

Identify resistance mechanisms against the cabbage whitefly *Aleyrodes proletella* in two *Brassica oleracea* cultivars

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Abstract

Whiteflies are phloem feeders that have a long and continuous interaction with the plant leading to specific defense mechanisms. Here we investigate resistance mechanisms against the whitefly *Aleyrodes proletella* in two *Brassica oleracea* cultivars: Rivera and Christmas Drumhead. Host plant preference and suitability showed that Rivera is resistant whereas Christmas Drumhead is susceptible in which the odor plays an important role. A first pre-induction by the whitefly itself showed no induced defense on survival and reproduction performance of whitefly for both cultivars. However, the pre-induction in Rivera led to a better performance of nymphs, whereas this was not the case in Christmas Drumhead. Transcriptional studies confirmed the occurrence of constitutive defenses but not the differences found between the two cultivars for nymphal stage. Both bioassay and transcriptional studies showed that pre-infestation by the caterpillar *Pieris rapae* had no effect on whitefly performance. This first approach narrows down coming research to understand the differences between those two cultivars.

Introduction

Whiteflies are polyphagous and can spread easily in an ecosystem and create complex interactions with other organisms at all trophic levels (Price *et al.*, 1980; Inbar and Gerling, 2008). Here we focus on the European whitefly *Aleyrodes proletella*, which has been reported recently to be a new pest in greenhouse and *Brassica* fields in Europe (Ramsey and Ellis 1996; Nebreda *et al.*, 2005).

Whiteflies use, like aphids, a stylet to take up large quantities of phloem sap, which causes damage to crops. This stylet navigates intercellularly and rarely damages epidermal or mesophyll cells prior to puncturing cells of the phloem. This presents a unique stress on plant fitness (Bryne *et al.*, 1991; Kempema *et al.*, 2007). While sucking phloem sap, the insect excretes a sugary substance that allows the growth of sooty molds, which can be a problem on Brussels sprout buttons and Kale. Whiteflies may also deplete plant reserves, reduce primary production, and cause indirect phytotoxic effects (Inbar and Gerling, 2008). When the whitefly population reaches enormous densities, it causes leaf chlorosis, leaf withering, premature dehiscence, defoliation, and plant death (Bryne *et al.*, 1991).

Whiteflies are holometabolists meaning that they metamorphose entirely during their life. The life cycle lasts about 28 days (Figure 1) (Kempema *et al.*, 2007; Heimpel *et al.*, 2008). The eggs, laid on the under part of the leaves, have a pedicel that provides a means of attachment either by insertion into a slit made by the ovipositor in the leaf surface (about 76% of species according to the literature) or into a stomata opening (24% of species according to the literature) (Bryne *et al.*, 1990; Bryne *et al.*, 1991). The total number of eggs per female is dependent on the temperature and the humidity, and varies between 48 and 390 (Bryne *et al.*, 1991; Nebreda *et al.*, 2005). The eggs do not seem to present a stress on the plant. They first hatch into crawling larvae for about three days; then legs atrophy and the larvae moult into sessile second instar larvae; this is followed by two further instars. At the fourth instar stage, called pupae, the insect stops feeding and metamorphoses into an adult, which is sexually mature and has functional wings (Ramsey and Ellis, 1996).

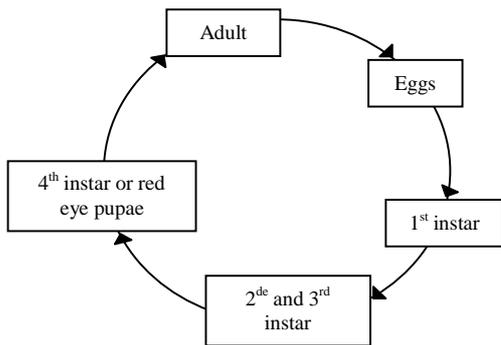


Figure 1: Life cycle of the *A. proletella*. The adults lay eggs which become nymphs (1st, 2^{de} and 3rd instar), which then become pupae that will give rise to a new adult.

Plants utilize both constitutive and induced defense systems. Constitutive defenses are always present in the plant and include physical barrier such as the leaf cuticle or cell wall (Kempema *et al.*, 2007). Induced plant responses are activated after attack by the insect. Induced defense can be direct and indirect (Inbar and Gerling, 2008). Direct includes for example accumulation of defensive compounds which will decrease feeding efficiency or reproduction success. The salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) dependent pathways are known to play an important role in the induced resistance against hemipterans (Kaloshian *et al.*, 2005). Indirect defense include for example volatiles that will attract natural enemies of the herbivore (Dicke *et al.*, 2003).

Regarding whiteflies, two types of plants resistance can occur: antixenosis, where host plant factors affect colonization by the insect (e.g. color, odor), and antibiosis, where host plant factors affect the insect after colonization (eg toxins, poor nutritional quality) (Ramsey and Ellis, 1996). From this approach, the plant defense can be divided into two layers (Figure 2):

- (1) Host plant selection that can be influenced by plant color or odor as a cue to select landing sites for feeding and oviposition. *A. proletella* was the only whitefly reported to respond to the odor of crushed cabbage leaves (Bryne *et al.*, 1991).
- (2) Host plant suitability, in which the plant prevents feeding damage, laying of eggs or development of nymphs.

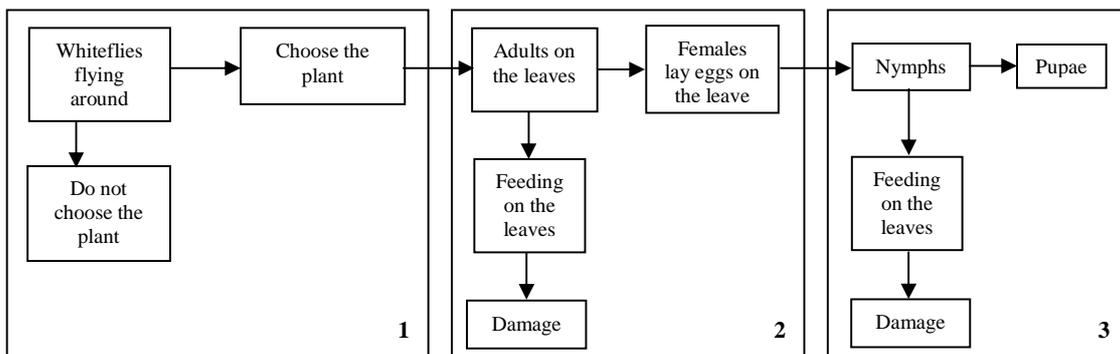


Figure 2: Overview of the interaction between plant and whiteflies. The number refers to the layer of defense of the plant: 1: Host plant selection, first layer of defense using odor or color to deter the whitefly. 2 and 3: Host plant suitability with 2: Second layer of defense once the whitefly is on the plant feeding and laying eggs and 3: Third layer of defense once nymphs come out and start to feed.

