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Genome-wide selection of potential target candidates for RNAi against *Nilaparvata lugens*

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Abstract

Background *Nilaparvata lugens* is one of the most destructive pests of rice. RNAi-based *N. lugens* control offers one alternative strategy to traditional chemical insecticides. However, selection of potential target for RNAi against *N. lugens* remains a major challenge. Only two target genes for nuclear transgenic *N. lugens*-resistant plants have been screened. Importantly, only one or few potential target genes against *N. lugens* were screened every time by knowledge of essential genes from model organisms in previous study.

Results Here, in silico genome-wide selection of potential target genes against *N. lugens* through homology comparison was performed. Through genome synteny comparisons, about 3.5% of *Drosophila melanogaster* genome was found to have conserved genomic synteny with *N. lugens* genome. By using *N. lugens* proteins to search *D. melanogaster* homologs defining lethal or sterile phenotype, 358 *N. lugens* genes were first screened as putative target genes. Transgenic rice lines expressing dsRNA of randomly selected gene (*NlRan* or *NlSRP54*) from 358 putative target genes enhanced resistance to *N. lugens*. After expression check and safety check, 115 *N. lugens* genes were screened as potential target candidates.

Conclusion The combined efforts in this study firstly provide one in silico genome-wide homology-based screening approach for RNAi-based target genes against *N. lugens*, which not only offer one new opportunity to batch select potential target candidates in pests of interest, but also will facilitate the selection of RNAi target in many pest species by providing more than one hundred potential target candidates.

Keywords *N. Lugens*, RNAi, Target gene, Homology, Rice

Introduction

Agricultural pests are severe threats to the global food security. Of these agricultural pests, *Nilaparvata lugens* is one of major delphacid planthoppers attacking rice, causing significant loss of rice yield [1]. Currently, *N. lugens* control is challenged by pesticide abuse and rapid adoption of *N. lugens* to host-plant resistance genes [2]. Especially, the rapid adoption of *N. lugens* to host-plant resistance genes may soon overcome host-plant resistance genes and then heavy *N. lugens* outbreaks and rice loss may be caused [3], necessitating the development of alternative, eco-friendly and economical strategies to control *N. lugens*. Among various strategies, RNA interference (RNAi)-based agricultural pest management, that is, generation of transgenic pest-resistant plant expressing pest double-stranded RNAs (dsRNA), has been considered as one green pest control strategy for sustainable agriculture [2]. RNAi is a sequence specific gene silencing process that is initiated by dsRNA. In RNAi-based

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agricultural pest control, through introducing the exogenous dsRNA targeting genes essential for pest's survival, development, growth, or specific biological processes, the introduced exogenous dsRNAs may specifically target the mRNA of essential genes in agricultural pests, which has the potential to diminish pests' fitness or even cause mortality [4]. Remarkably, transgenic crops expressing agricultural pest dsRNA successfully cause phenotype disturbance of agricultural pests [5, 6], opening a new era for controlling agricultural pests by RNAi in field conditions.

The crucial issue in the success of RNAi-based pest control is the careful selection of one effective target gene [7]. Effective dsRNAs are often discovered during large-scale RNAi-based analyses of gene functions, revealing phenocopies of mutations that affect survival or development of insects [8]. Although large-scale RNAi screens for gene function are normally performed in classical model organisms, selection of RNAi targets for non-model organisms can be initially guided by knowledge of essential genes in classical model organisms [9]. Alternatively, researchers and pest managers would develop on their own by large-scale RNAi screens or cDNA library screening for non-model organisms; however, which in most cases is time-consuming and laborious [9]. Nowadays, with the development of the omics technology, more sophisticated and high throughput approaches to screening effective target genes have been developed, including RNA-seq and digital gene expression tag profile technologies [10], coupled transcriptome with proteome data [11] and genome-wide RNAi screens [9, 12]. Although target genes against *N. lugens* such as *NlRan* [13], *NlCaM* [14], *NlEcR* [15], *NlCHSA* [16] and *NlGST* [17] have been screened, screenings for these target genes against *N. lugens* in most cases were guided by existing data from model organisms. Moreover, such screenings based on available data from model insect species in most cases are small-scale screenings and less effective [18], in which few or only one essential gene from model organisms were selected to search homologs from pest of interest every time. Therefore, more sophisticated and highly effective approaches to screening effective target genes against pest are needed to be explored. Ideally, genome-wide screenings offer the widest coverage of potential target genes against *N. lugens*. However, no genome-wide screenings have performed in *N. lugens* up to date. Alternatively, screenings guided by knowledge of essential genes in classical model organisms or existing data from closely related species can be used to identify target genes based on homology [9]; however, no existing data from closely related species is available for the non-model pest insect *N. lugens*. Of note, the first insect genome-wide RNAi screen was performed in S2 *Drosophila* cell culture [19]; these cell-based studies have paved a way for large-scale whole-genome RNAi screens in the classical model organism *D. melanogaster* [20], and *D. melanogaster* stock center in Europe has the world's largest collection of *Drosophila* RNAi lines with viable, sterile and lethal phenotype. These studies paved a way for in silico large-scale whole-genome RNAi screens in the non-model organisms such as *N. lugens*.

Effective screenings mainly depend on effective delivery of exogenous dsRNA targeting genes essential for pest's development or survival. Among various approaches for exogenous dsRNA delivery, transgenic plant expressing dsRNAs may be one feasible approach for plant protection from pests in the field [21]. Transgenic plant expressing dsRNAs includes nuclear and plastid transgenic plant expressing dsRNA [22]. Reports showed that success of transplastomic plants expressing dsRNA depends largely on the accessibility of the plastids to sap-sucking insect species [23, 24]. Although dsRNAs accumulating in plastids are accessible to the sap-sucking insect *N. lugens*, there is no successful story of transplastomic plants expressing dsRNA for *N. lugens* control [24]. On the contrary, there are two successful stories of nuclear transgenic plants expressing dsRNA for *N. lugens* control [15, 17].

