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Virus transmission efficiency: investigation on Chinese clone diversity of wheat aphids and role of aphid endosymbionts

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Abstract: Clone diversity in aphids (Hemiptera: Aphididae) was known to be related to acceptance and suitability of host plant. Occurrence of particular patterns of bacterial endosymbionts was demonstrated to specific plant – aphid interactions. Barley yellow dwarf virus (BYDY) has a high degree of vector specificity to be transmitted only by specific species of cereal aphids. BYDV and their aphid vectors are associated in a variety of complex interactions. In this context, the main objective of this dissertation was to understand the virus partners in host-aphid interactions, the role of endosymbionts pattern on virus transmission efficiency and (E)-β-farnesene (EBF) production.

Aphids were of closely association with bacterial endosymbionts; which substantially affect the physiology, ecology, reproduction and behaviors of aphids in a variety of way. Firstly, endosymbionts were selective eliminated by antibiotic, the EBF production was reduced; from the result of protein analysis, some protein from *Buchnera aphidicola* were found. It demonstrated that endosymbionts take part in the EBF production. Secondly, Fourteen populations of *Sitobion avenae* Fabricius (Hemiptera: Aphididae) originating from China were tested for their ability to transmit Barley yellow dwarf virus-PAV (BYDV-PAV, one isolate from Belgium, another from China) using wheat plants. All populations could transmit BYDV-PAV and variation in transmission rates ranged from 24.42% to 66.67% with BYDV-PAV-

Belgium and from 23.55% to 56.18% with BYDV-PAV-CN. Significant differences of percentages of transmission between the populations with BYDV-PAV-Belgium and BYDV-PAV-CN were observed. *Buchnera* and seven S-symbionts (PASS, PABS, PAUS, *Rickettsia*, *Spiroplasma*, *Wolbachia* and *Arsenophonus*) universally found in different local population with different infection frequencies. The endosymbionts in most and least efficient vector aphid clones were selectively eliminated by antibiotic, the BYDV transmission efficiencies were inhibited. Compared with the result of western blot, *Buchnera* plays an import role on BYDV transmission. Finally, according to the direct toxic effect of lectins on insect biological parameters but also to the potential competitive effect of lectins towards viral particles in virus transmission by aphids, GNA and PSL incorporated in an artificial diet, the BYDV transmission efficiencies were inhibited. The inhibition rate of GNA-treatment reached to 46.63% in STY-BYDV-PAV-CN treatment and PSL-treatment was 46.47% in STY-BYDV-PAV-CN treatment. It is demonstrated that lectins represent a very promising protein to control aphid pest damages in crops.

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Chapter I: *GENERAL INTRODUCTION*

There are about 4700 species of aphid in the world, which belong to the superfamily of Aphidoidea (the other two families are the Adelgidae and the Phylloxeridae). Of these, about 450 species have been recorded from crop plants, and about 100 species are reported as economically important pests on crops, not only directly by feeding on plant fluid nutrients from phloem of leaves, stalks and ears, but also indirectly by excreting honeydew and transmitting viruses. Among aphid species, the English grain aphid *Sitobion avenae* (Fabricius), is a monoecious aphid species with exhibit complex life cycles and has a wide host range, as well as has a cyclical parthenogenesis and wing dimorphism. It is an important pest in cereals, especially in wheat and barley under temperate climates mainly in America, Europe and Asia.

Barley yellow dwarf viruses (BYDVs, family *Luteoviridae*) are one of the most important cereal diseases worldwide. BYDVs are transmitted by aphids in a persistent and circulative manner. To obtain maximum transmission rate, it is required that aphid feeding in virus infected plant for 48 hours or longer for acquisition and infection. Once acquired, the virus is retained for a relative long time, often the rest of the vector’s life duration. The circulative route of virus movement through the aphid body has been partially characterized. Each BYDV strain only transmitted efficiently by corresponding aphid species; one species aphid usually can

efficiently transmit more than one virus strain. *S. avenae* is considered to be an important vector of BYDV, especially spreads the virus from winter hosts (wheat and barley) to spring barley and corn in the spring.

Almost all of the aphids contain the intracellular symbionts in the hind gut. The obligate "primary" symbiont *Buchnera aphidicola*, supplement the host insects' diet through the provision of essential amino acids, sterol and vitamin, they were found in almost all aphids and housed in the bacteriocytes or mycetocytes cells; in many but not all lineages of aphids contain the facultative "secondary" bacteria, which are additional types of vertically transmitted endosymbiotic bacteria to the aphid offspring, their presence is not universal and found in tissues surrounding the bacteriocytes and in specialized secondary bacteriocytes, they have polyphyletic evolutionary origins. Secondary symbionts are not required for survival or reproduction, but they have been show to help aphids adjust to ward off parasitoids, heat stress, fungal infection and can alter host plant use.

To understand the interaction of endosymbiont, aphid and BYDY, we designed some tests using molecular biology techniques to do investigations: detecting vector aphid, *S. avenae* clones on the intra-specific variations of BYDVs transmission efficiency, finding the impact

of aphid endosymbiont and lectins on BYDV transmission, and expounding relationship of EBF, antibiotic and endosymbiont through checking the pathway leading to alarm pheromone biosynthesis.

Chapter II: *Transmission of barley yellow dwarf virus by aphids: a review of virus-vector interactions*

Transmission of barley yellow dwarf virus by aphids: a review of virus-vector interactions

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Abstract: Barley yellow dwarf viruses (BYDVs) are economically important viruses that infect cereal crops worldwide. Aphids are important vectors of BYDVs in temperate countries. BYDVs have a high degree of vector specificity and are transmitted only by specific species of cereal aphids. BYDVs and their aphid vectors are associated in a variety of complex interactions. Investigations of BYDV variants and their vectors have been undertaken for several years, in order to elucidate their interactions. Nevertheless, some important questions remain to be answered. In recent years, most studies on aphid-BYDV relationships have been focused on variations in species specific transmission among aphid species. Intra-specific variations in BYDV transmission efficiency has become a new focus for analyzing the efficiency of aphid transmission, for identifying virus migration paths and for assessing significant variations in virus transmission by aphids.

Keywords: BYDV, *Sitobion avenae*, transmission efficiency

Introduction:

Barley yellow dwarf viruses (BYDVs, family *Luteoviridae*) are one of the most important cereal diseases worldwide. BYDVs are transmitted by aphids in a persistent and circulative manner. In order to obtain the maximum transmission rate, a feeding period of 48 hours or more is required for acquisition and infection. Once acquired, the virus is retained for a relative long period, often for the rest of the vector's life span. The circulative route of virus movement through the aphid body has been partially characterized (Gray and Gildow, 2003). Each BYDV strain is only transmitted efficiently by its corresponding aphid species; one aphid species is usually able to efficiently transmit more than one virus strain (Rochow, 1969). Virus-aphid specificity seems to be related to the recognition of particular receptors in the accessory salivary glands of aphids (Bencharki, *et al*. 2000). The English grain aphid, *Sitobion avenae*, widespread throughout the temperate climates of the northern and southern hemispheres, is one of the most common and destructive pests attacking wheat. *S. avenae* exhibits complex life cycles and has a wide host range. It also exhibits a cyclical parthenogenesis and wing dimorphism. *S. avenae* is considered to be an important vector of BYDV,

especially in the spring when it spreads the virus from winter hosts (wheat and barley) to spring barley and corn (Dedryver, *et al*., 2005).

In this review, we focus on the damage caused by *S. avenae* and BYDVs, and variations in BYDV transmission by aphid species. Our aim is to gain a better understanding of virus-aphid interactions and to propose new insights for future epidemiological scenarios.

1 Aphids

About 4700 species of aphid are known around the world, which belong to the superfamily of Aphidoidea (the other two families are the Adelgidae and the Phylloxeridae). Of these, about 450 species have been recorded from crop plants, and about 100 species are reported as the significant agricultural economic importance (Remaudière and Remaudière, 1997).

1.1Biology of aphids

Aphids (Order Hemiptera; superfamily Aphidoidae) are small, soft-bodied insects, sap-sucking in the plant phloem with complex life cycles and a wide host range. There are two major types of aphid life cycle: host alternating (heteroecious) and non-host alternating (monoecious) (Figure 1). Host-alternating aphids have at least two unrelated host plants, a primary host plant (woody) and one or more secondary host plant

(herbaceous). Approximately 10% of aphids employ this life cycle, producing eggs on primary host plant in winter, migrating to secondary host plant(s) in summer, and coming in autumn back to the primary host plant. Non-host-alternating aphids feed on either the same host plant or migrate among a range of related host plants (herbaceous) throughout the year (Williams and Dixon, 2007). Aphids are well-known for cyclical parthenogenesis, displaying a high reproductive rate asexually. The bird cherry-oat *Rhopalosiphum padi* (L.) has been proven three peculiarities of their reproductive biology for the high reproductive rate. Firstly, female aphids obviate the need for males reproduce parthenogenetically during the spring and summer months. Secondly, the embryos initiate development immediately after the budding of the oocyte from the germarium and young aphids' larvae develop into the adult stage during spring and summer when days are long. Finally, the oldest embryos also contain embryos and each adult female can give birth by viviparity, so that adult parthenogenetic aphids carry not only their daughters but also some of their granddaughters within them. In autumn, when the day length shortens, the development of sexual females and males are induced by declining daily photoperiod and temperature. These sexual aphids mate and fertilized females lay yolk-rich eggs that undergo diapause to make them through winter (Simon, *et al*., 2002). Aphids exhibit a high degree of polymorphism with wing dimorphism, as another feature of the

complex life cycles. Wing polyphenism occurs primarily among parthenogenetic females, while wing polymorphism has been found only in males. The morphological differences between winged and apterous phenotypes usually correlate with differences in life history. Winged morphs typically possess a full set of wings that are adapted to flight and reproduce in new locations. They have longer nymphal development and reproductive durations, lower offspring production and prolonged longevity than apterous morphs. In contrast, the latter show adaptation to maximize fecundity (Hazell, *et al*., 2005).

1.2Impacts of aphid pests

Aphids are responsible for major crop losses in world agriculture, not only directly by feeding on leaves, stalks and ears, but also indirectly by excreting honeydew and transmitting viruses (Rabbinge, *et al*., 1981). *S. avenae* (Fabricus) is a monoecious aphid species with a complex life cycle on members of the *Poaceae* family. It is an important pest in cereals, especially in wheat and barley under temperate climates mainly in America, Europe and Asia.

Damages induced by *S. avenae* have been investigated since the late 1960s. It may reduce cereal yield and grain quality in diverse ways. As the ear generally remains physiologically active longer than the leaf, compared to other species, *S. avenae* can maintain it longest in the crop,

with a multiply twice as quickly on the ear as on the flag leaf. The quantity of phloem sap ingested by aphids depends on the nitrogen content of the sap, because high nitrogen level promotes aphid growth, increases the reproductive rate, shortens development time, and inhibits wing formation in cereal aphids; *S. avenae* injects saliva in plant phloem elements and grapes plant nutrients. The saliva can change plant physiology, while the plant nutrients can potentially reduce several indicators, such as number of heads, number of grains per head, and grain/seed weight (usually expressed as 1000-grain weight) (Kolbe and Linke, 1974). With strong preference for the ear, *S. avenae* feeds on the rachis and base of the spikelets, which leads to substantial yield loss. Besides *S. avenae* also secretes honeydew and transmissions viruses, which causes indirect damages. Honeydew, a sugar-rich aphid secretion, indirectly causes physiological changes by covering large areas of a plant's epidermis, inducing chlorotic symptoms in leaves, hampering photosynthesis, affecting net carbon dioxide assimilation in wheat, and promoting the growth of saprophytic fungi (black filamentous saprophytic ascomycetes) which may also have a negative effect on photosynthesis and leaf duration. In field experiments, 72% of the yield losses could be attributed to aphid feeding and honeydew, while 28% to saprophytic fungi (saprophytic fungi feeding on aphid honeydew may

have caused a loss of about 200 kg ha $^{-1}$ out of 800 kg) (Rabbinge, *et al*., 1981).

S. avenae is considered as an important vector of barley yellow dwarf virus, especially when it transfers from winter hosts (mostly wheat and barley) to spring hosts (mostly barley and corn) in the spring (Dedryver, et al., 2005); BYDV depends on S. avenae for transmission, not only among other parts of the same plant, but also more distant hosts. Winged aphids can move considerable distances by drifting in the prevailing winds, transmitting viruses to cereal fields. Once occurred, BYDV can be propagated by apterous aphids multiplying in the fields and moving from plants to plants. The intensity and pattern of BYDV dissemination, or secondary spread, depends on interactions among virus, host-plants, vectors and climates (conditions of infestation of both crops and aphids, of viral multiplication in the crops, and of vector production), it is a major component of yield losses, but also of inoculum production for further infection.

2 Barley yellow dwarf virus

Barley yellow dwarf (BYD) is also called cereals yellow plague, cereal yellow dwarf, yellow dwarf or red leaf. As one of the most widespread and damaging viral diseases of grasses and cereal crops in the world, affects more than 100 species in the family *Poaceae*, including

wheat (*Triticum aestivum* L., *Triticum durum* Desf.), barley (*Hordeum vulgare* L.), oats (*Avean sativa* L.), rice (*Oryza sativa* L.), rye (*Secale cereal* L.), sorghum (*Sorghum vulgare* L.) and plenty wild grasses. Belonging to the family *Luteoviridae*, BYDV is transmitted in a circulative pattern and a persistent manner by more than 25 species of aphid living on *Poaceae* with a variable degree of vector specificity (D'Arcy and Burnett, 1995). The virus is caused by a group of related single-stranded RNA viruses assigned to the *Luteovirus* (Barley yellow dwarf virus) or *Polerovirus* (Cereal yellow dwarf virus) genera, or those unassigned to a genus in the family *Luteoviridae*. Phloem-limited in host plants, it cannot be mechanically transmitted, with typical symptoms including yellowing of leaves and stunting of whole plants (Miller, *et al*., 1988).

2.1 The history and distribution of BYDV

Barley yellow dwarf disease is ubiquitous across the globe where *Poaceae* (both wild and cultivated species) are grown. Since been firstly recognized by Oswald and Houston as a new virus in 1951, it was subsequently found to have a worldwide distribution, affecting nearly all members of the *Poaceae*. Possibly the earliest record of this disease was in 1890 in North America (D'Arcy and Burnett, 1995). The BYDV

history and distribution in Northern America, Europe, Asia and Australia has been shown in Table 1.

2.2 Genus members of BYDV

Five different strains (RPV, RMV, MAV, PAV, and SGV) from New York transmitted preferentially by their different primary aphid vectors were characterized by Rochow (1969 and 1987). Each virus was defined based on the abilities of particular aphid species to acquire and transmit (Table 2), which were initially classified by their biological properties. Already, the strain designations now used seem to be broken down. The type isolate of PAV, described from parts of Europe and the Mediterranean was transmitted by *R. maidis*, while the isolate of RPV was transmitted nonspecifically by both *S. avenae* and *S. graminum* in California (Creamer and Falk, 1989). Several such examples have been reported (Rochow, *et al*., 1987). In addition, it is reported recently that isolates from other parts of the world may differ serologically from these five serotypes, for example, those from China. There were four BYDV (GPV, PAV, GAV, and RMV) isolates in China according to Rochow's system. BYDV-GAV is transmitted nonspecifically by the *S. avenae* and *S. graminum* aphids, which is similar to BYDV-MAV; BYDV-GPV is transmitted by both *S. graminum* and *R. padi*, which is more closely related to BYDV-RPV, BYDV-PAV is transmitted efficiently by *R. padi*

and *S. avenae*, while BYDV-RMV is transmitted efficiently by *R. maidis* (Zhou, *et al*., 1987; Wang and Zhou, 2003). Moreover, the BYDV-GPV strain has only been reported in China (Zhou, *et al*., 1987).

Presently, the BYDVs were divided into two major sub-groups based on serological relationships, cytopathological and differences in genome organization, which were subsequently reclassified as separate species (Table 3). The International Committee on the Taxonomy of Viruses (ICTV) Working Group on *Luteoviruses* reclassified the subgroup I serotypes would be called BYDV, and members of subgroup II were given a new name cereal yellow dwarf virus (CYDV). Currently, only BYDV-MAV (transmitted specifically by *S. avenae)* and BYDV-PAV (transmitted efficiently by *S. avenae* and *R. padi*) are barley yellow dwarf viruses (Smith and Barker, 1999).

2.3 Yield losses caused by BYDV

BYD disease can infect 97 susceptible species from 34 genera of the family *Poaceae* and about 100 species of grasses. BYDVs were transmitted by several aphid species. The extensive host range and numerous aphid species enable the virus to survive in different environments. Consequently, BYDV was regarded as the most significant viral pathogen affecting cereal crops today (D'Arcy and Burnett, 1995). BYDV was a serious threat to the cultivation of cereal crops in the world,

because it occurred wherever cereals were grown, transmitted by aphids varying from field to field and year by year. To estimate yield losses of cereal crops caused by BYDV, researchers used a few parameters, such as symptomatology, grain yield, harvest index, spike weight, biomass and plant height, and a combination of these factors.

BYDV had considerable economic significance worldwide, particularly in higher rainfall regions where yield losses in wheat have been reported to be as high as 40-50%. Due to the natural BYDVs infection, global average yield losses can range from 11% to 33%, whereas in some areas the losses reach up to 87%. BYDV-PAV caused substantial losses throughout the world in barley (15%), wheat (17%), and oats (25%) (D'Arcy and Burnett, 1995). In USA, yield losses attributed to BYDV infection in large production areas was from 25% up to 74%. Linear relationship was found between disease incidence and yield loss, 1% incidence causing 30 - 60 kg/ha loss in oats, 20 - 50 kg/ha in wheat. Hewings and Eastman (1995) calculated that hypothetical 5% losses caused by BYDVs would result in yield losses valued at $847.0 million for corn, $48.5 million for barley, $387.1 million for wheat and $28.0 million for oats in the United States in 1989. Yount *et al*. (1985) estimated that yield loss ranged between 45% and 75% for two-row barleys, six-row barleys, winter wheat and spring wheat resulting from BYDV in Montana, USA. In Canada, cereal crops, such as bread wheat,

durum wheat, oats and triticale, were all infected with BYDV. Haber (1997) developed a yield prediction model for spring bread wheat following BYDV infection, in average conditions, economic loss could reach up to 25%. In Europe, a very severe epidemic occurred in different barley varieties in Hungary, with ranging from 27% to 100% in 1982 (Pocsai and Kobza, 1983). In Denmark the record was as high as 80% in 2000 (Gron, *et al*., 2000). A serious outbreak of BYDV was on spring and winter wheat in northern Germany from 1988 to 1990, south-western Germany in 1991 as well. The BYDV caused considerable yield losses in central regions of Germany, especially on winter wheat and winter barley (Habekuß and Schliephake, 2002). In European Russia, small grain yields decreased 90% during the epidemic between 1988 and 1991. BYDV was found in all of more than 1000 samples from six provinces of four Asiatic Russian regions and from twenty provinces of eight European Russia regions between 1996 and 2002 (Mozhaeva and Kastal'yeva, 2002). In the Czech Republic, BYDV has caused significant yield losses, particularly in winter crops over recent decades, meanwhile; PAV was regarded as the only species inducing 5 to 80% yield losses, with an average of 30% in affected fields (Perry, *et al*., 2000). BYDV was widespread but serious losses were sporadic in Britain, only some coastal and low-lying areas of southern Britain were infected the diseases every year. The average yield increased from 3.5 to 5 t/ha for barley and from 4

to 6.5 t/ha for wheat over the past 30 year, respectively. From the 1940s to the 1960s, BYDV occurred occasionally in southwest England, while the BYDV problem had become more widespread since the mid-1970s (Knight, *et al*., 1996). In Asia, BYDVs were main diseases of wheat throughout the northern and northwestern provinces in China for the last two decades (Zhou, 1987). From 1966 to 1978, the diseases reached epidemic proportions over a vast area including Anhui, Gansu, Guizhou, Heilongjiang, Henan, Hebei, Jiangsu, Jilin, Liaoning, Qinghai, Shannxi, Shanxi, Shandong, Sichuan provinces and Ningxia Hui, Xinjiang, Xizang and the Inner Mongolian autonomous regions. Zhang, *et al*. (1983) estimated that the yield losses in wheat were between 20% and 30%. Recently, Wang and Zhou (2003) had also observed crop losses from 20% to 30% for many years in Shaanxi and Gansu provinces, the serious epidemic happened in Shanxi, Shaanxi, Gansu, and Hebei provinces as well as Ningxia Hui and the Inner Mongolian autonomous regions in 1998.

3 BYDV transmission efficiency of aphids

About 94% arthropods and 6% nematodes can transmit plant viruses; the most common plant virus vectors are arthropods, the great majorities (99%) of which are insects from Hemiptera order. In addition, 55% of insect vectors are aphids. For example, from 288 aphid species potential

vectors, 277 were found to be able to transmit at least one plant virus, most of which were in the *Aphidinae* sub family (Ng and Falk, 2006). Different plant viruses are transmitted by different vectors, and different aphid clones (genotypes) have different abilities to transmit viruses in many instances.

