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Identification of the *CesA7* Gene Encodes Brittleness Mutation Derived from IR64 Variety and Breeding for Ruminant Feeding

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Abstract: Rice straw presents challenges as livestock feed due to its low digestibility and the presence of chemical residues. One potential solution is to focus on breeding brittle varieties that possess disease-resistance traits. In this study, AZ1803, a brittle mutant line isolated from the IR64 mutant pool, was chosen for gene identification and breeding. The AZ1803 mutant was crossed to the TNG67 variety to generate a mapping population and to the CS11 variety for fine mapping and breeding. The gene was mapped on chr. 10 between RM467 and RM171 SSR markers and was narrowed down to RM271 and RM5392 with 600 kb proximately interval. The AZ1803 and IR64 sequencing results revealed a substitution mutant in the Exon 9th of the *OsCesA7* gene, resulting in an amino acid mutation at the end of the transmembrane domain 5th of the CESA7, responsible for cellulose synthesis for the secondary cell wall. The cellulose content of AZ1803 was reduced by 25% compared with the IR64. A new brittle and disease-resistant variety was bred by using developed markers in marker-assisted selection. In addition, bending tests and bacterial blight inoculation were applied. The bacterial lesion length of the bred variety is 64% lower than that of AZ1803. The rice straw of the new variety can be used for livestock feeding, which increases farmer income and reduces pesticide residues and air pollution from straw burning.

Keywords: *Oryza sativa*; brittle culm; cell wall composition; breeding; bacterial blight

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1. Introduction

Rice is cultivated on more than 160 million ha worldwide, making it a staple crop of global importance [1]. In the same planting area, farmers typically cultivate similar rice varieties, a practice that leads to the spread of disease [2]. For disease control, pesticides are extensively used and result in chemical residues in the rice grain and straw [3]. Rice straw is a by-product that is produced in a similar amount to grain [4] and can be used to feed livestock. The rice straw of the brittle mutant (BM) showed significantly improved digestion compared with normal rice straw by in situ digestion [5]. However, its utilization may pose risks to both livestock and human health as well as to the environment because of the potential pesticide residues [6,7]. In addition, the process of disposing of rice straw is not easy. Due to its slow degradation, a significant amount of straw waste in paddy fields is burnt to prepare for the new crop season, resulting in air pollution and the release of greenhouse gases [4,8].

Brittle culm mutant shows easily breakable rice tissues, including culm, leaf, sheath, and node due to the reduced cell wall thickness [9–11]. Leaving brittle rice straw in the paddy field after harvesting can help avoid burning and increase organic matter. To increase farmer profit, farmers can sell brittle rice straw at higher prices for livestock due to its increased nutritional value and digestibility compared with normal straw [5]. The reduction of cell wall thickness and better digestibility were a result of an alteration

of cell wall composition in rice straw. The brittle culm mutant arises from mutations in genes associated with cell wall composition, specifically cellulose, which serves as the backbone of mechanical strength in the secondary cell wall structure [12]. Previously, brittle culm mutants were reported and many attributed to *OsCesA4*, *OsCesA7*, and *OsCesA9* (*cellulose synthase catalytic subunit*). Mutants that encode the *OsCesA4* gene include *bc7*(*t*) [13], *bc11* [14], *fc17* [15], and *bc19* [16], among others, while mutants that encode the *OsCesA9* gene are, for instance, *Bc6* [17], *bc88* [18], *bc13* [19], *S1-60* [20], *bc-s1* [21], *dwf1* [22], and *bc26* [23]. However, only *S1-24* and two *Tos17* inserted mutants encode the *OsCesA7* gene [11,24]. All brittle culm mutants showed reduced cellulose content compared with their wild types, while an increase in hemicellulose enhances the value of rice straw. Despite numerous reports on brittle culm mutants, there are only few commercially available brittle rice varieties, notably in China, such as Kefujing No. 7, Kecuijing No. 1, and Zhongcui A CMS line [25]. Previous reports have indicated that brittle rice mutants exhibit dwarfism, spread plant type, and sterility, among other traits [11,20,24].

Conversely, the reduction of cell wall thickness of the brittle culm mutants observed in brittle culm mutants may render them vulnerable to disease attacks, particularly bacterial blight (*Xanthomonas oryzae* pv. *oryzae*), which often penetrates through leaf wounds. [26]. Bacterial blight disease is a significant threat to rice crops and can lead to yield reductions of up to 50% [27]. While pesticide application is a common method to manage this disease, it poses health and financial concerns [28]. Hence, disease resistance is a crucial trait in rice breeding, as resistant varieties can help alleviate production costs. Although applied to a resistant gene, it is easy to break down the resistance [29,30]. An army of resistant genes within a variety helps extend the time before pathogens overcome the host plant's resistance, providing broader spectrum resistance. An efficient method to select many target genes as gene pyramiding is molecular breeding [31]. Numerous bacterial blight disease-resistant genes totaling at least 38 genes have been identified [32]. The pyramiding of *Xa* genes increases resistance against various races. For example, IRBB66, which carries five *Xa* genes (*Xa4*, *xa5*, *Xa7*, *xa13*, and *Xa21*), is commonly used as a resistant genes donor parent and is resistant to all races [33–35].

