Intron-containing hairpin RNA interference vector for OBP8 show promising mortality in peach potato aphid

Amber Afroz1 · Safeena Aslam1 · Umer Rashid1 · Muhammad Faheem Malik2 · Nadia Zeeshan1 · Muhammad Ramzan Khan3 · Muhammad Qasim Shahzad But1 · Sabaz Ali Khan4

Received: 18 May 2021 / Accepted: 15 September 2021 / Published online: 23 September 2021
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Abstract

Myzus persicae is a devastating pest affecting potato production. Intron-containing hairpin RNA (ihpRNA) silenced the odorant-binding protein 8 (OBP8) for enhanced protection against Myzus persicae in potatoes. OBP8 was isolated from M. persicae, sequenced, with the allotment of GenBank ID. ERNAi was used to design siRNA targets from OBP8 with no off-targets. Multiple Sequence Alignment show M. persicae OBP8 resemblance with Acyrthosiphon pisum, Rhopalosiphum maidis, Aphis fabae, and Sitobion avenae. dsRNA-OBP8 (7 µg/µL) oral feeding resulted in a 69% mortality, and 58% OBP8 reduced expression 8D post-feeding compared to control. Golden Gate cloning is used for RNA interference taking advantage of type IIs restriction enzyme Eco31I. ihpRNA-OBP8 introduced by agroinfiltration in Solanum tuberosum. Transgenic S. tuberosum fed Myzus show 57.6% mortality and 49% reduction in OBP8 expression 8D post-feeding, compared to control. This work proves OBP8 as promising ihpRNA targets in potato and related crops for whom Myzus is a destructive pest.

Key message

IhpRNAi-OBP8 construct is used for silencing OBP8 using pRNAiBAB7ihp developed in our lab. Aphids feeding on transgenic potato show mortality and reduced OBP8 expression.

Keywords

M. persicae · Odorant binding protein · ihpRNAi · dsRNAi · Agroinfiltration

Abbreviations

RNAi RNA interference
dsRNA Double-stranded RNA
siRNA Small interfering RNA
hpRNA Hairpin RNA
ihpRNA Intron-containing hairpin RNA
LIC Ligation independent cloning
PCR Polymerase chain reaction
GG Golden gate
OBPs Odorant binding proteins

Communicated by Henryk Flachowsky.
Introduction

With the growing world population, there is an intense need of increasing crop production. Bacterial, fungal, viruses, and insect pests are a threat to crop production. Hemipteran insects like Myzus persicae (Green peach aphid) adapted to varying environmental conditions by switching between the primary and secondary hosts, making it a successful pest, resulting in crop loss (Liu et al. 2020). It is a pest affecting more than 40 plant families for parthenogenetic and sexual reproduction; either by direct feeding damage to plants or by indirect transmittance by 100 plant viruses such as cucumber mosaic virus, and tobacco rattle virus (Faisal et al. 2019; Mulot et al. 2016). M. persicae RNAi targets are important against forty different host varieties, including S. tuberosum, Triticum aestivum, Hordeum vulgare, Brassica oleracea, B. napus, and S. lycopersicum.

Insecticides are not only the biggest threat to the environment but can also affect the non-target organisms and the beneficial soil microbes (Bass et al. 2014). Transgenic approaches are utilized but remain non-efficient because of the non/off-targets. Plant lines expressing the mt-2 and chitinase gene; resulted in nymph reduction and enhanced fecundity but were not efficient for all lines (Mottaghinia et al. 2011; Saguez et al. 2005, 2010). RNAi provides an alternative by oral administration, feeding on transgenic hosts, or siRNA microinjection in M. persicae (Mulot et al. 2016; Saguez et al. 2010; Tariq et al. 2019). Potential RNAi genes targeting in aphids is crucially essential for RNAi-mediated knockdown to assess the flexibility of the target gene and its off-target effects (Vogel et al. 2019). Double-stranded RNA (dsRNA), Small interfering RNA (siRNA), or hairpin RNA (hpRNA) mediated interference; reported for studying the gene function along with gene knockdown function (Liu et al. 2020; Mulot et al. 2016). Intron-containing hairpin RNA (ihpRNA) gene silencing initially involved the multiple restriction ligation steps, followed by gateway cloning with head to head or tail to tail combination without restriction ligation reactions (Wesley et al. 2001; Jiang et al. 2013). Ligation independent cloning (LIC) was not a single-step reaction as it requires two rounds of PCR (Yan et al. 2009), pRNAi-Golden gate (GG); with a single restriction-ligation step with a PCR product flanked with type II restriction enzyme recognition site, for ihpRNA the formation finally used (Marillonnet and Grutzner 2020; Xu et al. 2010; Yan et al. 2012). Here type II restriction enzyme is used for both sense and antisense orientations for ihpRNA of OBP8 gene in a single tube.

Plant-mediated gene silencing is a protective tool in agriculture against insects (Poreddy et al. 2017). To test a target for silencing agroinfiltration can give a good picture of whether to produce stable transformants or not (Hoffmann et al. 2006). RNAi reported against fungal aflatoxins and increasing iron availability in maize (Thakare et al. 2017), cucumber mosaic virus in tomato and tobacco (Ntui et al. 2014; Guo et al. 2021), rice stripe virus in Arabidopsis (Sun et al. 2020), whitefly (Raza et al. 2016), Fusarium head blight in wheat with β-1, 3-glucan synthase gene (Chen et al. 2016), F. graminearum in cereals (Jiao and Peng 2018), tomato chlorosis and yellow leaf curl virus in tomato (Jin et al. 2020) and Colletotrichum gloeosporioides in chilli and tomato (Mahto et al. 2020). F. graminearum α/β hydrolase gene silencing up to 50% using agroinfiltration assay in cereals is reported (Jiao and Peng 2018).

