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Strategies for the Control of *Brassica juncea*-Parasite Plant *Orobanche aegyptiaca*

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Abstract

Due to eco-geographical variation and human selection *Brassica juncea* has various forms and most commonly grown in North America and Europe as a kitchen herb, on the Indian subcontinent for seed oil and the Far East as a vegetable. The plant broomrape, *Orobanche aegyptiaca* is the major weed which drastically diminishes the yield of world-wide economically important crops from 5%-50%, whereas it reduces the yield of Indian mustard up to 30%. The *Orobanche aegyptiaca* is a holo-parasite as well as non-photosynthetic having a complex lifestyle. Various methods such as chemical, physical & biological have been applied and suggested for the management of *Orobanche aegyptiaca*. An integrated approach can be one of the most preferable and effective methodologies used to control the plant parasite. We have identified few gene candidates which can be used further to control the holoparasite *via* siRNA technology. Also, one more step is added to the integrated approach i.e. Bio-briquette machine in which one can harvest the weeds and use it for domestic purpose. The role of fungal agents has been reviewed in the mechanism of signal transduction of *Orobanche aegyptiaca* and *Brassica juncea*.

Also, it took in consideration about the photosynthetic genes which are present in the holoparasite even though the *Orobanche aegyptiaca* is a non- photosynthetic plant. It has been checked whether these genes are pseudo-gene or not, using PAML and HYPHYMP package. Based on these results, it is hypothesized that these genes play a crucial role in early development of *Orobanche aegyptiaca* before developing mature haustorium. The study proposes a new insight into fungal-fungal signal transduction in a plant-parasite-host relationship that can lead future aspects in science.

Keywords: Haustorium; HGT; (Integrated Weed Management) IWM; Plant parasite; siRNA; Allelopathy; Selection

Introduction

Weed management and weed control strategies in agriculture

The researchers are consecutively reporting about new techniques and publishing various articles on weed control in different species including vegetables as well as in economically important species. Consequently, one should concentrate more on the uncertainty of crop-weed relationship and less on weed mortality rate [1]. In the current findings, it has been found that an Integrated Weed Management (IWM) strategy can be more reliable other than using chemical strategies. IWM includes techniques, strategies and creative plans as well as processes in which one can avoid the use of chemical herbicides. The recently launched bio-briquette machine can also contribute to weed management [2]. There are various methods for weed detection and preventive weed management which can be effective for weed management. As another alternative 'organic farming' is also an integrated system that avoids the use of synthetic fertilizer and promotes the use of natural herbicide, biodegradable mulch, mulching, soil solarization, hot water and agronomic practices such as competitive varieties stale seedbed which has a significant impact on weed. It has been found that the *Orobanche aegyptiaca* (Figure 1) is an invasive plant species. Discovery of herbicides is not so much effective over the knowledge-based-approach used by farmers. To overcome this problem weed science came into existence in which one can harness the techniques that execute practical weed management. The population of herbicide resistant plants is increasing gradually that is why weed biology is become more necessary [3]. The molecular biodiversity and horizontal gene transfer are widely accepted reasons for the evolution of herbicide resistance plant, weed's success and failure of weed management strategies. A weed can show a different kind of diversity at various levels. Biodiversity can be present in weeds populations at all level of plant organization, from molecular to global. There are various modes including genetic variation within the species, a somatic variation of plant parts, temporal adaptation within the community and

Figure 1: The picture of *Orobanche aegyptiaca* (Source: https://alchetron.com/Orobanche-aegyptiaca).

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floristic diversity of a community at a higher level than the species. It also has been found that weeds evolve in response to cropping system by adapting and occupying the niches left available in an agroecosystem [4]. So far various control methods were suggested and applied for *Orobanche* management which can be classified into resistant varieties, cultural (crop rotation), physical (weed handling), biological and chemical control by herbicides [5-9]. "Weed production has a direct correlation with reduction in crop yields". In order to control the weed, farmers are needed to be educated in crop techniques and alternate ways which can enhance the crop production. Farmers are informed about the weeds and kind of weed which can affect the crop [10].

Allelopathy contributes to weed control. "Rice (1984) defined 'Allelopathy' as *the effect of one plant (including microorganisms) on another plant through the release of a chemical compound into the environment*". Allelopathy plays a critical role in a different kind of cropping systems such as mixed cropping, multiple cropping, cover cropping and crop rotation [11]. It has been revealed that the *Orobanche* and *Phelipanche* species are distributed in Southern and Eastern Europe, the Middle East and North Africa and have recently been reported in USA, Australia and some Asian countries which is depicted in (Figure 2) [12]. The main problem for mitigating these species is that seeds of these species remain viable for decades in the field. Complete mitigation by chemical control of broomrapes is exceedingly difficult to achieve because these are anatomically as well as physiologically well connected to the host [13]. Based on the various past studies including greenhouse experiments and pot studies, it has been shown that synthesizing amino acid biosynthesis-inhibiting herbicides can be an acceptable control strategy [14].

Weed, herbicides, *Orobanche aegyptiaca* and its control strategies in different crops

Weeds are prolific seed producers. The research findings show "there are invasive weeds which are non-native plants introduced to North America, Europe, and Asia" (Fundamentals & Ecosystems, n.d.). Many weeds are crop specific and/or location specific and many of them affect the crop productivity of mustard, potato, tobacco, tomato and many more. For instance, *Orobanche aegyptiaca*, *Chenopodium*, *Asphodelus*, *Melilotus* and *Trianthema spp*, cause severe yield loss in mustard crop production [15]. Based on past studies it has been revealed that weeds cause ~50% crop yield loss across the world [16]. Due to such devastating economic consequences, the last two decades have seen increasing use of herbicides [4]. The herbicide is used for increasing the productivity in the crop field and gain yield. These compounds affect the parasite's susceptibility to host plant [17,18]. Among herbicides, the non-selective herbicides are more frequently used. Few herbicides are widely used named glyphosate (non-selective agent), sulfuric acid acetolactate synthase and acetyl coenzyme A carboxylase inhibitor, 2, 4-D [19]. Glyphosate is one of the most widely used herbicides in different species including *Orobanche aegyptiaca* as a weed control agent [20]. Research findings have revealed that the herbicide resistant plants are increasing and that can be due to evolution and growth in selection pressure of herbicide resistance plant [4]. The research findings (pot studies and petri-dish experiments in tomato and potato crops) revealed that there are some herbicides named sulfosulfuron, imidazolinones etc., which are used to mitigate the *Orobanche aegyptiaca* but have side effects on the host plant [14].

Some scientific studies suggested the use of biocontrol agents. For example, the bacteria (*Pseudomonas aeruginosa* QUBC1, *P. fluorescens* QUBC3, *Bacillus atrophaeus* QUBC16 and *B. subtilis*) can be used as biocontrol agents against the elongation of *O. aegyptiaca* and *O. cernua* [21]. As an another alternative 'organic farming' is also an integrated system that avoids the use of synthetic fertilizer and promotes the use of natural herbicide, Biodegradable mulch, mulching, soil solarization, hot water and agronomic practices such as competitive varieties stale seedbed which has a significant impact on *Orobanche aegyptiaca*.