The brittle mutant line, AZ1803, was a mutant line obtained from the IR64 mutant pool generated by sodium azide (NaN_3) mutagenesis. However, as the AZ1803 mutant line exhibited a spread plant type, sterility, low yield, and no strong bacterial blight disease resistance, farmers are hesitant to cultivate it. Therefore, our studies aimed to eliminate the limitations of the AZ1803 mutant line by identifying its brittleness gene, developing the linkage markers for molecular marker-assisted selection, and eventually utilizing the developed linkage markers in the breeding program. The bacterial blight disease-resistant variety was selected, as the brittleness trait may increase the opportunity for infection. An SSR marker was used due to its simplicity and utility. Our breeding objectives aimed at producing the long-grain rice (*indica*) that is widely consumed worldwide. Additionally, given that the smallholders especially in Southeast Asia utilize the rice straw as livestock feed, our objective was also to introduce this new rice variety with the brittleness trait and enhanced digestibility properties. We aim to support farmers by providing pesticide-free rice straw, potentially suitable for livestock feed use.

2. Materials and Methods

2.1. Rice Materials and Cultivation

Forty-six F_2 individuals from AZ1803 and TNG67 crossing were used for coarse mapping. During the breeding processing, 155 of 600 F_2 individuals from AZ1803 and CS11 crossing were used for fine mapping. The breeding parent used was CS11 (Chang-Sen 11), the lab-bred variety which carries most of Tainan 11 (TN11) background and four bacterial blight disease-resistant genes from IRBB66.

All materials were planted in a paddy field in Wufeng District, Taichung City, Taiwan. The rice was planted by transplanting with two- to three-week-old seedlings in the paddy field, which was plowed three times before transplanting, as part of our regular opera-

tion. The fertilizer was applied four times as a foundation, first-, second-dressing, and panicle initiation fertilizer for a total of 125 kg of nitrogen, 75 kg of phosphorus, and 50 kg of potassium.

2.2. Genetic Mapping by SSR Markers and Software

The 73 simple sequence repeat (SSR) markers having a clear polymorphism between AZ1803 (*indica*) and TNG67 (*japonica*) were used for genotyping. The brittleness appearance of 46 F_2 individuals from AZ1803/TNG67 was investigated for phenotyping. The genotyping and phenotyping data were applied in MapDisto (version 2.0) [36] and MAPMAKER (version 3.0) [37] software for coarse mapping. The mapping was operated with the default setting. After obtaining the mapped region of the brittleness gene, 20 SSR markers within the mapped region were used to find polymorphic markers. Eight markers showed an obvious polymorphism between AZ1803 and CS11 (*japonica*). Therefore, the mapped region was narrowed down using the 155 F_2 individuals.

2.3. Gene Sequencing

Primers to clone the candidate gene were designed using the *OsCesA7* sequence of IR64 downloaded from the ROOTomics (https://rootomics.dna.affrc.go.jp/, accessed on 10 June 2022) by VectorNTI (version 8) [38] software. Three pairs of primers were designed (Table S1). The PCR reaction (30 cycles) was performed by a Veriti™ 96-Well Fast Thermal Cycler with a Takara Ex Taq kit. The PCR product was recovered by a DNA clean/extraction kit (GeneMark, GMbiolab Co., Ltd., Taichung City, Taiwan). The sequencing was carried out by a 3730xl DNA Analyzer. The sequencing of IR64 and AZ1803 was contig using ContigExpress function and compared using AlignX function in the VectorNTI software.

2.4. Cell Wall Composition Analysis

Rice straw at the maturity stage was collected and dried in a hot air oven at 70 °C for 1 h, followed by 55 °C until dry (approx. 48 h). The dried rice straw was separated into leaves only for the precision of analysis. The tissue was ground into powder and was sieved by 40 mesh (0.4 mm). Approximately 0.5 g (±0.0005) powder (n = 3) was filled in the filter bag (F57, Ankom technology, New York, NY, USA). The filter bag was placed into the analyzer machine (Ankom fiber analyzer A200, Ankom technology, New York, NY, USA) to perform the neutral detergent fiber (NDF) analysis for the weight of all cellulose, hemicellulose, lignin, and ash; then, acid detergent fiber (ADF) analysis was performed for the weight remaining of cellulose, lignin, and ash. Thereafter, the cellulose was washed with acid detergent lignin (ADL) for lignin and ash weight. Finally, the sample after ADL analysis was burnt in a furnace at 550 °C for 8 h for ash weight [39].

