

# Induced responses in willow determine feeding success of a gall-forming insect

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## *Induced responses in willow determine feeding success of a gall-forming insect*

### **Abstract**

In the willow *Salix viminalis* L. (Salicaceae), growth and defence are key responses determining feeding success of the gall midge *Dasineura marginemtorquens* Bremi (Diptera: Cecidomyiidae). The interaction includes induced growth (gall formation), and induced defence (hypersensitive response, HR). *Salix viminalis* exhibits large genotypic variation in resistance (larval mortality) against *D. marginemtorquens*.

This thesis focuses on testing hypotheses developed from field observations of interactions between the willow and the gall midge. A specific aim was to gain insight into the mechanism(s) of resistance.

Combined greenhouse and field experiments were used to test whether abiotic factors induce susceptibility in otherwise resistant genotypes at the time of gall initiation. The results suggest that plasticity in plant resistance is linked to ambient conditions, probably light.

Resistance was associated with HR, but to different degrees among willow genotypes. Hydrogen peroxide, a marker of HR, was induced in genotypes expressing HR but not in resistant genotypes not showing symptoms.

The genetic architecture of plant traits that determine larval mortality and HR was investigated by means of a QTL analysis. The presence of one particular allele at one single locus was enough to determine life or death of the gall midge.

The defence hypothesis was not supported when examining the homologous region in the poplar genome. However, an auxin gene was located within the QTL area, lending some support for the starvation and anti-manipulation hypothesis.

The place of birth (leaf position) was found to be important for insect fitness, and the quality of a gall seemed to be associated with the duration of the site as a resource sink.

In conclusion, data suggest that the mechanism of resistance involves anti-manipulation of growth rather than defence. Such, nonreactivity genes may have pleiotropic effects, and the frequency of genes insensitive to manipulation might be restricted in nature due to selection compared with the gall stimulus phenotype.

*Keywords:* *Salix viminalis*, gall midge, HR, fitness, QTL, developmental resistance, hypersensitive response, defence, growth, plant-insect interaction.

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## Dedication

To myself, for finding an alternative explanation...

*Från det Stora Ovan lade Inanna sitt öra mot det Stora Nedan*

Tolkning av Gunilla Hultgren

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## List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

- I Höglund S, Larsson S (2005). Abiotic induction of susceptibility in insect-resistant willow. *Entomologia Experimentalis et Applicata* vol(115), 89-96.
- II Höglund S, Larsson S, Wingsle G (2005). Both hypersensitive and non-hypersensitive responses are associated with resistance in *Salix viminalis* against the gall midge *Dasineura marginemtorquens*. *Journal of Experimental Botany* vol (56) no 422, 3215-3222.
- III Höglund S, Rönnerberg-Wästljung A-C, Lagercrantz U, Larsson S  
A rare major plant QTL determines resistance against a gall-forming insect in willow (Submitted manuscript).
- IV Höglund S. Birthplace determines fitness of a galling insect on willow. (manuscript)

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Related publication

Ollerstam, O., Rohfritsch, O., Höglund, S. & Larsson, S. 2002. A rapid hypersensitive response associated with resistance in the willow *Salix viminalis* against the gall midge *Dasineura marginemtorquens*. *Entomologia Experimentalis et Applicata* 102, 153-162.





# 1 Introduction

The basket willow *Salix viminalis* L. is a rapidly growing plant that has, in recent years, been used in short rotation forestry for biomass production. The gall midge *Dasineura marginemtorquens* Brems (Diptera, Cecidomyiidae) is commonly found in plantations, and can occasionally attain population levels high enough to reduce plant productivity. The interaction between *S. viminalis* and *D. marginemtorquens* includes phenomena that, in general, are central to our understanding of the resistance and susceptibility of plants to insects and pathogens.

Induced plant responses in the basket willow are associated with susceptibility and resistance to the gall midge. The interaction between these two organisms is complex and includes symptoms known to enhance insect performance by gall formation, as well as those that reduce it through the hypersensitive response (HR). Resistance is expressed as the rapid mortality of neonate larvae at the time of gall initiation. Thus, larval mortality may either be due to a noxious diet, or the absence of a functioning gall leading to starvation.

The aim of this thesis was to further test hypotheses based on observations of the interaction between the willow and its specialized gall-forming insect. A specific aim was to better understand the mechanism(s) of resistance in this gall midge – host plant system.

## Induced plant responses in *Salix viminalis*

Growth – gall formation



Defence – hypersensitive response (HR)



## 2 Background

### 2.1 Scientific models

The distribution and abundance of species in nature is a matter of great concern. Depending on socio-economic or ethical considerations, we may consider some species to be too abundant, i.e. as pests; others too rare, i.e. to be in danger of extinction. Two sub-disciplines of biology, viz. ecology and plant pathology, emphasize the necessity of understanding interactions between organisms. Ecology is the scientific study of interactions among species and their environments, whereas phytopathology focuses on a sub-sample of those interactions, namely those interactions that impair the normal functioning of the host plant. However, ecology and plant pathology have each developed from different historical backgrounds and belong to different thought collectives, which can influence the ways in which phenomena are interpreted (Fleck, 1935).