Orobanche sp., simply called "Broomrapes" are holo-parasitic as well as both obligate and facultative plant parasites those parasitize many vegetables and economically important crops including mustard

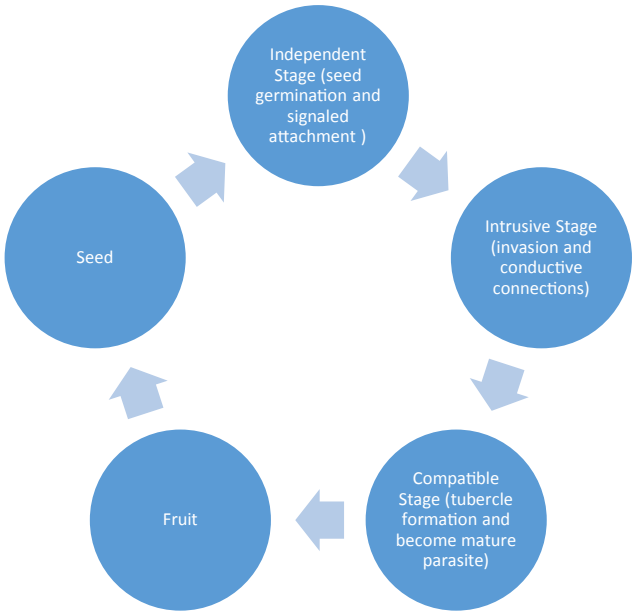

Figure 2: It describes the life cycle of *Orobanche aegyptiaca* including different stages.

(*Brassica juncea*, *Braasica napus*), tobacco, leguminous plants and medicinal plants causing severe losses in crop yield [6].

The Genus *Orobanchaceae* consists of 200 species including *Orobanche aegyptiaca*, *Orobanche ramosa* as well as *Orobanche cernua* and many more species [22]. The parasitic plant *Orobanche aegyptiaca*, *Orobanche ramosa* and *Orobanche cernua* use Haustorium (modified structure) to tap in to photosynthetic plant (*Brassica juncea* and many more hosts) for extracting water and nutrients [23]. Many experimental studies have been done on *Orobanche aegyptiaca* with model plant *Arabidopsis thaliana*. Consequently, it has been found that the broomrape *Orobanche aegyptiaca* manipulates the host by acting as a sink for auxin [24]. The strigolactone is a major chemical compound that helps in seed germination of both the parasite *Orobanche* and *Phelipanche*. The strigolactone secreted by model plant are carotenoid derived phytohormones regulating the shoot branching and stimulating germination of root parasite [25]. The plant parasite *Orobanche aegyptiaca* has a broad range of host specificity in term of seed germination. It has been found that several key genes are involved in seed germination, and these genes are associated with changes in the plant hormones. These genes may be developed as new targets to control *O. aegyptiaca* [26]. Apart from model plant *Arabidopsis thaliana*, Parsley (*Petroselinum crispum*) can be used for *Orobanche* studies. The Parsley is a host of the angiosperm root holo-parasite *Orobanche crenata* and *Orobanche aegyptiaca* [27]. Many chemical compounds have been synthesized for controlling plant parasites, but field studies do not show much good results [9]. In India, *Orobanche aegyptiaca* is extremely harmful and noxious weed to economically important crop 'Indian mustard (*Brassica juncea*)'. This plant parasite has various regional names including Gulli, Khumbhi, Rukhri etc., [28]. It has been revealed that the silencing of parasite genes by producing siRNA in the host gives a novel strategy for controlling parasitic weeds. The research findings have shown that the expression of siRNA silenced a homologous GUS gene in parasitic plant *Triphysaria* which belongs to family Orobanchaceae [29]. In view of this one can design small interfering RNA to silence a gene that is necessary for an important metabolic activity of parasitic plant. It is essential to identify those candidates as powerful tool for targeting through designing siRNA [30]. Small interfering RNAs down regulate the gene expression guided by sequence complementarity [31].

Genetics of *Orobanche aegyptiaca*

Orobanche aegyptiaca (synonymous *Phelipanche aegyptiaca*) is closely related to *Orobanche ramosa* but it is more robust [12]. It is 20-40 cm high and flowers are 20 mm long (Figure 3). *Orobanche*

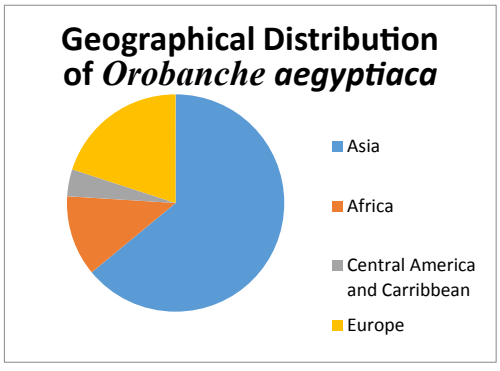

Figure 3: Geographical distribution of *Orobanche aegyptiaca* (Source: Http://www.cabi.org/isc/datasheet/37742, 2014).

has 1.4-1.5 Gbps genome size. The *Orobanche aegyptiaca* consists of 24 chromosomes and it is a diploid broomrape plant (Http://www.cabi.org/isc/datasheet/37742, n.d.). It adapts gene from its host through horizontal gene transfer [32]. There are genes which adapted by *Orobanche* through HGT from different hosts including Brassicaceae family [33]. There are various ways of acquiring new genes, which act as novel genes and horizontal gene transfer is one of the most common mechanisms in plants. More than 45 expressed and functional horizontal gene transfer events have been identified in family Orobanchaceae [34]. It has been rarely reported that the nuclear genome is rarely transferred through HGT in plants, but it has been revealed that the Stricto-Synthase Like gene (SSL) found in nucleus of Brassicaceae plant, is co-opted by *Orobanche aegyptiaca* [33,35,36]. The evolutionary analysis has revealed that *Orobanche aegyptiaca* has evolved the terminal haustoria and loss of photosynthesis genes independently [37]. Some experimental evidences have shown that it lost the function of photosynthesis genes not the whole set of genes [38]. Those genes may present as pseudo-genes or might play role in early development of plant parasite [39].

Horizontal gene transfer

Horizontal gene transfer is the movement or transmission of genetic material and genomic integration between non-mating organisms/offspring including unicellular and multicellular organisms. HGT (Horizontal Gene Transfer) serves as driving force for prokaryotic evolution. It is the way of acquiring new genes which give the novel phenotype to parasite plant *Orobanche aegyptiaca*, *Cuscuta australis* and many more [40,41]. The nuclear genome is more commonly transferred to parasitic plants *Orobanche aegyptiaca* from its host and it show heterotrophic dependence towards its host (*Brassica juncea*). The research findings show that the *Orobanche aegyptiaca* gets genes transferred from rosids, monocots. HGT probably is one of the reasons for acquiring genes and adaptation of *Orobanche aegyptiaca* [32]. It has been found that two nuclear related genes named BO genes cited as hAT superfamily of class II transposon endows from Brassicaceae to both the genus *Orobanche and Phelipanche via* horizontal gene transfer. This BO gene is actively expressed in *Orobanche aegyptiaca* but not transcribed in Brassicaceae [42].