In 2007, Broekgaarden *et al.*, (submitted), studied the intraspecific variation in herbivore community composition and the transcriptional profiles in field grown *B. oleracea* cultivars in the Netherlands. More than 50 individuals of *A. proletella* were found per plants on white cabbage cultivar Christmas Drumhead and close to 0 on white cabbage cultivar Rivera. It is not known if this difference was because of resistance mechanisms or due to preference. A choice and non-choice experiment with *A. proletella* on these two

white cabbage cultivars has been done in the field in 2008 (unpublished data). The choice experiment showed that Rivera harboured the lowest, while Christmas Drumhead harboured the highest numbers of whitefly adults. The number of adults did not change on Rivera during the season, but it slightly decreased for Christmas Drumhead. Christmas Drumhead may have a resistance mechanism which is switched on later upon whitefly feeding. In the non-choice experiment, Rivera showed the highest rate of mortality compared with Christmas Drumhead. Rivera also harbored the lowest number of laid eggs and pupae per female. Therefore, Rivera and Christmas Drumhead seem interesting cultivars to study defense mechanisms upon *A. proletella* feeding.

In this study, we investigated the interaction between *A. proletella* and the two cultivars Rivera and Christmas Drumhead in more detail. We examined (1) host plant selection, (2) host plant suitability and (3) induced plant responses. The results will help to see if the results on *A. proletella* performance found in the field are reproducible in the glasshouse at a different stage of development; if the defense is constitutive or induced (i.e. induction by the whitefly itself or by other insect) and finally which pathways are involved in the *B. oleracea* - *A. proletella* interaction.

Materials and Methods

1. Experiments in the greenhouse

1a. Whitefly rearing and cultivation of *B. oleracea* plants

Whiteflies *Aleyrodes proletella* are originated from a field site in the surrounding of Wageningen, The Netherlands. They were maintained on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv. Cyrus) in a greenhouse compartment with a 16H day and 8H night period (Day: 22 °C, Night: 16°C, 40% relative humidity). Indeed, under good climate and host plant availability condition, the population grows exponentially (Iheagwam *et al.*, 1978; Bryne *et al.*, 1991). Seeds of eight white cabbage cultivars were used. Rivera, Lennox, Bartolo were obtained from Bejo. Galaxy was obtained from Seminis. Christmas Drumhead, BadShip, Bewama and Langedijker Bewaar were obtained from the Center of Genetic Resources, the Netherlands (CGN). Seeds were germinated in potting compost (Lentse Potgrond®), two-week old seedlings were transferred to 1.45L pots containing the same potting compost. Plants were grown in a glasshouse compartment at Wageningen University, the Netherlands in the beginning of 2009 with a 16H day and 8H night period (Day: 22 °C, Night: 16°C, 40% relative humidity). All plants were watered every day. No chemicals control was applied for diseases or pests.

1b. Whiteflies performance under choice experiment

Ten pre-selected whiteflies females (using a microscope and CO₂ to keep them asleep during the selection) were placed in clip cages. Two clip cages were then put on the youngest leaves of six weeks old plant. The cultivars have been positioned in a random design with five replicates per cultivars. At seven days post infection (dpi), the whiteflies were removed and the number of dead adults and eggs were counted. At 28 dpi the number of nymphs and pupae were counted.

1c. Whiteflies performance under no-choice experiment

Two experiments of this type have been done in January and March 2009. Around 1000 whiteflies were released randomly in the greenhouse. The eight cultivars have been positioned in a random design with five replicates per cultivar. At seven dpi, the whiteflies were removed and the number of adults and eggs were counted.

1d. Whiteflies performance under induction experiment

For each induction experiment, ten six-week old plants of Rivera and Christmas Drumhead were divided into five control and five infested plants. The cultivars have been positioned in a random design. The insects used for the infestation were the whitefly *Aleyrodes proletella* and the caterpillar *Pieris rapae*. The caterpillars were obtained from the laboratory of Entomology, Wageningen University, The Netherlands. Ten or 20 (depending on the experiment carried out) pre-selected whiteflies females (using a microscope and CO₂ to keep them asleep during the selection) were placed in clip cages. Two clip cages were then put on the youngest leaves. Ten caterpillars were placed in big clip cages. One clip cage was then put on the

youngest. Empty clip cages were put on the control plant. At three dpi (for the experiment with 20 whiteflies and five caterpillars) or seven dpi (for the experiment with ten whiteflies); the insect were removed. Then, ten new pre-selected whiteflies females were placed in clip cages. Two clip cages were put on the leaves preinfested by the whiteflies and one clip cage was put on the leaf preinfested by the caterpillar. The control plant i.e. not infested yet, were also infested. At 7 dpi, the whiteflies were removed and the number of dead adults and eggs were counted. At 28 dpi, the number of pupae was counted.

1e. Collection of material

For the gene expression analysis, samples have been taken at two different time points: at 7 dpi i.e. to study the effect of feeding and laying eggs and at 28 dpi i.e. to study the effect of nymphs. One leaf disc (diameter 2-3 cm) was harvested at the site of infestation by the insect (local leaf area). The samples were immediately put in liquid nitrogen and stored at -80°C.

1f. Odor experiment

A small Y tube olfactometer was used to investigate the behavior of whiteflies towards plant volatiles. By using air pressure, an air flow was generated through activated charcoal for each odor source container. The two odors flowed into each branch of the Y. Air was extracted at the base of the olfactometer with a vacuum system to remove odor from the Y and create the airflow (Takabayashi and Dicke, 1992; Mc Gregor and Gillespie, 2004; Nomikou *et al.*, 2005). Aluminum was put on pots to prevent odor from the soil to interact with odor from the plant. The whole 6-week old plant was put as source of odor. Only pre-selected females were used for this experiment. Females were tested one by one. Ten females were tested for one pair of plants. Ten pairs of plants were tested: Rivera vs. Christmas Drumhead; Rivera vs. clean air; Christmas Drumhead vs. clean air. The Y tube was cleaned every five tests in order to prevent any signals let by the whiteflies. Odor sources were switched between left and right side arms for each trial to minimize any special choices effects. Females that did not make a choice after five minutes were remove form the statistical analysis.

2. Quantitative RT-PCR

The following methods were done according to Broekgaarden *et al.*, (2008). Total RNA was extracted with TRIzol reagent (Invitrogen) followed by a purification using RNeasy Plant Mini kit (Qiagen). One µg of total RNA was treated with DNaseI (Invitrogen) according to the manufacturer's instructions. The RNA was then converted into cDNA using the iScript cDNA synthesis kit (BioRad) according to the manufacturer's instructions. Gene specific primers have been already used with success by Broekgaarden *et al.*, (submitted). The primers sequences are shown in table 1.

Table 1: Sequences of *B. oleracea*-derived primers used in quantitative real-time PCR analyses.

Gene name	Forward primer (5'→ 3')	Reverse primer (5'→ 3')
GADPH	AGAGCCGCTTCCTTCAACATCATT	TGGGCACACGGAAGGACATACC
<i>CTR1</i>	AAATCAGCGGTTCTCCAC	GCTCACGAGGCATGTACCTT
<i>LOX2</i>	CAGAGTTGTCAAAGCTGTTGCT	ACCATAAACCGCAGGGTCT
<i>PR1</i>	TCCACCATGTTACACCTTGC	GGCCTTATGGAGAGA AACTTGG
<i>TPI</i>	TGGTGACAAGTAGCTGTGGTG	TCCAAGTTATGGGCAGTGG

RT-PCR analysis was performed in optical 96-well plates with a MylQ Single Color real time PCR detection system (BioRad), using SYBR Green to monitor dsDNA synthesis. Each reaction contained 10 µg 2x SYBR Green Supermix Reagent (BioRad), 10 ng cDNA, and 300 nM of each gene-specific primer to make in total a final volume of 20 µl. All the samples studied were performed in duplicate. The following PCR program was used for all PCR reaction: 3 min 95°C; 40 cycles of 30 sec 95°C (denaturation) and 45 sec 60 °C (annealing and elongation). The threshold cycle (Ct) values have been calculated using Optical System software, version 2.0 for MylQ (BioRad). Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of the constitutively expressed gene GADPH (glyceraldehyde-3-phosphate dehydrogenase) from the Ct value of the gene of interest. Indeed, this gene is known to be a good housekeeping gene in *B. oleracea* (Broekgaarden *et al.*, 2008). The absolute expression levels of GADPH were similar for all our sample of the same development stage in our study (data not shown).