The concepts used in ecology are related to those used in economics. In each of these disciplines, trade-off is a fundamental idea in which the loss of one quality, or aspect of something, is traded-off against the gain of another. Such a trade-off may often be seen in plants, which, under certain conditions, may be considered as having a ‘choice’ to expend resources in either growth or defence (Coley *et al.*, 1985); a choice that, indirectly, will have consequences for the performance of an attacking organism (but cf. Mooney *et al.*, 2010).

Phytopathology is the study of plant diseases and is a field of research closely related to medicine, but which focuses on protection. Plant defences rely on the presence of plant chemicals and physical barriers that limit and

restrict the establishment and growth of disease organisms and parasites. The sessile life style of plants makes them particularly vulnerable to attack from such organisms, compared with other life-forms that are able to escape from their enemies (Howe & Jander, 2008). The need to withstand attack from diseases and pests suggests that resistance is very common in nature (Bolter & He, 2009) and the innate responses of plants towards pathogens and herbivores are comparable to the immune system developed by animals against infectious diseases (Pieterse *et al.*, 2009).

The ecological/economical model, and the pathological/medical model, which focus on trade-offs and protection, respectively, have each developed different concepts to describe the participating organisms and the outcomes of interactions between them. The present investigation of the mechanism of resistance in the *S. viminalis* / *D. marginemtorqueens* system deals with plant traits associated with both the ecological and the pathological model. Although it may be questioned how well these models contribute to our understanding of the outcomes of this interaction, the key point is to discriminate between two alternative hypotheses that may explain the mechanism of resistance: an inability of the insect to attract food resources to its site of feeding, which is linked to plant growth; and the noxiousness of the food resource, which is linked to the plant's defence.

## 2.2 Plant growth

Growth in plants is a response governed by light, water, and nutrients, with overall growth being regulated by the availability of the scarcest resource according to Leibig's 'Law of the Minimum' (Austin, 2007; Junk, 2009). The rate and timing of growth are triggered and controlled through various external environmental signals that are transmitted between and within cells (Wolters & Jürgens, 2009). The three-dimensional organisation of a plant, in terms of its architecture, represents the pattern by which resources have been allocated to the various tissues within the plant (Weiner, 2004). It is genetically determined and regulated at numerous levels through such processes as apical dominance, meristem determinacy, and the differential growth of stems (Wang & Li, 2008). A plant's modules (Harper, 1977), such as the leaf, develop as part of a dynamic process with young, heterotrophic leaves acting as sinks dependent on carbohydrates imported from other parts of the plant (Turgeon, 2006), while mature, autotrophic leaves photoassimilate an excess of carbohydrates for transport to other parts of the plant (Turgeon, 1989). The transition of tissues from being sinks to sources

begins shortly after the leaf has begun to unfold, and starts at the leaf apex (Turgeon, 1989).

Cell growth is largely a property of the plant's cell wall (Cosgrove, 2005). The cell's internal turgor pressure is thought to drive all growth (Uyttewaal *et al.*, 2010) by stimulating cell wall synthesis and remodeling so allowing the cell to increase in size (Uyttewaal *et al.*, 2010). Thus, at the cellular level, this mechanical feedback between cell wall expansion and turgor pressure is central to growth (Geitmann & Ortega, 2009). Reactive oxygen species (ROS) are known, among other molecules, to affect cell wall remodeling, and to regulate cell wall plasticity and growth rates (Cosgrove, 2005). Phytohormones also regulate many aspects of plant growth and development (Wolters & Jürgens, 2009). Auxins, have been implicated in the regulation of plant architecture as well as being associated with the loosening of cell walls, cell expansion, and organ development (Benjamin & Scheres, 2008).

### 2.3 Plant defences

Unlike the products of primary metabolism common to all plants, secondary metabolites differ markedly among plant species (Bennet & Wallsgrove, 1994). Secondary metabolites are often important regulators of biochemical processes (Seigler & Price, 1976) and many are highly toxic and thus act as defensive agents against insects and other attacking organisms (Bennett & Wallsgrove, 1994; Chen, 2008). There is generally considered to be a trade-off between growth and the production of secondary chemicals for defence (Coley, *et al.*, 1985; Herms & Mattson, 1992). Inherently fast-growing plant species tend to inhabit resource-rich environments and have lower levels of chemical defences compared with inherently slow-growing species (Coley *et al.*, 1985). The duration of growth is genetically pre-determined and is classified into two main types: indeterminate growth, in which growth is not terminated; and determinate growth, which ceases once a structure has been completely formed, i.e., before external factors such as frosts terminate any plant growth (Barthélémy & Caraglio, 2007). The initiation and duration of growth during the phenological development of a plant have important influences on the performance of insects feeding on it (Yukawa, 2000).

## 2.4 Induced plant responses

### *Gall formation*

Some locally induced plant responses deviate from the normal pattern of plant development. The formation of galls, or tumours, by insects, fungi, or bacteria, is one such response in which the plant produces an abnormal tissue growth in response to stimuli from the attacking organism. Galls are an expression of a particularly close association between an attacking organism and the plant; so close that galling insects, such as *D. marginemtorquens*, cannot survive without a functional gall. For gall-forming insects, the gall enhances food availability and quality, provides shelter from harsh abiotic conditions, and affords protection from natural enemies (Price *et al.*, 1987). Many galling insects are economically important crop pests (Harris *et al.*, 2003).