Odorant factors play a crucial role in the insect’s choice of food, host searching, mating, and defence, initiated by chemical signals/odorants detection from the environment (Carey and Carlson 2011; Reisenman et al. 2016). The first odorant binding protein (OBP) was reported in insects about 39 years ago; highly expressed in sensilla; chemo-receptive with considerable sequence diversity within members of the same family (Vogt and Riddiford 1981; Vieira and Rozas 2011). Insect feeding assay regulated by odorant detectors in the host plant and acts as potential RNAi targets (Swarup et al. 2011; Reisenman et al. 2016). M. persicae transcriptomics and genomic analysis show their important role in olfactory function (Wang et al. 2019). OBP2 serves as a potent target in RNAi-based gene inhibition in cotton aphids (Rebijith et al. 2016).

Literature has demonstrated deep insights into the roles of different families of OBPs (Venthur and Zhou 2018; Wang et al. 2019; Xue et al. 2016). The study has targeted the OBP8 gene as an RNAi target against M. persicae is not reported before. Here, we describe a rapid, single-step method for making ihpRNA constructs in new plant RNAi vector pRNAiBAB7ihp, introduced in potato. Promising cloning efficiency in the vector observed along with efficient transient OBP8 silencing and mortality in Myzus. The result suggested reliability and reproducibility for making ihpRNA-OBP8 resulting in peach potato aphid mortality. Agrobacterium-mediated in planta transformation, for transient OBP8 silencing in potato, was used.

Materials and methods

Insect sampling and rearing on potato plants

Potato leaves grown in different rural regions in north-east Punjab (Sialkot & Gujrat) were the source of M. persicae.
collection in 1.5 mL sterilized centrifuge tubes. *Solanum tuberosum* pure line Desiree (red variety) planted in plastic pots (LxW: 3 × 5") with a potting mixture containing mixing sand, clay, and farmyard manure in the ratio of 1:1:1. This potting mixture was sterilized with formalin for adult apherous *M. persicae* maintenance on 3-W-Old in vivo potato plants grown under white fluorescent light (300 µmol/m²/s, 16 h light/8 h dark at 22 °C and 70% relative humidity) and was covered with insect-proof mesh to avoid contamination. Adults from a single colonial lineage were transferred to fresh 3-W-Old potato plants and allowed to reproduce within 24 h. The new nymphs produced were transferred to another potato plant until becoming the third instar for RNA extraction.

**Total RNA extraction and complementary DNA (cDNA) synthesis**

*M. persicae* (50 mg), RNA extraction was performed by PureLink™ RNA Mini Kit (Cat # 12,183,018 A Thermo Scientific) (https://www.thermofisher.com/order/catalog/product/12183018A#/12183018A). Full-length cDNA produced from RNA via RevertAid First Strand cDNA Synthesis Kit (Cat # K1621; Thermo Scientific™) (https://www.thermofisher.com/order/catalog/product/K1621#/K1621).

**Reverse transcriptase polymerase chain reaction (RT-PCR)**

OBP8 partial mRNA FASTA sequences retrieved from NCBI for primer designing using Primer 3. Primers Tm, GC content, 3’ and 5’ modifications, and hairpin formation: confirmed on OligoCalc (Oligonucleotide Properties Calculator online) (Table 1). To amplify OBP8, reverse transcriptase-polymerase chain reaction (RT-PCR) was performed with 1.5 µL template cDNA, 2 µL Taq buffer, 1 µL MgCl₂, 0.5 µL dNTP mixture, 0.5 µL of forward and reverse primer, 1 µL Taq polymerase and 13 µL nanopure water. The RT-PCR reaction included denaturation at 95°C for 3 min, 35 cycles of annealing (94 °C for the 40 s, 55 °C for 45 s and 72 °C for 40 s), with the final extension carried out 72 °C for 10 min. 5 µL of product with 1 µL of 6× loading dye was loaded on 1% agarose gel along with a 1 kb DNA ladder.

**Cloning of OBP8**

OBP8 was purified from the gel by GenElute™ Gel Extraction Kit (Sigma Aldrich: NA1111-1KT) (https://www.sigmaaldrich.com/PK/en/product/sigma/na1111?context=product) and cloned in pTZ57R/T by Fermentas InsTA clone PCR Cloning Kit (Cat #K1214) (https://tools.thermofisher.com/content/sfs/manuals/MAN0012706_InSTAclone_PCR_Cloning_UG.pdf). DH5α competent cells primary culture prepared by heat shock method, placing individual colonies in a lysogeny broth liquid medium followed by incubation at 37 °C. Heat-Shock was used to transform pTZ57R/T ligated with target genes in competent cells. Plasmid preparation was performed; after colony PCR (GeneJET Plasmid Miniprep kit; Cat # K0502) (https://tools.thermofisher.com/content/sfs/manuals/MAN0012655_GeneJET_Plasmid_Miniprep_UG.pdf).

| Table 1 | Primers used in amplification of odorant binding protein 8 (OBP8), heat shock protein 60 KDa (HSP 60 KDa) and Actin |
| --- | --- |
| S. no. | Gene | Primers | Description |
| 1. | OBP8 F | TTAAAGTGGCGCTGCTCTGTC | OBP8 sequence in *M. persicae* |
| 2. | OBP8 R | TGGCTCTGAATTTCGGTGA | dsRNA assay |
| 3. | OBP8 promoter | TAAATACGACTACTATAGGGTTAAAGTGGCGCTGCTCTGTC | dsRNA assay |
| 4. | OBP8 promoter | TAAATACGACTACTATAGGGTGCGCTCTGAATTTCGGTGA | dsRNA assay |
| 5. | GFPF + promoter | GATCCTTAAATACGACTACTATAGGGTGCGCTCTGAATTTCGGTGA | dsRNA assay |
| 6. | GFPF + promoter | GATCCTTAAATACGACTACTATAGGGTGCGCTCTGAATTTCGGTGA | dsRNA assay |
| 7. | Actin-F | GGGTGTCCTCACACACAGTGCC | Negative control for RT PCR |
| 8. | Actin-R | CGGCCTGGGCTGGTGAAGCTG | |
| 9. | OBP8Res1 | ATTCGGCTCTCAATCGAGATCTGCTCTTATAGGG | Insertion into vector |
| 10. | OBP8Res2 | ATTCGGCTCTCAATCGAGATCTGCTCTTATAGGG | Insertion into vector |
| 11. | Primer 1 | GACGTAAAGGGATGACGCA | Confirmation of sense arm |
| 12. | Primer 2 | TTTTCACCCGAACACGCGC | Confirmation of sense arm |
| 13. | Primer 3 | TGCGAAATTCGGTGGTCAAG | Confirmation of antisense arm/Pdk intron |
| 14. | Primer 4 | CACCGCGCGCATAATT | Confirmation of antisense arm/Nos terminator |
Sequencing and multiple sequence alignment