3. Data analysis

The analysis was performed using Genestats. The number of eggs per female was calculated by the number of eggs per the average of total females and females alive. This was done to compensate for the fact that it was not known when the female died. From the qRT PCR, normalized gene expression was obtained from the equation $2^{-\Delta Ct}$. When it was needed, $\log_{10}(X)$ or $\log_{10}(X+1)$ transformation was applied. Extreme values have been removed. One way analysis of variance (ANOVA) in combination with LSD or T tests was used to analyze the results. When the data could not be normalized, the non parametric test of Wilcoxon has been used. When mentioned, a two way ANOVA was done in order to take into account a non predicted block effect, the average was then predicted to take into account this undesirable block effect. For the odor experiment, a $K\chi^2$ test was used to analyze the data. $\alpha=0.05$ was applied for each test.

Results

1. Host plant selection

Host plant selection was studied with a choice experiment in order to see the level of preference of the whitefly for Rivera and Christmas Drumhead within six other white cabbage cultivars. An odor experiment was also carried out for Rivera and Christmas Drumhead.

1a. Choice experiment

In the two choice experiments, Rivera and Christmas Drumhead belonged respectively to the resistant and susceptible group. Christmas Drumhead was by far the most susceptible. Rivera always belonged to the most resistant group (Table 2). Three groups of resistance could be identified: (1) resistant, including Bewama, Galaxy, Bartolo and Rivera; (2) partial resistant, including Lennox and LangBew; (3) susceptible, including BadShip and Christmas Drumhead.

Table 2: Whiteflies preference for eight white cabbage cultivars. A and B refer to two distinct experiments done in the same conditions, but at different times. Experiment A: One way ANOVA, LSD method, $\alpha=0.05$. Experiment B: Two way ANOVA (due to unexpected block effects), LSD method, $\alpha=0.05$. Different letters mean significant differences between values for one column. P-value refers for the cultivar effect only.

Cultivar	Number whiteflies		Number eggs	
	A	B	A	B
Bewama	5.8 ^a	5.78 ^a	111.4 ^a	130.7 ^a
Galaxy	6.0 ^a	28.54 ^{ab}	129.0 ^{ab}	360.1 ^a
Bartolo	7.6 ^a	22.34 ^{ab}	214.4 ^{ab}	330.5 ^a
Rivera	8.2 ^a	24.20 ^{ab}	229.2 ^a	407.6 ^a
Lennox	8.6 ^{ab}	25.43 ^{ab}	352.4 ^{ab}	450.8 ^a
LangBew	9.0 ^{ab}	23.27 ^{ab}	259.6 ^{ab}	415.1 ^a
BadShip	23.4 ^{bc}	39.40 ^b	502.0 ^{bc}	473.1 ^a
Christmas Drumhead	42.6 ^c	66.85 ^c	756.8 ^c	1288.4 ^b
F value	5.53	8.52	6.10	5.62
p value	< 0.001	< 0.001	< 0.001	0.004

1b Odor experiment

Whiteflies preferred significantly Christmas Drumhead instead of Rivera. Christmas Drumhead did not attract significantly the insect, i.e. there was no difference between clean air and Christmas Drumhead. Rivera deterred significantly the insect via odor, i.e. the whitefly preferred clean air instead of Rivera (Figure 3).

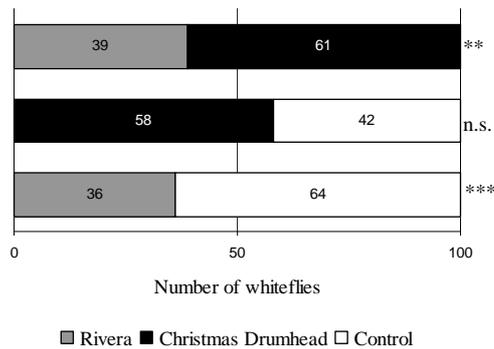


Figure 3: Whiteflies performance under a choice experiment with a Y tube, i.e. only according to the odors emit by the cultivar, between Rivera and clean air (Control); Christmas Drumhead and clean air (Control); Rivera and Christmas Drumhead. χ^2 test, $\alpha=0.05$, ***: $p<0.01$, **: $p<0.05$, n.s.: $p>0.05$: non significant.

2. Host plant suitability

Host plant suitability was studied with a no-choice experiment in order to see the level of suitability of the whitefly for Rivera and Christmas Drumhead within six other white cabbage cultivars under controlled condition. From the percentage of survival ($F=2.25$; p value=0.002); Rivera and Christmas Drumhead belonged respectively to the resistant and susceptible group (Table 2). Concerning the oviposition rate, nothing can really be conclude because of the lack of significance ($F=1.53$; p value=0.192). Nevertheless, a tendency can be made: Rivera and Christmas Drumhead belonged respectively to the resistant and susceptible group (Table 3). From the nymphs and pupae per eggs ratio ($F=3.98$; p value=0.003); Rivera and Christmas Drumhead belonged respectively to the resistant and susceptible group; although they were not significantly different for this trait (Table 3). Three groups of resistance can be made: (1) resistant, including Rivera, Bewama and Galaxy; (2) partial resistant, including Lennox and Bartolo; (3) susceptible, including Christmas Drumhead, LangBew and BadShip.

Table 3: Whiteflies performance under a no-choice experiment for eight white cabbage cultivars. One way ANOVA, LSD method, $\alpha=0.05$. Different letters mean significant differences between values for one column.

Cultivar	% survival	eggs/female	nymphs and pupae/eggs
Rivera	56.4 ^a	14.06	0.63 ^{abc}
Bewama	56.5 ^a	13.95	0.51 ^{ab}
Galaxy	65.0 ^{ab}	16.38	0.50 ^a
Lennox	68.0 ^{abc}	16.71	0.50 ^a
Bartolo	69.7 ^{abc}	18.08	0.64 ^{abc}
Christmas Drumhead	79.0 ^{bcd}	17.20	0.84 ^{cd}
LangBew	82.0 ^{bcd}	19.46	0.74 ^{bcd}
BadShip	90.0 ^d	18.26	0.92 ^d
F value	2.25	1.53	3.98
p value	0.002	0.192	0.003

3. Induced plant responses

3.a Transcriptional responses after whitefly feeding

Upon adults feeding *CTR1* was not differentially expressed in both cultivars (Figure 4). Upon nymphs feeding *CTR1* was down regulated in Rivera, but was not differentially expressed in Christmas Drumhead. *LOX2* was up regulated in both cultivars upon adult feeding, whereas it was not induced by nymphs feeding. There was no change of *TPI* expression upon both adults and nymphs feeding. Upon adults and nymphs feeding *PRI* expression did not change in both cultivars, but the expression of *PRI* was higher in Rivera than in Christmas Drumhead in the adult feeding control ($p=0.003$) (Figure 4).

