Elimination of an unfavorable allele conferring pod shattering in an elite soybean cultivar by CRISPR/Cas9

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Abstract Pod shattering can lead to devastating yield loss of soybean and has been a negatively selected trait in soybean domestication and breeding. Nevertheless, a significant portion of soybean cultivars are still pod shattering-susceptible, limiting their regional and climatic adaptabilities. Here we performed genetic diagnosis on the shattering-susceptible trait of a national registered cultivar, Huachun6 (HC6), and found that HC6 carries the susceptible genotype of a candidate Pod dehiscence 1 (PDH1) gene, which exists in a significant portion of soybean cultivars. We next performed genome editing on PDH1 gene by clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein 9 (Cas9). In T2 progenies, several transgene-free lines with pdh1 mutations were characterized without affecting major agronomic traits. The pdh1 mutation significantly improved the pod shattering resistance which is associated with aberrant lignin distribution in inner sclerenchyma. Our work demonstrated that precision breeding by genome editing on PDH1 holds great potential for precisely improving pod shattering resistance and adaptability of soybean cultivars.

Keywords Genome editing, Pod shattering, Soybean, Precision breeding, Marker assisted selection, CRISPR/Cas9

Dear Editor,

Conventional breeding has been playing a fundamental role in crop improvement in the past century. Nevertheless, incorporating beneficial genetic variations while excluding unfavorable alleles remains a major challenge, impacted by recombination rates, population size, allelic variations, and effectiveness of phenotypic selection (Lyzenga et al. 2021). As a result, an elite cultivar may take 5–10 years to develop, yet still carry substantial unfavorable allelic variations. Genome editing technologies, particularly clustered regularly interspaced short palindromic repeats (CRISPR)/associated (Cas) nucleases (CRISPR/Cas), can facilitate knock-out, knock-in, and base-editing of target genes, opening up new possibilities to precise improvement of crops (Cai et al. 2020; Chen et al. 2019; Li et al. 2020; Tang et al. 2019; Wang et al. 2020b). The development of CRISPR/Cas-based gene editing has created an avenue for creation of favorable alleles or elimination of maladapted genetic variations in germplasm, before or after the breeding cycle (Lyzenga et al. 2021).

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Pod shattering has been an unfavorable trait in soybean (*Glycine max*) breeding, yet is inapparent in humid climate. As a result, a significant portion of soybean cultivars and landraces are still pod shattering-susceptible in areas with higher humidity (Zhang and Singh 2020). The adaptability of shattering-susceptible varieties is severely limited by local climates and is not suitable for introduction to arid regions. For instance, Huachun 6 (HC6) is a national registered cultivar in south China featuring good yield performance and high protein content, yet is shattering-susceptible. The manual harvesting practice and high humidity in south China helped avoid shattering, so yield performance of HC6 is not significantly affected (Fig. 1A). However, in HuangHuaIai (HHH) region where HC6 can be adapted as summer sowing variety, the low humidity and machine harvest can cause severe yield losses of HC6 (Fig. 1B), limiting its regional adaptability.

To diagnose the genetic basis of pod shattering susceptibility in HC6, we performed QTL mapping with a recombinant inbred line (RIL) population of HC6 and pod shattering-resistant JD12. A reproducible major QTL controlling shattering resistance was mapped to chromosome 16, which overlapped with the previously reported qPDH1 QTL (Fig. 1C). The putative PDH1 gene was proposed as *Glyma16g25580* (Wm82.a1.v1) encoding a dirigent (DIR) family protein expressed in the inner sclerenchyma of pod walls in shattering susceptible varieties (Funatsuki et al. 2014). We sequenced *Glyma16g25580* and found a SNP in JD12 (chr16-29944393, A/T (HC6/JD12); Wm82.a2.v1) leading to a nonsense variant, consistent with a previous report that the presence of *pdh1* gene is highly associated with curling of pod walls (Funatsuki et al. 2014). According to the nonsense SNPs A-T, we surveyed the haplotypes of PDH1 gene among resequencing data from 1080 soybean cultivars. The proposed shattering-resistant H-T haplotype is largely fixed in regions with low relative humidity and mechanic harvesting, including 94.50% in northeast China and 85.17% in HHH region (Fig. 1D). In contrast, the shattering-susceptible H-A haplotype is retained in areas with relatively high humidity and/or manual harvesting, including 59.72% of south China, 83.64% of Japan, 81.51% of Korea, and 65.71% of southeast Asia cultivars (Fig. 1D). This result suggested that the presence of *Glyma16g25580* gene is highly associated with the relative humidity and harvesting mode shaped pod shattering tract.

