Short Title: Long distance movement of small RNAs in Host-Parasitic complexes

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**Research area:**

**Signalling and response**

**Long distance movement of naturally occurring small RNAs in Host-parasitic plant complexes generating from common source to elucidate common function.**

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One sentence summary: Small RNAs generating from orthologous genes are moved to long distance tissues in bidirectional manner for their common target in *Cuscuta japonica*- *Glycine max* and *Cuscuta campestris*-*Arabidopsis thaliana* complexes.

**Author contributions**

**Footnotes**

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**Abstract**

*Cuscuta*(Dodder)are holo-parasitic plants grow on host plants through haustorial formation to uptake water and nutrients for their survival and growth. Plant endogenous mRNAs and proteins are also moved in bi-directionally through parasitic interface tissues. Recent report reveals that accumulation of interface tissue specific miRNA of parasitic plantsoccurs to control/or regulate host gene through secondary siRNA production during host-parasitic plant interaction. However, there is no direct evidence thatlong distance movement of naturally occurring small RNAsoccur. Here, in this paper, we report that naturally occurring small RNAs of both host plant and parasitic plants moved long distance through interface tissues. Analyses of sRNAs of different species of Cuscuta (*C*. *japonica* and *C*. *campestris*) parasitizing on different hosts (*Glycine max* and *Arabidopsis thaliana* respectively) reveals that naturally occurring mobile sRNAs are generating form several common (orthologous) gene familyas well as some sRNAs are also targeting orthologous genes and moved to long distance tissue to control trans-species gene regulation.

Key words: Long distance movement, Small RNA, Ortholous gene

**Introduction:**

*Cuscuta* (Dodder), a holoparaiticplant belongs to the family Convolvulaceae, including 150 species, naturally attack domesticated and non-domesticated plants and suppress the host growth and sometimes leading to host death (Alakonya et al, 2012). Dodder seed germinate in the soil by inductionof strigolactone and other hormones with thread-like stem containing little cholorophyll for few days’ survival. Non parasitic dodder seedlings search nearby host plants to parasitize with them. Volatile oil of host plants induces the dodder attachment to host followed by parasitization through haustorial formation (singular: Haustorium) and continuation of the process. Long, unicellular hyphae are produced in hausotia which help them to connect host vascular tissues of host plant to withdraw water and nutrients from hosts for their survival growth.Vascular connection also allowsdoddermacromolecules such as mRNAs (Roney et al., 2007; LeBlanc., 2008; David-Schiwartz., 2013), proteins (Haupt., 2001), pathogens like viruses and viroids (Brischwilks., 2006).

A large number of mRNAs exchange occur through interface tissue in bidirectional manner in *Cuscuta pentagona-Arabidopsis thaliana* parasitic plant complex. Similar phenomenon was also observed in Cuscuta-Tomato complex. Directionality of mRNA movement was first reported by (Kim et al, 2014) although their function is not clear. This finding demonstrates that RNA-based mechanism may involve in host-parasitic interaction to establish the connection and continue the process.In addition to mRNA exchange there is a strong possibility movement of short length RNAs like tRNA, piwiRNA, miRNA and small RNA etc. which can directly involve in gene regulation. Here, in this paper we are emphasized on the study and analyses of small RNAs.

From the biogenesis, regulatory mechanism and functional aspects, small RNAs are categorized into different groups: micro RNAs, trans-acting small interfering RNAs (ta-siRNAs), natural *cis*-antisense RNAs (cis-nat-siRNAs) and heterochromatic small interfering RNAs (hc-siRNAs) (vaucheret., 2017), are involved in transcriptional gene silencing (TGS) and post-transcriptional gene silencing (PTGS) (Axtell et al., 2011; Li et al., 2011) through inactivation, translational repression and degradation of mRNAs. miRNAs are produced from nuclear genomic loci as pri-miRNAs as intermediate product. Dicer like-1 (DCL1) is responsible for the formation of miRNA duplex which cleave mRNA through RNA-induced silencing complex (RISC) formation with Argonaut-1(AGO1) protein (Axtell et al., 2011), (Li et al., 2011). miRNA also target ta-siRNA loci (*TAS*)which are transcribed from nuclear gene to generate ta-siRNAs. RNA dependent RNA polymerase (RDR6) and Suppressor of gene silencing (SGS3) transformed single stranded TAS cleavage product to double stranded RNA duplex. DCL4 subsequently processed dsRNAs into 21-nt ta-siRNAs (Gasciolli et al., 2005). Cis-nat-siRNAs are produced from natural antisense transcripts (NATs) by the endogenous function of DCL2 reported in Arabidposis (Borsani et al., 2005). Small RNAs not only control endogenous gene but moved to long distance tissue. However, althoughbiogenesis pathway of small RNAs are little known,their regulation, role in cross-kingdom RNAi and mode of transportation is still subject of investigation. The scope of understanding of long distance movement of small RNAsand their mediated gene regulation during plant-plant association and/or plant-pathogen interaction is focused on here.

