panicle [36,46]. Because R382 is taller and had thicker stalks than Huazhan, its HT and FWT values were significantly greater than those of Huazhan, and thus the BM values were much greater than those of Huazhan (Figures 1 and 2, Table S3). The experimental data in 2022 showed that the BM of N1 and N2 of R382 exceeded those

of Huazhan by 20.3% and 23.1%, respectively (Table S3). Similarly, the 2023 data showed that the BM of R382 was 53.0% and 52.8% greater than that of Huazhan for N1 and N2, respectively (Table S3). As mentioned earlier, R382 also had significantly larger BS values than Huazhan (Figure 4a). From the data of 2022 and 2023, the LI of R382 was significantly lower than that of Huazhan, which indicated that R382 was more resistant to lodging than Huazhan (Table S3).

3.4. Structural Carbohydrate Content Analysis

Moreover, the lignin, cellulose and hemicellulose accumulated significantly more in R382 than Huazhan (Figure 5). The lignin content of R382 was 57.9% (N1) and 218.7% (N2) higher, while the cellulose content was 50.5% (N1) and 39.9% (N2) higher, and the hemicellulose content was 28.2% (N1) and 30.1% (N2) higher than that of Huazhan, respectively (Figure 5). In all, R382 accumulated more structural carbohydrates at the basal internodes than Huazhan, and this difference in chemical component content for cell wall may be one of the reasons for the more robust R382 stalks.

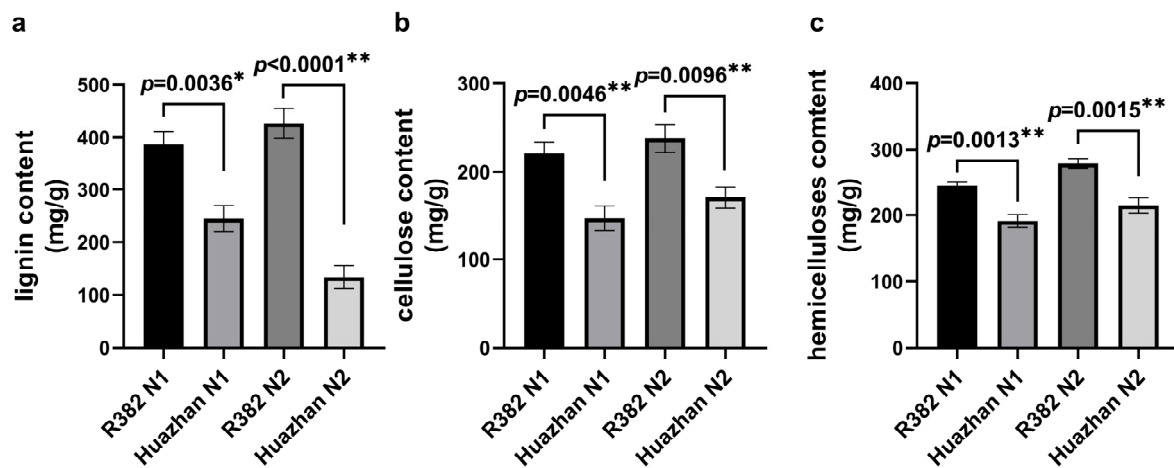


Figure 5. (a) Lignin, (b) cellulose, (c) hemicellulose content of the basal internodes was measured. Values are shown as means $\pm$ SE of 5 replicates. * $p < 0.05$, ** $p < 0.01$ (compared with Huazhan; Student's *t*-test).

