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Improving Seed Shattering Resistance in Wild *O. alta* Rice with Mesoporous Silica Nanoparticle Delivery Systems

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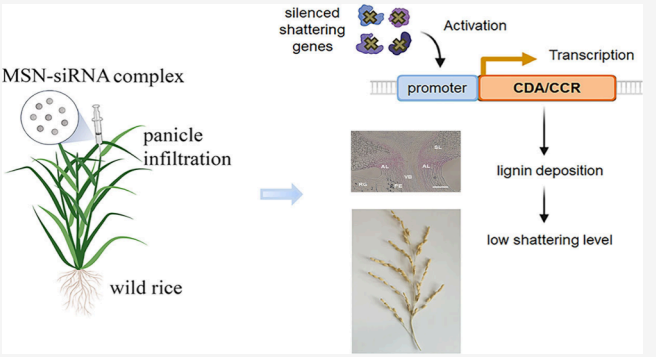
ABSTRACT: To address food security challenges and climate change, the polyploid wild rice *Oryza alta* has been explored as a potential crop, although it suffers from seed shattering. We employed mesoporous silica nanoparticles (MSNs) to deliver small interfering RNAs (siRNAs) for targeted gene silencing. Foliar spraying of MSN-siRNA complexes effectively delivered siRNA, resulting in up to 70% gene silencing of the *PDS* gene and 75% silencing of the transgenic *Ruby* gene. Additionally, MSN-siRNAs were infiltrated into the panicles of *O. alta* to target four seed shattering major genes every other day for 2 weeks until heading outdoors. This method silenced all four shattering genes ranging from 10.7% to 49.4% and significantly reduced the formation of the abscission layer between rice grains and pedicels, which enhanced pedicel tensile strength. Our MSN-siRNA system provides a flexible, nonpermanent approach to modifying crop traits, offering a promising tool for sustainable agricultural practices.



KEYWORDS: *MSN delivery, siRNA delivery, seed shattering, wild O. alta rice, gene modulation*

Climate change poses significant threats to crop productivity and global food security, creating an urgent need for sustainable agricultural practices.[1,2] The globally growing population further intensifies the demand for sustainable agriculture.[3] Rice (*Oryza sativa* L.), a staple food crop supporting approximately half of the global population,[4] is particulary vunerable to these challenges. The *Oryza* genus is diverse, including 11 genome types, six diploid (AA, BB, CC, EE, FF, GG) and five polyploid (BBCC, CCDD, HHJJ, HHKK, KKLL).[4,5] Among there, polyploid species like *Oryza alta* (*O. alta*) possess the CCDD genome and exhibit desirable traits like larger biomass (higher plant height and dry weight) as well as enhanced resistance to both abiotic and biotic stresses.[6−8]

A critical challenge in utilizing wild and weedy rice species like *O. alta* in agriculture is seed shattering—a natural process where seeds are dispersed when mature, a trait detrimental to yield.[9,10] The physical structure of shattering is the abscission layer (AL) in the pedicels, whose formation is influenced by environmental factors and phytohormone changes.[11] Seed shattering is regulated by genes as quantitative trait loci (QTLs), including major genes (MG) controlling AL formation and pleiotropic genes (PG) affecting the process.[3,12] In *O. alta*, four potentially principal QTLs named *SH01*−*SH04*—orthologue genes corresponding to alleles *SH4*, *qSH1*, *SH1*, and *SH5*—might be significant contributors to this trait in allotetraploid.[11,13−15]

Recent advances in genetic engineering offer potential solutions to mitigate seed shattering by genetic modification.[16,17] *Agrobacterium*-mediated gene knockouts and targeted mutations in genes such as *SH4* have successfully produced nonshattering phenotypes in rice cultivars.[11,18,19] However, these genetic interventions can affect other vital plant functions; for example, the knockout of *SH1* in African rice reduces lignin biosynthesis, crucial for plant structure and stress response.[20,21]

In response to these challenges, our research has pivoted to nanotechnology—an innovative approach associated with traditional genetic engineering.[22,23] Nanotechnology has shown promise in agriculture by enhancing resistance to salinity and drought,[24] improving carboxylation reactions and CO_2 uptake,[25] and mitigating heavy metal toxicity.[26] Moreover, nanotechnology-based gene delivery methods have been developed for plant genetic engineering.[27] Carbon nanocarriers