Plant pathogenic fungus *Botrytis cinerea*generate small RNAs which target host mRNAs during pathogenic interaction to hijack host immunity (Weiberg et al., 2013). Conversely, host-induced gene silencing (HIGS) and siRNA-induced transgenes silencing is also reported against nematodes (Huang et al., 2006), insects (Baum et al., 2007), fungi (Nowara et al., 2010) and the parasitic plant *Cuscuta pentagona* (Alakonya et al., 2012). Father more, study on fungal infection caused by *Verticillium dahliae* on cotton plants report that host miRNA accumulates and export to fungal hyphae for specific silencing. So, there is indication that small RNAs can move in bidirectional manner.

In recent study, miRNA study of host-parasitic Plant complex (*Cuscuta campestris –Arabidopsis thaliana* and *C. campestris- Nicotiana benthamiana* parasitic complexes) report that 22nt long miRNA are accumulated from parasitic plants in interface tissue which cleave host mRNA and produce secondary siRNA in hosts (Sahid et al., 2018). Although they report on parasitic miRNA target host mRNA in adjacent tissues it is not clear whether they moved to long distance or not. The response of host against parasitic plant and role of host generating small RNAs in host-parasitic complex is unknown. Now, the questions are arise- does small RNA also exchange in host-parasitic complex? The naturally occurring small RNAs directionality and movement is selective in host-parasitic complex? How small RNAs function in natural condition in host-parasitic interaction?

Here, in this paper, we are focusing on long distance movement of naturally occurring small RNAs of both hosts and parasitic plants in different host-parasitic plant complexes. Two species of *Cuscuta* as parasitic plant (*Cuscuta japonica* and *Cuscuta campestris*) grows on different hosts (*Glycine max* and *Arabidopsis thaliana*) respectively. The validation and mobility of mobile small RNAs are checked in plant complexes. We are also trying to find out commonality of mobile small RNAs of these complexes i.e. mobile small RNAs are generating from orthologous gene as well as mobile small RNAs targeting orthologous gene after movement.

**Materials and methods:**

**Plant materials:** Parasitic plant of cuscuta (*Cuscuta japonica* and *Cuscuta campestris*) grows on two different hosts (*Glycine max* and *Arabidopsis thaliana* respectively) are used for experimental analyses.

**Seed germination and plant parsitization:** Dry seeds of *C. japonica* and *C*. *campestris* seed are treated with concentrated Sulfuric acid (192-04696, Wako Pure Chemical Industries Ltd., Osaka, Japan) for 15 and 30 minutes respectively to soften the seed coat, followed by repeated washing with distilled water until pH comes to normal (pH -7). Then seeds were placed on wet paper in fiber Petri dishes (GA-100, Toyo-Roshi Kaisha, Ltd., Tokyo, Japan) at 25ᵒC for 1 weak. The seedlings were then placed on vermiculate and grown in controlled environment (16h light/8h dark cycle, 25ᵒC). These parasitic plant seedlings are used for plant parasitization with host plants.

*Glycine max* seeds were germinated through conventional germination protocol in ambient condition of light, temperature and relative humidity in growth chamber. Seedlings were grown on soil (Sukoyaka-baido, Yammar Co. Ltd., Osaka, Japan) with same volume of vermiculate mixture (GS30L, NITTAI Co., Ltd., Osaka, Japan) supplemented with 1000 times diluted Hyponex (S8038-1, HTPONeX JAPAN CROPS Ltd., Osaka, Japan) in 16h light/8h dark cycle (Plant-lux, Fl40S-BRN, TOSHIBA Crop., Ltd., Osaka, Japan) at 25ᵒC. 14 days old seedlings will be selected for parasitization. For *A*rabidopsis *thaliana*, seeds were germinated and grown on vermiculate supplemented with Hyponex (1000-fold dilution) in 16h light/8h dark cycle at 22ᵒC and 25ᵒC. Flowering individuals were selected for parasitiztion.