Briefly, RNAi-based *N. lugens* control offers an alternative strategy to traditional chemical insecticides; however, the selection of RNAi target genes remains a major challenge. The major weakness in selection of target genes in previous study is that only one or few target genes were screened every time by using the known essential gene(s). To overcome the hurdle, in this study, by using *N. lugens* proteins to search homologs defining sterile and lethal phenotype in *D. melanogaster* on a genome-wide scale, employing identity and coverage as important measures of similarity, a total of 358 genes in *N. lugens* were thereby screened as putative target genes. Transgenic rice lines expressing dsRNA of randomly selected gene (*NlRan* or *NlSRP54*) from these 358 putative target genes were then generated. The enhanced resistance of transgenic rice lines suggested that these 358 putative target genes would be potential plant-mediated RNAi target genes in *N. lugens* management. After expression check and safety check, 115 genes from 358 putative target genes were screened as potential target candidates against *N. lugens*. The combined efforts in this study not only firstly provide one in silico genome-wide potential target candidates screening approach in *N. lugens*, but also provide more than one hundred potential target candidates for plant-mediated RNAi-based *N. lugens* control.

Materials and methods

Plant and insect materials

Taichung Native 1 (TN1) and Nipponbare, *N. lugens*-susceptible rice cultivars, were used for rearing *N. lugens*

and transgenic analysis, respectively. Seeds of TN1 were sown and grown in plastic boxes (40×30×12 cm^3) containing soil from rice paddy fields in a climate-controlled chamber, 27±2 °C, 90% relative humidity and 16 h light/8 h dark. The *N. lugens* insects were collected from rice paddy fields in Zhejiang Province, China in 2022 and maintained on TN1 plants in Zhejiang Normal University. The population that had been reproduced for over 10 generations on TN1 seedlings was used in the subsequent experiments.

Genome-wide protein homology comparison between *D. Melanogaster* and *N. lugens*

As described previously [25], whole genomic DNA sequence, whole-genome protein sequence, and genome annotation in GFF format of *D. melanogaster* (Genbank: Release 6 plus ISO1 MT) and *N. lugens* (Genbank: GCA_014356525.1) were downloaded from NCBI (www.ncbi.nlm.nih.gov). To screen the potential target genes through homology comparison, genome synteny between *D. melanogaster* and *N. lugens* were firstly analyzed by using MCScanX software (version 1.1.11) with default parameters [26] and were visualized using SynVisio (https://synvisio.github.io/#/) [27]. Then the whol e-genome protein sequences of *D. melanogaster* and *N. lugens* were used to perform protein homology analysis by employing "Two Sequence Files" tool in the "BLAST GUI Wrapper" function of the alignment toolkit TBtools (TBtools-II, Windows-x64, version 2.030, https://tbtools.cowtransfer.com/s/fd65f874240044) [28], and the alignm ent results were shown in Table format. For each of the annotated proteins of *N. lugens*, we performed BLASTp against the *D. melanogaster* protein data set using TBtools [28], and declared a provisional homologous pair (each pair consists of a *N. lugens* protein and a homologous *D. melanogaster* protein) if the *E*-value of the match was $\leq 10^{-5}$. In addition to identity, coverage defining as percentage of alignment length of protein length was also used to estimate the protein homology.

In silico genome-wide screening of target genes

Access the Vienna *Drosophila* Resource Center homepage at https://shop.vbc.ac.at/vdrc_store/, download Excel file of current *Drosophila* RNAi lines by following the hyperlink of the "RNAi Stocks". The Excel file includes detailed information on stock numbers, genotypes and their associated genes of more than 30,000 independent *Drosophila* RNAi lines. In the Excel file, the genotype was classified into three categories (viable, sterile and lethal) in the light of viability. Sort the Excel file and genes were so classified into three groups, namely, viable, sterile and lethal group, respectively. Download *D. melanogaster* protein sequences of sterile and lethal group, respectively from http://flybase.org/download/sequence/batch/. By employing "Two Sequence Files" tool in the "BLAST GUI Wrapper" function of the alignment toolkit TBtools [28], *N. lugens* proteins having *D. melanogaster* homologs were used as queries in BLASTp searches against the *D. melanogaster* proteins defining sterile and lethal phenotype, respectively. The alignment results were shown in Table format.

Detect repeats in protein sequences using the search and sequence analysis tools services from EMBL-EBI in 2022 (https://www.ebi.ac.uk/Tools/pfa/radar/). To readily categorize the screened putative target genes, the putative target genes defining lethal or sterile phenotype were represented in a Venn diagram using the Venn diagram web tool (http://bioinformatics.psb.ugent.be/webtools/Venn/). To perform safety check, that is, to avoid putative target genes targeting non-target organisms, the RNA sequences of the putative target genes were used as queries to BLASTn searches against the NCBI genome database of rice, maize, soybean, human beings, *Anagrus nilaparvatae*, *Cyrtorhinus lividipennis*, coccinellidae and spider. Because recent studies have shown that a 100% complementary sequence length of ≥19 nucleotides may effectively reduce the corresponding mRNA levels [29], the sequences of putative target genes should not contain more than 19 consecutive bases homologous to genomic sequences of rice, maize, soybean, human beings, *A. nilaparvatae*, *C. lividipennis*, coccinellidae and spider.