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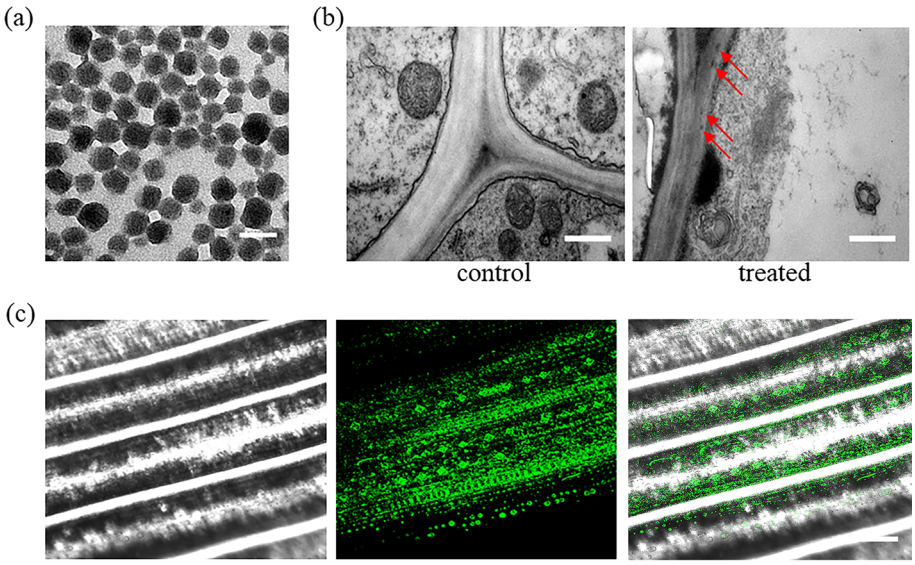

Figure 1. MSN-siRNAs delivery in wild rice leaves. (a) Representative TEM images of synthesized MSN. Scale bar: 60 nm. (b) Representative TEM images of wild rice leaves 24 h postspray with siRNA-functionalized MSN. Scale bars: 600 nm. (c) Representative confocal images of MSN delivery FAM modified scramble siRNA foliar sprayed on wild rice leaves. Scale bar: 150 μm.

of SWNT with siRNA (siRNA-SWNT) infiltrate leaves of *Nicotiana benthamiana* (*N. benthamiana*) that transiently knock down *mGFP5* gene as detected by qPCR.[28] And the methods provide a species-independent manner in *N. benthamiana*, arugula, wheat, and cotton without DNA integration.[29] Nanomaterials could address challenges of the CRISPR-Cas systems for plant genetic engineering through improvements in cargo delivery, species independence, and gene editing efficiency.[30]

Mesoporous silica nanoparticles (MSNs) have emerged as versatile tools for plant biotechnology. MSN was used as a fungicide by surface modification without delivery.[31] MSNs capped by gold nanoparticles can deliver DNA into intact plant cells by a gene gun system.[32] Cre protein was loaded inside the pores of gold-plated MSNs, and these particles were delivered by the biolistic method to maize cells.[33] In our preliminary studies, MSNs have been used for the targeted delivery of siRNAs to *N. benthamiana* by foliar spray, demonstrating their potential for transient gene silencing.[34] Building on these findings, we explored the use of MSNs to suppress key genes responsible for AL formation, thereby improving the shattering trait. Our approach involves applying MSN-loaded siRNAs via foliar sprays to verify the delivery system and panicle infiltration to transiently inhibit genes responsible for seed shattering. Our initial findings in wild rice have validated the effectiveness of this approach, successfully targeting and silencing multiple genes linked to seed shattering and preventing the formation of the AL of panicles at crucial developmental stages. This novel application of MSNs not only circumvents the drawbacks of permanent genetic modifications but also introduces a flexible, reversible tool for crop trait improvement, setting a new precedent in the field of agricultural biotechnology.

MSNs were synthesized by using a positively charged hexadecyl trimethylammonium bromide (CTAB) template and NH_4OH as reported in our previous work.[34] The morphology and particle sizes of the synthesized MSNs were analyzed with electron microscopy. Through transmission electron microscopy (TEM) (Figure 1a) and scanning electron microscopy (SEM) imaging (Figure S1a, Supporting Information), the 8-aminopyrene-1,3,6-trisulfonic acid trisodium salt (APTS) modified MSNs displayed a uniform size and spherical shape. The TEM images reveal that the mean particle diameters are about 30 nm. The size of MSN we used in wild *O. alta* rice had been verified to be effective in *N. benthamiana* in our previous work. The N_2 adsorption−desorption isotherms exhibit a steep adsorption behavior at P/P_0 around 0.35, known as a type IV isotherm (Figure S1b). The ζ potential value of APTS modified MSNs (17.1 mV) was much more positive than that of MSNs (−32.9 mV) as revealed in Figure S1c, showing abundant primary amines on the surface of MSNs-APTS. This ensures binding to negatively charged nucleic acids.