Brassica juncea

After soybean and palm, the rapeseed-mustard which belongs to family Brassicaceae (Syn. Cruciferae), is the important oilseed crop across worldwide and in India it is second most important edible oilseed after groundnut [43]. Among rapeseed-mustard, *Brassica juncea* known as Indian mustard, is an important and essential winter oilseed crop of India [44]. Due to eco-geographical variation and human selection the Indian mustard (Brown mustard) has various morphological forms and most commonly grown in North America and Europe as a kitchen herb, on the Indian subcontinent for seed oil and far east as a vegetable [45]. The research findings revealed that in India the broomrape infestation caused ~30% yield loss in rapeseed-mustard [43]. *Brassica juncea* (chromosome number 36, genome AABB) is the result of hybridization between *Brassica rapa* (chromosome number: 20, genome AA) and *Brassica nigra* commonly known as black mustard (chromosome number 16, genome BB) and it has various local and/or regional names including Rai, Raya, Laha and Banga Sarson [46-48]. It has been found that mycorrhizal fungi symbiotic association help to enhance nutrient uptake range for host *Brassica juncea*. A case study has been done in India which revealed about such fungi [49].

Materials and Methods

Bioinformatics retrieval of sequences for photosynthetic genes and horizontally transferred genes

The gene candidates which are horizontally transferred were obtained from the github https://github.com/dePamphilis/HGT_PNAS_2016. The other genes sequences were taken from two main sources: NCBI and ENSEMBL Plants. The annotated genome sequence was obtained from the ppgp project (http://ppgp.huck.psu.edu/). BLAST search is done to validate the photosynthetic gene candidate. The NCBI ORF Finder is used to get the open reading frame for the sequences of photosynthetic genes obtained from the plant parasite genome project.

Alignment

Alignments for various gene candidates were performed using MAFFT, Clustal Omega [50], PRANK [51], MUSCLE [52], KALIGN [53], T-Coffee [54]. The paml format is obtained by using PAL2NAL [55] and then used in PAML for purifying selection of photosynthetic genes.

Phylogenetic analysis

For each alignment the phylogenetic analyses are performed to explore the topology of the tree and the effect of inclusion or exclusion of various groups of sequences. The Fast Tree version 2.1.8 Double precision (No SSE3) is used for building the phylogenetic trees. The purifying selection is done using PAML and hyphymp (http://datamonkey.org/). Trees were visualized and modified using FigTree v1.4.2.

Online tool for designing siRNA; Si-Direct

The si-Direct online server is used to find out candidates for design of si-RNA with high accuracy and efficiency. It gave candidate for si-RNA with melting temperature and seed sequence. [56]. The list of siRNAs is provided in supplementary material.

Strategies

Strategy I (Genes present in multiple parasites)

In this strategy few important genes are collected based on articles, reviews and scientific research literatures which tell that these genes play role in various stages of parasite development. Those genes were refined and checked for their presence in multiple parasites. A list is made up of those genes obtained from multiple parasites including *Cuscuta pentagona*, *Cuscuta japonica*, *Rafflesia cantleyi*, *Arceuthobium sichuanense*, *Triphysaria versicolor*, *Striga hermonthica*, *Santalum album*, *Orobanche cumuna*, *Orobanche ramosa*, *Orobanche aegyptiaca*. BLASTN, BLASTP and SRA BLAST against genome of *Brassica juncea*, *Brassica rapa*, *Brassica olerecea* is done to find out any similarity which can help us to in designing siRNA to target those candidates with this techniques. The alignment and phylogenetic analysis are done using above mentioned programs and tools. After doing analysis siRNAs are designed for those candidates using *in-silico* method i.e. siDirect (Figure 4). Various candidates are found those can be targeted using this strategy but potentially cytochrome p450 and carotenoid biosynthesis genes are identified as target candidates through this step. These candidates are included in Table 1 as target candidates through production of siRNA. Some regions are marked in the alignment of these genes sequences which shows the dissimilarity with its host plant. Based on that the list of siRNAs is prepared.

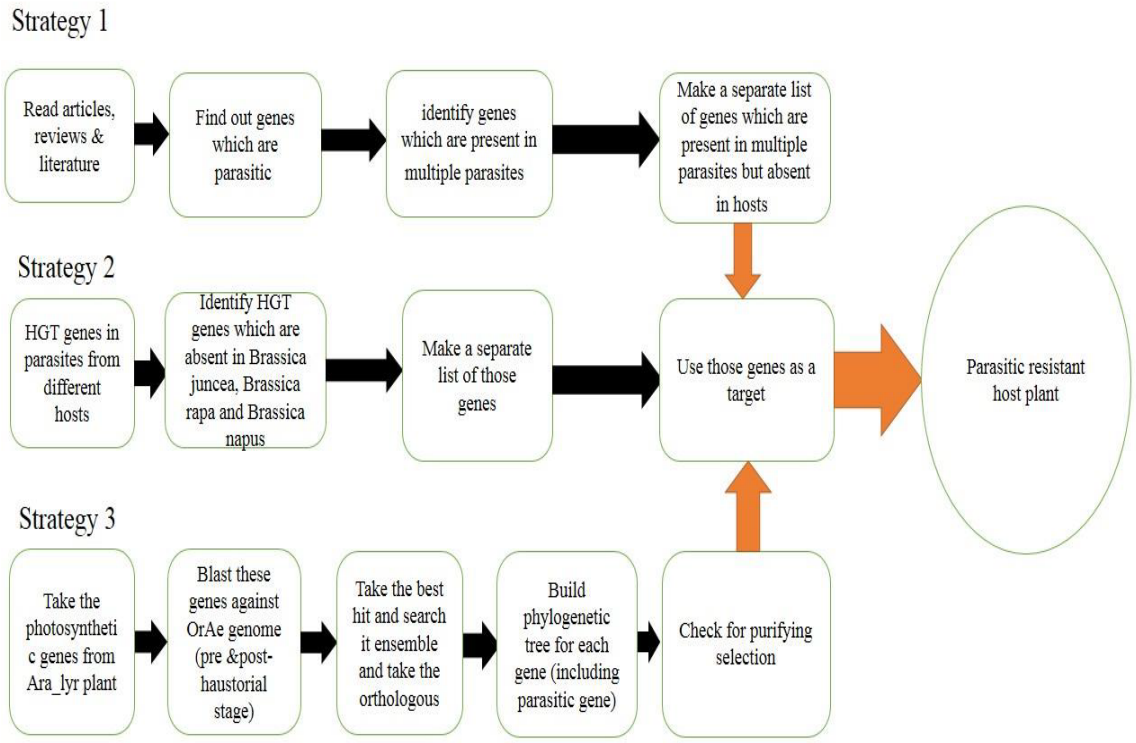

Figure 4: Flow chart which shows various suggested strategies used in our *In-Silico* method.