2.5. Investigation of Brittleness Traits

The brittleness appearance was investigated at 45 (tillering stage) days after transplanting by the bending method. The breaking force of the fresh flag leaf (n = 9) at the maturity stage was measured by using an iDealTA texture analyzer (Horn Instruments Co., Ltd., Taoyuan City, Taiwan) with a cut leaf blade [40]. The highest force required to break the tissue was utilized. The dissection for secondary cell wall thickness was investigated by histological technique [41]. The tissue was fixed in the FAA (10% of formaldehyde, 5% of acetic acid, 50% of alcohol, and 35% of double-distilled water) fixing solution. The tissue in the FAA solution was vacuumed until the tissues sank into the solution and no bubbles emerged from the tissue. The tissue was dehydrated by soaking in a series of different EtOH concentrations (i.e., 30%, 50%, 70%, 85%, 95%, and 99.9% EtOH). Thereafter, the tissue was soaked in a series of different xylene concentrations (EtOH: xylene (2:1, v/v), (1:1, v/v), (1:1, v/v), and pure xylene). For paraffin infiltration, the tissue was soaked in a series of different paraffin concentrations (i.e., xylene: paraffin (2:1, v/v), (1:1, v/v), (1:2, v/v), and pure paraffin) under 60 °C condition. The tissue was soaked in paraffin overnight and then solidified paraffin in a block at room temperature. The tissue was sectioned by

microtome and placed on a slide. The tissue on the slide was washed with xylene and rehydrated by a series of different EtOH concentrations (i.e., 99.9%, 95%, 70%, 50%, 30%, and 0% EtOH). After staining, the tissue was observed under 400× by the light microscope.

2.6. Evaluation of Morphological Traits

The morphological traits were investigated following the standard evaluation system (SES) guideline from the International Rice Research Institute (IRRI). The qualitative traits were investigated using a single plant, while the quantitative traits were investigated by twenty plants.

2.7. Inoculation of Bacterial Blight and Resistance Investigation

At 45 days after transplanting (the maximum tillering stage), the rice leaves were inoculated with bacterial blight *Xanthomonas oryzae* pv. *oryzae* (Xoof2 and XF89b strains) using the leaf clipping method [42]. The field condition was logged with 3 cm of water. The success of inoculation was confirmed by the presence of bacterial ooze under a light microscope on the leaf three days after the inoculation. The lesion length was investigated at 21 days after inoculation. The lesion length was determined by measuring the distance from the end of the cut leaf to the furthest point of infection, characterized by yellowing or dryness. The lesion length was an average of five leaves from five plants.

2.8. Statistic Analysis

Student *t*-test was calculated using "*t*-test: Two-Sample Assuming Equal Variances" in Microsoft Excel to compare the mean of two samples.

3. Results

3.1. Characteristics of AZ1803 Brittle Mutant Line from IR64 Mutant Pool

AZ1803 was a brittle mutant line screened from the IR64 mutant pool mutagenized by sodium azide (NaN_3) using finger bending (Figure 1a). Moreover, the tissues including, leaf blade, leaf sheath, and panicle were brittle. The sclerenchyma cells of the culm in AZ1803 and its wild type (IR64) were observed under a light microscope. AZ1803 was found to have a thinner cell wall compared with the wild type (Figure 1b,c). The force required to break the flag leaf was measured using an iDealTA texture analyzer with a rounded knife edge. The force required to break the AZ1803 flag leaf was significantly lower than that required for the wild type (Figure 1d). Using NDF, ADF, and ADL analysis, it was observed that cell wall compositions of AZ1803 underwent changes compared with the wild type. Specifically, cellulose decreased by 25%, while hemicellulose increased by 16% (Figure 1e). These results indicated that the AZ1803 brittle mutant line altered its cell wall composition in its secondary cell wall and cell wall thickness, resulting in the loss of their mechanical strength. On the other hand, the morphological traits of AZ1803 were different from the wild type. For instance, the single plant grain yield and fertility percentage of AZ1803 were lower than IR64 by 51% and 56%, respectively (Figure 1f,g). Moreover, leaf and culm length of AZ1803 decreased when compared with the wild type (Table S2). These results indicated that the traits may be an infliction from brittleness or mutation in other genes that control morphological traits simultaneously.

