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Identifying potential RNAi targets in aphid species (*Macrosiphum rosae, Rhopalosiphum maidis, Sitobion avenae, Toxoptera aurantii*) in sub-tropical region with extreme summer

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Abstract

RNA interference is a useful and efficient tool that had been used to incorporate tolerance against different stresses. Five unigene sequences were selected from exotic grain aphid that were reported to be an ideal RNAi targets. Aphids (*Macrosiphum rosae, Rhopalosiphum maidis, Sitobion avenae, Toxoptera aurantii*) were reared on *Rosa indica, Zea mays, Triticum aestivum, Chrysanthemum hibiscus, Solanum melongena, Abelmoschus esculentus*. PCR of unigene 28469 and 21789 was positive for all aphids and there is no ortholog found for them so unique and effective to be used in RNAi technology. Cytochrome c oxidase was found to be positive for *M. rosae, S. avenae, T. aurantii* and negative for *R. maidis.* Zinc finger protein was found to be positive for *R. maidis, S. avenae* and negative for *M. rosae*, and *T. aurantii.* Cuticular proteins was found to be positive for *S. avenae* and *M. rosae* and negative for *R. maidis* and *T. aurantii*. Genes identified in the aphids are defense and as important structural genes and their suppression with RNAi technology will be important target to have insect resistant crops. From these gene sequences cytochrome c oxidase is reported as bar coding gene and can be used in future for interspecific genetic variation in these aphids. Results revealed the high expression of these sequences in local aphid species and can be used as RNAi target for them. That can be used in future or applications as pest management, monitoring and plant quarantine.

Keywords: Aphids**,** *Sitobion avenae*, RNA interference, Cytochrome c oxidase.
Abbreviations: RNA interference (RNAi); *Bacillus thuringiensis* (Bt); Double stranded RNA (dsRNA); messenger RNA (mRNA); RNA-dependent RNA polymerase (RdRP); Cytochrome c oxidase (CO-1); Cuticular proteins (CPs).

Introduction

Aphids are chief agricultural pests, plays an important role in the destruction of crops including cereals, oilseeds, timber and fruit and important medical plants all over the world through the direct effects of feeding and by vectoring debilitating plant viruses (Dedryver et al., 2010; Bhatia et al., 2011). Annual worldwide crop losses due to aphids are estimated at hundreds of millions dollars (Blackman and Eastop, 2000; 2006, Oerke et al.,1994). 11 subfamilies and 4,400 species of aphids, which are known all belongs to the family aphidoidea, and among them 250 species are serious pests and causes yield loss (Blackman and Eastop, 2006). One of the important species infesting wheat is grain aphid, most dominant and destructive, affecting 65% of wheat production areas in China, Europe and America (Stoger et al., 1999; Zhang et al., 2009). Small amount of data is available for the Homoptera order of aphids *Diuraphis noxia*, *Schizaphis graminum* and *Rhopalosiphum padi* affecting wheat crops in hot tropical areas of Punjab (Muhammad et al., 2013; Khan et al., 2012; Aslam et al., 2004; Shahzad et al., 2013). Selection of the RNAi targets for grain aphid is constraint because of lack of genomic information and small alimentary canal (Xu et al., 2014). Agrochemical insecticides are intimidated by development of insecticide resistance, human health, environmental hazards, non-specificity, and toxicity (Usta, 2013). Therefore, targeted alternative is the need of the hour. For the last few decades, conventional breeding programs have been undertaken in an attempt to increase wheat aphid resistance worldwide (Stoger et al., 1999). However, due to the lack of effective aphid resistant germplasm, the complexity of plant-aphid interactions and the rapid development of resistant pest biotypes, occurrence of aphids causing substantial losses of wheat continue to be reported regularly. Breeders and growers are still struggling to find an efficient genetic strategy for aphid control in wheat (Yu et al., 2012).