### *The hypersensitive response*

The hypersensitive response (HR) is another plant response that diverges from a plant's normal pattern of growth. With HR, dead plant cells appear to be associated with restricted growth of the pathogenic organism (Heath, 2000). The invading organism triggers the HR, which benefits the plant because the HR cascade restricts further development of the inducing agent, although the consequence for the plant is the death of a few cells (Richael & Gilchrist, 1999). However, the cause and effect relationship between cell death and the restriction of pathogen growth is not clear (Richael & Gilchrist, 1999). The production of ROS occurs early in HR. Besides being toxic, ROS also function as signalling agents regulating many biological processes and are important modulators of programmed cell death (PCD) (Lam, 2004). The rapid death of host cells that are associated with resistance is known to occur in response to microorganisms (Heath, 1998) and to insects (reviewed by Fernandes, 1990; Ollerstam *et al.*, 2002).

## 2.5 The plant as a food resource

The nutritional requirements of herbivorous insects are similar to those of other animals, although they may differ greatly among insect species and developmental stages (Schoonhoven *et al.*, 1998). Primary and secondary metabolites fundamentally affect the food quality of plants to insects: primary metabolites, such as nitrogen and carbohydrates, being crucial for insect growth (Schoonhoven *et al.*, 2005) as is water (Henriksson *et al.*, 2003),

while secondary metabolites, such as phenolics, terpenoids, and alkaloids, affect insects in a number of diverse ways. For example, for insects that specialize on a certain type of plant, secondary metabolites may guide the insect to the plant and act as feeding stimuli (Chapman, 2003). However, many secondary compounds are more or less toxic depending on an insect's ability to digest, metabolize, sequester or secrete them, processes that may lead to corresponding reductions in growth and survival (Rosenthal & Berenbaum, 1992; but cf. Carmona *et al.*, 2010).

## 2.6 The interaction of *S. viminalis* and *D. marginemtorquens*

*Salix viminalis* exhibits a large degree of genotypic variation in resistance against *D. marginemtorquens*. In a common-garden experiment, Strong *et al.* (1993) found certain genotypes to host only a few galled leaves, whereas other genotypes had 20-30 galled leaves per shoot. It was found that the lack of galls on certain genotypes could not be explained by midges discriminating against them but rather by different mortality rates among neonate larvae (Larsson & Strong, 1992). On resistant genotypes, necrotic lesions, which are interpreted to be caused by a hypersensitive response (HR), are induced by the larvae (Ollerstam *et al.*, 2002). On other resistant genotypes, however, no visible plant responses occur (Ollerstam *et al.*, 2002).

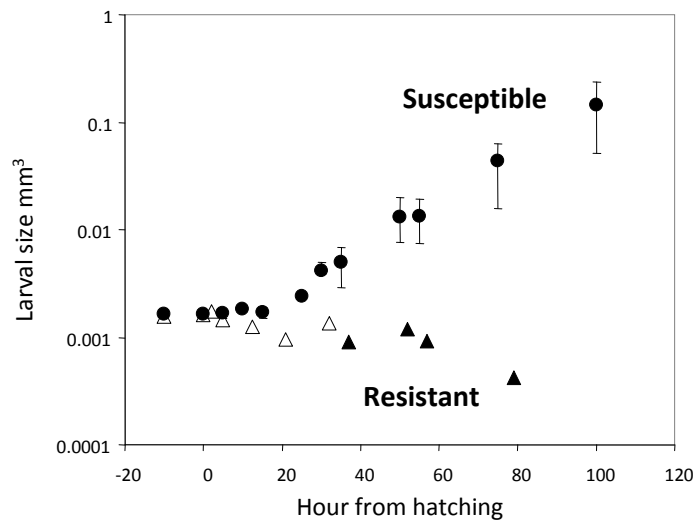


Figure 1. Size development of neonate *Dasineura marginemtorquens* larvae on susceptible (dots) and resistant (triangles) *Salix viminalis* genotypes. Open triangles indicate living larvae; filled triangles indicate that all larvae are dead. Adapted from Ollerstam *et al.* (2002) Ent. Ex. Appl.



### 3 Aims of the present study

The aim of the present study was to further test hypotheses based on observations of the interaction between the willow and its specialized gall-forming insect. A specific aim was to better understand the mechanism(s) of resistance in this gall midge – host plant system. The following are short introductions to each of the four papers included in the thesis.

#### *Abiotic induction of susceptibility in insect-resistant willow (Paper I)*

The observation of high, but incomplete, resistance formed the basis for Paper I. Specifically, the aim was to determine which factors underlie the putative plasticity of the suitability of leaves for gall formation on resistant genotypes. In the experiment reported by Strong *et al.* (1993) the number of galled leaves varied greatly among genotypes. However, most of the resistant individuals still harboured a small number of galls on a few leaves. Thus, resistant genotypes were not completely immune. Paper I reports the testing of the hypothesis that random biotic or abiotic events induce a susceptibility to attack in willow shoots that is expressed later, e.g. when leaves develop from the shoot in the following season. A second experiment combined field and greenhouse experiments to test the possibility that susceptibility is induced by abiotic factors acting on the target leaves immediately before, and during, gall formation, i.e., that susceptibility is rapidly induced.

