Intron-Containing Hairpin RNA Interference Vector for OBP8 Show Promising Mortality in Peach Potato Aphid

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Abstract

Myzus persicae is a devastating pest affecting potato production. RNA Interference technology is used against essential odorant binding protein 8 (OBP8) to enhance protection against *Myzus persicae* in potato. Gene was isolated, sequenced and GenBank IDs were allotted and 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 (7 µg/µl) oral acquisition had resulted in 69% mortality and 58% reduction in OBP8 expression 8D post dsRNA feeding in comparison to control. Golden Gate (GG) cloning based RNAigg is used for RNA interference taking advantage of type IIs restriction enzyme Eco31I. Agro infiltration assay was used for introduction of intron-containing hairpin RNA (ihpRNA) in *Solanum tuberosum*. Aphids feeding on transgenic *S. tuberosum* show 57.6% mortality and 49% reduction in OBP8 expression 8d post-feeding in comparison to control. This work proves OBP8 as promising ihpRNA targets in potato and related crops for whom *Myzus* is a devastating target.

Introduction

With the growing world population there is increasing need of increased crop production. Biotic stresses such as bacterial fungal, viral pathogens along with the insect pests are the big threat to crop production. Hemipteran insects like *Myzus persicae* (Green peach aphid) adapt to varying environmental conditions by switching between the primary and secondary hosts for its survival; thus responsible for great 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, tobacco rattle virus (Faisal et al. 2019; Bwye et al. 1997; Mulot et al. 2016). *M. persicae* RNAi targets can be used broadly as it fed on forty varieties of hosts, economically important included *S. tuberosum*, *Triticum aestivum*, *Hordeum vulgare*, *Brassica oleraceae*, *B. napus* and *S. lycopersicum*.

Insecticides are not only the biggest threat to the environment but can also affects the non-target organisms and the beneficial soil microbes (Bass et al. 2014). Transgenic approaches are not so efficient being nor targeted with plants exhibiting the mti-2 and chitinase gene show nymph reduction in a few lines/ enhanced fecundity (Mottaghinia et al. 2011; Saguez et al. 2005; 2010). RNAi is the only alternative and it had been successfully recorded by oral administration, or feeding on transgenic hosts or siRNA micro-injection in *M. persicae* (Mulot et al. 2016; Saguez et al. 2010; Tariq et al. 2019). The identification of potential genes 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). The RNA interference (RNAi) by double-stranded RNA (dsRNA), Small interfering RNA (siRNA), or hairpin RNA (hpRNA) had been 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 with multiple restriction ligation steps was initial 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 introduced; but

still it was not single step reaction as it requires the two rounds of PCR (Yan et al. 2009). It was followed by pRNAi-GG with single restriction-ligation step with a PCR product flanked with type IIs restriction enzyme recognition site for formation of ihpRNA (Marillonnet and Grutzner 2020; Xu et al. 2010; Yan et al. 2012). Here type IIs restriction enzyme is used for at both sense and antisense orientations for ihpRNA of OBP8 gene in single tube.

Genetic modification using RNAi had been reported for increasing iron availability using low phytic acid contents, against fungal aflatoxins in maize (Thakare et al. 2017), using partial replicase gene and elevated CO_2 level against cucumber mosaic virus in tomato and tobacco respectively (Ntui et al. 2014; Guo et al. 2021), rice stripe virus coat protein as RNAi target in *Arabidopsis* (Sun et al 2020), against whitefly using aquaporin and α glucosidase (Raza et al. 2016), against *Fusarium* head blight in wheat using β-1, 3-glucan synthase gene (Chen et al. 2016), against fungal gene in *Colletotrichum gloeosporioides* conidial morphology 1 gene (CgCOM1) in chilli and tomato (Mahto et al. 2020). Jiao and Peng (2018) reported against *F. graminearum* in silencing of α/β hydrolase gene infecting cereals with reduction in gene expression up to 30–50% using agroinfiltration assay. RNAi expression vector of tomato, the coat protein (CP) genes of tomato chlorosis virus (ToCV) and tomato yellow leaf curl virus were selected in this study (Jin et al. 2020).

Odorant factors play a crucial role in the choice of food, host searching, mating, and defense in insects; initiated by the detection of chemical signals/odorants 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). So, it is proposed that insect feeding assay is regulated by odorant detectors in the host plant and act as potential RNAi targets (Swarup et al. 2011; Reisenman et al. 2016). Three odorant binding proteins (OBPs) and chemosensory proteins were reported for their roles in insect sensory function. *M. persicae* transcriptomics and genomic analysis show their important role in their olfactory function (Wang et al. 2019). OBP2 is reported to serve 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 OBP8 gene as RNAi target against *M. persicae* not explained before.

Agrobacterium mediated transformation is a natural, easy, minimum number of copies of gene integration make it advantageous over the other methods. It had been reported extensively in monocots and dicots. Potato is a model crop to check the transformation efficiency; so will be used to check the transformation efficiency of the RNAiGG OBP8 based vector.