Transgenic crops are an option for insecticidal proteins production for e. g lectin and protease inhibitor (Wang et al., 2005; Hossain et al., 2006; Rahbe et al., 2003). Characterization and expression study of chitin-binding lectin, was associated with resistance against Hessian fly and grain aphid (Giovanini et al., 2007; Repellin et al., 2001). Altpeter et al. (1999) introduced barley trypsin inhibitor into wheat against grain moth (*Sitotroga ceralella*) a major pest of stored wheat grain. Li et al. (2014) reported the harpin protein (Hpa1) with manifold effects in wheat, promoting plant growth with increased crop yield, and inducing resistance to insects. In related study wheat was genetically modified to release insect pheromone (farnesene synthase) for defense (Bruce et al., 2015). *Bacillus thuringiensis* (Bt) toxin as a pesticide replaced use of chemical insecticides on range of crops and Bt resistant crops had been developed as well. However, Bt toxins, is found to be not efficient for

chewing-type insects, and development of resistance against toxins had been reported (Mao et al., 2007).

RNA interference (RNAi) is important phenomenon playing vital role as reverse genetics to study gene function. RNAi was described as post transcriptional gene silencing; done by transmitting single stranded RNA or double stranded RNA (dsRNA) for silencing toxin receptors, pigment or target genes (Kumar et al., 2009; Zha et al., 2011). With RNAi method dsRNA lowers transcript abundance of target gene when injected in organism, cultured cells or feeding on artificial diet containing dsRNA (Shakesby et al., 2009; Fire et al., 1998). Down regulation of targeted genes by using plants mediated RNAi was thru (Pitino and Hogenhout, 2013). Digestive proteolytic activity of aphid nymphs mainly relies on enormous serine proteases. So, protease inhibitors induction in tissues is reported for production of immunity against *Myzus persicae* in *Arabidopsis* (Bhatia et al., 2012). RNAi involves cleavage of dsRNA into small interfering RNA of approximately 21-23 nucleotides by enzyme dicer (Meister and Tuschl, 2004). These siRNAs than incorporate into RNA-induced silencing complex. RISC catalytic contents use siRNA as template to recognize and degrade complementary messenger RNA (mRNA) (Meister and Tuschl, 2004). Host-mediated delivery of dsRNA is an attractive paradigm for developing aphid resistance inspite of elusive mechanistic details governing the uptake of dsRNA in aphids. Successful knockdown of target genes through RNAi was also observed in pea and peach aphid (Mutti et al., 2006; Whyard et al., 2009; Possamai et al., 2007; Shakesby et al., 2009; Pitino et al., 2011), however, systemic RNAi assay evidence was missing in aphid species. Orthologs of RNA-dependent RNA polymerase (RdRP) are present in nematodes, plants and higher animals, the presence of RdRP was never confirmed in insects (Jose and Hunter, 2007; Tomoyasu et al., 2008; Richard et al., 2008). So, systemic RNAi probably does not exist in insects. Systemic RNAi twigs both cell-self signal and environmental RNAi signal in which: silencing signal is transported from the parent cell (dsRNA is applied) to other cells/ tissues (Huvenne and Smagghe, 2010). Gene knockdown effects exhibited by injecting and/or feeding dsRNA to insects was limited to cells that have taken up dsRNA (Price and Gatehouse, 2008; Zha et al., 2011). So, it will require continuous input of dsRNA to persist. Systemic RNAi has been demonstrated in some insect species, such as *Hyalophora cecropia* and *Bombyx mori*, in which injection of dsRNA into the larvae, demonstrating a persistence of the RNAi signal throughout the larval and adult stages and systemic spread of RNAi signal from the gut to the antennae (Terenius et al., 2011; Turner et al., 2006). Recently a comprehensive transcriptomic data analysis was done to find out all the unigenes expressed in grain aphid after wheat feeding. 30,427 novel unigenes were identified in grain aphids as successful target of RNAi after gene expression analysis of their alimentary canal. From all these unigenes, 5 were reported to favor minimal risk of non-specific target but also maintain the low efficacy of lethal dose in comparison to previous studies (Zhang et al., 2013; Pitino et al., 2011). These target genes after their confirmation and introduction in our local wheat varieties will be efficient for aphid control in local agricultural practice. Therefore, area of interest is

(a) To find out these unigene sequences causing RNAi assay in local species of aphids. (b) To find the expression of those genes by doing RTPCR analysis.