OBP8-purified plasmid DNA (40 µL); was sent to Macrogen Korea for sequencing. Bioinformatics tools used after sequencing; for phylogenetic analysis, structural, and RNAi target prediction. The phylogenetic tree; was constructed to find out the evolutionary relationship with other aphid species using the Maximum Likelihood method via Phylogeny.fr (http://www.phylogeny.fr/simple_phylogeny.cgi). Multiple Sequence Alignment (MSA); was carried out to find conservancy among proteins of closely resembled aphid species (https://www.genome.jp/tools-bin/clustalw).

siRNA target prediction

Potential RNAi targets of OBP8 find by the ERNAi, an in-silico approach (Boutros lab, E-RNAi-Version 3.2) (Horn and Boutros 2010). It helped to find out suspected siRNA targets produced by dsRNA; their position, off-targets, and graphical view of designed targets (https://www.dkfz.de/signaling/e-rna3/evaluation.php).

Artificial feed stabilization and dsRNA assay

Control aphids percentage survival, was calculated for 8D with 20% sucrose used as a negative control. cDNA as a template was used, for OBP8-RTPCR, with primers having T7 promoter region (5’TATACTACGTATAG 3’) for optimum Tm (Table 1). PCR products after purification from 1% agarose gel; were used for the dsRNA preparation according to kit instructions (MEGAscript™ T7 Transcription Kit; Catalog number: AM1334). Single-stranded RNA along with DNA, removed from the transcription reaction by DNase I and RNase treatment (https://www.thermofisher.com/order/catalog/product/AM1334#/AM1334). dsRNA-OBP8 added to the artificial diet (15 ng/µL + 20% sucrose). M. persicae mortality assay performed in triplicate with ten aphids/replicate, used for the dsRNA feeding between 2-8D (Tariq et al. 2019).

OBP8 expression analysis by quantitative real-time PCR (RT-qPCR)

To analyze the OBP8 expression level before and after feeding dsRNA-OBP8, sucrose (negative control), and dsRNA-GFP (positive control), quantitative reverse transcription PCR (RT-qPCR), was performed. Primers for the OBP8, GFP, and actin gene as an internal control, were designed online using Primer 3 (Table 1). RT-qPCR reactions; were performed in an Applied Biosystems (Step One™ Real Time PCR: Applied Biosystems USA), using SYBR Green supermix PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific: Cat #: A25779) according to the protocol (https://www.thermofisher.com/order/catalog/product/A25779#/A25779) following the manufacturer’s instructions. PCR conditions were 95 °C for 10 min, 40 cycles with (95 °C (15 s), 65 °C (30 s), and 72 °C (30 s). RT-qPCR; was performed in triplicate for statistical analysis and confirmation.

RNAi vector construct for OBP8 silencing

Solanum tuberosum seeds (pure line Desiree) were planted in the potting mixture, containing equal amounts of sand, soil, and manure in the greenhouse under standard conditions (16 h light/8 h darkness at 25 ± 1 °C and 70% relative humidity). Agroinfiltration with Agrobacterium (EHA105) carrying ihpRNAi, performed on 4-week-old S. tuberosum pure line Desiree. Post-transcriptional gene silencing using long intron-containing plasmid constructs was found most effective in plants (Zhao et al. 2020). Agroinfiltration performed with EHA105; carrying pRNAiBAB7ihp vector, made in the Department of Biochemistry and Biotechnology. It had two copies for replacement of lac Z reporter gene, which when replaced can give white colonies on selection medium (Fig. 1). A unique set of primers were designed to add adapters to the amplified product of the gene of interest. Primers; were designed; in addition to the sequences of OBP8, the restriction site of enzyme Eco3I and the 5’ end an adapter sequence (enzyme restriction site) was added; for gene insertion in vector (Table 1). Restriction, and ligation was done in a single tube using 25 µL restriction ligation reaction; containing 75 ng plasmid, 75 µg PCR product, 0.1× Ligase buffer, 1000 U T4 DNA Ligase, and 30U Eco3I. The tube incubation was for 2 h; (37 °C and 23 °C consecutively) followed by 60 °C incubation (5 min) for enzyme inactivation. The resulting mixture was transformed into electro-competent cells of Escherichia coli, followed by blue-white screening. The white colonies were the recombinants with replaced LacZ gene. The gene insertion and correct orientation, confirmed by the PCR (Table 1; Fig. 3). Primer 1 and primer 4; were used for the confirmation of recombinants. Primer 1 and Primer 2; used for checking the sense; Primer 2 and Primer 4 were for antisense strand. Primer 3; was used to check the orientation of the pdk intron (Figs. 2 and 3). OBP8 gene presence as an inverted repeat flanking the pdk intron; was confirmed by sequencing with an M13 primer set. pRNAiBAB7ihp was transformed into Agrobacterium tumefaciens (EHA105) and was used for the agroinfiltration.

Infiltration procedures and to generation

The Agrobacterium strain; containing pRNAiBAB7ihp cultured in a lysogeny broth (LB) medium, with kanamycin (100 mg/L) and hygromycin (50 mg/L) at 28 °C, 200 rpm (OD600 = 0.6). The Agrobacterium cells, centrifuged at 3000×g and suspended in buffer (200 µM...
acetosyringone + OD600 = 0.2) for syringe agroinfiltration. The *Agrobacterium* transformed cells were injected in tobacco leaves using a syringe. Agroinfiltred *S. tuberosum*, taken as T₀ generation and leaves were harvested for RT-qPCR assay.

