Both hypersensitive and non-hypersensitive responses are associated with resistance in *Salix viminalis* against the gall midge *Dasineura marginemtorquens*

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Abstract

Hypersensitivity responses (HR) play a major role in plant resistance to pathogens. It is often claimed that HR is also important in plant resistance to insects, although there is little unambiguous documentation. Large genotypic variation in resistance against the gall midge *Dasineura marginemtorquens* is found in *Salix viminalis*. Variation in larval performance and induced responses within a full-sib *S. viminalis* family is reported here; 36 sibling plants were completely resistant (larvae died within 48 h after egg hatch, no gall induction), 11 plants were totally susceptible, 25 plants were variable (living and dead larvae present on the same plant). Resistance was associated with HR, but to different degrees; 21 totally resistant genotypes showed typical HR symptoms (many distinct necrotic spots) whereas the remaining 15 genotypes showed no, or very few, such symptoms. Hydrogen peroxide, used as a marker for HR, was induced in genotypes expressing HR symptoms but not in resistant genotypes without symptoms, or in susceptible genotypes. These data suggest that production of hydrogen peroxide, and accompanying cell death, cannot explain larval mortality in the symptomless reaction. Another, as yet unknown, mechanism of resistance may be present. If so, then it is possible that this unknown mechanism also contributes to resistance in plants displaying HR. The apparent complexity observed in this interaction, with both visible and invisible plant responses associated with resistance against an adapted insect species, may have implications for the study of resistance factors in other plant–insect interactions.

Key words: Cecidomyidae, HR, hypersensitive response, induced resistance, insect, plant/insect interaction, ROS, Salicaceae, symptomless response, willow.

Introduction

The hypersensitive response (HR) is a major form of resistance in plants against pathogens (Goodman and Novacky, 1994). HR includes the rapid death of cells at the infection site associated with defence gene expression (Heath, 2000). The classical view of HR is that it isolates the intruder from the surrounding area and causes it to starve to death (Agrios, 1997). A number of biochemical changes, however, coincide with or relate to the process of cell death, for example, the presence of an oxidative burst, and production of phytoalexins, hydrolytic enzymes, pathogenesis–related proteins, salicylate, proteinase inhibitors, and the deposition of lignin and callose (Richael and Gilchrist, 1999, and references therein; Heath, 2000). Because many of these responses are induced simultaneously with the cell death, it has been difficult to determine the causality between cell death and resistance (Heath, 1999; Richael and Gilchrist, 1999). Thus, it is possible that HR could be the consequence of a biochemical process that is actually killing both host and those of the infecting organism (Dangl et al., 1996).

Models of HR dynamics are almost exclusively based on interactions between plants and pathogens. Much less is
known about HR as a resistance trait in plants against insects. Early studies, based on observations of necrotic tissue, reported HR to be present in a number of plant/insect interactions (reviewed by Fernandes, 1990). In some cases, cell death appears to be due to responses induced against pathogens associated with the insect, i.e. bark beetles (Christiansen et al., 1999). Most reports on the association between HR and insects, however, are based on field observations with limited possibilities to conclude about mechanisms (Fernandes and Negreiros, 2001; Fernandes et al., 2003). It is often difficult to determine if plant cell death is actually causing insect mortality, or if dead cells result from disrupted feeding after the insect has been killed by some other agent.

It is generally believed that most insect species damage plant tissue in such a way as to trigger wound responses, thought to be fundamentally different from the pathogen-induced changes leading to HR (Gatehouse, 2002). Feeding by insect species with a sedentary life form, and a piercing/sucking feeding mode, however, results in plant responses that are, in many ways, similar to those by pathogens (Walling, 2000). Still, few studies have unequivocally documented HR as a response to feeding by piercing/sucking insects (Walling, 2000).