To confirm whether MSN enters the cells and the specific location, we conducted TEM and energy-dispersive X-ray (EDX) analysis on the ultrathin section of treated leaves. The TEM analysis showed that MSN particles, confirmed by the Si element in the EDX result (Figure S2), evenly distributed along and passing through the cell walls could be captured (Figure 1b). To evaluate the internalization and siRNA delivery efficiency of MSN in wild *O. alta* rice, we first assessed their penetration through the leaf's waxy surface via foliar spray. The MSN-siRNAs solutions (Supporting Information) were sprayed on leaves and sampled after 24 h. SEM analysis indicated that the wax layer on the surface of rice leaves did not show an observable change after treatment with MSN (Figure S3a). For further analysis of MSN internalization, we sprayed wild *O. alta* rice leaves with fluorescein isothiocyanate (FITC) labeled MSN and then performed confocal microscopy. FITC, serving as a fluorescent marker, was uniformly distributed along the veins (Figure S3b), which is attributed to the three-dimensional structure of the veins in the rice leaves. Subsequently, the leaves were treated with MSN loaded with fluorescein amidite (FAM) modified scramble siRNA. Fluorescence microscopy revealed that the FAM signal was distributed along the cell contours (Figure 1c), suggesting effective delivery and distribution within the leaf cells. These results suggest that MSN can effectively deliver siRNAs into the mature plant cells of wild *O. alta* rice.

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To evaluate the siRNA delivery efficiency of MSN, we targeted the phytoene desaturase (*PDS*) gene in wild rice and the *Ruby* transgene in transgenic wild *O. alta* rice. In wild rice, we foliar sprayed leaves with MSN encapsulating siRNA against *PDS* (MSN-siPDS) and a control group using MSN with scrambled siRNA. We collected the treated leaves and extracted total RNA 24 h after spraying to assess gene silencing efficiency via quantitative PCR (qPCR). The sampling time was confirmed by several genes in *O. alta* that target genes were silenced in 1 day and recovered to expression in 2 and 3 days by once treated and sequential 3 days sampling (Figure S4). The MSN-siPDS treated leaves showed a 70% reduction in *PDS* mRNA transcription levels compared to the controls including free-siPDS treatment (Figure 2a, Figure S5a).

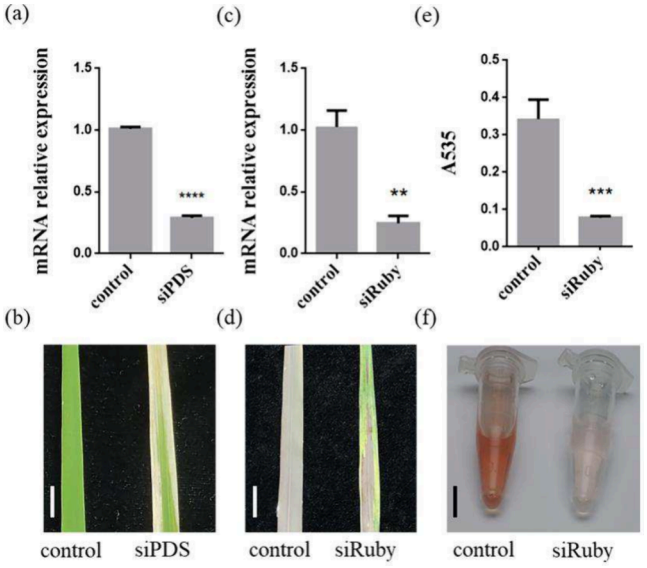

Figure 2. MSN mediated gene silencing by spraying on wild rice leaves. (a) PDS siRNA delivered to leaves by MSNs via spray. The PDS was silenced effectively compared with controls measured by qPCR. (b) Bleaching phenotype of rice leaves after MSN-siPDS sprayed 15 days. Scale bar: 2 cm. (c) qPCR analysis for Ruby mRNA fold changes at 24 h post spray. The mRNA expression level of Ruby was apparently silenced. (d) Phenotype of Ruby overexpression rice leaves after MSN-siRuby sprayed 3 days. The treated leaves turned to green from purplish red. Scale bar: 2 cm. (e) Betacyanin extracted from control and MSN-siRuby treated leaves. An absorbance at 535 nm was detected and the treated group showed a low content of betacyanin. (f) Betacyanin extracted from control and MSN-siRuby treated leaves. Scale bar: 0.8 cm. Data are means ± SD. Asterisks represent significant differences determined by Student's *t* test. $^{**}p < 0.01$. $^{***}p < 0.001$. $^{****}p < 0.0001$.