BYDVs are persistently transmitted by aphids to all common small grain cereals but not transmissible through seed, soil or sap. It displays a high degree of vector specificity among different aphid species living on *Poaceae*, and each virus is only transmitted by one or a few aphid species. It is well documented that the various aphid species differ in their abilities to transmit the various variants of BYDV, a virus isolate can be transmitted with different efficiency by different clones of aphid species, and similarly, an aphid clone can transmit different virus isolates with different efficiencies (Bencharki, *et al*., 2000). At present, the understanding of intra-specific variation of BYDVs transmission has caught particular attention. Rochow (1960) reported strains of the greenbug *S. graminum* (Rondani) (Florida, Wisconsin and Illinois) differing in their ability to transmit a BYDV-SGV isolate in the United States. Four biotypes of the corn leaf aphid, *R. maidis* were shown to differ in their abilities to transmit the AG-1 strain of BYDV; Biotype KS-4 was consistently less efficient (28%), followed by biotypes KS-1 and K8-3 (44 and 46%, respectively), Biotype KS-2 was a highly efficient

vector (87%) (Saksena, *et al*., 1964). Rochow and Eastop (1966) reported variations in transmission abilities of two morphologically different clones of *R. maidis* (New York clones and Kansas clone) for BYDV-RMV virus, and noticed that differences between clones were less pronounced when experiments were conducted at 30℃ than at lower temperatures. Seventeen *R. padi* clones originating from Europe, North America and North Africa, were evaluated by transmitting two BYDV isolates (serotypes MAV2 and MAV11). Both isolates were rather well transmitted by one clone named Rp5. Isolate MAV2 was transmitted at a very low percentage (<5%) by all other clones tested, while isolate MAV11 was not transmitted by eight clones and poorly transmitted by two clones. European clones transmitted significantly better than North America ones, and holocyclic clones transmitted significantly better than the others (Sadeghi, *et al*., 1997). Similar results have been reported for the transmission of BYDV-RMV isolates by *R. padi* in New York. Several field populations of *R. padi* differed in their ability to transmit the BYDV-RMV isolates and the transmission efficiency was increased at higher temperatures (Lucio-Zavaleta, *et al*., 2001). Vector efficiency of 44 clones of *S. avenae* belonging to 31 different genotypes originating from Western France was evaluated by transmitting BYDV-PAV4 isolate; variation in transmission rates from 4% to 93% were observed (Dedryver, *et al*., 2005). In China, Du, *et al*. (2007) reported that BYDV-PAV was

efficiently transmitted by *R. padi*-La and *S. avenae*-La but poorly by *S. graminum*-La; *S. graminum*-La, *S. avenae*-La and *M. dirbodum* showed transmission rates of 75% with BYDV-GAV; BYDV-GPV was transmitted by *S. graminum*-La and *R. padi*-La, with a frequency of about 80% and 50%, respectively.

Little is known about the reason of the diversity in transmission ability. In aphid, the virus were ingested from the host plant into the lumen of aphid's alimentary canal, crossed from hindgut into hemocoel, retention in the tissues and hemocoel, then transmitted through salivary gland into phloem(Gray and Gildow, 2003). Thus, it is likely that several barriers and several genetic loci are responsible for vector competence. In some cases, gut membrane seems to regulate transmission efficiency. Bencharki, *et al*. (2000) suggested that the observed intraspecific variability in efficiency of transmission might be related, at least in part, to differences in ability of the movement of BYDV virus throughout the epithelial cell barriers at the hindgut and/or the accessory salivary glands in the different clones or to the stability of the virus in the hemolymph. Van den Heuvel *et al*. (1997) have demonstrated that symbionin, a GroEL homologous protein synthesized by endosymbiotic bacteria and secreted into the aphid hemolymph, was essential for efficient *Luteovirus* transmission. He showed that symbionin from different aphid species binding *in vitro* with different affinities to *Luteoviridae*. Possibly,

symbionin of the inefficient aphid sub-populations was released at low concentrations or had low binding affinity for the virus. In *S. avenae*, two proteins (SaM35 and SaM50) had been isolated from head tissues and identified as potential receptors for BYDV-MAV by Li and his colleagues (2001). A protein (P50) from *S. graminum* and *S. avenae*, situating in the plasma membrane surrounding the accessory salivary glands in the head tissues, binding with a Chinese variant of BYDV-GAV is probably related with virus transmission (Wang and Zhou, 2003). Nevertheless, no accurate identification of functional proteins was achieved at that time. Further identification assays should be performed based on the availability of aphid sequenced genome databaseof *A. pisum*.

Important changes of aphid behaviors can be observed in relation to the absence/occurrence of virus in plants and affect the virus transmission efficiency rate; some aphids feed preferentially on virus-infected plants while others preferentially orient toward virus free plants. Du and his colleagues (2007) showed that the aphid populations which have highest transmission efficiency were from high prevalent area for BYDV. Aphid-BYDV associations are characterized by interactions among numerous host plants, vector and virus relations exhibiting different degrees of host specialization. Assessingnot only variation for virus transmission by an aphid species but also clones is of special interest when epidemiological studies have to be developed to predict virus spreading in crops.

4 Conclusions

As an unachieved work, the analysis on aphid virus transmission efficiencies of aphid species and clones should be promoted for the important significance on understanding the virus migration path in aphid and further propose of new potential tools to control virus transmission. Indeed, identification of molecular receptors in aphid would allow potential findings of virus competitors (such as glycolysed protein like lectins) leading to the non binding of virus and reduction of virla transmission. Also, the assessment of virus transmission variation according to aphid species and clones is of special interest in epidemiological studies. Indeed, future epidemiological scenario should consider the transmission abilities of prevalent vector genotypes, the probability of emergence of new transmission phenotypes by local sexual reproduction, migration or mutation, and their success in the face of selection. Here, the review was focused on biology and damage by aphid and transmission efficiency related to geographic aphid species. Further investigations should be proposed for a better understanding of the virus-aphid interactions and a new insight in future epidemiological and virus control strategies.

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Table 1 History and distribution of Barley Yellow Dwarf Virus in the world - Distribution et historique du virus de la jaunisse nanisante de l'orge dans le monde

Year of event	Distribution of Barley yellow dwarf virus disease	References
1951	California	Oswald and Houston (1951) Plant Dis Rep. 11:471–475
1955	New Zealand	Smith (1999) CABI Publishing. Wallingford
1957	Australia	Smith (1999) CABI Publishing. Wallingford
1957	United Kingdom	Watson and Mulligan (1957) Plant Pathology. 6: 12-14
1958	Belgium	Roland (1960) Parasitica 16 : 62-65
1960s	China	Zhou, *et al*. (1987) Scientia Agricultura Sinica. 20 (4): 7-12
1963	India	Nagaich and Vashisth (1963) India phytopathol. 16: 318-319
1967	Hungary	Szirmai (1967) Magyar Mezõgazdaság. 22:19
1968	France	Bogavac, *et al*. (1968). Ann. Epiphytis. 19 : 275-277
1969	USA	Rochow (1969) Phytopathology. 59: 1580-1589.
1972	Italy	Osler (1984) Situation reports. Italy. In barley yellow dwarf. CIMMYT. Mexico.p190
1978	Poland	Hoppe, *et al*. (1983) Zeszyty Problemowe Posepow Nauk Polniczych. 291: 119-129
1981	Switzerland	Gugerli and Derron (1981) Revuesuisse Agric. 13, 5 : 207-211
1984	German Democratic	Proeseler, *et al*. (1984) Schucssfolgerungen aus dem Auftreten des Gerstengelbverzwergungs virus von 1982 bis 1984 in der DDR. 38: 9, 199-200
1988	Australia	Miller, *et al*. (1988) Nucleic Acids Research. 16(13): 6097-6111.
1997	USA	McGrath, *et al*. (1997). European Journal of Plant Pathology. 103(8): 695-710.
2002	France	Papura, *et al*. (2002). Archives of Virology. 147(10): 1881-1898.
2003	China	Wang and Zhou (2003). Chinese Science Bulletin. 48(19): 2083-2087.
2004	Latvia and Sweden	Bisnieks, *et al*. (2004). Archives of Virology 149(4): 843-

		853.
2006	China	Yan, *et al*. (2006). Chinese Science Bulletin. 51(19): 2362-2368.
2007	China	Du, *et al*. (2007). Journal of Plant Pathology. 89(2): 251-259.
2012	China	Liu, *et al*. (2012). Journal of General Virology 93(Pt 8): 1825-1830.

Table 2 Genus members of Barley Yellow Dwarf Virus and their vectors characterized by Rochow (1969 and 1987) - Genres de virus de la jaunisse nanisante de l'orge et de leurs vecteurs par Rochow (1969 et 1987)

Species name	Vectors	Genus member
Barley yellow dwarf virus PAV (BYDV-PAV)	*Rhopalosipham padi*; *Sitobion avenae* and other aphids	*Luteovirus*
Barley yellow dwarf virus MAV (BYDV-MAV)	*Sitobion avenae*	*Luteovirus*
Barley yellow dwarf virus SGV (BYDV-SGV)	*Schizophis graminum*	*Luteovirus*
Breley yellow dwarf virus RPV (BYDV-RPV)	*Rhopalosipham padi*	*Polerovirus*
Barley yellow dwarf virus RMV (BYDV-RMV)	*Rhopalosipham madis*	*Polerovirus*

Table 3 Genus subgroup of Barley Yellow Dwarf Virus – Sous-groupe de virus de la jaunisse nanisante de l'orge

Subgroup	Species name	Genus member
I	Barley yellow dwarf virus PAV (BYDV-PAV)	*Luteovirus*
I	Barley yellow dwarf virus MAV (BYDV-MAV)	*Luteovirus*
I	Barley yellow dwarf virus SGV (BYDV-SGV)	*Luteovirus*
I	Soybean dwarf virus (SbDV))	*Luteovirus*
II	Cereal yellow dwarf virus RPV (CYDV-RPV)	*Polerovirus*
II	Cereal yellow dwarf virus RMV (CYDV-RMV)	*Polerovirus*
II	Beet western yellow virus (BWYV)	*Polerovirus*
II	Potato leaf roll	*Polerovirus*
II	Carrot red leaf virus	*Polerovirus*
II	Pea enation mosaic virus (PEMV)	*Enamovirus*
II	Southern bean mosaic sobemovirus	

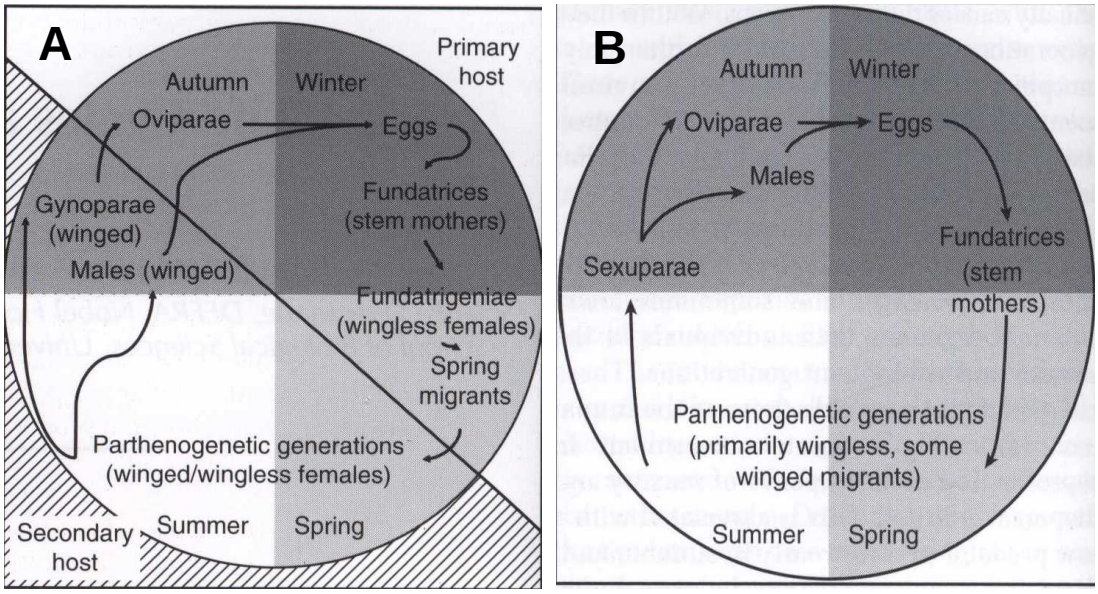

Figure1 Life cycles of host-alternating aphids and non-host-alternating aphids (Williams and Dixon, 2007) - Cycles de pucerons alternant ou non de plantes hôtes (Williams and Dixon, 2007)

A: A generalized life cycle of host-alternating aphids – cycle general de pucerons dioeciques

B: A generalized life cycle of non-host-alternating aphids – cycle general de pucerons monoeciques

Chapter III: *OBJECTIVES*

The aim of this thesis was to investigation on Chinese clone diversity of wheat aphids and role of endosymbionts on virus transmission efficiency. Clone diversity in aphids was known to be related to acceptance and suitability of host plant. Occurrence of particular patterns of bacterial endosymbionts was demonstrated to specific plant – aphid interactions. In a further approach including virus partners in host-aphid interactions, the role of endosymbiont pattern on virus transmission efficiency is to be investigated. After a selection and mass rearing of grain aphid, *Sitobion avenae* (Hemiptera: Aphididae) from different geographic field areas in China, several clonel lines (clones) will be tested (from Anhui-Bengbu, Henan-Dengzhou, Henan-Luoyang, Henan-Xinxiang, Hubei-Danjiangkou, …) for their potential virus transmission efficiency using barley yellow dwarf virus (BYDV). After comparing the differential transmission efficiency of BYDV among *S. avenae* clones, the most and least efficient vector aphid clones will be selected for further molecular investigations focusing on proteomic (gel electrophoresis coupled with mass spectrometry, chromatography purification techniques) and genomic (Polymerase Chain reaction based techniques) approaches. The aim of these biochemical and molecular

investigations will be to determine the virus transmission mechanisms focusing on the aphid strains by comparing and identifying the role of aphid symbiotic bacteria and related produced proteins (transport proteins and receptors) in efficiency. The findings would lead to a better understanding of the virus-aphid interactions and to propose new insight in virus transmission control in crop protection.

In the fourth chapter of this thesis, we studied on the alarm pheromone production in *S. avenae* in relation with symbiotic presence in the aphid. In order to investigate the aphid endosymbionts role, aphids were reared on artificial diet including antibiotics to selectively eliminate the bacterial endosymbionts for two kinds of investigations:1)a proteomic approach (2 dimension electrophoresis coupled with Maldi mass spectrometry, Liquid chromatography associated with electro spray mass spectrometry) to characterize the proteomic pattern related to antibiotic treatment and the suppression of endosybionts; 2)the assessment of EBF production by the aposymbiotic aphids to correlate the protein expression pattern changes and the ability to produce EBF.

In the fifth chapter of this thesis, we investigated differential transmission efficiency of BYDV among *S. avenae* clones. After a

selection and mass rearing of *S. avenae* from different geographic field areas in China, all clones were tested for their potential virus transmission efficiency using BYDV-PAV (one isolate from Belgium, another from China). After comparing the differential transmission efficiency of BYDV among clones, the most and least efficient vector aphid clones will be selected for further molecular investigations.

In the sixth chapter of this thesis, we investigated the endosymbionts effect on transmission efficiency of BYDV. After endosymbionts were detected in the most and least efficient vector aphid clones were rearing on artificial diet with or without antibiotics, proteomic and genomic approaches were applied to compare and identify the role of aphid symbiotic bacteria and related produced proteins (transport proteins and receptors) in efficiency.

Finally, in the last chapter of this thesis, we detected the BYDV transmission efficiency after the most and least efficient vector aphids were reared on artificial diet including lectins, and tried to propose a new insight in virus transmission control in crop protection.

Chapter IV: *Role of aphid endosymbionts on alarm pheromone production in Sitobion avenae*

General Introduction to Chapter IV

Aphids (Homoptera: Aphididae: Aphidinae) are among the most abundant and destructive insect pests of agriculture, particularly in temperate regions, causing direct damage to arable and horticultural crops by sucking plant nutrients as well as serving as vectors for many important plant diseases.

Aphids have some special characterization in defending pathogens, parasitoid wasps, predators and parasites. Almost all aphids closely associated with bacterial endosymbionts, specifically with *Buchnera aphidicola*, a primary, obligatory species which synthesize essential amino acids and other nutrients for their host aphids. A number of aphids harbours several inherited secondary or facultative symbionts (S-Symbiont) in addition to *Buchnera aphidicola*, which are not strictly required for host survival, but can provide a selective advantage in certain aspect. These symbionts may be involved in aphid defense against pathogens and parasitoid wasps. In response to attack by predators or parasites, aphid produced and utilized an alarm pheromone with the most common component being the sesquiterpene (*E*)-β-farnesene (EBF), this alarm pheromone is secreted droplets from the cornicles causing nearby

aphids to disperse from the area. In addition to warning of the presence of immediate danger, aphid alarm pheromones play a number of additional roles in aphid ecology, including as key foraging cues for many aphid predators.

As these characterizations, we try to find some pathway connecting the endosymbionts and (*E*)-β-farnesene production.

Role of aphid endosymbionts on alarm pheromone production in *Sitobion avenae*

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Abstract: In response to attack by predators or parasites, this alarm pheromone is secreted droplets from the cornicles causing nearby aphids to disperse from the area, alarm pheromone with the most common component being the sesquiterpene (*E*)-β-farnesene (EBF). Aphids closely associated with bacterial endosymbionts, which take part in many metabolic pathways in a variety of way. Our results showed that *Buchnera* aphidicola and S-symbionts (PASS, PAUS, *Rickettsia*, *Spiroplasma* and *Wolbachia*) universally found in Belgium and Chinese local population with different infection frequencies. Endosymbionts were selective eliminated from Belgium population and Chinese populations by rifampicin; the mortality of Belgium/Chinese aphids showed higher significant difference with negative control and has fewer offspring than that negative control. In addition, the (E)-β-farnesene (EBF) production were reduced significantly. Protein analysis showed eighteet *Buchnera* aphidicola were detected from the 2D-gel, which take part in many metabolic pathways, such as Carbohydrate metabolism, Energy metabolism, Amino acid metabolism, Protein synthesis, Stress response, Nucleotide metabolism and Membrance transport. They may provide energy, material and enzyme in the EBF production. From these result we conclude that endosymbiont bacteria play a role in EBF production, especially *Buchnere* do indeed play a crucial role in EBF production.

Key words: *Sitobion avenae*; (E) - β-farnesene; endosymbionts; 2D-electrophoresis; GC-MS

Introduction

Aphids (Homoptera: Aphididae: Aphidinae) are among the most serious agricultural insect pests, particularly in temperate regions. They cause major economic losses in several arable and horticultural crops worldwide, directly because of their feeding and indirectly by serving as vectors for many important plant diseases (e.g., viruses). Aphids include a great number of species (*Sitobion avenae* Fabricius, *Acyrthosiphon pisum* Harris, *Myzus persicae* Sultzer, and *Aphis fabae* Scopoli), most aphid species produced and utilized an alarm pheromone with the most common component being the sesquiterpene (*E*)-β-farnesene (EβF) (Bowers, Nault et al. 1972; Pickett and Glinwood 2007). The sesquiterpene, (E)-β-farnesene, has been isolated from several economically important species of the subfamilies Aphidinae and Chaitophorinae and identified as the primary component of alarm pheromone (Bowers, Nault et al. 1972; Edwards, Siddall et al. 1973; Wientjens, Lakwijk et al. 1973; Nault and Bowers 1974). Recently, Francis et al. (2005) (Francis, Vandermoten et al. 2005) characterized the volatile emissions of crushed aphids, out of 23 species examined, 21 contain EβF in their volatile chemical emitted pheromone, while EβF was the only or the major volatile compound in 16 of them, and a minor component for another five species. In response to attack by predators or parasites, this alarm pheromone is secreted droplets from the cornicles

causing nearby aphids to disperse from the area (Kislow and Edwards 1972; Nault, Edwards et al. 1973; Goff and Nault 1974).