An intimate relationship between the insect and its host plant seems to promote HR, such as in the case of gall-inducing insects (Fernandes, 1990; Fernandes et al., 2000). Morphological and biochemical responses of an HR type have been observed to be induced by economically important gall midge species, i.e., Mayetiola destructor, the Hessian fly, Oresolia oryzae, the rice gall midge, and Sitodiplosis mosellana, the wheat gall midge (Harris et al., 2003, and references therein). However, whether or not these responses actually cause larval death, or occur after some other mechanism has killed the insect, is not known (Harris et al., 2003).

Large genotypic variation in resistance has been documented in the basket willow Salix viminalis L. against the gall midge Dasineura marginemtorquens Bremi (Strong et al., 1993). Resistant willow genotypes respond to gall midge attack with a HR-like reaction, i.e. rapid cell death, accumulation of phenolic compounds (Ollerstam et al., 2002) and salicylic acid (Ollerstam and Larsson, 2003). On the resistant genotypes 100% of the neonate larvae fail to induce galls and die within 48 h after hatching from the eggs (Ollerstam et al., 2002).

Preliminary data suggest that certain S. viminalis genotypes are completely resistant against D. marginemtorquens, and still show no sign of HR (Ollerstam et al., 2002). Reports on local symptomless reaction associated with plant resistance are rare (Yu et al., 1998). One example is the man-made Arabidopsis dnd ‘defence, no death’ mutant against the bacterium, Pseudomonas syringae (Yu et al., 2000), which demonstrate that changes within a few genes may explain the loss of HR. Another example is from rice where resistant varieties respond against the rice gall midge Orseolia oryzae in the same way as resistant genotypes of S. viminalis against D. marginemtorquens, i.e. some with a symptomless response, and other with HR (Sardesai et al., 2001).

An early feature of the HR is the rapid generation of reactive oxygen species (ROS) (Dixon and Harrison, 1994; Levine, 2004). ROS compounds are in themselves toxic and can kill cells and microorganisms (Baker and Orlandi, 1995), but ROS may also act as signalling molecules involved in the regulation of plant development and pathogen protection (Dixon and Harrison, 1994; Lamb and Dixon, 1997; Wojtaszek, 1997; Apel and Hirt, 2004). Hydrogen peroxide is an important ROS acting as a signalling molecule in plant responses as well as having antimicrobial properties (Baker and Orlandi, 1995; Wojtaszek, 1997; Apel and Hirt, 2004).

The aim of this study was to examine preliminary observations of a range of resistance responses in S. viminalis against D. marginemtorquens. Salix viminalis is a rapid grower used in short rotation forestry for biomass production (Ledin, 1992) and produces new leaves throughout the growing season. Genotypes are easily propagated from stem cuttings. Dasineura marginemtorquens is monophagous on S. viminalis. The midge is an ephemeral 2–3 mm insect whose larva induces a gall on young unfurled S. viminalis leaves. The mature gall is a 5–10 mm pocket (Mani, 1964) along the leaf margin and consists of enlarged plant cells.

The distribution of living and dead larvae and induced plant responses was first documented by means of biotest within a full-sib family of S. viminalis. The hypothesis that resistance against D. marginemtorquens can be expressed without symptoms associated with HR was then tested. In these experiments, hydrogen peroxide was used as a marker for HR. Finally, it was investigated whether or not there were any differences in the rate by which resistance is expressed. It was expected that gall midge larvae would respond by losing size and dying more quickly on genotypes with HR symptoms, i.e. lesion formation and the production of hydrogen peroxide, compared with resistant genotypes lacking these symptoms.

**Materials and methods**

**Intraspecific variation in induced responses and larval survival**

Strong et al. (1993) reported on variation in resistance, i.e. variation in number of galled leaves, to Dasineura marginemtorquens among 40 full-sib Salix viminalis families. Six offspring from each family were tested. In the current study, a new crossing was conducted in order to determine the level of variation in larval performance and induced responses for a higher number of siblings within a family. The crossing was between genotype 78-0-195 as the mother and genotype 81-0-084 as the father (equivalent to family no. 46, a family with some resistant siblings, in Strong et al., 1993). The genotype
Genotypes were still alive and the leaves with larvae were still furled, in the experiment in order to verify the time of larval hatching.