Genome-wide in silico prediction of expression of the putative target genes

To readily examine the expression of the putative target genes in *N. lugens*, the genome-wide in silico prediction of expression of the putative target genes was performed. The sequence of the putative target genes in *N. lugens* was used as a query in BLASTn search against transcriptome of *N. lugens* as follows. Information of the available transcriptome of *N. lugens* in public databases was collected from InsectBase 2.0 (http://v2.insect-genome.com/Transcriptome) [30]. In the subsequent resulting page, orderly follow the hyperlink of BioProject, SRA Experiments in Project Data, and Runs, then follow the hyperlink of BLAST in Experiment to access to the Blast window. The BLAST results were downloaded in Table format.

Molecular cloning

Among the putative target genes having 90-100% and 80-90% amino acid identity to *D. melanogaster*, respectively, one putative target gene *NlRan* encoding GTP-binding nuclear protein (XM_022340841.2) and one putative target gene *NlSRP54* encoding signal recognition particle 54 kDa protein (XM_022328877.2), were randomly selected to determine whether the putative target genes would be potential plant-mediated RNAi targets in *N. lugens* management. The sequence of *NlRan*

(XM_022340841.2) and *NlSRP54* (XM_022328877.2) was downloaded from NCBI. The accuracy of *NlRan* and *NlSRP54* was thereafter validated by polymerase chain reaction (PCR).

RNAi vector construction and plant transformation

A fragment containing a 454 bp of *NlRan* cDNA was amplified by PCR for RNAi vector construction of *NlRan* (named as *Ran*) (Table S1a). Meanwhile, a fragment containing a 218 bp of *NlSRP54* cDNA was amplified by PCR for RNAi vector construction of *NlSRP54* (named as *SRP54*) (Table S1b). Each fragment was inserted into pCAM1300-GUS-NOS vector in opposite orientations flanking intron sequence (to form RNAi expression cassette), and the expression of RNAi expression cassette was under the control of cauliflower mosaic virus 35s RNA promoter (Table S1a and S1b). Transgenic rice was obtained from mature embryos of Nipponbare by *Agrobacterium tumefaciens*-mediated genetic transformation method [31]. The positive rice transformants were determined via PCR using gene-specific primer pair Hyg-F/Hyg-R (Hpt-F, 5'-CTGCCCGCTGTTCTACAACCG G-3'; Hpt-R, 5'-GGAGCATATACGCCCGGAGTC-3') and GUS staining.

Evaluation of resistance of transgenic rice to *N. lugens*

To estimate the plant-mediated RNAi efficiency in *N. lugens* control, the resistance of transgenic rice to *N. lugens* was examined. The resistance of transgenic rice to *N. lugens* was evaluated as previously described [32]. Briefly, twenty one-day old seedling of Nipponbare and transgenic rice line in a 9-cm plastic cup (six seedlings per cup) was covered by an inverted 9-cm plastic cup and infested *N. lugens* nymphs (third- to fourth-instar) at six insects per seedling. The *N. lugens* was allowed to continuously feed on the plants. The control Nipponbare was maintained in parallel with *N. lugens* introduction. Three replicates per Nipponbare and transgenic rice line, respectively. Once all the control Nipponbare seedlings had died, the transgenic rice line was assigned a numerical score between 0 and 9.

Results

Genome synteny between *D. Melanogaster* and *N. lugens*

To determine whether *D. melanogaster* and *N. lugens* share collinear genes which can help to predict functional relationships between genes of *D. melanogaster* and *N. lugens*, the chromosome distribution of synteny blocks between *D. melanogaster* and *N. lugens* was firstly examined by McScanX. Through genome synteny comparison, about 3.5% of the *D. melanogaster* genome was found to have conserved genomic synteny with *N. lugens* genome (Fig. 1). Although the synteny between *D. melanogaster* and *N. lugens* was low (3.5%), the synteny existing between *D. melanogaster* and *N. lugens* showed potential for in silico genome-wide screening of target genes for RNAi-based *N. lugens* control through homology comparison.

Homologous pairs between *D. Melanogaster* and *N. lugens* at the protein level

Because detectable synteny existed between *D. melanogaster* and *N. lugens* at the genome scale, we thus adopted the strategy of identifying *D. melanogaster* and *N. lugens* homologs at the protein level [25] and examined the homologous pairs via BLASTp. Our data sets for this study consisted of all the annotated *D. melanogaster* and *N. lugens* genomic sequences available in NCBI database as of July 2022. The corresponding *D. melanogaster* and *N. lugens* protein data set consisted of 30,794 and 32,453 annotated proteins, respectively. Through examining the homologous pairs via BLASTp, a total of 3,570,217 putative homologous pairs were found (Table S2a). The found *D. melanogaster* homologs of each *N. lugens* protein vary from 0 to 7,596. Of the 32,453 annotated *N. lugens* proteins, 8,732 (26.91%) have no *D. melanogaster* homolog. Among the top three *N. lugens* proteins having *D. melanogaster* homologs, the most (7.27%), second (7.09%) and third (4.4%) *N. lugens* proteins have one, two and three *D. melanogaster* homologs, respectively, together accounting for about 18.76% (Table S2a).