3.5. Correlation Analysis of Lodging Resistance-Related Traits

A Pearson correlation analysis was conducted to figure out the relationships between M and LI, the vertical and horizontal morphology of stems, stem mechanical strength parameters and culm structural carbohydrate content of N1 (Figure 6) and N2 internodes (Figure S1). Differences in lodging resistance characterized by M-values should be attributed mainly to differences in the sectional modulus (SM1, $R = 0.97$, $p = 2.56 \times 10^{-6}$), the outer diameter of stems with leaf sheaths (DWS1, $R = 0.93$, $p = 7.93 \times 10^{-5}$), culm thickness (CT1, $R = 0.92$, $p = 1.51 \times 10^{-4}$), fresh weight of the part from the basal node to spike tip (FWT1, $R = 0.88$, $p = 7.86 \times 10^{-4}$), cellulose content (CC1, $R = 0.77$, $p = 9.71 \times 10^{-3}$), and breaking stress (BS1, $R = 0.74$, $p = 1.45 \times 10^{-2}$) (Figure 6). The negative indicator for lodging resistance LI was negatively correlated with lignin content (LC1, $R = -0.64$, $p = 0.045$) (Figure 6). Interestingly, experimental data based on R382 and Huazhan showed that LI was negatively correlated with plant height (PH), whereas the M value was positively correlated for PH, which was contrary to previous findings [46]. Correlation studies on N2-related parameters yielded similar results (Figure S2). Principal component analysis (PCA) confirmed that the M1 was closely correlated with SM1, DWS1, CT1, FWT1, CC1 and BS1, while the M2 was closely correlated with DWS2, FWT2 and BS2 (Figure S3). It was suggested that R382 is more resistant to lodging not because it is shorter and has a lower above-ground load, but because the physical structure and chemical composition of its basal stems are more optimized.