Extra plants were exposed to ovipositing females at the start of the experiment. Midge larvae in galled leaves were collected, as pupae or third instar larvae in galled leaves, from galls that were collected at Bjelkesta, 25 km south of Uppsala, Sweden. Galled leaves were placed in Petri dishes and adults were collected as they emerged and then introduced to caged plants for controlled inoculations as earlier described (Larsson et al., 1995). Five females per plant were allowed to lay eggs for 24 h.

Five days after egg hatch (9 d from inoculation) one leaf, positioned in the middle of the egg-bearing leaves along the shoot axis, was examined on each plant. The number of living and dead larvae, lesions (areas with dead cells), and young galls were counted under a stereomicroscope. Data were possible to obtain from 72 out of 79 siblings; the remaining siblings died before the biotest was conducted. From six of the 72 siblings only data from one replicate was obtained owing to absence of larvae. On average, there were 30.6 (SD 29.84) larvae on the inspected leaves. In total 4250 larvae were examined with an average larval survival of 27.4%.

The resistant genotypes, i.e. genotypes without surviving larvae, were classified according to the presence of necrotic lesions. Genotypes were designated ‘Resistant Few Lesions’ (RFL) genotypes when there were fewer lesions than larvae (in many cases no lesions at all), and ‘Resistant Many Lesions’ (RML) genotypes when there were more lesions than larvae.

The consistency in expression of responses over time was investigated by repeating the biotest in 2002 and 2003 on a subsample of genotypes. Six genotypes per category (RML genotypes with the highest number of lesions per larva, RFL with zero lesions, and randomly selected susceptible (SUS)) were tested in two blocks (benches) in the greenhouse. Cuttings for these experiments were obtained from a research plot at Pustnäs, Uppsala, where overwintering from the crossing made in 2001 had been planted. The handling of plants, execution of the biotest, and origin of the midges were the same as described above, except for origin of the midges in 2003 when galled leaves were collected at Bjelkesta, 25 km south of Uppsala. Also, the number of living and dead larvae, and the number of necrotic lesions were counted on three leaves from each plant, instead of one leaf as in 2001.

Presence of ROS: a marker for the hypersensitive response

The connection between formation of necrotic lesions and production of hydrogen peroxide was investigated in a greenhouse experiment in 2003. Seven genotypes each of RML, RFL, and SUS, replicated twice, were tested regarding production of hydrogen peroxide. Stem cuttings used to produce test plants were harvested in the winter 2002/2003 from the research plot at Pustnäs and stored at 4 °C until used. The midges were collected, as pupae or third instar larvae in galled leaves, from outbreak populations at Bjelkesta, 25 km south of Uppsala. Handling of midges and plants were the same as earlier described. One plant of each genotype was inoculated on two consecutive days. Extra plants were exposed to ovipositing females at the start of the experiment in order to verify the time of larval hatching.

About 20 h after egg hatch, when the larvae on the resistant genotypes were still alive and the leaves with larvae were still furled, one leaf with larvae was removed from each plant and tested for the presence of hydrogen peroxide. The leaf was pasted on an object glass, two cuts 5 mm apart were made with a razor blade from the leaf scroll to the midrib (but not through the midrib). The scroll on that leaf piece was then carefully unrolled and glued to the glass. Only those leaf pieces having one or at the most two larvae were selected for the analysis.

A fluorescent probe (DCFH-DA, 2′,7′-dichlorofluorescein diacetate), dissolved in methanol, was employed to detect the presence of hydrogen peroxide (Wolfe et al., 2000). A detergent (Tween 20, diluted in distilled water to a concentration of 1%) was added in order for the solution to penetrate through the hairs on the underside of the leaf. A droplet (10 μl) of the probe, at a final concentration of 1 μM, was added to the selected leaf section.