**OBP8 expression post *S. tuberosum* feeding**

RT-qPCR used for OBP8 expression analysis; after 2, 4, 6, and 8D of feeding transgenic *S. tuberosum* (T₀ plants). Primers for the OBP8, actin, and GFP genes used, are given in Table 1. RT-qPCR reactions performed (StepOne™ Real Time PCR: Applied Biosystems USA). RT-qPCR using PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific: Cat #: A25779) according to the protocol (https://www.thermofisher.com/order/catalog/product/A25779#/A25779). The experiment was done in triplicate using n = 50 *M. persicae* per replicate post-feeding. The reaction mixture consists of cDNA (25 ng), forward and reverse primers (0.5 µM), SYBR Green PCR master mix (10 µL) with a total volume of 20 µL. PCR conditions were 95 °C for 10 min along with 40 cycles (95 °C for 15 s, 60 and 72 °C for the 30 s).
**Statistical analysis**

*M. persicae* mortality with dsRNA feeding/artificial feeding assay in triplicate (20 aphids/replicate); statistically significant difference, was found with mean and standard deviation (STDEV) calculation on MS Excel. *M. persicae* mRNA expression after feeding control and the transgenic potato; was done in triplicate (40 aphids/replicate), followed by RT-qPCR. Means and STDEV used; for error bars in MS Excel. One-way analysis of variance (ANOVA); was used to find the significant difference between the positive control and dsRNA-artificial diet assay, control, and transgenic potato feeding assay. Different alphabets are representing the significant difference of the control with feeding assays.

**Results**

**GenBank IDs of OBP8 acquired after cloning**

RNA extraction, cDNA synthesis was followed by PCR. Tm optimized for OBP8 (465 bp) was at 54°C (Fig. 4B). OBP8 was purified and inserted in pTZ57R/T followed by the transformation in DH5α (Fig. S1A; S1B). OBP8 was confirmed; by colony PCR (Fig. S1C; S1D). OBP8 was sequenced, deposited in NCBI, and the accession number obtained was MN611704. The OBP8 gene analyzed by MSA was to identify the individual homology of this gene in the evolutionarily related aphid species. That can help to avoid the off-targets/broad-targeted approach for agronomic crops safety.

**MSA and phylogenetic analysis of OBP8 genes**

MSA and Phylogenetic analysis of *M. persicae*-OBP8 mRNA/protein present its alignment with *A. pismum, S. avenae, A. fabae, R. maidis*, and *Megoura vicieae (> 93%)* (Fig. 5, S2). MSA of OBP8 used to align the sequences
show resemblance to the 6 Hemipterans and one Homopterans (A. *pisum*). The resembling targets can be potential RNAi targets for all these aphid species (Black bean aphid, grain aphid, corn aphid, rose-grain aphid, and green aphid). The evolutionary history was speculated by a phylogenetic tree using the Maximum Likelihood method (Fig. 5).

**siRNA target prediction**

OBP8 potential siRNAi targets analyzed by the ERNAi; an in-silico approach. For the said purpose A. *pisum*, was selected in sequence identifier; because of *M. persicae*-OBP8 similarity (˃90%) with the A. *pisum*-OBP8 (Fig. 5, S2). The map produced consists of a gene sequence, transcript sequence of A. *pisum*, and followed by highlighted siRNA targets (Fig. S3; Table S1). siRNAs designed against target genes checked for the non-target effects; show no off-targets (Kola et al. 2015) (Fig. S3; Table S1).

**In-vitro RNAi via dsRNA feeding assay**

The highest mortality by the dsOBP8 gene was 69% 8D post-feeding in comparison to the positive (DsGFP) and negative control (20% sucrose) (Fig. 6a). ANOVA calculated for three replicates with aphids per replicate was 30; show significantly different results of aphid mortality in comparison to control (95% confidence interval) (Fig. 6a). The artificial diet is natural phloem sap containing essential amino acids, carbohydrates, minerals and sucrose. Sucrose (20–30%) for *M. persicae* artificial feeding assays; is reported (Tariq et al. 2019; Puterka et al. 2017).

**mRNA expression after 2, 4, 6 and 8 days of dsRNA feeding**

mRNA expression; was calculated after 2-8D of the ds-OBP8 feeding experiment (Fig. 6b). Ds-GFP was a positive control, and 20% sucrose without the dsRNA feeding was negative control. OBP8 expression was reduced to 30% 2D post-feeding and 50–58% 4-8D post-feeding. ANOVA calculated for three replicates with aphids per,

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**Fig. 5** Schematic diagram of ihpRNA construction with ihpRNA vector. The cassette of ihpRNA. The CaMV 35S enhanced promoter, two copies of OBP8 gene, the Pdk intron, Eco31I and an adapter sequence where the enzyme will cut were also added to the 5 prime end. Adapters with the same color have the same sequences but opposite orientation. The target fragment of the gene of interest is PCR amplified using gene-specific primers carrying Eco31I sites and adapters complementary to the appropriate sequences on the vector. The purified PCR product is mixed, in one tube, with ihpRNA vector, Eco31I enzyme and T4 ligase for a one-step restriction-ligation reaction.

**Fig. 6** Cloning of intron-containing hairpin RNA (ihpRNA) constructs. A Colony PCR of pRNAi-OBP8 using primes 2 and 4; which can amplify the two arms simultaneously with a difference of 357 bps in length (L2, L3, L4). Primer 1 and 2 amplified the sense strand (158 bps) (L6); while intron orientation confirmation is by Primer 3 and 4 with product size of 647 bps (L5 and L7), L8 is 1 kb Bioline DNA marker.
replicate was 30; show significantly different results of OBP8 expression in comparison to control (95% confidence interval) (Fig. 6b). OBP8 quantitative decrease expression by artificial diet assay is not reported before against *M. persicae*.