Continuous treatment every other day for 2 weeks led to visible photobleaching, with the leaf blade edges showing an albino appearance starting at about 12 days due to chlorophyll deficiency (Figure 2b, Figure S5b). The free-siRNA did not silence endogenous *PDS*. We hypothesize that the main reason might be environmental factors and the structure of the leaves. Strong sunlight exposure can easily shorten the half-life of siRNA. Additionally, the waxy covering of rice leaves may lead to the degradation of siRNA before it can penetrate the leaf. In the case of *Ruby* transgenic *O. alta* rice, developed through *Agrobacterium*-mediated transformation and expressing betacyanin, leaves were sprayed with an MSN-siRuby delivery system and a control (Figure S6a,b). Post-treatment RNA extraction and qPCR analysis 24 h later revealed a 75% reduction in *Ruby* mRNA levels (Figure 2c). Subsequent sprays led to a shift in leaf color from beet red to green within 5 days, directly observable and attributed to betacyanin reduction (Figure 2d). Quantitative analysis of betacyanin by the absorbance of betacyanin solutions at 535 nm confirmed a reduction to 22.8% of the control level (Figure 2e). Besides, the betacyanin solutions showed a macroscopic difference (Figure 2f). These experiments underscore the potential of MSN-siRNA delivery systems for targeted genetic interventions in crop species, demonstrating their efficacy in silencing both endogenous and transgenic genes.

Having established that MSN can penetrate the waxy layer of rice leaves and deliver siRNA effectively, we demonstrated that the MSN-siRNA delivery system can achieve long-term and multigene silencing in wild *O. alta* rice. This system specifically targets seed shattering, which is a critical trait for crop improvement (Figure 3a). The physiological basis of seed shattering involves the AL, which is composed of a small, dense cytoplasm and flat parenchyma cells at the junction of the sterile lemma and rudimentary glume. With shattering gene expression closely linked to AL development, our goal was to silence these genes before their mass upregulated expression in

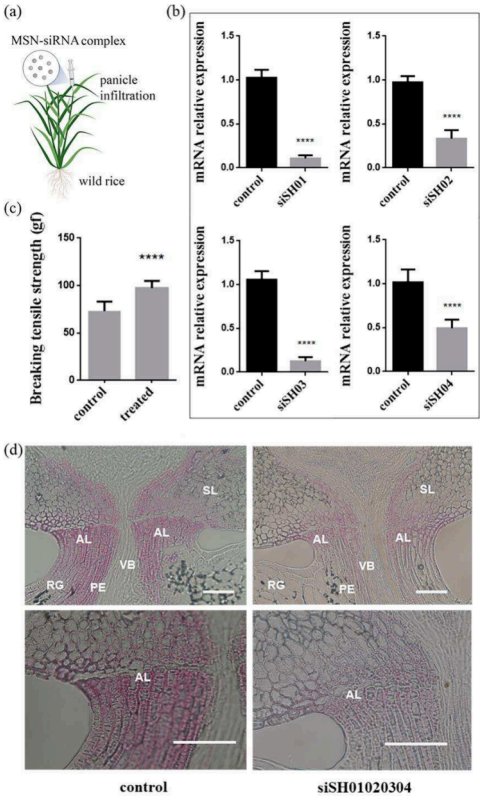

Figure 3. MSN-siRNAs targeted to multiple genes of shattering character of wild rice. (a) Schematic of MSN-siRNAs infiltrated into rice panicles. (b) All four shattering genes were apparently silenced by qPCR analysis in panicles. (c) The breaking tensile strength of control and MSN-si shattering rice grains disconnected from panicles. Data are means ± SD ($n > 15$ tillers). (d) Lignin of control and si shattering panicles stained with phloroglucinol. The bottom panels show enlarged images of boxed areas in top panels. Scale bars: 100 μm. Abbreviations: AL, abscission layer; PE, pedicel; RG, rudimentary glume; SL, sterile lemma; VB, vascular bundle. Asterisks represent significant differences determined by Student's *t* test. $^{****}p < 0.0001$.