After 3–6 min, the leaf section was examined. The number of greenish spots, visible in fluorescent light, were counted on a transect (about 5 mm in length) from the leaf edge to the midrib at ×50 magnification in a combined fluorescence and light microscope (Leitz dialux 20, with an attachment of Ploemopak 2.3). Immediately after, the number of lesions visible in light on the same transect was counted.

The exact time for examining leaf samples had to be based on calibration of single leaves. Production of hydrogen peroxide is triggered in the veins when leaves are removed from the plant (Orozco-Cardenas and Ryan, 1999) and found to be triggered in the trichomes as well (S Höglund, unpublished data). The background radiation from veins and trichomes that is induced because of the removal of the leaf, can influence the possibility of detecting other sources of hydrogen peroxide. In order to standardize the time period from adding the probe to examining the leaf, a mechanical wound was added with a fine needle to the leaf tissue (leaf tissue producing hydrogen peroxide in connection with wounding, (Orozco-Cardenas and Ryan, 1999). The leaf was examined when the wound was observed in fluorescence light. Data were obtained from seven genotypes of each category but not from all replicates due to the absence of larvae (n=12, 11, and 11 plants from RML, RFL, and SUS, respectively). Lesions were counted on fewer plants (n=11, 10, and 7 plants from RML, RFL, and SUS, respectively).

To exclude the possibility that the observed positive reaction from DCFH-DA staining was caused by autofluorescence from substances concurrently induced by hydrogen peroxide, a non-fluorescent probe (2 mM DAB, 3,3′-diaminobenzidine) dissolved in 50 mM TRIS-HCl buffer was used to detect the presence of hydrogen peroxide (Thordal-Christensen et al., 1999). Twenty μl of the probe was added in the same way as described earlier for DCFH-DA and incubated for 8 min. Leaf tissue was cleared in ethanol (Orozco-Cardenas and Ryan, 1999) and examined under a stereomicroscope (Leica Wild M10). The DAB-staining technique is a less sensitive method and more difficult to employ on young unfurled leaves than the DCFH-DA method and therefore, was not used throughout the investigation.

Larval performance on willow genotypes with and without symptoms

The effects of lesion formation on larval growth was studied in a laboratory experiment where the size of 30-h-old larvae reared on RML plants was compared with larvae of the same age reared on RFL plants (four genotypes of each category with three replicates). Larvae on susceptible plants were used as controls (two genotypes with three replicates). Extra plants were inoculated in order to measure the body size of newly hatched larvae (NEW), i.e. measured immediately after leaving the egg. The plants were exposed to ovipositing females during a very short period (2 h) in order to obtain as uniform a larval age as possible. After being oviposited upon, the plants were placed in a growth chamber with 18/6 h light/dark...
photoperiod, at 21/20 °C, 80% RH, and 300–600 μE m⁻² s⁻¹ light intensity. Thirty h after egg hatch the body size of six larvae was determined from one leaf per plant. Larval size was estimated by body volume. The length and width of individual larvae were measured by means of a stereomicroscope. Body volume (approximated to be a cylinder) was calculated as

$$v = l \times (w/2)^2 \times \pi$$

where $v$ is the volume; $l$ is the length, and $w$ is the width. The status of the resistant genotypes was verified by counting the number of lesions on one of the remaining leaves with larvae 10 d after egg laying. Data were obtained from four genotypes of RML ($n=12$ plants) and RFL ($n=10$) and two genotypes of SUS ($n=5$). The size of newly hatched larvae was estimated by measuring 17 larvae on three resistant plants.

**Results**

_Intraspecific variation in induced responses and larval survival_

There was great variation in neonate larval survival among sibling plants (Fig. 1). Out of 72 sibling plants, 36 (50%) had no surviving larvae, hereafter referred to as resistant genotypes. In 11 genotypes (15%), all larvae survived, they are referred to as susceptible. In the 25 (35%) remaining genotypes there were living and dead larvae on the same leaf, and these will be referred to as variable genotypes.