Aphids feed on the phloem sap of plants (Pollard 1973), which is an unbalanced diet. It is rich in sugars and usually dominated by sucrose (Ziegler 1975), but free amino acids and most or all of the essential amino acids (viz. leucine, valine and phenylalanine) that aphids cannot synthesize are present at very low concentrations (Rahbé, Delobel et al. 1990; Girousse, Bonnemain et al. 1991; Douglas 1993; Febvay, Rahbe et al. 1999). However, Some of endosymbiont bacteria play an important role in providing the aphids with essential amino acids to compliment the deficiency in phloem sap, it products several kinds of amino acids (Dadd and Krieger 1968; Mittler 1971), fatty acids (Houk, Griffiths et al. 1976) and cholesterol (Griffiths and Beck 1977) for host growth, development, differentiation and fecundity. Almost all of the aphids contain the intracellular symbionts; they are located within specialised aphids' cells in the abdomen. The obligate "primary" symbiont *Buchnera aphidicola* supplement the insects' diet through the provision of essential amino acids, sterol and vitamin, they were found in almost all aphids and housed in the bacteriocytes or mycetocytes cells; in many but not all lineages of aphids contain the facultative "secondary" bacteria which additional types of vertically transmitted endosymbiotic bacteria to the aphid offspring,

their presence is not universal and found in tissues surrounding the bacteriocytes and in specialized secondary bacteriocytes, they have polyphyletic evolutionary origins (Buchner 1965; Fukatsu and Ishikawa 1993; Douglas 1998; Fukatsu and Nikoh 1998; Fukatsu, Nikoh et al. 2000; Fukatsu 2001; Sandström, Russell et al. 2001; Moran, Tran et al. 2005). Secondary symbionts are not required for survival or reproduction, but they have been show to help aphids adjust to ward off parasitoids, heat stress, fungal infection and can alter host plant use (Chen, Montllor et al. 2000; Montllor, Maxmen et al. 2002; Oliver, Russell et al. 2003; Ferrari, Darby et al. 2004; Tsuchida, Koga et al. 2004; Oliver, Moran et al. 2005; Scarborough, Ferrari et al. 2005; Ferrari, Scarborough et al. 2007; Schmid, Sieber et al. 2012).

1 Materials

Samples of *S.avenae* used in this study were collected from Belgium (wheat fields of Gembloux Agro-Bio Tech, University of Liege) and China (wheat fields of the Institute of Plant Protection, the Chinese Academy of Agricultural Sciences, Beijing), which were reared on wheat plants (*Triticum aestivum L.*) in a culture room under the following conditions: temperature, 22℃±1; relative humidity (RH), 60-70 %; and photoperiod, 16/8 hr.

2 Methods

2.1 Antibiotic treatment

The 2nd instars nymphs *S. avenae* (24 h old) were fed on artificial diet including 50μg ml -1 rifampicin (Sigma) in a 15% sucrose-containing solution through paraffin membrane (two layers of parafilm enclosing 200 μl of diet) for 48 hr (named rifampicin-diet), transfer the nymphs to wheat seedlings, every 20 nymphs put in one pot in thirty replicates (total of 600 aphids), the artificial diet absent rifampicin was negative control (named rifampicin-free). Aphid mortality rates were recorded and collected living imago aphids and offspring aphids after 5days.

2.2 DNA extraction and Specific PCR detection

Total DNA was isolated from *S.avenae* individuals (Belgium/Chinese imago aphids and offspring aphids with/without antibiotic), using the DNeasy Tissue Kit (QIAGEN) according to manufacturer's instructions.

For detection of respective endosymbiotic bacteria, diagnostic PCR analysis was conducted using the specific primer according to Tsuchida *et al*. (2002). PCR reactions were conducted using 10× Taq Buffer 5μL, Mg2+ 4μL, dNTP 1μL, Forward Primer (10mM) 2μL, Reverse Primer

(10mM) 2μL, Taq Polymerase 0.5μL, DNA 1μL, ddH2O 34.5μL. The cycling conditions were as follows: 95℃ for 4 min, 40 cycles of 95℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s; final extension at 72℃ for 5 min. The amplified product was checked by gel.

2.3 Volatile qualitative analysis

For each replicate, every 5 Belgium/Chinese *S.avenae* (feed with/without antibiotic) were introduced into a clean 20ml glass vial for 1hour and air was sampled, and then crushed aphids' bodies with a glass pestle. Ten replications were performed. The crushed samples were maintained at 30 ± 0.2 ℃ in thermostated glass tubes for 30 min and directly check (E)-β-farnesene (EβF). EβF released by *S. avenae* aphids was analyzed using an electronic nose, zNose®, model 7100. The zNose® is equipped with an injector, pump, six-port GC valve, Tenax-trap, column (DB5 Column SYS4300C5, 1m, film thickness 0.25μm, ID 0.25mm) and SAW (Surface Acoustic Wave) detector. The set-up temperatures were at 200°C for inlet port, 160°C for valve and 40°C for sensor. For quantification, standard solutions (5, 2.5, 1, 0.5 and 0.1ng/10μl) were prepared by diluting pure EβF with hexane. The headspace vapor of 10μl of each solution was collected on TenaxTM absorbent. The trapped compounds were heated quickly to 225°C to vaporize the adsorbed materials. Subsequently, the helium carrier gas

(3ml/min) transported the material down to the capillary column which was programmed from 40 to 180°C at 5°C s-1. Separated compounds were sequentially detected by the SAW detector through a deviation from its resonance frequency. After each data sampling period, the system needed a 5s baking period, in which the detector was shortly heated to 120°C and after which the temperature conditions of the inlet, column and sensor were reset to the initial conditions.

2.4 2-D polyacrylamide gel electrophoresis

Fresh aphids (Belgium/Chinese imago aphids feed with/without antibiotic) were collected from artificial diet and 20 mg samples were crushed in a 20mM UT buffer (7 M urea, 2 M thiourea, 20 mM Tris, pH 8.5 buffer including 2% CHAPS), centrifuged at 15000g, 4℃ for 15 min. Supernatants were collected and proteins were extracted using the 2D-Clean-UP Kit according to the manufacturer's instructions (GE Healthcare). Quantification of the precipitated proteins was realized using the RC DC Protein Assay and RC DC quantification kit from the same company. The protein extracts (samples of 12.5μg) were labelled with one of three Cydyes (GE Healthcare) following standard DIGE protocol. Two samples corresponding to two different treatment groups (Belgium aphids or China aphids without or with antibiotics) labeled either with Cy3 or Cy5 were mixed an internal reference standard protein mixture

(pooled from 3.125µg Belgium aphids without/with antibiotics and 3.125µg Chinese aphids without/with antibiotics) labeled with Cy2. A conventional dye swap for DIGE was performed by labeling two replicates from each treatment group with one dye (Cy3 or Cy5) and the third replicate with the other of the two Cydyes. A non-labeled 500µg sample of aphid protein mixture was added in one of the analytical gel and the protein spots were excised from that gel using an Ettan spotpicker robot (GE Healthcare). The 2D polyacrylamide gel electrophoresis method and protein identifications were similar to the one used by Francis, et al. (2010)(Francis, Guillonneau et al. 2010). Experimental and Mascot results molecular weights and *pI* were also compared. To categorize the identified proteins based on metabolic function, searches were performed using the KEGG pathway database and Expasy Proteomic tools, in particular the Biochemical–Metabolic pathway sections.

2.5 Statistical analysis

Results are expressed as means ± MSE. Analysis of variance (ANOVAs) was performed using the general linear model (GLM) procedure in the SAS system (SAS Institute Inc. 2001). The normalized data was deal with Ducan's multiple-range test at $P = 0.05$.

Belgium/Chinese *S.avenae* feed with antibiotic were compared to negative control using the method of two-sample *t* test.

3 Results

3.1 Mortality of *S. avenae*

Mortality assays were carried out to assess the potential effect of the endosymbiont on *S. avenae* (Table 1). The mortality of Belgium/Chinese aphids fed on artificial diet containing rifampicin was higher than that negative control. A one-way variance analysis of mortality showed a high significant effect of Belgium imago aphids (df = 58, MS= 0.02, F=17.25, P < 0.0001), there were high significant difference (t=4.153, *P*< 0.0001) between Rifampicin-diet treatment and Rifampicin-free treatment. The mortality of China imago aphids was very higher, reached to 62.50%. High significant differences in mortality were observed in China imago aphids (df = 58, MS= 0.013, F=13.91, P < 0.0005), two-sample *t* test showed high significant difference (t=3.175, *P* < 0.003) among the two treatments. In addition, Belgium/Chinese aphids fed on artificial diet containing rifampicin have fewer offspring than that negative control. High significant differences in the number of Belgium offspring aphids were observed between the two treatment (df = 58, MS= 145.66, F=20.96, P < 0.0001), two-sample *t* test showed high significant difference (t=4.578, *P* < 0.0001), too. The same condition occurred on the number

of China offspring aphids, high significant differences were observed between the two treatment (df = 58, MS= 177.75, F=55.33, P < 0.0001), two-sample *t* test showed significant difference (t=5.548, *P* < 0.0001).

3.2 PCR detection of S-symbionts in *S. avenae*

To investigate the secondary endosymbiotic bacteria in Belgium/Chinese populations feed with/without antibiotic of *S. avenae* were subjected to specific PCR detection. As expected, P-symbiont *Buchnera* was detected in all the samples, including the aphids fed with rifampicin. PABS, *Spiroplasma*$_2$ and *Arsenophonus* were not detected at all samples. PASS1, PAUS, *Rickettsia*$_1$ and *Spiroplasma*$_1$ exhibited in all the test samples. PASS2 was not detected in imago of Belgium/Chinese populations deal with antibiotic, but detected in all the offspring aphids. *Rickettsia*$_2$ was not detected in imago and offspring of Belgium/Chinese populations deal with rifampicin. *Wolbachia* was just detected in offspring of Belgium and imago of Chinese populations from negative control (Table 2).

3.3 Qualitatively analyzed of (E)-β-farnesene (EBF)

Using the zNose®, the only volatile chemical found in the headspace of *S. avenae* was (E)-β-farnesene (EBF). The amount of EBF was reduced significantly in Belgium population feed with rifampicin,

just accounted for 6.08% in negative control; there was high significant difference (t=-3.558; df=18; p=0.0046) between rifampicin-diet treatment and rifampicin-free treatment. The same condition occurred in Chinese population feed with rifampicin, accounted for 22.89% in negative control; two-sample *t* test showed significant difference (t=-3.177; df=18; p=0.0188) (Table 3). The Belgium population with rifampicin-free diet released more EBF than Chinese one, high significant difference were observed in the two treatments (F=10.3; df=18; p=0.0059). But, no significant difference were observed in Belgium population with rifampicin-diet and Chinese one (F=0.35; df=18; p=0.5634).

3.4 2-D polyacrylamide gel electrophoresis

More than 1000 spots were visualized on the stained with Cy2 reference gel and matched across all 2D gels in the experiment (Fig. 1). Quantitative differences in spot intensity were observed among the three gels of two treatment groups. Student's t-tests were performed to analyze the following comparisons among the treatment groups: Belgium *S. avenae* aphids reared on artificial diet with rifampicin and Belgium *S. avenae* aphids reared on artificial diet without rifampicin. According to our statistical threshold ($p < 0.05$, Student's t-test), a total of 67 proteins exhibited differences in normalized spot volume ratios exceeding 1.5 between two treatment groups. From the varying 67 protein spots, eleven

of the differentially regulated proteins could be identified (Table 3). Altogether, 56 of the differentially regulated proteins were identified and checked the related metabolic pathways showed by Table 1 and Table 2, eighteen proteins of putative bacterial endosymbionts origin differed in abundance between the two treatments in this study, these differentially regulated proteins derived from the primary symbiont *Buchnera* aphidicola (Table 2).

4 Discussions

Almost all aphids (Hemiptera: Aphididae) harbours endosymbionts, several inherited secondary or facultative symbionts (S-Symbiont) in addition to *Buchnera* aphidicola (Buchner, 1965). *Buchnera* aphidicola and S-symbionts (PASS, PAUS, *Rickettsia*, *Spiroplasma* and *Wolbachia*) universally found in Belgium and Chinese local population with different infection frequencies. The endosymbionts show remarkable differences in morphology, quantity, and localizations between lineages, and are thought to be of polyphyletic evolutionary origins, which are not strictly required for host survival, but can provide a selective advantage in certain aspect (Augustinos, et al., 2011).

Aphids feed on the phloem sap of plants, which is an unbalanced diet. It is rich in sugars, but free amino acids and most or all of the essential amino acids (viz. leucine, valine and phenylalanine) that aphids

cannot synthesize are present at very low concentrations (Rahbé, Delobel et al. 1990; Girousse, Bonnemain et al. 1991; Douglas 1993; Febvay, Rahbe et al. 1999). However, endosymbiont bacteria play an important role on synthesizing essential amino acids and other nutrients for their host aphids to compliment the deficiency in phloem sap (Douglas, 1998). Deprivation of *Buchnera* by antibiotic treatment results in retarded growth, sterility and/or death of the host aphids (Houk and Griffiths 1980). Recently, novel antibiotic-based selective elimination techniques were devised in *A. pisum*; moderate rifampicin treatment selectively eliminated the obligate symbiont *Buchnera* from the aphids (Koga, Tsuchida et al. 2003). Endosymbionts were selective eliminated from Belgium population and Chinese populations by rifampicin; the mortality of Belgium/Chinese aphids showed higher significant difference with negative control and has fewer offspring than that negative control. In addition, the (EBF) productions were reduced significantly. (Jayaraj, Ehrhardt et al. 1967) repotted that the populations of *Aphis fabae* on *Vicia faba* was in greatly reducing when sprayed antibiotic on larvae on and off the plant. The insects were not killed, but their fertility was decreased by over 97% in the first generation, leading to total sterility in the second generation. In our study, the S-symbiont *Rickettsia* was eliminated by antibiotic, but it was not impact on the reproduction, but often negatively affected on the host fitness components. A previous work showed

that *Rickettsia* infection does not affect the host reproductive mode under different pea aphid genotypes (Simon, Sakurai et al. 2007; Simon, Boutin et al. 2011). So *Buchnera* play an important role on the reproduction, fitness components and EBF production.

Nearly one fourth of the proteins were derived from known bacterial symbionts. These proteins differed in rifampicn-treatment and firampicin-free-treatment in Bulgium population; they are all from *Buchnera aphidicola*. More than two million cells of the primary endosymbiont *Buchnera aphidicola* are estimated to be housed within the a single pea aphid, it is not surprising in light of the symbionts' importance in aphid biology, and their abundance within aphids (Wilkinson and Douglas 1998). Evidence that the isoprenoid components of the aphid alarm pheromone including EBF are produced by a pathway linked to juvenile hormone (Gut, Harrewijn et al. 1987; van Oosten, Gut et al. 1990). Pyruvate dehydrogenase is the necessary enzyme for synthesizing acetyl-coA, which is the important component in the pathway (Lamelas, Gosalbes et al. 2011). We found pyruvate dehydrogenase E2 component from *Buchnera aphidicola*.

Aphids' associations with bacteria have been studied for many years, but the relationship between aphid bacterial symbionts and alarm pheromone has few reports. Our study indicates that *Buchnera* take part

in the EBF production, but the mechanisms remain to be researched. This proteomic study also illustrates the cross links in the metabolism of aphids and their bacterial symbionts, and the responsiveness of endosymbionts to the environment of their aphid hosts.

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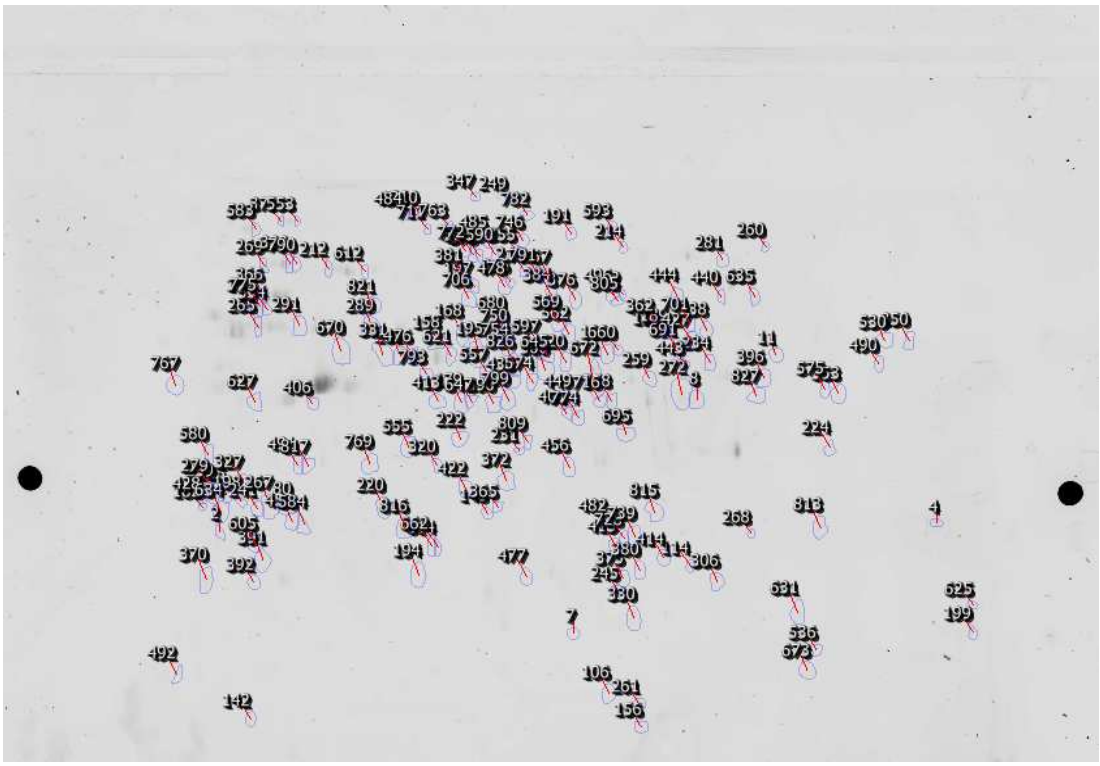

Figure 1 Proteomic profile of Belgium *Sitobion avenae* feed with/without antibiotics as revealed by 2D DIGE analysis. A 2D-PAGE of Belgium *Sitobion avenae* feed with/without antibiotics separated on a 12.5% acrylamide gel. Identified proteins showed significant expression level. Numbered spots corresponded to proteins significantly varying according to antibiotic feeding; complete properties are given in Table 4, Table 5 and Table 6.

Table 1 The Mortality of imago and the Number of offspring of *S. avenae* after feeding with/without RIFAMPICIN

The Mortality of imago (%) and the Number of offspring								
	Rifampicin-Diet	Rifampicin-Free	F value and Significance of difference			T value and Significance of difference		
			F	df	Pr>F	t	df	Pr>t
Belgium imago aphids	19.33±1.88	34.50±3.13	17.25**	58	0.0001	4.153**	58	0.0001
Chinese imago aphids	46.25±2.92	62.50±2.97	13.91**	58	0.0005	3.175**	58	0.003
Belgium offspring aphids	40.233±2.2635	25.967±2.1418	20.96**	58	0.0001	4.578**	58	0.0001
Chinese offspring aphids	43.400±3.2746	14.600±2.2892	55.33**	58	0.0001	5.548**	58	0.0001

** indicate significant differneces at $P < 0.01$, respectively.

Table 2 The endosymbiont detected in Belgium/Chinese local populations of *S. avenae* of rearing on with/without RIFAMPICIN

	Belgium/Chinese local populations of *Sitobion. Avenae*							
Endosymbiont	BI	BI+Rif^{+}	BS	BS+Rif^{+}	CI	CI+Rif^{+}	CS	CS+Rif^{+}
Buchnera	+	+	+	+	+	+	+	+
PASS1	+	+	+	+	+	+	+	+
PASS2	+		+	+	+		+	+
PAUS	+	+	+	+	+	+	+	+
PABS								
*Rickettsia*1	+	+	+	+	+	+	+	+
*Rickettsia*2	+		+		+		+	
*Spiroplasma*1	+	+	+	+	+	+	+	+
*Spiroplasma*2								
Wolbachia			+		+			
Arsenophonus								

+ indicates that strain was examined for endosymbiont, blank means no endosymbiont was detected.

BI: Imago Belgium *S. avenae* fed rifampicin-Free; BI+Rif^{+}: Imago Belgium *S. avenae* fed rifampicin-diet; BS: The offspring of Belgium *S. avenae* fed rifampicin-Free; BS+Rif^{+}: The offspring of Belgium *S. avenae* fed rifampicin-diet; CI: Imago China *S. avenae* fed rifampicin-Free; CI+Rif^{+}: Imago China *S. avenae* fed rifampicin-diet; CS-

F: The offspring of China *S. avenae* fed rifampicin-Free; CS+Rif^{+}: The offspring of China *S. avenae* fed rifampicin-diet.