**ihpRNA constructs for OBP8 silencing**

Agrobacterium-mediated transformation; using T-DNA RNAi vector used, using ihpRNAi assembly. For the said purpose intron, was flanked with the sense and antisense strands of OBP8 in T-DNA. The vector gene sequence consists of the OBP8 gene under CaMV 35S promoter followed by the Pdk intron, OBP8 gene antisense gene and the Nos terminator (Fig. 2). Two Eco31I sites, followed by the adaptor sequence (marks the restriction site), flanking the OBP8 gene. The Eco31I on the left and right of the OBP8 gene are in different orientation resulting in the ihpRNAi construct. Amplification with primer 2 and 4; represent the correct orientation of antisense primer (Product size 357 bps). While the correct intron orientation; disclosed by primer 3 and 4 (Product size: 647 bps). The OBP8 sense strand orientation was deliberated by primer 1 and 2 (158 bps) (Fig. 3). PCR product of OBP8 genes with sense and antisense orientations; forms arms of a hairpin. Incubation of the purified PCR product and ihpRNA in the presence of the Eco31I enzyme and T4 ligase generates the desired vector (Fig. 3). The mixture was transformed in DH5α, followed by a selection of recombinants. ihpRNA-OBP8 was transformed into *Agrobacterium* for *S. tuberosum* transformation.

**Silencing of OBP8 by agroinfiltration**

Agroinfiltration assay had been widely used for transient assays and for checking RNAi efficacy; transient assays. Here also pRNAiBAB7ihp-OBP8 ability to gene silencing by Agrobacterium-mediated transient expression was checked. Different leaves of *S. tuberosum* (*T₀* plants); infiltrated by Agrobacterium tumefaciens (with pRNAiBAB7ihp-OBP8). *A. tumefaciens* mediated transformation is natural, easy, with a minimum number of gene copies integration (Afroz et al. 2011). OBP8 gene knock-down was quantified and confirmed by RT-qPCR. OBP8-mRNA; reduced to 49% compared to the control samples, 8D post-feeding (Fig. 7a). *M. persicae* show significant mortality of 57.6% in comparison to control 8D post-feeding (Fig. 7). ANOVA with a 95% confidence interval gave significantly different results for mortality assay in comparison to control. The OBP8-RNAi plants no 1, 3, and 5 show the highest reduction in OBP8 transcript levels and thus were selected for subsequent use in potatoes. Pdk intron in the vector had similar RE IIs sites at both ends. Intron-specific primers 3 and 4 confirmed the intron orientation (Figs. 2 and 3).

Fig. 7  **a** Double stranded RNA feeding along with control experiment for diet optimization. Effect of artificial diet optimized along with 7 µg/µL of Control and Odorant Binding Protein 8 (OBP8 gene) Gene 2D, 4D, 6D and 8D after feeding dsRNA. **b** Fold increase in mRNA expression (determined by of Quantitative Reverse Transcription PCR (RT-qPCR) for Odorant Binding Protein 8 (OBP8 gene) 2D, 4D, 6D and 8D post-feeding in comparison to negative control (*M. persicae* without dsRNA feeding assay). Means are significantly different at 0.05 probability in comparison to negative control
Discussion

RNA interference (RNAi) has become an indispensable tool for functional genomic studies by gene silencing across eukaryotes and plant systems. The implication of RNAi lies in the exploration of genes playing significant roles in the survival of insects. Two important pest management targets; that is environmentally friendly are OBP8s and odorant receptors are located in insect antennae. OBP8s are involved in odorants transport to receptors in a signal transduction pathway (Venturh and Zhou 2018). Severe insecticide applications of peach potato aphids caused the resistance to the wide range of insecticides (Bass et al. 2014). Identification of the active chemicals targets insect olfaction systems/silencing of the olfactory gene; cause the insect reluctance toward the important food crops; will change sexual behaviour (Carey and Carlson 2011). So the silencing of the OBP8s via dsRNA feeding assay is an important target to control peach potato aphids (Pitino et al. 2011).

In this study, pRNAiBAB7ihp is for silencing the OBP8 is designed and tested against *M. persicae* for the first time in one restriction ligation cloning step. ihpRNA construct; efficiently suppressed the OBP8 (up to 49%) and caused 57.6% mortality. ihpRNAi reported and highlighted; for targeted gene silencing and functional genomics. The cloning strategy based on type IIs restriction enzymes; help the assemblage of multiple DNA fragments in a restriction-ligation reaction (Marillonnet and Grutzner 2020). The adapter sequences employed; is smaller in comparison to the att adaptors used in previous protocols. The universal primer set for specific genes used for silencing will make it cost-effective. T4 DNA polymerase for plasmid construct; with single PCR reaction and cloning step in *E. coli* is required. The procedure did not require any PCR product sub cloning; resulted in targeted cloning along with good ihpRNA efficiency. This method will be proved promising for large-scale analysis of RNAi and mortality in peach potato aphids.

After the complete information display of the model aphid; aphid pathogens like peach aphid, grain aphid, potato aphid complete genomic/transcriptomic information are missing or unpublished; required for the comparative genomic analyses and RNAi targets prediction. OBP8 is an important RNAi target connected to aphid virulence against important economic crops (Reisenman et al. 2016; Joga et al. 2016). OBPs genes similarities to the aphid species; show similar host-aphid interactions (Figs. 5, S2). This study reports dsRNAi-OBP8 silencing using artificial feeding and transgenic potato transient assay; in *M. persicae*. The current research had successfully proved the role of dsRNA-OBP8 and ihpRNA-OBP8 target for *Myzus*-resistant potato.