Different genotypes displayed dissimilar responses to larval gall initiation attempts (Fig. 2a, e). Within the group of resistant genotypes, there was variation in the number of necrotic lesions formed, despite the fact that all larvae died. The number of lesions in relation to the number of larva ranged from zero to 12.3 (Fig. 3). RML genotypes had between 1.7 and 12.3 lesions per larva ($\bar{x}=7.1$, SD=3.00, $n=21$ genotypes) compared with RFL genotypes that had between zero and 0.9 lesions per larva ($\bar{x}=0.19$, SD=0.29, $n=15$ genotypes). RML genotypes represented 58% of all resistant genotypes. On variable genotypes, the proportion of living larvae ranged from 0.05 to 0.97 and, in most cases, few lesions per larva were formed ($\bar{x}=0.89$, SD=2.42, $n=25$ genotypes). The susceptible genotypes responded with gall development (Fig. 2h) and no visible signs of necrotic lesions ($n=11$ genotypes). Galls always appeared together with living larvae.

![Fig. 2. Induced responses of Salix viminalis leaves attacked by neonate Dasineura marginemtorquens larvae. Plant responses on the resistant RML genotype (a–d) and the RFL genotype (e–g) show presence of lesions and markers for hydrogen peroxide in the case of RML and absence of lesions and markers in the case of RFL. The plant response on susceptible genotypes (h) shows formation of young galls on the underside of the leaf. Lesions were visible at the upper side of the leaf in stereomicroscope in the case of RML (a) but absent in the case of RFL (e). Green spots, indicating presence of hydrogen peroxide, were visible in fluorescence microscopy with DCFH staining in the case of RML (b) but absent in the case of RFL (f). The same tissue under light microscopy showed the presence of lesions in RML (c) and the absence of lesions in RFL (g). In the case of RFL (g) the presence of two larvae is indicated with dashed lines. Brown lesions indicated the presence of hydrogen peroxide in RML (d) with a non-fluorescent DAB staining. The presence of a young larva is indicated with a dashed line (d). Scale bars represent 0.5 mm.](image-url)
Larval density (ranging from 2–131 larvae per leaf) did not affect larval survival, or the presence of lesions (larval survival: $R^2=0.011$, $n=138$, $P>0.05$, number of lesions per larva: $R^2=0.008$, $n=138$, $P>0.05$).

There was a strong consistency in plant responses over time. Averaged over three years, the RML plants had 12 (SD=7.97) lesions per larva ($n=12$, 8, and 12 plants in 2001, 2002, and 2003, respectively) compared with the RFL plants that had 0.05 (SD=0.056, $n=12$, 10, and 12 plants); (Kruskal-Wallis Test, $H=44.34$, df=1, $P<0.001$). No living larvae and no galls were ever found on the resistant genotypes ($n=66$ plants, $n=5220$ larvae [697, 423, and 4100, in 2001, 2002, and 2003, respectively]), in contrast to susceptible genotypes ($n=12$, 7, and 6 plants in 2001, 2002, and 2003, respectively) where all larvae were alive, galls were formed, and no necrotic lesions were visible.

**Presence of ROS: a marker for the hypersensitive response**

Spots developed by adding DCFH-DA, and the fact that they were visible in fluorescence microscopy indicated the presence of hydrogen peroxide in the RML plants in connection with gall initiation attempts (Table 1; Fig. 2b). By contrast, the absence of spots in RFL plants (Fig. 2f) indicated no production of hydrogen peroxide in the symptomless responses (Table 1). Neither was there any formation of hydrogen peroxide in susceptible genotypes in connection with gall formation (Table 1). There were no differences between the number of visible lesions and the number of spots visible in fluorescent light in RML genotypes (Table 1, paired $t$-test: $T=−1.86$, $n=10$, $P>0.05$; Fig. 2b, c). The lack of spots in RFL was not due to the absence of larvae (Fig. 2g). The possibility that the spots observed with DCFH-DA staining were caused by auto-fluorescence was dismissed by the result from the DAB-staining technique. The positive reaction, dark spots visible in light microscopy (Fig. 2d), confirmed that hydrogen peroxide was induced together with lesion formation.

