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Induced Resistance in Basket Willow Against a Gall Midge

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Abstract

Plants are defended against herbivores and pathogens by means of a vast array of mechanisms such as physical obstacles, chemical defences, phenological escape, and attraction of predators. Plant defence mechanisms may be present independently of the enemy attack, *i.e.*, constitutive, or initiated by the activity of the enemy, thus referred to as induced resistance. This thesis focuses on induced resistance in basket willow (*Salix viminalis*, Salicaceae) against the leaf roller gall midge *Dasineura marginemtorquens* (Diptera: Cecidomyiidae). Observations from the field revealed an, for plant/insect systems, unusually great variation among *S. viminalis* genotypes in resistance to *D. marginemtorquens*. This great genotypic variation in combination with the fact that *D. marginemtorquens* is a leaf galler with a relatively sessile life style make this system especially suitable for studies on local resistance reactions in the plant to attack by the insect. We tested the hypothesis that the hypersensitive response (HR), a type of plant-programmed localised cell death, is involved in the resistance. We found support for the hypothesis. Local HR-like necroses were found in resistant but not in susceptible *S. viminalis* genotypes after larval attempts to initiate galls. The importance of defensive plant signalling transduction pathways was investigated in several experiments. We found that the HR-associated salicylic acid (SA)-dependent pathway mediates the resistance. In contrast, the jasmonic acid (JA)-dependent pathway does not seem to be important in this system either as a mediator of resistance or through suppression of the SA-dependent pathway (negative "cross-talk"). Furthermore, total peroxidase activity was associated with the resistance. As such, the resistance in *S. viminalis* to *D. marginemtorquens* seems to be of an HR-type associated with SA-dependent responses including induced peroxidase activity. Thus, great similarity exists between plant resistance in the *S. viminalis*/*D. marginemtorquens* system and plant/pathogen systems with regards to activation of the HR, the SA-dependent pathway, and peroxidase activity.

Key words: *Salix*, induced resistance, hypersensitive response, gall midge, willow, salicylic acid, herbivory, HR, SA, peroxidase

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Key words: induced resistance, hypersensitive response, gall midge, willow, salicylic acid, herbivory, HR, SA, peroxidase

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Glädje kraften är och bliver
I en växlingsrik natur.
Glädje, glädje hjulen driver
Kring i världens stora ur.
Blommor hon ur fröet trollar
Solar hon på fästet sår
Och i rymden sfärer sållar
Dem ej forskarns fjärrglas når.

strof ur von Schillers ode "An die Freude"
(Alfred Victorins tolkning)

Contents

Appendix

Papers I-IV

The present thesis is based on the following papers, which will be referred to by their Roman numerals.

I. 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* (in press)

II. Ollerstam, O. & Larsson, S. Salicylic acid mediates resistance in the willow *Salix viminalis* against the gall midge *Dasineura marginemtorquens* (submitted manuscript)

III. Ollerstam, O. & Höglund, S. Peroxidase activity is induced in gall midge-resistant genotypes of basket willow and increases after stress (manuscript)

IV. Ollerstam, O. Interactions between a gall midge, host-plant genotype, and other herbivorous arthropods – role of signalling pathways (manuscript)

Study organisms and background observations

A plant gall is the product of a delicate interaction between a gall inducer (*e.g.*, an insect) and its host plant. By responding with gall formation, the host plant provides the parasitising gall inducer with food and shelter (Price *et al.*, 1986). The gall inducer usually depends on successful gall initiation in order to survive, and lives a, more or less, sessile life in the gall. Gall inducers manipulate the growth and differentiation of the host-plant tissue in, usually, species-specific ways. Furthermore, most gall-inducing insects are specialised herbivores restricted to one or a few host-plant species within a single genus (Rohfritsch & Shorthouse, 1982).

Most galls induced by gall midges (Diptera: Cecidomyiidae) are highly organised structures with several well-defined cell layers (Rohfritsch, 1992). In addition to histological changes in the host plant, the gall inducer also manipulates the chemical composition of the host tissue (Hartley, 1998; Nyman & Julkunen-Tiitto, 2000). In gall midges, it is the larva that initiates the gall. A typical mature cecidomyiid gall consists of plant tissue that has, through increased growth around the gall-initiation spot, finally completely surrounded the larva (Rohfritsch, 1992). Gall midge larvae do not consume the gall tissue, but instead seem to feed on cell content that leaks from the host tissue (Grover Jr., 1995). It is not known how the gall inducer manipulates its host to form a gall, but it is generally believed that some chemical elicitor is transferred from the gall inducer to the host (Hori, 1992). Recently, the chemical structures of a group of extremely potent insect-derived plant-growth elicitors were identified, the "bruchins", produced by a pea weevil (*Bruchus pisorum*) (Doss *et al.*, 2000). Although the bruchins do not induce galls, but rather neoplastic growth, they represent an example of insect-derived plant-growth regulators.