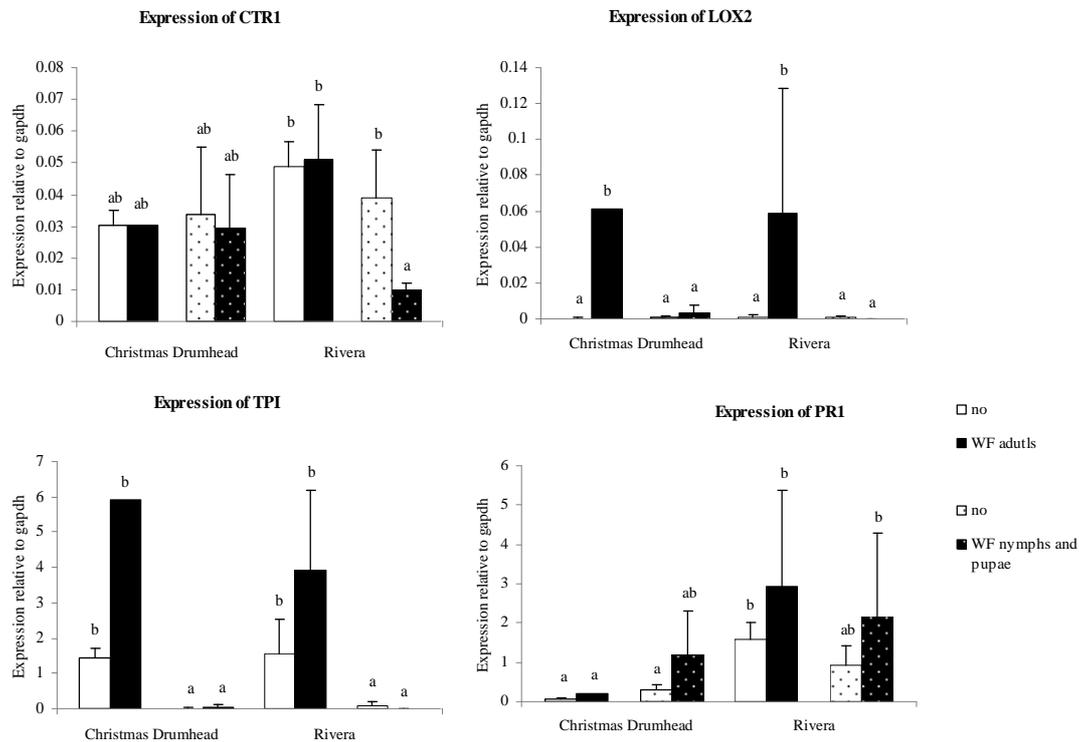


Figure 4: Gene expression relative to gapdh between control (white bars) and adults feeding (black bars) and between control (white with black dotted bars) and nymph and pupae feeding (black with white dotted bars). One way ANOVA for each gene, LSD method, $\alpha=0.05$. Different letters mean significant differences between values. CTR1: $F=3.125$, $p=0.016$; LOX2: $F=6.292$, $p<0.001$; TPI: $F=12.896$, $p<0.001$; PR1: $F=4.205$, $p=0.003$.

3.b Pre-infestation with whiteflies

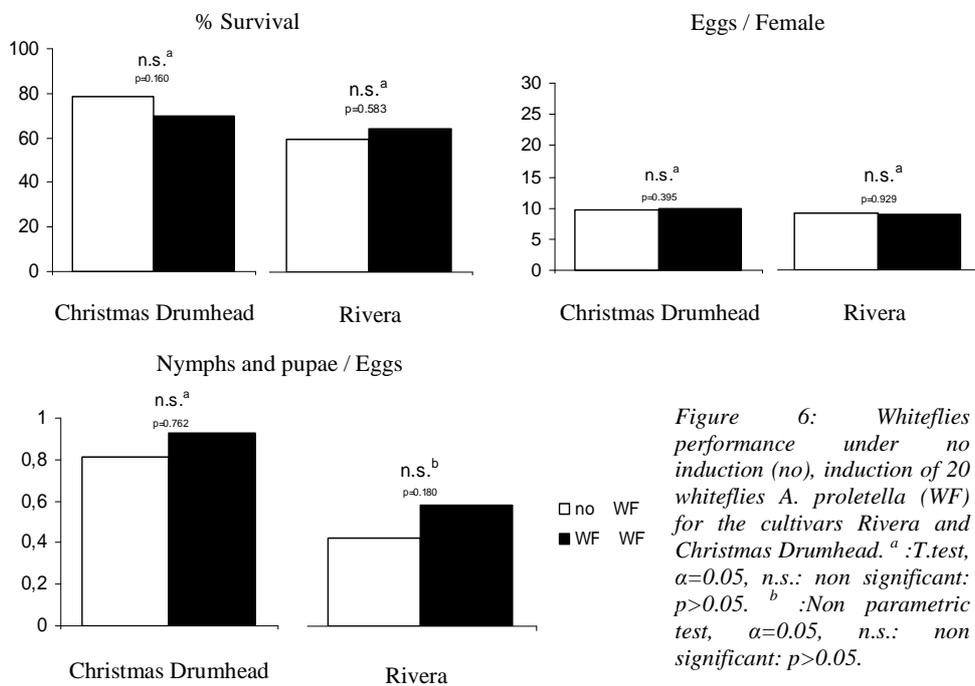
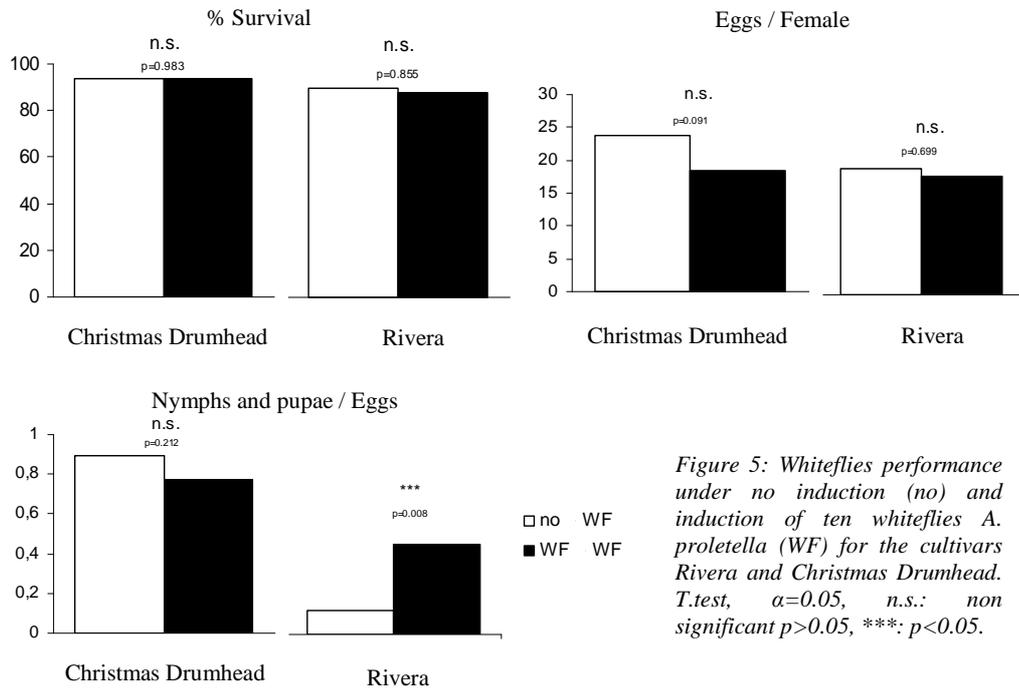
3bi.bioassay

For both pre-infestation with ten and 20 whiteflies, there were no significant differences between the control and the pre-infested plants for the survival rate and oviposition rate of whiteflies from a second infestation for both Rivera and Christmas Drumhead (Figure 5 and 6). However, with the ten whiteflies pre-infestation, there were less (but not significantly) survival and eggs per female after induction for both cultivars (Figure 5).