**Larval performance on willow genotypes with and without symptoms**

Larvae displayed negative growth on both RML and RFL plants (12% and 22%, respectively) during 30 h from egg hatch as compared with newly hatched larvae (Fig. 4) (One sample $t$-test $H_o=0.0014$; RML: $T=−4.12$, $n=12$ plants, $P<0.01$; RFL: $T=−11.44$, $n=10$ plants, $P<0.001$). Contrary to expectations, larvae on RML were larger than larvae reared on RFL (RML: $x=0.0012$ mm$^3$, SD 0.0041, $n=12$ plants; RFL: $x=0.00109$ mm$^3$, SD 0.00009, $n=10$ plants; two-sample $t$-test; $T=2.76$, df=18, $P<0.05$) (Fig. 4). As expected, larvae on susceptible genotypes had a positive growth (130% larger after 30 h) (Fig. 4). Examination of the leaves confirmed that more lesions were formed on the RML compared with the RFL plants (RML: $x=11.8$ lesions per larva, SD=5.87, $n=8$ plants; RFL: $x=0.25$ lesions per larva, SD=0.28, $n=7$ plants; Kruskal-Wallis Test $H=10.52$, $P<0.01$).

**Discussion**

This study documents great variation in Dasineura marginemtorquens larval survival (ranging from zero to 100%) among full siblings of Salix viminalis (Fig. 1). Resistant S. viminalis genotypes varied greatly in their responses (lesions per larva) to D. marginemtorquens attack (Figs 2, 3). The formation of lesions was associated with the production of hydrogen peroxide (Table 1), one key element in the hypersensitive response (HR) (Lamb and

![Number of lesions per Dasineura marginemtorquens larva on resistant genotypes of Salix viminalis (sorted after number of lesions). Each data point represents an average number of lesions divided by the number of larvae from one leaf per plant from two plants of each genotype. Vertical lines indicate standard deviation. ($n=36$ genotypes, 67 plants; 5 genotypes have only one observation)](image)

**Fig. 3. Number of lesions per Dasineura marginemtorquens larva on resistant genotypes of Salix viminalis (sorted after number of lesions). Each data point represents an average number of lesions divided by the number of larvae from one leaf per plant from two plants of each genotype. Vertical lines indicate standard deviation. ($n=36$ genotypes, 67 plants; 5 genotypes have only one observation).**

**Table 1. Number of spots and lesions, counted in fluorescence- and light-microscopy after adding the fluorescent probe DCFDA, on Salix viminalis leaves attacked by larvae of Dasineura marginemtorquens**

<table>
<thead>
<tr>
<th></th>
<th>Number of spots in fluorescence microscopy</th>
<th>Number of lesions in light microscopy</th>
</tr>
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<tbody>
<tr>
<td>Resistant genotypes with few lesions response (RFL)</td>
<td>0 a (0) $n=12$ plants</td>
<td>0 a (0) $n=11$ plants</td>
</tr>
<tr>
<td>Resistant genotypes with many lesions response (RML)</td>
<td>4.6 b (3.67) $n=11$ plants</td>
<td>5.7 b (2.71) $n=10$ plants</td>
</tr>
<tr>
<td>Susceptible genotypes (SUS)</td>
<td>0.2 a (0.40) $n=11$ plants</td>
<td>0.1 a (0.38) $n=7$ plants</td>
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Induction of lesions per se did not negatively affect larval performance. By contrast, 30-h-old larvae on resistant genotypes with lesions were, in fact, bigger than larvae on resistant genotypes without lesions (Fig. 4). In the end, however, all larvae died on both kinds of resistant genotypes.

