

Phloem-specific resistance in *Brassica oleracea* against the whitefly *Aleyrodes proletella*

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Abstract

The cabbage whitefly [*Aleyrodes proletella* L. (Hemiptera: Aleyrodidae)] is becoming a serious pest in *Brassica oleracea* L. (Brassicaceae) crops. However, almost nothing is known about the interaction of this insect with its host plants. Previous studies have shown differences in the natural occurrence of adults, eggs, and nymphs on the closely related *B. oleracea* cultivars Christmas Drumhead and Rivera grown in the field. In this study, we aimed to identify the nature of these differences and to gain insight into the resistance mechanisms against *A. proletella*. We used no-choice experiments on field- and greenhouse-grown plants to show that the differences between the two cultivars are mainly based on antibiosis (traits that reduce herbivore performance) and not on antixenosis (traits that deter herbivory). This was further supported by laboratory choice experiments that indicated little or no discrimination between the two cultivars based on plant volatiles. We showed that resistance is dependent on plant age, that is, resistance increased during plant development, and is mainly independent of environmental factors. Analysis of probing behaviour revealed that the resistance trait affects *A. proletella* at the phloem level and that morphological differences between the two cultivars are most likely not involved. We suggest that compounds present in the phloem reduce sap ingestion by the whitefly and that this explains the observed resistance.

Introduction

The cabbage whitefly, *Aleyrodes proletella* L. (Hemiptera: Aleyrodidae), is becoming a serious pest of *Brassica oleracea* L. (Brassicaceae) crops, causing substantial losses to especially kale, Brussels sprouts, and Savoy cabbage in Europe (Ramsey & Ellis, 1996; Trdan & Papler, 2002). Damage caused by *A. proletella* is mainly cosmetic, but strongly reduces the marketability of the crop. Adult females lay eggs in circular patterns, embedded in a circle of wax, on the lower leaf surface. Eggs hatch into crawling nymphs that move a few centimetres on the leaf surface to locate a suitable feeding place. Once such a place is found, crawlers penetrate the plant tissues by probing intercellularly through epidermal and mesophyll cell layers and

ultimately feed from the phloem sieve elements (Byrne & Bellows, 1991). Once a feeding site is established, crawlers moult into sessile nymphs and feed at this site almost continuously throughout their development. Both adults and nymphs suck phloem sap and excrete honeydew, a sugary substance that allows the growth of moulds. Control of *A. proletella* is based mainly on the use of insecticides, but alternative means of control are being sought because of environmental concerns (Lewis et al., 1997; Huang et al., 2009).

Host plant resistance is an effective form of insect control and offers a very good alternative to the use of insecticides (Ramsey & Ellis, 1996; Broekgaarden et al., 2011). To be able to develop insect-resistant varieties, it is essential to identify effective sources of resistance and to characterize the mechanisms behind this resistance. One well-studied example of effective host plant resistance involves the *Mi* gene from tomato that confers resistance against several isolates of the potato aphid [*Macrosiphum euphorbiae* (Thomas)] (Rossi et al., 1998).

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The resistance mechanism against this phloem-feeding insect is based on antibiosis, that is, it reduces survival and reproduction of the insect (Goggin, 2007). The *Mi* gene also confers resistance to a certain biotype of the whitefly *Bemisia tabaci* (Gennadius). The mechanisms behind this are based on a combination of antibiosis and antixenosis, where the latter depends on traits that deter insects (Jiang et al., 2001; Nombela et al., 2003). Another example involves effective resistance against the blue-green aphid (*Acyrtosiphon kondoi* Shinji) that has been identified in a *Medicago truncatula* (Gaertn) cultivar (Klingler et al., 2005). In this case, the resistance mechanism is also based on a combination of antibiosis and antixenosis (Goggin, 2007; Walling, 2008). Unfortunately, effective sources of resistance against *A. proletella* have not been identified so far. Furthermore, there is only limited knowledge on the biology and ecology of *A. proletella* and its interaction with host plants (Ramsey & Ellis, 1996; Nebreda et al., 2005).

In a previous field study, we observed clear differences in the natural occurrence of *A. proletella* on two *B. oleracea* cultivars. On cv. Rivera, low numbers of whitefly adults and eggs were found and, interestingly, no nymphs were present on the leaves. Conversely, cv. Christmas Drumhead was heavily infested with adults, eggs, and nymphs of this insect species (Broekgaarden et al., 2010). The observed differences in whitefly occurrence between these two closely related cultivars provide the opportunity to identify the mechanisms of resistance against *A. proletella* in a crop species of economic interest. The objectives of the present study were to identify the nature, that is, antixenosis or antibiosis, of the differences observed between the cultivars and to get insight into the mechanism behind them. The resistance was characterized at multiple levels, including abundance and performance under field conditions, as well as performance, response to host plant odours, and probing behaviour under greenhouse conditions. We present evidence for a phloem-localized resistance mechanism that interferes with whiteflies' sap ingestion.

Materials and methods

Plant material

Seeds from white cabbage (*B. oleracea capitata* var. *alba*) cultivars Rivera (F1 hybrid) and Christmas Drumhead (open-pollinated) were obtained from Bejo Zaden B.V. (Warmenhuizen, The Netherlands) and the Centre of Genetic Resources (CGN, Wageningen, The Netherlands) respectively. For all experiments, seeds were germinated on potting compost (Lentse Potgrond, Lent, The Netherlands) in a greenhouse compartment at 20 ± 2 °C with an L16:D8 photoperiod and 40–70% r.h.

Plants for all field experiments were individually transferred to peat blocks 10 days after germination. Three-week-old plants were allowed to acclimatize to field conditions by placing them outside the greenhouse. Plants were watered every other day and received no chemical control for pests and diseases. Five-week-old plants were transplanted to the field with their peat blocks.