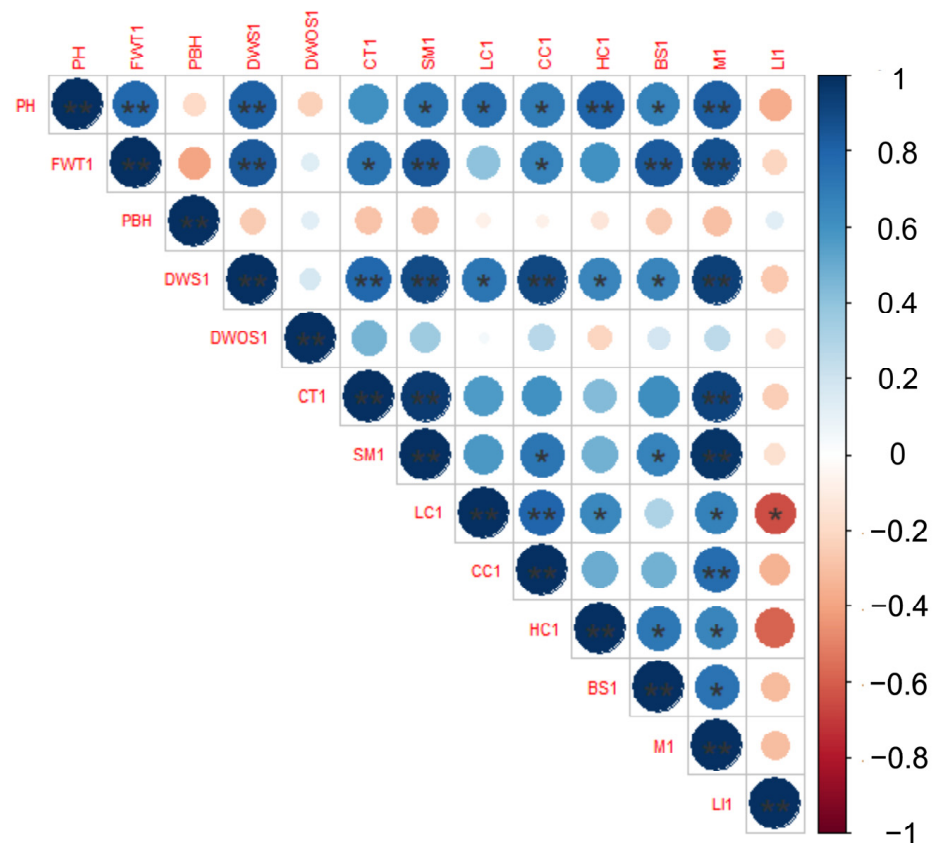


Figure 6. Correlation matrix diagram showing the correlation coefficients among 13 traits. Most traits were measured for N1 internodes. PH, plant height; FWT1, fresh weight from the basal node to panicle tip; PBH, the proportion of the three basal sections to the overall height of the plant; DWS1, the outer diameter of stem with leaf sheath; DWOS1, the diameter of stem without leaf sheath; CT1, culm thickness; SM1, section modulus; LC1, lignin content; CC1, cellulose content; HC1, hemicellulose content; BS1, bending stress; M1, the bending moment at break; LI1, lodging index. The circle with greater and greater color intensity represents a greater absolute correlation coefficient value. * $p < 0.05$, ** $p < 0.01$.

3.6. Expression Levels of Genes Related to the Chemical Composition of Stems

As shown in Figure 7, the expression levels of lignin synthesis-related genes including *OsCAD2*, *OsCOMT*, *CCoAOMT*, *Os4CL5*, *Os4CL3*, *OsPAL6* in R382 were dramatically higher than that in Huazhan at 12–15 d after jointing. It was suggested that the high lignin content in R382 may be due to the elevated expression of genes which encode enzymes related to lignin synthesis in R382 (Figures 5a and 7a–f). The expression level of the gene encoding cellulose synthase (*OsBCIL4* and *OsCESA4*) in R382 was also significantly higher than that of Huazhan on days 12–15 after the jointing stage (Figures 5b and 7g,h). In addition, the expression levels of three other genes (*OsMYB103L*, *OsMYB46*, and *OsSWN1*) related to the secondary cell wall formation of stems were also significantly higher in R382 than in Huazhan at the jointing stage, which may be one of the reasons for the thicker culms of R382 (Figures 2c and 7i–k). In addition, the *OsSUT1* gene, which is important for sucrose transport into stems [56,57], was expressed at a significantly higher level in R382 than in Huazhan, which may explain why the accumulation of starch granules in the internodes of R382 was higher than that of Huazhan (Figures 3b and 7l).