Materials And Methods

Insect sampling and rearing on potato plants

M. persicae was collected into 1.5 mL sterilized centrifuge tubes from potato leaves grown in different rural regions in the cities of north-east Punjab (Sialkot & Gujrat). *Solanum tuberosum* pure line Desiree (red variety) were sown in plastic pots (LxW: 3x5") with potting mixture containing mixing sand, clay, and farm yard manure in the ratio of 1:1:1. The mixture was then sterilized with formalin and adult apterous *M. persicae* (collected from fields) were maintained on 3-W-Old *in-vivo* potato plants grown under white fluorescent light (300 µmol m^{-2} s^{-1}, 16 h light/8 h dark at 22°C and 70% relative humidity) and were covered with insect-proof mesh to avoid contamination. Adults obtained from a single colonial lineage were transferred to fresh 3-W-Old potato plants and allowed to reproduce within 24h, after which the new nymphs were transferred to another potato plant; until reach the third instar. 50-100 mg of these aphids were used for RNA extraction.

Total RNA Extraction and Complementary DNA (cDNA) Synthesis

RNA from *M. persicae* was extracted by PureLink™ RNA Mini Kit (Cat # 12183018A; Thermo Scientific™). RNA was converted to full-length cDNA via RevertAid First Strand cDNA Synthesis Kit (Cat # K1621; Thermo Scientific™).

Polymerase Chain Reaction (PCR)

FASTA sequences of partial mRNA of OBP8 were retrieved from NCBI, and primers were designed using Primer 3. The primers were checked for their Tm values, GC content, 3' and 5' modifications and potential hairpin formation on OligoCalc (Oligonucleotide Properties Calculator online) (Table 1). To amplify OBP8 reverse transcriptase (RT) PCR reaction was carried out using Taq DNA Polymerase, recombinant (5U/µL) kit (Thermo ScientificTM). For the amplification of OBP8 cDNA, the initial denaturation step was carried out at 95ºC for 3 min, followed by 35 cycles of annealing involving heating at 94ºC for 40s, 55ºC for 45s and 72ºC for 40s. The final extension step was carried out 72ºC for 10 min. 5 µL of product with 1 µL of 6X loading dye was loaded on 1% agarose gel along with 1kb DNA ladder.

Cloning of OBP8

OBP8 was purified from gel by (GEL DNA Extraction Kit; Molecular Diagnostics) and cloned in pTZ57R/T by Fermentas InsTA clone PCR Cloning Kit (Cat #K1214). DH5α competent cells were prepared by heat shock method. The primary culture was prepared by placing individual colony in 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. After colony PCR plasmid preparation was done (GeneJET Plasmid Miniprep kit; Cat # K0502).

Sequencing and Multiple Sequence Alignment

Purified plasmid DNA (40µL); with gene of interest was sent to Macrogen Korea for sequencing. After sequencing, bioinformatics tools were used for phylogenetic analysis, structural and RNAi target prediction was done. Phylogenetic tree was constructed to find out evolutionary relationship with other

aphid species using Maximum Likelihood method via Phylogeny.fr. Multiple Sequence Alignment (MSA) was performed to find conservancy among proteins of closely resembled aphid species.

siRNA Target Prediction

Potential RNAi targets of OBP8 was find out by ERNAi tool using *in-silico* approach (Boutros lab, E-RNAi-Version 3.2) (Horn and Boutros 2010). Tool helped to find out suspected siRNA targets produced by dsRNA; their position, off targets and graphical view of designed targets.

Artificial feed stabilization and dsRNA Assay

Percentage survival of control sample was calculated for 8D on basis of time and sucrose concentration consumed by aphids. For dsRNA preparation: cDNA was used as a template for OBP8 gene amplification with primers attached with T7 promoter region (5'TAATACGACTCACTATAG 3') (Table 1). RTPCR was run for candidate genes amplification using T7 promoter containing primers with optimum T_m (57°C) for OBP8 (Table 1). These PCR products were then purified from 1% agarose gel as above and processed according to instructions (MEGAscript® kit (Ambion) and stored at -80°C for subsequent dietary experiments. DNA and single-stranded RNA were removed from the transcription reaction by DNase I and RNaseA treatment. For dsRNA feeding assays; dsRNA-OBP8 were added to the artificial diet (15ng/µL+ 20% Sucrose) without dsRNA as **-ve** control. Ten aphids replicated were used per dsRNA treatment along with control. The mortality rate was checked for aphids and nymphs between 2-8D (Tariq et al. 2019).

OBP8 expression analysis by Quantitative Real-Time PCR (RT-qPCR)

Quantitative reverse transcription PCRs (RT-qPCR) was performed to analyze the expression level of OBP8 before and after feeding dsRNAOBP8, sucrose and dsRNAGFP. Primers for the OBP8, GFP and Actin gene as internal control were designed online using Primer 3 (Table 1). RT-qPCR reactions were performed using SYBR Green supermix in an Applied Biosystems RT qPCR (Thermo Fisher Scientific), following the manufacturer's instructions. PCR conditions were 95°C for 10min, 40 cycles with (95°C (15s), 65°C (30s), and 72°C (30s)). The expression analysis was done in triplicate for statistical analysis and confirmation.

