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Summary of thesis

博士の専攻分野の名称：　博　士（農学）　　氏名 Wikum Harshana Jayasinghe

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Evolution of symbiotic interactions to enhance aphid transmission of plant viruses and satellite RNAs (ウイルスやそのサテライト RNA のアブラムシ伝搬を促進する共生相互作用の進化)

Introduction

The idea of onward transmission of plant viruses and satellite RNAs is indispensable for their survival. For the movement of plant viruses from one host to another most viruses seek assistance of Homopteran insects, and aphids have become the most important plant virus vector. In this thesis, two symbiotic interactions, which coevolved targeting the upsurge of aphid transmissibility, are documented.

CHAPTER 1: Helper component protease (HC-Pro) of leek yellow stripe virus facilitate the aphid transmission of an onion yellow dwarf virus isolate with a mutated HC-Pro gene: A symbiotic association evolved among two potyviruses

It has been previously reported that garlic plants in Aomori Prefecture, Japan were infected with a spontaneous mutant of the OYDV, which was named as isolate G79. This OYDV isolate lacks 92 amino acids in the N-terminal of HC-Pro (Takaki et al., 2006). In a recent survey using garlic cultivated in Hokkaido and a few other regions in Japan, it has been found that OYDV was always associated with LYSV in garlic and in the sequence information of several HC-Pro genes from

OYDV isolates from Japanese garlic showed that all of the HC-Pro proteins lacked about 100 amino acids in the N-terminal region (Kim et al., 2020). The OYDV isolates with an N-terminal deletion (about 100 amino acids) are hereafter called OYDV-S in this report.

Since the missing sequences of N-terminal of HC-Pro of the OYDV isolated form Japanese garlic include the KITC motif, it is assumed that those isolates have lost the ability to be aphid transmissible (Kim et al., 2020). The OYDV isolates without a deletion at the N-terminus (intact HC-Pro) should be transmitted by aphids. These OYDV isolates with an intact HC-Pro are hereafter called OYDV-L in this report. Considering that OYDV-S was always associated with LYSV, it was hypothesised that OYDV-S strains may be transmitted by aphids only when they are coinfecting garlic with LYSV.

Table 1. List of primers used for virus detection

Virus	Primer Name	Primer Sequence (5' - 3')
LYSV	LT5P	AATCTCAACACAACTTATRC
	LY2M	AGTACGTTGCCTGCTCTGTAG
OYDV	OYDV-CP-FW1	AATTGGACAATGATGGACGG
	OYDV-CP-RV1	GTTACCATCCAGGCCAAACA
Allexivirus	al-CP5-750	TGGRCNTGCTACCACHHYGG
	al-CP3-750	CCYTTCAGCATATAGCTTAGC
GLV	GLV-5-1000	AGGTGCATTGTTATCATTACTGG
	GLV-3-1000	GTATGCAACTTAAATATAGCACGC
GCLV	GCLV-5-1070	GCTAGACATTGGTAGCCTTAGG
	GCLV-3-1070	GTCAGTAGGCACGCCACTAT

Two evaluate our hypothesis, we purchased commercially available virus-free garlic bulbs and confirmed that they not infected with five garlic viruses (LYSV, OYDV, allexiviruses, garlic latent virus [GLV] and garlic common latent virus [GCLV]) by RT-PCR using the primer set

shown in Table 1, following the procedures described previously (Kim et al., 2020). Secondly, we wanted to acquire plants infected with OYDV alone from the plants coinfected with OYDV and LYSV. We prepared the inoculum with OYDV alone by use some onion cultivars as a propagation host plant. Onions are easily infected with OYDV but rarely infected by LYSV, Allexiviruses and carlaviruses although some onion cultivars have been reported to be susceptible to these viruses (Ward et al. 2009). Accordingly, we first produced an onion plant infected with OYDV alone using the strategy shown in Fig. 1, and used its sap as the inoculum for a subsequent inoculation of virus-free garlic.