Results

RNAi is a useful and efficient generic tool that had been used in the field of insect functional genomics. This technology had been used for the screening of efficient and new insect resistance gene and to create crops having resistance against aphids. Aim of research was to explore most effective unigene sequences from samples collected from local weather environment, These 5 unigenes were reported to be efficient in causing mortality of grain aphid. Other than this there were 2-3 factors for the choice of these unigenes as RNAi targets. All the differentially expressed unigenes directly or indirectly involved in the activity of oxidation-reduction processes like NADH dehydrogenase, oxidoreductase, so their suppression will be excellent approach for transgenic crop production especially wheat. Secondly artificial feed also because high mortality within 24 hours comparable to microinjection and low dosage of the construct in artificial diet was found to be efficient for aphid mortality. The 5 unigene sequences selected will be used for RNAi based assay by producing transgenic tobacco followed by transgenic corn and wheat (Zhang et al., 2013).

DNA extraction from aphids *Macrosiphum rosae, Rhopalosiphum maidis, Sitobion avenae, Toxoptera aurantii* reared on *Rosa indica, Zea mays, Triticum aestivum, Chrysanthemum hibiscus, Solanum melongena, Abelmoschus esculentus* was done (Fig 1).

DNA extraction was done using CTAB-PVP DNA extraction protocol with freshly collected preserved specimens in 70 % ethanol, which didn't give satisfactory results. So phenol chloroform method was then used for the DNA extraction (Fig S1A). Both quality and quantity of extracted DNA was improved with this method. DNA extraction from old specimens might have been poor result due to degradation of DNA in the dead samples but, in our case we have good results in both fresh and preserved samples (Hajibabaei et al., 2006; Wang et al., 2013).

Incubation of sample with lysis buffer gives good quality pallet of DNA. Multiple Methods were used for the extraction of RNA from samples of aphids show positive results in aphids. Best results were obtained with the Trizol reagent extraction (Tri Reagent: Sigma-Aldrich. Product No. T9424). RNA extraction is done for checking the expression of 5 unigene sequences within our collected species, by running the sample on gel electrophoresis. A band with a little smear confirms the presence of RNA in sample (Fig S1B, S1C). CDNA was prepared from these RNA and utilized for PCR amplification. Positive results were shown from both set of extracted RNA from gut region and from whole aphids. (Fig S1D). To find presence of desire sequences five set of unigene primers were used (Table No 2). T_m optimized for each primer is given in Table 2.

Presence of unigenes was confirmed in all five namely (*M. rosae, R. maidis, S. avenae, T. aurantii* reared on *R. indica, Z. mays, T. aestivum, C. hibiscus, S. melongena, A. esculentus*) at optimized conditions. Size of the DNA bands (159 kb) for all sequences was confirmed with 100 bp ladder (Fig 2A, 2B, 2C & Fig 3).

Unigenes 21789 and 28469

Unigenes 21789 and 28469 had no orthologues identified in aphids or wheat. PCR results were found to be positive with all four aphid strains e.g. *M. rosae, R. maidis, S. avenae, T. aurantii* with 211 bps and 257 bps respectively. (Fig 2A; Table 3).

Table 1. Name of the Aphids collected from their respective host plant.

Aphid Species	Host plant
Macrosiphum rosae	*Rosa indica*
Rhopalosiphum maidis	*Chrysanthemum, Hibiscus rosa-sinensis, Solanum melongena, Abelmoschus esculentus*
Sitobion avenae	*Chrysanthemum, Hibiscus rosa-sinensis, Solanum melongena, Abelmoschus esculentus, Triticum aestivum*
Toxoptera aurantii	*Citrus sinensis*