#### *Both hypersensitive and non-hypersensitive responses are associated with resistance in *S. viminalis* against the gall midge *D. marginemtorquens* (Paper II)*

The aim of the study reported in Paper II was to better document some preliminary observations of a range of resistance responses in *S. viminalis* against *D. marginemtorquens*. The distributions of living and dead larvae, and

induced plant responses, were recorded by means of biotests conducted on 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 by using hydrogen peroxide as a marker for HR. Finally, differences in the rate at which any resistance might be expressed was investigated. It was hypothesised that the response of gall midge larvae on genotypes with HR would be a reduction of size and more rapid death, when compared with larvae on resistant genotypes lacking HR symptoms, i.e., genotypes that did not form lesions or produce hydrogen peroxide.

*A rare major plant QTL determines mortality of a gall-forming insect in willow (Paper III)*

In Paper III the mechanisms that underlie the remarkable degree of variation of resistance expressed by *S. viminalis* against *D. marginemtorquens* were studied. Specifically, quantitative trait locus (QTL) analysis was used to investigate the genetic architecture of plant traits that determine larval mortality of gall midges, and HR expression. The main goal was to estimate the number of QTLs that determine larval mortality and HR, and from the estimated positions of the QTLs on the chromosomes, to infer whether resistance is caused by starvation due to a failure to initiate gall formation, i.e. anti-manipulation; or by plant defence reactions rendering the plant noxious to the midge. In addition, a biotest was performed in order to determine the frequency of highly resistant genotypes in a nearby willow population.

*Birthplace determines fitness of a galling insect on willow (Paper IV)*

The aim of the study reported in Paper IV was to investigate the conditions that determine the performance of the gall midge on leaves of susceptible genotypes. The main objective was to investigate the extent to which the growth of apparently suitable leaves for gall initiation varies within shoots, and if so, whether this variation affects insect fitness. Specifically, the aim was to understand whether a temporal decline in leaf suitability might be explained by one of the alternative hypotheses: developmental resistance or diminishing growth.

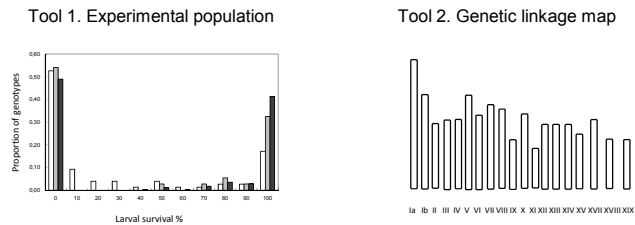
## Study organisms

The gall midge *Dasineura marginemtorquens* is monophagous on *Salix viminalis*. The 2-3 mm midge is an ephemeral insect, whose larvae induce galls on *S. viminalis* leaves. The life cycle takes about three weeks, with the midge able to complete 3-4 generations per year in Sweden (Glynn & Larsson, 1994). The female lays 20-50 eggs along the midribs of unfurled leaves in the leaf bud. Several young leaves per shoot are accepted as oviposition sites each day (Larsson & Strong, 1992). Several females can lay eggs on the same leaf at the same time. After 3-5 days, depending on the temperature, the eggs hatch and the first instar larvae crawl into the scrolls of the unfurled leaves where they induce a gall in the young leaf tissue. The mature gall is a 5-10 mm pocket formed from enlarged plant cells (Mani, 1964) along the leaf margin. Each leaf can harbour up to 60 galls, and at high densities the leaf becomes twisted resulting in a reduced photosynthetic leaf area.

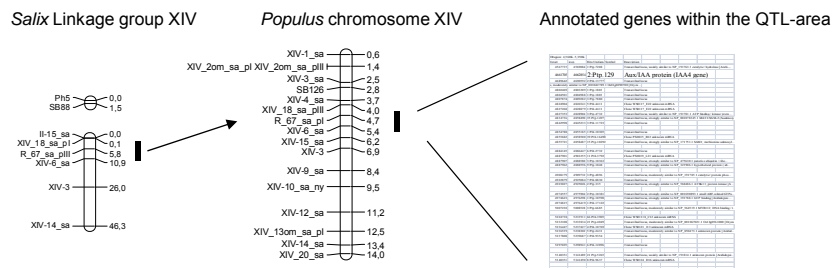
*Salix viminalis* grows along the banks of streams and lakes in southern Sweden. During the last 25 years *S. viminalis* has been used in short rotation forestry to produce biomass (Ledin, 1992). Although introduced into Sweden from eastern Europe and central Asia (Jonsell, 2000), it has spread and become part of the natural vegetation. The first record of *S. viminalis* in Sweden dates from the eighteenth century (Leche, 1774; referenced in Hylander, 1971). *Salix viminalis* grows rapidly and produces new leaves throughout the growing season. When cultivated for biomass production, 15 cm long cuttings are planted in rows at a density of one cutting per m<sup>2</sup>. Each rooted cutting produces several shoots known as a stool. *Salix viminalis* can easily be propagated from stem cuttings, and is thus an ideal plant in experimental research.