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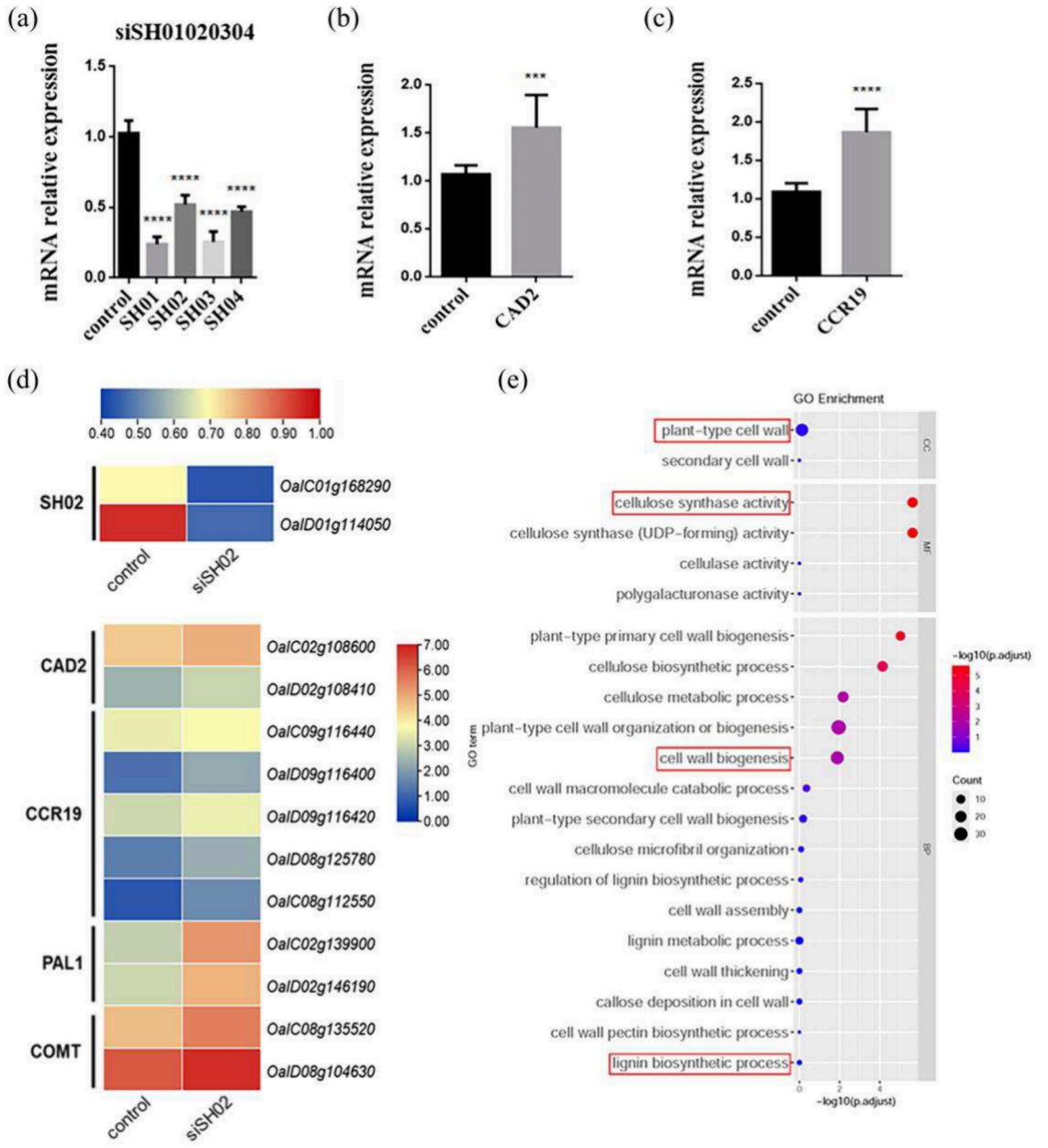

Figure 4. MSN-siRNAs with various combinations silencing shattering genes of panicles. (a) The expression pattern of four shattering genes after all four shattering genes were silenced. (b,c) The expression of the lignin deposition related genes *CAD2* and *CCR19* upregulated in four shattering gene silenced wild rice. (d) Heat map showing expression patterns of DEGs involved in lignin sythase genes of MSN-siSH02 treated plant. (e) Gene ontology term analysis of DEGs enrichment in MSN-siSH02 treated plant. Asterisks represent significant differences determined by Student's *t* test. ****$p < 0.0001$. ***$p < 0.001$.

wild rice panicles to disrupt AL formation and improve the seed shattering trait. We started to treat panicles about 16−20 days before the rice heading. Continuous infiltration to panicles was performed every other day for about 16 days and panicle samples were collected to detect gene silencing after 24 h. The time interval of retreatment and sampling was confirmed by silenced duration of shattering genes (Figure S4). The remaining panicles were collected for spikelet slices and breaking tensile strength (BTS) experiment after a week according to AL development progress.