RNAi vector construct for OBP8 silencing

Solanum tuberosum seeds were planted under standard conditions in the potting mixture of sand, soil and manure in equal amounts in a greenhouse (16 h light/8 h darkness at 25±1^{o}C and 70% relative humidity). Plant mediated gene silencing can be used as a protective tool in agriculture for the control of insects (Poreddy et al. 2017). To test a target for silencing agroinfiltration can give a good picture whether to produce stable transformants or not (Hoffmann et al. 2006). The 4-week-old plants were used for agroinfiltration. Post transcriptional gene silencing by using a long intron containing constructs are found be most effective in plants (Zhao et al. 2020). In the present study construct, pRNAiBAB7ihp made in the Department of Biochemistry and Biotechnology, was used. For the insertion of gene in vector, primers were designed where in addition to the sequences of OBP8, restriction site of enzyme Eco31I and an

adapter sequence where the enzyme will cut were also added to the 5 prime end (Table 1). As with the Golden Gate (GG) technology, restriction and ligation can be done in a single tube. 25 µl restriction ligation reaction was prepared which contained 75ng of Plasmid, 75ng of PCR product, 0.1 X Ligase buffer, 1000 U of T4 DNA Ligase and 30U of Eco31I. The tube was incubated for two hours at 37˚C followed by two hours at 23^{o}C. Enzyme inactivation was done at 60^{o}C for 5 min. The mixture was transformed into electro competent cells of *Escherichia coli* followed by plating for blue white screening. The white colonies were the recombinants where LacZ gene was replaced. The insertion and correct orientation was confirmed by the Polymerase Chain Reaction (Table 1; Fig 5). Primer 1 and primer 4 were used for the confirmation of recombinants. Primer 1 and Primer 2 were used to check the sense strand while it's antisense by Primer 2 and Primer 4. Primer 3 was used to check the orientation of pdk intron (Fig 4; 5). OBP8 gene presence as an inverted repeat flanking the pdk intron was confirmed by sequencing with M13 primer set. pRNAiBAB7ihp was transformed into *Agrobacterium tumefaciens* (EHA105) and was used for the agro infiltration.

Infiltration Procedures and To Generation

The *Agrobacterium* strain containing pRNAiBAB7ihp was cultured in lysogeny broth medium containing kanamycin (100 mgL^{-1}) and hygromycin (50 mgL^{-1}) was cultured at 28^{o}C at 200 rpm until OD_{600} reached 0.6. The *Agrobacterium* cells were centrifuged at 3000g followed by suspension in buffer containing 200 µM acetosyringone and OD_{600} of 0.2; used for syringe agroinfiltration. The *Agrobacterium* transformed cells were injected in tobacco leaves using a syringe. Agroinfiltred *S. tuberosum* was taken as T_o generation; leaves were harvested for RT-qPCR assay.

OBP8 expression post *S. tuberosum* feeding

Expression of OBP8 was analyzed after 2, 4, 6 and 8 days feeding of transgenic potato by RT-qPCR. Primers for the OBP8, actin and GFP genes were used as before in dsRNA feeding assays (Table 1). RT-qPCR reactions were performed using Applied Biosystems RT qPCR using SYBR Green PCR Supermix (Thermo Fisher Scientific) according to the protocol. The experiment was done in triplicate using n= 50 *M. persicae* per replicate post feeding. The reaction mixture consist of cDNA (25 ng), forward and reverse primers (0.5 µM), SYBR Green PCR master mix (10 µL), and cDNA template (2 µL) with total volume of 20 µL. PCR conditions were 95°C for 10 min along with 40 cycles (95°C for 15s, 60°C and 72°C for the 30s).

Statistical Analysis

Mortality using artificial diet feeding assay statistically significant difference was find out by using MS Excel by calculation of the means and standard deviation (STDEV) among the 3 replicate experiment using 20 aphids per replicate. The mRNA expression after ds RNA feeding of the artificial diet and the transgenic potato was done in triplicate. Again 40 aphids per replicate were used to collect mRNA followed by RT qPCR. STDEV was used for the error bars in MS Excel. One-way analysis of variance (ANOVA) was used to find out the significant difference between the **+ve** control and artificial diet dsRNA

assay as well 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, and cDNA synthesis was followed by PCR and T_m for OBP8 (465 bp) was found 54ºC (Fig 1B). OBP8 were purified and inserted in pTZ57R/T followed by transformation in DH5α (Fig S1A; S1B). Colony PCR was conducted for confirmation of target genes (Fig S1C; S1D). OBP8 was sequenced and deposited in NCBI and the accession numbers obtained was (MN611704). The identified genes sequences were analyzed by MSA to find out individual homology of these genes in evolutionarily related aphid species for broad-targeted approach in potato crops safety.

MSA and Phylogenetic analysis of OBP8 genes

MSA and Phylogenetic analysis of OBP8 encoding mRNA/protein from *M. persicae* was found to be aligned greatly to *A. pisum*, *S. avenae*, *A. fabae, R. maidis* and *Megoura viciae* (>93%) (Fig 2, 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 target for all of these aphid species (Black bean aphid; grain aphid, corn aphid, rose-grain aphid, large green aphid). The evolutionary history was inferred with phylogenetic tree using Maximum Likelihood method (Fig 2).

siRNA Target Prediction

Potential siRNAi targets of OBP8 was find out by ERNAi tool an *in-silico* approach. For OBP8 siRNAi targets finder map included *A. pisum* found in the submitted sequence was of OBP8 of pea aphid (Zhou et al. 2010). It was followed by transcript sequence; which is hypothetical protein of *A. pisum* in map (Fig S3; Table S1). As pea is a model plant and MSA show resemblance of the *Myzus* OBP8 more than 80% to *A. pisum*. SiRNAs designed against target genes were cross-checked for non-target effects using model pea plant having 90% homology with *Myzus* (Kola et al. 2015) (Fig S3; Table S1).