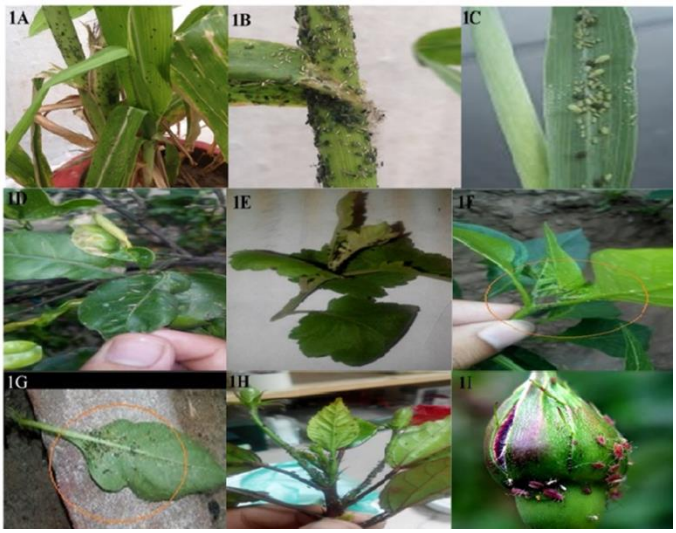

Fig 1. Sampling of *Macrosiphum rosae, Rhopalosiphum maidis, Sitobion avenae, Toxoptera aurantii* reared on *Rosa indica, Zea mays, Triticum aestivum, Chrysanthemum hibiscus, Solanum melongena, Abelmoschus esculentus.* (1A & 1B) Samples of *R. maidis* on *Z. mays*. (1C) Samples of *S. avenae* on *T. aestivum*. (1D and 1E) Samples of *T. aurantii* on *C. sinensis* and *Chrysanthemum* (1F and 1G) Samples of *S. avenae* on *S. melongena & C. annum*. (1H) Samples of *S. avenae* on *H. rosa-sinensis* (1I) Samples of *M. rosae* on *R. indica*.

Unigene 21088

Unigene sequences 21088 showed positive PCR results with *M. rosae, T. aurantii and S. avenae* but no results were shown for *R. maidis* with 198 bps (Fig 3).

Unigene 23028

Unigene 23028 expressed positive PCR results with *R. maidis, S. avenae* but no results were showing with *M. rosae* and *T. aurantii* with 195 bps (Fig 2C).

Unigene 8273

Unigene 8273 give results with *M. rosae* and *S. avenae* no results were found with *R. maidis* and *T. aurantii*. Overall results from above mentioned reactions are presented (Table 3).

All 5 unigenes (21789, 28469, 21088, 23028 & 8273) sequences were found in *S. avenae* isolated from *S. melongena, C. annum, T. aestivum* and *H. rosa-sinensis*, similar to unigenes isolated from *S. avenae* reared on wheat (Zhan et al., 2013). Unigene sequences were also found to be present in *S. avenae* isolated from chilies, brinjal, Gul-Lala and local wheat plants. While other aphids represent variable results (Fig 2B).

Discussion

In Pakistan other than wheat major staple crop, fruits, vegetables, brinjal, are main host of aphid causes 10-90 % damage to crops every year (Irshad, 2001). Severity of aphid infestation 70 -80% loss in yield exceeded all over the world (Dedryver et al., 2010; Bhatia et al., 2011; Blackman and Eastop, 2000; 2006). *R. maidis, S. avenae, M. rosae* and *T. aurantii* were reported to be important graminaceous, fruits, vegetable and rose aphids in northern areas and subtropical regions of Punjab, Pakistan (Hamid, 1983; Wains et al., 2014; Mani, 1987; Quratulain, 2015). *D. noxia, S. graminum* and *R. padi* are some other aphids affecting wheat crops in tropical areas of Punjab areas (Muhammad et al., 2013; Khan et al., 2012; Aslam et al., 2004; Shahzad et al., 2013; Faheem et al., 2015). RNAi technology is widely reported for pest control by knocking down vital insect genes. Among insect pests, aphids also respond to the successful delivery of siRNA or dsRNA by triggering RNAi responses (Mutti et al., 2006; Possamai et al., 2007). The cells of the insect gut lumen take up dsRNA either by oral delivery or through artificial diet or ingestion of bacteria expressing dsRNA (Turner et al., 2006; Huvenne and Smagghe, 2010; Tian et al., 2009). Although mechanistic details governing the uptake of dsRNA in aphids still remain elusive, host-mediated delivery of dsRNA is still an attractive paradigm for developing aphid resistance. Transgenics expressing aphid inhibitors have also shown deterrent effect against aphids in feeding trials (Carrillo et al., 2011; Tran et al., 1997; Rahbe et al., 2003). Present study was first time carried out in Gujrat, Pakistan to determine the presence of unigene sequences in local aphid species which will help further in the incorporation of RNAi in aphids via transgenic plants and to produce resistance crop line by using this method. Systemic RNAi comprehend both cell internal signal/ environmental RNAi signal for its valid efficacy. This is an important step for their control in crop system, should be follow by their control measures.

PCR of unigene 28469 and 21789 was positive for all *M. rosae, R. maidis, S. avenae, T. aurantii*. These two unigene sequences were found to be novel and found to be present in

Table 2. Primers used for PCR amplification of required gene region.