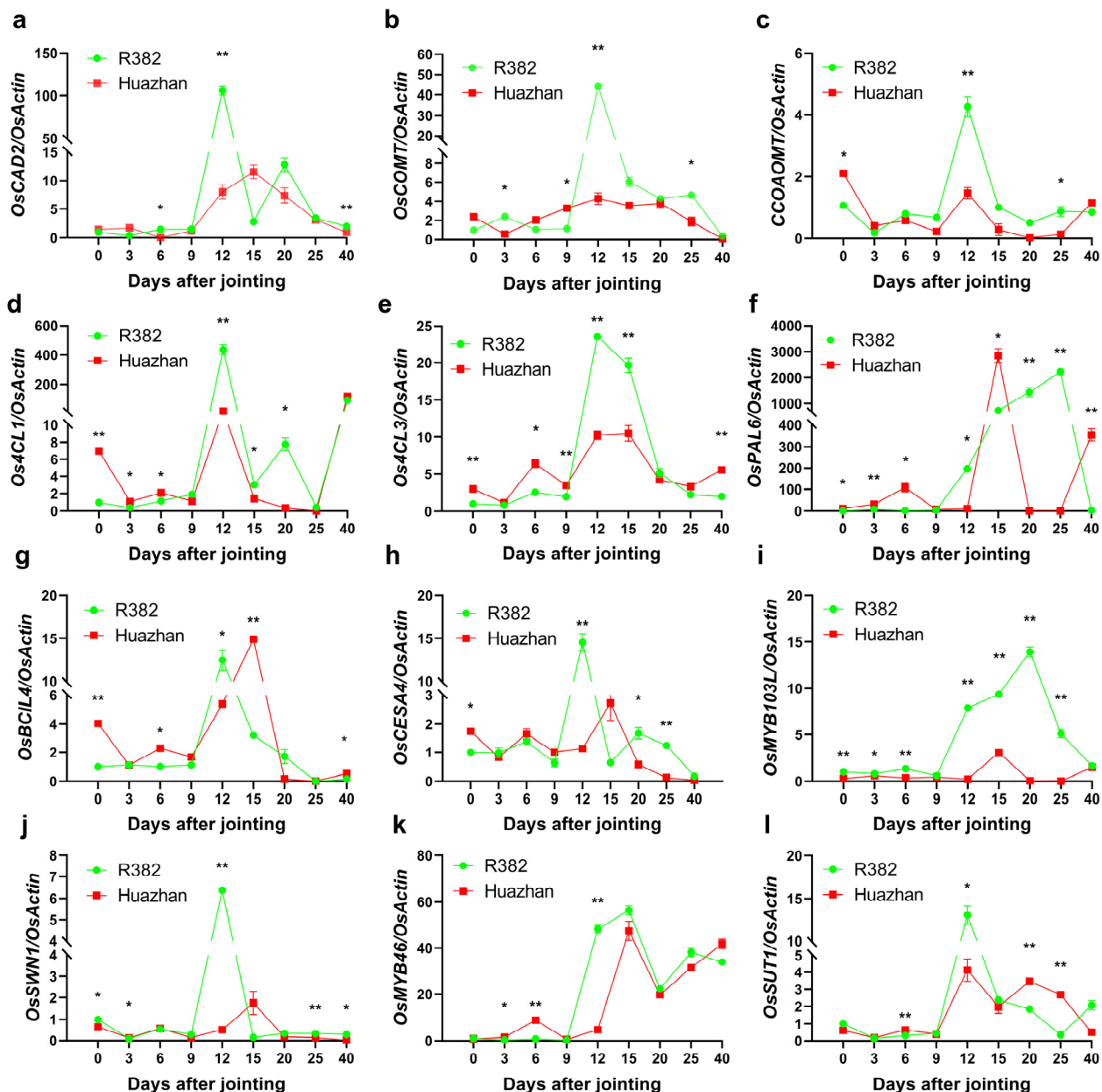


Figure 7. The expression levels of (a) *OsCAD2*, (b) *OsCOMT*, (c) *CCoAOMT*, (d) *Os4CL5*, (e) *Os4CL3*, (f) *OsPAL6*, (g) *OsBC1L4*, (h) *OsCESA4*, (i) *OsMYB103L*, (j) *OsSWN1*, (k) *OsMYB46*, and (l) *OsSUT1* in R382 and Huazhan. At 0, 3, 6, 9, 12, 15, 20, 25, and 40 days after the jointing stage, RNA was extracted from the N1 internodes. *OsActin* gene was used as the control and the value obtained on day 0 of the R382 jointing stage was set to 1. Data are shown as means $\pm$ SE of 3 replicates, * $p < 0.05$; ** $p < 0.01$ (compared with Huazhan, Students' *t*-test).

3.7. Yield Traits

Rice yield is mainly determined by the panicle number per plant, seed-setting rate, and 1000-grain weight. To find out whether R382's enhanced greater resistance to lodging came at the expense of yield, we compared the number of panicles per plant, seed-setting rates, and 1000-grain weight of them. The results showed that the seed-setting rate of R382 was slightly higher than Huazhan in both years, and R382 had a higher 1000-grain weight than Huazhan, but Huazhan had significantly more panicles than R382, which ultimately resulted in a non-significant difference in yield per plant (Figure 8). It was indicated that the yield of R382 is comparable to that of the widely used and well-known restorer line

Huazhan, but it is more resistant to lodging, and has the potential to breed lodging-resistant hybrid combinations with a high yield.