Table 3 GC-MS qualitatively analyzed of (E)-β-farnesene (EBF) in Belgium/Chinese local populations of *S. avenae* of rearing on with/without RIFAMPICIN

	The amount of (E)-β-farnesene (EBF)		
	RIFAMPICIN-Free	RIFAMPICIN-Diet	T value and Significance of difference
Belgium *Sitobion avenae*	304.1±89.59Aa	18.5±7.03Aa	t=-3.558**; df=18; p=0.0046
Chinese Sitobopn avenae	61.9±17.82Bb	14.1±4.03Aa	t=-3.177*; df=18; p=0.0188
F value and Significance level	F=10.3; df=18; p=0.0059	F=0.35; df=18; p=0.5634	

Horizontal: Two-sample t test: * and ** indicate significant differneces at $P < 0.05$ and $P < 0.01$, respectively

Vertical: Significantly different are indicated by different letters, and means within columns followed by the same letter are not significantly different (one-way ANOVA; Duncan's multiple-range test). "AB" and "ab" indicate significant differneces at $P < 0.05$ and $P < 0.01$, respectively

Table 4 A list of identified proteins and related metabolic pathways in *S. avenae* aphids that differ in abundance between two treatment groups

Spot Number	MW	pI	Mowse	MS cov	Peptide Number	Protein Identification	Accession	Organism
Carbohydrate metabolism								
156	44555	5.85	81	19	7/18	phosphoglycerate kinase-like	XP_001944310.1	Acyrthosiphon pisum
7	35584	7.98	43	23	5/37	fumarylacetoacetate hydrolase	XP_001949600.1	Acyrthosiphon pisum
106	57748	5.89	56	14	7/29	u4/U6 small nuclear ribonucleoprotein Prp4	XP_001949663.1	Acyrthosiphon pisum
673	39871	6.62	72	31	8/49	fructose-bisphosphate aldolase-like isoform 1	XP_001951517.1	Acyrthosiphon pisum
536			92	28	9/31	fructose-bisphosphate aldolase-like isoform 1	XP_001951517.1	Acyrthosiphon pisum
Protein synthesis								
220	62774	5.53	54	10	7/34	30S ribosomal protein S1	NP_240132.1	Acyrthosiphon pisum
816	35448	9.33	32	10	3/8	39S ribosomal protein L19, mitochondrial	NP_001155559.1	Acyrthosiphon pisum
168	24648	6.53	43	15	3/15	GTP-binding nuclear protein Ran	NP_001155556.1	Acyrthosiphon pisum
575	30361	10.77	46	17	4/14	50S ribosomal protein L2	NP_240328.1	Acyrthosiphon pisum
496	41157	9.07	38	16	3/11	translation-associated GTPase	NP_240022.1	Acyrthosiphon pisum
782	21674	6.96	56	16	4/17	GTP-binding protein SAR1b-like	XP_001943743.1	Acyrthosiphon pisum
590	89285	5.10	98	14	10/21	transitional endoplasmic reticulum ATPase TER94-like	XP_001949588.1	Acyrthosiphon pisum
406	22395	5.86	55	25	4/10	oligoribonuclease, mitochondrial	XP_001653487.1	Aedes aegypti
Amino acid metabolism								
265	31971	9.43	30	5	2/3	diaminopimelate epimerase	NP_240392.2	Acyrthosiphon pisum
11	60165	8.02	45	7	3/7	cysteinyl-tRNA synthetase	XP_003395414.1	Acyrthosiphon pisum
672	54707	8.95	35	5	3/7	2-isopropylmaltate synthase	AAG31386.1	Acyrthosiphon pisum
821	30102	9.17	32	12	3/14	diaminopimelate epimerase	NP_660893.2	Acyrthosiphon pisum
194	20590	8.19	41	9	3/7	ubiquitin-conjugating enzyme m-like	NP_001155460.1	Acyrthosiphon pisum
279	119417	8.07	31	3	3/6	ATP-dependent RNA helicase spindle E-like isoform 2	XP_003243800.1	Acyrthosiphon pisum
Energy metabolism								
331	47031	7.07	86	20	9/27	short/branched chain specific acyl-CoA dehydrogenase	XP_001604014.2	Acyrthosiphon pisum
362	57682	8.22	33	17	3/10	fatty acyl-CoA reductase 1-like isoform 1	XP_001948060.2	Acyrthosiphon pisum
214			39	8	4/9	ACYPI006470	BAH71927.1	Acyrthosiphon pisum
805	52522	6.14	57	15	6/17	NADH dehydrogenase [ubiquinone] iron-sulfuprotein 2	XP_001947632.1	Acyrthosiphon pisum
557	26357	6.06	48	30	4/6	mitochondrial ATP synthase gamma-subunit	NP_001119637.1	Acyrthosiphon pisum
38	33019	9.34	46	17	4/11	mitochondrial ATP synthase gamma-subunit	NP_001119637.1	Acyrthosiphon pisum
701	59795	9.14	85	14	8/14	ATP synthase subunit alpha	XP_001943349.1	Acyrthosiphon pisum
220	51948	9.05	41	8	4/10	flagellum-specific ATP synthase	NP_239910.1	Acyrthosiphon pisum
584	11412	5.70	45	27	3/15	dynein light chain roadblock-type 2-like	XP_001947855.2	Apis mellifera
Co-factors and vitamins								

754	59169	6.68	42	6	4/7	membrane-bound alkaline phosphatase-like	XP_001943536.2	Acyrthosiphon pisum
Cytoskeleton								
774	38200	5.36	76	30	8/33	actin related protein 1	NP_001136108.1	Acyrthosiphon pisum
4	41785	5.29	90	36	14/39	actin related protein 1	NP_001136108.1	Acyrthosiphon pisum
366	41785	5.29	55	14	5/14	actin related protein 1	NP_001136108.1	Acyrthosiphon pisum
Nucleotide metabolism								
555	63513	5.13	33	5	3/6	eukaryotic peptide chain release factor GTP-binding	XP_001949512.2	Acyrthosiphon pisum
Hormone biosynthesis								
413	30614	6.91	46	8	4/7	cytochrome P450 302a1	XP_001948299.2	Acyrthosiphon pisum
Stress response								
3	71442	5.34	49	7	3/7	heat shock 70 kDa protein cognate 4-like isoform	XP_001951233.1	Acyrthosiphon pisum
327	83417	4.94	163	19	20/31	heat shock protein 83-like	XP_001943172.1	Acyrthosiphon pisum
Xenobiotic degradation								
251	23438	5.18	30	19	3/6	glutathione S-transferase-like	XP_003245722.1	Acyrthosiphon pisum
Membrance transport								
809	30068	8.88	35	7	3/5	septum site-determining protein	YP_004590056.1	Acyrthosiphon pisum

MW, molecular weight; pI, isoelectric point; Mowse, Mowse score according to Mascot search; MS cov, percentage of the protein sequence identified; Peptide number, number of peptide hits for each protein; Accession, accession number on NCBI; Organism, related original organism for the protein identification.

Table 5 A list of identified proteins and related metabolic pathways in bacterial endosymbionts that differ in abundance between two treatment groups

Spot Number	MW	pI	Mowse	MS cov	Peptide Number	Protein Identification	Accession	Organism
Carbohydrate metabolism								
440	47697	9.58	40	900	4/11	pyruvate dehydrogenase E2 component	YP_004589984.1	*Buchnera* aphidicola
612	52297	9.08	65	2900	10/53	dihydrolipoyllysine-residue succinyltransferae	XP_001944020.2	*Buchnera* aphidicola
Protein synthesis								
422	6729	10.23	31	2500	3/5	50S ribosomal protein L30	NP_240313.1	*Buchnera* aphidicola
Amino acid metabolism								
791	31102	9.17	41	800	4/5	diaminopimelate epimerase	NP_660893.2	*Buchnera* aphidicola
627	41478	6.03	50	1800	5/19	glutamine synthetase 2	NP_001153848.1	*Buchnera* aphidicola
224	22027	8.81	34	1800	3/4	Histidinol dehydrogenase	Q9RQ82.1	*Buchnera* aphidicola
Energy metabolism								
350	30604	9.44	43	1000	4/20	NAD synthetase	NP_660524.1	*Buchnera* aphidicola
191	33019	9.34	48	1700	4/11	mitochondrial ATP synthase gamma-subunit	NP_001119637.1	*Buchnera* aphidicola
Co-factors and vitamins								
212	24343	5.21	49	1500	4/10	ras-related protein Rab-11A-like isoform 1	XP_001950094.1	*Buchnera* aphidicola
Nucleotide metabolism								
767	48782	5.62	58	2100	10/23	ATP-dependent RNA helicase WM6-like	XP_001942765.1	*Buchnera* aphidicola
80	192809	8.29	49	500	8/21	chromodomain-helicase-DNA-binding protein	XP_001946846.2	*Buchnera* aphidicola
477	95448	6.86	43	400	3/5	DNA repair endonuclease XPF-like	XP_001121259.2	*Buchnera* aphidicola
Stress response								
763	55992	5.22	103	39	13/49	GroEL	ABW81768.1	*Buchnera* aphidicola
410	60452	5.06	58	2000	8/37	60 kDa heat shock protein	XP_003241704.1	*Buchnera* aphidicola
71	60452	5.06	118	3700	19/66	60 kDa heat shock protein	XP_003241704.1	*Buchnera* aphidicola
Membrance transport								
695	32274	5.61	58	1700	8/17	guanine nucleotide-binding protein	XP_001947878.1	*Buchnera* aphidicola
Signaling pathway								
1660	29373	4.77	54	1800	5/20	14-3-3 protein epsilon	NP_001155476.1	*Buchnera* aphidicola
427	28225	4.72	70	24	7/18	14-3-3 protein zeta	NP_001156510.1	*Buchnera* aphidicola

Table 6 List of some reference identified proteins between treatment groups

Spot Number	MW	pI	Mowse	MS cov	Peptide Number	Protein Identification	Accession	Organism
8	44745	6.46	52	11	4/20	nitrogen permease regulator 2-like protein	XP_001945394.2	Acyrthosiphon pisum
222	49036	5.35	64	18	7/41	26S protease regulatory subunit 7-like	XP_001948503.1	Acyrthosiphon pisum
197	18180	9.64	34	11	3/3	polypeptide deformylase	NP_778040.1	Acyrthosiphon pisum
289	48003	8.09	38	18	3/3	histidinol dehydrogenase	AEO08463.1	Acyrthosiphon pisum
79	62678	9.37	53	10	5/14	ubiquitin carboxyl-terminal hydrolase 2-like	XP_001944500.1	Acyrthosiphon pisum
775			41	10	4/11	hypothetical protein LOC100574382	XP_003240429.1	Acyrthosiphon pisum
574	37170	5.43	55	23	6	Peptidase : ubiquitin carboxyl-terminal hydrolase	gi|:157127884|	Aedes aegyptii
799	46663	6.38	40	20	3/10	paxillin-like isoform 2	XP_003244023.1	Acyrthosiphon pisum
55	59954	5.36	109	26	14/38	t-complex protein 1 subunit alpha-like	XP_001943068.1	Acyrthosiphon pisum
876	25561	9.53	38	7	3/4	protease specific for YgjD	AEO08676.1	Acyrthosiphon pisum
372						hypothetical protein LOC100164681	XP_001946008.2	Bombus terrestris

Chapter V: *Variation in transmission of barley yellow dwarf virus-PAV by different Sitobion avenae clones in China*

General Introduction to Chapter V

Barley yellow dwarf viruses (BYDVs, family *Luteoviridae*) is also called cereals yellow plague, cereal yellow dwarf, yellow dwarf or red leaf. It is one of the most widespread and damaging viral diseases of grasses and cereal crops in the world, which can affect more than 100 species in the family *Poaceae*, including wheat (*Triticum aestivum* L., *Triticum durum* Desf.), barley (*Hordeum vulgare* L.), oats (*Avean sativa* L.), rice (*Oryza sativa* L.), rye (*Secale cereal* L.), sorghum (*Sorghum vulgare* L.) and plenty wild grasses. BYDV has considerable economic significance worldwide, particularly in higher rainfall regions where yield losses in wheat have been reported to be as high as 40-50%. Global average yield losses due to the natural BYDVs infection can range from 11% to 33%, whereas in some areas the losses reach up to 87%. BYDV-PAV caused substantial losses throughout the world in barley (15%), wheat (17%), and oats (25%). BYDVs are transmitted by aphids in a persistent and circulative manner. Each BYDV strain only transmitted efficiently by corresponding aphid species; one species aphid usually can efficiently transmit more than one virus strain. The English grain aphid, *Sitobion avenae*, widespread throughout China, is one of the most

common and destructive pest attacking wheat. It is considered to be an important vector of BYDV, especially in the spring when it spreads the virus from winter hosts (wheat and barley) to spring barley and corn.

As an unachieved work, the analysis on aphid virus transmission efficiencies according to aphid species and clones should be promoted according to the important significance on understanding the virus migration path in aphid and further propose of new potential tools to control virus transmission.

Variation in transmission of barley yellow dwarf virus-PAV by different *Sitobion avenae* clones in China

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Abstract: Fourteen *Sitobion avenae* Fabricius (Hemiptera: Aphididae) clonal lines (clones) originating from China were tested for their ability to transmit BYDV-PAV (one isolate from Belgium and another from China) using wheat plants. By sequence analysis, the coat protein gene of BYDV-PAV-BE was distinguishable from BYDV-PAV-CN. All of the clones could transmit BYDV-PAV, and the transmission varied from 24.42% to 66.67% with BYDV-PAV-BE and from 23.55% to 56.18% with BYDV-PAV-CN. These data suggest that *S. avenae* has no specialty in BYDV-PAV isolate. Significant differences in the transmission frequencies between the clones with BYDV-PAV-BE and BYDV-PAV-CN were observed. The transmission efficiencies of aphid clones from the middle-lower reaches of Yangtze River (AH, HD, HDE, HZ, JZ, JY and SJ) and Yunnan province (YH) were similar. Nevertheless, differences in the virus transmission efficiencies of the clones from northern (ST and STA) and northwestern (QX, SB and XS) regions were assessed. The transmission efficiency of *S. avenae* from northern and northwestern China, where BYDV impact is more important, was higher than that from the middle-lower reaches of the Yangtze River and Yunnan province. This work emphasizes the importance of considering aphid vector clonal diversity in addition to virus strain variability when assessing BYDV transmission efficiency.

Key words: transmission efficiency, barley yellow dwarf virus, *Sitobion avenae*, RT-PCR, TAS-ELISA

Introduction

Barley yellow dwarf is one of the most damaging cereal diseases worldwide. It is caused predominantly by different viruses currently classified into two genera, *Luteovirus* and *Polerovirus* of the plant virus family *Luteoviridae* (Mayo and D'Arcy, 1999). BYDVs display a high degree of vector specificity among different aphid species. Each BYDV strain is transmitted efficiently by only a limited number of aphid species. Nevertheless, one aphid species can efficiently transmit more than one virus strain (Rochow, 1959). Four BYDV (GPV, PAV, GAV, and RMV) isolates exist in China according to Rochow's system (Zhou *et al*., 1987; Wang *et al*., 2001; Liu *et al*., 2007). BYDV-GAV is similar to BYDV-MAV, which is transmitted nonspecifically by the *Sitobion avenae* and *Schizaphis graminum* aphids (Wang *et al*., 2001), whereas the BYDV-GPV strain is more closely related to BYDV-RPV, which is transmitted by both *S. graminum* and *Rhopalosiphum padi* (Wang *et al*., 1998). *R. padi* and *S. avenae* efficient transmit PAV, whereas RMV is best transmitted by *R. maidis* (Wang and Zhou, 2003, Zhou *et al*., 1984; Zhou *et al*., 1987; Wang and Zhou, 2003). Lastly, the BYDV-GPV strain has only been observed in China (Zhou *et al*., 1987).

BYDVs are transmitted by aphids in a persistent or circulative manner. Acquisition and infection feeding periods of 48 hours or more

are required to maximize the transmission rate. Once acquired, the virus is retained for numerous days, often for the entire life of the vector. The circulative viral route through the aphid body has been partially characterized. Aphids acquire the viruses from infected phloem cells while feeding using their piercing-sucking stylets. The virions travel up the stylet food canal and into the aphid's gut lumen. Subsequently, the virions traverse the lining of the hindgut, are released into the body cavity (hemocoel), and begin to circulate in the hemolymph. The virions suspended in the hemolymph that contact the paired accessory salivary glands (ASG) are actively endocytosed into the ASG cells, transported into the salivary duct, from which they can infect potential host plants (Gildow, 1985; Gildow, 1993; Gildow and Gray, 1993; Yang *et al*., 2008). Virus-aphid specificity likely results from the recognition between virions of a specific isolate and the viral receptors in the accessory salivary glands of a particular aphid species (Gildow and Rochow, 1980; Gildow and Gray, 1993; Peiffer *et al*., 1997; Bencharki *et al*., 2000).

Although the viral transmission efficiency is well known to differ between aphid species (even if the molecular mechanisms are still unknown), the mechanisms that differentiate the ability of distinct aphid clones to transmit various BYDV variants remain unknown. At present, studies on intra-specific variation of BYDV transmission that were performed mainly with *S. graminum* and *R. padi* always used one virus

strain, which did not always correspond to origin of the tested aphid clones (Gildow and Rochow, 1983; Guo *et al*., 1996; Guo *et al*., 1997a; Guo *et al*., 1997b; Bencharki *et al*., 2000; Smyrnioudis *et al*., 2001; Papura *et al*., 2002; Dedryver *et al*., 2005; Du *et al*., 2007).

Given that *S. avenae* is one of the most common and destructive wheat pests and is a primary vector for BYDV-PAV, we assessed the viral transmission efficiency of a diversity of aphid clones and virus strains using a wheat model in this study. A large collection of *S. avenae* aphid clones throughout China were attained and tested for BYDV-PAV transmission in two different geographic isolates, one from China and one from Europe (Belgium).

1 Materials and Methods

1.1 Collection and rearing of *S. avenae*

Fourteen S. avenae clones were collected from winter wheat (Triticum aestivum L.) fields in the wheat-growing areas of China, including the Huang-Huai winter (autumn sowing) wheat area, Yangtze River winter (autumn sowing) wheat area, Xinjiang winter-spring wheat area, Qinghai-Tibet spring-winter wheat area and the Southwest winter (autumn sowing) wheat area (Figure 1) in 2009. All clones were maintained separately on seedlings from a susceptible aphid wheat cultivar (cv. Toison d'or). All of the aphids were reared under conditions

that minimized the contamination risk between clones, i.e., the aphid isolates were transferred to pots of wheat seedlings at the second stage, and each pot was separated with a transparent plastic cylinder cage covered with gauze (12cm height and 24cm in diameter). The aphids and plants were maintained in a greenhouse compartment at 22°C±1, 60-70 % relative humidity and a 16 hour light photoperiod.

1.2 Virus strains

The BYDV strains were obtained from Belgium, Louvain-la-Neuve (BYDV-PAV-BE) in 2009 and China, Yangling - Shannxi province (BYDV-PAV-CN) in 2011. They were maintained separately on wheat seedlings cv. Toison d'or infested with S. avenae in a greenhouse compartment at 20 ± 1°C and a 16hour light photoperiod.

1.3 RT-PCR

RT-PCR was used to identify the BYDV strain using the primer pair P5 (5'-CCAGTGGTTGTGGTC-3') and P3 (5'-GGAGTCTACCTATTT-3') (Du *et al*., 2007). Total RNA was extracted from the plant material using the RNeasy plant mini-kit (Qiagen, Germany) following the manufacturer's instructions. cDNA synthesis with RT-PCR and PCR were performed as described by Robertson *et al*. (1991) and Du *et al*. (2007), respectively. The amplified products were purified and sequenced, and the sequences were aligned using Clustal W. The aligned RNA

sequences were imported into MEGA4.0 (Tamura *et al*., 2007) for sequence comparison and variation analysis.

1.4 Virus transmission efficiency assays

The plants were assessed for the presence of the virus with DAS-ELISA (DSMZ, Braunschweig). Plants with similar optical densities (OD) were used as virus inoculums in the transmission experiments. Two-day-old *S. avenae* nymphs were fed an artificial diet (infected tissue ground in a 15% sucrose-containing solution) through paraffin membrane for a 48 hour acquisition access period. Aphids were removed from the membrane, and three were transferred onto each test plant. Thirty plants were used for each condition. After a 72 hour infection access period, the aphids were killed. The wheat plants were stored in a greenhouse for 15 days before observation. Transmission by each *S. avena*e clone was repeated in three separate experiments.

The presence of the BYDV-PAV virus in the leaves of infected plants was assessed using DAS-ELISA following the manufacturer's instructions (Dr S. Winter, DSMZ, Braunschweig, Germany). The samples were considered positive when the OD values were greater than three times the mean of the results from uninfected control leaves.

1.5 Data analyses

An analysis of variance (ANOVAs) was performed using the GLM procedure in the SAS system (SAS Institute Inc. 2001). A one-way analysis of variance with treated aphid clones was conducted for all variables, and the means were separated by Tukey's studentized range test (HSD) at $P = 0.05$.