Strategy II (Genes which are transferred via HGT)

Past few years back an extensive study had been done on horizontally transferred genes in parasitic plants especially obligate parasite. Based on summarized those studies and it has been found that there are around 42 genes which horizontally transferred from host to family Orobanchaceae including *Orobanche aegyptiaca* from different rosids which are distantly related to parasitic plants. Some of them has been taken and checked for their presence in host plant *Brassica juncea* by using BLASTP, BLASTN and SRA BLAST on NCBI. Some are present but have some sequences similarity and some of the sequences were restricted only to parasitic plants meaning that they are absent in host plant. These sequences are put on screening and selected according to their role in parasite development. The siRNA is designed for those genes which can be used more potentially as new targets. Albumin I and cytochrome p450 (Table 1) are two of them. The alignment and phylogenetic analysis are done using above mentioned softwares and tools.

Strategy III (Photosynthetic genes found in Orobanche aegyptiaca)

It was well known that the plant *Orobanche aegyptiaca* is non-photosynthetic and it has lost its photosynthetic genes. But recent studies have proved that it has photosynthetic genes and it has lost their functions. This information is summarized for further analysis. Those genes are taken and checked under selection. The purifying selection is done using Arabidopsis thaliana as model plant. 42 genes are found which are chloroplastic in *Orobanche aegyptiaca*. Many hits are found in *in-silico* studies, but study is restricted to only 42 genes because they are chloroplastic. It might be possible that *Orobanche aegyptiaca* may use these genes for its machinery in early development i.e. before developing mature haustoria. Based on these assumptions these candidates are used for purifying selection and it is done using PAML and data-monkey (online server) and HYPHYMP. The NCBI ORF finder is used for getting open reading frame for our genes. The PPGP plant website is taken for consideration for getting genome of *Orobanche aegyptiaca*. The Arabidopsis lyrata from ENSEMBL plants website is used for validating our photosynthetic genes from the genome assembly of *Orobanche aegyptiaca* and *Arabidopsis lyrata* interface. In the analysis above mentioned tools are used for alignment, BLAST, siRNA design and sequence retrieval.

Results

Uniquely present HGT genes

The study has done for the genes which are commonly present in parasitic plant *Orobanche aegyptiaca* and host plant *Brassica juncea* as well as its other relatives i.e. *Brassica napus*, *Brassica olerecea* and *Brassica rapa*. Few candidates which are uniquely present in *Orobanche aegyptiaca* are found and those candidates can be used for targeting through small interfering RNA (siRNA). Apart from that there sixteen genes which are present in all *Brassica* host plants those can be used for further analysis of HGT in parasite from mustard species. Figure 5 and Table 2 show the name of genes and number of genes which are found in all above-mentioned species.

Comparative study for HGT genes by different alignment softwares

As earlier the term horizontal gene transfer is introduced in family Orobanchaceae, those genes are taken and aligned using MAFFT,

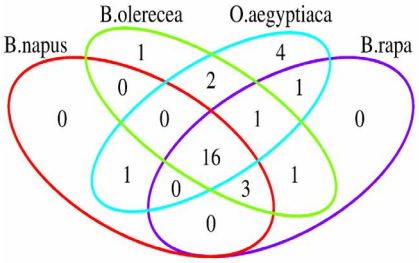

Figure 5: Venn diagram: It shows the candidates which are horizontally transferred from distantly related rosids to orobanchaceae plants. Here we have looked those in mustard specially which are economically important. This figure gives an idea about the presence of these genes in host plants and parasitic plant.

Sl.no	Gene Name	Target position	Target sequence 21nt, target + 2nt overhang	Guide strand 5'-3'	Passenger strand 5'-3'	Tm (guide strand, passenger strand
1	*Cytochrome p450*	312-334	TAGACTTTCTTACAATTTTTTGG	AAAAAAUUGUAAGAAAGUCUA	GACUUUCUUACAAUUUUUUGG	-12.0 ◦C,17.7 ◦C
		1-23	ATCAAAATGGCTACATTTATATG	UAUAAAUGUAGCCAUUUUGAU	CAAAAUGGCUACAUUUAUAUG	-1.8 ◦C, 11.3 ◦C
		111-133	TCCAATTGTTGGCAATTTACACC	UGUAAAUUGCCAACAAUUGGA	CAAUUGUUGGCAAUUUACACC	-0.3 ◦C, 5.3 ◦C
		47-69	CTCTATTTCTCATCTTTTTAAAG	UUAAAAAGAUGAGAAAUAGAG	CUAUUUCUCAUCUUUUUAAAG	-3.8 ◦C ,6.9 ◦C
2	*Albumin I (28)*	182-204	AAGATTATATGATTAATAAGTTT	ACUUAUUAAUCAUAUAAUCUU	GAUUAUAUGAUUAAUAAGUUU	-2.3 ◦C, -8.6 ◦C
		368-390	TCGTTAAAGCTTTCTTAAAAATT	UUUUUAAGAAAGCUUUAACGA	GUUAAAGCUUUCUUAAAAAUU	-3.8 ◦C ,13.7 ◦C
3	*Methionyl-tRNA synthetase*	681-703	GAGCCGAAATGCTATTAATATAA	AUAUUAAUAGCAUUUCGGCUC	GCCGAAAUGCUAUUAAUAUAA	-8.0 ◦C ,20.7 ◦C
4	*carotenoid cleavage dioxygenase-7(MAX3/CCD7)*	1385-1407	TTGGATTTCATGGATTTTGGTCT	ACCAAAAUCCAUGAAAUCCAA	GGAUUUCAUGGAUUUUGGUCU	11.3 ◦C ,16.3 ◦C
		1412-1434	ATGTTTCACGCAGTACTGTAAAT	UUACAGUACUGCGUGAAACAU	GUUUCACGCAGUACUGUAAAU	20.2 ◦C ,21.1 ◦C
		1427-1449	CTGTAAATAGCCCAGTTTTAAAG	UUAAAACUGGGCUAUUUACAG	GUAAAUAGCCCAGUUUUAAAG	4.9 ◦C, -2.3 ◦C

Table 1: The final list of designed siRNAs for the gene candidates.

MUSCLE, T-coffee, KALIGN, Clustal omega and bullied phylogenetic trees using FastTree. Topology has been checked by calculating Robinson foulds distance (Figure 6 and Table 3). Also, their significance level is checked by performing both kind of analysis, parametric (Wilcoxon test) and non- parametric (Kruskal Wallis test). The horizontally transferred genes can be used as a candidate for targeting through siRNA. In the study it has been shown that MAFFT is better to infer HGT compare to other software.