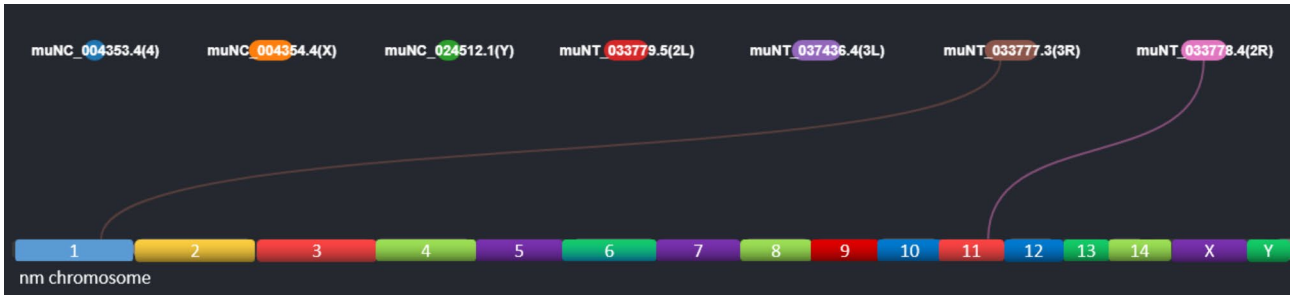

Fig. 1 The synteny blocks for *D. melanogaster* and *N. lugens* genomes. mu, *D. melanogaster*; nm, *N. lugens*

The identity and coverage of the putative homologous pairs

It is commonly accepted that sequence similarity is the best marker for substantiating homologous relationships [33]. Therefore, the identity of the 3,570,217 putative homologous pairs is firstly noted in this study. According to result of BLASTp, the identity of *N. lugens-D. melanogaster* pairs varied from 13.703 to 100% (Table S2b-S2d). When the putative homologous pairs sharing more than 80% amino acid sequence identity to each other [25] were aligned by BLASTp again to screen the putative target genes, it was striking to note that it was a fragment of protein but not the whole length of protein that shared 100% amino acid sequence identity in some putative homologous pairs (Table S2e), e.g., XP_039285266.1 and NP_001027285.1, therefore, the coverage was thereby used as another important measure of similarity when performing the in silico genome-wide screening of target genes through homology comparison.

The putative target genes defining lethal phenotype

To readily screen the putative target genes defining lethal phenotype, the *N. lugens* proteins having *D. melanogaster* homologs were used as queries in BLASTp searches against the *D. melanogaster* proteins defining lethal phenotype. In the examination of the homologous pairs via BLASTp, the corresponding *D. melanogaster* and *N. lugens* protein data set consisted of 11,240 and 23,721 annotated proteins, respectively. Through examining the homologous pairs via BLASTp, a total of 1,570,860 putative homologous pairs were found (Table S3a). To effectively screen the putative target genes defining lethal phenotype, the putative homologous pairs having more than 80% amino acid sequence identity [25] with high (more than 90%) coverage of *N. lugens* proteins and *D. melanogaster* homologs were screened as putative target genes. As shown in Table S3c, 950 putative homologous pairs were screened as putative target genes. Of note, among homologous pairs having more than 80% amino acid sequence identity and 99.8–100% coverage of *D. melanogaster* but less than 90% coverage of *N. lugens* proteins, some *D. melanogaster* proteins were found to be one part (e.g., XP_039292495.1 and FBpp0301732) (Table S3d) or one repeat (e.g., XP_039285264.1 and FBpp0085250) (Table S3e) of *N. lugens* proteins. Considering that *D. melanogaster* protein is lethal, therefore, these 81 putative homologous pairs were also screened as putative target genes. Together, 1,031 putative homologous pairs were screened as putative target genes defining lethal phenotype (Table S3f).

After removing redundancy, a total of 358 *N. lugens* proteins were screened as putative target genes defining lethal phenotype. According to gene function annotation by NCBI (Table S3g), among these 358 putative target genes defining lethal phenotype, some ones including *v*-ATPase A, actin and tubulin are known target genes for nuclear or plastid transformation-mediated dsRNA-expressing transgenic plants in pest control [34]; some ones are known potential target for non-transgenic RNAi-based (by feeding, injection or spray methods) *N. lugens* control, including genes encoding NlRan (XP_022196533.1) [13], ribosomal proteins [35], calbindin [14], chromatin remodeling protein [36], guanine [37], heat shock protein [38], myosin protein [39] and calcium sensor [40]; some ones are known potential target for non-transgenic RNAi-based pest control, including genes encoding signal recognition particle 54 kDa protein [41], argonaute [42] and *S*-adenosyl-L-homocysteine [43].

The putative target genes defining sterile phenotype

The sterile phenotype caused by the plant mediated RNAi can decrease the production of offspring, which thus can be an alternative approach to controlling the pests, therefore, the putative target genes defining sterile phenotype were also screened. In the examination of the homologous pairs via BLASTp, the corresponding *D. melanogaster* and *N. lugens* protein data set consisted of 3,823 and 23,721 annotated proteins, respectively. Through examining the homologous pairs via BLASTp, a total of 570,049 putative homologous pairs were found (Table S4a).

To effectively screen putative target genes defining sterile phenotype, the putative homologous pairs having more than 80% amino acid sequence identity [25] with high (more than 90%) coverage of *N. lugens* proteins and *D. melanogaster* homologs were also preliminarily screened as putative target genes. As shown in Table S4c, 431 putative homologous pairs were screened as putative target genes. Of note, among homologous pairs having more than 80% amino acid sequence identity and 99.8–100% coverage of *D. melanogaster* but less than 90% coverage of *N. lugens* proteins, some *D. melanogaster* proteins were found to be one part (e.g., XP_039292496.1 and FBpp0077741) (Table S4d) or one repeat (e.g., XP_039285264.1 and FBpp0085250) (Table S4e) of *N. lugens* proteins. Considering that the *D. melanogaster* protein is sterile, therefore, these 57 putative homologous pairs were also screened as putative target genes. Together, 488 putative homologous pairs were screened as putative target genes defining sterile phenotype (Table S4f).

After removing redundancy, a total of 157 *N. lugens* genes were screened as putative target genes defining sterile phenotype. According to gene function annotation by NCBI (Table S4g), among these 157 putative target genes defining sterile phenotype, some ones were reported to be essential for fertility, such as 14-3-3

epsilon protein [44], calcineurin [45], myosin [46], cofilin/actin-depolymerizing factor [47], heat shock protein [48] and ras-related protein [49]. Strikingly, all the 157 putative target genes defining sterile phenotype were found to be common to 157 of 358 putative target genes defining lethal phenotype (Fig. 2). Altogether, a total of 358 *N. lugens* genes were screened as putative target genes defining lethal or sterile phenotype.