2 Results

2.1 Molecular diagnosis of BYDV strain

Two unique 534 bp and 503 bp RT-PCR product were amplified for the BYDV strain from Belgium and China (Figure 2) and were separately sequenced. In comparisons with other known *Luteoviridae* members sequences, the BYDV strain sequence from Belgium was similar to the BYDV-PAV-Sweden isolate (Accession number: AJ563413) sequence, and the BYDV strain sequence from China was similar to the BYDV-PAV-CN isolate (Accession number: EU332318.1) sequence. This study employed the BYDV-PAV strain. Genetic distance analysis based on the coat protein gene sequences indicated that the BYDV-PAV-BE isolate was distinguishable from the BYDV-PAV-CN isolate (Figure 3), but the nucleotides of the two BYDV-PAV gene were 78% similar (Figure 4).

2.2 Transmission efficiency of different aphid populations

Fourteen geographically separate *S. avenae* clones were submitted to transmission experiments. All aphid clones transmitted the BYDV-PAV-BE isolate and BYDV-PAV-CN isolate (Table 1). The average transmission rate of BYDV-PAV-BE isolate was 42.06%. A one-way variance analysis of transmission rates revealed a significant effect for the *S. avenae* clone ($df = 13$, $MS= 42.326$, $F = 10.36$, $P < 0.001$). The STA clone transmitted at 66.67±3.84%, whereas the HDE clone transmitted at only 24.42±2.21%. The most efficient *S. avenae* clone transmitted BYDV-PAV approximately three times more efficiently than the least efficient aphid strain. The average transmission rate for the BYDV-PAV-CN isolate was 35.08%, which was lower than for the BYDV-PAV-BE isolate. A significant effect of the clone on transmission rate was observed ($df = 13$, $MS= 8.219$, $F = 23.5$, $P < 0.001$). The STA clone transmitted at 56.18±5.22%, wherease the HDE clone transmitted at only 23.55±1.36%. The most efficient *S. avenae* clone transmitted the BYDV-PAV approximately 2.4 times more than the least efficient clone. The transmission efficiency of the clones from the middle-lower reaches of the Yangtze River (AH, HD, HDE, HZ, JZ, JY and SJ) and the Yunnan province (YH) were not significantly different, and neither were the transmission efficiencies of the clones from the northern (ST and STA) and northwestern (QX, SB and XS) regions. Lastly, the transmission efficiency of *S. avenae* from the northern and northwestern regions was

higher than from the middle-lower reaches of the Yangtze River and the Yunnan province.

3 Discussion

BYDVs have been previously demonstrated to display high degrees of vector specificity among different aphid species, where each virus is transmitted efficiently by only one or a few aphid species. In China, Du *et al*. (2007) reported that BYDV-PAV was efficiently transmitted by *R. padi*-La and *S. avenae*-La but poorly by *S. graminum*-La. *S. graminum*-La and *S. avenae*-La. *M. dirbodum* exhibited transmission rates of 75% with BYDV-GAV, and BYDV-GPV was transmitted by *S. graminum*-La and *R. padi*-La at approximately 80% and 50%, respectively. The Chinese BYDV-PAV and BYDV-GAV isolates are members of *Luteovirus* and distinguishable from their relatives isolated in other countries. BYDV-GPV is a distinct virus in China. It harbors a polerovirus-like coat protein gene and is closest to CYDV-RPV and CYDV-RPS but lacks a serological relationship all U.S. isolates (Du *et al*., 2007).

In this study, the *S. avenae* clones were from China, and the BYDV-PAVs were from China and Belgium, Europe. The genetic distance analysis based on the coat protein gene sequences indicated that the BYDV-PAV-BE isolate was distinguishable from the BYDV-PAV-CN isolate, but the nucleotide sequences of the two BYDV-PAV genes were

78% similar. The Belgian virus strain was used to assess the transmissibility of non-vector-associated aphid clones. Our results demonstrated that all Chinese clones were able to transmit BYDV-PAV-BE at different efficiency rates. The transmission rates varied from 24 to 67% with the BYDV-PAV-BE isolate and from 23 to 56% with the BYDV-PAV-CN isolate. These results are partly consistent with previous BYDV-PAV transmission studies using different *S. avenae* clones. Guo *et al*. (1996; 1997a; 1997b) observed a transmission range of 0 to 76% among 21 clones collected from 4 French regions. Papura *et al*. (2002) tested 39 F1 progeny and observed a 0% to 88% of viral transmission rates. Notably, the BYDV-PAVs did not possess any non-vector strains in this study. Indeed, all strains transmitted both BYDV-PAV isolates at least at a 20% transmission rate, whereas an extremely large area for aphid strain collection was investigated. This finding provides some context to the results from Bencharki *et al*. (2000), who reported that the transmission efficiency of *S. avenae* was generally dependent upon the PAV isolate. Considering a pool of aphid clones, the final transmission rate was extremely similar even for different BYDV-PAV strains.

Little is known about why such diversity in transmission ability exists. van den Heuvel *et al*. (1997; 1994) have demonstrated that symbionin, a GroEL homologous protein synthesized by endosymbiotic bacteria and secreted into the aphid hemolymph, is essential for efficient

Luteovirus transmission. Additionally, they revealed that symbionin from different aphid species could bind to *Luteoviridae* in vitro with different affinities. Bencharki *et al*. (2000) suggested that the observed intraspecific variability in transmission efficiency may be related, at least in part, to differences in the movement ability of the BYDV virus throughout the epithelial cell barriers at the hindgut and/or the accessory salivary glands in the different clones or to the stability of the virus in the hemolymph. Symbionin of the inefficient aphid sub-clones may be released at low concentrations or exhibit a low binding affinity for the virus. Although endosymbionts may participate in stabilizing virus particles, Burrows *et al*. (2007) did not believe that they determine vector competence because they observed that the hindgut and accessory salivary gland barriers to transmission were genetically controlled and separated in F2 *S. graminum* hybrid. Further functional experiments using omics tools are merited to elucidate the molecular mechanisms of the virus-aphid interactions and to characterize the participating proteins.

Taken together, the results suggest that both the *S. avenae* clone and the BYDV-PAV strains must be considered when assessing aphid-virus interactions. Both the aphid speciesand the viral clones should be considered for viral transmission assessment. Moreover, the heterogeneity of aphid clones from a common given species, such as *S. avenae*, is extremely high and thus must be significantly sampled for

thorough representation. Indeed, if most of the clones displayed extremely similar virus transmission efficiencies between the BYDV-PAV-BE and BYDV-PAV-CN strains, where half of the clones displayed a 10% or less changes in viral transmission efficiency, and more than a quarter of the clones (4 of 14 clones) exhibited a 20 to 30% virus transmission variation among the BYDV-PAV strains. Moreover, two clones (from Shaanxi Baoji and Qinghai Xining) displayed highly significant changes (from 40 to 90% rate increases) when switching from a BYDV-PAV to an other strain. These results were systematically observed with BYDV-PAV-BE, which is a strain that does not occur co-geographically with other tested aphid clones. This finding is important given that aphids are dispersed regionally and internationally. Because a two to three fold increase in viral transmission is due to a new efficient aphid clone in a particular area, extensive damage and yield losses could occur. In this study, the transmission efficiency of *S. avenae* from northern and northwestern China was higher than that from the middle-lower reaches of the Yangtze River and Yunnan province. Coincidentally, BYDVs predominantly caused wheat yellow dwarf diseases throughout the northern and northwestern provinces of China (Jin *et al*., 2004). Du and colleagues (2007) also demonstrated that the clones that display the highest transmission efficiency were from a highly prevalent BYDV area. These results suggest that assessing variation for viral transmission by

identifying the aphid species will be immensely interesting for epidemiological studies.

Analysis of aphid transmission efficiencies is particularly significance for researching its migration path. Using microsatellite markers, Wang (2007) inferred that: (i) *S. avenae* are highly migratory throughout the middle-lower reaches of the Yangtze River wheat growing area of China; (ii) *S. avenae* are highly migratory throughout the north and northwest wheat growing area of China; (iii) the microsatellite marker analyses did not support the conclusion that *S. avenae* overwinters in the south and migrates north in the spring. The above conclusion coincides with the result of our study: (i) the transmission efficiencies of the clones from the middle-lower reaches of the Yangtze River (AH, HD, HDE, HZ, JZ, JY and SJ) did not differ; (ii) the transmission efficiencies of the clones from the northern (ST and STA) and northwestern (QX, SB and XS) areas of China did not differ; (iii) and the transmission efficiency of *S. avenae* from the northern and northwestern regions was higher than from the middle-lower reaches of the Yangtze River.

In conclusion, further analysis of the virus-aphid interactions are merited to elucidate the mechanisms of virus acquisition, transport and availability in the aphid vectors by integrating high and low transmitting efficiency clones and comparing them. Further research may provide

further context for monitoring the occurrence of important transporters and/or receptors in efficient aphid vectors and for identifying potential inhibitor/competitors of virus-binding proteins to control virus dispersion. A better understanding of viral transmission efficiency in aphids may change epidemiological models that are applied to plant viruses in regional areas and may improve control strategies for aphid-virus associations.

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Figures and tables

Figure

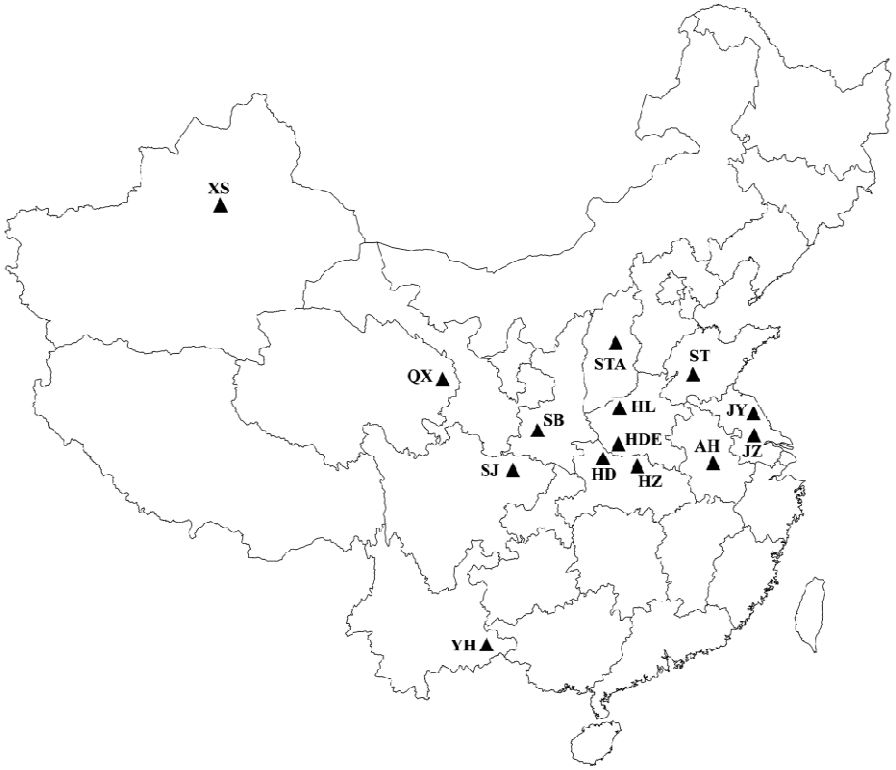

Figure 1. Locations (province & city) where *Sitobion avenae* populations have been collected in China.
AH, Anhui Hefei; HD, Hubei Danjiangkou; HDE, Henan Dengzhou; HL, Henan Luoyang; HZ, Hubei Zaoyang; JY, Jiangsu Yancheng; JZ, Jiangsu Zhenjiang; QX, Qinghai Xining; SB, Shaanxi Baoji; SJ, Sichuan Jiangyou; ST, Shandong Taian; STA, Shanxi Taiyuan; XS, Xinjiang Shihezi; YH, Yunnan Honghe.

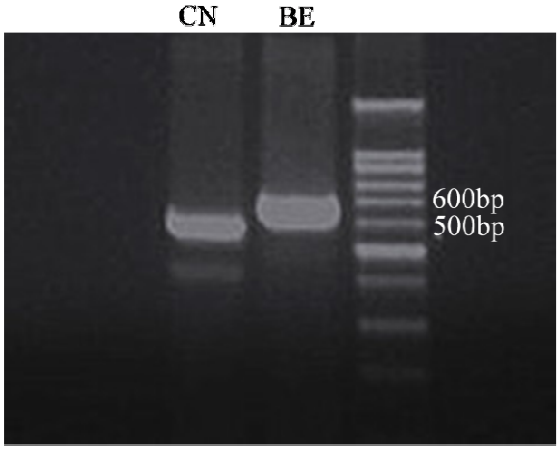

Figure 2. RT-PCR products of coat protein gene from BYDV-PAV isolate in this study using the primer pair P5 and P3.
CN: BYDV-PAV-China isolate (503bp);
BE: BYDV-PAV-Belgium isolate (534bp).

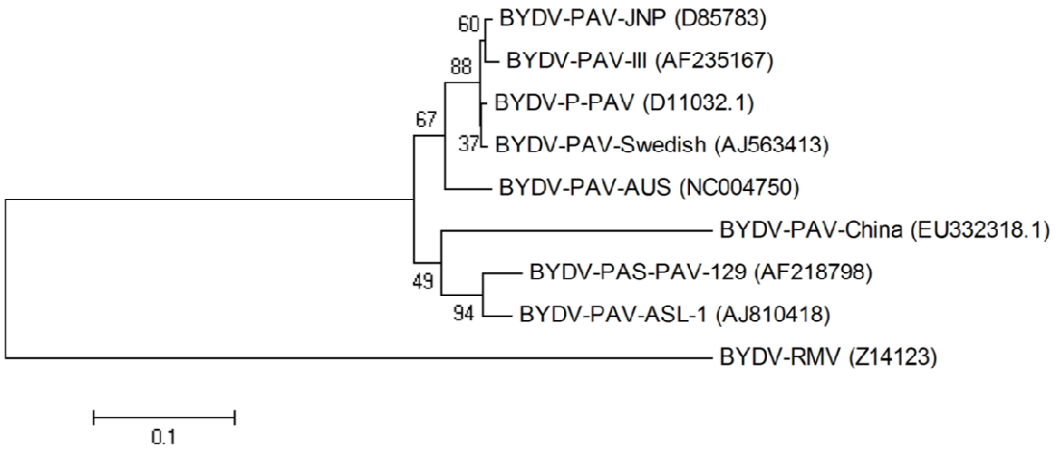

Figure 3. Genetic distance analysis for BYDV-PAV in this study and other PAV isolates based on coat protein gene sequences. Accession numbers of previously sequenced BYDV coat protein gene sequences are shown in parentheses. BYDV-RMV (Z14123) was utilized as an outgroup. Their genetic distances were analyzed with MEGA 4.0 by neighbor-joining method with a bootstrap value of 1000)

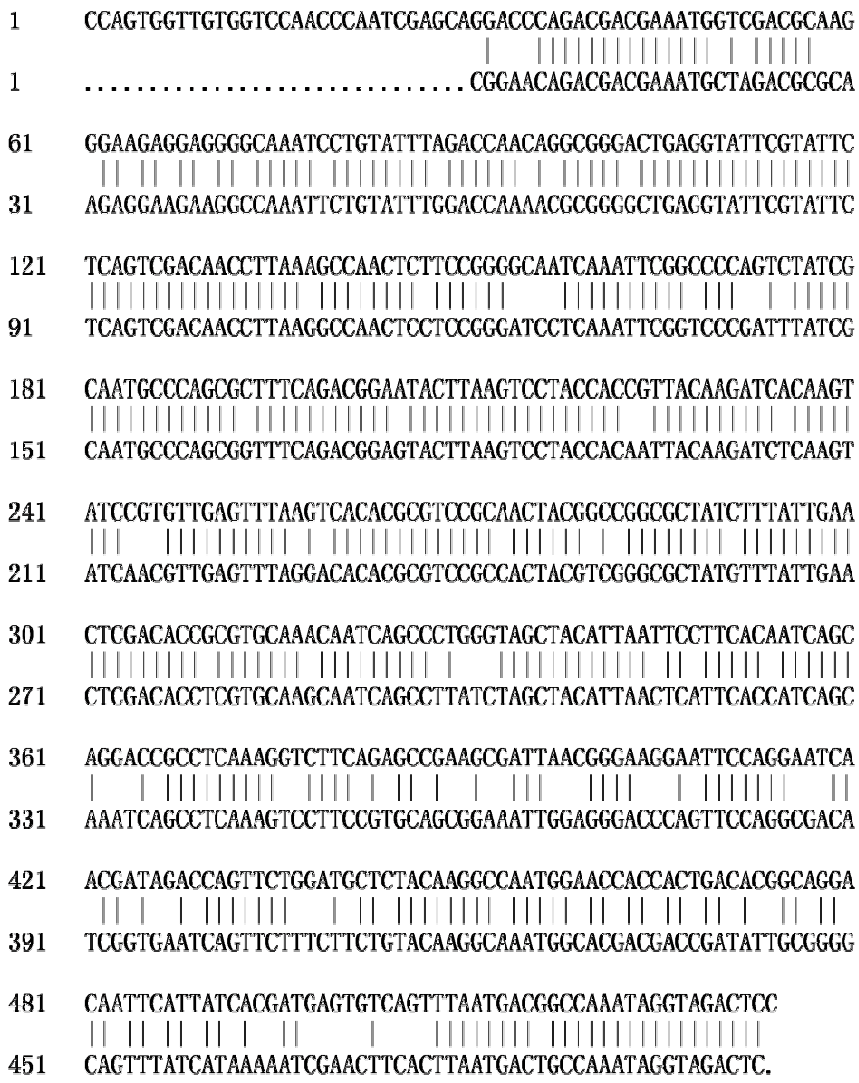

Figure 4. Alignment of BYDV-PAV-Belgium and BYDV-PAV-YL-CN
Upper line: BYDV-PAV-Belgium, lower line: BYDV-PAV-YL-CN
Identity=77.93%(392/503) Gap=5.81%(31/534)

Table 1. Comparison of transmission efficiency of BYDV-PAV between *Sitobion avenae* populations

Locality	Code	Transmission rates of BYDV-PAV-BE (%)				Transmission rates of BYDV-PAV-CN (%)			
		Repeat 1	Repeat 2	Repeat 3	Mean ± SE[a]	Repeat 1	Repeat 2	Repeat 3	Mean ± SE[b]
Huang-Huai winter (autumn sowing) wheat area and Yangtze River winter (autumn sowing) wheat area									
Anhui Hefei	AH	40.00 (10[c])	33.33 (10)	40.00 (10)	37.78±2.22 CDE	34.19 (10)	32.96 (10)	30.83 (10)	32.66±1.70 C
Henan Dengzhou	HDE	26.67 (8)	26.67 (8)	20.00 (6)	24.42±2.21 E	22.89 (10)	25.11 (10)	22.65 (9)	23.55±1.36D
Henan Luoyang	HL	40.00 (10)	46.67 (10)	40.00 (10)	42.23±2.23 BCDE	31.61 (10)	29.32 (10)	37.76 (10)	32.90±4.36 C
Hubei Danjiangkou	HD	33.33 (10)	26.67 (8)	40.00 (10)	33.32±3.85 DE	27.59 (10)	29.36 (10)	30.79 (10)	29.54±1.60 CD
Hubei Zaoyang	HZ	40.00 (10)	33.33 (10)	26.67 (8)	33.33±3.85 DE	30.16 (10)	31.08 (10)	34.23 (10)	31.82±2.13 CD
Jiangsu Yancheng	JY	40.00 (10)	40.00 (10)	33.33 (10)	37.78±2.22 CDE	37.30 (10)	36.82 (10)	38.03 (10)	37.38±0.61 C
Jiangsu Zhenjiang	JZ	26.67 (8)	26.67 (10)	40.00 (10)	31.12±4.44 DE	29.61 (10)	31.10 (10)	33.35 (10)	31.35±1.88 CD
Shaanxi Baoji	SB	66.67 (10)	53.33 (10)	60.00 (10)	60.00±3.87 AB	30.61 (8)	28.31 (10)	36.91 (10)	31.94±4.45 CD*
Shandong Taian	ST	46.67 (10)	53.33 (10)	46.67 (10)	48.89±2.21 ABCD	46.46 (10)	49.65 (10)	42.72 (10)	49.61±3.13 B

Shanxi Taiyuan	STA	73.33 (10)	66.67 (10)	60.00 (10)	66.67±3.84 A	50.36 (10)	60.45 (10)	57.74 (10)	56.18±5.22 A
Xinjiang winter-spring wheat area									
Xinjiang Shihezi	XS	53.33 (10)	46.67 (10)	33.33 (10)	44.43±5.87 BCD	29.39 (10)	30.83 (10)	36.44 (10)	32.22±3.72 C
Qinghai-Tibet spring-winter wheat area and Southwest winter (autumn sowing) wheat area									
Sichuan Jiangyou	SJ	46.67 (10)	40.00 (10)	40.00 (10)	42.23±2.23 BCDE	36.77 (10)	36.81 (10)	36.64 (10)	36.74±0.09 C
Qinghai Xining	QX	66.67 (10)	53.33 (10)	46.67 (10)	55.56±5.89 ABC	38.13 (10)	34.12 (10)	37.90 (10)	36.72±2.25 C*
Yunnan Honghe	YH	26.67 (10)	40.00 (10)	26.67 (8)	31.12±4.44 DE	30.65 (10)	28.83 (10)	27.73 (10)	29.07±1.47CD

Horizontal: Two-sample *t*-test; *Significantly different transmission efficiency between the two BYDV-PAV isolate are indicated by "*" (n = 3, $P < 0.05$).