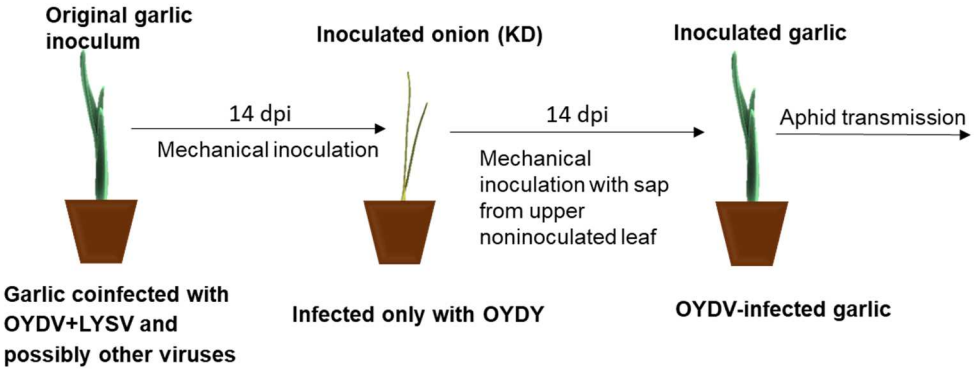

Figure 1 Strategy to obtain OYDV-only-infected plants from mixed-infected garlic

We conducted a series of aphid transmission assays. As shown in Table 2, the RT-PCR results showed that the infection rates of LYSV and OYDV-S were 61% and 17%, respectively. Of 18 tested plants, 9 plants were infected only with LYSV, while 3 plants were coinfected with LYSV and OYDV-S. No plants were infected with only OYDV-S. (Table 2, Exp1). As shown in the experiment aphids could transmit OYDV-L from infected onion to both onion and garlic at the rate of 17% and 30%, respectively (Table 1, Exp3). These results clearly indicate that the OYDV-S was not transmitted to garlic by aphids when it was not coinfected with LYSV (Table 2, Exp 2). In contrast, OYDV-L was readily transmitted to both garlic and onion by aphids. On the other hand, aphids could transmit OYDV-L from infected onion to both onion and garlic at the rate of 17% and 30%, respectively (Table 2, Exp3).

Table 2 Aphid transmission of OYDV-S and OYDV-L

Exp.	Inoculum (Virus)	Inoculated plants[a] (Number)	No. of infected plants (Percentage)				
			OYDV	LYSV	Allexivirus	GCLV	GLV
1	Garlic (OYDV-S + LYSV)	Garlic (18)	3[b] (17%)	11 (61%)	–	–	–
2	Garlic (OYDV-S)	Garlic (16)	–	–	–	nt	nt
	Onion (OYDV-S)	Garlic (14)	–	–	nt	nt	nt
3	Garlic (OYDV-L)	Garlic (10)	3 (30%)	–	–	–	–
		Onion (6)	1 (17%)	–	nt	nt	nt
	Onion (OYDV-L)	Garlic (7)	2 (29%)	–	nt	nt	nt

OYDV-S, OYDV with short HC-Pro; OYDV-L, OYDV with long HC-Pro; –, not detected; nt, not tested, [a]Plants were virus-free, [b]Plants were coinfected with LYSV and OYDV-S

These results clearly indicate that the OYDV-S was not transmitted to garlic by aphids when it was not coinfected with LYSV. We then investigated the most plausible hypothesis of the molecular mechanism by which LYSV aids aphid transmission of OYDV which may be by OYDV CP binds to LYSV HC-Pro.

First, we attempted to detect the binding between the two proteins by the pull-down assay with His-affinity gel. Two proteins were synthesized in *Nicotiana benthamiana* plants by the agroinfiltrating Ti-plasmids with the respected protein genes. The input proteins used for the pull-down assay were detected on Western blots (Fig. 2a). The HC-Pro and CP bands are indicated by arrows in Fig. 2a. As shown in Fig. 2b, the OYDV CP was detected in the pull-down fraction of HC-Pro.

To detect the direct binding of both proteins, we then synthesized them as a fusion protein with maltose-binding protein (MBP) in *E. coli.* The coding cDNA sequences of OYDV CP, LYSV HC-Pro and LYSV HC-Pro mutants (MBP-HC900, MBP-HC700 and MBP-HC460*)* (Fig. 4) were

cloned into the plasmid, pMAL-c2x. The HC-Pro proteins contain the C-terminal FLAG-tag. The MBP-fused proteins were purified through the column following the manufacturer's instruction.