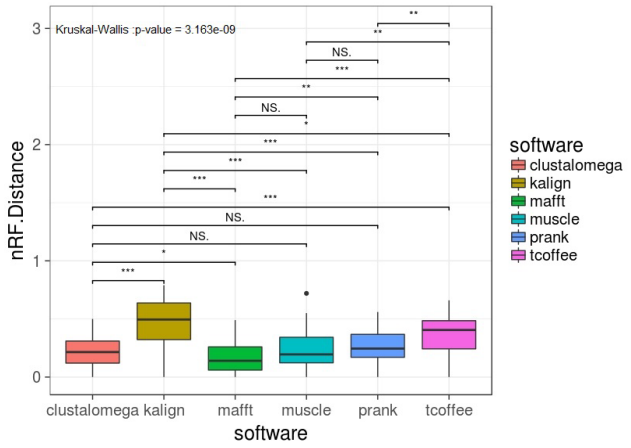

Figure 6: Boxplot which shows the distribution of normalized Robinson Foulds distance calculated by ete3 which gives an idea of tree topology. Here the Kalign (alignment tool) gives higher distance.

Gene Mannose 6-phosphate reductase in *Brassica rapa* and *Orobanche aegyptiaca*

Based on previous results it has been revealed that M6PR is essential for parasite root development and targeted through the production of homologous dsRNA in tomato plant. This gene sequence is taken, and alignment is performed with the gene sequence of its orthologous in *Brassica rapa* and *Orobanche aegyptiaca* (Figure 7). It found that one can use it and design siRNA as well as target the parasite through disruption of this gene function in parasite.

Parasitic genes present in multiple parasites

According to the third strategy genes are taken from different parasites including root & shoot parasites. Around 24 genes are selected and looked for their presence in various parasites including shoot plant parasite and root plant parasites. Various gene candidates are found which are specifically present. These data are plotted for few gene candidates in Figure 8 and the number of genes is given in Table 4. Some of them are commonly found in all selected plants. The study was restricted only those candidates which play role in the development of parasite in some way. Here wit can be can concludes that one can take commonly present candidates and design siRNA. That siRNAs will be used to treat all parasites and saved host from destroying them by parasites. So, these candidates can be used widely across the globe against controlling the plant parasites.

Photosynthetic genes in *Orobanche aegyptiaca*

It had been accepted earlier that the parasite had lost its photosynthetic genes during the evolution but after reading articles it is decided to look for photosynthetic genes *Orobanche aegyptiaca*. All the annotated sequences and assembly are downloaded from plant parasite

Brassica napus	***Brassica rapa***	***Brassica olerecea***	***Orobanche aegyptiaca***
Cytochrome P450	*Cytochrome P450*	*Cytochrome P450*	*Cytochrome P450*
Cysteine-rich receptor-like kinase	*Cysteine-rich receptor-like kinase*	*Cysteine-rich receptor-like kinase*	*Cysteine-rich receptor-like kinase*
BTB/POZ	*BTB/POZ*	*BTB/POZ*	*Proteasome subunit alpha type*
Disease resistance protein	*Disease resistance protein*	*Disease resistance protein*	*Disease resistance protein*
Ankyrin repeat family protein	*Ankyrin repeat family protein*	*Ankyrin repeat family protein*	*Hyoscyamine 6-dioxygenase-like*
Valyl-tRNA synthetase	*Kelch modif related to galactose oxidase*	*Kelch modif related to galactose oxidase*	*Ankyrin repeat family protein*
Methionyl-tRNA synthetase	*Valyl-tRNA synthetase*	*Valyl-tRNA synthetase*	*Kelch modif related to galactose oxidase*
Histidine-tRNA ligase	*Methionyl-tRNA synthetase*	*Methionyl-tRNA synthetase*	*Poly(A) polymerase*
Ribosomal protein S13	*tRNAHis guanylyltransferase*	*Histidine-tRNA ligase*	*Nucleolin 2-like*
ABC transporter C family member 3	*Histidine-tRNA ligase*	*Ribosomal protein S13*	*Valyl-tRNA synthetase*
Cytosolic purine 5-nucleotidase	*Ribosomal protein S13*	*ABC transporter C family member 3*	*Methionyl-tRNA synthetase*
Alpha/beta-Hydrolases	*ABC transporter C family member 3*	*Cytosolic purine 5-nucleotidase*	*tRNAHis guanylyltransferase*
Tubulin-specific chaperone D	*Cytosolic purine 5-nucleotidase*	*Alpha/beta-Hydrolases*	*Histidine-tRNA ligase*
Poly (ADP ribose) glycohydrolase	*Alpha/beta-Hydrolases*	*Tubulin-specific chaperone D*	*Ribosomal protein S13*
Nuclear pore complex protein	*Tubulin-specific chaperone D*	*Poly (ADP ribose) glycohydrolase*	*ABC transporter C family member 3*
Ubiquitin-like–specific protease 1	*Poly (ADP ribose) glycohydrolase*	*Nuclear pore complex protein*	*Cytosolic purine 5-nucleotidase*
Zinc finger, GRF-type	*Nuclear pore complex protein*	*Zinc finger, GRF-type*	*Albumin I (28)*
FBD-associated F-box protein	*Zinc finger, GRF-type*	*FBD-associated F-box protein*	*Alpha/beta-Hydrolases*
	FBD-associated F-box protein	*Uroporphyrinogen-III synthase*	*Tubulin-specific chaperone D*
	Uroporphyrinogen-III synthase	*hAT transposon*	*Poly (ADP ribose) glycohydrolase*
	Valyl-tRNA synthetase	*Putative harbinger Transposase-derived nuclease*	*Nuclear pore complex protein*
	Methionyl-tRNA synthetase	*MULE transposase*	*Ubiquitin-like–specific protease 1*
			FBD-associated F-box protein
			hAT transposon
			MULE transposase

Table 2: HGT genes from *Orobanche aegyptiaca* to *Brassica napus*, *Brassica olerecea* and *Brassica rapa*.