Vertical: One-way ANOVA, Tukey's studentized range test (HSD); [a] Means within columns followed by the same letter are not significantly different (*df* = 13, MS= 42.326, F = 10.36, $P < 0.0001$); [b] Means within columns followed by the same letter are not significantly different (*df* = 13, MS= 8.219, F = 23.5, $P < 0.0001$)

[c] No. of viruliferous aphids

Chapter VI: *Endosymbiotic bacteria in Sitobion avenae and its effect on transmission efficiency of BYDV*

General Introduction to Chapter VI

BYDVs are persistently transmitted by aphids to all common small grain cereals and are not transmissible through seed, soil or sap. It displays a high degree of vector specificity among different aphid species living on *Poaceae*, and each virus is only transmitted by one or a few aphid species. It is well documented that the various aphid species differ in their abilities to transmit the various variants of BYDV, a virus isolate can be transmitted with different efficiency by different clones of aphid species, and an aphid clone can transmit different virus isolates with different efficiencies. At present, the understanding of intra-specific variation of BYDVs transmission has caught particular attention. Little is known about the reason of the diversity in transmission ability. In aphid, the virus were ingested from the host plant into the lumen of aphid's alimentary canal, and cross from hindgut into hemocoel, retention in the tissues and hemocoel, then transmission through salivary gland and into phloem. Thus, it is likely that several barriers and therefore several genetic loci are responsible for vector competence. In some cases, gut membrane seems to regulate transmission efficiency. Some reports have demonstrated that symbionin, a GroEL homologous protein synthesized

by endosymbiotic bacteria and secreted into the aphid hemolymph, is essential for efficient *Luteovirus* transmission and symbionin from different aphid species bound *in vitro* with different affinities to *Luteoviridae*.

As this reason, we tried to find the effect of endosymbionts on transmission efficiency of BYDV by proteomic (gel electrophoresis coupled with mass spectrometry, chromatography purification techniques) and genomic (Polymerase Chain reaction based techniques) approaches.

Endosymbiotic bacteria in *Sitobion avenae* and its effect on transmission efficiency of BYDV

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Abstract : Aphids (Hemiptera: Aphididae) are the biological models for studies of insect-plant interactions, symbiosis and virus vectoring. Almost all aphids closely associated with bacterial endosymbionts, and aphids as an important vector of barley yellow dwarf virus, so understanding the relationship of endosymbiont and transmission efficiency of BYDV become necessary. The S-symbionts in *Sitobion avenae* local clones (21 clones) from China were detected by a specific PCR; it's the first extensive and systematic survey of multiple S-symbionts in natural *S. avenae* clones. Primary endosymbiont (*Buchnera*) and seven S-symbionts (PASS, PABS, PAUS, *Rickettsia*, *Spiroplasma*, *Wolbachia* and *Arsenophonus*) universally found in different local clone with different infection frequencies. Feeding aphid nymphs on an antibiotic-containing artificial diet prior to BYDV acquisition, endosymbiont were selectively eliminated, *Buchnera* was reduced by rifampicin-treatment, *Rickettsia* was eliminated by ampicillin-treatment and rifampicin-treatment, separately; and the transmission efficiencies of BYDV were all inhibited. From these result we concluded that endosymbiont bacteria play a role in transmitting BYDV, *Buchnere* and *Rickettsia* do indeed play a crucial role in BYDV transmission. The analysis on aphid virus transmission efficiencies according to endosymbiont should be promoted to understanding the virus migration path in aphid and further propose of new potential tools to control virus transmission.

Key Words: *Sitobion avenae*, transmission efficiency, barley yellow dwarf virus, endosymbiont

Introduction

In China, wheat (*Triticum aestivum*) is the most widely grown food crop that is apt to be injected by barley yellow dwarf virus (BYDV). BYDV is an important pathogen that belongs to the genus *Luteovirus* (family *Luteoviridae*), affects nearly all members of the *Gramineae*, leading to severe crop yield losses worldwide (D'Arcy and Burnett, 1995). BYDV is strictly transmitted by aphid species to all common small grain cereals in a persistent and circulative manner. To obtain maximum transmission rate, a feeding periods of 48 hours or more for acquisition and infection are required. Once acquired, the virus is retained for a relative long time, often the rest of the vector's life duration. Rochow (1969) and others distinguished five different strains of the BYDV by their primary aphid vector; each BYDV strain only transmitted efficiently by corresponding aphid species. It is well documented that the various aphid species differ in their abilities to transmit the various variants of BYDV (Bencharki, *et al.*, 2000), a virus isolate can be transmitted with different efficiency by different clones of aphid species, and an aphid clone can transmit different virus isolates with different efficiencies. At present, the understanding of intra-specific variation of BYDVs transmission has become a particular focus. The circulative route of virus movement through the aphid body has been partially characterized (Gray

and Gildow, 2003; Yang, *et al*., 2008). Ultrastructural studies indicate that all *luteovirids* follow a similar pathway through their aphid vectors. Subsequently, one protein was identified as symbionin, an *Escherichia coli* GroEL homologue produced by the aphid endosymbiont *Buchnera aphidicola*, which was capable of binding potato leafroll virus from *Myzus persicae* (van den Heuvel, *et al.*, 1994). Symbionin may protect the virus from recognition by the aphid immune system (Filichkin, *et al.*, 1997; van den Heuvel, *et al*., 1999). Symbionins are molecular chaperonins produced by intracellular endosymbiotic bacteria and are the most abundant proteins found in aphid's hemocoel (Ishikawa, 1984; Ishikawa and Yamaji, 1985). Aphids treated with antibiotics, presumably killing or inhibiting endosymbionts, which were unable or less able to transmit some *luteoviruses* (van den Heuvel, *et al.*, 1994).

Almost all aphids (Hemiptera: Aphididae) closely associated with bacterial endosymbionts, specifically with *Buchnera aphidicola* (Gamma proteobacteria), a primary, obligatory species which resides in the cytoplasm of mycetocytes, hypertrophied cells in the abdomen. These bacteria are passed from mother to eggs during oogenesis in sexual forms and directly to developing embryos during embryogenesis of asexual morphs (Miure, et al., 2003). They synthesize essential amino acids and other nutrients for their host aphids that are deficient in their plant sap diet (Douglas, 1998); and deprivation of *Buchnera* by antibiotic treatment

results in retarded growth, sterility and/or death of the host aphids (Houk and Griffiths, 1980). A number of aphids harbours several inherited secondary or facultative symbionts (S-Symbiont) in addition to *Buchnera aphidicola* (Buchner, 1965), they are facultative and can undergo low levels of vertically transmitted by which they can colonize new hosts. Some S-symbionts have been reported, such as *Serratia symbiotica*, *Hamiltonella defensa*, *Regiella insecticola*, *Wolbachia sp.*, *Rickettsia sp.*, *Spiroplasma sp.*, *Arsenophonus sp.* (Chen, *et al.*, 2000; Darby, *et al.*, 2001; Fukatsu, *et al.*, 2001; Sandström, *et al.*, 2001; Haynes, *et al.*, 2003; Russell, *et al.*, 2003; Moran, *et al.*, 2005; Guay, *et al.*, 2009). These S-symbionts show remarkable differences in morphology, quantity, and localizations between lineages, and are thought to be of polyphyletic evolutionary origins, which are not strictly required for host survival, but can provide a selective advantage in certain aspect. These symbionts may be involved in aphid defense against heat stress, fungal pathogens and parasitoid wasps (Augustinos, *et al.*, 2011). Whether their effects are beneficial, detrimental or nearly neutral, many of these symbionts substantially affect the physiology, ecology, reproduction and behaviors of their hosts in a variety of way; for example, *Arsenophonus* can increase host survival or reproductive output (Dale and Moran, 2006; Oliver, *et al*., 2010).

Given that *S. avenae* (Fabricus) is one of the most common and destructive wheat pests and is a primary vector for BYDV-PAV; a large collection of *S. avenae* aphid clones throughout China were attained and tested for BYDV-PAV transmission in two different geographic isolate, one from China and one from Europe (Belgium). In this study, the possible role of endosymbiont in BYDV transmission efficiency was examined, we conducted special PCR to detect endosymbiont bacteria in *S. avenae*, and utilised antibiotic-based to identify their effects on transmission efficiencies of BYDV-PAV.

1 Materials

1.1 Collection and rearing of *S. avenae*

Samples of *S. avenae* used in this study were collected from fields of winter wheat (Triticum aestivum L.) at 21 localities covering a wide area of China including Huang-Huai winter (autumn sowing) wheat area, Yangtze River winter (autumn sowing) wheat area, Xinjiang winter-spring wheat area, Qinghai-Tibet spring-winter wheat area and Southwest winter (autumn sowing) wheat area in 2009 (Figure 1). To reduce the risk of collecting the same genotype multiple times, individual aphids were collected from plants growing at least 10 m apart. All populations were separately maintained on seedlings of a susceptible aphid wheat cultivar (cv. Toison d'or). All aphids were reared under conditions that

minimized the risk of contamination between populations, i.e. the aphid isolates were transferred to pots of wheat seedlings at second stage, and each pot was separated with a transparent plastic ventilated cylindrical cage (size: 10 cm×30 cm) covered with gauze on the top (12 cm height and 24 cm in diameter). Aphids and plants were maintained in greenhouse compartment at 22°C±1, 60-70 % relative humidity (RH) and photoperiod, 16/8 hr.

1.2 Virus strains

The BYDV strains were obtained from Belgium, Louvain-la-Neuve (BYDV-PAV-BE) in 2009 and China, Yangling - Shaanxi province (BYDV-PAV-CN) in 2011. They were maintained separately on wheat seedlings cv. Toison d'or infested with *S. avenae* in a greenhouse compartment at 20 ± 1°C, with a 16hour light photoperiod.

2 Methods

2.1 DNA extraction and Specific PCR detection

Total DNA was isolated from S.avenae individuals (21 clones), using the DNeasy Tissue Kit (QIAGEN) according to manufacturer's instructions.

For detection of respective endosymbiotic bacteria, diagnostic PCR analysis was conducted using the specific primer according to Tsuchida et

al. (2002). PCR reactions were conducted using 10× Taq Buffer 5μL, Mg2+ 4μL, dNTP 1μL, Forward Primer (10mM) 2μL, Reverse Primer (10mM) 2μL, Taq Polymerase 0.5μL, DNA 1μL, ddH2O 34.5μL. The cycling conditions were as follows: 95℃ for 4 min, 40 cycles of 95℃ for 30 s, 55℃ for 30 s and 72℃ for 30 s; final extension at 72℃ for 5 min. The amplified product was checked by 2% agarose gel.

2.2 Virus transmission efficiency assays

To eliminate *Buchnera* or S-symbiotic selectively, the 2nd instars nymphs *S. avenae* (24 h old) were treated with ampicillin or rifampicin, respectively, using an artificial diet technique. The *S. avenae* (STY and HDZ) were fed on artificial diet including 50μg ml -1 rifampicin/ampicillin (Sigma) in a 15% sucrose-containing solution through paraffin membrane (two layers of parafilm enclosing 200 μl of diet) for 48 hr (named Rifampicin-diet/Ampicillin-diet), transfer the nymphs to the new artificial diet (BYDV infected tissue ground in a 15% sucrose-containing solution) for 48 hour acquisition access period. Aphids were transferred to seven day old healthy wheat plants (one aphid was transferred onto each test plant) and covered with a plastic jacket. Fifty plants were used for each condition. After 5 days infection access period, aphids were killed. Wheat plants were kept in greenhouse for 15

days before observation. The artificial diet absent antibiotic was negative control (named Rifampicin-free/Ampicillin-free).

Detection of BYDV-PAV virus in leaves of infected plants was performed by standard double antibody sandwich (DAS)-ELISA according to provider instructions (Dr S. Winter, DSMZ, Braunschweig, Germany). Plants with similar optical densities (OD) were used as virus inoculums for transmission experiments. Samples were considered positive when OD values were greater than three times the mean of the results from uninfected control leaves. The inhibition rate = (treatment-control)/control ×100%.

2.3 Data analyses

Results are expressed as means ± MSE. Analysis of variance (ANOVAs) was performed using the GLM procedure in the SAS system (SAS Institute Inc. 2001). The normalized data was deal with Student t-test at P = 0.05.

3 Results

3.1 S-symbionts in *Sitobion avenae* of different Chinese clones

To investigate the secondary endosymbiotic of *S. avenae* in Chinese clones, 21 aphid clones originating from main wheat produce region were subjected to specific PCR detection. As expected, the essential

intracellular symbiotic bacterium *Buchnera* was detected in all the clones examined, whereas others were not detected at all. PASS was just found in Huang-Huai winter wheat area (including HLY clone and HDJK clone). PAUS, PABS and *Rickettsia* were not detected in Xinjiang winter-spring wheat area, but were found in Huang-Huai winter wheat area, Yangtze River winter wheat area, Qinghai-Tibet spring-winter wheat area and southwest winter wheat area. *Spiroplasma* was found in many localities except HDZ clone, HDJK clone, HZY clone, JYC clone, QXN clone and YYX clone. *Wolbachia* were just detected in Southwestern wheat winter wheat area (SJY clone). *Arsenophonus* were discovered in Huang-Huai winter wheat area, Yangtze River winter wheat area (Table 1). PASS, PAUS, PABS, *Rickettsia*, *Sprioplasma*, *Wolbachia* and *Arsenophonus* showed infection frequencies of 9.52% (2/21), 57.14% (12/21), 42.86% (9/21), 66.67% (14/21), 71.43% (15/21), 4.71% (1/21) and 33.33% (7/21), respectively.

The composition and frequency of the S-symbionts were often different among closely located geographical population. Four individual *S. avenae* clones from Henan province were compared in the S-symbiont infection (Fig. 2). HXX clone and HZK clone harbored the same endosymbionts; they harbored *Buchnera* and five S-symbionts (PAUS, PABS, *Rickettsia*, *Spiroplasma* and *Arsenophonus*). Whereas the endosymbionts infected with HDZ clone and HLY clone were different

from the forward two clones. HDZ clone only harbored *Buchnera* and *Rickettsia*; HLY clone harbored *Buchnera* and five S-symbionts (PASS, PABS, *Rickettsia*, *Spiroplasma* and *Arsenophonus*).

3.2 Transmission efficiency of BYDV-PAV by high/poor vector feed with/without antibiotic

The most efficient vector (STY clone) and the least one (HDZ clone) of BYDV-PAV transmission efficiency were chosen to treat with antibiotic (Data not show), then, the endosymbiont and transmission efficiency of BYDV-PAV were detected.

From the result of specific PCR detection, it was only found *Buchnera* in HDZ clone, and *Rickettsia* was successfully removed via Ampicillin-diet/Rifampicin-diet. In STA clone, *Buchnera*, PABS, *Spiroplasma1* and *Arsenophonus* were retained, *Rickettsia* and *Spiroplasma2* were successfully eliminated via Ampicillin-diet; whereas *Buchnera* and PABS were retained, *Rickettsia*, *Spiroplasma* and *Arsenophonus* were successfully elininated via Rifampicin-diet (Table 2).

Rifampicin could selectively remove the obligate symbiont *Buchnera* from the host insect (Koga, *et al*., 2003), but the result of PCR showed Rifampicin-treatment aphids still harbored it. So the yield and purity of the DNA samples were estimated by respectively measuring OD260 and OD260/280, and diluted to the concentration of 500 ng/μL, then detected the *Buchnera* by PCR (Fig. 3). The band of Rifampicin-

treatment was lighter than Rifampicin-free-treatment in the STY clone, the some condition occurred on HDZ clone.

The transmission efficiencies of BYDV-PAV were all inhibited when *S. avenae* treated with antibiotics. When *S. avenae* infected with BYDV-PAV-CN isolate, the inhibition rate of STY clone treated with ampicillin was -44.2% and -25.01% with rifampicin, *t*-test revealed a significant effect between the two treatments (df=82; t=7.935; p=0.0001). The inhibition rate of HDZ clone treated with rifampicin (-23.88%) more than aphids treated with ampicillin (-14.19%), significant difference between the two treatments (df=59; t=4.370; p=0.0001); and only twenty wheat plants were positive. The transmission rate of STY clone treated with ampicillin was inhibited more than other treatments. Whereas aphids infected with BYDV-PAV-BE isolate, the inhibition rates of HDZ clone were very low, aphids treated with ampicillin was -3.45% and -3.896% with rifampicin, no difference between the two treatments (df=92; t=-0.199; p=0.8425). The inhibition rate of STY clone treated with ampicillin (-25.84%) and rifampicin (-21.44%)I was not significantly different (df=98; t=1.786; p=0.0772).

A significant effect of the STY clone and HDZ clone on inhibition rate was observed (t=-17.858; df=76; p=0.0001) when aphids treated with ampicillin and infected with BYDV-PAV-CN isolate; whereas the inhibition rates of STY clone and HDZ clone treated with rifampicin and

infected with BYDV-PAV-CN isolate were not significantly different (t=-0.349; df=65; p=0.7282). The results indicated that the inhibition rates of the STY clone which treated with ampicillin and infected with BYDV-PAV-BE isolate was significantly correlated with HDZ clone (t=10.183; df=93; p=0.0001); in addition, significantly different were observed for the inhibition rate of STY clone and HDZ clone treated with rifampicin and infected with BYDV-PAV- BE isolate (t=7.189; df=97; p=0.0001).

4 Discussions

Five facultative S-symbionts (PASS, PAUS, PABS, *Rickettsia* and *Spiroplasma*) have been characterized from the pea aphid *Acyrthosiphon pisum*, in addition to the essential symbiont *Buchnera* in Japan and provided a full picture of the inter- and intra-cloneal endosymbiotic diversity in a particular region (Tsuchida, *et al*., 2002). However, no studies have reported that the distribution of S-symbionts was characterized from *S. avenae* in China. This study is the first extensive and systematic survey of multiple S-symbionts in natural *S. avenae* clones. We found that the *S. avenae* harbored the S-symbiont at different frequencies. A great amount of *S. avenae* clones (14 clones) harbored *Buchnera* and at least two S-symbionts, a small part of *S. avenae* clones just harbored *Buchnera* and one S-symbiont, including HDZ clone

(*Rickettsia*), JZJ clone (*Spiroplasma*), STA clone (*Spiroplasma*), XSHZ clone (*Spiroplasma*), QXN clone (PAUS), YHH clone (*Spiroplasma*) and YYX clone (PABS). These results demonstrated that the S-symbionts infections are prevailing in Chinese *S. avenae* clones, and exhibit different infection frequency. It is interesting that *Wolbachia* was not detected from *S. avenae* except SJY clone, because it exhibits high infection frequencies in *S. miscanthi* from China (WANG, *et al*., 2009). But our result was similar to the previous report of (Augustinos, *et al*., 2011); out of 425 samples from Spain, Portugal, Greece, Israel and Iran, only 37 were found to be infected with *Wolbachia*. The reason may be due to low titer, genetic variability and lack of optimized identification and classification tools. It is surprising that we found *Arsenophonus* in *S. avenae* and it had a high frequency in China, because it was not been reported from *S. avenae* in any geographical regions. The diversity of infection with endosymbiont bacteria in China suggests that symbiont microbiota in *S. avenae* clones may be significantly different between distant geographical regions.