In-vitro RNAi via dsRNA Feeding Assay

Highest mortality of was observed by dsOBP8 gene (69%) in comparison to the **+ve** (DsGFP) and **-ve** control (20% sucrose) 8d post-feeding (Fig 3a). ANOVA was calculated with 95% confidence interval for 3 replicates with number of aphids per replicate was 30. ANOVA show significantly different results for mortality assay in comparison to control presented by different alphabets (Fig 3a). Identification of OBP8 quantitative decrease in their expression by artificial diet assay is not reported before against *M. persicae*. The artificial diets are based on the natural phloem sap containing essential amino acids, carbohydrates, minerals and sucrose. Sucrose diet (20-30%) is reported for maximum survival of *M. persicae* (Tariq et al. 2019; Puterka et al. 2017).

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

mRNA expression of said genes was quantified after 2-8D of feeding experiment (Fig 3b). GFP was taken as **+ve** control along with **-ve** control without the dsRNA feeding. OBP8 expression was reduced to 30% 2D post-feeding followed by 50-58% 4-8D post-feeding respectively. ANOVA was calculated with 95% confidence interval for 3 replicates with 25 aphids per replicate. ANOVA show significantly different results for OBP8 gene expression in comparison to control presented by different alphabets (Fig 3b).

ihpRNA constructs for OBP8 silencing

Agrobacterium mediated transformation using T-DNA RNAi vector was used using GG assembly. For the said purpose intron region was flanked with the sense and antisense strands of OBP8 in TDNA. The vector gene sequence consist of OBP8 gene under CaMV 35S promoter followed by the Pdk intron, OBP8 gene antisense gene and the Nos terminator (Fig. 4). Two Eco31I sites are 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 hpRNAi construct. Amplification with primer 2 and 4 show the correct orientation of antisense primer (Product size 357 bps). While the intron correct orientation is shown by primer 3 and 4 (Product size: 647 bps) and sense strand orientation of OBP8 is shown by primer 1 and 2 (158 bps) (Fig 5). PCR product of OBP8 genes with sense and antisense orientations; forms arms of hairpin. Incubation of the purified PCR product and pRNAi-GG in the presence of Eco31I enzyme and T4 ligase generates the desired vector (Fig 5). The mixture was transformed into DH5a; and only recombinants were recovered. pRNAi-GG constructs were transformed directly into *Agrobacterium* for *S. tuberosum* transformation.

Silencing of OBP8 by agroinfiltration

Agroinfiltration assay had been widely used for transient assays as well as to check the efficacy of RNAi constructs mediated silencing. Here also pRNAiBAB7ihp-OBP8 ability to gene silencing by *Agrobacterium*-mediated transient expression was checked. *Agrobacterium* cultures, (with pRNAiBAB7ihp -OBP8) were infiltrated to different leaves of *S. tuberosum* To plants. To confirm the knock-down of the OBP8 gene at the molecular level, RT-qPCR was performed. The results presented that OBP8 mRNA levels were reduced to 49%; compared to the control samples 8d post-feeding (Fig. 6a). Mortality of *M. persicae* was observed 57.6% 8d post-feeding (Fig 6b; 7). ANOVA with 95% confidence interval gave significantly different results for mortality assay in comparison to control shown by different alphabets. The OBP8-RNAi plants no 1, 3 and 5 show greatest reduction in OBP8 transcript levels and thus were selected for subsequent use in potato. Pdk intron in the vector had similar RE IIs sites at both ends. The intron direction was confirmed by the PCR by intron specific primer 3 and 4 (Fig 4; 5).

Discussion

RNAi has become an indispensable tool for gene silencing studies across a wide range of eukaryotes and is now extended to plant systems for functional genomic studies. The implication of RNAi lies is the

exploration of vital genes playing significant roles in the survival of insects. RNA silencing induced by hpRNA is one of the most important tools for targeted gene silencing and studying functional genomics. In this study new pRNAiBAB7ihp is made, for making silencing OBP8 gene in *M. persicae* for the 1st time in one restriction ligation cloning step. ihpRNA constructed based on GG cloning method, efficiently suppressed the OBP8 genes (up to 49%) and caused 57.6% reduced expression. Cloning strategy is based on type IIs restriction enzymes helps assemblage of multiple DNA fragments in a restriction-ligation reaction (Marillonnet and Grutzner 2020). The adaptor sequences used over here are smaller in comparison to the att adaptors used in previous protocols along with the universal primer set for specific gene used for silencing will make it cost effective. Along with this study just require T4 DNA polymerase for plasmid along with single PCR reaction and single cloning step in E.coli is required. Procedure did not requires 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 plant physiology in response to the RNAi.