# From herbivore abundance to candidate gene

1. Observation: Genotypic variation in herbivore abundance
2. Question: What is the mechanism of resistance (larval mortality)?
3. Method: Estimating the genetic architecture of resistance by QTL-analysis



4. Results: One R-allele (on LGXIV) responsible for ≈70% of the variation in larval mortality



5. Conclusion: Biased, linked to scientific model → Presence of defence- noxious diet ?  
Absence of growth initiation – starvation ?

Figure 2. From variation in herbivore abundance to candidate gene. The investigation of the interaction between *Salix viminalis* and the gall midge *Dasineura marginemtorquens* (Paper III). 1. Observation: Outbreak in a common-garden experiment (Strong *et al.* 1993). 2. The low density of galls on certain genotypes due to neonate larval mortality (Larsson & Strong, 1992). 3. Quantitative trait locus (QTL) analysis; tool A: Experimental population with suitable phenotypic variation, tool B: Genetic linkage map of *Salix*. Markers developed by using the Populus genome as a reference (Berlin *et al.*, 2010). 4. Results of the QTL analysis: One major QTL on LG XIV. About 100 genes in the poplar genome within the QTL area. 5. Conclusion: Associated with scientific model. Presence of an auxin/IAA gene within the QTL-area.

## 4 General methods

The basic method used to study the interaction between gall midge and willow was a biotest, in which plant quality was evaluated according to its impact on larval mortality. To this end, specific willow plants were crossed in order to obtain a high number of offspring with the desired phenotypic variation necessary for certain experiments. A genetic linkage map that had been previously constructed for this experimental population (Berlin *et al.* 2010) was used to investigate the genetic background of gall midge resistance and HR as described in Paper III. The genetic markers used to construct the linkage map were developed from the poplar genome. Biomarkers were used in order to document the presence of hydrogen peroxide in the HR and the symptomless response (Paper II).

The experimental plant population was created by crossing two diploid *S. viminalis* genotypes [78-0-195 (female) and 81-0-084 (male)]; the genotype numbers refer to the classification used within the Swedish Energy Forest Project (Sirén *et al.*, 1987)]. This particular family was included also in the study by Strong *et al.* (1993), (there labelled # 46) who found it to exhibit high levels of variation in its resistance to *D. marginemtorquens* among the six tested siblings. The crosses in the present study were conducted on two occasions, one in 2000 resulting in 72 offspring, and another in 2004 resulting in 210 offspring, thus giving a total full-sib family of 282 individuals. The crosses were performed at the Department of Plant Breeding and Forest Genetics, SLU. The plants were used to establish a plant archive at Pustnäs, 1 km southeast of Uppsala.

Naturally growing willow trees along the River Fyris just south of Uppsala were investigated in order to record the frequency at which resistant genotypes occur naturally. This was an admixed population of

diverse geographic origin (Lascaux *et al.*, 1996). The plants were maintained in an archive at the Department of Plant Biology and Forest Genetics, SLU, Uppsala.

Over a number of years, our research group at the Department of Ecology (formerly the Department of Entomology) SLU has developed a reliable protocol for the biotest. The protocol includes methods of growing the plants, locating fields where galls naturally occur on willows, techniques for collecting galled leaves and handling midges, and a body of knowledge describing how and when to collect data on larval performance and induced plant responses.

Handling of plants: When used in greenhouse experiments, cuttings were planted in pots filled with soil (“Hasselfors Garden Special”) and given a supply of optimal nutrient solution (Wallco 51-10-43+ micronutrients, 100 mg N l<sup>-1</sup>), the technique of nutrient supplying being adapted to the maximum growth rate (Ingestad, 1987). Plants were watered and fertilized daily until they were about 50 cm high.

Handling of midges: Midges used in biotests were collected in the field as pupae or third instar larvae in galled leaves, from natural populations around Uppsala. Due to variation in gall midge population densities among fields and years, galled leaves were collected from different fields in different years. In the laboratory, galled leaves were kept in Petri dishes with moist filter paper. After emerging, the adults were caught with an aspirator, and transported to experimental cages covered with muslin in which female midges were allowed to oviposit for 24 hours. The prevalence of virulence among midge populations (Harris *et al.*, 2003) is a potentially confounding factor. We investigated this possibility in a separate experiment where, based on earlier experience, willow genotypes known to be resistant or susceptible were exposed to ovipositing midges from the sampled populations.

## 5 Results and discussion

### 5.1 The mechanism of resistance

In order to understand the mechanism of resistance in this system, and in particular which plant traits contribute to rapid larval mortality, the following two alternative hypotheses were addressed: 1) the food quality hypothesis; and 2) the starvation hypotheses.