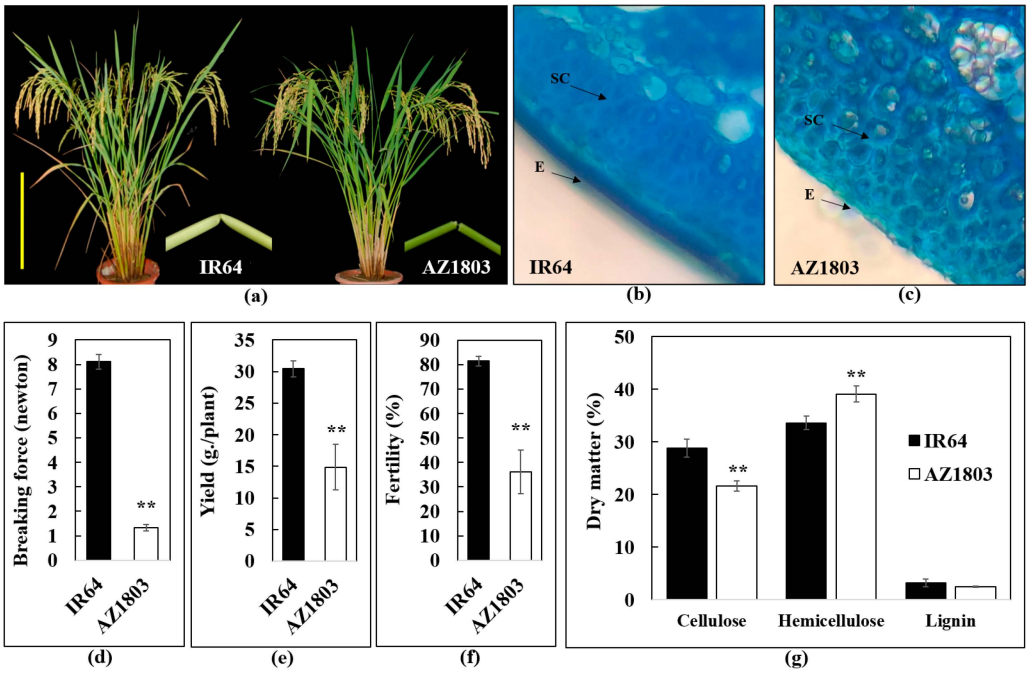

Figure 1. Characterization of AZ1803 mutant line compared with the IR64 (wild type). (**a**) Plant architecture and brittleness after finger bending in AZ1803 and its wild type. (**b**,**c**) Cell wall thickness of sclerenchyma cells of the culm in AZ1803 and its wild type. E; epidermis, S; sclerenchyma. (**d**) Breaking force of fresh flag leaf in AZ1803 and its wild type. (**e**,**f**) Yield and percentage of fertility of AZ1803 and its wild type. (**g**) Cell wall compositions of the dried leaf blade in AZ1803 and its wild type. ** $p < 0.01$, Student *t*-test.

3.2. Identification of Brittleness Gene in AZ1803 Mutant Line

Mapping and identification of the brittleness gene in AZ1803 were employed to develop molecular markers for the brittle rice breeding program. The AZ1803 mutant line (*indica* background) was crossed by TNG67 (*japonica*) to generate a high segregation F_2 population. The F_1 plant was non-brittle, but F_2 plants showed a non-brittle and brittle ratio of 3:1. Therefore, the brittleness gene of AZ1803 was controlled by a single recessive gene. To rapid the coarse mapping process, only 46 F_2 individuals were genotyped with 73 polymorphic SSR markers, which were distributed on 12 rice chromosomes. The genotype and phenotype data of 45 F_2 and their parent were applied for gene mapping using MapDisto software and MAPMAKER. The brittleness gene of AZ1803 was mapped between RM467 and RM171 markers on chr. 10 within an approximate 5.5 Mb interval (Figure 2a). Another 600 F_2 individuals from the breeding population derived from AZ1803 crossed to CS11 (*japonica*) were applied to narrow down the region with more density of molecular markers. The recombinants of polymorphic markers were observed in 155 brittle F_2 individuals. The results showed that the brittleness gene was located between RM271 and RM5392 (~600 kb region) (Figure 2b). The Q-TARO database (QTL Annotation Rice Online, http://qtaro.abr.affrc.go.jp/, accessed on 10 June 2022) was used to find the candidate gene within the region. Among them, the *Cellulose synthase A catalytic subunit 7* (*CesA7*, LOC_Os10g32980), which had been reported as a brittle culm mutant gene, was found. Therefore, the *CesA7* gene in AZ1803 was sequenced by PCR cloning to compare with IR64. A nucleotide substitution was found at exon ninth (at 3879 bp), resulting in an amino acid at 931 changed from Gly to Asp (Figure 2c). In the protein structure, the mutation point is located at the end of the fifth transmembrane domain (Figure 2d). Moreover, the position of previous mutations of this gene also were labeled.