This is a normal protocol that after the complete information display of the whole model aphid species; other important pathogen aphid species like peach aphid, grain aphid, potato aphid; which can help not only for the comparative genomic analyses but also for most suitable RNAi targets. Odorant binding proteins were reported to be important for establishment of aphid virulence against important economic crops (Reisenman et al. 2016; Joga et al. 2016). It had been reported that these genes had similarity with many aphid species show the common method for host aphid interaction (Fig. 2, S2). In this study, dsRNAi-based silencing using artificial feeding and transgenic potato transient assay of OBP8, is reported in *M. persicae* for the sustainable protection of potato crops. The current research had successfully manipulated the role of OBP8 dsRNA and OBP8ihpRNA target for *Myzus*-resistant potato.

ERNAi tool an *in-silico* approach is used for OBP8 siRNAi targets prediction; with pea as reference; as MSA show 90% resemblance 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. viciae*. The resembling targets can be potential RNAi target for black bean aphid; grain aphid, corn aphid, rose-grain aphid, and large 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. So that will minimize the chances of non-target affects (Mogren and Lundgren 2017). As the siRNAs can have the match with many consensus sequences; that makes the chances of off-targets for most eukaryotic and prokaryotic genomes (Qiu et al. 2005). E-RNAi analysis tool was reported for the design of dsRNA in peach potato aphid and five hundred siRNAs with efficiency score of 53.26 and 52.23; so with possibility of off-target effects (Tariq et al. 2019). dsRNA size of 140–500 nucleotides along with its stability and effective uptake by the target species are important for successful RNAi (Joga et al. 2016). In hemipteran species extra-oral salivary degradation of dsRNAs 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). 69% mortality and 59% reduction in OBP8 expression was observed by dsOBP8 gene was observed in comparison to

the control 8d post-feeding (Fig. 3a, 3b). OBPs generally play an important role for the survival of insects and had been explored as crucial target for beneficial pest management (Venthur 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 aphid. 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 + ve 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 GG strategy for cloning of the intron-containing inverted repeat inserts into the vectors, with pRNAi-GG. Type IIs restriction enzymes Eco31I was used to produce the hairpin of OBP8 gene and final elimination from the vector after digestion and ligation of products (Fig. 4). The mixture was transformed directly into DH5a, and recombinants containing both arms were recovered followed by transformation in *Agrobacterium* for *S. tuberosum* transformation. Transformed potato To plants show promising mortality of aphids in comparison to the control plants (Fig. 7a, b, c). *Agrobacterium*- mediated transient expression of a transgenic tomato achieves highest level in 2–3 days after Argo infiltration; followed by decrease of its 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. Similar result was observed in the vector based on the GATEWAY system. The reverse orientation of intron in recombinant ihpRNA was considered negative for silencing efficiency; RNAi vectors with spacer fragments with two introns in opposite orientations was reported for atleast one intron in forward orientation (Marillonnet and Grutzner 2020). But still effect of intron orientation on gene silencing is not confirmed. In summary, rapid and reliable method for making ihpRNA constructs is developed; will prove promising for the large-scale analysis of plant functional genomics.

Conclusion

Efficient construction of intron-containing hpRNA (ihpRNA) vector with type IIs restriction enzyme based on the GG cloning. Is reported. A single PCR product of the gene of interest flanked with Eco31I recognition sequence, was cloned in pRNAi-GG at both sense and antisense orientations for formation of ihpRNA construct. RNAi GG assembly was found affective for interfering OBP8 followed by its introduction in *S. tuberosum* with Agro infiltration assay. This method provides a novel and high-throughput platform for large-scale analysis of plant functional genomics. DsRNAi assay artificial feeding assay show promising mortality and more than half reduction in OBP8 expression 8d post

feeding. While agroinfiltration assay followed by transgenic *S. tuberosum* feeding show 57.6% mortality. MSA of *M. persicae* show conserved amino acid sequences with hemipterans and homopterans for OBP8. The phylogenetic analysis supports OBP8 ihpRNA target for black bean aphid; grain aphid, corn aphid, rose-grain aphid, and large green aphid.

Abbreviations

RNA interference (RNAi); Double-stranded RNA (dsRNA); Small interfering RNA (siRNA); Hairpin RNA (hpRNA); intron-containing hairpin RNA (ihpRNA); Ligation independent cloning (LIC); Golden Gate; (GG); Odorant Binding Proteins (OBPs); Multiple Sequence Alignment (MSA), Quantitative Real-Time PCR (RT-qPCR).

Declarations

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Declaration of interests

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.

Author Contributions

Authors contributed in experimentation and its design is as follows. Experimental Design and Concept: [Amber Afroz; Umer Rashid, Muhammad Faheem Malik]; Experiment execution [Safeena Aslam, Amber Afroz]; Formal analysis and investigation: [Muhammad Ramzan Khan, Muhammad Qasim Shahzad Butt]; First draft preparation, Funding source & Supervision [Amber Afroz]; Editing: [Sabaz Ali Khan]; Resources: [Nadia Zeeshan] and all authors read and approved the final manuscript.

Ethical Statement

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

Consent to participate

The research do 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.

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Tables

Table 1: Primers used in amplification of odorant binding protein 8 (OBP8), heat shock protein 60KDa (HSP 60KDa) and Actin.