Through sequence alignment, we proposed four shattering genes (*SH01*−*SH04*) in wild *O. alta* rice, analogous to the known shattering genes in other cultured species. Given the allotetraploid nature of *O. alta*, each gene has two sequences across the CC and DD subgenomes. We synthesized siRNAs targeting each sequence and coincubated them with MSN before infiltrating them into rice panicles (Figure S7a). The qPCR analysis performed 24 h post-treatment revealed significant silencing of all four shattering genes, with knockdown efficiencies ranging from 10.7% to 49.4% (Figure 3b, Figure S7b,c). Continuous treatment until heading suppressed gene expression, and a BTS experiment showed that treated spikelets exhibited higher tensile strength (Figure 3c) and immature and asymmetric AL formation (Figure 3d), compared to controls where one to three genes were silenced (Figure S8). Among the four shattering genes only *SH02* (*qSH1*) has been identified as a significant contributor to the shattering in *O. alta*.[8] The above results further determined the

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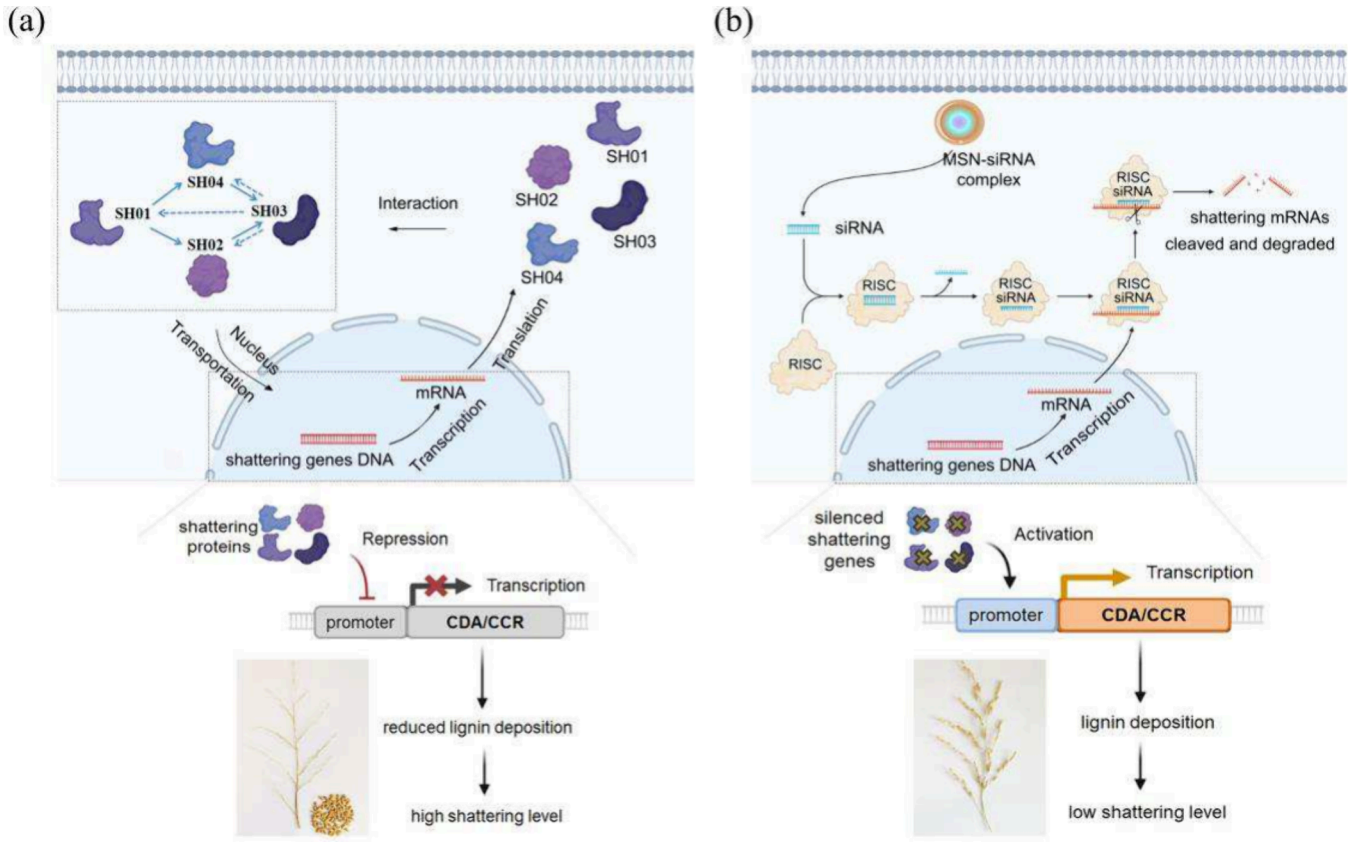