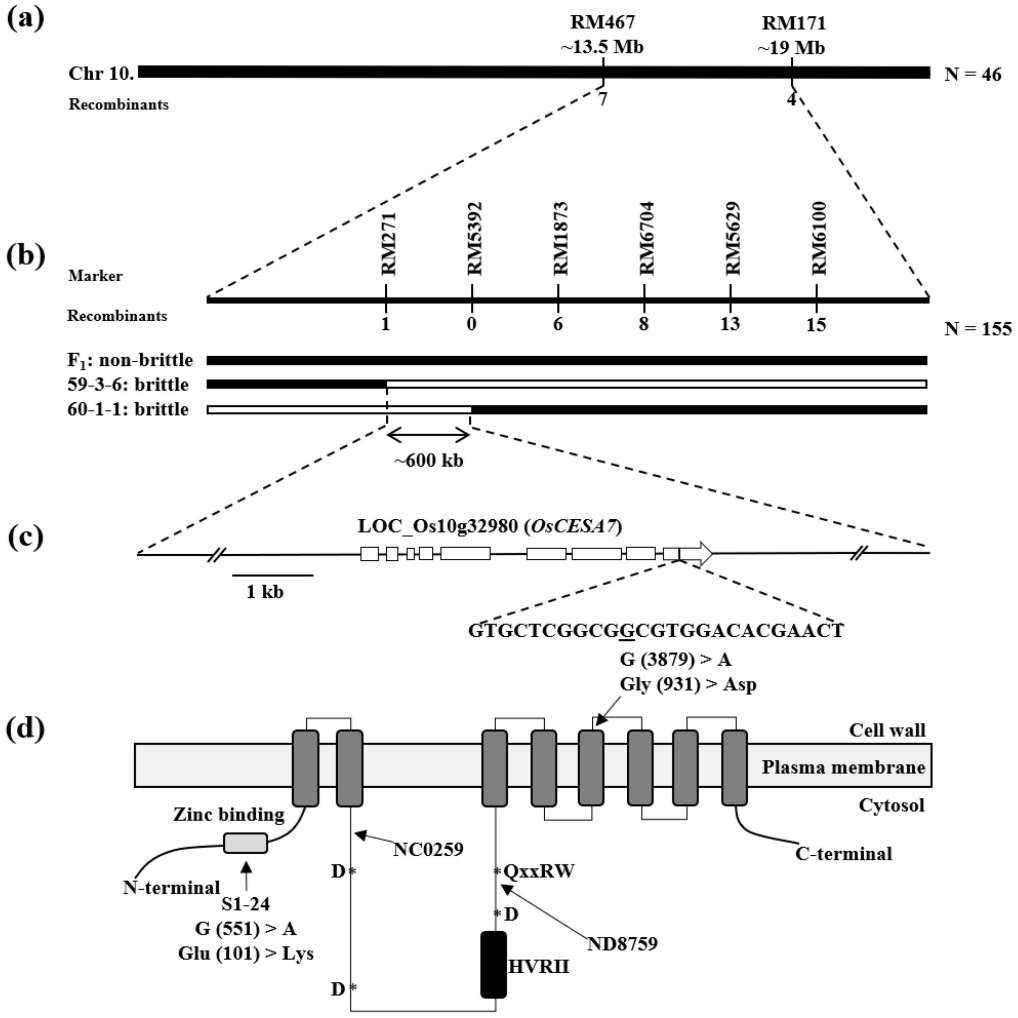

Figure 2. Molecular characterization of the brittle mutant gene of AZ1803. (**a**) The brittle mutant gene was mapped on the chr. 10 between RM467 and RM171. (**b**) Fine mapping narrowed down the mutant gene between RM271 and RM5392 with a region of 600 kb proximately. (**c**) Within the region, the LOC_Os10g32980 gene encoding the *Cellulose synthase A catalytic subunit 7* (*CesA7*) was cloned and sequenced. A SNP (G to A) was found at 3879 bp, resulting in changing an amino acid (Gly to Asp) at 931 aa. The mutation position located at the end of fifth transmembrane domain. (**d**) The positions of mutation points (in this study and previous reports) were drawn on the protein structure. "*" in CESA protein structure indicated positions of Asp (D) residue and QXXRW motif [11,24].