S. No	Gene	Primers	Description
1.	OBP8 F	TTAAAGTGGCGTGTCTGTGC	OBP8 sequence in *M. persicae*
2.	OBP8 R	TGCGTCTGAATTTCGGTGAA	
3.	OBP8F+Promoter	TAATACGACTCACTATAGGG TTAAAGTGGCGTGTCTGTGC	dsRNA assay
4.	OBP8R+Promoter	TAATACGACTCACTATAGGG TGCGTCTGAATTTCGGTGAA	
5.	GFPF+ Promoter	GGATCCTAATACGACTCACTATAGGAAGAGTGCCATGCCCGAAGGT	dsRNA assay
6.	GFPR+ Promoter	GGATCCTAATACGACTCACTATAGGAAAGGACAGGGCCATCGCCAA	
7.	Actin-F	GGTGTCTCACACACAGTGCC	-ive control for RT PCR
8.	Actin-R	CGGCGGTGGTGGTGAAGCTG	
9.	OBP8Res1	ATTCGGTCTCAATCGGATGTTCGTTCTTAAAGTGG	Insertion into vector
10.	OBP8Res2	ATTCGGTCTCGGCTAACGTCGATGAGGTCCTTTGCCTT	Insertion into vector
11.	Primer 1	GACGTAAGGGATGACGCACA	Confirmation of sense arm
12.	Primer 2	TTTTCACCGAAAACGACGGC	Confirmation of sense arm
13.	Primer 3	TGGGAAATTGGGTTCGAAGG	Confirmation of antisense arm/Pdk intron
14.	Primer 4	CACCGCGCGCGATAATTT	Confirmation of antisense arm/Nos terminator

Figures

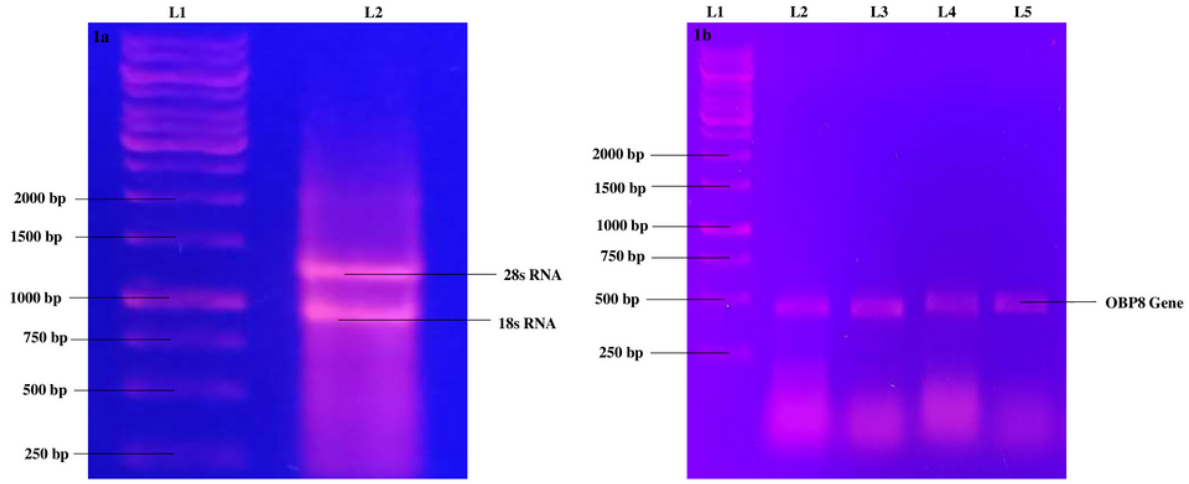

Fig 1

Figure 1

(1A) RNA extraction of Myzus persicae (PureLink™ RNA Mini Kit) (L1) 1 Kb ladder; (L2) 18S, 28S RNA (1B) Reverse Transcriptase Polymerase chain reaction (RTPCR) of Odorant Binding Protein 8 (OBP8 gene) (455 bp) (L1) 1 Kb ladder, (L2, L3, L4, L5) OBP8 gene.