In silico genome-wide prediction of expression of the putative target genes defining lethal or sterile phenotype

Because RNAi is defined as sequence-specific silencing of target gene, one ideal target gene for RNAi must express. Therefore, the expression of the putative target genes defining lethal or sterile phenotype was predicted through BLASTn against the collected transcriptome of *N. lugens.* The transcriptome of *N. lugens* in InsectBase 2.0 database [30] consisted of 124 records and 110 transcriptome of *N. lugens* were available. According to results of BLASTn against 110 transcriptome data available for stage-, sex- and tissue-specific transcription of *N. lugens*, as shown in Table S5, besides 4, 26, 2 and 8 putative target genes were not detected in the transcriptome of pupa (BioProject: PRJNA451497), adult female head and first foreleg (BioProject: PRJNA514182), female ovary (BioProject: PRJNA480530) and BioProject: PRJNA629998, respectively, all remaining putative target genes had BLAST hits with 100% identity to the transcriptomes, indicating that all remaining putative target genes expressed in different stage, sex and tissue. Considering that these 40 putative target genes were just not expressed in pupa, female head and first foreleg, female ovary of *N. lugens*, these 40 putative target genes were still considered as ideal gene targets for RNAi. Together, all the 358 putative target genes passed the expression check in the light of transcript.

Resistance of the transgenic rice plants to *N. lugens* attack

To determine whether these putative target genes would be potential plant-mediated RNAi targets in *N. lugens*

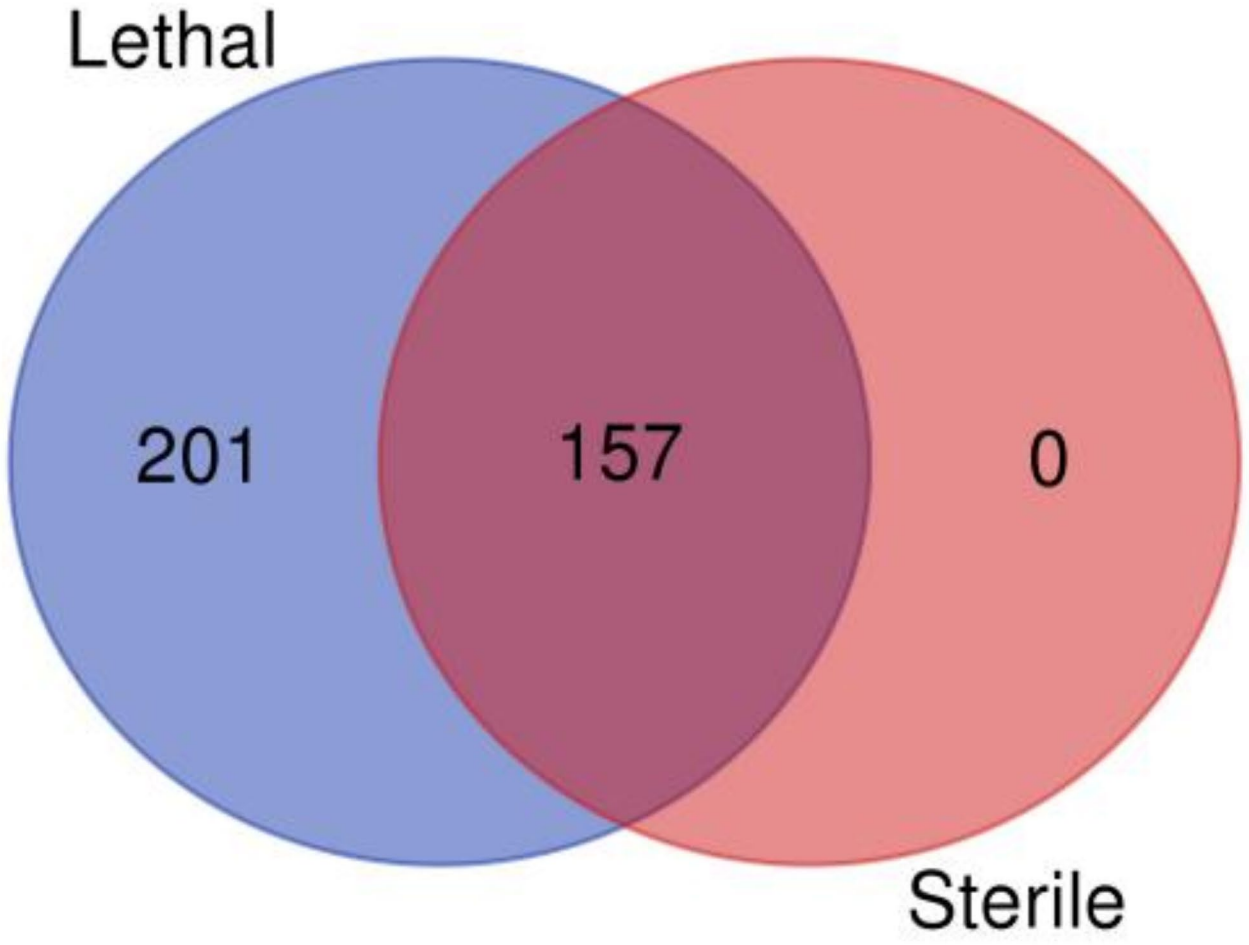

Fig. 2 Venn diagram showing the target genes defining lethal and sterile phenotype. The number in the Venn diagram is the number of the target genes that belong to its corresponding category of the Venn diagram

management, the resistance of transgenic rice lines expressing dsRNA of *NlRan* or *NlSRP54* was evaluated. After *N. lugens* infestation for about 10 d, entire control rice plants withered and died, whereas transgenic rice expressing dsRNA of *NlRan* or *NlSRP54* remained healthy with a numerical score 2±0.72 and 3±0.98, respectively (Fig. 3). Thus, transgenic plants expressing *NlRan* or *NlSRP54* dsRNA are more resistant than control, demonstrating that transgenic expressing ds*NlRan* or ds*NlSRP54* increases resistance of rice to *N. lugens*. These results indicate that these putative target genes would be potential target genes for plant-mediated RNAi-based *N. lugens* management.