Multiple endosymbionts commonly coexist in the same aphid, which play some role in host fitness, reproduction, parasitoid resistance and the others are unknown (Oliver, *et al*., 2010; Łukasik, *et al*., 2013; Łukasik, *et al*., 2013). In order to obtain an understanding of the biological roles of the individual symbionts in such complex systems, it’s important to

selectively remove one or some specific symbiont from the host. Recently, novel antibiotic-based selective elimination techniques were devised in *A. pisum*: moderate rifampicin treatment selectively eliminated the obligate symbiont *Buchnera* from the aphids (Koga, *et al*., 2003), and ampicillin treatment selectively eliminated the facultative symbiont *Regiella* and *Serratia* (Leonardo, 2004; Leonardo and Mondor, 2006). In this study, antibiotic treatment was used to selectively eliminate the symbiont from naturally infected lineages. However, *Buchnera* was found in all treatments; we speculated that rifampicin-treatment might reduce symbiont density without fully removing the symbiont. The result of PCR demonstrated the speculation was right, the band of antibiotic-treatment was lighter than free-treatment. Because *Buchnera* is always intracellular (Koga, *et al*., 2012) and we applied artificial diet with a low concentration of antibiotic to feed aphids, the rifampicin can't completely remove all the *Buchnera*. Whether *S. avenae* fed with ampicillin-diet or rifampicin-diet, *Rickettsia* all was eliminated. Sakurai (2005) identified the localization of the *Rickettsia* symbiont in secondary mycetocytes and sheath cells, which was quite similar to the localization of other secondary symbionts in the *γ-proteobacteria* (PASS, PAUS, and PABS) of *A. pisum* (Tsuchida, *et al*., 2005). These S-symbionts might share some common molecular and cellular mechanisms for their infection and maintenance in the aphid (Sakurai, *et al*., 2005). So ampicillin can

remove *Rickettsia* successfully. However, we found PABS in the STY clone of ampicillin-treatment; we suspected that antibiotic treatment might reduce symbiont density without fully removing the symbiont. Because PABS were localized in secondary mycetocytes and sheath cells and also in hemolymph (Fukatsu, *et al*., 2000; Sandström, *et al*., 2001; Koga, *et al*., 2003; Tsuchida, *et al*., 2005); we applied a low concentration of artificial diet to feed aphids, most of the antibiotic transmitted in the digestive system and can't reach the haemolymph. *Arsenophonus* and *Spiroplasma* were successfully eliminated in rifampicin-treatment; they were remained in ampicillin-treatment. The result similar to previous research on *Bemisia tabaci*, the inactivation rate of *Arsenophonus* was higher with rifampicin than ampicillin (Ahmed, *et al*., 2010). This might be partly explained by the different mechanisms of the antibiotics. Rifampicin inhibits DNA-dependent RNA polymerase in bacterial cells by binding to the beta subunit, thus preventing transcription of messenger RNA (mRNA) and subsequent translation to proteins, while ampicillin inhibits bacterial cell-wall synthesis in S-symbionts (Campbell, *et al*., 2001).

The destruction of the endosymbionts by antibiotic is likely to have effects on the metabolism and physiology of the aphids (Wilkinson ,1998), and these changes may directly or indirectly be responsible for the effects on BYDV survival and transmission. Feeding *S. avenae* on an

antibiotic-containing artificial diet prior to BYDV acquisition reduced virus transmission. As expected, the result showed the virus transmission was inhibited by antibiotic-treatment. Endosymbiont presumably was killed or inhibited, which were unable or less able to transmit BYDV. The result similar to previous research of van den Heuvel *et al*., (1994), *M. persicae* treated with tetracycline, PLRV transmission by the antibiotic-treatment aphids was reduced by more than 70%. In HDZ clone with BYDV-CN isolate, density of *Buchnera* was reduced by rifampicin, but remained by ampicillin, the inhibition of rifampicin-treatment was more than ampicillin-treatment. *Buchnera* produce copious amounts of a protein named symbionin, a homologue of the GroEL protein of *Escherichia coli* (Baumann, *et al*., 1995; Filichkin, *et al*., 1997). GroEL (also termed symbionin), a protein is essential for *Luteovirus* transmission (Baumann, *et al*., 1996). As the virus particles transit the haemolymph from gut to salivary gland, they bound to GroEL, which protects BYDV from aphid immune attack in the aphid haemolymph (Filichkin, *et al*., 1997). Virus binding to *Buchnera* GroEL is a phenomenon common to all plant viruses transmitted by aphids in a circulative nonreplicative manner (Gildow, 1987). Native *Buchnera* GroEL, that consists of 14 identical subunits of 60 kDa arranged in two stacked heptameric rings (Braig, *et al*., 1994; Filichkin, *et al*., 1997; van den Heuvel, *et al*., 1997; Hogenhout, *et al*., 1998). Both the subunits and the native 14-meric

protein have been shown to bind *Luteovirus*es in different ligand assays (van den Heuvel, *et al*., 1994; Filichkin, *et al*., 1997; van den Heuvel, *et al*., 1997). It has demonstrated binding in vitro of BYDV to the GroEL protein at a single epitope that has also been identified as a PLRV-binding site (Hogenhout, *et al*., 2000; Bouvaine, *et al*., 2011). The readthrough domain (RTD) of a *Luteovirus*, determines the interaction with *Buchnera* GroEL, which exposed on the surface of a *Luteovirus* particle and contains determinants necessary for virus transmission by aphids (Jolly and Mayo, 1994; Brault, *et al*., 1995; Chay, *et al*., 1996; Bruyère, *et al*., 1997; Filichkin, Brumfield *et al*. 1997). Moreover, treatment of *M. persicae* larvae with antibiotics that significantly reduced *Buchnera* GroEL levels in the haemolymph, inhibitsed transmission efficiency and results in the loss of capsid integrity in the haemolymph (van den Heuvel, *et al*., 1994).These observations indicate that the *Luteovirus*–GroEL interaction is essential for virus retention in the haemolymph of the aphid (van den Heuvel, *et al*., 1997).It is speculated that if aphids harbored more GroEL, GroEL would protect more virus from degradation, and then the transmission efficiency of BYDV will become higher. GroEL may function as a chaperonin to preserve or change the structure of the capsid and facilitate virus movement into the accessory salivary gland. When aphids were treatment with antibiotic, which interfere with prokaryotic protein synthesis, the virus was poorly

transmitted by these antibiotic-treated aphids (van den Heuvel, *et al*., 1994; Hogenhout, *et al*., 1996). The transmission efficiency of BYDV was also inhibited in ampicillin-treatment; these results suggested that *Rickettsia*, the only symbint absent from the sample, may be an important factor in facilitating BYDV transmission. Sakurai (2005) investigated the *Rickettsia* symbiont was specifically localized in secondary mycetocytes and sheath cells, and virus-like particles were sometimes observed in association with the *Rickettsia* cells by electron microscopy. In STY clone with BYDV-CN isolate, compared to the ampicillin-treatment, the rifampicin-treatment eliminated the *Rickettsia*, *Spiroplasma* and *Arsenophonus*, reduced the density of *Buchnera*, but the inhibition of rifampicin-treatment was lower than ampicillin-treatment. The results suggested that *Spiroplasma* and *Arsenophonus* may not take part in BYDV transmission, directly.

Although the coexistence of symbiotic organisms in arthropods is well documented (Buchner, 1965; Ishikawa, *et al*., 1989), studies in which their ability to influence vector transmit virus is reported are scarce. It is expected, however, that this field of research will become increasingly important in the near future. Our research may provide insight into the relationship between endosymbiont and *Luteovirus* transmission. *Buchnera* and *Rickettsia* do indeed play a crucial role in virus transmission, the function of other S-symbiont need deeper

research. The analysis on aphid virus transmission efficiencies according to endosymbiont should be promoted to understanding the virus migration path in aphid and further propose of new potential tools to control virus transmission. Indeed, identification of molecular receptors in aphid would allow potential findings of virus competitors (such as antobiotic) leading to the non binding of virus and reduction of virla transmission.

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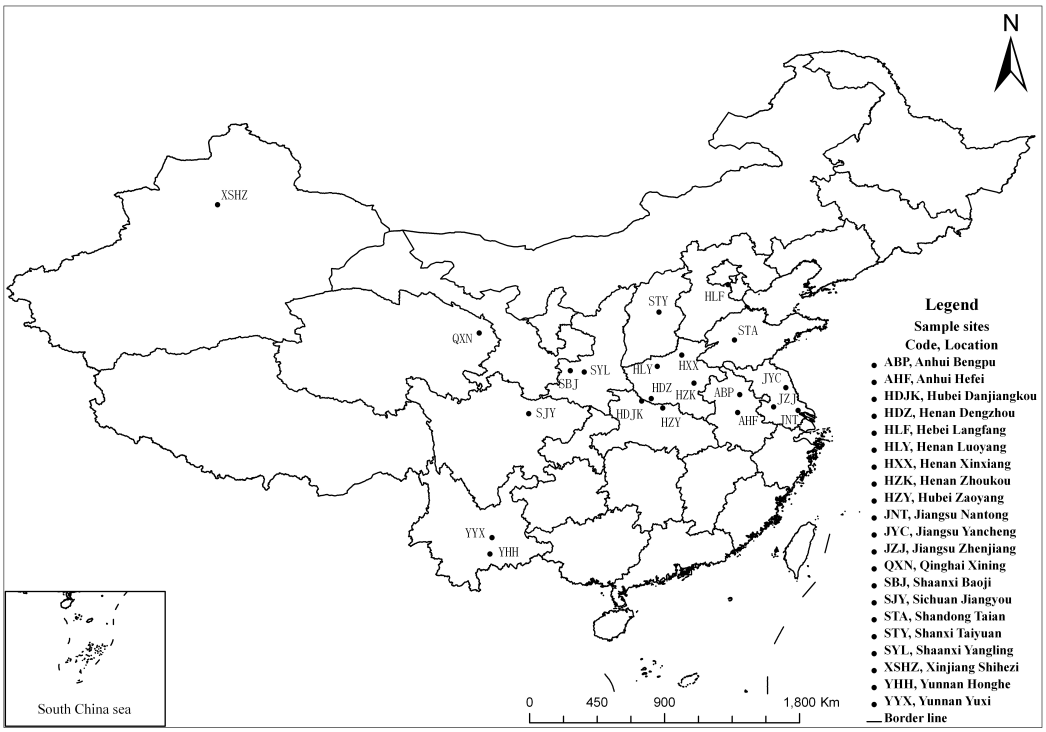

Figure 1 Locations (province & city) where *Sitobion avenae* populations have been collected in China.

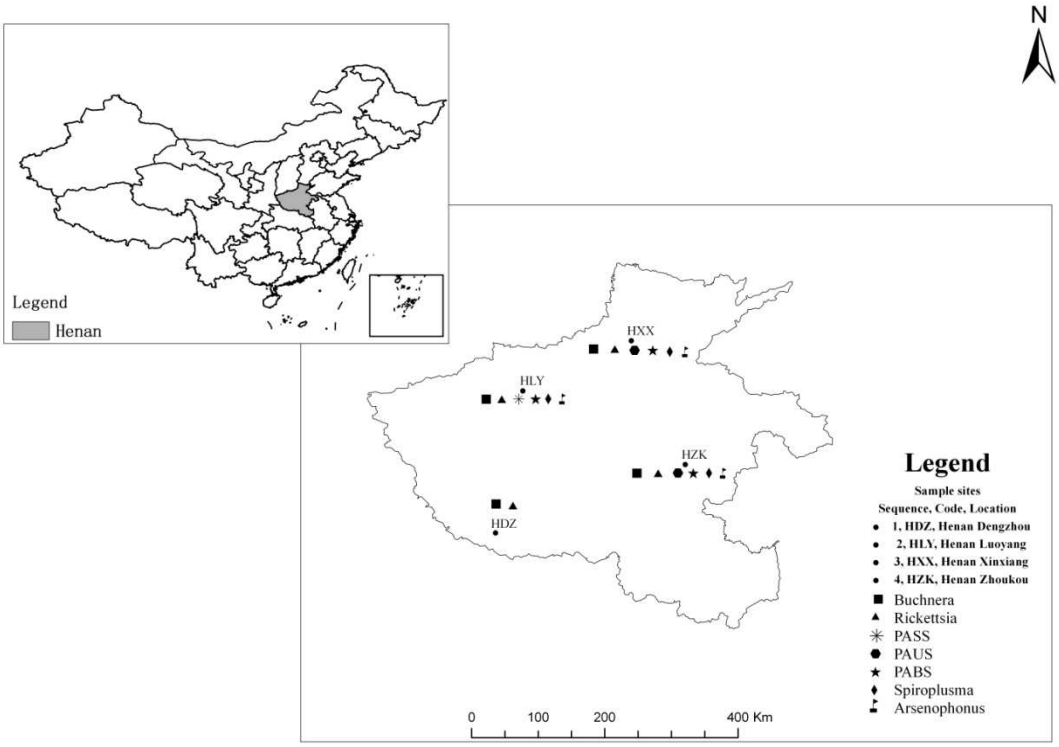

Figure 2 Diversity of endosymbionts in Henan province

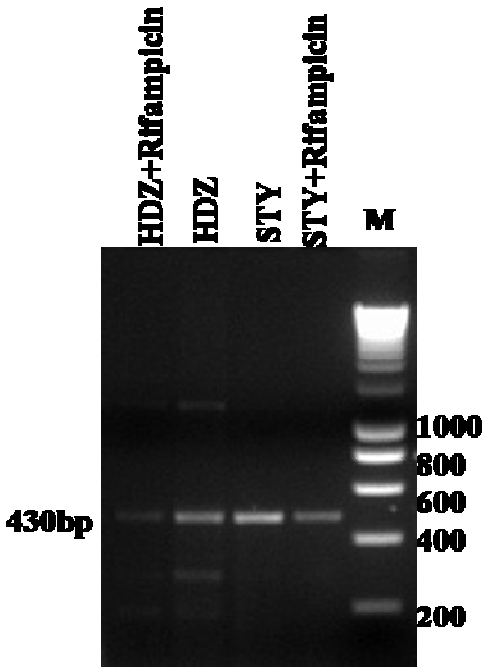

Figure 3 Diagnostic PCR analyses to confirm selective elimination of *Buchnera*

Table1. The endosymbiont detected in different Chinese local populations of osymbiont detected in different Chinese local populations of *S. avenae*

Location		Endosymbiont										
		Buchnera	PASS1	PASS2	PAUS	PABS	*Rickettsia*1	*Rickettsia*2	*Spiroplasma*1	*Spiroplasma*2	*Wolbachia*	*Arsenophonus*
Huang-Huai winter (autumn sowing) wheat area and Yangtze River winter (autumn sowing) wheat area												
ABP	Anhui Bengpu	+			+	+	+		+			+
AHF	Anhui Hefei	+			+				+			
HLF	Hebei Langfang	+			+	+	+		+			+
HDZ	Henan Dengzhou	+					+					
HLY	Henan Luoyang	+	+			+	+		+			+
HXX	Henan Xinxiang	+			+	+	+		+			+
HZK	Henan Zhoukou	+			+	+	+		+			+
HDJK	Hubei Danjiangkou	+		+	+		+					
HZY	Hubei Zhaoyang	+			+		+					

JNT	Jiangsu.Nantong	+		+	+	+			+					
JYC	Jiangsu Yancheng	+		+		+								
JZJ	Jiangsu Zhenjiang	+							+					
STA	Shandong Taian	+							+					
STY	Shanxi.Taiyuan	+			+	+			+	+			+	
SBJ	Shannxi Baoji	+				+			+					
SYL	Shannxi Yangling	+		+	+	+			+				+	
Xinjiang winter-spring wheat area														
XSHZ	Xinjiang Shihezi	+							+					
Qinghai-Tibet spring-winter wheat area and Southwest winter (autumn sowing) wheat area														
QXN	Qinghai Xining	+		+										
SJY	Sichuan Jiangyou	+		+		+		+	+			+		
YHH	Yunnan Honghe	+							+					
YYX	Yunnan Yuxi	+			+									

+ indicates that strain was examined for endosymbiont, blank means no endosymbiont was detected.

Table2 The endosymbiont detected in STY local population and HDZ local population of *S. avenae* which feed with antibiotic

	Treatments					
Endosymbiont	HDZ-free	HDZ-Amp	HDZ-Rif	STY-free	STY-Amp	STY-Rif
Buchnera	+	+	+	+	+	+
PASS1						
PASS2						
PAUS					+	
PABS				+	+	+
*Rickettsia*1	+			+		
*Rickettsia*2						
*Spiroplasma*1				+	+	
*Spiroplasma*2				+		
Wolbachia						
Arsenophonus				+	+	

Table3 The inhibition rate of BYDV transmission of Shanxi Tanyuan and Henan Dengzhou from AMPICILLIN/RIFAMPICIN

Vector	Virus	Control (%)	Inhibition rate by Ampicillin (%)	Inhibition rate by Rifampicin (%)	T value and Significance of difference
Shanxi Taiyuan	BYDV-CH	36.14 (0/50[a])	-44.20±3.83 (13/50)	-25.01±14.29 (3/50)	t=7.935**; df=82; p=0.0001
Hennan Dengzhou	BYDV-CH	24.57 (0/50)	-14.19±9.55 (9/50)	-23.88±3.65 (30/50)	t=4.370**; df=59; p=0.0001
Shanxi Taiyuan	BYDV-EU	32.95 (0/50)	-25.84±10.64 (0/50)	-21.44±12.97 (0/50)	t=1.786; df=98; p=0.0772
Hennan Dengzhou	BYDV-EU	25.75 (0/50)	-3.450±10.56 (5/50)	-3.896±11.11 (1/50)	t=-0.199; df=92; p=0.8425

[a]No. of non-viruliferous aphid/no. of detected aphids

Significantly different transmission efficiency between the two antibiotics is indicated by "**" (Student's t-test, $P < 0.01$)

Chapter VII: *Effect of lectins on barley yellow dwarf virus transmission efficienty by Sitobion avenae*

General Introduction to Chapter VII

Lectins have the ability to bind carbohydrates that are widely distributed in nature and play different roles and functions in biological processes such as recognition molecules within the immune systemin animals and as storage proteins or in defence mechanisms against pest and pathogens in plant. Lectins have obvious potential as insect control agents although knowledge as to the mechanisms of lectin action is limited. Some previous studies have been conducted which have shown mannose-binding plant lectins with antimetabolic properties towards Hemiptera insect pests including aphids, planthoppers, and leafhoppers. Lectins have been suggested as one of the promising agents against insect pests and have been engineered successfully into a variety of crops including wheat, rice, tobacco, and potatoes. For example, Production of *Rhopalosiphum maidis* nymphs were significantly reduced on Galanthus nivalis agglutinin-expressing plants.

Among the insect pest orders, some Hemiptera cause serious damage to many crop plants by directly extracting the nutrients from the plants but also by acting as virus vectors. BYDVs caused substantial losses throughout the world in barley, wheat, and oats, which were transmitted by aphid species. According to the direct toxic effect of lectins on insect biological parameters but also to the potential competitive effect of

lectins towards viral particles in virus transmission by aphids, lectins represent a very promising protein to control aphid pest damages in crops.

In order to confirm the speculation, the lectin was incorporated into an artificial diet at a single concentration, and then detected the transmission efficiency of BYDV. At last, we tried to propose a new insight in virus transmission control in crop protection.

Effect of lectins on barley yellow dwarf virus transmission efficiency by *Sitobion avenae*

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Abstract: *Sitobion avenae* (Fabricus) is considered as an important vector of barley yellow dwarf virus (BYDV); BYDV has considerable economically important viruses that infect cereal crops over the world. In order to reduce the impact of BYDV, we tried to find a new way to control the BYDV transmission. Feeding aphid nymphs on an artificial diet-containing lectin prior to BYDV acquisition, the transmission efficiencies of BYDV-PAV were all inhibited. The inhibition rate of GNA-treatment was a little more than PSL-treatment in each group; and STY population has a higher inhibition than HDZ population in the same lectin-treatment. The inhibition rate of GNA-treatment reached to 46.63% in STY-BYDV-PAV-CN treatment and PSL-treatment was 46.47% in STY-BYDV-PAV-CN treatment. It is demonstrated that lectins can inhibit BYDV transmission and represent a very promising protein to control aphid pest damages in crops.

Key Words: *Sitobion avenae*; BYDV; lectin; transmission efficiency

Introduction

In recent years there has been much interest in the potential of lectins in crop protection. The term “lectin” was coined by William Boyd in 1954 from the Latin word “legere”, which means “to select” or “to bind”. Lectins and is proteins/glycoprotein, which have at least one non-catalytic domain that exhibits reversible binding to specific monosaccharide or oligosaccharides. They can bind to the carbohydrate moieties on the surface of erythrocytes and agglutinate the erythrocytes, without altering the properties of the carbohydrates (Lis and Sharon, 1986). The ubiquitous occurrence of lectins-in plants, animals, and microorganisms-has been firmly established. The number of purified lectins has increased to well over 100. Lectins are widely distributed in nature and play different roles and functions in biological processes such as recognition molecules within the immune systemin animals (Kilpatrick, 2002) and as storage proteins or in defense mechanisms against pest and pathogens in plants (Gatehouse, *et al*., 1984; Peumans and Van Damme, 1995; Rüdiger and Gabius, 2001; Down, *et al*., 2003; Lam and Ng, 2011). Lectins have been suggested as one of the promising agents against insect pests and have been engineered successfully into a variety of crops including wheat, rice, tobacco, and potatoes. This approach could be used as a part of integrated pest management strategies and caveat pest attack,

although knowledge as to the mechanisms of lectin action is limited. Lectins demonstrate anti-insect activity. They increase the mortality or delay the development of insect (Sauvion, *et al*., 2004). Production of *Rhopalosiphum maidis* nymphs was significantly reduced on *Galanthus nivalis* agglutinin (GNA)-expressing plants (Wang, *et al*., 2005). Insecticidal lectins have been shown to be bound to midgut epithelial cells in a variety of pest species (Habibi, *et al*., 2000). GNA was found bound to glycoproteins that can be found in the guts of larvae of *Adalia bipunctata*, *Chrysoperla carnea*, and *Coccinella septempunctata* (Hogervorst, *et al*., 2006).