The gall midge *Dasineura marginemtorquens* Bremi (Fig. 1) is in Sweden monophagous on *Salix viminalis* L., and induces irregular roll galls (Mani, 1964), which are atypical for gall midges (Rohfritsch, 1992), in young leaf tissue (Figs 1 & 2). Females tuck eggs into the scrolls of unfurling leaves in terminal buds. The eggs hatch within three to four days (at 20 °C), and first-instar larvae can crawl several centimetres over the hairs on the undersides of the leaves before starting to initiate the galls (Glynn & Larsson, 1994). Thus, as in other cecidomyiids, it is the larva, rather than the ovipositing female, that initiates the gall. The larvae retain their ability to move around for at least one day after egg hatch (S. Höglund, unpubl.). The galls are 5-10 mm long thickened pockets along the leaf edges, and are essentially composed of enlarged cells (I). Larvae pupate in these galls, and adults emerge about two weeks after oviposition (at 20 °C). *D. marginemtorquens* over-winters as diapausing larvae behind buds on willow stems or in the soil. The adults emerge in May. Under Swedish field conditions the generation time is about one month. Thus, up to four generations of the gall midge per season may occur.

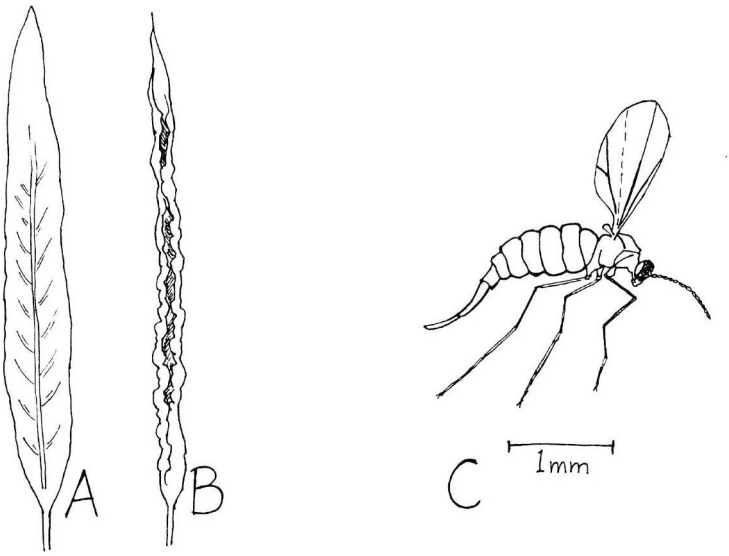

Figure 1. *Salix viminalis* leaves that are attacked by *Dasineura marginemtorquens* remain partly unfolded due to the roll galls along the leaf edge. A= mature *S. viminalis* leaf; B= leaf with many roll galls induced by *D. marginemtorquens* along both edges; C= schematic illustration of adult *D. marginemtorquens* female (Zhiwei Liu).

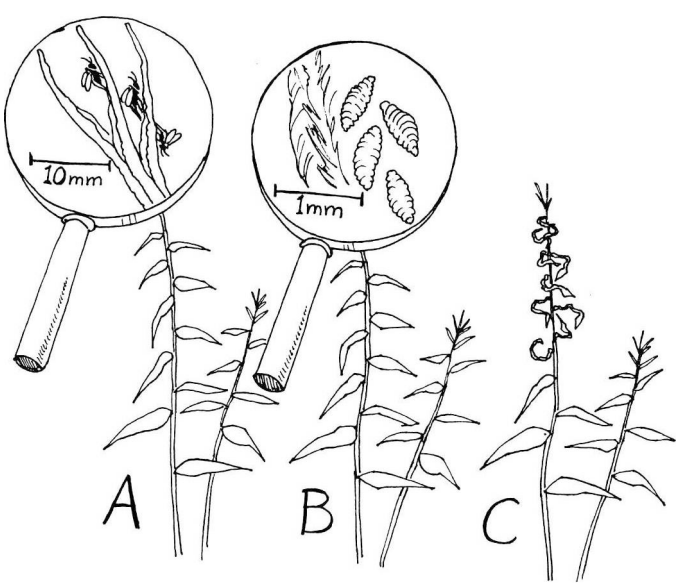

Figure 2. Biology of *Dasineura marginemtorquens.* Females oviposit in the young leaves of terminal buds of *Salix viminalis* (A). Within three to four days (at 20 °C) the larvae hatch and crawl to the leaf edge where they initiate the galls (B). Galling causes twisting of the leaves, and thus, the leaf area available for photosynthesis is reduced (C).