The food quality hypothesis states that larvae are rapidly killed because of a noxious diet, and thus there is no time for a gall to develop. This hypothesis is supported by data from other systems in which plant quality has been widely accepted as a factor that explains performance variability among herbivores (Schoonhoven *et al.*, 2005). In our system, the presence of HR in certain resistant genotypes seemingly supported the defence hypothesis, but at the same time it was found that the expression of HR varied among genotypes that were nevertheless still resistant. Thus we suspected that resistance might be caused by several factors. Rapid cell death, however, is a very common mechanism of resistance both in animals and plants (Lam, 2004). Therefore, we have conducted intensive investigations to discover whether or not HR is a major driver in this system by using several approaches: i) the use of biomarkers in order to record the presence of toxic products (hydrogen peroxide) that accompany HR; ii) testing whether resistance, in terms of larval mortality, is more effective if HR is present; and iii) examining whether QTLs associated with resistance and cell death are co-located. The data suggest that the production of hydrogen peroxide, and the accompanying cell death, cannot be the only explanatory factors for resistance (Paper II). Contrary to our prediction, larvae lost weight faster on genotypes with a symptomless response than on genotypes

with HR, although ultimately larvae died on both kinds of resistant genotypes. Thus, the production of hydrogen peroxide, and the accompanying cell death, cannot explain larval mortality in the case of a symptomless reaction. The QTL study revealed that the life or death of this gall midge was determined by a major R-allele at a single locus (Paper III). Furthermore, suppression and not stimulation of HR seemed to be the trait that was co-located with resistance according to the QTL analysis. We found no support for the defence hypothesis when examining the homologous region in the poplar genome (Paper III). Genes with a known defence function in poplar were not present in the target QTL area.

The starvation hypothesis implies that a larva starves to death because it is unable to attract resources when gall initiation fails. In a susceptible interaction, the enlarged cells designated as nutritive tissue cells characterise gall initiation (Rohfritsch, 1992), and provide *D. marginemtorquens* larvae with food (Ollerstam *et al.*, 2002). However, much less is known about how nutritive tissue associated with gall formation is induced, than is known about the conventional aspects of food quality. When examining the homologous region in the poplar genome, an auxin/IAA (indole-acetic acid) gene was located within the QTL area, so lending some support for the starvation hypothesis (Paper III). Auxin is a plant hormone that controls cell elongation (Christian *et al.*, 2006) and the initiation of plant organ development (Benjamins & Scheres, 2008), processes that are of the utmost importance for gall formation (Stone & Schönrogg, 2003).

Because of the very small size of the midge larva, physical barriers such as leaf toughness and trichome density might render sensitive areas difficult to reach and so the midge from successfully initiating gall formation. Contrary to this hypothesis, tougher leaves and higher trichome densities were associated with induced susceptibility in otherwise resistant genotypes (Paper I).

To summarize, experimental evidence suggests that the mechanism of resistance represents non-responsiveness and acts through starvation rather than through a noxious diet. The major QTL was the result of one male allele, which had a decisive impact on larval survival (Paper III). The large difference in effect between male alleles (88 %), and the low value of the interaction term (4 %), suggests that the major mortality QTL (the R-allele) represents a dominant trait. Thus, the presence of one particular allele at a single locus is enough to determine the life or death of a specialized insect on this host plant.



## 5.2 Phenotypic plasticity

We investigated whether or not the induction of susceptibility could account for the occasional presence of galled leaves on resistant genotypes. We found support for rapidly induced susceptibility, but not for delayed induced susceptibility (Paper I). Delayed induced susceptibility results from abiotic or biotic events occurring in the season(s) that precede the time of attack, and may thus affect future herbivore generations (Haukioja, 1990; Karban & Baldwin, 1997). Although we found support for the rapid induction of susceptibility, resistance remained strong. Significantly more leaves with surviving larvae and galls were found on potted plants that had been moved to the field, than on identical plants that had remained in the greenhouse. The susceptibility must therefore have been induced by an environmental factor experienced during the two weeks when the pots were positioned in the field. Thus, we can exclude soil moisture, nutrient availability, and the plants' history as factors explaining the induction of susceptibility. The question remains as to what kind of abiotic conditions could have been associated with induction of susceptibility in this case. One of the main differences between the greenhouse and field environments was light quality. We therefore tentatively suggest that the level or quality of radiation interferes with the expression of resistance, and that plasticity may be linked to the ambient light conditions.

The repeated QTL analysis revealed that certain mortality QTLs were only present in one of two investigated years, suggesting that these QTLs represent induced resistance traits (Paper III). The interaction between these QTLs, as well as the alleles within the QTLs, suggests the existence of a complex genetic pattern. Variable genotypes, i.e. plants with both living and dead larvae, seemed to be common among naturally growing *S. viminalis*; 19 out of 73 individuals were classified as being variable (Paper III). Our results suggest that variable resistance might be the effect of an interaction between two QTLs, and when present, have substantial effect on the larval mortality of this gall-forming insect.

The degree to which insect resistance in natural plant populations is plastic is not well understood. For most of the time truly resistant individual plants will harbour very few, if any, insects that can be observed, and are thus difficult to study. Hence, data on resource heterogeneity tends to relate exclusively to susceptible plants, and refer primarily to variation caused by a decrease in suitability (Schultz, 1983; Agrawal & Karban, 1999; Orians & Jones, 2001). We have shown that certain genotypes are partly resistant with high levels of variation in resistance, but also that highly resistant plants are