Plants for all greenhouse and laboratory experiments were individually transferred to 1.45-l pots containing potting compost 2 weeks after germination. Eight-week-old plants were transferred to bigger pots (5 l) containing potting compost to allow continuous growth and to avoid stress due to lack of space. Plants were grown at 20 ± 2 °C (L16:D8 photoperiod; 40–70% r.h.), watered every other day, and fertilized with 2.5 mg l^{-1} Kristalon Blauw (N-P-K-MgO, 19-6-20-3; Hydro Agri, Rotterdam, The Netherlands) every 3 weeks from the age of 4 weeks onwards.

Insects

Cabbage whiteflies, *A. proletella*, were reared on Brussels sprouts (*B. oleracea* var. *gemmifera* cv. Cyrus) in a climate chamber at 20 ± 2 °C with an L16:D8 photoperiod and 40–60% r.h. This population originated from adults collected in 2008 from a white cabbage field in Wageningen, The Netherlands (51°57'N, 5°38'E). Whiteflies were reared under conditions in which there was always sufficient foliage for feeding and oviposition. For all experiments, adult whiteflies of assorted ages were collected from the rearing chamber using an aspirator.

Whitefly population dynamics in the field with early planting

To analyse the population dynamics of whiteflies on white cabbage, a field experiment was conducted to monitor the natural in-flight. The field site was located in the neighbourhood of Wageningen, The Netherlands (51°57'N, 5°38'E). Plants were sown in week 13 (end of March) and transplanted to the field in week 18 (end of April) of 2009. The experimental site was divided into separate square plots of 4.5×4.5 m (7×7 plants) each containing a monoculture of 49 plants of one of the *B. oleracea* cultivars with a spacing of 75 cm between plants. The field site contained eight plots per cultivar which were organized in a square. A strip of 6 m sown with a grass mixture of *Lolium* and *Poa* species separated the plots. From week 23 (early June) until week 36 (early September) the nine central plants of each plot were monitored weekly for the presence of *A. proletella* adults, eggs, and nymphs. We also monitored the abundance of other herbivorous insects and their natural enemies, the results of which are described in Kos et al. (2011). For each week, the numbers of whitefly adults, eggs, and nymphs were averaged per plot before being averaged per cultivar.

Whitefly abundance in the field after late planting

To reduce the effect of differences in development between the two cultivars and to allow for testing plants before head formation, another field site was established in which plants were sown later and planted in the field shortly before whiteflies become active. For this field experiment, plants were sown in week 23 (beginning of June) and transplanted to the field in week 28 (middle of July) of 2009. The experimental site consisted of one plot of 12 × 10 m (22 × 14 plants) containing five plants per cultivar with a spacing of 70 cm between plants in a single block randomized design. The plot contained the two *B. oleracea* cultivars together with six other white cabbage, 11 Brussels sprouts, 10 kale (*B. oleracea acephala*), and 21 Savoy cabbage (*B. oleracea capitata* var. *sabauda*) cultivars that showed considerable differences for whitefly resistance (C Broekgaarden, G Steenhuis, RE Voorrips & B Vosman, unpubl.). The plot was surrounded by a single row of all the *B. oleracea* cultivars present in the experimental field in a random order. Plants were monitored in week 30 (middle of July) and week 34 (middle of August) for the presence of *A. proletella*.

Whitefly performance in the field

Whitefly performance was monitored in the field during 2 years, 2008 and 2009, to test for consistency of our results over time. In 2009, we monitored whitefly performance on five Rivera and five Christmas Drumhead plants in the field site with the late planting described above. In 2008, the experimental setup was equal to that of the late planting experiment in 2009 with the only exception that the plot was smaller (9 × 6 m; 12 × 8 plants) and contained, besides Rivera and Christmas Drumhead, 10 Brussels sprouts cultivars that were all susceptible to *A. proletella* (G Steenhuis, C Broekgaarden, RE Voorrips & B Vosman, unpubl.). In week 35 (late August) of both years, when plants were 12 weeks old, female whiteflies were confined to the lower surface of leaves using clip cages (Ø 2 cm, height 1.2 cm). Whiteflies were briefly (<30 min) anaesthetized with a gas mixture (N₂:H₂:CO₂, 80:10:10; Linde Gas Benelux, Schiedam, The Netherlands; flow of 10 cm s⁻¹) to enable selection and transfer of females to the clip cages (Bethke et al., 1991). Plants received two clip cages, each containing five females, on two young, fully expanded leaves. Whiteflies were allowed to feed and oviposit for 7 days, after which adult survival was monitored and remaining females were removed. Subsequently, the number of eggs was counted. After an additional period of 2 weeks, the number of nymphs on every plant was recorded. Values were averaged per plant prior to statistical analysis.

Whitefly performance in the greenhouse

Six- and 12-week-old greenhouse-grown plants were used to examine whitefly performance under more controlled conditions. Plants received two clip cages, each containing 10 whitefly females, on two young, fully expanded leaves. Five plants per cultivar were used for both plant ages. The experiment was performed and analysed as described above for whitefly performance in the field.

To evaluate the effect of epicuticular wax on the performance of whiteflies, half of the lower part of a young, fully expanded leaf of a 16-week-old plant was gently rubbed with wet cotton to remove the wax layer. Two clip cages containing 10 whitefly females each were placed on the treated and the untreated part of the leaf respectively. Whitefly performance was subsequently recorded on five plants per cultivar as described above for whitefly performance in the field.