Aphids (order Hemiptera; superfamily Aphidoidae) are pests of cultivated and wild plants. They are responsible for major crop losses in world agriculture, not only by direct effects on plant growth, but also by acting as vectors for transmission of viruses. The English grain aphid, *Sitobion avenae* (Fabricus) is considered as an important vector of barley yellow dwarf virus (BYDV), especially when it transfer from winter hosts (mostly wheat and barley) to spring hosts (mostly barley and corn) in the spring (Dedryver, *et al*., 2005); BYDV depend on *S. avenae* for transmission, among not only other parts of the same plant, but also more distant hosts. BYDV belongs to the family *Luteoviridae* and is transmitted in a circulative fashion and a persistent manner (D'Arcy and Burnett, 1995). It is phloem-limited in host plants and cannot

mechanically transmitted, with typical symptoms including yellowing of leaves and stunting of whole plants (Miller, *et al*., 1988). BYDV has considerable economically important viruses that infect cereal crops over the world, particularly in higher rainfall regions where yield losses in wheat have been reported to be as high as 40-50%. Global average yield losses due to the natural BYDVs infection can range from 11% to 33%, whereas in some areas the losses reach up to 87%. BYDV-PAV is one isolate of BYDV, which caused substantial losses throughout the world in barley (15%), wheat (17%), and oats (25%) (D'Arcy and Burnett, 1995).

According to the direct toxic effect of lectins on insect biological parameters but also to the potential competitive effect of lectins towards viral particles in virus transmission by aphids, lectins represent a very promising protein to control aphid pest damages in crops. This paper examines the effects of GNA and PSL on BYDV transmission efficiencies, when incorporated in an artificial diet, and we tried to propose a new insight in virus transmission control in crop protection.

1 Materials

1.1 Collection and rearing of *S. avenae*

Samples of *S. avenae* used in this study were collected from fields of winter wheat (Triticum aestivum L.) at Dengzhou-Henan (HDZ) and Taiyuan-Shanxi (STY) in 2009. To reduce the risk of collecting the same

genotype multiple times, individual aphids were collected from plants growing at least 10 m apart. All populations were separately maintained on seedlings of a susceptible aphid wheat cultivar (cv. Toison d'or). All aphids were reared under conditions that minimized the risk of contamination between populations, i.e. the aphid isolates were transferred to pots of wheat seedlings at second stage, and each pot was separated with a transparent plastic ventilated cylindrical cage (size: 10 cm×30 cm) covered with gauze on the top (12 cm height and 24 cm in diameter). Aphids and plants were maintained in greenhouse compartment at 22°C±1, 60-70 % relative humidity (RH) and photoperiod, 16/8 hr.

1.2 Virus strains

The BYDV strains were obtained from Belgium, Louvain-la-Neuve (BYDV-PAV-BE) in 2009 and China, Yangling - Shannxi province (BYDV-PAV-CN) in 2011. They were maintained separately on wheat seedlings cv. Toison d'or infested with *S. avenae* in a greenhouse compartment at 20 ± 1°C, with a 16 hour light photoperiod.

2 Methods

2.1 Virus transmission efficiency assays

The 2nd instars nymphs *S. avenae* (24 h old) were fed on artificial diet including 50μg ml -1 Galanthus nivalis agglutinin (GNA)/ Pisum sativum lectin (PSL) (Sigma) in a 15% sucrose-containing solution through paraffin membrane (two layers of parafilm enclosing 200 μl of diet) for 48 hr (named GNA-diet/PSL-diet), transfer the nymphs to the new artificial diet (BYDV infected tissue ground in a 15% sucrose-containing solution) for 48 hour acquisition access period. Aphids were transferred to seven day old healthy wheat plants (one aphid was transferred onto each test plant) and covered with a plastic jacket. Fifty plants were used for each condition. After 5 days infection access period, aphids were killed. Wheat plants were kept in greenhouse for 15 days before observation. The artificial diet absent antibiotic was negative control (named GNA-free/PSL-free).

Detection of BYDV-PAV virus in leaves of infected plants was performed by standard double antibody sandwich (DAS)-ELISA according to provider instructions (Dr S. Winter, DSMZ, Braunschweig, Germany). Plants with similar optical densities (OD) were used as virus inoculums for transmission experiments. Samples were considered positive when OD values were greater than three times the mean of the results from uninfected control leaves. The inhibition rate = (treatment-control)/control ×100%.

2.2 Data analyses

Results are expressed as means ± MSE. Analysis of variance (ANOVAs) was performed using the GLM procedure in the SAS system (SAS Institute Inc. 2001). Effect of the studied concentrations of phytoheamagglutinin on grain aphid performance and influence of GNA/PSL on BYDV transmission were deal with Student t-test at P = 0.05.

3 Results

3.1 Transmission efficiency of BYDV-PAV by high/poor vector feed with/without lectin

We chosen the most efficient *S. avenae* population (STY) transmitted BYDV-PAV and the least efficient vector (HDZ) to feed with lectin (date not show), and then detected the transmission efficiency of BYDV-PAV by DAS-ELISA.

The transmission efficiencies of BYDV-PAV were all inhibited when *S. avenae* fed with lectin. The inhibition rate of GNA-treatment was a little more than PSL-treatment in each group; and STY population has a higher inhibition than HDZ population in the same lectin-treatment.

In STY population, we found thirty-three BYDV infected plants in GNA-BYDV-PAV-CN treatment, the inhibition rate was 46.63%, and

twenty-nine infected plants were detected in PSL-BYDV-PAV-CN treatment, the inhibition rate was 46.47%; there is no difference in the two treatment (df=60; t=0.564; p=0.5751). When aphids fed with BYDV-PAV-EU isolate, the transmission efficiencies and the inhibition rates of lectin were lower than BYDV-PAV-CN treatments. Twenty-five infected plants were found in GNA-treatment, the inhibition rate was 32.64%; and seventeen infected plants in PSL-treatment were detected, the inhibition rate was 32.02%. There is still no difference in the two treatment (df=40; t=0.033; p=0.9735).

In HDZ population, aphids fed with BYDV-PAV-CN isolate, the inhibition rate of GNA-treatment was 21.13% and thirty-four plant was infected, thirty-eight plants was infected in PSL-treatment and the inhibition rate was 17.64%, Student t-test showed a significant effect in the two treatments (df=70; t=3.251; p=0.0018). About BYDV-PAV-EU isolate, twenty-three infected plants were found and the inhibition rate was 19.82% in GNA-treatment; sixteen infected plants were detected and the inhibition rate was 18.36% in PSL-treatment, There is still no difference in the two treatment (df=37; t=-0.984; p=0.3317).

4 Discussions

BYDV displays a high degree of vector specificity among different aphid species living on Poaceae, and each virus is only transmitted by one

or a few aphid species. It is well documented that the various aphid species differ in their abilities to transmit the various variants of BYDV, a virus isolate can be transmitted with different efficiency by different clones of aphid species, and an aphid clone can transmit different virus isolates with different efficiencies (Bencharki, et al., 2000). BYDV is phloem-limited in host plants and cannot mechanically transmit. Artificial diet approach was introduced in this study, feeding aphid nymphs on an artificial diet-containing lectin prior to BYDV acquisition. Artificial diet bioassays have shown that plant lectins, including the mannose-specific lectin from snowdrop (Galanthus nivalis agglutinin, GNA), are effective in decreasing survival, development and fecundity in aphids (Rahbé and Febvay, 1993; Sauvion, et al., 1996) and other Homopterans (Habibi, et al., 1993; Powell, et al., 1993). The mechanism of lectin toxicity in insects is not clear, but seems to involve binding to the gut surface (Eisemann, et al., 1994). So we used artificial diet-containing lectin to feed aphids is the right way. Aphids sucked lectin from artificial diet, lectin was transmitted and bonded to gut.

Galanthus nivalis agglutinin (GNA); snowdrop lectin, derived from bulbs of the snowdrop lily (G. nivalis L., Amaryllidaceae) (Van Damme, et al., 1987), which exhibiting a strict specificity for alpha-d-mannose and had a significant effect on parthenogenetic fecundity as well as on

insect development (Sauvion, et al., 1996), has received the most attention since it acts on sap-feeding insects. GNA is toxic towards a number of important insect pests; these include Homoptera such as aphids, Coleoptera such as bruchid beetles, and Delphacidae belonging to the Hemiptera (Powell, et al., 1993; Gatehouse, et al., 1995; Rahbé, et al., 1995; Sauvion, et al., 1996; Powell, et al., 1998). However, the effects of GNA ingestion vary from species to species of insect. For example, GNA incorporated in artificial diet at 0.1% w/v concentration had only marginal effects upon survival of glasshouse potato aphids (Aulacorthum solani), although it significantly decreased both the development and fecundity (Down, et al., 1996). But it had a significant effect upon both development and survival of rice brown planthopper at the same concentration in artificial diet, with 90% corrected mortality observed over 5 days (Powell, Gatehouse et al. 1993). It is worth to concern that the effects of GNA ingestion vary from in different local population of *S. avenae*. The inhibition rate of STY-GNA-BYDV-CN treatment and HDZ-GNA-BYDV-CN treatment was -46.63% and -21.13%; STY-GNA-BYDV-EU treatment and HDZ-GNA-BYDV-EU treatment was -32.64% and -19.82%. The reason of the different effect may relate to endosymbionts. STY population harbored much S-symbiont, which play a complex role on the BYDV transmission (the date not show). There is no evidence for GNA toxicity towards mammals and higher animals

(Pusztai, et al., 1990), so this protein has the potential to be suitable for incorporation into a transgenic crop.Transgenic tobacco and potato plants expressing GNA have been produced and have been shown to decrease growth and fecundity of aphids feeding on them (Down, et al., 1996; Couty, et al., 2001). Potatoes expressing GNA at levels of 0.3–1.5% of total soluble protein (in leaves) decreased fecundity, but not survival, of both Myzus persicae and Aulacorthum solani (Down, et al., 1996). These results taken together with data from artificial diet studies suggest that GNA could inhibit the BYDV transmission, and it is incorporation into a transgenic crop, which could protect the plants against cereal aphids.

Pea (Pisum sativum) lectin (PSL) is a dimeric protein, composed of two identical monomers, and is specific for d-mannose/d-glucose (Trowbridge, 1974). It is a metalloprotein containing Mn^{2+} and Ca^{2+}, and has a single carbohydrate-binding site per monomer. After synthesis, the lectin is processed but not glycosylated, yielding a protein with a total molecular mass of about 49 kDa, consisting of two small α- and a β-chain. The crystal structure shows that the α- and β-chains are closely interdigitated in the structure of one subunit (Einspahr, et al., 1986). The interface in the “canonical dimer” is composed almost exclusively of the β-chain (Lam and Ng, 2011). The lectin is encoded by one functional gene, is very abundant in pea seeds, and is produced and secreted in small

amounts by pea roots (Dazzo, et al., 1978; Kamberger, 1979; Driessche, et al., 1981; Díaz, et al., 1990; Hoedemaeker, et al., 1994). The PSL would inhibit BYDV transmission, and have different efficiencies in the four treatments. Variability in the inhibit effects of lectin between inter-species aphids may be accounted for by differences in the mechanisms involved in lectin action, which remain to be clarified.

Some aphids cause serious damage to many crop plants by directly extracting the nutrients from the plants but also by acting as virus vectors. According to the direct toxic effect of lectins on insect biological parameters (Sauvion, Nardon et al. 2004) but also to the potential competitive effect of lectins towards viral particles in virus transmission by aphids, the lectin could be used as a part of integrated pest management strategies, and represent a very promising protein to control aphid pest damages in crops.

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Table 1 The inhibition rate of BYDV transmission of Shanxi Tanyuan and Henan Dengzhou from GNA/PSL

Vector	Virus	Control (%)	Inhibition rate by GNA (%)	Inhibition rate by PSL (%)	T value and Significance of difference
Shanxi Taiyuan	BYDV-CN	36.14 (50/50)[a]	-46.63±8.23 (33/50)	-46.47±4.97 (29/50)	t=0.564; df=60; p=0.5751
	BYDV-EU	32.95 (50/50)	-32.64± 5.29 (25/50)	-32.02± 8.83 (17/50)	t=0.033; df=40; p=0.97356
Hennan Dengzhou	BYDV-CN	24.57 (50/50)	-21.13± 4.47 (34/50)	-17.64± 10.84 (38/50)	t=3.251**; df=70; p=0.0018
	BYDV-EU	25.75 (50/50)	-19.82± 4.03 (23/50)	-18.36± 4.74 (16/50)	t=-0.984; df=37; p=0.3317

[a]No. of viruliferous aphid/no. of detected aphids

Significantly different transmission efficiency between the two antibiotics is indicated by "**" (Student's t-test, $P < 0.01$)

Chapter VIII: *GENERAL CONCLUSIONS,*

DISCUSSIONS AND PERSPECTIVE

Almost all aphids closely associated with bacterial endosymbionts, which substantially affect the physiology, ecology, reproduction and behaviors of their hosts in a variety of way. Aphids were considered as an important vector of BYDV. Clone diversity in aphids was known to be related to acceptance and suitability of host plant. Occurrence of particular patterns of bacterial endosymbionts was demonstrated to specific plant – aphid interactions. So virus partners in host-aphid interactions, the role of endosymbiont pattern on virus transmission efficiency is to be investigated.

Firstly, our results showed that *Buchnera aphidicola* and S-symbionts (PASS, PAUS, *Rickettsia*, *Spiroplasma* and *Wolbachia*) universally found in Belgium and Chinese local population with different infection frequencies. Endosymbionts were selective eliminated from Belgium population and Chinese populations by rifampicin; the mortality of Belgium/Chinese aphids showed higher significant difference with negative control and has fewer offspring than that negative control. In addition, the (E)-β-farnesene (EBF) production were reduced significantly. Protein analysis showed eighteet *Buchnera* aphidicola were detected from the 2D-gel, which take part in many metabolic pathways, such as Carbohydrate metabolism, Energy metabolism, Amino acid metabolism, Protein synthesis, Stress response, Nucleotide metabolism

and Membrance transport. They may provide energy, material and enzyme in the EBF production. From these result we conclude that endosymbiont bacteria play a role in EBF production, especially *Buchnere* do indeed play a crucial role in EBF production.

Secondly, fourteen populations of *S. avenae* originating from China were tested for their ability to transmit BYDV-PAV (one isolate from Belgium, another from China) using wheat plants. By sequence analysis, the coat protein gene of BYDV-PAV-Belgium was distinguishable from BYDV-PAV-CN. All populations could transmit BYDV-PAV and variation in transmission rates ranged from 24.42% to 66.67% with BYDV-PAV-Belgium and from 23.55% to 56.18% with BYDV-PAV-CN. It suggests *S. avenae* has no specialty in BYDV-PAV isolate. Significant differences of percentages of transmission between the populations with BYDV-PAV-Belgium and BYDV-PAV-CN were observed. Transmission efficiency of the populations from the middle-lower reaches of Yangtze River (AH, HD, HDE, HZ, JZ, JY and SJ) and Yunnan province (YH) were not differences. Nevertheless, differences in virus transmission efficiencies from the populations from the northern (ST and STA) and northwestern (QX, SB and XS) regions were determined. Transmission efficiency of *S. avenae* from northern and northwestern China regions, where BYDV impact is more important, was

higher than that from the middle-lower reaches of Yangtze River and Yunnan province. Investigations on the virus-aphid interactions should be performed to better understand the different steps of virus acquisition, transport and availability in the aphid vectors by integrating high and low efficient transmitting clones and comparing them.

Thirdly, the S-symbiont in Chinese local populations (21 clones) of *S. avenae* were detected, it' the first extensive and systematic survey of multiple S-symbionts in natural populations of *S. avenae*. One primary endosymbiont (*Buchnera*) and seven S-symbionts (PASS, PABS, PAUS, *Rickettsia*, *Spiroplasma*, *Wolbachia* and *Arsenophonus*) universally found in different local population with different infection frequencies. Feeding aphid nymphs on an antibiotic-containing artificial diet prior to BYDV acquisition, endosumbiont were selectively eliminated, *Buchnera* was reduced by rifampicin-treatment, *Rickettsia* was eliminated by ampicillin-treatment and rifampicin-treatment, separately; and the transmission efficiencies of BYDV were all inhibited. These result demonstrated that endosymbiont bacteria play a role in transmitting BYDV, *Buchnere* and *Rickettsia* do indeed play a crucial role in BYDV transmission. The analysis on aphid virus transmission efficiencies according to endosymbiont should be promoted to understanding the virus migration

path in aphid and further propose of new potential tools to control virus transmission.

Finally, Feeding aphid nymphs on an antibiotic-containing lectin diet prior to BYDV acquisition, the transmission efficiencies of BYDV-PAV were all inhibited. The inhibition rate of GNA-treatment was a little more than PSL-treatment in each group; and STY population has a higher inhibition than HDZ population in the same lectin-treatment. The inhibition rate of GNA-treatment reached to 46.63% in STY-BYDV-PAV-CN treatment and PSL-treatment was 46.47% in STY-BYDV-PAV-CN treatment. It is demonstrated that lectins can inhibit BYDV transmission and represent a very promising protein to control aphid pest damages in crops.

According to study of this dissertation, these biochemical and molecular investigations would lead to a better understanding of the virus-aphid interactions and to propose new insight in virus transmission control in crop protection.

Chapter IX: *LIST OF PUBLICATIONS ORAL*

PRESENTATIONS AND POSTE

1 Publications

1. **Wenjuan Yu**, Julian Chen, Dengfa Cheng, Jingrui Sun. The method of *Cdc42* gene accurate positioning and quantitative analysis of the organization in the wheat aphids, *Sitobion avenae* [J], Chinese Bulletin of Entomology, 2010, 47(6):1095-1103.
2. VANDERMOTEN Sophie, **YU Wenjuan**, CHEN Julian, YONG Liu, HAUBRUGE Eric, FRANCIS Frédéri, Investigation of mevalonate pathway in aphids, 6th International Symposium on Molecular Insect Science.
3. **Wenjuan Yu**, Zhaohuan Xu, Julian Chen, Claude Bragard, Yong Liu, Dengfa Cheng, and Frédéric Francis, Variation in transmission of barley yellow dwarf virus-PAV by different populations of *Sitobion avenae* in China. Journal of Virological Methods. (Accepted)
4. **Wen-Juan YU**, Ju-Lian CHEN, Min CAO , Hong-ling JIANG, Deng-Fa CHENG, Ya–Zhong CAO, Xi-Wu GAO, Han-Xiang NI. Cloning and Function Verification of Wing Differentiation Related Gene *Cdc42* from Wheat Aphid. Insect Molecular Biology. (under review)

5. **Wen-Juan Yu**, Emilie Bosquée, Ju-lian Chen, YONG Liu, BRAGARD Claude, Frédéric Francis. Transmission of Barley yellow dwarf virus by Aphids: a review on virus-vector interactions. Biotechnol. Agron. Soc. Environ. (under review)
6. **Wen-Juan YU**, Ju-Lian CHEN, Frédéric Francis, Deng-Fa CHENG, Jing-Rui SUN, Yong LIU. The impact of juvenile hormone and its analogue ZR-515 on wing dimorphism and *Cdc42* gene expression in the English grain aphid, *Sitobion avenae*. Insect Science. (under review)
7. Jia Fan, Yong Zhang, **Wenjuan Yu**, Shanshan Jiang, Julian Chen. Identification of interaspecies alarm pheromone and two conserved odorant binding protein associated with EBF response in *Rhopalosiphum padi* Linnaeus. Insect Science. (Under review)
8. **Wenjuan Yu**, VANDERMOTEN Sophie, CHEN Julian, YONG Liu, BRAGARD Claude, HAUBRUGE Eric, FRANCIS Frédéric. Role of aphid endosymbionts in leading to alarm pheromone production in *Sitobion avenae*.
9. **Wenjuan Yu**, Emilie Bosquée, Julian Chen, Yong Liu, Claude Bragard, Frederic Francis. Endosymbiotic bacteria in *Sitobion*

avenae and its effect on transmission efficiency of BYDV.

10. **Wenjuan Yu**, Qing Deng, Emilie Bosquée, Sophie Vandermoten, Julian Chen, Yong Liu, Claude Bragard, Eric Haubruge, Frederic Francis. Effect of lectins on BYDV transmission efficienty by *Sitobion avenae*.

2 Poster

Wenjuan YU, Zhaohuan XU, Julian Chen, Claude BRAGARD, Frédéric FRANCIS, Yong LIU, Dengfa CHENG. Variation in transmission of barley yellow dwarf virus-PAV by different populations of *Sitobion avenae* in China. The 9th International Symposium on Aphids (9ISA 2013), Beijing, China.2-6 Jun, 2013.