Intraspecific variation in *D. marginemtorquens* gall density on *S. viminalis* has earlier been documented to be genetically determined (Strong et al., 1993). This study documents how the early stages in the resistance, expressed as neonate larval mortality, vary among siblings within a *S. viminalis* family. Commonly, intraspecific variation in plant quality has moderate effects on the performance of adapted insect species, for example with respect to developmental time, body size, and survival (Larsson et al., 1992; Osier et al., 2000). Most insect herbivores consume existing plant tissue, in contrast to galling insects that manipulate normal plant growth in order for a gall to develop and feeding to be possible. This difference, between a free-living insect and a gall-inducer, may explain why there is such a big effect (all or nothing) on performance in the case of neonate *D. marginemtorquens* larvae compared with free-living larvae on willow (Häggström and Larsson, 1995).

Many *S. viminalis* genotypes were indeed homogenous regarding the effects on larval performance, i.e. they were either susceptible or resistant. A fair number of genotypes (35%), however, showed intra-plant heterogeneity, i.e. living and dead larvae occurred on the same plant and even on the same leaf. This could be the result of either variable midge genotypes or variable intra-plant quality. In plant/pathogen interactions, within-plant heterogeneity is often explained by the presence of virulent and avirulent pathogens (Agrios, 1997). In the case of *S. viminalis/D. marginemtorquens*, the high correlation (Pearson 0.74, *P* < 0.001) between the first and the second test of siblings classified as variable suggests that the outcome of the interaction is more likely to be explained by intra-plant variation than intra-midge variation. At present, however, the mechanism behind the intra-plant variation is not known.

The density of induced lesions varied among resistant willow genotypes (ranging from zero to 12.3 lesions per larva) (Fig. 3). This type of lesion has earlier been interpreted as hypersensitive responses (HR) (Ollerstam et al., 2002). An intriguing question is why there are more lesions than larvae on some of the genotypes. Contrary to pathogens, gall midge larvae, although they are regarded as sessile insects, have a certain degree of mobility as neonates that allow them to attack several plant cells. The high number of lesions per larva on RML plants may reflect the number of unsuccessful attempts that larvae made in order to induce a gall before dying. But why then do RFL genotypes have very few lesions, or no lesions at all? Histological data suggest that the young larva indeed tries to induce a gall on RFL genotypes; a few damaged cells can be found on the leaf close to where the larva stays (Ollerstam et al., 2002). Therefore, it is concluded that the difference in the number of lesions does not reflect dissimilar larval behaviour on RML as compared with RFL plants, but rather is explained by plants responding differently to gall initiating attempts.

Differences in expression of HR, similar to those found in this study, have been observed in plant/pathogen interactions. In barley (three resistant lines), the proportion of HR induced by the same powdery mildew fungus race differs greatly (between 1% and 78% of the interactions induced HR) (Hückelhoven et al., 1999). In *Arabidopsis thaliana* mutants, there is great variation in their ability to express HR (no HR, mixed HR, and HR) against *Pseudomonas syringae* (Yu et al., 2000).

The difference in visible responses to larval attack among resistant willow genotypes was consistent over time; averaged over three years RFL genotypes had very few lesions (\(\bar{x}=0.05\) per larva) as compared with RML genotypes (\(\bar{x}=12\) lesions per larva). The difference in larval performance among resistant and susceptible genotypes was also consistent through the years; no larvae survived on the resistant genotypes whereas all larvae survived on the susceptible genotypes. Thus, it is confidently concluded that the induced responses associated with resistance, and the plant-mediated effect on larval performance, are inherited plant traits.