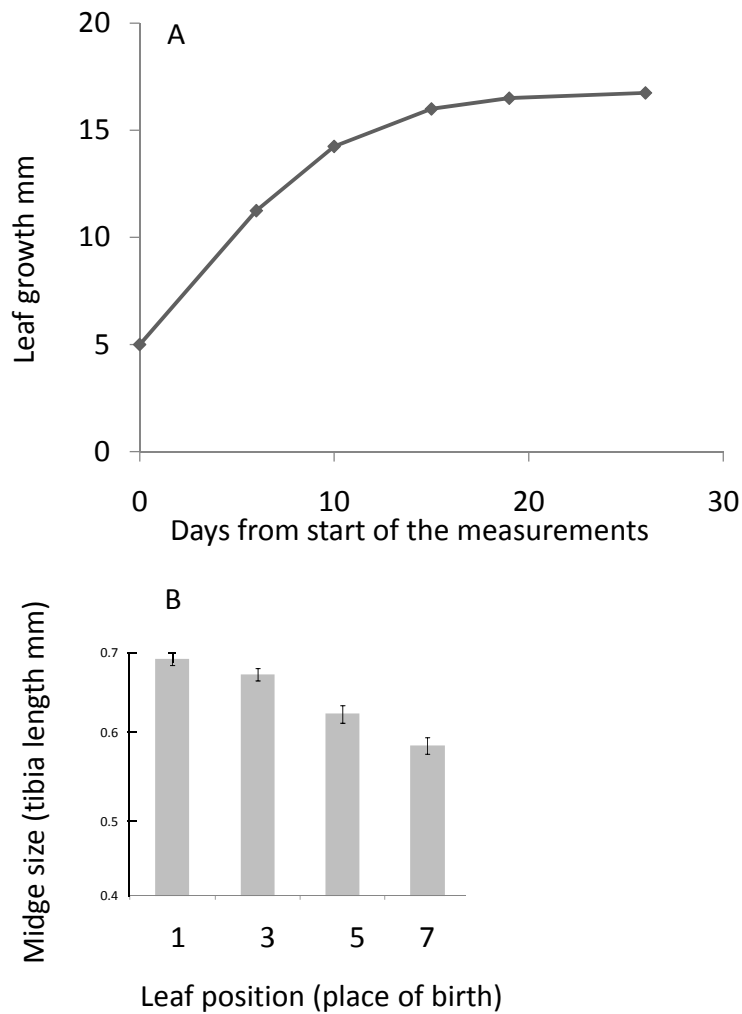


Figure 3. The association between the developmental growth of a young *Salix viminalis* leaf and the fitness of the gall midge *Dasineura marginemtorquens* (Paper IV). A. The growth of a young developing leaf from its beginning in the leaf bud. Two marks, 5 mm apart, were made to the base of the leaf margin at the start of taking measurements.

B. The tibial length of the gall midges developed from leaves at different positions, along the shoot of susceptible *Salix viminalis* genotypes. Leaf position 1 corresponds to the position of the young leaf at the start of the experiment in Fig. 2A.

plastic in their response and that under certain environmental conditions susceptibility can be induced, even though in this case resistance was still very high.

### 5.3 The distribution of resistance in nature

Serendipity played a role in the discovery of the large effect that those heritable traits seen in *S. viminalis* had on the population density of *D. marginemtorquens*. An outbreak of the gall midge occurred in a common-garden experiment (Strong *et al.*, 1993). Our further investigations, based on the results from the QTL analysis and the biotest of naturally growing *S. viminalis* individuals, showed that there were three types of plant response to the same external stimulus of an attempt to initiate gall formation by *D. marginemtorquens* larvae: i) unconditional susceptibility – all attempts to modify plant growth were successful, which is the most common situation in nature; ii) unconditional resistance (single gene) – most of the attempts to modify plant growth were unsuccessful, which is very rare in nature; and iii) environmentally induced resistance (interaction between two loci) – which is more common in nature than qualitative resistance (Paper III).

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 more common in natural plant populations than had been previously thought. Insect-plant systems with R-genes are generally thought to be rare in nature (Mitchell-Olds & Bergelson, 2000). An intimate physiological relationship between the insect and its host seems to promote the incidence of R-genes (Mattson *et al.*, 1988; Yencho *et al.*, 2000). Major genes affecting insect resistance in plants have mainly been identified as acting against species within the orders Homoptera and Diptera (Cecidomyiidae) (Yencho *et al.*, 2000). Because most R-genes have been found in crop plants, rather than in wild plants, it has been suggested that human selection has had a strong influence on shaping crop resistance traits (Heath, 1991). This suggestion, however, might be biased because resistance is mainly sought in agricultural systems (Harris *et al.*, 2003). Our results demonstrate that a major R-gene against a gall midge is rare, but that it does occur in a plant population that has not been intentionally selected for resistance against insect attack.