ERNAi is an in-silico approach; used for OBP8-siRNAi targets prediction with pea aphid as a reference. MSA shows a 90% resemblance of *A. pisum*-OBP8 with *Myzus*-OBP8. MSA and phylogenetic analysis of OBP8 was more than 93% similar to *A. pisum, S. avenae, A. fabae, R. maidis*, and *M. vicieae*. The resembling targets can be potential RNAi targets for black bean, grain, corn, rose-grain, and green aphid (Kirfel et al. 2020). One of the biggest challenges in RNAi; is the identification of the targets that don’t interfere with the non-target species. Different bioinformatics tools are available for RNAi target prediction from the given gene sequence that identifies and compares this with all the genome databases available with the software. It will minimize the chances of non-target effects. As the siRNAs can match aphids/plant sequence; makes the chances of off-targets for most eukaryotic and prokaryotic genomes (Mogren and Lundgren 2017). By E-RNAi, 500 siRNA targets; for peach potato aphid with no off-targets are reported (with an efficiency score of 53.26 and 52.23) (Tariq et al. 2019). For successful RNAi, dsRNA size (140–500 nucleotides), stability, and effective uptake by the target species are important (Joga et al. 2016). In hemipteran species, extra-oral salivary dsRNAs degradation and blockage of cellular uptake is reported (Singh et al. 2017). These factors need to be addressed for efficient uptake of RNAi by the plants in field conditions to elicit RNAi (Joga et al. 2016). Eight days post-feeding, 59% OBP8 expression suppression was observed by the dsRNA-OBP8 gene compared to the control (Fig. 6a and b). OBPs are crucial for *Myzus* survival and pest management (Venturh and Zhou 2018). Zhang et al. (2013) reported 5 effective RNAi targets among the selected 16 up-and down-regulated genes. Tariq et al. (2019) reported a 2.5 fold decrease in dsMPnAv expression and 65.7% mortality in peach potato aphids. Pitino et al. (2011) reported knocked down of *M. persicae* transient MpCOO2 dsRNA expression in transgenic tobacco.

Faisal et al. (2019) had reported acetylcholinesterase 1 gene silencing of *M. persicae* had resulted in 37.5% and 26.4% lower fecundity in the tomato (var. Jamila and Tomaland) respectively. Prentice et al. (2016) reported dsRNA assay against Coleoptera class potato weevil and found 12 out of 24 targets to be as lethal as the positive control *Snf7*. Zhang et al. (2018) reported RNAi of glutathione S transferase against lepidopterans Asian corn borer and reported 54% mortality. Mulot et al. (2016) reported silencing against tobacco rattle virus source was *M. persicae*, of Eph and ALY transcripts was achieved using RNA hairpin targeting tobacco.

DS-RNAOBP8 assay was followed by cloning of the intron-containing inverted repeat inserts into the vectors, with ihpRNA-OBP8. Type IIs restriction enzymes Eco31I were used to produce the hairpin of the OBP8 gene and final elimination from the vector after digestion and ligation of products (Fig. 2). The mixture transformed directly to

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DH5α and recombinants containing both arms; were used for Agrobacterium-mediated transformation in *S. tuberosum*. Transformed potato plants (T0) show promising aphid mortality compared to the control (Fig. 8a–c). Agrobacterium-mediated transient expression of a transgenic tomato achieves the highest level in 2–3 days after Argoinfiltration, followed by a reduced expression (Voinnet et al. 2003). Saguez et al. (2010; 2005) reported RNAi of chitinases in potato transformation. Eco31I type IIs restriction enzyme site on both sides of intron resulted in recombinant ihpRNA vectors with intron with forward/reverse orientation. The intron reverse orientation in a recombinant ihpRNA was –ve for RNAi efficiency. RNAi vectors with spacer fragments with two introns in opposite orientation are important, but only one intron in the forward direction is reported (Marilolonnet and Grutzner 2020). But still, the effect of intron orientation on gene silencing is not confirmed. In summary, a rapid and reliable method for making ihpRNA constructs is developed; will prove promising for the large-scale analysis of plant functional genomics.

Conclusions

OBP8 gene was identified, cloned, sequenced and deposited in GenBank. OBP-8 DsRNAi based artificial feeding assay shows promising mortality and more than half reduction in OBP8 expression 8D post-feeding. The ihpRNA-OBP8 construct was found effective for silencing the OBP8 gene. The construct introduced in *S. tuberosum* with Agroinfiltration assay followed by feeding resulted in 57.6% mortality. Efficient construction of intron-containing OBP8-hpRNA vector with type IIs restriction enzyme reported.

Supplementary Information. The online version contains supplementary material available at https://doi.org/10.1007/s11240-021-02174-4.

Author contributions. Authors contributed in experimentation and its design is as follows. Experimental Design and Concept: [AA; UR, MFM]; Experiment execution [SA, AA]; Formal analysis and investigation: [MRK, MQSB]; First draft preparation, Funding source & Supervision [AA]; Editing: [SAK]; Resources: [NZ] and all authors read and approved the final manuscript.

Funding. The research funding is supported by National Research Program for Universities (NRPU 6506), HEC Pakistan at Department of Biochemistry and Biotechnology, University of Gujrat, Gujrat Pakistan.

Declarations

Conflict of interest. The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Consent to participate. The research does not involve human subjects, so consent for participation is not required in the manuscript.

Consent for publication. Corresponding author had taken consent from all co-authors to submission and publication of their data in Plant Cell Tissue and Organ Culture Journal.

Ethical statement. This article does not contain any studies with human participants or animals performed by the authors.

References

Afroz A, Chaudhry Z, Rashid U, Ali GM, Nazir F, Iqbal J, Khan MR (2011) Enhanced resistance against bacterial wilt in transgenic tomato (*Lycopersicon esculentum* cvs) lines expressing the Xa21 gene. Plant Cell Tissue Organ Cult 104:227–237. https://doi.org/10.1007/s11240-010-9825-2

Bass C, Puinean AM, Zimmer CT, Denholm I, Field LM, Foster SP, Gutbrod O, Nauen R, Slater R, Williamson MS (2014) The evolution of insecticide resistance in the peach potato aphid, *Myzus persicae*. Insect Biochem Mol Biol 51:41–51. https://doi.org/10.1016/j.ibmb.2014.05.003

Bwye AM, Proudlove W, Berlandier FA, Jones RAC (1997) Effects of applying insecticides to control aphid vectors and cucumber...
mosaic virus in narrow-leaved lupins (*Lupinus angustifolius*). Aust J Exp Agric 37:93–102

Carey AF, Carlson JR (2011) Insect olfaction from model systems to disease control. Proc Natl Acad Sci USA 108(32):12987–12995

Chen W, Kashtner C, Nowara D, Oliveira-Garcia E, Ruten T, Zhao Y, Deising HB, Kumlehn J, Schweizer P (2016) Host-induced silencing of *Fusarium culmorum* gene protects wheat from infection. J Exp Bot 67(17):4979–4991. https://doi.org/10.1093/jxb/erw263