Response of whiteflies to plant odours

The attraction of females to the cultivars was assessed with a Y-shaped glass tube olfactometer (Koschier et al., 2000). In this set-up, individual whitefly females were offered a choice between odour-loaded air from cv. Rivera in one arm and odour-loaded air from cv. Christmas Drumhead in the other arm of the Y-tube. A 6-week-old plant was placed in a glass jar that was connected to the Y-tube arms and pots were covered with aluminium foil to avoid contamination of odours coming from the soil. The length of the arms and base of the Y-tube was 5 cm, with an inner diameter of 0.5 cm. To establish airflow through the Y-tube, air was sucked at the base of the Y-tube by means of a membrane pump that produced an airflow of 20 cm s⁻¹. The Y-tube was illuminated with artificial light and situated in a closed room at 20 ± 2 °C. Whitefly females were briefly anaesthetized as described above, transferred into the base of the Y-tube and allowed to make a choice within 5 min after waking up. Females that did not make a choice were excluded from the statistical analysis. After testing five insects, the odour sources were interchanged to avoid position effects and the plants were replaced by new ones after testing 10 insects. The experiment was terminated after 100 whiteflies had made a choice. The experiment was repeated using 12-week-old plants of the two cultivars. For 6-week-old plants, comparisons were also made between a plant and clean air. For that purpose, pots containing soil only were covered with aluminium foil and placed in one of the glass jars whereas a 6-week-old plant of one of the cultivars was placed in the other glass jar.

Whitefly survival over time

Whiteflies were confined to 12-week-old greenhouse-grown plants and monitored every day to determine their

survival. Plants received two clip cages, each containing five whiteflies, on two young, fully expanded leaves. Five plants per cultivar were used in this experiment. Whiteflies were also kept in a Murai cage (Murai & Loomans, 2001) closed with a double layer of parafilm with water in between as a no-food control.

Whitefly probing behaviour

Whitefly probing activities on greenhouse-grown, 12-week-old plants were studied using the Electrical Penetration Graph (EPG) technique (Tjallingii, 1978). We monitored probing of adult females for 8 h during the day at 20 ± 2 °C. One Rivera and one Christmas Drumhead plant were placed in a Faraday cage and two whiteflies, one on each plant, were recorded simultaneously. Plants and whiteflies were only used once. Whiteflies were placed on the lower side of a young, fully expanded leaf. Before exposure to the plant, a 2–3 cm long 18 μ m diameter gold wire was attached to the dorsal surface of the whitefly's abdomen using water-based silver glue (EPG Systems, Wageningen, The Netherlands). Whiteflies were anaesthetized as described above and subsequently held in place by a vacuum suction device (van Helden & Tjallingii, 1993). Before being glued, the wax layer covering the whitefly's abdomen was removed using a fine brush and water. The other end of the gold wire was attached to a copper wire (3 cm long \times 0.2 mm in diameter) and connected to a Giga-4 direct current amplifier with four channels and 1 giga-ohm input resistance and 50 \times gain (manufactured by Wageningen University). A copper rod (10 cm long \times 2 mm in diameter) inserted into the soil of the potted plant closed the electrical circuit. We recorded whitefly probing behaviour on five plants per cultivar.

Data acquisition and waveform analysis were mediated by Probe 3.0 software (Laboratory of Entomology, Wageningen University, The Netherlands). The position of the whitefly stylets and their feeding activities were interpreted according to EPG waveforms previously defined by Tjallingii (1978) and Lei et al. (1996). Baseline voltage occurs when the whitefly does not have its stylet inserted into the plant and is referred to as 'non-probing'. Waveform C represents 'pathway phase', when the whitefly is penetrating with its stylet through the leaf tissue. 'Phloem phase', when the stylet is inserted into a phloem sieve element, is composed of waveforms E1 (salivation) and E2 (sap ingestion). Waveform G represents 'xylem phase' when the whitefly inserts its stylet into the xylem of the plant. Both sequential and non-sequential parameters (Sarria et al., 2009) were used to characterize probing behaviour of *A. proletella* (Supporting information, Table S1).

Whitefly post-access behaviour

To evaluate the lasting effects of feeding from the resistant plants on whitefly behaviour, we performed a transfer experiment. Adult whiteflies were caged, 10 in a clip cage, on a fully expanded young leaf of 12-week-old Rivera or Christmas Drumhead plants. After 24 h the living whiteflies were anaesthetized as described above and four of them were carefully transferred to a leaf of the other cultivar or to another position on the same leaf as a control. Whiteflies were confined to the leaves in a clip cage and monitored daily for their survival on five plants per cultivar.

Statistical analysis

Statistical analyses were performed using SPSS for Windows (15th edition; Chicago, IL, USA). For population dynamics, the data were analysed using structured repeated measurements mixed models ANOVA with the repeated structure type AR (1). The dependent variable (average number of adults, eggs, or nymphs per plant in a plot) was modelled by the factors cultivar, week, and the factorial interactions.

Data obtained from field-grown plants, that is, whitefly abundance and performance in the field with late planting, and from the performance tests on greenhouse-grown plants were $\log_{10}(x + 0.1)$ -transformed or, in case of percentages, arcsin square-root transformed to obtain a normal distribution. Comparisons between the two cultivars were subsequently made using independent sample t-tests. Whitefly preference in the Y-tube experiment was statistically analysed using a χ^2 test with the null-hypothesis that whiteflies did not have a preference for one of the two odour sources. Survival curves of whiteflies on 12-week-old plants and of those in the transfer experiment were analysed using general linear model (GLM) repeated measures ANOVA followed by LSD tests. Day was considered a within-subjects factor and cultivar/treatment a between-subjects factor. The EPG data were analysed using Mann-Whitney U-tests to make comparisons between the two cultivars, as a normal distribution could not be obtained for these data.