For the nymphs and pupae per eggs ratio; whitefly-induced Rivera plants harbored more pupae per eggs than the control plants, this was significant with ten whiteflies infestation and almost significant with 20 whiteflies infestation. On the other hand, for Christmas Drumhead both pre-infestations showed no significant differences between the control and the pre infested plant (Figure 5 and 6).

3bii. Transcriptional responses

Upon pre-infestation, CTR1, LOX2 and PR1 were not differentially expressed. The expression of TPI did not change in Christmas Drumhead, but was up regulated in Rivera (Figure 7).



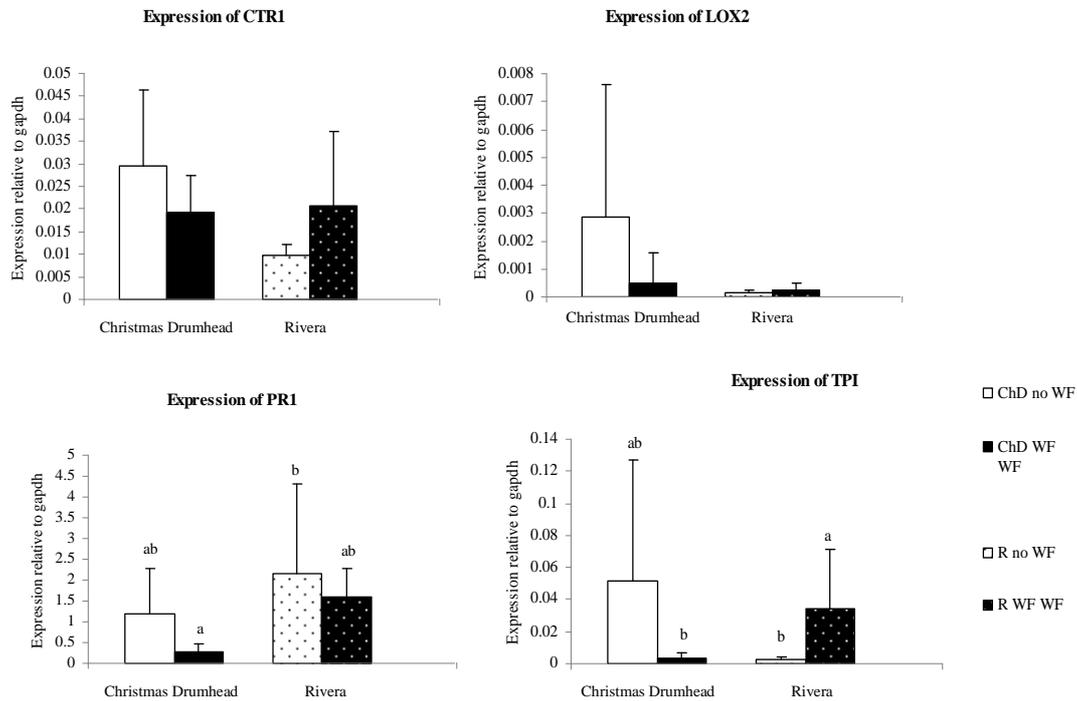


Figure 7: Gene expression relative to gapdh between not pre-infested (no WF) and pre-infested by *A. proletella* (WF WF) for Rivera (R) and Christmas Drumhead (ChD). One way ANOVA for each gene, LSD method, $\alpha=0.05$. Different letters mean significant differences between values. No letters mean no differences. CTR1: $F=1.864$, $p=0.174$; LOX2: $F=0.591$, $p=0.629$; TPI: $F=2.291$, $p=0.115$; PR1: $F=2.420$, $p=0.102$.

3c. Pre-infestation with caterpillars

3ci.bioassay

For both Rivera and Christmas Drumhead, there were no significant differences between the control and the pre infested plant for the survival rate and the oviposition rate. For the nymphs and pupae per eggs ratio; induced plants harbored significantly more pupae per eggs than the control on Rivera. For Christmas Drumhead there was no significant difference between the control and the pre infested plants (Figure 7).

3cii Transcriptional responses

Upon pre-infestation, *PR1*, *LOX2* and *TPI* were not differentially expressed in both cultivars. *CTR1* expression did not change in Rivera, but was down regulated in Christmas Drumhead (Figure 8).

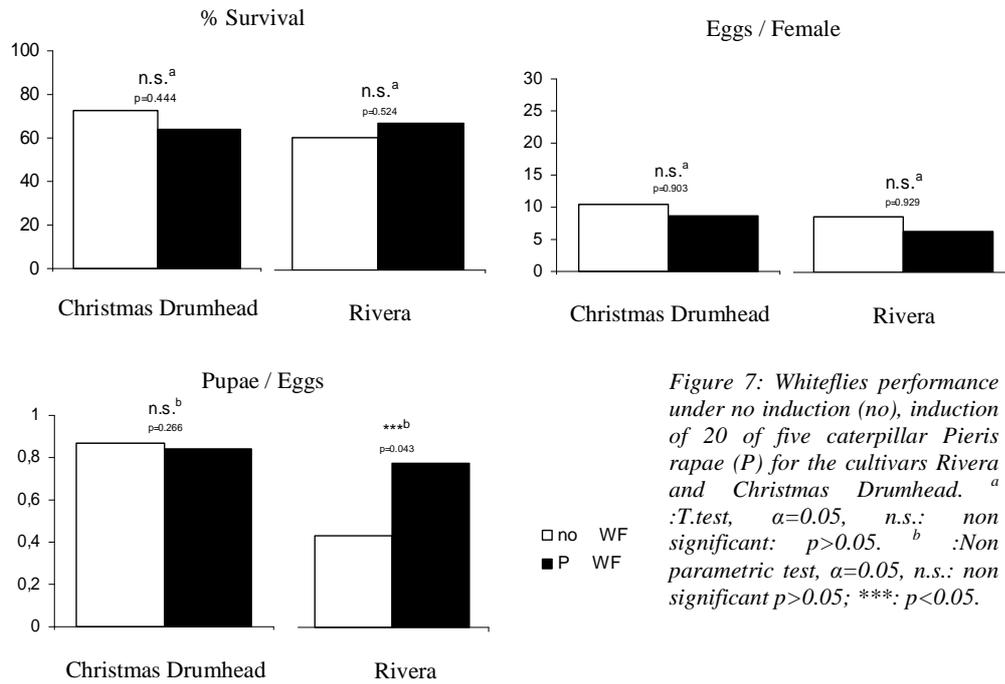


Figure 7: Whiteflies performance under no induction (no), induction of 20 of five caterpillar *Pieris rapae* (P) for the cultivars Rivera and Christmas Drumhead. ^a:T.test, $\alpha=0.05$, n.s.: non significant: $p>0.05$. ^b:Non parametric test, $\alpha=0.05$, n.s.: non significant $p>0.05$; ***: $p<0.05$.