Safety check

Safety to non-target organisms is one critical issue for plant-mediated RNAi approach for controlling pests. One ideal target gene for RNAi should not to target gene(s) of plant or host. In this study, to avoid target genes from targeting host gene(s), homologous examination of these putative target genes against 48 rice genomes available in NCBI genome database was then performed. As shown in Table S6b, 31 putative target genes were found to be homologous to rice genes with 72.553-100% identity and 28–1526 bp alignment length. In addition, food safety and environment risk of genetically modified crops have attracted serious public attention. To avoid these target genes from targeting gene(s) of human beings and main crops such as maize and soybean, homologous examination of these putative target genes against 9 human representative genomes, 22 representative maize genomes and 20 available soybean genomes in NCBI genome database was also performed. As shown in Table S6c-6e, 76 putative target genes were found to be homologous to human genes with 72.709-100% identity and 28–2060 bp alignment length; 20 putative target genes were found to be homologous to maize genes with 71.816-100% identity and 28–1504 bp alignment length; 39 putative target genes were found to be homologous to soybean genes

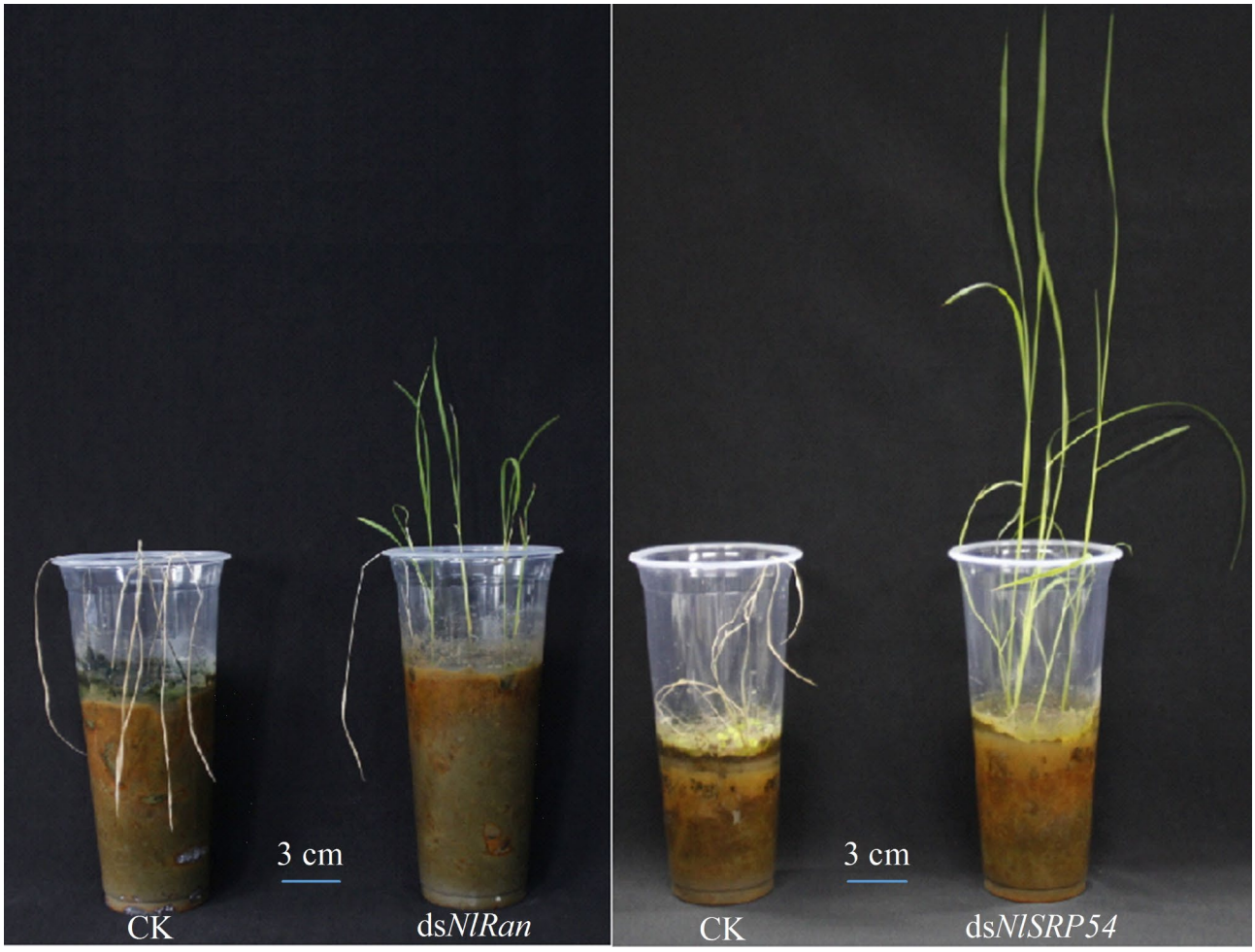

Fig. 3 The phenotype of rice plants that were treated with *N. lugens*. CK, control plants; ds*NlRan* and ds*NlSRP54*, transgenic rice plants expressing ds*NlRan* and ds*NlSRP54*, respectively

with 72.973-100% identity and 28–1818 bp alignment length.