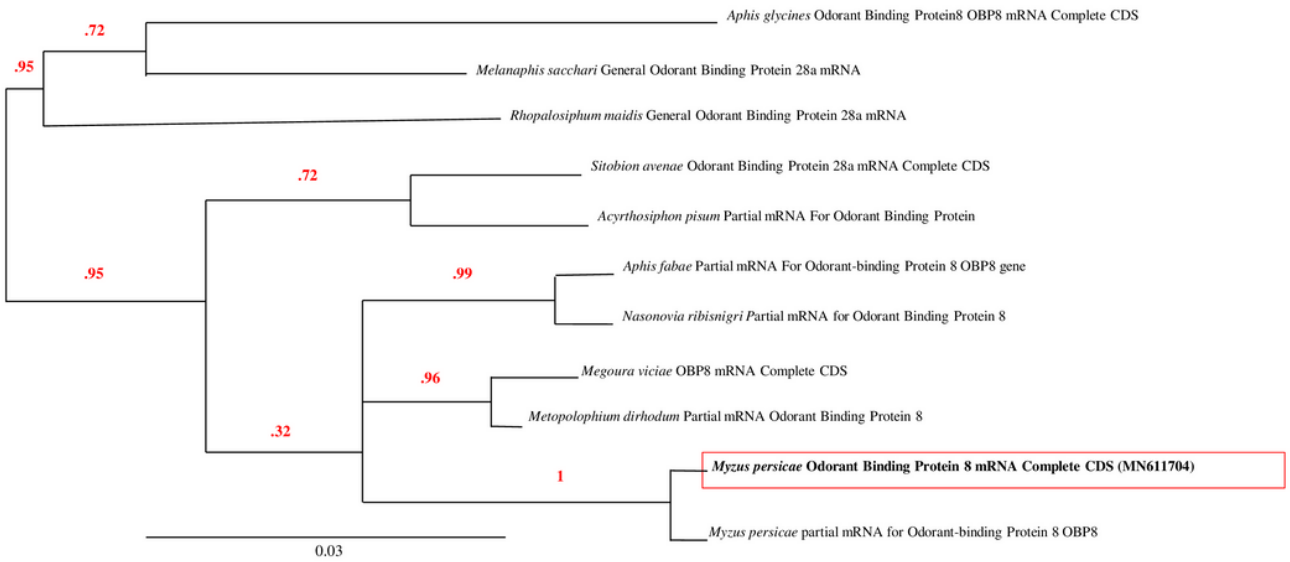

Fig 2

Figure 2

Phylogenetic tree of Odorant Binding Protein 8 (OBP8 gene) after BLAST search and selection of closest homology (more than 83%) by phylogeny.fr with molecular sequences of related and homologous organisms.

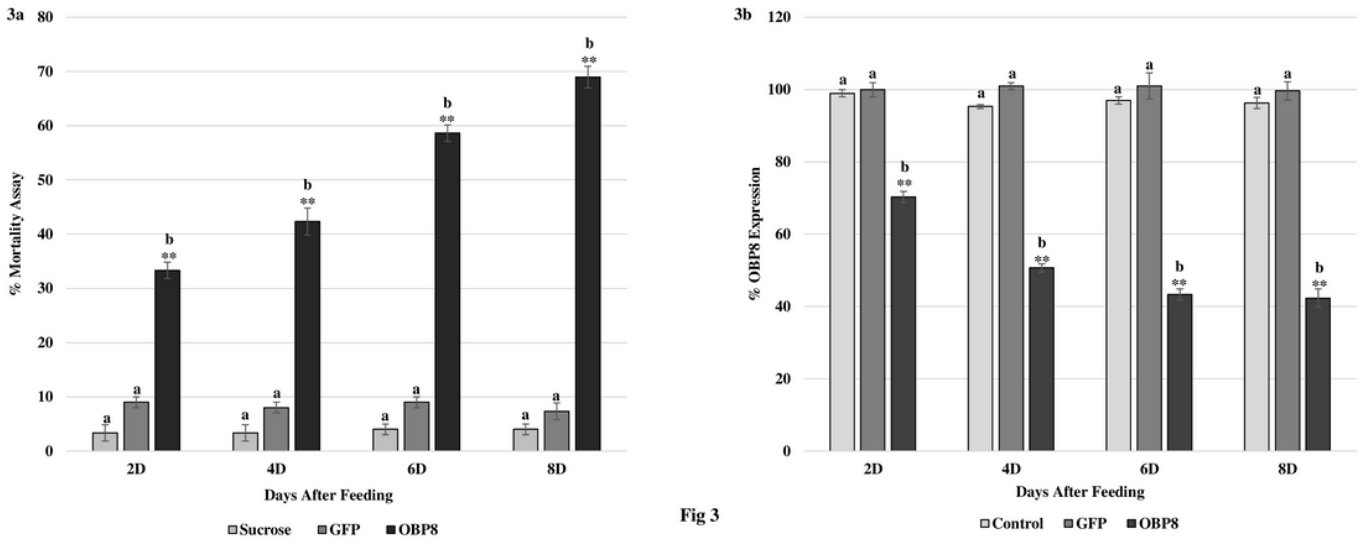

Fig 3

Figure 3

(3a) Double stranded RNA feeding along with control experiment for diet optimization. Effect of artificial diet optimized along with 7 µg/µl of Control (GFP) gene and Odorant Binding Protein 8 (OBP8 gene) Gene 2D, 4D, 6D and 8D after feeding dsRNA (3b) Percentage increase in mRNA expression (determined by of Quantitative Reverse Transcription PCR (qRT-PCR) for Odorant Binding Protein 8 (OBP8 gene) 2D, 4D, 6D and 8D post-feeding in comparison to +ve control (GFP) and -ve control (M. persicae without dsRNA feeding assay). Means are significantly different at 0.05 probability in comparison to GFP (+ve control) and (-ve) control.