HGT Genes	Clustal Omega	Kalign	MAFFT	Muscle	Prank	T-Coffee
ABC transporter C family member 3	0.06	0.05	0.06	0.06	0.03	0.43
Histidine-tRNA ligase	0.21	0.72	0.15	0.28	0.46	0.44
Tubulin-specific chaperone D	0.18	0.43	0.2	0.25	0.41	0.34
Kelch motif related to galactose oxidase	0.5	0.69	0.49	0.72	0.53	0.66
Hyoscyamine 6-dioxygenase-like	0.3	0.64	0.26	0.22	0.26	0.42
Putative harbinger Transposase-derived nuclease	0.12	0.53	0.07	0.35	0.21	0.47
Hypothetical protein	0.22	0.34	0.17	0.24	0.26	0.52
Ribosomal protein S13	0.2	0.2	0.2	0.16	0.16	0.16
MULE transposase	0.07	0.48	0.07	0.1	0.12	0.22
POSUnknown	0.46	0.51	0.44	0.5	0.47	0.62
hAT transposon	0.26	0.7	0.12	0.15	0.16	0.28
Valyl-tRNA synthetase	0.06	0.47	0.09	0.13	0.17	0.43
Cysteine-rich receptor-like kinase	0.39	0.63	0.37	0.55	0.38	0.57
Ankyrin repeat family protein	0.33	0.47	0.12	0.17	0.41	0.23
Methionyl-tRNA synthetase	0.31	0.52	0.31	0.17	0.24	0.4
Proteasome subunit alpha type	0.1	0.32	0.06	0.18	0.28	0.37
Poly (ADP ribose) glycohydrolase	0.17	0.54	0.04	0.13	0.17	0.24
tRNA His guanylyltransferase	0.22	0.6	0.4	0.44	0.27	0.47
FBD-associated F-box protein	0.42	0.79	0.49	0.52	0.51	0.62
hAT transposon	0.47	0.72	0.33	0.38	0.38	0.49
Uroporphyrinogen-III synthase	0.21	0.25	0.12	0.1	0.19	0.23
Poly(A) polymerase	0.31	0.75	0.14	0.45	0.56	0.64
Unknown	0.33	0.66	0.17	0.32	0.24	0.41
BTB/POZ	0.12	0.38	0	0.12	0	0
hAT transposon	0.4	0.67	0.26	0.44	0.33	0.58
hAT transposon	0.11	0.72	0	0.06	0.11	0.61
Albumin I (28)	0.21	0.05	0.05	0.11	0.21	0.11
Zinc finger, GRF-type	0.3	0.35	0.22	0.32	0.3	0.49
Unknown	0.2	0.4	0.13	0.27	0.33	0.27
Unknown	0.28	0.55	0.21	0.21	0.17	0.46
Unknown (26)	0.29	0.53	0.16	0.25	0.2	0.46
Unknown	0	0.25	0	0	0	0.25
Unknown	0	0	0	0	0	0
Disease resistance protein	0.14	0.29	0	0.14	0.21	0.29
Unknown	0	0	0	0	0.5	0
Cytochrome P450	0.12	0.09	0.07	0.05	0.1	0.12
Alpha/beta-Hydrolases	0.29	0.33	0.28	0.36	0.3	0.37
Cytosolic purine 5-nucleotidase	0.5	0.59	0.15	0.16	0.25	0.36

Table 3: Robinson Foulds distance calculated by ete3 for HGT gene ortho groups.

Figure 7: The alignment of gene M6PR sequence taken from *Brassica rapa* and *Orobanche aegyptiaca*. The yellow color represents the region which can be targeted by siRNA.

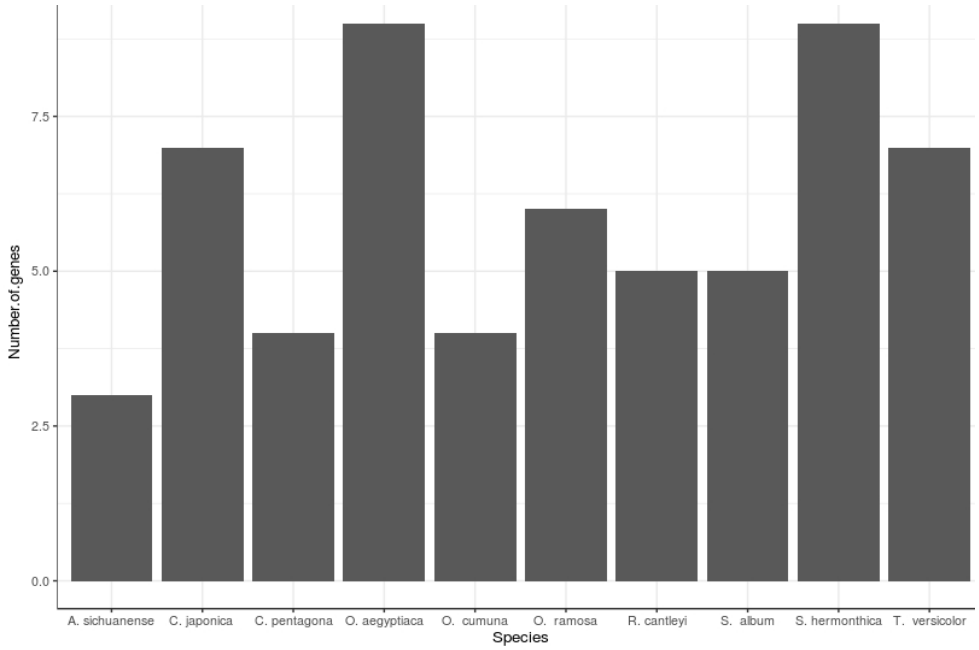

Figure 8: We have looked for genes commonly present in parasites and built a bar plot. There are genes at least 3-4 those are present in all parasites and play crucial role in their development. By designing siRNA, those genes can be targeted.

C. japonica	*R. cantleyi*	*A. sichuanense*	*T. versicolor*	*S. hermonthica*	*O. aegyptiaca*	*S. album*	*O. ramosa*
0	1	0	1	0	0	1	0
1	0	0	0	1		0	0
0	0	0	0	1		1	1
0	0	0	0	0	0	0	0
0	0	0	0	0	1	0	0
1	1	1	1	1	1	1	1
0	0	0	0	0	0	0	0
0	0	0	0	1	1	0	0
0	0	0	0	0	0	0	0
0	0	0	1	0	1	0	1
0	0	0	0	1	1	0	0
0	0	0	1	0	0	0	0
1	0	0	1	0	0	0	0
0	0	0	0	0	0	0	0
1	1	0	0	0	0	0	0
0	0	0	0	0	0	0	0
0	0	0	0	1	0	0	0
1	0	0	0	0	0	0	0
1	1	1	1	1	1	1	1
1	1	1	1	1	1	1	1
0	0	0	0	0	1	0	1
1	0	0	0	1	1	0	0
0	0	0	0	0	0	0	0

Table 4: Genes which are common in various parasites. (0 means absent, 1 means present).

genome project. There are 42 genes which are photosynthetic as well as chloroplastic in this plant parasite. These genes were searched in ensembl plant website but some of them are not chloroplastic. Some of the accession numbers were present more than one times. After refining them some candidates are selected and checked for pseudo genes by doing blast search (Table 5a). Also, the analysis is done using PAML and calculated branch omega for each branch and plotted those values here in Figure 9. The Significance level is checked by likelihood ratio test using PAML. The results have shown strong purifying selection in these genes. Most of the genes in selected data show less than one omega value for those genes which revealed that these genes are not pseudo genes. The details are provided in Tables 5a and b. Based on