Primer ID	Non-redundant protein database	Primers	Tm
Unigene 23028 (F)	gi193695320	5-3' TTCAACTTCAGTCAACGGGATA	56.3°C
Unigene 23028 (R)		5-3' TGTTGAACCCTTCTGACACG	57.8°C
Unigene 28469 (F)	No ortholog found	5-3' AGCCAGGATGTTGGCTTAGA	57.8°C
Unigene 28469 (R)		5-3' CTCATGTCAGCATTCGCACT	57.8°C
Unigene 8273 (F)	gi193716064	5-3' AGACGAACCAATTAGAGCAG	55.7°C
Unigene 8273 (R)		5-3' GTTTCTTACAGCTCCACACC	57.8°C
Unigene 21789 (F)	No ortholog found	5-3' CGGCAACAATTGACTCTAA	53.2°C
Unigene 21789 (R)		5-3' GAAGTCACATGTTCGCACTA	55.7°C
Unigene 21088 (F)	gi254281286	5-3' TTGTTGAACCCTTCTGACA	53.2°C
Unigene 21088 (R)		5-3' TGATTCAACTTCAGTCAACG	53.7°C

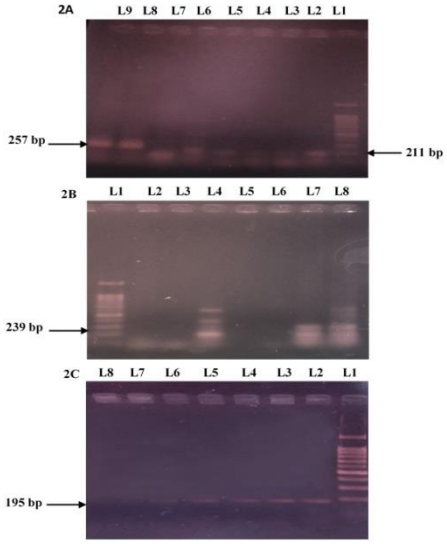

Fig 2. PCR of unigene 28469 and 21789. (A) PCR results were positive for all *Macrosiphum rosae*, *Rhopalosiphum maidis*, *Sitobion avenae*, *Toxoptera aurantii* reared on *Rosa indica*, *Zea mays*, *Triticum aestivum*, *Chrysanthemum hibiscus*, *Solanum melongena*, *Abelmoschus esculentus.* PCR of unigene 21789. L1. 100 bp Ladder L2: *T. aurantii*; L3: *S. avenae*; L4: *R. maidis*; L5: M. rosae. PCR of unigene 28469. L6: *T. aurantii*; L7: *S. avenae*; L8: *R. maidis*; L9: *M. rosae*. (B) PCR of unigene 8273: PCR results were found to be positive for *S. avenae* and *M. rosae* and negative for *R. maidis* and *T. aurantii* reared on *R. indica*, *Z. mays*, *T. aestivum*, *C. hibiscus*, *S. melongena*, *A. esculentus*. PCR of unigene 8273. L1. 100 bp Ladder; L2, 3, *T. aurantii*; L4: *S. avenae*; L5: *R. maidis*; L7, 8: *M. rosae*. (C) PCR of unigene 23028: PCR results were found to be positive for *R. maidis*, *S. avenae* and negative for *M. rosae*, and *T. aurantii* reared on *R. indica*, *Z. mays*, *T. aestivum*, *C. hibiscus*, *S. melongena*, *A. esculentus*. PCR of unigene 23028: L1. 100 bp Ladder; L7: *M. rosae*; L3 & L4: *R. maidis*; L2: *S. avenae*; L8: *T. aurantii*.

Table 3. Presence of selected unigenes in local aphid species (*Macrosiphum rosae, Rhopalosiphum maidis, Sitobion avenae, Toxoptera aurantii)* reared on *Rosa indica, Zea mays, Triticum aestivum, Chrysanthemum hibiscus, Solanum melongena, Abelmoschus esculentus,* confirmed by RTPCR analysis.