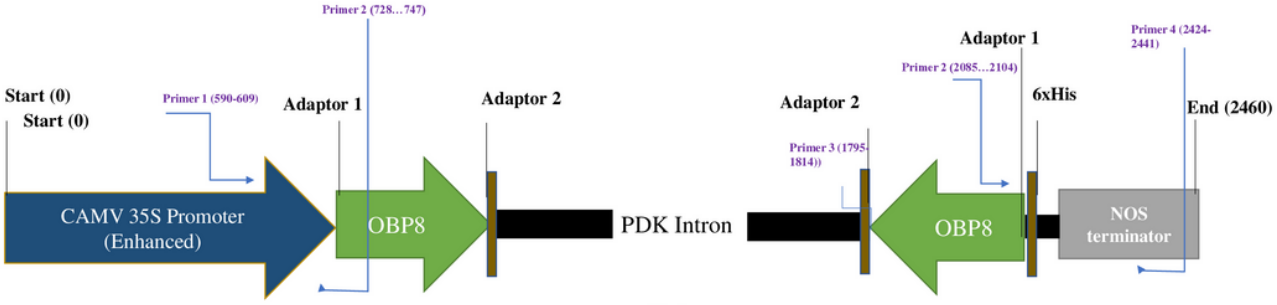

Fig 4

Figure 4

Schematic diagram of ihpRNA construction with pRNAi-GG (Golden Gate) vector. The cassette of pRNAi-GG. 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. Adaptors 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 adaptors complementary to the appropriate sequences on the vector. The purified PCR product is mixed, in one tube, with pRNAi-GG vector, Eco31I enzyme and T4 ligase for a one-step restriction-ligation reaction.

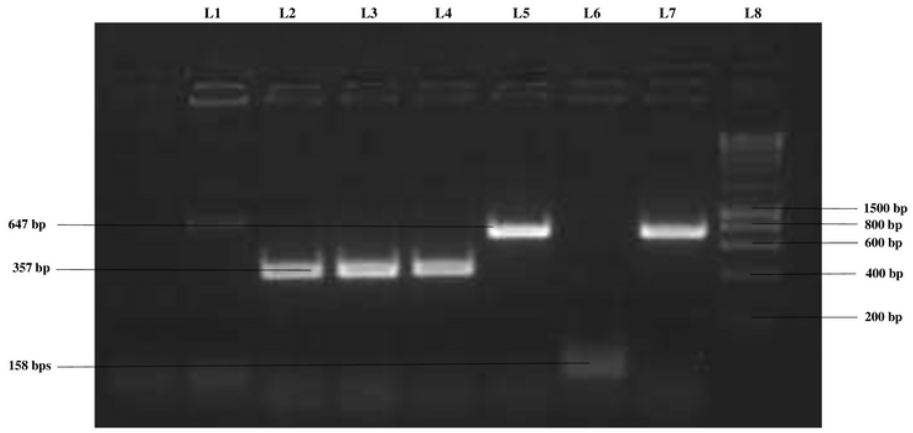

Fig 5

Figure 5

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 I kb Bioline DNA marker.

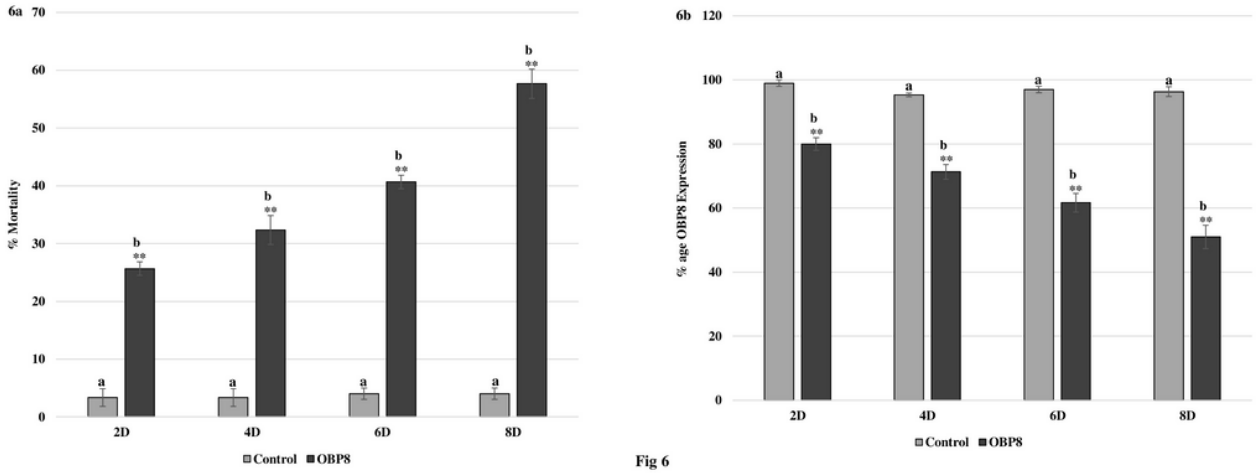

Fig 6

Figure 6

(6a) 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 (6b) Fold increase in mRNA expression (determined by of Quantitative Reverse Transcription PCR (qRT-PCR) for Odorant Binding Protein 8 (OBP8 gene) 2D, 4D, 6D and 8D post-feeding in comparison to -ve control (M. persicae without dsRNA feeding assay). Means are significantly different at 0.05 probability in comparison to -ve control.

Fig 7

Figure 7

(7a) M. persicae on 3-W-Old Solanum tuberosum pure line Desiree control potato plant (7b) Myzus persicae nymphs (7c) M. persicae after eating transgenic Solanum tuberosum pure line Desiree.

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