Safety to natural enemies is another critical issue in plant-mediated RNAi approach for controlling pests. To prevent off-target effects on the natural enemies of *N. lugens* such as *A. nilaparvatae*, *C. lividipennis*, coccinellidae and spider, homologous examination of these putative target genes against 1 *A. nilaparvatae* genome, 1 *C. lividipennis* genome, 13 coccinellidae genome and 8 spider genomes in NCBI genome database was then performed. As shown in Table S6f, 104 putative target genes were found to be homologous to *A. nilaparvatae* genes with 73.497-100% identity and 28−1,478 bp alignment length. Meanwhile, 59 putative target genes were found to be homologous to *C. lividipennis* genes with 76.147-89.344% identity and 74−1,404 bp alignment length; 145 putative target genes were found to be homologous to coccinellidae genes with 71.73-100% identity and 28−2,739 bp alignment length; 65 putative target genes were found to be homologous to spider genes with 71.717-100% identity and 28−1,663 bp alignment length (Table S6g-6i).

After removing redundancy, a total of 243 putative target genes were homologous to genes of rice, maize, soybean, human beings, *A. nilaparvatae*, *C. lividipennis*, coccinellidae and spider (Table S6a), accounting for nearly two-thirds of the 358 putative target genes. Together, the remaining 115 putative target genes passed the safety check by homologous examination against rice, maize, soybean, human, *A. nilaparvatae*, *C. lividipennis*, coccinellidae and spider genome. As shown in Table S6a, a total of 237 putative target genes were homologous to genes of *N. lugens*' natural enemies, accounting for 97.53% of the 243 putative target genes which were homologous to genes of non-target organisms. Remarkably, 17 putative target genes have *A. nilaparvatae* homologs with 100% identity and 28–553 bp alignment length (Table S6f). On the contrast, a total of 96 putative target genes were homologous to genes of rice, maize, soybean, and human beings, accounting for 39.51% of the 243 putative target genes which were homologous to genes of non-target organisms. Therefore, to reduce the off-target effects, issues of safety to natural enemies of *N. lugens* should be taken into careful consideration. Moreover, for some target genes having homologs of non-target organisms with high identity but short alignment length, such as XM_022332702.2 (with 100% identity of rice and *A. nilaparvatae* and 28 bp alignment length), although the short alignment length may be excluded when designing dsRNA specific to the *N. lugens*, to reduce the possibility for biosecurity problems, such putative target genes were still discarded in this study. Altogether, 115 putative target genes passed safety check and screened as potential target candidates for plant-mediated RNAi-based *N. lugens* control (Table S7).

Discussion

RNAi targets are often discovered during the large-scale RNAi-based analyses of gene functions [7]. Large-scale RNAi screens for gene function are normally performed in classical model organisms such as *D. melanogaster* [20]. Although large-scale RNAi screens can provide a source of target genes that might be exploited, such large-scale manufacturing at appropriate cost is still a major challenge. Therefore, screening the target genes in non-model organisms guided by existing data from model organisms or closely related species is recognized as one economical strategy. However, in many cases such large-scale RNAi data of closely related species is not available, e.g., neither large-scale nor genome-wide RNAi data of closely related species is available for *N. lugens*. Generally, screenings guided by existing data from model organisms or closely related species are homology-based [9]. However, there are still many genes that lack homology with genes in other evolutionary lineages [50]. Consistently, the synteny between *N. lugens* and the model insect *D. melanogaster* is low (3.5%), and only 1,859 (about 5.73%) proteins of *N. lugens* share homology with proteins of *D. melanogaster* having more than 80% amino acid sequence identity (Table S1g), which may be one reason why there are only two successful stories of nuclear transgenic plants expressing dsRNA for *N. lugens* control in which the target gene is successfully discovered based on homology [15, 17]. In this study, by using *N. lugens* proteins to search homologs defining sterile and lethal phenotype from *D. melanogaster* on a genome-wide scale, a total of 358 genes in *N. lugens* were screened as putative target genes; transgenic rice lines expressing dsRNA of randomly selected gene (*NlRan* or *NlSRP54*) from these 358 putative target genes enhanced resistance to *N. lugens*. Therefore, although using pre-screens in closely related species may work better than those more evolutionarily distant to achieve high rate of success of target genes selection [51], in the case of no data of closely related species is available, using pre-screens in model insects such as *D. melanogaster* which is distantly related species may be still proposed as a feasible and economical strategy for selection of potential target genes in the pests of interest.

The success of plant-mediated RNAi-based pest management primarily depends on selection of target genes. Several categories of essential insect genes have served as successful targets of RNAi [21, 25], including ① lethal genes; ② immunity-related genes, including modulator and effector genes and genes involved in immune recognition, signal transduction and melanization; ③ growth and development genes; ④ sterility genes; ⑤