**Host-parasitic interaction and sample harvest:** 7days old of *C. japonica* seedlings are parasitized on 14 days old *of G. max* with 1h exposure of Far-red light (FL20S-FR-74, TOSHIBA Crop.) as a stimulant, followed by 23h of darkness (Tada *et al*, 2000). For *A. thaliana* - *C. campestris* plant parasitic complex, blue light (BC-BML4 λp=450 nm, Biomedical science K.K., Tokyo, Japan) was used instead of Far-red light and parasitization procedure is same as above. Sample was harvested according to (Kim et al., 2014) with little modification. In brief, following tissues were harvested from10daysold host-parasitic plant complex: Junction parasitic sample (JPS) also known as Interphase tissue;*C. japonica* parasitic sample (CJPS) - 1 cm away from the interphase tissue which only contain parasitic tissue and G. max parasitic sample (GMPS) - 1 cm away from interphase tissue which only contain host tissues. *Glycine max* non parasitic sample (GMNS), which are not parasitized to *C. japonica* and 10 day old *C. japonica* non parasitic sample (CJNS), grown on vermiculate are also harvested as control.

Harvested tissues of *C. campestris* – *A. thaliana* complexes are named as JPS - Junction parasitic sample, CCPS - *C. campestris* parasitic sample, ATPS – *A. thaliana*parasitic sample, CCNS - *C. campestris*non-parasitic sample and ATNS – *A. thaliana*non-parasitic samples. All these tissue specific samples were harvested by pooling from different associations at a time.

**RNA isolation and small RNA sequencing:** Total RNA was isolated from all sets of samples using miRCURYTMRNA isolation kit- Cell & Plant, following manufacture’s protocol including β-mercaptoethanol and RNase inhibitor in the extraction buffer. Total RNA was treated with DNase to remove residual DNA. RNA quality and integrity was checked by Agilent High Sensitivity RNA ScreenTape 2200 (Agilent,CA). Samples with two replications of each tissues which have integrity number above 6.5 are used for sequencing. Small RNA-seq of *G. max*–*C. japonica* parasitic complex was performed at ……………*A. thaliana*–*C. campestris* parasitic complex’s small RNA sequence was performed at…Small RNA-seq was performed from total RNA following the manufacture’s protocol of TruSeq Small RNA kit (Illumina) on Hiseq2500 instrument (Illumina). Small RNAs are isolated from 6% TBE Urea gel and SYBR Gold (Invitrogen) Agilent High Sensitivity DNA kit and KAPA Library Quantification Kit (For Illumina) are used for preparation of small-RNA-seq libraries.

**Sequence pre-processing and filtering:** Raw data in FASTQ format are processed by using several softwares. Quality and read length distribution of raw data are checked by FastQC(<https://www.bioinformatics.babraham.ac.uk/projects/fastqc/>). Barcode remove and adapter trimming was performed by FASTX-Toolkit (<http://hannonlab.cshl.edu/fastx_toolkit/download.html>)followed by quality control (-q 20 –p 80). Sequences are filtered 15-25 nt fragments by Prinseq*-*lite (<https://sourceforge.net/projects/prinseq/files/>) and small RNA reads are mapped on transcripts with zero mismatch by NCBI-Magic blast (<https://ncbi.github.io/magicblast/>). Glycine max transcripts (GCF\_000004515.4\_Glycine\_max\_v2.0) and *Cuscuta japonica* transcripts are used for mapping of *G.max - C. japonica* parasitic complex derived sRNAs. The mapped sRNA reads of non-parasitic and parasitic tissues of both Host and parasitic plants are analyzed for mobile sRNAs. For *A. thaliana - C. campestris* parasitic complex, TAIR10 and Cuscuta campestris version-3 (Ccv3) transcripts are used for mapping.

**Target prediction of mobile sRNAs and searching common target (orthology genes) in different parasitic complexes:** Parasitic plant’s (*C. japonica* and *C. campestris)* mobile sRNAs target prediction was done by psRNATarget software (<http://plantgrn.noble.org/psRNATarget/home>) with default setting using mobile sRNA reads as queries and opponent plant’s genes (*G. max* and *A. thaliana* transcripts respectively) as target. The output data are filtered by (Expectation value, 2.5). To find the orthologous genes of hosts been targeted by parasitic mobile sRNAs, hosts genes of interest (*G. max and A. thaliana* transcripts) are used as queries for a BLASTX analyses against 37 eudicot proteomes available on Phytozome-12 (<https://phytozome.jgi.doe.gov/pz/portal.html>) as mentioned by (Shahid et al., 2018). On the other hand, *A. thaliana* and *G. max* transcripts are BLASTX against TAIR10 protein database. Common results of these two BLAST search against different datasets are considered as common target of hosts by parasitic plant’s mobile sRNAs.