Results

Whitefly population dynamics in the field with early planting

Whitefly adults were first observed on plants by the end of July (week 30; Figure 1A), 2 months after planting. Whitefly population sizes, expressed as adult, egg, and nymph numbers per plant, differed between the two cultivars (repeated measures ANOVA, adults: $F_{1,37.249} = 245.145$; eggs: $F_{1,24.220} = 105.618$; nymphs: $F_{1,36.124} = 15.396$, $P < 0.001$ for all life stages; Figure 1). During the whole growing season hardly any whitefly adults were observed

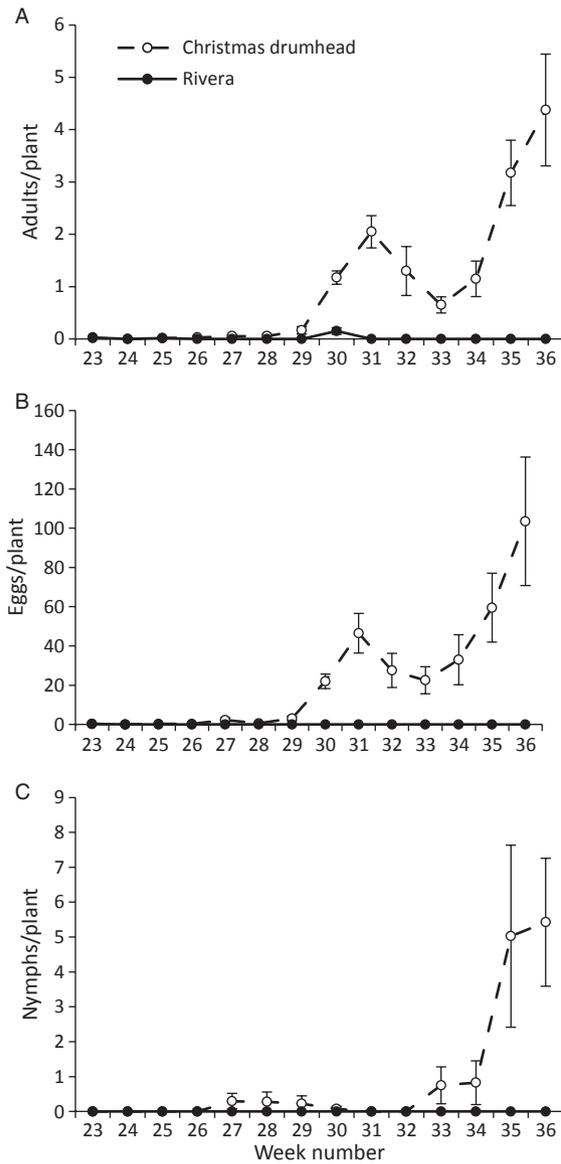


Figure 1 Population dynamics of *Aleyrodes proletella* on two *Brassica oleracea* cultivars over time in a common garden experiment in 2009. Data represent mean \pm SE ($n = 8$) numbers of (A) adults, (B) eggs, and (C) nymphs per cultivar monitored over 14 weeks.

on cv. Rivera and there were no eggs and nymphs present on this cultivar. Conversely, many eggs were observed on cv. Christmas Drumhead and nymphs emerged from these eggs a few weeks later (Figure 1). In this field experiment, there were also clear differences in developmental stages between the two cultivars. Cultivar Christmas Drumhead started to develop a head in week 30 whereas cv. Rivera developed a head about 5 weeks later (Kos et al., 2011).

Whitefly abundance and performance in the field

To reduce the developmental variability between the two cultivars, we conducted a field experiment in which plants were sown later and planted in the field shortly before whiteflies become active. Natural occurrence of whitefly adults and eggs did not differ on 7-week-old plants, but clear differences were found between 11-week-old plants of the two cultivars (independent sample t-test, adults: $t = 3.704$, d.f. = 4.908, $P = 0.014$; eggs: $t = 5.211$, d.f. = 4.221, $P = 0.006$). Hardly any adults and eggs were found on Rivera whereas Christmas Drumhead was heavily infested (Table 1). As in the early planting experiment, no nymphs were found on Rivera at any time point during the season whereas they did occur on Christmas Drumhead ($t = 14.822$, d.f. = 4.0, $P < 0.001$; Table 1).

We evaluated whitefly performance in the field on 12-week-old plants under no-choice conditions in 2008 and 2009. In 2008, whitefly survival on Rivera was about 50% lower than on Christmas Drumhead ($t = 4.862$, d.f. = 4.862, $P = 0.001$). In addition, the daily number of eggs per female was about three times lower on Rivera compared with Christmas Drumhead ($t = 5.549$, d.f. = 5.549, $P = 0.001$). All nymphs that hatched from the eggs died on Rivera (Table 2). In 2009 the differences were even more extreme, as all females died on Rivera within 1 week after infestation and no eggs were laid on this cultivar, resulting in a complete absence of nymphs (Table 2). Conversely, 60% of the females released on Christmas Drumhead were still alive after 1 week and they laid about one egg per day. Furthermore, ca. 70% of all the laid eggs on this cultivar hatched and nymphs were able to develop into the adult stage (Table 2).

Whitefly performance under controlled conditions

To test under more controlled conditions, whitefly performance was evaluated on 6- and 12-week-old plants grown in the greenhouse. Performance was only slightly different on 6-week-old plants of the two cultivars, but showed clear differences on 12-week-old plants (Table 2). On 12-week-old plants, female and nymph survival was clearly lower on Rivera than on Christmas Drumhead (independent sample t-test, adult: $t = -13.351$, d.f. = 8; nymph: $t = -11.062$, d.f. = 5.86, $P < 0.01$ for both life stages). The difference for daily egg production per female was also very clear, with very low rates on Rivera compared with Christmas Drumhead ($t = -4.360$, d.f. = 4.665, $P = 0.009$).

Whitefly performance was also monitored on leaves of 16-week-old plants with and without epicuticular wax to test for the influence of this morphological trait on whitefly resistance. For both cultivars, the removal of epicuticular wax did not affect adult survival, oviposition rate, or nymph development (adult: $t = -6.781$, d.f. = 8; eggs:

Table 1 Abundance of *Aleyrodes proletella* on *Brassica oleracea* cultivars Rivera and Christmas Drumhead in a common garden experiment with late planting in 2009

Plant age	Adults/plant			Eggs/plant			Nymphs/plant		
	Rivera	Christmas Drumhead	P ¹	Rivera	Christmas Drumhead	P ¹	Rivera	Christmas Drumhead	P ¹
7 weeks	0.4 ± 0.4	0.6 ± 0.2	0.36	0 ± 0	3 ± 2	0.21	ND	ND	
11 weeks	0.6 ± 0.4	4.6 ± 1.4	0.014	2 ± 1	82 ± 13	0.006	0 ± 0	13 ± 3	<0.001

Values are means ± SE (n = 5). ND = not determined, as there were no nymphs in the field at that time.