D. marginemtorquens sometimes occurs at pest densities in Swedish *S. viminalis* plantations (Forsberg *et al.*, 1991). The galling inhibits normal unrolling of the leaves, and heavy infestation results in severe twisting of the leaves, thus reducing the leaf area available for photosynthesis. The midge occasionally reaches outbreak population levels resulting in up to 85 % of the leaves in the plantation being severely damaged (S. Larsson & S. Höglund, unpubl.). Attack by *D. marginemtorquens* can result in up to 30 % growth loss in *S. viminalis* plants (Glynn *et al.*, 1998).

S. viminalis is native to central Eurasia (Skvortsov, 1999), and has been used in basket weaving since the time of the Roman Empire (Pohjonen, 1984). *S. viminalis* grows rapidly, resprouts readily after coppicing, and can easily be cloned simply by propagating stem cuttings. It is an indeterminate grower, providing young leaf tissue to *D. marginemtorquens* throughout the growing season. The species is introduced to Sweden, but has been naturalised since the mid 1700´s (Hylander, 1971). Today, *S. viminalis* is used in Swedish short-rotation forestry for production of biomass for central-heating of buildings (Ledin, 1992). An established willow plantation produces enough biomass per hectare and year to warm up about 1.5 one-family houses in a northern climate (Johansson, 1995). In addition, other uses are investigated, such as phytoredmediation of heavy metals on cadmium and lead contaminated land (Bertholdson, 2001).

Strong *et al.* (1993) reported great genetic variation in resistance in field-grown *S. viminalis* against *D. marginemtorquens*. On resistant *S. viminalis* genotypes, the great majority (>90%) of first-instar *D. marginemtorquens* larvae die. In contrast, on susceptible genotypes mortality is less than ten percent (Larsson & Strong, 1992). In addition to the resistant and susceptible *S. viminalis* genotypes defined above, moderately resistant genotypes exist on which *D. marginemtorquens* larval survival is highly variable among plant individuals of the same genotype (S. Larsson & S. Höglund, unpubl. data). This variation among cloned plants has been difficult to explain.

Scope of the study

The great genotypic variation in *S. viminalis* in resistance to *D. marginemtorquens* (Strong *et al.*, 1993), with several well-defined resistant and susceptible plant genotypes, provides an unusually good opportunity to study plant resistance responses to an insect herbivore. Furthermore, because ovipositing *D. marginemtorquens* females do not distinguish between resistant and suscepitble *S. viminalis* genotypes, despite the low larval survival on resistant plants (*i.e.*, an example of oviposition mistakes) (Larsson & Strong, 1992), controlled experimental inoculations are easily performed.

This thesis focuses on whether the resistance in *S. viminalis* to *D. marginemtorquens* is induced or constitutive, and whether the resistance is active (*e.g.*, intoxication of larvae) or passive (non-recognition of galling signal). To answer these questions and characterise the resistance, we compared resistant and susceptible *S. viminalis* genotypes with respect to histological, enzymatic, and biochemical-signalling responses to attack by *D. marginemtorquens*.

Among insects, gall inducers stand out because they depend on active responses from their hosts, *i.e.*, successful gall formations in order to survive. However, if the plant responds to a gall-initiation attempt with local necrosis, the gall inducer will be excluded from food and shelter, and, most probably, die (Fernandes, 1990; Bronner *et al.*, 1991a, Fernandes & Negreiros, 2001). In plant pathology, such rapid local plant-programmed cell death associated with resistance is referred to as hypersensitive response (HR) (Stakman, 1915; Heath, 1998). The HR has also been reported as being active against certain gall inducers (Bronner *et al.*, 1991a). Preliminary observations suggested that local leaf necrosis sometimes is associated with larval mortality on resistant *S. viminalis* genotypes (S. Höglund, pers. comm.). Thus, we hypothesised this induction of necroses after larval feeding to be a feature of resistant *S. viminalis* genotypes, and we found support for this hypothesis (I). This larval-induced necrosis showed interesting similarities with pathogen-induced HR; it was rapid, local, associated with accumulation of phenolics, and restricted to resistant plant genotypes (Dangl *et al.*, 1996; Lam *et al.*, 2001).