Faisal M, Abel-Salam EM, Alatar AA, Saquib Q, Alwathnani HA, Canto T (2019) Genetic transformation and siRNA-mediated gene silencing for aphid resistance in tomato. Agronomy 9:893. https://doi.org/10.3390/agronomy9120893

Guo H, Ge P, Tong J, Zhang Y, Peng X, Zhihua Zhao Z, Ge F, Sun Y (2021) Elevated carbon dioxide levels decreases cucumber mosaic virus accumulation in correlation with greater accumulation of rgs-CaM, an inhibitor of a viral suppressor of RNAi. Plants 10:59. https://doi.org/10.3390/plants10100059

Hoffmann T, Kalinowski G, Schwab W (2006) RNAi-induced silencing of gene expression in strawberry fruit ( Fragaria × ananassa) by agroinfiltration: a rapid assay for gene function analysis. Plant J 48(5):818–826

Torn H, Boutros M (2010) E-RNAi: a web application for the multi-species design of RNAi reagents-2010 update. Nucleic Acids Res. https://doi.org/10.1093/nar/gkq317

Jiang Y, Xie M, Zhu Q, Ma X, Li X, Liu Y, Zhang Q (2013) One-step cloning of intron-containing hairpin RNA constructs for RNA interference via isothermal in vitro recombination system. Planta 238(2):325–330. https://doi.org/10.1007/s00425-013-1896-y

Jiao J, Peng D (2018) Wheat microRNA1023 suppresses invasion of *Fusarium graminearum* via targeting and silencing FGSG-03101. J Plant Interact 13(1):514–521. https://doi.org/10.1080/17429145.2018.1528512

Jin F-M, Song J, Xue J, Sun HB, Zhang Y, Wang S, Wang Y-H (2020) Successful generation of anti-ToCV and TYLCV transgenic tomato plants by RNAi. Biol Plant 64:490–496

Joga MR, Zotti MJ, Smagghe G, Christiaens O (2016) RNAi efficiency, systemic properties, and novel delivery methods for pest insect control: what we know so far. Front Physiol 7:553. https://doi.org/10.3389/fphys.2016.00553

Kirsch P, Skaljac M, Grotmann J, Kessel T, Seip M, Michaelis K (2020) Inhibition of histone acetylation and deacetylation enzymes affects longevity, development and fecundity in the pea aphid (*Acrithosiphon pisonum*). Arch Insect Biochem Physiol 103:e21614. https://doi.org/10.1002/arch.21614

Kola VSR, Renuka P, Madhav MS, Mangrauthia SK (2015) Key enzymes and proteins of crop insects as candidate for RNAi based gene silencing. Front Physiol 6:1–15

Liu S, Jaouannet J, Coustau C, Kogel K-H (2020) RNA-based technologies for insect control in plant production. Biotechnol Adv 39:107463

Mahto BK, Singh A, Pareek M, Rajam MV, Dhar–Ray S, Reddy PM (2020) Host-induced silencing of the *Colletotrichum gloeosporioides* conidial morphology 1 gene (*CgCOM1*) confers resistance against Anthracnose disease in chilli and tomato. Plant Mol Biol. https://doi.org/10.1007/s11103-020-01046-3

Marillonnet S, Grutzner R (2020) Synthetic DNA assembly using golden gate cloning and the hierarchical modular cloning pipeline. Curr Protoc Mol Biol 130:e115. https://doi.org/10.1002/cpmh.115

Mogren CL, Lundgren IG (2017) *In-silico* identification of off-target pesticial dsRNA binding in honey bees (*Apis mellifera*). Peer J 5:e4131. https://doi.org/10.7717/peerj.4131

Mottaghinia L, Razmjou J, Nouri-Ganbalani G, Rafiee-Dastjerdi H (2011) Antibiosis and antixenosis of six commonly produced potato cultivars to the green peach aphid, *Myzus persicae* Sulzer (Hemiptera: Aphididae). Neotrop Entomol 40(3):380–386

Mula M, Boissinot S, Monsion B, Rastegar M, Clavijo G, Halter D, Bochet N, Erdinger M, Brault V (2016) Comparative analysis of RNAi-based methods to down-regulate expression of two genes expressed at different levels in *Myzus persicae*. Viruses 8(11):316. https://doi.org/10.3390/v8110316

Ntui VO, Kong K, Azadi P, Khan RS, Chin DP, Igawa T, Mii M, Nakamura (2014) RNAi-mediated resistance to cucumber mosaic virus (CMV) in genetically engineered tomato. J Integr Plant Biol 5:554–572

Pitzino M, Coleman AD, Maffei ME, Ridout CJ, Hogenhout SA (2011) Silencing of aphid genes by dsRNA feeding from plants. PLoS ONE 6:e25709

Poreddy S, Li J, Baldwin IT (2017) Plant-mediated RNAi silences midgut-expressed genes in congeneric lepidopteran insects in nature. BMC Plant Biol 17(1):1–10

Prentice K, Christiaens O, Pertry I, Bailey A, niblett C, Ghislain M, Gheyseb G, Smagghe G (2016) RNAi-based gene silencing through dsRNA injection or ingestion against the African sweet potato weevil *Cylas puncticollis* (Coleoptera: Brentidae). https://doi.org/10.1002/pes.4337

Puterka GJ, Nicholson SJ, Cooper W (2017) Survival and feeding rates of four aphid species (*Hemiptera: Aphididae*) on various sucrose concentrations in diets. J Econ Entomol 110(4):1518–1524

Raza A, Malik HJ, Shafiq M, Amin, Scheffler JA, Scheffler BE, Scheffler BE, Mansoor S (2016) RNA interference based approach to down regulate osmoregulators of whitefly (*Bemisia tabaci*) potential technology for the control of whitefly. PLoS ONE 11(4):e0153883. https://doi.org/10.1371/journal.pone.0153883