¹Probability levels of independent sample t-tests for comparisons between the two cultivars.

Table 2 Performance of *Aleyrodes proletella* on *Brassica oleracea* cultivars Rivera and Christmas Drumhead under field and controlled conditions

	% adult survival			Daily eggs/female			% nymph survival		
	Rivera	Christmas Drumhead	P ¹	Rivera	Christmas Drumhead	P ¹	Rivera	Christmas Drumhead	P ¹
Field condition									
2008	34 ± 9	82 ± 4	0.001	1.5 ± 0.3	3.9 ± 0.3	<0.001	0 ± 0	32 ± 10	0.006
2009	0 ± 0	60 ± 7	<0.001	0 ± 0	1.1 ± 0.1	<0.001	0 ± 0	68 ± 8	<0.001
Greenhouse condition									
6-week-old plants	56 ± 2	72 ± 5	0.03	2.0 ± 0.2	2.4 ± 0.3	0.49	63 ± 12	76 ± 14	0.27
12-week-old plants	2 ± 2	88 ± 4	<0.001	0.6 ± 0.3	6.1 ± 1.0	<0.001	1 ± 1	48 ± 6	<0.001

Values are means ± SE (n = 5). Plants under field conditions were 12 weeks old.

¹Probability levels of independent sample t-tests for comparisons between the two cultivars.

t = -6.349, d.f. = 8; nymphs: t = -8.224, d.f. = 4, P>0.05 for all life stages; Table 3).

Response of whiteflies to plant odours under controlled conditions

To determine if host plant colonization by whitefly females is influenced by their response to olfactory cues, we conducted a choice assay using a Y-tube olfactometer. The odours emitted by 6-week-old Rivera attracted slightly fewer adults than odours emitted by Christmas Drumhead of the same age ($\chi^2 = 4.84$, d.f. = 1, P = 0.028; Figure 2). To test if this was due to the influence of attractant and/or repellent volatiles, we also tested the response of females

when offered one of the cultivars against clean air. Whitefly females did not discriminate between Christmas Drumhead and clean air, but they had a slight preference for clean air over Rivera (Figure 2). When offering 12-week-old plants, females did not differentiate between odour sources from the two cultivars ($\chi^2 = 1.44$, d.f. = 1, P = 0.23; Figure 2).

Whitefly survival and oviposition on 12-week-old plants

Daily monitoring of the survival of whitefly adults on 12-week-old plants revealed that 50% of the whiteflies placed on Rivera died within the first 2 days, increasing to more

Table 3 Performance of *Aleyrodes proletella* on *Brassica oleracea* cultivars Rivera and Christmas Drumhead with and without epicuticular wax

	% adult survival		Daily eggs/female		% nymph survival	
	Untreated	No wax	Untreated	No wax	Untreated	No wax
Rivera	1 ± 1	2 ± 2	0.18 ± 0.03	0.50 ± 0.07	0 ± 0	0 ± 0
Christmas Drumhead	68 ± 12	73 ± 16	10.98 ± 0.40	9.07 ± 0.36	79 ± 10	64 ± 17

Values were not significantly different between treatments (independent sample t-test: P>0.05).

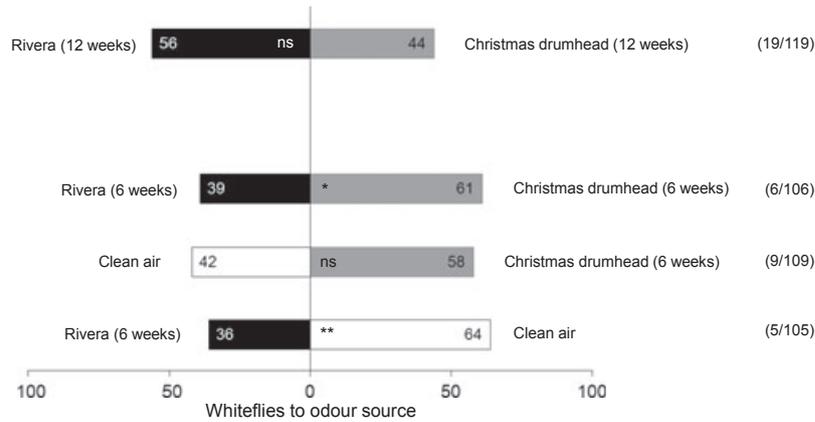


Figure 2 Response of *Aleyrodes proletella* to volatiles emitted by *Brassica oleracea* cultivars Rivera (black bars) and Christmas Drumhead (grey bars) as assessed in the Y-tube olfactometer. Data represent the number of females that made a choice for that specific cultivar. The top set of bars presents a choice between 12-week-old plants. The lower three sets of bars present choices between 6-week-old plants of Rivera (black bars) and Christmas Drumhead (grey bars), or between a 6-week-old plant and clean air (white bars). * $P < 0.05$, ** $P < 0.01$, ns = no significant difference. The number of whiteflies that did not make a choice and the total number of whiteflies tested are given in parentheses.