Accession No	Gene Name	E-value (BLAST search)	BLASTN hits
AT1G74960	chloroplastic	4.00E-64	2
AT1G77130	not chloroplastic	4.00e-32, 4.00e-54, 4.00e-12 , 1.00e-24	4
AT2G21340	chloroplastic	3.00E-64	1
AT2G36390	chloroplastic	8e-08, 8e-26	2
AT3G02780	not chloroplastic	6e-11, 5e-22, 2e-32	3
AT3G11420	not chloroplastic	0.0001	0
AT3G12120	not chloroplastic	5e-22, 5e-32, 7e-04	3
AT3G12120Arath	not chloroplastic	4e-36,4e-36, 1e-24	3
AT3G15940	not chloroplastic	1e-12, 1e-12	2
AT1G52420	not chloroplastic	0.18	0
AT3G20440	chloroplastic	7.00E-42	1
AT3G25110	chloroplastic	4.00E-19	1
AT3G25780	chloroplastic	1.00E-16	1
AT3G25860	chloroplastic	3e-32, 4e-12, 4e-18, 1e-36	4
AT3G53520	not chloroplastic	1e-64,1e-32,2e-18, 7e-55, 6e-05	5
AT3G56910	chloroplastic	4.00E-16	1
AT3G60750	chloroplastic	1.00E-29	1
AT3G62830	not chloroplastic	6e-56, 6e-52, 3e-56, 4e-22, 1e-64	5
AT4G00300	not chloroplastic	0.007	0
AT4G04770	chloroplastic	2e-40, 5e-04	2
AT4G16390	not chloroplastic	0.04	0
AT4G18240	chloroplastic	2.00E-32	1
AT4G20010	chloroplastic	1e-43, 1e-43	2
AT4G25100	chloroplastic	7.00E-33	1
AT4G39030	not chloroplastic	0.066	0
AT5G23310	not chloroplastic	0.003	0
AT5G24300	not chloroplastic	0.011	0
AT5G24300Arabidopsis_thalia	not chloroplastic	0.065	0
AT5G59290	not chloroplastic	4e-55, 2e-53,7e-46	6
OrAeIntArathGB1_6547	not chloroplastic	3e-86,3e-86, 7e-82	3
OrAeIntArathGB1_8294	chloroplastic	1.00E-32	1
OrAeIntArathGB1_15711	chloroplastic	1.00E-19	1
OrAeIntArathGB1_33338	not chloroplastic	3.00E-44	3
OrAeIntArathGB1_36601	not chloroplastic	0.14	0

Table 5a: Genes searched on ensemble plant and their BLASTN results on NCBI GenBank.

those results it can concluded that these genes play crucial role in early development of plant parasite before developing mature haustorium.

Discussion

The family Orobanchaceae consists a wide range of plant parasites which devastatingly destroy the economically important crops across the world including India. This plant family is nexus for future studies because it has plants those have different characteristics and affecting other plants in various degrees. So far various strategies including chemical, physical and biological have been applied to control orobanchaceae plants in different crops including potato, tomato, tobacco, mustard etc. The oilseed mustard devastatingly affected by this plant family. Many herbicides like glyphosate, sulfosulfuron, and imidazolinones have been used under the chemical studies. All these compounds are synthesized based on pot studies and it has been seen that these compounds work at certain level but also have side effects on the crops. Also, these compounds are too costly in the market. In many regions of India, farmers adopt weed handlings to remove this plant. But at a large scale this is not considered an effective way of removing parasites completely. Various methodologies and techniques have suggested and some of them have been used in different economically important crops to escape Orobanchaceae plants. The *Orobanche aegyptiaca* has its various stages during its germination so based on that one can think about to stop it at different levels.

Accession No	dN/dS
AT1G52420	1.0709
AT3G15940	0.807175
AT3G53520	0.5287
AT3G56910	0.41492
AT4G00300	0.315188
AT4G04770	0.423511
AT4G18240	3.109
AT4G20010	0.357928
AT4G25100	0.429679
AT5G23310	0.901834
AT5G59290	0.0001
AT2G21340	0.957701
AT3G02780	0.415677
AT1G74960	0.4646723
AT3G20440	0.1810371
AT1G77130	0.938029
AT3G11420	0.409604
AT3G12120	0.0001
AT3G62830	0.54137
AT2G36390	0.441619

Table 5b: dN/dS for each branch for selected genes.

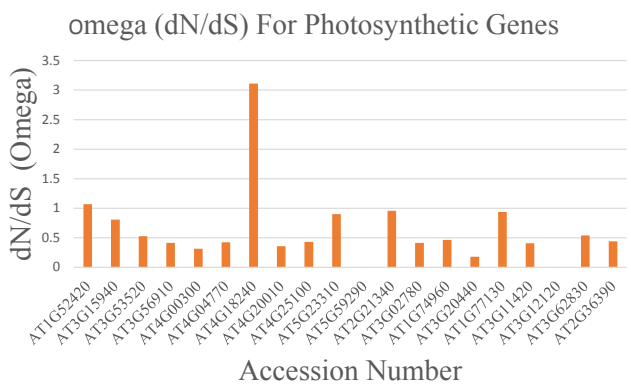

Figure 9: dN/dS values were plotted for genes those are beneficiary in photosynthesis and heme cofactor. Most of the genes show omega (dN/dS) less one which reveal purifying selection.

Some studies have suggested biological agents for destroying it. There are few fungal species which are specific to *Orobanche aegytpica* and caused diseases. *Fusarium* species are one of them. A case study has been done on *Fusarium oxysporum orobanche sp.* and it has been found that the fungal agents caused diseased specifically to *Orobanche aegyptiaca*. Along with this no disease have been caused in host plant. But much study had not done in *Brassica juncea* and *Orobanche aegyptiaca* in this regard. This also can be a good idea, but the fungal agents can cause any kind of disease to *Brassica juncea*. So, a details study needs to do in this field.

Gene targeting along with integrated approach can be one of the best methods to control the plant parasites of the family Orobanchaceae. For this we need to understand all possible combinations and candidates which can be effective in this case. The selection of gene candidate is very tough task. A broad study has been done in tobacco with respect to *Orobanche ramosa* and *Orobanche aegyptiaca* but no study has been conducted in context of *Brassica juncea* and *Orobanche aegyptiaca*. Based on research articles, literatures, reviews, case studies and scientific reports, here it is concluded that there is specific fungus to both *Orobanche aegyptiaca* and *Brassica juncea*.

Throughout the study it is found that there can be few possibilities while selecting candidates for gene targeting methodology. For instances, fungal agents may be used for our target and itself *Orobanche aegyptiaca* can be used for this purpose. A deep study is done on many research articles, reviews, literature and scientific reports. After getting idea from them various gene candidates are selected and analysis is performed. A temporary list of candidates is prepared which can be differentiate just based on their presence or absence in both the plants *Orobanche aegyptiaca*, *Brassica napus* and *Brassica juncea* as well as *Brassica rapa* list of candidates. Then, few genes are selected and used for study. Using blast search, alignment with *Brassica juncea* sequences, strongly gone through for siRNA designing for those candidates. Effective candidates naming Cytochrome p450, Methionyl-tRNA synthetase, Albumin I, carotenoid cleavage dioxygenase-7 (MAX3) are fund for designing siRNA. Some of them are horizontally transferred. The siDirect online tool is used for designing siRNA as discussed above in material & methods section. The Table 6 gives a list of that candidates those can be used to target plant parasite through the production of siRNA [1]. The above-mentioned gene candidates involve in the development of plant parasite growth.