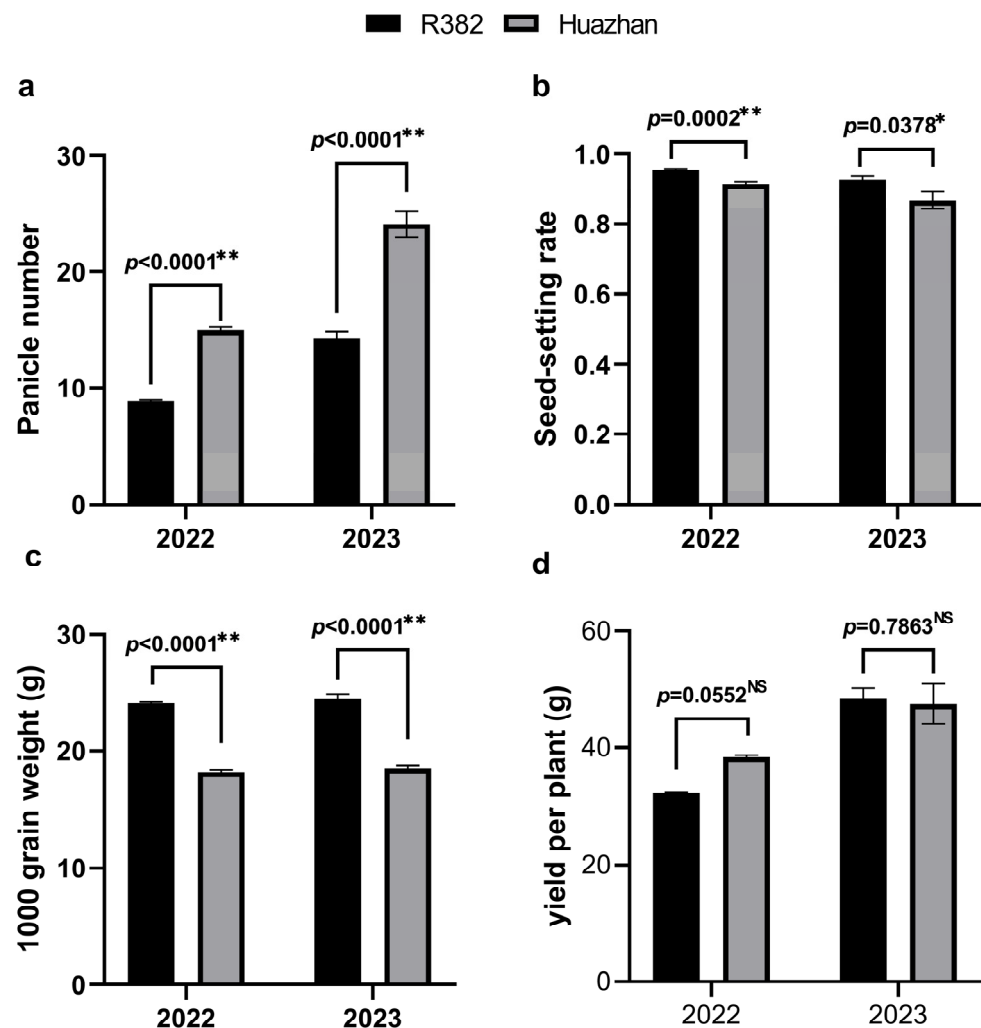


Figure 8. Comparison between R382 and Huazhan on (a) the number of panicles per plant, (b) the seed setting rate, (c) 1000-grain weight, (d) yield per plant in 2022 and 2023. Values are shown as means $\pm$ SE of 12 replicates. NS, no significant, * $p < 0.05$, ** $p < 0.01$ (compared with Huazhan; Student's *t*-test).

4. Discussion

Rice is one of the most important crops in the world. China is both the largest consumer and producer of rice worldwide; more than 65% of Chinese households consume rice as the staple source in their diet, and the nation produces more than 28% of the total global rice supply [58]. The rice production of China in 2023 is about 206.6 Mt (https://www.stats.gov.cn/sj/sjtd/202401/t20240118_1946695.html (accessed on 10 June 2024)). Hybrid rice technology increases yield dramatically, thus contributing significantly to food security [59]. However, the breeding of hybrid rice, especially super hybrid rice, requires an even higher yield while preventing rice from lodging due to excessive grain weight during the mature stage. Although the introduction of a *semi dwarf* gene (*sd1*) via the Green Revolution has led to a decrease in plant height and greatly reduced the risk of lodging, the goal of higher yields requires more stem and leaf growth to provide energy sources [4,60]. Meanwhile, in order to save costs and improve efficiency, there is an urgent need to develop complete mechanized rice production [59]. Compared to traditional transplanting, hybrid rice under a mechanical direct seeding pattern is more prone to lodging [32]. Mechanical harvesting also requires a stronger resistance to lodging,