3.3. Marker-Assisted Breeding for Brittleness and Bacterial Blight Resistance

AZ1803 is a mutant line that exhibits certain inferior traits, such as low yield caused by low fertility, open plant type which makes harvesting using the combiner more difficult, and moderate susceptibility to bacterial blight disease (*Xanthomonas oryzae* pv. *oryzae*), a significant concern in rice production. Therefore, the AZ1803 was crossed to the CS11 variety, which carries four bacterial blight disease-resistant genes (*xa5*, *Xa7*, *xa13*, and *Xa21*), close plant type, and high fertility (Figure 3a). In the F_2 generation, the linkage markers RM271 and RM5392 were applied to select the brittleness gene (*AZ1803*) on chr. 10 (Figure 4) together with the phenotyping by the finger bending. For the *Xa* genes selection, the linkage markers were used together with inoculation in the paddy field to ensure the resistance. SSR markers RM153 and RM611 were used to screen *xa5* on chr. 5, RM20582 and RM3138 were applied to select *Xa7* on chr. 6, RM149 and RM3761 were used to genotyping *xa13* on chr. 8, and RM16914 and PTA248 were employed to observe *Xa21* on chr. 11 (Table S3). In addition, every individual was inoculated by Xoof2 and XF89b strains of bac-

terial blight using the leaf clipping method. The 99 F_2 plants from 600 total F_2 population, which carry the *AZ1803* gene in homozygous from AZ1803 and homozygous or heterozygous *Xa* genes from CS11, were selected using the SSR markers in marker-assisted selection (MAS). In the F_3 generation, the 66 plants that carried the homozygous target alleles of *AZ1803*, *xa5*, *Xa7*, *xa13*, and/or *Xa21* and with obviously brittle and bacterial blight resistance, were selected. Although using MAS, at least three resistant genes were enough for strong resistance.

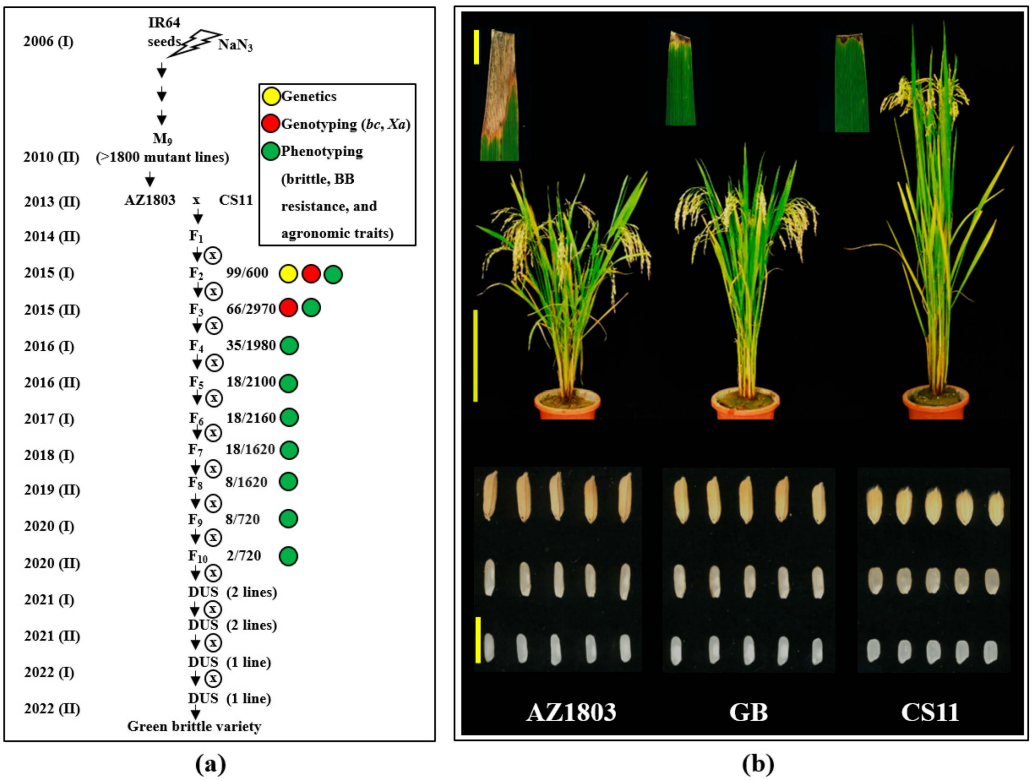

Figure 3. Breeding scheme of green brittle (GB) variety. (**a**) Flowchart of breeding for brittleness and bacterial blight disease resistance. The brittle mutant line, AZ1803, isolated from the IR64 mutant pool was used as the maternal donor of the brittleness gene. The CS11 variety was used as a paternal donor of the bacterial blight disease-resistant genes. The progeny was selected for the target genes using MAS. After the yield trial, a bred line was named as the "green brittle" (GB) variety. (**b**) Architecture, bacterial disease lesion, and grain appearance of green brittle compared with its parents.