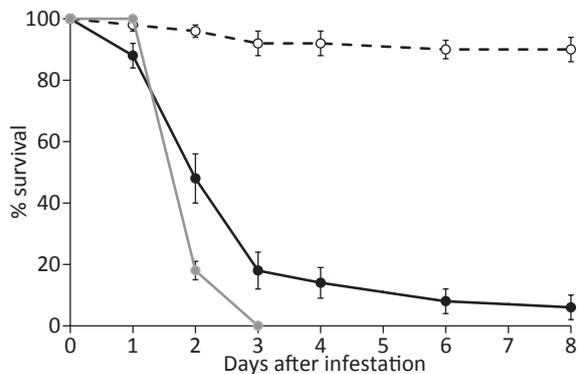


Figure 3 Survival of *Aleyrodes proletella* adults on leaves of *Brassica oleracea* cv. Rivera (solid black line) and Christmas Drumhead (solid grey line). The dashed line represents the no-food control. Values are mean (\pm SE) numbers of survivors per plant ($n = 5$).

than 90% after 6 days. In contrast, 90% of the whiteflies on Christmas Drumhead were alive after 8 days (Figure 3). The GLM model, used to compare survival curves from the three food sources (Rivera, Christmas Drumhead, no-food), showed a significant difference between these sources (GLM repeated measures: $F = 107.777$, $d.f. = 2$, $P < 0.001$). Whitefly survival was lower on Rivera leaves than on Christmas Drumhead leaves ($P < 0.001$), but larger than on the no-food control ($P = 0.025$).

More detailed observations on the behaviour of whitefly females on the two cultivars revealed clear differences in

oviposition behaviour. On Christmas Drumhead females deposited their eggs in circles covered with a layer of wax whereas eggs were deposited singly on Rivera and no circles of eggs were observed on this cultivar (Figure 4). For the images, whitefly females were allowed to feed for 1 day in which they laid about 10 eggs on Christmas Drumhead. In the field experiments, we saw circles of about 20 eggs on Christmas Drumhead plants (data not shown).

Whitefly probing behaviour on 12-week-old plants

Quantification of EPG results (Table S1) was used to determine the probing activities of whitefly adults on the two cultivars. All whiteflies started to penetrate the leaf on which they were placed for EPG recording. The frequency and duration of EPG parameters associated with stylet pathway behaviour before phloem contact did not differ between the cultivars (Table S1). Moreover, whiteflies on the two cultivars did not differ in the time from the start of stylet penetration to the first recorded phloem salivation. For all the recorded whiteflies, each period of phloem salivation was followed by passive sap consumption. The total duration of phloem salivation was not significantly different between the cultivars (Mann–Whitney U-test: $U = 4$, $d.f. = 8$, $P > 0.05$; Table S1), but the total duration of phloem consumption was shorter on Rivera than on Christmas Drumhead ($U = 0$, $d.f. = 8$, $P = 0.016$; Figure 5A). Furthermore, the number of sustained phloem consumption events per whitefly and the number of probes after the first phloem event were significantly lower on Rivera than on Christmas Drumhead

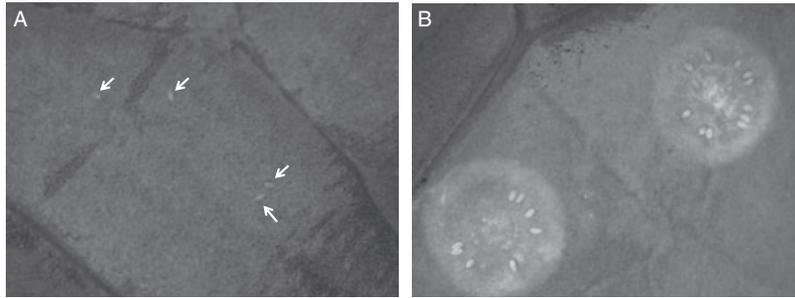


Figure 4 Whitefly oviposition patterns on *Brassica oleracea* cv. (A) Rivera and (B) Christmas Drumhead. In the experiment, whitefly females were allowed to feed and oviposit on leaves of the two cultivars for 1 day. White arrows in the left photo point out the eggs.

($U = 2$, d.f. = 8, $P = 0.032$ for both events; Figure 5B). Several whiteflies on Rivera also showed xylem ingestion whereas this type of behaviour was not seen on Christmas Drumhead (Figure 5B). Periods of extracellular phloem salivation and derailed stylet mechanics were not observed in our recordings.

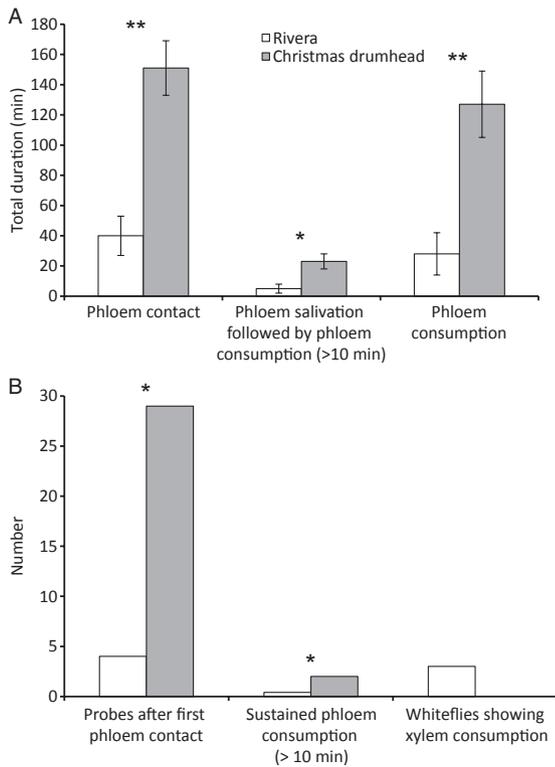


Figure 5 Electrical Penetration Graph (EPG) parameters and associated behaviour of *Aleyrodes proletella* on *Brassica oleracea* cultivars Rivera and Christmas Drumhead. Values are (A) mean (\pm SE; $n = 5$) total duration (min) of a certain event and (B) the number of times that a certain event occurred. Asterisks indicate significant differences (Mann–Whitney U-test: * $P < 0.05$; ** $P < 0.01$).