otherwise the efficiency of mechanical harvesting will be reduced or even impossible to carry out [61]. Therefore, the breeding of lodging-resistant hybrid rice varieties is highly desirable. Backbone parents are the foundation to successful hybrid rice breeding. In the process of hybrid rice utilization in China, the restorer line Huazhan is well known for its superiority in yield traits. There have been 111 hybrid combinations (including two-line and three-line) and 15 derived restorer lines have been bred using Huazhan as the male parent [62]. These rice materials derived from Huazhan are popular in the market, but Huazhan's resistance to lodging needs to be further improved.

In this study, it was identified that although the newly created restorer line R382 has higher plant height and fresh weight above-ground, its lodging index is significantly lower mainly due to stronger stem strength compared to Huazhan (Figures 1, 4 and 6, Supplemental Table S1). It is certainly uncontroversial that the main parameter characterizing stem strength, bending moment at breaking (M), is positively correlated with the outer diameter of the basal stem, culm thickness, sectional modulus, bending stress, and the content of the various chemical constituents that make up the cell wall (Figures 6, S2 and S3), since all of these traits will result in enhanced stalk strength [19,26,41,58]. Our results are similar to those of the previous study on the effect of nitrogen fertilizer application, in which it was found that an increase in nitrogen application led to a reduction in culm thickness, and changes in the number and area of vascular bundles, resulting in less lignin and cellulose deposition, an increase in the hollow area of the stem, and a higher likelihood of lodging in rice [20]. However, our study differs from previous cultivation studies in that our focus is on the differences between rice varieties, with the long-term goal of resolving the molecular genetic mechanisms of lodging resistance of restorer lines used in hybrid rice production, in order to breed germplasm resources that stably maintain strong resistance to lodging. Therefore, we further conducted a series of gene expression dynamics analyses related to cell wall component metabolism, sugar transport, and secondary cell wall synthesis during the jointing stage of the two cultivars (Figure 7), thus providing ideas for future genetic analyses.

The structural compounds in the cell wall play a decisive role in the mechanical strength of the stem, among which cellulose and lignin are not only the backbone support of the cell wall, but also promote growth and development [63–65]. Our results showed that the cellulose, lignin and hemicellulose contents of the basal internodes of R382 were higher than those of Huazhan, and the genes that led to more chemical components for the cell wall formation and increased accumulation of starch granules were expressed more highly in the basal stem of R382 (Figures 4 and 7). However, we are not aware of the detailed molecular mechanism underlying these differences, and future research needs to map the genes that lead to differences in lodging resistance between the two. This could be achieved through forward genetic research; for example, constructing a genetic population derived from the cross of R382 and Huazhan, and choosing extreme individual plants in the F₂ genetic population for BSA sequencing analysis to preliminarily map QTLs related to lodging resistance. The standard for choosing extreme individual plants can be bending stress or out diameters of the basal internodes, which are the two most-correlated indicators with M (Figures 6, S2 and S3), and their measurement methods are relatively simple and fast. The causal gene will be identified via fine-mapping through the construction of higher generation populations such as the nearly isogenic lines (NILs). In the future, through the functional studies of the target genes, it may be possible to reveal new molecular mechanisms underlying the regulation of lodging resistance.