Unigenes	Annotations	*M. rosae*	*R. maidis*	*S. avenae*	*T. aurantii*
21789	No orthologs identified	Present	Present	Present	Present
28469	No orthologs identified	Present	Present	Present	Present
21088	Cytochrome c oxidase subunit VIIc precursor (*Acyrthosiphon pisum*).	Present	Absent	Present	Present
23028	Similar to zinc finger protein (*A. pisum*)	Absent	Present	Present	Absent
8273	Cuticular proteins (*A. pisum*)	Absent	Present	Present	Absent

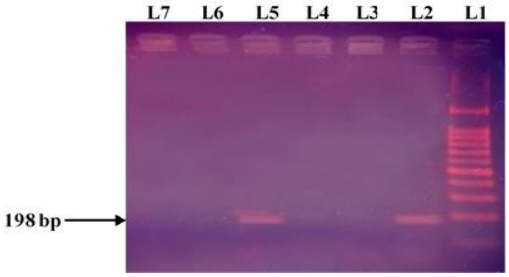

Fig 3. PCR of unigene 21088. PCR was found to be positive for *M. rosae*, *S. avenae*, *T. aurantii* and negative for *R. maidis* reared on *R. indica*, *Z. mays*, *T. aestivum*, *C. hibiscus*, *S. melongena*, *A. esculentus*. PCR of unigene 21088. L1. 100 bp Ladder; L2: *S. avenae*; L4, L6: *R. maidis*; L5: *T. aurantii*.

all four aphids and had not the possibility to interfere with the wheat genome as well so could be important for broad resistance against grain aphids. Very small amount of dsRNA (7.5 ng/µl) as compared to previous data was found to be efficient for RNAi mediated silencing using these selected unigenes. So, these unigenes also favor minimal risk of non-specific target but also maintain the low efficacy of lethal dose. 4 d later, the mortality levels of aphids fed with dsRNAs of C002 and unigenes 21088, 21789, 23028, 28469 and 29698 were between 60% and 90% after correction. This low dose effectiveness not only maintain the minimal risk of non-specific effects but also facilitate the application of plant-mediated RNAi silencing of these target genes for aphid control in agricultural practice. dsRNA could be taken by digestive system and was not localized to the midgut and temporally limited, could spread to the whole body tissues in grain aphid, then lead to a down-regulation/knock out of the target gene expression and finally to the development retarding and/or death of grain aphid. Number increases after feeding.

PCR of unigene 21088 was found to be positive for *M. rosae, S. avenae, T. aurantii* and negative for *R. maidis.* It encodes for cytochrome c oxidase (CO-1) subunit VIIc precursor of (*Acyrthosiphon pisum*) and its expression reduced significantly reduced to half after feeding on artificial diet with this protein as RNAi target. CO-1 is used as bar coding gene for elucidation of cryptic aphid species of order Hemiptera (Rebijith et al., 2013). CO-I gene sequence as DNA barcoding is highly reported as effective tool for species identification (Foottit et al., 2008; Glover et al., 2010; Lee et al., 2010). In addition, CO-I may be suitably employed to elucidate the prevalence of biotypes and for the discovery of new species within the Aphididae (Shufran et al., 2000).

PCR of unigene 23028 was found to be positive for *R. maidis, S. avenae* and negative for *M. rosae*, and *T. aurantii*. Unigene was zinc finger protein (*Acyrthosiphon pisum*) expression reduced drastically upon eating on artificial diet with RNAI target. Zinc finger protein form one of the largest group of proteins in small grain aphids (Nicholson et al., 2015). Zinc finger protein is reported as defense protein in plants and insects in response to grain aphids (Gupta et al., 2012; Guan et al., 2015).

PCR of unigene 8273 was found to be positive for *S. avenae* and *M. rosae* and negative for *R. maidis* and *T. aurantii*. Cuticular protein (CPs) decreases after feeding on artificial diet with this protein as RNAi target. CPs include non-structural and structural proteins. CPs Include RR-1 isolated from soft or flexible cuticles, and RR-2 proteins are more often associated with hard cuticles (Willis et al., 2005). It had been reported that cuticular protein is associated with seasonal variation and help the aphids to withstand the harsh conditions (Gallot et al., 2010). Synthesis of cuticular proteins is linked with the tyrosine synthesis occurred in early embryonic stages of pea aphid and is major precursor for cuticle maturation (Rabatel et al., 2013). Silva et al. (2012) reported culticular protein along with other enzymes related to energy metabolism, detoxifying enzymes, proteins of extracellular transport, and peptidases as defense mechanisms in response to insecticide in *M. persicae*.