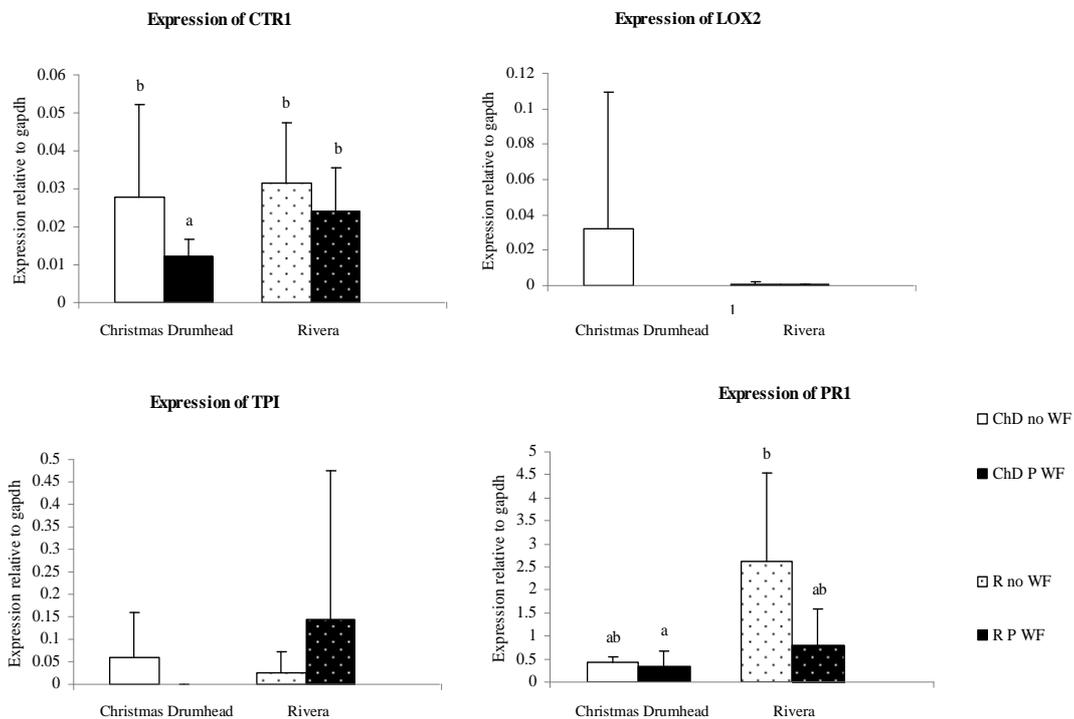


Figure 8: Gene expression relative to *gapdh* between not pre-infested (no WF) and pre-infested by *p. rapae* (P WF) for Rivera (R) and Christmas Drumhead (ChD). One way ANOVA for each gene, LSD method, $\alpha=0.05$. Different letters mean significant differences between values.No letters mean no differences. CTR1: $F=2.510$, $p=0.091$; LOX2: $F=1.345$, $p=0.293$; TPI: $F=1.351$, $p=0.293$; PR1: $F=4.050$, $p=0.024$.

Discussion

1. *A. proletella* performance differs on Rivera and Christmas Drumhead: Host plant selection and Host plant suitability.

Plant mediated interactions are important in whitefly biology because of three main things. First, whiteflies are polyphagous which create the opportunity to form complex interactions with numerous herbivores and natural enemies on a variety of plant species in different habitats. Secondly, whiteflies can induce significant responses and manipulate host plant physiology. Thirdly, whitefly immatures are mostly sessile (i.e. they can not move) (Inbar and Gerling, 2008). The plant is under stress once whitefly adults and/or nymphs are present. Some studies have been done already in order to find out suitability upon whiteflies feeding, mostly with the whitefly *B. tabbaci* or *B. argentifolii* (Mayer *et al.*, 2002; Leite *et al.*, 2006; Kakimoto *et al.*, 2007; Jindal *et al.*, 2007) but only a few with *A. proletella* (Nebreda *et al.*, 2005).

In this study, host plant selection has been studied with two choice experiments in order to see the level of preference of the whitefly for Rivera and Christmas Drumhead. The first choice experiment was carried out in the greenhouse for Rivera and Christmas Drumhead within six other white cabbage cultivars and showed clear differences in preference for these two cultivars. Christmas Drumhead was the most susceptible among all the cultivars, whereas Rivera belonged to the resistant group. The terms resistance or susceptibility are used here as a way of comparison. An odor experiment was carried out for Rivera and Christmas Drumhead in order to see if repellent or attractant odors could be involved in the difference in preference. The results showed that whiteflies preferred Christmas Drumhead over Rivera. This was due to a repellent effect of Rivera and not to attractiveness of Christmas Drumhead.

Most studies have been focusing on Host Induced Plant Volatiles (Van Poecke *et al.*, 2001; Dicke *et al.*, 2009) but not many studies include constitutive plant volatiles. Nevertheless, Poelman *et al.* (2009) found interesting blends of volatile compounds for clean plants of Rivera and Christmas Drumhead. From this blend, 27 volatiles showed a higher concentration in Rivera than in Christmas Drumhead. One or more of these volatile compounds may explain that Rivera deters the whitefly. Most of these volatiles belong to the class of monoterpenes (between 3 and 4.5 fold higher in Rivera than in Christmas Drumhead) like for example camphene (4.5 fold), α -terpinene (3.8 fold) or α -thujene (3.7 fold) (Poelman *et al.*, 2009). However, this study does not show all chemicals involved in the preference process and much more needs to be done. Odor repellents are really interesting and are already studied as a good first layer of defense for eco friendly pest management (Al-mazra'awi *et al.*, 2009). Other interesting characteristic, besides odor, of the plants may also play a role in host plant selection. Color can also be a reason for selection (Ramsey and Ellis, 1996). Further studies for the color as well as other chemical volatiles are interesting.

Host plant suitability has been studied with a no-choice experiment in the greenhouse for Rivera and Christmas Drumhead within six other white cabbage cultivars. Rivera belonged to the resistant group for all traits: % survival, eggs per female and nymphs/pupae per eggs. Christmas Drumhead belonged to the most susceptible cultivars. Those differences can be due to leaf structure (e.g. wax component) or induced defense mechanisms switched on by the plant. The accepted hypothesis is that the wax components rarely deter herbivore attack and more commonly stimulate whiteflies to feed (Leite *et al.*, 2006).

From the choice experiment, it is clear that Christmas Drumhead is the most susceptible among all the cultivars, which is not the case for the no-choice experiment. Therefore, the host plant selection seems the main weakness of Christmas Drumhead. Since this cultivar does not deter the whiteflies, lots of them come on the plant. Even if Christmas Drumhead is able to defend itself more than some other cultivars once the whiteflies are on the plant (cf no-choice experiment); the number of whiteflies is already very high and finally the damage will be high. On the other hand, Rivera seems resistant at each stage: its odor deters the insect, which is a good first barrier, and once the whiteflies are on the plant Rivera is still able to defend itself very effectively.