Target prediction of host’s mobile sRNAs and orthologues search of predicted targets of parasitic plants (*C. japonica* and *C. campestris*) are also analyzed by above mentioned methods. In brief, *G. max* and *A. thaliana* sRNAs target predictions are done against *C. japonica* and *C. campestris* transcriptsrespectively followed by BLASX search of targeted transcripts against Phytozome12 of 37 eudicot proteomes and *C. campestris* proteomes obtained from *Cuscuta campestris* genome project (<https://www.plabipd.de/project_cuscuta2/start.ep>). Common results of these two BLAST search against different datasets are considered as common (orthologues) targets of parasitic plants by host’s mobile sRNAs.

**Confirmation of small RNA sequence by stem-loop PCR:** Presence of small RNAs are confirmed by stem-loop PCR described earlier by (van Kleeff et al. 2016, Kramer, 2011 and Varkonyi-Gasic et al. 2007). Total RNA was used for a reverse transcription reaction ReverTra Ace –α– (Toyobo) with sRNA specific RT-primers, complimentary with 3’ end of sRNA. The cDNA was used stem-loop RT-PCR using 5’ sRNA specific forward primer and universal reverse primer (KOD-plus). In reaction mixture, 1 µl of cDNA used as a templet for the 20µl total volume of reaction for 40 cycle with an annealing temperature 58°C for 30 second and 68°C for 05 second of extension. PCR products were run on 5% agarose gel and cloned with Zero Blunt TOPO PCR cloning kit followed by sanger sequencing.

**Confirm the mobility by cross species checking:**

**qRT-PCR:** Accumulation level of mRNAs are checked by qRT-PCR as mention by (Shimizu et al., 2018). Briefly, Total RNA was used for cDNA synthesis using oligo(dT) primer by ReverTra Ace –α– (Toyobo). Fast SYBRTM Green Master Mix (Thermo Fisher Scientific) was used for Real-time PCR on Step One Plus Real-Time PCR System (Thermo Fisher Scientific). CjRPS18, CcRPS18, GmTUA4 and AtActin genes are used as reference genes to measure relative expression of respective plants. cDNA dilution series are used to draw the standard curve. PCR condition was…….

**Mutant analyses:** Arabidopsis sgs3-2 (AT5G23570) and rdr6-2 (AT3G49500) mutants are purchased. Arabidopsis dcl1-9, dcl2-1, dcl3-1 single mutant and dcl2-1 dcl4-2t double mutants are gifted from Dr. Iwata and dcl4-2t mutant seeds are gifted from Dr. Mochizuki of Osaka Prefecture University are used for mutant analyses. All the mutants are on Arabidopsis Col-0 background.

**Results:**

**Small RNA accumulation in Host-parasitic plant complexes:** Small RNA seq analyses of *G. max -C. japonica* parasitic complex comparing with non-parasitic tissues shows that- in *C. japonica* non-parasitic tissues, on an average 3412833 sRNA reads are mapped to transcripts wherein parasitic tissue average reads are 7278249. In G. max, 2155817 and 3023141 sRNA reads are mapped from non-parasitic and parasitic tissues respectively. On the other hand, *A. thaliana - C. campestris* parasitic complex sRNA seq analyses, in *C. campestris*, 13591151 and 12042058 sRNA reads are mapped to transcripts in non-parasitic and parasitic tissues respectively. In Arabidopsis, 6007813 and 6695539 number of sRNA reads are mapped to transcripts in non-parasitic and parasitic condition respectively. In the parasitic condition, sRNAs accumulation are increased.

1. **Small RNAs are moved in bidirectional manner:** Small RNA analyses of *G. max -C. japonica* parasitic complex predict that 83 of C. japonica sRNA reads are mobile to *G. max* long distance tissue and 6872 of *G. max* sRNA reads are moved to long distance tissue of *C. japonica*. Moreover, sRNA analyses of *A. thaliana - C. campestris* parasitic complex showed that 14 of *C. campestris* sRNA and 17 of A. thaliana sRNA are mobile. Long distance movement of sRNA occurs in bi-directional manner in both host-parasitic plant complexes.
2. **Mobile small RNAs target orthologous genes of opponent plants:** *C. japonica* and *C. campestris* mobile sRNA target hosts genes when parasitic connection was established. We searched sRNA-targeted orthologs between two different parasitic plant complexes. In hosts,20 of *G. max* genes and 9 of *A. thaliana* genes are aligned into 20 pair of orthologues are been targeted by parasitic mobile sRNAs. On the other hand, G. max and A. thaliana mobile sRNA target parasitic plant’s genes. In parasitic plants, 14 of *C. japonica* genes and 13 of *C. campstris* genes result into 14 pair of orthologues are been targeted by host’s mobile sRNAs.
3. **Mobile sRNA accumulation host-parasitic plant complexes:**
4. **Change of mRNA accumulation level of target genes in host-parasitic plant complexes:**