Figure 5. Schematic of MSN-siRNAs silenced shattering genes of panicles. (a) Seed shattering phenotype with high expression of shattering genes and suppressed lignin synthesis and deposition. (b) Seed shattering improved by seed shattering genes silenced by MSN-siRNAs, lignin synthesis, and deposition suppress shattering.

shattering function of the other three genes including *SH01* (*SH4*), *SH03* (*SH1*), and *SH04* (*SH5*) in *O. alta* by experimental verification.

In the regulatory mechanisms of grain shattering, multiple studies have indicated that certain genes implicated in this process can suppress lignin synthesis by inhibiting the expression of lignin-related genes, leading to decreased lignin deposition in the AL, and consequently enhanced shedding. To evaluate the efficacy of the MSN-siRNA delivery system in reducing shedding traits, we assessed the expression of lignin biosynthesis genes *CAD2* and *CCR19* in wild rice specimens with concurrent silencing of four shedding genes (Figure 4a). The outcomes demonstrated significant upregulation of both *CAD2* and *CCR19* (Figure 4b,c). The results showed that lignin biosynthesis genes were downregulated by shattering genes, and after shattering genes were silenced, lignin could be biosynthesized and deposited in the AL, further improving seed shattering.

Further, to ascertain the specificity of the shedding gene siRNA, we targeted *SH02*, an analogue of the well-documented rice gene *qSH1*,[13] also functionally identified in *O. alta*,[8] using MSN-siRNA-mediated knockdown. Subsequent RNA sequencing of the treated samples revealed 126 differentially expressed genes, including a reduction in the expression of *SH02* (Figure 4d), thus validating the siRNA's specificity. Notably, upregulated genes encompassed *CAD2*, *CCR19*, *PAL1*, and *COMT*, among others implicated in lignin production, which aligns with experimental observations indicating that lignin-related genes are upregulated subsequent to the knockdown of shattering genes (Figure 4d). A gene ontology (GO) enrichment analysis of these differentially expressed genes (DEGs) elucidated their primary localization within cell walls and involvement in molecular functions like cellulose synthase activity. Biological processes were predominantly associated with cell wall biogenesis and lignin biosynthesis, corroborating prior research (Figure 4e). Other DEGs pointed to roles in photosynthesis, chloroplast organization, and carbohydrate biosynthesis, hinting at a broader involvement of shedding genes in essential physiological and metabolic pathways.

We also probed the effects of various constituents of the MSN-siRNA delivery system on plants via RNA sequencing. Notably, treatments with the delivery buffer alone and with added MSNs caused changes in 5 and 12 genes, respectively, indicating minimal impact on plant transcriptomes, considering the backdrop of about 55000 transcripts in wild rice specimens (Figure S9a,b). Conversely, the addition of Tween 20 to the delivery buffer markedly increased the number of DEGs to 941 (Figure S9c), representing 1.7% of the total gene pool, with DEG enrichment in the redox state, transcription initiation, transcription factor activity, and RNA polymerase binding—effects likely attributable to Tween 20's properties as a nonionic surfactant[35,36] (Figure S9e). In contrast, the full MSN-siRNA delivery system, inclusive of scramble siRNA, led to a more pronounced alteration with 1472 genes differentially expressed (Figure S9d), encompassing 2.6% of the genome. The induced pathways in these samples were substantially different from those affected by Tween 20 alone, with a marked enrichment in pathways related to bacterial response and defense as well as carbohydrate binding, suggesting that the full delivery system may trigger plant defense mechanisms

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(Figure S9f). This analysis indicates that the MSN-siRNA delivery system might be operating by overcoming plant cellular barriers, including the cell wall and membrane, to deliver siRNA effectively and induce specific physiological responses.[37,38]

This study demonstrates the effective use of MSNs as carriers for siRNAs to modulate gene expression in wild *O. alta* rice. The application of MSNs through foliar spraying allows for efficient delivery without harming the wax on leaf surfaces. Specifically, the *PDS* gene was knocked down by 70% within 24 h, and the *Ruby* gene was suppressed in transgenic plants, illustrating the system's efficacy. Furthermore, *SH01*, *SH03*, and *SH04* were verified as shattering genes among multiple seed shattering genes in *O. alta*, highlighting the potential of MSN-siRNAs for both exploring plant gene functions and improving traits.