Broekgaarden *et al.* (unpublished data) did a choice and no-choice experiment on 10 weeks old *B. oleracea* cultivars in the field. In the field, plants are under environmental pressure, i.e. plants are always exposed to biotic and abiotic stress and always on the alert This was not the case in our study. Broekgaarden *et al.*, (unpublished data) showed with a choice experiment, that Rivera was the most resistant whereas Christmas Drumhead was the most susceptible. This matches with the present results found in the greenhouse. A no-

choice experiment was also carried out in the field. It is interesting to see that Rivera is the most resistant cultivar in the field and belongs to the resistant cultivars in the greenhouse. Remarkably, no pupae have been found on Rivera in the field, which was not the case in the greenhouse. Christmas Drumhead belongs to the most susceptible cultivars in both the field and the greenhouse. Environmental effect as well as the age may play a role in the disparities of the cultivars.

2. The difference in whitefly performance before the stage nymphs and pupae between Rivera and Christmas Drumhead is due to constitutive defense.

The choice and no choice experiments showed that Rivera is resistant while Christmas Drumhead is susceptible to whiteflies. It is interesting to see if the gene expression between those two cultivars differs and could explain such differences. The expression of four genes have been studied: *LOX2*, *PR1*, *CTR1* and *TPI*. Lipoxygenase (*LOX2*) is a key gene involved in the synthesis of the phytohormone JA, *PR1* is involved in the SA pathway and *CTR1* is down regulated by the phytohormone ethylene (Lietchi and Farmer, 2002; Zarate *et al.*, 2007). The SA, JA and ET dependent pathways are known to play an important role in the induced resistance against hemipterans (Bostock *et al.*, 2005; Kaloshian *et al.*, 2005). Trypsin and Protease Inhibitor (*TPI*) provide protection against the constitutive and induced complexity of gut proteinases and reduce growth and development of herbivores (Glawe *et al.*, 2003; Telang *et al.*, 2003).

It is interesting to see that both cultivars induced the JA-responsive gene *LOX2* at the same level upon adults feeding, but no changes were detected for this gene upon nymphs feeding. This suggests that both cultivars activate the JA pathway upon adult feeding. The expression of *CTR1* was repressed upon nymphs feeding, but not upon adults feeding in Rivera. This suggests an activation of the ET pathway as ET represses the activity of *CTR1*. Van de Ven *et al.*, (2000) have found that in squash, whitefly adults and nymphs of the same species can trigger different responses in the plant. The SA dependent pathway and *TPI* does not seem to play a role in a defense process in both cultivars. *TPI* was not induced by adults and nymph feeding. This gene does not seem involve in the defense. *PR1* was higher expressed in Rivera than in Christmas Drumhead control plants, suggesting a higher constitutive level of SA dependent pathways in Rivera. This may explain a constitutive defense higher in Rivera than in Christmas Drumhead.

Broekgaarden *et al.*, (2007) who did microarray analysis between control Rivera and control Christmas Drumhead did not find any genes that were clearly associated with an higher constitutive level of direct defense. This differ in our study where the SA dependent pathway seem constitutively higher expressed in Rivera than in Christmas Drumhead. Those differences can be due to threshold in the statistic analysis. The present data may also not be representative due to the lack of replicates and the big variation met.

Induction by the whiteflies itself have been investigated to see if an induced effect could be find. The bioassay showed that there were no differences in survival and eggs per females between pre-induced and control plants by both ten and 20 whiteflies. Unfortunately, transcriptional analysis for the stage adults for the induction experiment has not been done. Therefore, the non differences can not be explained by any gene expression analysis. This remains to be done.

According to the transcriptional analysis and the bioassay, the defense seems constitutive upon whiteflies feeding for both Rivera and Christmas Drumhead. An idea of threshold in the number of whiteflies up to which a defense is switch by the plant does not seem valid, i.e. ten or 20 whiteflies for the pre-infestation do not change anything. It has also to be kept in mind that the qRT PCR is a really sensitive technique and that the standard deviation is really big in all experiments. The variation intra cultivar is really high. Nevertheless, those results match up with Broekgaarden *et al.*, (2007) who did microarray analysis between control Rivera and control Christmas Drumhead but did not find any genes that were clearly associated with a higher constitutive level of direct defense.

In this study we looked only for the pathogenesis-related (PR) protein *PR1* but did not look for any others. Mayer *et al.*, (2002) found that the silver leaf whitefly induces a number of host plant defenses, including PR protein accumulation (e.g., chitinases, b-1,3-glucanases, peroxidases, chitosanases, etc.). Induction of plant-defensive proteins by SLW feeding was both local and systemic. It would be interesting also to look for other PR protein upon adult feeding.

3. Differences in defense happen during nymph feeding between Rivera and Christmas Drumhead.

According to the number of nymphs and pupae per eggs, interesting and surprising results happened. On pre-induced plants with ten whiteflies, Rivera had significantly more nymphs and pupae per eggs than the control. The same tendency is observed with 20 whiteflies but it is not significant. This means that the insect, in its nymphal stage, can develop itself easier once the plant has been already infected by whiteflies. However, this is not the case for Christmas Drumhead, there were no differences between control and pre-induced plants, the nymphs do not induce any responses.

Gene expression has been studied at the stage nymphs and pupae in order to investigate this different response in Rivera and Christmas observed in the bioassay. The SA responsive gene *PR1* and the JA responsive gene *LOX2* did not change upon nymphs feeding in both cultivars. JA and SA dependent pathway do not seem to play a role in the differences observed. A tendency can be made for *CTR1* and *TPI* expression (respectively $p=0.174$ and $p=0.115$). The main differences between Rivera and Christmas Drumhead are that *TPI* and the ET dependent pathway (oppositely correlated with the ET responsive gene *CTR1*) seem up-regulated in Rivera but do not change in Christmas Drumhead.

Microarray analysis on *A. thaliana* show that *B. tabaci* nymphs suppresses JA and induces SA related pathways, while ET related pathways are not involved in the process (Kempema *et al.*, 2007). The plant synthesizes JA upon the attack. The insect interfere in the plant defense by inducing SA pathway. This has been also checked via JA and SA mutants. Mutants that activate SA defenses (*cim10*) or impair JA defenses (*coi1*) accelerated *B.tabaci* nymphal development. Reciprocally, mutants that activate JA defenses (*cev1*) or impair SA defenses (*nPR1*, *NahG*) slowed *B.tabaci* nymphal development (Zarate *et al.*, 2007). Here this is not observed. The qRT PCR is a really sensitive technique and the standard deviation is really big in all experiments. The variation intra cultivar is really high. More replicates are needed to be done in order to find something straightforward. Treatment to stimulate SA pathway with the salicylate mimic, benzothiadiazole could be also interesting to investigate (Thaler *et al.*, 1999).

The significant differences seen in the bioassay can not be correlated with a significant difference in expression of genes. Here we are only talking about tendency. Those changes can be due to other genes or to an additive effect of several genes. The age may also have a role in the defense, the plant are young and may not be enough developed to defend effectively against the insect. The results in the field, on ten weeks old plant, showed on both cultivars less survival and eggs (Broekgaarden *et al.*, unpublished data). Age has been already found to have an effect on defense (Kus *et al.*, 2002; Fluch *et al.*, 2008). In the study of Kempema *et al.* (2007), the *A. thaliana* plants were five weeks old, i.e. close to the end of its life cycle (Yano and Ohsaki, 1993; Meinke *et al.*, 1998). Moreover, we can see that the level of expression of *PR1* in the control plant differs between the ages of the plant. Further experiments based on development stage would be really interesting to carry out.