host recognition- and adaptation-related genes; ⑥ ones encoding detoxification and digestive enzymes, and ⑦ ones encoding proteins that promote interactions with symbiotic bacteria. Currently, tens of genes of insects have been identified which are useful target for nuclear or plastid transgenic pest-resistant plants [21, 24, 34, 52], including *cytochrome P450 monoxygenase*, *v-ATPase*, *chitinase*, *β-actin*, *GST* (*glutathione S-transferase*), *EcR* (*ecdysone receptor*), *cuticular protein*, *acetylcholinesterase*, *zinc finger protein*, *Rack1* (*Receptor of Activated Kinase C 1*), *MpC002* (*Myzus persicae C002*), *MpDhc64C* (*Myzus persicaedynein heavy chain 64 C*), *hunchback*, *aquaporin*, *a-glucosidase*, *chitin synthase*, *cathepsin L*, *arginine kinase*, *serine protease*, *HMGR* (*HMG-CoA reductase*), *HR3* (*hormone receptor 3*), *hexose transporter*, *carboxypeptidase*, *trypsin-like serine protease*, *Gqa* (*α subunit of a subgroup of guanine nucleotide-binding proteins*), *coatomer subunitα*, *ribosomal protein P0*, *vitellogenin receptor*, *boule*, *SNF* (*transport III subunit SNF7*), *tubulin*, *plant defense suppression*, *antimicrobial peptide*, *sucrose 1*, *TP* (*tyrosine phosphatase*), *MSP* (*mitochondrial stress-70 protein precursor*) and *20-Hydroxyecdysone* and so on. In this study, some known target genes for nuclear or plastid transgenic pest-resistant plants such as ones encoding *v*-ATPase, actin, tubulin, arginine kinase and ribosomal protein were screened. Meanwhile, some novel target genes for nuclear or plastid transgenic pest-resistant plants conferred in this study such as ones encoding GTP-binding nuclear protein Ran and signal recognition particle 54 kDa protein were also screened. These results may suggest that function of these target genes screened based on homology may conserve among different species. To screen one target gene, it is still preferential for researchers and pest managers to search homologs in the pests of interest from model organisms or closely related species. Especially, the major weakness in selection of target genes in previous study is that only one or few target genes were screened every time by using the known essential gene(s). In this scenario, based on conservation of gene function among different species, in silico genome-wide homology-based selection approach in this study may offer a new opportunity to batch select target genes in the pests of interest with greater effectiveness and less expense.

Despite the homology lack, another hurdle for selection of RNAi targets guided by knowledge of essential genes is the miss of novel RNAi target genes. As shown above, about forty genes have been identified as useful target for nuclear or plastid transgenic pest-resistant plants [21, 24, 34, 52]. Compared with the number of genome genes of insect which universally is more than ten thousand, the percentage of the useful target genes in one genome genes of insect is far below 0.4%. Therefore, approach for screening target gene by testing genes where previous data indicated an essential function is a small-scale screen. Such knowledge-based approach will miss many novel RNAi target genes that have not yet been discovered in previous study, e.g., besides *NlEcR* [15] and *NlGST* [17], no more useful targets for nuclear transgenic *N. lugens*-resistant plants have been screened by knowledge-based approach in previous study. On the contrary, two randomly selected novel target genes (*NlRan* or *NlSRP54*) in this study were proved to be potential RNAi target genes for nuclear transgenic *N. lugens*-resistant plants. Therefore, the in silico genome-wide homology-based selection approach in this study may offer an alternative option to batch select novel RNAi target genes in the pests of interest with greater effectiveness and less expense.

Conclusion

Compared with selection of target genes in previous study in which only one or few target genes against *N. lugens* were screened every time by knowledge of essential genes from model organisms, in this study, by using *N. lugens* proteins to search homologs defining sterile and lethal phenotype in *D. melanogaster* on a genome-wide scale, such in silico genome-wide homology-based selection approach for RNAi potential target gene not only offers a new opportunity to batch select RNAi potential target genes in the pests of interest, but also offers an alternative option to batch select novel RNAi potential target genes in the pests of interest with greater effectiveness and less expense. The results present here will not only aid the screening of RNAi potential target candidates in many pest species, but also provide a manageable number of excellent potential target candidates to be tested in many pest species, which will promote the application of plant-mediated RNAi-based pest control from laboratory to field in future.

Abbreviations

RNAi	RNA interference
dsRNA	Double stranded RNAs
GST	*Glutathione S-transferase*
EcR	*Ecdysone receptor*
Rack1	*Receptor of Activated Kinase C1*
MpC002	*Myzus persicaeC002*
MpDhc64C	*Myzus persicaedynein heavy chain 64 C*
HMGR	*HMG-CoA reductase*
Gqaa	*Subunit of a subgroup of guanine nucleotide-binding proteins*
SNF	*Transport III subunit SNF7*
HR3	*Hormone receptor 3*
TP	*Tyrosine phosphatase*
MSP	*Mitochondrial stress-70 protein precursor*

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10940-9.

Supplementary Material 1: Table S1 Generation of RNAi vector expressing dsRNA against *NlRan* and *NlSRP54* gene, respectively.

Supplementary Material 2: Table S2 Identification of *N. lugens* proteins homologous to *D. melanogaster* proteins.

Supplementary Material 3: Table S3 The screening of putative target genes defining lethal phenotype.

Supplementary Material 4: Table S4 The screening of putative target genes defining sterile phenotype.

Supplementary Material 5: Table S5 Expression check by searching putative target genes against the available transcriptomes of *N. lugens* in public databases.

Supplementary Material 6: Table S6 Safety check by homologous examination of putative target genes against rice, maize, soybean, human beings, *A. nilaparvatae*, *C. lividipennis*, coccinellidae and spider genome.

Supplementary Material 7: Table S7 The detailed information on the potential target candidates.

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Author contributions
ZWL contributed to the study design and drafted the article. ZJ, LM and LJ analyzed the experiment results, prepared figures and tables. All authors read and approved the manuscript.

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Data availability
The datasets analysed during the current study are available in the NCBI repository (Genbank: Release 6 plus ISO1 MT for *D. melanogaster* and Genbank: GCA_014356525.1 for *N. lugens*). Meanwhile, the datasets generated during the current study are available in the supplementary material.

Declarations

Ethical approval and consent to participate
Not applicable. No animal was used in this study. Meanwhile, the *N. lugens* is pest of rice, and it is free to be collected from field.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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