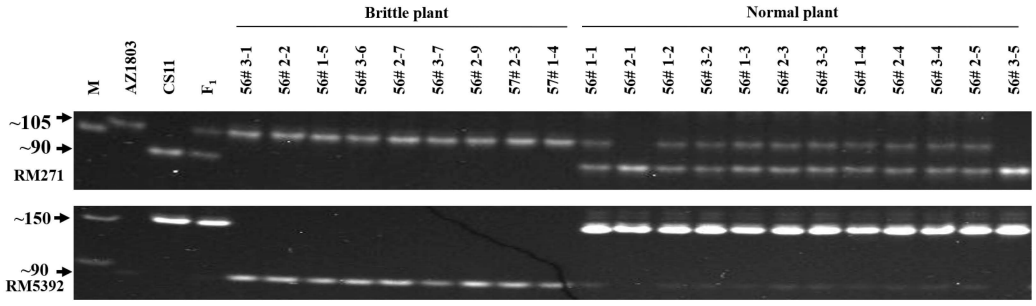

Figure 4. Genotyping of 21 F_2 individuals from AZ1803 and CS11 crossing. RM271 showed the genotype of brittle F_2 progeny similar to AZ1803 (~105 bp). RM5392 showed the genotype of brittle F_2 progeny similar to AZ1803 (~90 bp).

3.4. Agronomic Traits between Brittle Bred Line, Its Parent, and Check Line

During the traits fixing (F_4 to F_6), plants exhibiting desirable morphological traits including erect plant type, well-exerted panicle without awns to facilitate harvesting, high fertility rate, and strong resistance to bacterial blight to enhance yield potential, and long grains similar to *indica* type, were harvested. At the F_6 generation, there were 18 bred lines (1# to 18#) having satisfied traits. At the F_{10} generation, two bred lines (8# and 13#) were selected for distinctness, uniformity, and stability (DUS) testing. Both bred lines were planted on a large scale for two years (i.e., four crop seasons) (Figure 3a). The 13# bred line showed more stable traits and yield trials. The bred line showed long grain as indica like AZ1803 (Figure 3b). Therefore, the line was named "Green brittle" (GB, Lù-Cuì) variety. The yield trail of the GB variety was improved from the brittle donor parent (AZ1803) by 88%. The filled grain number and percentage of fertility were improved (80% and 77%, respectively) by the reducing of sterile spikelet number (43%) when compared with the AZ1803. The bacterial blight disease lesion length of the GB variety decreased by 64% of the AZ1803, as short as the CS11-resistant parent (Table 1).

Table 1. Comparison of agronomic traits and genotypes of green brittle (GB) and its parents, AZ1803 and CS11.

Line	Grain Yield (g/Plant)	Filled Grain No./Plant	Sterile Spikelet no./Plant	Fertility (%)	BB Length (cm)	Resistant Genes
AZ1803	14.88 ± 3.59	579.33 ± 26.10	1022.67 ± 33.56	36.16	2.59 ± 0.60	-
CS11	30.79 ± 2.68	1074.00 ± 28.28	300.00 ± 171.10	78.16	0.53 ± 0.14	*xa5*, *Xa7*, *xa13*, *Xa21*
GB	28.02 ± 1.30	1042.00 ± 78.25	585.33 ± 185.60	64.03	0.94 ± 0.44	*xa5*, *xa13*, *Xa21*

4. Discussion

4.1. AZ1803 Brittle Mutant Line from IR64 NaN_3- Generated Mutation Pool

To expand diversity, mutagenesis can generate desirable traits. Despite the fact that the most common mutagenesis is ethylmethanesulfonate (EMS) [43,44], sodium azide (NaN_3) mutagenesis is also wildly used in rice [45–48]. In this study, a brittle mutant line was isolated from the IR64 mutant pool mutagenized by NaN_3. A variety of mutagenesis have been reported to generate brittle culm mutants in rice but not NaN_3 mutagenesis. NaN_3 has been reported to generate mutant lines that are resistant to blast disease [48] and contain high iron and zinc contents [49]. The AZ1803 mutant line showed a decline in breaking force as well as in decreases in cellulose and cell wall thickness, and, in addition, changes in morphological traits, plant height, and fertility. These phenotypes were similar to previous reports that those were pleiotropic of brittle mutants [11,18,20,50]. However, those traits can be discarded during the breeding, indicating that the trait might not be pleiotropic. Moreover, there were many brittle culm mutants that did not show any penalty trait [16,17,19].