Pathogen-induced HRs are often associated with activation of the salicylic acid (SA)-dependent defensive signal transduction pathway (Rasmussen *et al.*, 1991; McDowell & Dangl, 2000). Thus, we investigated whether activation of the SA-dependent pathway occurred in attacked resistant *S. viminalis* genotypes, and found this to be the case (II). Because induced accumulation of pathogenesis-related (PR) proteins often occurs in association with activation of the HR (*e.g.*, Van Loon, 1997), we investigated the enzymatic activity of two PR proteins in resistant and susceptible *S. viminalis* genotypes (III).

Finally, in an attempt to explain the observed variation in resistance among cloned plants of some *S. viminalis* genotypes (S. Larsson & S. Höglund, unpubl. data) we investigated how biotic and abiotic factors influenced the expression of resistance (III; IV).

Theoretical background

An herbivorous insect has to handle a wide range of more or less troublesome plant traits and responses in order to exploit the plant. The interactions between insect herbivores and plants are, to a large extent, shaped by hostile plant traits and the capacity of insects to overcome these. From an ecological perspective, plant traits known to influence insect preference and performance can be divided into factors related to primary metabolites, secondary metabolites, physical factors, and phenological factors (*e.g.*, Larsson, 2002). Primary metabolites, such as proteins and carbohydrates involved in plant growth, are rarely considered to be resistance traits. However, because nitrogen is often the limiting element for insect herbivore growth, it is reasonable to believe that the quality and quantity of nitrogen-containing compounds might be related to plant resistance (Mattson & Scriber, 1987). Secondary metabolites are defined as chemical compounds that do not have any known direct role in plant growth (Bernays & Chapman, 1994). Many studies have verified the importance of secondary metabolites such as terpenoids, phenolics, and alkaloids in resistance to insects (Bernays & Chapman, 1994). However, a secondary metabolite that is deterrent or toxic to one insect might be, to another insect an attractant, or even useful in the insect's own defence against predators (Rowell-Rahier & Pasteels, 1992).

Plant resistance factors are often grouped into constitutive (present in the plant independently of the attacker) and induced (present in the plant only after attack) (*e.g.*, Karban & Baldwin, 1997), although the limit between the two groups is not rigid. Trichomes, for instance, are often present in the plant before herbivory, but may increase in density after herbivory by some chewing insects (Baur *et al.*, 1991; P. Dalin & C. Björkman, unpubl.). This thesis focuses on induced resistance in the willow *Salix viminalis* to the gall midge *Dasineura marginemtorquens*. Resistance is here defined in consistence with the terminology used by Karban & Baldwin (1997), where resistance is defined as reduced performance of the attacking insect on resistant plants. This definition of resistance makes no assumptions about the evolution of the plant trait that confers resistance. Thus, a thick cuticle, for instance, might have evolved to minimise desiccation or pathogen infection but may, at the same, time provide efficient resistance against certain insects. Neither does the definition of resistance make any assumption about the impact of the herbivore on plant fitness. Thus, a resistant plant might even suffer a higher degree of damage due to compensatory feeding by the herbivore than a susceptible plant.

Induced resistance occurs at different spatial and temporal scales. Locally induced resistance (LIR) is the term used to describe responses in the attacked tissue, in contrast to systemically induced resistance (SIR) that occurs throughout the plant, even in non-attacked plant organs. In this thesis, LIR is studied with respect to histological and chemical responses in resistant *S. viminalis* genotypes. Haukioja & Neuvonen (1987) introduced the terms rapid induced response (RIR) with effects on insect individuals in the attacking generation, and delayed induced response (DIR) affecting insects in the following generation(s). In addition to the direct effects of plant resistance on the feeding insect, indirect effects, although not studied here, of induced plant resistance involving the third trophic level (*e.g.*,

predators and parasitoids) are probably important in many systems (Dicke, 1999; Thaler, 2002).

In recent years, molecular biologists have applied the knowledge gained from studies on plant resistance to pathogens on plant/insect interactions (*e.g.*, Walling, 2000). For instance, studies on the induction of defensive signal transduction pathways, known to be pathogen-induced, in response to attack by insects have revealed that plants respond differently to chewing and sap-sucking insects (Karban & Baldwin, 1997; Walling, 2000).

Results and discussion

Histological plant responses in willow genotypes resistant to the gall midge

In the first study we investigated in what ways the responses of leaf tissue of resistant *Salix viminalis* genotypes differ from those of susceptible genotypes after attack by the gall midge *Dasineura marginemtorquens* (I).