Post-access behaviour

To evaluate if there were any lasting effects of Rivera on whitefly behaviour, we performed a transfer experiment. Performance after change of host-plant or host-leaf negatively affected the whiteflies as survival rapidly decreased equally in all treatments during the 1st day after the transfer. Survival of whiteflies transferred from Rivera to Christmas Drumhead was comparable to survival of whiteflies transferred from Christmas Drumhead to Christmas Drumhead (Figure 6) and females started to lay eggs in circular patterns after both treatments. Whiteflies transferred from Christmas Drumhead to Rivera all died within 5 days, which was comparable to survival of whiteflies transferred from Rivera to Rivera (Figure 6). The GLM model, used to compare survival curves from the four treatments (Rivera to Rivera, Christmas Drumhead to Christmas Drumhead, Rivera to Christmas Drumhead, or Christmas Drumhead to Rivera), showed a significant difference between these sources (GLM repeated measures: $F = 13.443$, d.f. = 3, $P < 0.001$). Survival of whiteflies trans-

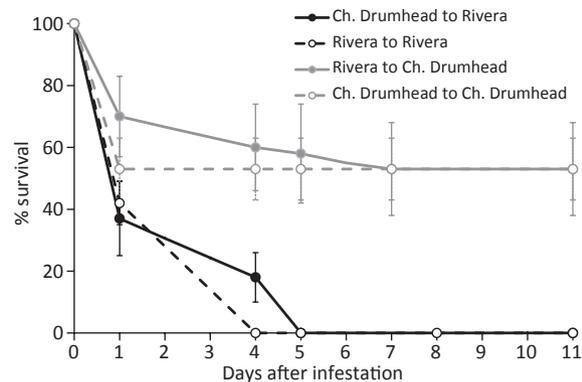


Figure 6 Survival of *Aleyrodes proletella* adults on *Brassica oleracea* cv. Rivera and Christmas Drumhead with transfers from one cultivar to the other (solid lines) or from one leaf to another of the same cultivar (dashed lines). Values are mean (\pm SE) numbers of survivors per plant ($n = 5$). Ch. = Christmas.

ferred to Rivera was lower than those transferred to Christmas Drumhead ($P \leq 0.001$).

Discussion

Natural inflight of whiteflies occurs late in the season

In the Netherlands, the natural inflight of whiteflies in a cabbage field containing cultivars Rivera and Christmas Drumhead started in the middle of summer, that is, end of July. Population development started right after the first eggs were laid by whitefly females and continued to increase until at least the end of the summer, that is, early September. Although we present data on 1 year only, the observations are in line with unpublished results of four other years as well as published results (Broekgaarden et al., 2010). The data show that whiteflies become active in the field later than most other herbivorous insects that infest cabbage cultivars (Poelman et al., 2009; Broekgaarden et al., 2010; Kos et al., 2011).

Whiteflies are not able to colonize cv. Rivera in the field

Once whiteflies became active in the field, clear differences in abundance of adults were seen between the two cultivars. Hardly any whitefly adults were observed on Rivera whereas Christmas Drumhead was heavily infested. The most interesting observation was the complete absence of nymphs on Rivera. These observations are consistent with results obtained in 2007 (Broekgaarden et al., 2010). However, in the field experiment with early planting there was a clear difference in developmental stage between the two cultivars by the time whiteflies became active, and this may have influenced whitefly abundance. At that point, Christmas Drumhead had already developed a head whereas Rivera was still completely open. Therefore, we conducted field experiments with late sowing and planting to reduce the differences in development between the two cultivars at the time whiteflies become active in the field. Four weeks after planting, when plants were 11 weeks old, Rivera harboured almost no adults and eggs, whereas large numbers of whiteflies were found on Christmas Drumhead. Similar abundance of whiteflies on these cultivars has been observed in field experiments conducted in four other years (C Broekgaarden, EH Poelman, G Steenhuis and KTB Pelgrom unpubl.) indicating the reliability of our observations. These results indicate that Rivera is not colonized by *A. proletella* under field conditions. Although host suitability of *B. oleracea* for *A. proletella* has been studied before (Ramsey & Ellis, 1996; Nebreda et al., 2005) our study is the first to identify such low numbers of *A. proletella* individuals on a plant of this species under natural conditions in the field.

Antixenotic factors do not explain the difference in whitefly abundance between the cultivars

Host plant selection by whitefly females is the first stage of colonization and plays a major role in determining whitefly populations in the field (Byrne & Bellows, 1991). Dual choice tests were done to determine if olfactory cues play a role in the observed differences in host plant colonization between the two cultivars. The results showed that whitefly females are slightly deterred by volatiles emitted from 6-week-old Rivera plants, but that this effect disappears during plant development as females did not discriminate between 12-week-old plants from both cultivars. The slight selection difference between greenhouse-grown, 6-week-old plants of the two cultivars did not affect the abundance of whiteflies on 7-week-old plants in the field. The similar numbers of whiteflies on both cultivars at the first monitoring date may therefore be due to the low numbers of whiteflies present in the field at that time. More importantly, the results show that antixenosis is not the main factor in creating the difference for whitefly abundance on 11-week-old plants in the field. This is supported by the presence of adults and eggs on Rivera in the field, which suggests that whiteflies do select this cultivar as a possible host. However, the complete absence of nymphs indicates that the whiteflies are not able to develop a population on Rivera plants. As whiteflies oviposit while feeding from the sieve elements (Byrne & Bellows, 1991), it is most likely that adults taste the phloem sap, lay a few eggs, and then decide that Rivera is not a suitable host to develop a population on. This is supported by the rapid adult and nymph mortality on field-grown Rivera plants under no-choice conditions. Therefore, we hypothesize that the resistance mechanism of Rivera is mainly based on antibiosis and that antixenosis is not, or only partly, involved.