A high yield is the primary goal of hybrid rice breeding, and therefore an important criterion for parental selection in hybrid rice [66,67]. The yield is mainly achieved through photosynthesis, which forms assimilates and then transfers nutrients to the panicle through vascular tissue for nutrient accumulation during grain filling [57,68]. However, the transport of assimilates to grains is affected by various factors [57,69,70]. At the late grain-filling stage, the accumulation of assimilates in the stem increases, thereby maintaining the upright position of the stem and improving its lodging resistance to cope with

the increased pressure burden on the spike [67]. This is confirmed by our experimental data, such as the basal stem of R382 having more assimilate derivatives such as starch granules and cellulose, lignin, and hemicellulose (Figures 3b and 4). The stem would use a lot of assimilates to enhance mechanical strength, and the accumulation of assimilates in grains would be negatively affected. Thus, there is usually an antagonistic relationship between crop yield and resistance to lodging stress, as both require nutrients and energy. However, R382 improved its lodging resistance while maintaining a yield equivalent to that of Huazhan (Figure 8). This indicated that the photosynthetic efficiency of R382 is high enough to simultaneously meet the needs of seed maturation and stem strength formation.

Along with theoretical research, the value of this study in breeding practice could be realized in two ways in the future. Firstly, multiple hybrid combinations can be produced using R382 as the male parent. Secondly, the construction of NILs in the background of Huazhan can improve the lodging resistance of Huazhan. Based on the DNA sequence differences in the target gene in the two restorers, molecular markers could be designed for the marker-assisted improvement of other rice materials.

5. Conclusions

In sum, although R382 was taller, with a higher fresh weight and a yield comparable to Huazhan, its stalks were more resistant to lodging because of the high mechanical strength of its basal internodes. The high stem mechanical strength was likely due to higher quantities of and larger vascular bundles as well as more starch granules in the culm. Furthermore, the elevated expression of genes contributing to the accumulation of cell wall components in R382 at the jointing stage may lead to more cellulose, lignin and hemicellulose in stems, which in turn resulted in higher bending stress and thus lodging resistance. Thus, this study identified a new restorer line with improved lodging resistance, which can be used as an excellent parent for hybrid rice.

In the future, further research is needed on the genetic mechanism of increased stem strength in R382, mapping genes, and designing molecular markers based on excellent genotypes in R382. R382 will be used as a donor to improve other restorer lines through marker assistant selection (MAS).

Supplementary Materials: The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/agronomy14061291/s1>, Figure S1: Daily highest and lowest temperature records of rice materials after transplantation in 2022 and 2023; Figure S2: Correlation matrix diagram showing the correlation coefficients among 13 traits of N2 internodes. The circle with greater and greater color intensity represents a greater absolute correlation coefficient value. PH, plant height; FWT2, fresh weight from the basal node of N2 to panicle tip; PBH, the proportion of the three basal sections to the overall height of the plant; DWS2, the outer diameter of N2 with leaf sheath; DWOS1, the diameter of N2 without leaf sheath; CT2, culm thickness of N2; SM2, section modulus of N2; LC2, lignin content of N2; CC2, cellulose content of N2; HC2, hemicellulose content of N2; BS2, bending stress of N2; M2, the bending moment at break of N2; LI2, lodging index calculated based on FWT2 and BS2; Figure S3: Principal component analysis (PCA) plot in the traits affecting lodging resistance of (a) N1 and (b) N2 internodes. Table S1: The growth period from sowing to maturation of R382 and Huazhan in 2022 and 2023; Table S2: Primers used in this study; Table S3: Traits related to lodging index of R382 and Huazhan.

Author Contributions: X.L. (Xiaoyi Liu), J.S. and J.X. carried out the experiment; X.L. (Xiaoyi Liu) also prepared the draft manuscript; G.Z. and H.D. helped experimental design and assisted in data analysis; W.T. conceived the idea and coordinated funding to support the study; X.L. (Xuedan Lu) designed the experiments, revised and finalized the manuscript. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Natural Science Foundation of China (No. 32100257), Hunan Science and Technology Innovation Program (No. 2021NK1011, 2021NK1001, 2021JC0007 and 2023NK1010).

Data Availability Statement: The original contributions presented in the study are included in the article/Supplementary Materials, further inquiries can be directed to the corresponding author.

Conflicts of Interest: The authors declare no conflicts of interest.

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