Based on field and pot studies through-out the study it has been suggested that there are bacterium and fungal agents those inhibit tubercle growth of *Orobanche aegyptiaca*. Few fungal species attacks on parasite directly and make diseased to it (Table 6). Apart from that various research articles provide a deep insight into post transcriptional gene silencing using viroid RNA.

A host plant produces strigolactone as signal through that signal it receives nutrient in more amount. It has been noted that both *Orobanche aegyptiaca* and *Brassica juncea* have arbuscular mycorrhizal fungal association to increase the range of receiving nutrients (Table 6). Both plants have specific fungus. It is hypothesized that there can be situation where host plant produces signal which passes through its fungus and received by *Orobanche aegyptiaca* through its specific fungus. Here fungus might be a traffic for signal coming from the host to parasite. As it is mentioned above, few fungi are specifically diseased to *Orobanche aegyptiaca*. These candidates can be used to control the parasite.

Bacteria	Fungus
B. subtilis QUBC18	*Fusarium oxysporum* in OrAe
Bacillus atrophaeus QUBC16	*F. oxysporum f. sp.* Orthoceras in OrCu
P. fluorescens QUBC3	*F. oxysporum f. sp.* Orobanchein in OrAe
Pseudomonas aeruginosa QUBC1	*Fusarium arthrosporioides* in OrAe

Table 6. The fungal and bacterial agents used against plant parasites.

Recently, bio briquetting machine is new creation for reducing pollution. This machine will also help to reduce dispersion of plant parasites at certain level by which income of farmers can be increased that is an extra advantage of using parasite.

The evolutionary studies have revealed that the plant parasite *Orobanche aegyptiaca* is a non-photosynthetic plant & have lost its photosynthetic genes. Few years back it also has been found that *Orobanche aegyptiaca* consists photosynthetic genes. There can be some possibilities, one is that *Orobanche aegyptiaca* uses its genes to synthesize food in its pre-haustorial attachment stage. The second possibility is that it might be uses those genes for other functions. It also has been revealed that the photosynthetic genes may be present but bypass the product of their original product and contribute in producing some other product. The third one is that whether those genes are pseudo-genes. To identify a pseudo gene is somewhat tough but at certain level one can assure based on some previous studies to identify them. The results are confirmed using PAML and HYPHY. It is suggested that those genes do function during early development of parasite. The likelihood ratio test is done to check significance level for photosynthetic genes. Based on the study it is concluded that they are not pseudo-genes and they play role at certain level in development of root parasite plant *Orobanche aegyptiaca*.

Conclusion

In the conclusion one can say that there are several key genes involved in seed germination, root development and growth which are associated with changes in the plant hormones and other pathways. There are genes which are acquired through horizontal gene transfer by plant parasite from the host plant. These genes may be developed as new targets to control *Orobanche aegyptiaca*. These genes were validated by q-PCR and their levels were verified in seed germination experiments. Few genes are identified and designed siRNA with their melting point and reduced seed sequences. Those genes have crucial role in plant parasite development. The candidates found for target in our study includes Cytochrome p450, Methionyl-tRNA synthetase, Albumin I, carotenoid cleavage dioxygenase-7 (MAX3). Using siRNA, designed for these candidates can be used to treat plant parasite *Orobanche aegyptiaca* so that one can save the economically crops especially *Brassica juncea* from this parasite and enhance the production as well as improve the fertility of those economically important crops. The above-mentioned gene candidates helps in signal transduction, plant growth and plant hormonal pathway.

Based on the results found in the study one can hypothesize that the gene which encodes for mannose-6 phosphate reductase enzyme is transferred horizontally from *Brassica rapa*. Further, this can be validated by q-PCR and can be used as a target against *Orobanche aegyptiaca*.

The plant parasite *Orobanche aegyptiaca* has photosynthetic genes. In the study it is concluded that *Orobanche aegyptiaca* uses photosynthetic genes in early development. The formation of terminal haustorium may include signal coming from photosynthetic genes. The photosynthetic genes encode chlorophyll and heme. It has been

concluded that in *Orobanche aegyptiaca* photosynthetic genes may help in the production of heme formation and chlorophyll a and chlorophyll b. It means photosynthetic gene have alternate function in *Orobanche aegyptiaca*. In plant parasite photosynthetic genes are not pseudo-genes. They are crucial for plant at certain degree. Second hypothetical view is that those genes are showing their presence because they are evolving, and it can be possible that in future they become non-functional completely. These genes can be used as new target for control of parasite. Throughout the study it is found that there are many fungal agents which are associated with both *Orobanche aegyptiaca* and *Brassica juncea*. Few fungi are specifically localized with plant parasite and caused diseased to plant parasite. It is hypothesized that fungal association might be traffic for signal transduction between host and parasite. In future, one should look in to those candidates like fungus and include them as new target. It can become a hallmark in this field. Instead of that, there are some enzyme present on fungus and bacteria which degrade cell wall of plant cell specifically. Those candidates can be used potentially.

Future Perspectives

The plant-plant interaction is one of the most interesting field of biology. Along with this, people are trying to see signal transduction mechanism associating with plant parasite. In future, the role of fungal association in signal transduction can be explained in host-parasite system. Some diseased caused fungus can be used as target and can be useful to control of *Orobanche aegyptiaca* infestation in crops and it can be challenged to identify suitable strategies on those agents. Apart from that fungal enzymes can be used to degrade cell wall of plant parasite specifically. There are many fungal enzyme databases along with all information. But it would be a challenge to identify the suitable candidates and stratifies for those candidates. Also, in future it needs high profile technology and tools as well as algorithm for finding those candidates and applying technology on them. Plant parasites have photosynthetic genes and those are not pseudo-genes. So, these genes can be used to alter any function of plant parasite so that it leaves the host plant. A detail study is needed with respect to this plant-host-parasite system. One possibility is that these genes can have some other functions, due to that they show their presence. This can be validated if further research carries out in this regard. Another hallmark regarding plant parasite is that it acquires DNA, mRNA and protein from many hosts. But no study is reported from host *Brassica juncea*. It can be possible that this plant parasite follows horizontal gene transfer. So, it is still a question how the plant parasite transports horizontally transferred mRNA, protein and nuclear as well as mitochondrial DNA from various hosts to its other parts. One possibility is that they use plasmodesmata for transferring any kind of material from its host. Further efforts are needed in this regard. Using fungal agents, one can think to design transgenic mustard like BT cotton. Apart from that the genome of *Orobanche aegyptiaca* is sequenced with low coverage. The N50 is around 244. So, in future the work can be carried out on it and genomes will be sequenced with high coverage value and good N50. Demographic population analysis can also be done on both parasite and host. In future, one can understand the demographic pattern in these plants and can infer some crucial conclusions. It will help to understand the relationship of these two plants at broad level. Based on those conclusions one can think to interfere with the machinery of plant parasite and increase the production as well as fertility of host plants.

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