Genome editing technologies, particularly clustered regularly interspaced short palindromic repeats (CRISPR)/associated (Cas) nucleases (CRISPR/Cas), can facilitate knock-out, knock-in, and base-editing of target genes, creating an avenue for elimination of maladapted genetic variations in germplasm (Cai et al. 2020; Chen et al. 2019; Li et al. 2020; Tang et al. 2019; Wang et al. 2020b). The *Glyma16g25580* (designated PDH1 thereafter) open reading frame is mainly responsible for the pod shattering susceptible trait. We then sought to generate mutation of PDH1 by CRISPR/Cas9 in HC6. We designed three sgRNAs, cloned into pGES701 vector individually, and pooled for Agrobacterium-mediated transformation (Fig. 1E–F). Among 23 T₀ transgenic plants, 17 lines contained sgRNA, 5 lines contained 2 sgRNAs, and 1 contained all sgRNAs. Hi-TOM (Liu et al. 2019) and sanger sequencing analysis showed that 10 of the 23 T₀ transgenic plants carried mutations in at least one target locus. In T₁ progenies, we characterized homozygous mutant plants from two lines, HC6*pdh1-5* with a 221 bp deletion and HC6*pdh1-9* with 1 bp deletion, respectively (Fig. 1G). qRT-PCR showed that the expression of PDH1 gene was diminished by homozygous mutations in both lines (Fig. 1H). In T₂ progenies, homozygous mutant plants without transgene were characterized in both lines (data not shown).

In a heat dried assay, HC6 pods displayed a high ratio of shattering (Ratio Pod Shattering = 63.75%, n = 8; Fig. 1I–J). In contrast, HC6*pdh1-5* and HC6*pdh1-9* exhibited significant resistance to pod shattering (RPS = 1.25%, n = 8; Fig. 1I–J). The dehisced pod walls of HC6*pdh1-5* and HC6*pdh1-9* exhibited much lower degrees of torsion than those of HC6, which is consistent with previous finding that qPDH1 locus was associated with curling of pod walls (Funatsuki et al. 2014). We then analyzed the anatomical characteristics of HC6*pdh1-5*, HC6*pdh1-9* and HC6 pods. We found that the lignin layer in inner sclerenchyma of HC6 tends to be thicker and looser. In contrast, the lignin layer in HC6*pdh1-5* and HC6*pdh1-9* appeared to be thinner and compact (Fig. 1L). This result demonstrated that genome editing of the PDH1 gene may affect the pod shattering resistance of HC6 by influencing the deposition of lignin layer in inner sclerenchyma. In 2021 summer, we performed a field trial at Shijiazhuang, Heibei, China. When harvesting was delayed for 2 weeks, HC6 exhibited significant pod shattering that caused substantial yield losses. In contrast, HC6*pdh1-5* and HC6*pdh1-9* barely showed shattering at the same condition (Fig. 1K). Meanwhile the genome editing of PDH1 did not significantly affect other agronomic traits, including plant height, branch number, pod number per plant, seed number per plant, 100-seed weight, and seed yield per plant (as determined before pod shattering occurs in HC6) (Fig. 1M).

Here we showcased the genetic diagnosis and "gene therapy" of the pod shattering trait of soybean by CRISPR/Cas9 genome editing. In comparison with
introduction breeding, genome editing approach could rapidly and precisely improve a trait, and is not limited by genetic diversity of breeding populations (Chen et al. 2019; Li et al. 2020; Manghwar et al. 2019). Therefore, genome editing can be integrated as a routine part of a breeding cycle to eliminate unfavorable alleles (such as PDH1) to facilitate the generation of a genetically superior cultivar.

MATERIALS AND METHODS

Genomic editing of soybean PDH1 by CRISPR/Cas9

CRISPR/Cas9 mutations of PDH1 in HC6 was performed using the protocol published previously (Bai et al. 2020), and a new CRISPR/Cas9 vector, pGES701 (Fig. 1E), was used for genome editing. The mixed Agrobacterium solution was transformed into the soybean cultivar HC6 via A. tumefaciens-mediated transformation, as described previously (Bai et al. 2020).

RNA extraction and real-time qPCR

To measure the expression of PDH1 gene in WT plants and mutants, real-time qPCR was performed using total RNA extracted from pod wall samples (3 weeks after flowering). Total RNA extraction, cDNA synthesis, and data analysis were performed as previously described (Wang et al. 2020a).

Evaluation of pod dehiscence percentage

The pod dehiscence percentage of WT and mutations was evaluated by heat treatment: ten fully matured pods of each plant were collected and kept in a circulation drier at 60 °C for 6 h and then counted the number of dehisced pods, respectively. Eighty plants per genotype were sampled. Fully matured pods of WT and mutations were examined for pod-wall lignification. Soybean pod was embedded in 7% agarose and cross-sections (80 μm thick) were stained with 10% toluidine blue, and observed under a microscope (Eclipse Ni-U, Nikon, Japan).

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Author contributions

YG designed research; ZZ, JW, HK, ZH, TK, PG, MB, SZ, XY, LC, HK, and SS performed experiments; ZZ, JW, HK, ZH, LY, and YG analyzed data; YG, LY, ZZ, and JW wrote the manuscript.

Declarations

Conflict of interest

The authors declare no conflicts of interest.

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