Hydrogen peroxide was produced during the formation of the HR in RML plants but not in the non-symptom response in RFL plants. Production of hydrogen peroxide is described as a biphasic event during plant/pathogen interactions. The first weak burst, which does not induce cell death, is followed, in the case of HR, by a second prolonged burst (Lamb and Dixon, 1997). A fast weak hydrogen

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**Fig. 4.** Body volume of neonate *Dasineura marginemtorquens* larvae from ‘few-lesions’ (RFL) and ‘many lesions’ (RML) resistant genotypes and susceptible (SUS) genotypes of *Salix viminalis* 30 h after hatching compared with newly (NEW) hatched larvae. Each bar represents average larval volume from six larvae per plant from four genotypes of each category (susceptible two genotypes), ± SD (RML: *n*=12 plants, four genotypes with three plants per genotype; RFL: *n*=10 plants, two genotypes with three plants, two genotypes with two plants; SUS: *n*=5 plants, two genotypes with three and two plants, respectively). Treatments with different letters differ significantly *P* <0.05.
peroxide response was not observed in the case of RFL or SUS. This could be because the method that was used did not detect a weak response in an environment with a high density of trichomes covering the reaction site. The number of trichomes did not differ among RFL, RML, and SUS genotypes (S. Högland unpublished results), and thus will not influence among-genotype comparisons with regard to hydrogen peroxide. Furthermore, because the first weak peak is regarded as a biologically non-specific reaction (Lamb and Dixon, 1997) the lack of a rapid weak response does not change the interpretation regarding HR. The absence of a prolonged oxidative burst in RFL genotypes indicates the presence of a non-hypersensitive response associated with resistance. Recently, effective pathogen resistance in the absence of virtual HR cell death has been shown in Arabidopsis dnd (defence no death) mutants (Yu et al., 1998; Jurkowski et al., 2004). Whether or not ROS is induced in these plant/pathogen interactions is not known.

The resistance was manifested as negative larval growth, as measured by larval body size 30 h after egg hatch, and at the end (48 h) the larvae died on both RML and RFL plants. Contrary to prediction, larvae on RML plants showed less negative growth than those on RFL plants (Fig. 4). This result may indicate that the mechanism of resistance acts earlier, or stronger, on the RFL than on the RML plants. A recent model of plant defence against pathogens focuses on the multitude of obstacles a pathogen has to overcome in order to cause disease within a plant (Thordal-Christensen, 2003). The model suggests that inducible cell barriers act earlier than recognition events leading to HR. Pathogens have to overcome both types of obstacle in order to attack the plant cell successfully. If this model is applicable on the D. marginemtorquens/S. viminalis system, then the non-hypersensitive response could be the first obstacle and the HR a later event that a larva has to overcome in order to induce a functional gall. The variation in larval size revealed in this study could then reflect the variation in time needed to induce different mechanisms of resistance.

Hypersensitive as well as non-hypersensitive responses associated with larval mortality have been found in the S.viminalis/D. marginemtorquens system. The resistance, defined as negative larval growth, was not strengthened by the production of hydrogen peroxide followed by rapid cell death in HR. There is considerable controversy in the literature as to whether the production of hydrogen peroxide, followed by rapid cell death, contributes to resistance, or if there are other simultaneously induced responses in HR that actually kill the attacking organism (Heath, 1999; Richard and Gilchrist, 1999). These data suggest that the production of hydrogen peroxide, and the accompanying cell death, cannot explain larval mortality in the symptomless reaction. Another, as yet unknown, mechanism of resistance has to be considered in these plants. If correct, then it is possible that this unknown mechanism also contributes to resistance in plants showing HR, and that the association between larval mortality, induction of hydrogen peroxide, and necrotic cells is spurious.

The presence of a powerful symptomless mechanism of resistance against a sessile insect in a plant species that has not been intentionally bred for resistance may suggest that this is a common phenomenon in natural plant populations. There are very few other observations on locally induced symptomless responses reported (Yu et al., 1998), none of which come from non-agricultural plants. Such a phenomenon, however, would be very difficult to observe unless, like in the present study, a plant population with enough variation in resistance traits is studied under controlled conditions.

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