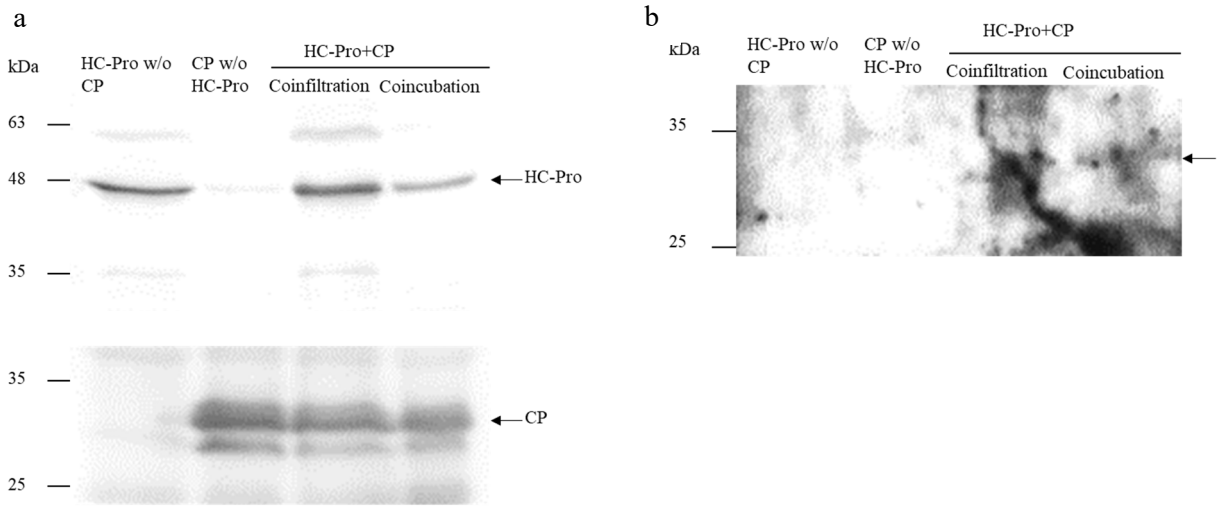

Figure 2 Far-Western dot blot assay. a, The input proteins used for the pull-down assay were detected on Western blots. The HC-Pro and CP bands are indicated by arrows. b, Pull-down assay with His-affinity gel. The detected CP band was indicated by arrow.

Far-Western dot blot assay was performed using purified HC-Pro and CP. The results showed that HC-Pro and CP bound to each other in a reciprocal combination (Fig. 3a,b). The input of MBP fusion proteins used for the Far-Western dot blot analysis is shown in Fig. 3c. With these data, we presumed that the two proteins were likely to bind.

The data revealed that the mutation in HC-Pro has resulted loss of aphid transmissibility. In the nature the loss of aphid transmissibility of OYDV isolate has overcome by the symbiotic interaction with leek yellow stripe virus (LYSV). We presume that LYSV HC-Pro works *in trans* as a platform that interlinks both LYSV and OYDV with the aphid stylet.