Differences in whitefly abundance are mainly due to age-dependent antibiosis in cv. Rivera

No-choice tests showed that adult survival was slightly lower on 6-week-old Rivera plants than on Christmas Drumhead plants from the same age and nymphs could fairly survive on both cultivars. Conversely, in accordance with the results on field-grown plants, clear differences between the cultivars were found for all whitefly life stages when plants were 12 weeks old. The similarity of the no-choice results obtained under field and greenhouse conditions indicate no or a weak effect of environmental factors on the resistance. Whitefly population development is clearly not possible on 12-week-old Rivera plants because nearly all adults and nymphs died within a few days after infestation or emergence respectively. This is similar to resistance against aphids in tomato (Kaloshian et al., 1997) and lettuce (van Helden et al., 1993), which caused

almost 100% mortality of aphids within 8 days. Our results support the hypothesis that antibiotic factors play a major role in the resistance of Rivera against *A. proletella* and that this resistance is strongly dependent on plant age. This pattern is consistent with results obtained for several tree species in which a positive correlation has been found between plant age and resistance against insects (Boege & Marquis, 2005). Increase of resistance with plant age has also been shown against a caterpillar species in tomato (Cipollini & Redman, 1999). This may be explained by the hypothesis that resources become more available for resistance when shoot:root ratio increases with plant growth (Boege & Marquis, 2005). However, Barton & Koricheva (2010) have shown that the plant developmental patterns of resistance depend on plant life-history, herbivore type, and defence traits.

Resistance in cv. Rivera is phloem based

Female oviposition behaviour can give an indication about the resistance mechanism, as whiteflies oviposit their eggs in a circle while their mouthparts remain inserted in the phloem (Byrne & Bellows, 1991). The circular egg patterns on Christmas Drumhead indicate that females can feed from the phloem without many problems. Conversely, the single eggs that were randomly distributed on the leaves of Rivera suggest the presence of phloem-specific factors that interfere with whitefly feeding.

To locate the tissues most likely to play a role in the resistance mechanism, we used the EPG method to record, in real time, the probing activities of whitefly adults (Janssen et al., 1989). None of the behavioural activities prior to the first phloem phase were significantly different between Rivera and Christmas Drumhead. Although several morphological traits, such as wax layer and leaf toughness, have been shown to differ between Rivera and Christmas (Kos et al., 2011), our EPG results suggest that these surface features do not affect whitefly probing behaviour. This is supported by the finding that removal of epicuticular wax from the leaf did not affect whitefly performance. Whiteflies were able to reach the phloem, puncture sieve elements, and ingest phloem sap, as indicated by the occurrence of phloem salivation and consumption events on both cultivars. The number and duration of salivation, which refers to the injection of salivary secretions into the sieve element, did not differ between the two cultivars indicating that elaborate sealing mechanisms of the phloem sieve pores, such as protein plugging and callose deposition, are not involved in the resistance against *A. proletella* (Will & van Bel, 2006; Will et al., 2007). The lower number of sustained phloem consumption events and shorter duration of this phase indicate that whiteflies had difficulty feeding for long periods

on Rivera. During sap ingestion, chemical solutes of the phloem sap may be detected by the whitefly and perhaps evoke the rejection of feeding on Rivera. The intake of such solutes seems likely as the number of probes after the first phloem event was much lower on Rivera than on Christmas Drumhead. These results indicate that the resistance is probably based on a component present in the phloem sap of Rivera that interferes with whitefly feeding. The phloem-specific resistance found in Rivera resembles that of *Mi*-mediated resistance against whitefly in alfalfa (Jiang & Walker, 2007), and against whitefly and aphids in tomato (Kaloshian et al., 2000; Jiang et al., 2001). Similar phloem-specific resistance has been shown for *Vat*- and *Nr*-mediated resistance against aphids in, respectively, melon and lettuce (van Helden & Tjallingii, 1993; Chen et al., 1996; Klingler et al., 1998). The melon *Vat* and tomato *Mi* genes have been cloned and were shown to be members of a family of plant resistance genes characterized by NBS-LRR motifs (Rossi et al., 1998; Kaloshian, 2004; Pauquet et al., 2004). Electrical Penetration Graph monitoring of aphids indicated that *Vat*-, *Mi*-, and *Nr*-mediated aphid resistance were all due to phloem-limited factors that shorten ingestion (van Helden & Tjallingii, 1993; Chen et al., 1996; Klingler et al., 1998; Kaloshian et al., 2000). Unfortunately, every attempt to isolate chemical compounds related to these aphids' resistances has failed (van Helden et al., 1995; Chen et al., 1997, 1999).

Our EPG results show that the resistance is probably based on a strong reduction of feeding on Rivera. The occurrence of xylem sap ingestion, which is thought to be a response of phloem-feeding insects that are dehydrated following a starvation period (Spiller et al., 1990; Powell & Hardie, 2002), also indicates that whiteflies on Rivera did not have sufficient food intake. This may well explain the observation that whiteflies on this cultivar survived a bit longer than starved whiteflies, but much shorter than whiteflies on Christmas Drumhead. The resumption of feeding when whiteflies were transferred from Rivera to Christmas Drumhead, as evidenced by the initiation of oviposition and reduced mortality, suggests that the resistance does not have lasting toxic effects on the whitefly. This has also been shown for lettuce and tomato where resistance did not have lasting toxic effects on the aphids as recovery was seen for aphids that were transferred from resistant to susceptible genotypes (van Helden et al., 1993; Kaloshian et al., 1997). This correspondence suggests that Rivera may also be resistant to aphids, which is supported by observations of natural occurrence of cabbage aphids on cabbage cultivars (Broekgaarden et al., 2008) and a high mortality of these aphids placed on Rivera plants in the field (Kos et al., 2011).

Conclusions

Our study shows that *B. oleracea* cv. Rivera is not a suitable host for *A. proletella*, which is the first to report such an effective resistance source against this herbivorous insect. We show that the resistance is mainly independent of environmental factors, but highly dependent on plant age. The resistance mechanism is probably located at the phloem sieve elements involving compounds that reduce sap ingestion by the whitefly. Future investigations will be focussed on a genetic unravelling of the resistance components present in Rivera using metabolomic, proteomic, and transcriptomic approaches.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Table S1 Description of electrical penetration graph (EPG) behavioural parameters considered and their relation to whitefly feeding activity.

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