The brittle rice straw is beneficial for livestock. The good roughage showed high NDF (content cellulose, hemicellulose, and lignin) [51], but a low ADF (content cellulose and lignin) value [52]. In addition, cellulose is harder to digest than hemicellulose and even lignin interrupts the digestibility in rumen [53]. Therefore, AZ1803 had lower cellulose and lignin than IR64 (low ADF), but a similar high total composition (NDF) indicated that the brittle rice straw (AZ1803) is beneficial to livestock [5,54].

4.2. A point Mutation Induces a Missense of Cellulose Synthase Catalytic Subunit

To date, understanding of cell wall synthesis is still lacking. Despite technological advancements, the availability and diversity of materials are very important [55–58]. Brittle culm mutants serve as ideal materials to gain a better understanding of the mechanical strength of plants and cell walls. Changes in cell wall composition reflect the function of the corresponding gene. Cellulose, one of the cell wall compositions, was synthesized by the CESA protein [11,14,20]. The CESA protein has a zinc finger domain, transmem-

brane domain, plant-conserved region, conserved D, D, D, QXXRW residues (motif), and class-specific region [16]. Only CESA4, CESA7, and CESA9 were reported to synthesize cellulose for secondary cell walls in rice. After gene identification of AZ1803, a missense mutation was found in the transmembrane domain of the CESA7 protein, whereas the *S1-24* brittle culm mutant changes an amino acid on the zinc finger domain [11]. The *bc11* and *S1-60* mutants were found in a mutation in the transmembrane domain of the CESA protein [14,20]. The same protein, but different traits, appear and may rely on the mutation site. Some mutants showed dwarfism, dry leaf tip, and spread plant type up to drooping [11,23,24]. The *Bc6* and *Bc19* mutant showed a missense mutation in the region that was predicted to be a cellulose synthesis site [16,17]. The comprehension of CESA protein and its domain functions was better.

4.3. Breeding of Brittle Rice Variety Using MAS

The brittleness observed in AZ1803 is not a genetic modification (GM) which can be released directly; however, the mutant line with some poor traits may fail to convince farmers [59]. For instance, based on our knowledge, the open plant-type characteristic of the brittle mutant can cause difficulties during harvesting using the combiner. In addition, farmers prefer high yield and a disease-resistant variety to ensure a stable income. Therefore, to introduce the bacteria blight disease resistance into the brittleness trait, the AZ1803 was crossed to CS11. The brittle gene was located on chromosome 10 close to the RM271 and RM5392 SSR markers, similar to the *fp2* brittle mutant. The markers were designed to select the brittleness gene in AZ1803 [60]. The GB variety increased hemicellulose by 12.75%, but decreased cellulose by 23.82%, similar to the change of the AZ1803 donor parent. To select bacterial blight disease-resistant genes (*xa5*, *Xa7*, *xa13*, and *Xa21*), linkage SSR markers were used. At least, three *Xa* resistant genes were strong enough to tolerate many races [31,35]. The effectiveness of the *Xa* genes selection through MAS is similar to the Jalmagna variety and NIL-IRBB lines which contain *xa5*, *xa13*, and *Xa21* and showed high levels of resistance against the bacteria blight disease [61,62]. In addition to genotyping, phenotyping was performed together to ensure the desirable resistance in breeding lines. The breeding of brittle rice makes the plant weaker than normal rice. Therefore, selection of the agronomic traits is also important. Agronomic traits such as high yield, plant type, and tiller number were also selected. Finally, the new brittle rice variety with bacterial blight disease resistance is bred and available for sustainable agriculture.

5. Conclusions

The green brittle rice variety is more suitable to feed the livestock because of its high nutrition value from brittleness and health from disease resistance. This superior variety was bred by marker-assisted selection using SSR markers. We were successful in identifying the brittleness gene in the AZ1803 and developed linkage markers. Using flanking markers and phenotypic selection, the brittle and three bacterial blight disease-resistant genes were selected efficiently. This pyramided variety must have the potential for ruminal animal feeding and sustainable agriculture.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14050706/s1, Table S1: Primers for the amplification of the *OsCesA7* gene; Table S2: Agronomic traits of the green brittle (GB) variety, its parents (AZ1803 and CS11), and check line (IR64) in 2021 first crop season; Table S3: The linkage SSR markers for the selection of *AZ1803* and *Xa* gene selection.

Author Contributions: A.S.: investigation, formal analysis, writing—original draft, writing—review and editing. T.-H.T. and W.-C.L.: investigation. C.-S.W.: conceptualization, supervision, resources, writing—review and editing. All authors have read and agreed to the published version of the manuscript.

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