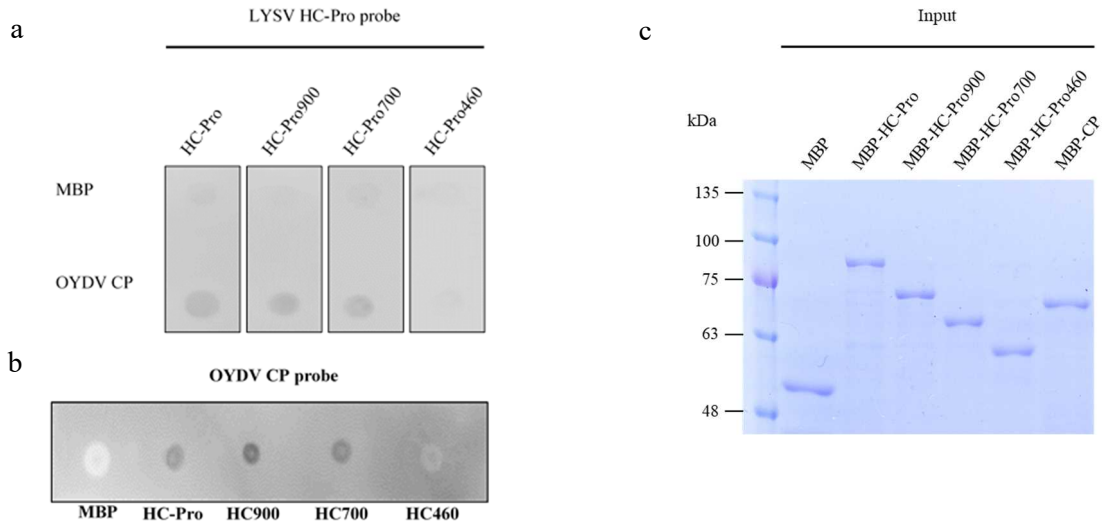

Figure 3 Far-Western dot blot analysis. a, OYDV CP-MBP was dotted onto the nitrocellulose membrane, overlaid with the MBP-C-terminal truncated LYSV HC-Pro proteins. b, The MBP-C-terminal truncated LYSV HC-Pro proteins were dotted onto the nitrocellulose membrane, overlaid with the MBP-OYDV CP. c, The input of MBP fusion proteins used for the Far-Western dot blot analysis.

CHAPTER 2: Evolution of a quadripartite symbiosis driven by a satellite RNA to promote aphid transmission

A virus is sometimes associated with a subviral non-cording RNA molecule such as a satellite RNA (satRNA) which can modify the accumulation levels and the symptoms caused by the helper virus(Shimura et al., 2011). Since viruses are differentially transmitted by insect vectors, any change in the virus-vector interactions would affect the natural selection of virus population. Cucumber mosaic virus (CMV) is one of the most successful viral pathogens as it can infect nearly 1200 plant species. Y satellite RNA (Y-sat) is a satRNA which depends on CMV for its replication and encapsidation. The presence of Y-sat in CMV infected plants down-regulates the *ChlI* mRNA, impairing the chlorophyll biosynthesis in *Nicotiana* plants (Shimura et al., 2011) causing bright yellow symptoms (Fig. 4). This Y-sat-mediated bright yellowing of leaves attracted our attention to decode the evolutionary standpoint of the above adaptation.

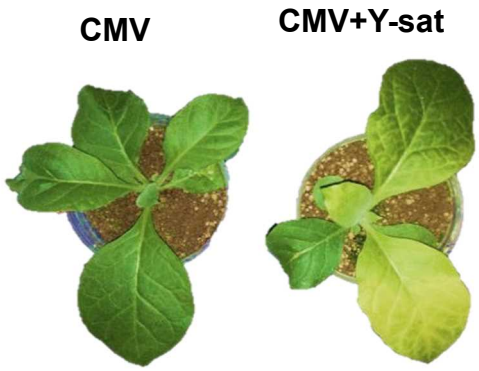

Fig. 4 Yellow symptoms on tobacco plants infected with CMV+Ysat.

A pairwise aphid attraction bioassay and Y-tube olfactory bioassay were conducted using healthy, CMV-infected and [CMV+Y-sat]-infected *Nicotiana* plants to observe the colour-dependent and odour-dependent aphid attraction, respectively. The results showed a significantly higher number of aphids was attracted by yellow of [CMV+Y-sat]-infected plants compared to that of CMV-infected plants. The olfactory bioassay showed that there was no significant difference in aphid attraction among all plant types, showing that neither CMV infection nor Y-sat infection can induce order-dependent attraction of aphids. The CMV accumulation levels in CMV-infected and [CMV+Y-sat]-infected *Nicotiana* plants were determined by RT-q-PCR. Our RT-q-PCR results revealed that the level of CMV in CMV-infected plants was more than 10X higher than that in [CMV+Y-sat]-infected plants.

The ability of the aphid to transmit the virus from either CMV-infected or [CMV+Y-sat]-infected *Nicotiana* plants was tested using *M. persicae* (green peach aphid), a natural vector of CMV. The transmission experiments resulted in 85% infection when aphids were transferred from CMV-infected to healthy plants, whereas we obtained 55% infection for [CMV+Y-sat]-infected plants (Fig. 4e). Therefore, we found that the CMV transmission rate was not strongly affected by the CMV level in [CMV+Y-sat]-infected plants although Y-sat normally reduces the CMV level down to less than 1/10 of the level in CMV-infected plants. Taken together, we concluded

that Y-sat dominated the epidemiology of the helper virus by attracting a significantly higher number of aphids ensuring Y-sat survival in the nature.

Finally, these two examples of symbiotic interactions driven by either a plant virus or a satellite RNA indicate how organisms have coevolved evolutionary-symbiotic-interactions to ensure their survival.

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