The targeted suppression of four identified seed shattering genes in *O. alta* revealed an intriguing internal regulation mechanism (Figure S10), where arbitrarily shattering gene expression correlated with decreased lignin synthesis in the AL, leading to increased seed shattering tendencies (Figure 5a). Conversely, silencing these genes enhanced lignin production, reducing seed shattering (Figure 5b), thereby illustrating a broader influence on plant physiology and metabolism.

The genome of *O. alta* is well-assembled, yet not all of the seed shattering genes have been fully annotated. Although four MGs influencing seed shattering have been identified based on sequence alignments with cultivated rice, silencing these genes temporarily may not fully prevent seed shattering. Our research seeks to enhance seed retention by targeting the formation of the abscission layer between the rice grain and the pedicel. We have demonstrated that the MSN-based siRNA delivery system effectively addresses this issue. This study not only highlights the potential of the MSN-siRNA system but also lays the groundwork for further exploration of seed shattering mechanisms in *O. alta*.

The MSN-siRNA delivery system proved effective at delivering siRNA across various plant structures and organs, including leaves and panicles of tobacco and rice. It can also simultaneously target multiple genes and control the duration of gene knockdown, making it a versatile tool for manipulating specific plant traits and developmental stages. RNA sequencing of rice leaves treated with different MSN-siRNA components shows that this system does not adversely impact plant growth or gene expression, confirming its biocompatibility and safety. Additionally, the biodegradable and absorbable nature of MSNs reduces the environmental impact. Since MSN-siRNAs are not transgenics, siRNAs are removed by cells after their effect, which could facilitate higher regulatory acceptance.

Despite the advantages of the MSN-siRNA delivery system, there are several limitations to this approach. The system currently targets traits regulated by stage-specific gene expression, rather than those affecting the growth cycle. The injection technique used for rice panicles is impractical for field production. Economic feasibility is also a challenge, as siRNA is expensive for large-scale use, even though bulk MSN synthesis is cost-effective. After the heading stage, rice requires about 40 additional days to mature, during which siRNA degrades due to environmental factors and its own chemical properties, reducing its effectiveness. Increased lignification of the panicle postheading further complicates results, and manual handling during treatment can exacerbate shattering, negatively affecting grain quality and yield. These limitations highlight the need for further research to improve the practicality and scalability of this method. While our study offers a foundational step toward advancing wild rice cultivation and sustainable agricultural practices, future research should focus on enhancing the system's applicability and efficiency, potentially expanding its use in plant biotechnology and field applications.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.4c02297.

All siRNA sequences and primer sequences, characterization of MSN, the Si element in the EDX result, wax layer of wild *O. alta* rice, confocal microscopy images of FITC-MSN on rice, temporal dynamics of genes expression, free siRNA of PDS sprayed to leaves, *Ruby* overexpression in wild rice, the shattering character of wild rice, AL location and BTS of silenced gene composition, schematic of shattering genes relation in *O. alta*, effect on *O. alta* of individual components in MSN-siRNAs delivery system, and Materials and Methods that provide a detailed explanation of the experiments, materials, and methodology including plant growth condition, MSN synthesis and characterization, MSN combined with siRNA, tracking MSN in rice leaves by TEM, *O. alta* treated with MSN-siRNA via spray or infiltration, histological analysis, fluorescence microscopy, BTS experiment, RNA extraction, RT-PCR and quantitative PCR, betacyanin extraction and detection, and transcriptome sequencing (PDF)

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Author Contributions

Z.L. and J.Z. contributed equally to this work. Yuhong Cao, J.L., X.M., and Z.L. conceived the idea and designed the study. Z.L. and J.Z. performed the majority of the experiments and data analysis. Z.L. and Yuhong Cao wrote or revised the manuscript. Yao Cai assisted in MSN synthesis. H.W. helped analyze the results. X.M. helped with plant seeding and maintenance. M.L. helped with RNaseq preparation. H.Y. helped to revise the manuscript. All authors edited the manuscript and approved the final version.

Notes

The authors declare no competing financial interest.

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