Rebijih K, Asokan R, Hanede HR, Kumar NK, Krishna V, Vinutha J, Bakhavatsalam N (2016) RNA interference of odorant-binding protein 2 (OBP2) of the cotton aphid, *Aphis gossypii* (Glover), resulted in altered electrophysiological responses. Appl Biochem Biotechnol 178(2):251–266

Reiseman CE, Lei H, Guerenstein PG (2016) Neuroethology of olfactory-guided behavior and its potential application in the control of harmful insects. Front Physiol 7:271

Saguez I, Hainez R, Cherqui A, Wuytswinkel OV, Jeanpierre H, Lebon G, Noiraud N, Beaujean A, Jouanin L, Laberche J-C, Vincent C, Giordanengo P (2005) Unexpected effects of chinatines on the peach-potato aphid (*Myzus persicae* Sulzer) when delivered via transgenic potato plants (*Solanum tuberosum* Linne) and in vitro. Transgenic Res 14(1):57–67

Saguez J, Cherqui A, Lehraiki S, Vincent C, Beaujean A, Jouanin L, Laberche J-C, Giordanengo P (2010) Effects of mti-2 transgenic potato plants on the aphid *Myzus persicae* (Sternorrhyncha: Aphididae). Int J Agron. https://doi.org/10.1155/2010/653431

Singh JK, Singh S, Mogilicherla K, Shukla JN, Palli SR (2017) Comparative analysis of double-stranded RNA degradation and processing in insects. Sci Rep 7:17059. https://doi.org/10.1038/s41598-017-17134-2

Sun F, Hu P, Wang W, Lan Y, Du L, Zhou Y, Zhou T (2020) Rice stripe virus coat protein-mediated virus resistance is associated with RNA silencing in *Arabidopsis*. Front Microbiol 11:591619. https://doi.org/10.3389/fmicb.2020.591619

Swarup S, Williams TI, Anholt RR (2011) Functional dissection of Odorant binding protein genes in *Drosophila melanogaster*. Genes Brain Behav 10(6):648–657. https://doi.org/10.1111/j.1601-183X.2011.00704.x

Tariq K, Ali A, Emyr Davies TG, Naz E, Naz L, Sohail S, Hou M, Tariq K, Ali A, Emyr Davies TG, Naz E, Naz L, Sohail S, Hou M, Ullah F (2019) RNA interference-mediated knockdown of voltage-gated sodium channel (*Mpnav*) gene causes mortality in peach-potato aphid, *Myzus persicae*. Sci Rep 9:5291. https://doi.org/10.1038/s41598-019-41832-8

Thakare D, Zhang J, Wing RA, Cotty PJ, Schmidt MA (2017) Atla-toxin-free transgenic maize using host-induced gene silencing. Sci Adv 3:e1602382
Venthur H, Zhou J-J (2018) Odorant receptors and odorant-binding proteins as insect pest control targets: a comparative analysis. Front Physiol 9:116
Vieira FG, Rozas J (2011) Comparative genomics of the odorant-binding and chemosensory protein gene families across the Arthropoda: origin and evolutionary history of the chemosensory system. Genome Biol Evol 3:476–490. https://doi.org/10.1093/gbe/evr033
Vogel E, Santos D, Mingels L, Verdonckt TW, Broeck JV (2019) RNA interference in insects: protecting beneficials and controlling pests. Front Physiol 9:1912. https://doi.org/10.3389/fphys.2018.01912
Vogt RG, Riddiford LM (1981) Pheromone binding and inactivation by moth antennae. Nature 293(5828):161
Voinnet O, Rivas S, Mestre P, Baulcombe D (2003) An enhanced transient expression system in plants based on suppression of gene silencing by the p19 protein of tomato bushy stunt virus. Plant J 33:949–956
Wang Q, Zhou JJ, Liu JT, Huang GZ, Xu WY, Zhang Q, Chen JL, Zhang YJ, Li XC, Gu SH (2019) Integrative transcriptomic and genomic analysis of odorant binding proteins and chemosensory proteins in aphids. Insect Mol Biol 28(1):1–22. https://doi.org/10.1111/imb.12513
Wesley SV, Helliwell CA, Smith NA, Wang MB, Rouse DT et al (2001) Construct design for efficient, effective and high-throughput gene silencing in plants. Plant J 27:581–590
Xu G, Sui N, Tang Y, Xie K, Lai Y, Liu Y (2010) One-step, zero-background ligation-independent cloning intron-containing hairpin RNA constructs for RNAi in plants. New Phytol 187:240–250
Xue W, Fan J, Zhang Y, Xu Q, Han Z, Sun J, Chen J (2016) Identification and expression analysis of candidate odorant-binding protein and chemosensory protein genes by antennal transcriptome of Sitobion avenae. PLoS ONE 25(8):11. https://doi.org/10.1371/journal.pone.0161839
Yan P, Shen W, Gao X, Duan J, Zhou P (2009) Rapid one-step construction of hairpin RNA. Biochem Biophys Res Commun 383:464–468
Yan P, Shen W, Gao X, Li X, Zhou P, Duan J (2012) High-throughput construction of intron-containing hairpin RNA vectors for RNAi in plants. PLoS ONE 7(5):e38186. https://doi.org/10.1371/journal.pone.0038186
Zhang M, Zhou Y, Wang H, Jones HD, Gao Q, Wang D, Ma Y, Xia L (2013) Identifying potential RNAi targets in grain aphid (Sitobion avenae F.) based on transcriptome profiling of its alimentary canal after feeding on wheat plants. BMC Genom 14:560
Zhang Y, Zhang Y, Fu M, Yin G, Sayre RT, Pennerman KK, Yang F (2018) RNA interference to control asian corn borer using dsRNA from a novel glutathione-S-transferase gene of Ostrinia furnacalis (Lepidoptera: Crambidae). J Insect Sci 18(5):1–9. https://doi.org/10.1093/jisesa/iey100
Zhao S, Luo J, Zeng X, Li K, Yuan R, Zhu L, Li X, Wu G, Yan X (2020) Rolling circle amplification (RCA)-mediated genome-wide ihpRNAi mutant library construction in Brassica napus. Int J Mol Sci 21(19):7243

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