All the unigenes were found to be present in *S. avenae* as this unigenes were selected from the study of *S. avenae* on wheat so unigenes are similarly expressed in local wheat aphids.

RNAi had been used as useful generic tool to produce insect resistant crops and was firstly introduced into the *C. elegans* to study the role of its individual gene (Fire et al., 1998). Success of RNAi experiments had been also observed in a number of lepidopteran species but variable rates of RNAi dependent knockdown had been observed (Price and Gatehouse, 2008; Terenius et al., 2011). Some genes used for dsRNA targets represents high mortality while others failed to be silenced. Secondly, vulnerability of novel targets to RNAi shows considerable variation in model species (Price and Gatehouse, 2008). Some proved to be completely recalcitrant to suppression as in neuronal expressed genes in *C. elegans* (Kennedy et al., 2004). Thirdly, risk of inadvertent cross-species silencing could be vital biosafety concern in RNAi mediated aphid resistance. Highly insect-specific genes with no good match to sequences in donor plants for engineering should be selected. Dosage of RNAi target is another important factor for targeted gene silencing. High variation subsists among different lepidopteran species, due to their sensitivity to systemic RNAi at adjustable levels. As, its not true that beyond optimal concentration results will be more efficient for silencing (Pitino et al., 2011; Shakesby et al., 2009; Terenius et al., 2011). Aphid injected with 5 nl C002 SiRNA (10µg/µl) led to knockdown of C002 gene and even lower doses observed in other cases (Mutti et al., 2006). In one of related study silencing of C002 gene and a gut-specific gene Rack-1 in peach aphid resulted in the knock down of these two genes by up to 60% after feeding on transgenic tobacco and *Arabidopsis* plants, with affected aphids producing less progeny (Shakesby et al., 2009).

Unigenes selected for the study were found to be friendly for wheat, diet containing them resulted in stunting & death of aphids. Of these RNAi targets two unigenes 21789 and 28469 had no orthologs identified; so were novel in grain aphid and important RNAi target genes for aphid resistance in transgenic plants.

Systemic RNAi pathway is not found in most insect species except some lepidopteran species. As RNA pathways are highly conserved in aphids, so successful knockdown of target genes through RNAi was also observed in pea and peach aphid (Pitino et al., 2011; Shakesby et al., 2009). Few cases are reported for plant-mediated RNAi for aphid control. Silencing of C002 gene and gut-specific gene Rack-1 in peach aphid resulting in 60% mortality after feeding on transgenic tobacco and *Arabidopsis* plants (Pitino et al., 2011).

We have found the expression of the variable expression of these unigenes in 4 local aphid species residing on variable host plants. Next target is to transcribe an antisense RNA from vector by cloning these targeted sequences in reverse orientation so that antisense RNA produced instead of Sense RNA. Vectors will be designed to express different siRNA molecules. This retrovirus-based vector will have two complementary sequences about 20 nucleotides in length that form a stem, separated by a loop region. When the vector will transform in cell, the shRNA will transcribed from these sequences and activates gene silencing. Transformation of model plant (tobacco) and cereals and vegetables will be done in future. Insect Bioassay will follow this. Aphids fed on transgenic cereals and vegetables with these RNAi targets will result in mortality of aphids along with down regulation of targeted sequences.

Materials and Methods

Collection of explants

Aphids are very host specific & plants sampled were *R. indica, Z. mays, T. aestivum, C. hibiscus, S. melongena* and *A. esculentus* (Table 01). Sampling was done from the different regions of district Gujrat from young branches of

plants with the help of very fine forceps. Samples were dislodged from the leaves or stems by using paintbrush. Some of aphids were stored in 70% alcohol and other were reared on wheat plants grown in pots at room temperature. Aphids were collected on their respective host plants. Aphid species were identified by Dr. Sumeira Afsheen (Associate Professor) Department of Zoology, University of Gujrat. We have found 4 different species of plants from their respective host. Compared with the entire region of Pakistan, the study area was very small, despite this, 4 different species were recorded (Table 1, Fig 1).