## 5.4 Growth and defence

Growth and defence are key plant traits that determine the success of an attack by herbivorous insects (Herms & Mattson, 1992). Induced growth responses, i.e. gall formation, improve food quality for the insect in comparison with unmodified plant tissue (Price *et al.*, 1987). HR, a commonly suggested defence trait, is associated with resistance against various pathogens (Agrios, 1997) and insects (Fernandes, 1990). The rapid temporal decline of leaves within shoots as suitable food, can be explained by a developmental resistance linked to defence (Kearsley & Whitham, 1989). The question remains as to what the alternative interpretation might be for those plant traits associated with reduced insect fitness, which are commonly thought to be associated with defence, i.e. HR and the developmental resistance of *S. viminalis* against *D. marginemtorquens*. Ollerstam *et al.* (2002) found that young *D. marginemtorquens* larvae on resistant *S. viminalis* genotypes react by losing size and dying after 48 h, whereas on susceptible genotypes larvae grow exponentially (Fig. 1). The induction of gall formation and HR, involve modification of corresponding plant cells (Ollerstam *et al.*, 2002). The target leaf tissue responds with enlarged (gall) or dead cells (HR) in susceptible and resistant genotypes, respectively (Ollerstam *et al.* 2002), which implies that the cellular response in both cases has a common ground. An interesting possibility is that the symptom expressed as HR could be a first step in a chain of intercellular communications linked to the initiation of nutritive tissue that, for some reason, fails to develop into a functional gall. Thus, in this system unsuccessful manipulation seems to be associated with gall midge resistance, and a gene involved in auxin synthesis may be a key factor behind the growth effect (Fig. 2) since auxin is involved in virtually every aspect of plant growth and development (Santner *et al.*, 2009). Thus, in addition to a role in gall initiation, auxin is likely to influence other growth processes. Indeed, the existence of such an interaction in this system is suggested by evidence from a field experiment in which genetic correlations were found between general growth traits linked to plant architecture (main stem length and number of stems) in the willow and resistance to *D. marginemtorquens* (Rönnerberg-Wästljung & Gullberg, 1999). Trade-offs between plant architecture and susceptibility to insect attack are known from other systems. For example, the Asian rice gall midge was considered to be a minor pest of rice until the mid 1960s when the “Green Revolution” in rice occurred in

Asia. The new high-yielding rice varieties were, and continue to be, highly susceptible to the Asian rice gall midge (Harris *et al.*, 2003). The new varieties, having an altered response to a plant hormone, are shorter, giving an increased grain yield at the expense of straw biomass (Peng *et al.*, 1999).

Developmental resistance is a common and predictable change in those traits that relate to the ontology and aging of a plant (e.g., Kearsly & Whitham, 1989). The study on susceptible willow shoots of the effect of leaf position on insect fitness, revealed that the feeding success of *D. marginemtorquens* was determined by the relative position of the galled leaves (Paper IV). The position on a plant where a larva hatched from its egg affected those life history traits important for the insect's capacity to disperse and propagate, such as staying alive until reproduction, developmental time, and final adult size. The declining trend of insect fitness coincided with the pattern of declining growth rates among leaves within shoots. This pattern of diminishing growth rates of leaves might explain the trend of declining insect fitness among leaves, since larvae on leaves at the lower positions might receive less nutrition at the end of the feeding period, compared with larvae that develop on leaves at higher positions along the shoots (Fig. 3).

Data suggest that leaf growth dynamics, but not leaf size, were associated with variation in insect fitness because there was no heterogeneity in leaf size among leaves within shoots. Plant module size is commonly assumed to be positively related to the fitness of galling insects (Price, 1991, but see Björkman, 1998). Indeed, shoot length has previously been shown to be associated with gall midge fitness in the system under study here; *D. marginemtorquens* midges developing on vigorous *S. viminalis* shoots in the field have been shown to be bigger than those developing on suppressed susceptible shoots (Glynn & Larsson, 1994). Intra-plant variation in midge size suggests other factors, as important as plant growth, may also determine insect fitness (Paper IV). The quality of galls, as estimated by insect fitness, seems to be associated with the duration of leaves as plant sinks rather than their final module size per se (Paper IV). Thus, the performance of *D. marginemtorquens* is likely to be related to processes associated with normal plant growth, in particular the transition of leaves from sink tissues to source tissues (Turgeon, 2006).

## 5.5 Conclusions

The interaction between the rapidly growing *S. viminalis* and the gall midge *D. marginemtorquens* is characterised by traits related to growth. Plant growth responses seem to be essential, irrespective of whether plant susceptibility or plant resistance is considered. In susceptible genotypes, insect fitness is linked to the natural processes of growth. The QTL analysis and the putative genes in the mortality QTL area suggest that the mechanism of resistance is linked to a plant hormone connecting gall midge resistance with plant architecture. The unusually strong resistance that we recorded in *S. viminalis* is difficult to understand in terms of it being an evolutionary response to resist attack by *D. marginemtorquens*. High population densities of the gall midge are rare in nature due to the controlling power of natural enemies (Stig Larsson, unpublished). Therefore, at least under present conditions, the selection pressure the midge exerts on the willow should be modest, although when grown more intensively as a crop, high gall midge densities can occasionally occur to the extent that they severely reduce plant production (Larsson & Höglund, unpublished). In conclusion, our data suggest that the mechanism of resistance involves anti-manipulation rather than defence. Because galling insects cannot survive without inducing a functioning gall, the ultimate defence of a plant should not be to respond to gall initiation attempts. However, such non-reactivity genes may have pleiotropic effects on the plant, and therefore the frequency of genes insensitive to manipulation might not be favoured by selection in natural populations compared with those that result in a phenotype that responds to the gall-forming stimulus (Weis *et al.*, 1988). Given that non-reactivity is an important resistance mechanism, current data, together with earlier results, suggest that genetic variation in resistance to galling insects is difficult to discover in natural plant populations.

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