4. Induction by *Pieris rapae* does not induce a defense on Rivera and Christmas Drumhead

Whiteflies have to be thought at the tritrophic level. Lots of interaction can occur in ecological communities between whiteflies, their predators, their plant feeding, etc (Price *et al.*, 1980). Food webs need to be investigated in order to emphasis direct, indirect and facilitative interactions (Ohgushi *et al.*, 2008). Since whiteflies are polyphagous, a broad range of organisms are related to it. Whiteflies interact with other herbivores. For example the presence of *B. tabaci* negatively affects the preference and performance of leafminers on various crops (Inbar and Gerling, 2008). Nevertheless, little is know about plant responses induced by a previous attack on whiteflies (Nombela *et al.*, 2009).

In the field, Rivera has no pupae; this differs from the greenhouse experiment. This may be due either to age or to an induction from an other species or abiotic factors. Broekgaarden *et al.*, (submitted), found at this moment of the season (middle of May) *P. rapae* caterpillars present on Rivera and Christmas Drumhead as well as eight other herbivores equally distributed over the two cultivars.

The effect of *P. rapae* on Rivera and Christmas Drumhead has been studied by Broekgaarden *et al.*, (2007). From this study on eight weeks old plant, it has been shown that Rivera was more resistant to the caterpillar than Christmas Drumhead. In both cultivars, *LOX2* was up regulated as well as a trypsin and protease inhibitor (*TPI*). *P. rapae* has also been found to induce JA responsive genes in *A. thaliana* (Reymond *et al.*, 2004; De Vos *et al.*, 2005).

We study here the “tripartite” interactions between the plant, caterpillars and whiteflies (Stout *et al.*, 2006). The induction by ten caterpillars shows no effect on whiteflies resistance for both Rivera and Christmas Drumhead compared to infestation with whiteflies in the bioassay. The genetic analyses show exactly the same pattern than the pre infestation by whiteflies for SA and JA dependent pathways and *TPI* expression. Nevertheless, it seems different for *CTR1* expression in Rivera. Therefore the induction by *p. rapae* did not induced any major changes in gene expression after whiteflies nymphs feeding compared to the induction by whiteflies. It can be concluded then that *p. rapae* does not induce a defence on the plant. The induced effect of the caterpillar on whiteflies resistance could be investigated on older plant. Further interaction could be also interesting to investigate between the whitefly and other herbivores found in the field by Broekgaarden *et al.*, (submitted).

5. Future perspectives: defense mechanisms are different between aphid and whitefly feeding.

Studies have been done in order to investigate interactions between crucifers and Hemipteran. Most of them were on aphids and the model plant *Arabidopsis thaliana* (Moran *et al.*, 2002; Kušnierczyk *et al.*, 2007; Thompson and Goggin, 2006). Since whiteflies and aphids belong to the same family and use the same feeding strategy, it is interesting to compare their effect on plants. While aphids have a short and mobile life story, whiteflies have a long and continuous interaction with the plant. Upon emergence from an egg, to the stage first instar to pupae, nymphs establish a feeding site that is used nearly continuously for over 28 days; stylets subsequently return to their established feeding site (Kaloshian *et al.*, 2005; Kempema *et al.*, 2007). Stylet penetration on resistant plants is often characterized by a higher frequency of short probes that do not successfully locate a sieve element, and a longer time to reach first phloem phase (Jiang *et al.*, 2007). Jiang *et al.*, (2007) found that resistance in alfalfa genotype against *B. argentifolii* appears to be expressed in the sieve elements themselves. In that study, most nymphs died on the resistant clone in the first instar, which let to think that resistance-based mortality is primarily a first-instar phenomenon. It would be really interesting to carry out such a study and also to differentiate each defense stage, i.e. adults, 1st instar, 2^{de} instar, 3rd instar and pupae. Indeed, under nymphs feeding, it is not know which instar stage induce (or not) the defense.

Gene expression responses induced by aphid feeding are most similar to SA-mediated gene induction, although expression of some JA and ET responsive genes are also upregulated as well as some other signaling pathway (De Vos *et al.*, 2007). Here, it is dependent on the cultivar, but SA, JA and ET mediated pathways seem to play a role in the defense upon whitefly feeding.

In Brassicaceae, secondary metabolites called glucosinates have an important role in pathogen and herbivore interactions. Upon cellular damage made by aphids, myrosinates are released from specialized myrosin cells and hydrolyze pounds such as nitriles, isothiocyanates, epithionitriles, and thiocyanates (De Vos *et al.*, 2007). But surprisingly, microarray analysis showed that whiteflies do not alter the expression of genes involved in sulfur and glucosinolate metabolism. In addition, on *A. thaliana* whiteflies do not induce cell death mechanisms but callose deposition has been detected (Kempema *et al.*, 2007). Hyper sensitive responses as well as callose deposition are still to be investigated upon *A. proletella* feeding.

Broekgaarden *et al.*, (2008) studied responses of young stage Rivera and Christmas Drumhead to infestation by the aphid *Brevicoryne brassicae*. It is interesting to compare this study with our results. No differences in host plant suitability were found between Rivera and Christmas Drumhead for aphid nymph mortality as well as for development time. However, Rivera supported slower aphid population development than Christmas Drumhead. On the other hand, there are differences in host plant suitability between Rivera and Christmas Drumhead for whiteflies percentage of survival. Unfortunately we can not

compare population development and development time. Still a lot needs to be done to study host plant suitability for *A. proletella*.

Microarray studies show that after aphid feeding a small number of genes overlap between Rivera and Christmas Drumhead. Quantitative Rt PCR show that *TPI* is significantly induced in Rivera but do not change in Christmas Drumhead. In our study, *TPI* does not change in Rivera and Christmas Drumhead upon adults and nymph feeding. But after pre-infestation, *TPI* is up regulated in Rivera and did not change in Christmas Drumhead. Therefore, both cultivars seem to respond in the same way than aphids once the plant is under stressed by pre-infestation.

Upon aphids, both cultivars induced gene responsive to oxidative stress. Rivera induced genes encoding aquaporines upon aphids feeding, which increase nutrient concentration and therefore profit to the insect. A xyloglucan endotransglucosylate (XTH6) was also up regulated in Rivera and Christmas Drumhead upon the aphid *B. brassicae* (Broekgaarden *et al.*, 2008). It may be interesting to investigate this further. XTHs cleave and re-attach xyloglucan polymers modifying hemicelluloses to strengthen cell walls (Campbell and Braam, 1999). Cell wall modification are know to be part of the local and systemic defense against aphid feeding by strengthening barriers against probing and feeding (Thompson and Goggin, 2006; Broekgaarden *et al.*, 2008).

To better understand the differences happening in Rivera but not in Christmas Drumhead during nymphs feeding; it would be interesting to investigate the expression of XTH6, gene responding to oxidative stress or genes encoding aquaporines. It would be interesting also to do the same experiment but with different plant stages to see if the age has to do with the defense.

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