DNA extraction

M. rosae, R. maidis, S. avenae, T. aurantii reared on *R. indica, Z. mays, T. aestivum, C. hibiscus, S. melongena, A. esculentus* were grinded with liquid nitrogen in clean mortar and pestle (after Black et al., 1992) or was purified using three phenol, chloroform DNA extraction methods (Sambrook et al., 1989; Milligan, 1992; De Barro et al., 1995). Samples were stored at 4°C prior to use. Confirmation of DNA was done by gel electrophoresis (Fig S1A).

Primer designing

Sequence of five unigenes provided by Zhang et al. (2013) were used to design relatively suitable length of PCR primers using primer 3 online software (version 0.4.0; Rozen and Skaletsky, 2000).

PCR reaction

Conformation of the desire sequences in extracted DNA from all species was done by running PCR reaction. 5 primers were used that are basically the unigene sequenced and are proved as successful target for RNAi in aphids (Table 2). Set of PCR reaction was run with an initial denaturation step for 4 min (95^oC), followed by 40 cycles of denaturation step for 30 seconds (95^oC), annealing temperature (as describe in Table 2) for 1.5 min and extension (72^oC) for 30 sec, and a final extension cycle of 7 min (72^oC). The amplified PCR products were analyzed by agarose gel electrophoresis.

Conformation of amplification

Gel electrophoresis (1% agarose gel) was conducted to confirm the PCR amplification. PCR amplified product (2µL) along with 4µL 6X loading dye was loaded on the gel while 100 bp (Sigma Aldrich) ladder was being loaded (2 µL) on a separate well.

Aphid dissection

M. rosae, R. maidis, S. avenae, T. aurantii reared on *R. indica, Z. mays, T. aestivum, **C.** hibiscus, S. melongena, A. esculentus* were harvested and sorted into different growth phases. Adult's aphids were dissected under a binocular microscope and their gut region was separated for RNA extraction. Dissected guts were kept on ice until adequate number had been collected and then stored at -20^oC. Total RNA was isolated from both, dissected and whole aphids. The aphids used were adults for RNA extraction.

RNA extraction, primer designing and unigene gene acquisition

Aphids total RNA will be isolated using TRIZOL Reagent (Qiagen, USA) and RNA will be converted into cDNA using cDNA synthesis kit (Invitrogen, USA). cDNA was PCR amplified with unigene-specific primers. Confirmation of the desirable sequences in extracted cDNA from all species will be done by running PCR reaction with five set of primers under specific conditions.

PCR analysis

20 ul PCR reaction was made containing 1.5 of the template cDNA, 2ul Taq buffer, 1ul $MgCl_2$, 0.5ul dNTP mixture, 0.5 ul of each forward and reverse primer, 1ul Taq polymerase and 13ul of nanopure water. PCR reactions were run with an initial denaturation step for 4 min (95^oC), followed by 40 cycles of denaturation step for 30 seconds (95^oC), annealing temperature (as describe in table) for 1.5 min and extension (72^oC) for 30 sec, and a final extension cycle of 7 min (72^oC). The amplified PCR products were analyzed by agarose gel electrophoresis.

Conclusion

ds RNA designed against insect target genes gave protection against pests through RNAi, and open the way for a new generation of insect-resistant crops (Price and Gatehouse, 2008). Based on this observation 5 major exotic unigenes explored via transcriptomic profiling of wheat aphids were selected (Zhang et al., 2013). These unigenes were confirmed in our local aphid strains such as *M. rosae, R. maidis, S. avenae, T. aurantii*) feeding on *C. sinensis, R. indica, S. melongen, Z. mays* and *T. aestivum.* Application of RNAi to aphids is somehow constrains due to vulnerability of novel targets to RNAi show considerable variation in target species. Unigenes selected were found to be friendly for wheat and by feeding on diet containing them resulted in stunting and death of aphids. Risk of inadvertent cross-species silencing could be vital biosafety concern in RNAi mediated aphid resistance. Insect-specific genes were selected with no good match to sequences in donor plants for engineering. Of these five RNAi targets, two unigenes 21789 and 28469 had no orthologs identified. These unigenes are novel in grain aphid, and important RNAi target genes for aphid resistance in transgenic cereals and vegetable plants. The unigenes favor minimal risk of non-specific target but also maintain the low efficacy of lethal dose. These unigenes will be used for production of transgenic plant with RNAi vector. CO-I gene find out was reported as DNA barcoding will be used for further species identification. CPs and zing finger proteins were other defense proteins identified to be important for the aphid knockdown.

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