The hypersensitive response (HR), has frequently been observed in plants infected by pathogenic microorganisms (*e.g.*, Dangl *et al.*, 1996). Unfortunately, no morphological feature that distinguishes cell death associated with the HR from cell death due to other reasons has been confirmed (Heath, 1998). It has often been observed, however, that an early, local accumulation of phenolic compounds at the infection site accompanies pathogen-induced HR (reviewed by Nicholson & Hammerschmidt, 1992). The HR has also been reported to be a mechanism of resistance in plants against higher organisms, including gall-inducing insects (reviewed by Fernandes, 1990; also *cf.* Fernandes & Negreiros, 2001). Because the HR had been implicated in a number of other plant/gall inducer interactions it seemed reasonable to hypothesise that the HR also is involved in the resistance in *S. viminalis* to *D. marginemtorquens*. In addition, during several years of work it has occasionally been possible to observe, by the naked eye, local necrotic lesions associated with resistance in *S. viminalis* to *D. marginemtorquens* (S. Larsson, S. Höglund & O. Ollerstam, unpubl.).

Two main questions were addressed. First, is the genotypic variation in resistance in *S. viminalis* against *D. marginemtorquens* correlated with the ability of different willow genotypes to mount an HR in response to infestation by the gall midge? Other studies on the HR and gall midges have either not investigated genotypic variation in the host's ability to express the HR (*e.g.*, Fernandes & Negreiros, 2001), or have only found weak correlations between the HR and resistance (Grover Jr., 1995; Bentur & Kalode, 1996). Second, if present, how rapid is the HR in the *S. viminalis*/*D. marginemtorquens* system? Previous reports on HR-based resistance against gall midges indicate that cell death does not occur until several days after egg hatch (Grover Jr., 1995; Bentur & Kalode, 1996).

As a first step to characterise responses to gall midge attack in resistant willow genotypes, leaf-tissue samples from attacked leaves were studied by means of

microscopy. We found that necrotic lesions containing phenolic compounds appeared rapidly (within 12 h) following gall-initiation attempts in a resistant but not in a susceptible genotype.

In plant pathology the HR is defined as a process occurring within a few hours after attack (*e.g.*, Vleeshouwers *et al.*, 2000). However, earlier reports on gall midge-induced HR, from the Hessian fly/wheat and rice gall midge/rice systems are inconsistent with this definition of the HR because the cell death has not been detected until days after attack (Grover Jr., 1995; Bentur & Kalode, 1996). In the *D. marginemtorquens/S. viminalis* system the observed necrosis seems to be similar to pathogen-induced HRs because it occurs on the same temporal scale.

Activation of defensive signal transduction pathways

After having established that HR-like necrotic lesions are associated with the resistance we turned to the biochemical signalling related to pathogen-induced HRs. The second study aimed to test if salicylic acid (SA) mediates the resistance.

Invading organisms induce plant responses via activation of several biochemical signal transduction pathways. Pathogen-induced signal transduction is mediated in the plant by jasmonic acid (JA), ethylene, and salicylic acid (SA) (Walling, 2000). Herbivorous arthropods also induce one or another of these signalling pathways, depending on feeding mode (Karban & Baldwin, 1997; Walling, 2000). For example, phloem-feeding insects, like pathogens, sometimes induce the SA-dependent pathway (reviewed by Walling, 2000). In general, activation of the SA-dependent pathway is associated with the HR (Rasmussen *et al.*, 1991; reviewed by Dangl *et al.*, 1996), and it has been proposed that SA acts synergistically with reactive oxygen intermediates (ROIs) and nitric oxide (NO) to trigger HR cell death (McDowell & Dangl, 2000).

Here we asked if SA accumulation is a feature of the resistance reaction, and if so, whether it is possible to make otherwise susceptible *S. viminalis* plants resistant by exposing them to SA.

Using high performance liquid chromatography (HPLC) and mass spectrometry (MS) an induced SA accumulation was detected in a resistant willow genotype, but not in a susceptible genotype, after gall midge attack. In addition, a susceptible willow genotype became more resistant when exposed to exogenous SA. Thus, we could verify that the previously reported gall midge-induced HR (I), like pathogen-induced HRs (Rasmussen *et al.*, 1991; Dangl *et al.*, 1996), is associated with SA accumulation. It was inferred that SA is active in the resistance and not simply correlating with the true resistance reaction. In contrast, previous studies on effects of exogenous SA, or SA-analogues, on performance of insects have come to quite different conclusions. Inbar *et al.* (2001) treated plants with BTH (a SA-analogue) and tested the effects on host-plant preference of the whitefly *Bemisia tabaci* and feeding efficacy of the cotton bollworm *Helicoverpa armigera*. It was concluded that BTH has a negligible effect on the insects. Similarly, Bi *et al.* (1997) detected no effect on the performance of the cotton bollworm *Helicoverpa zea* when SA was exogenously applied to cotton plants. Furthermore, Thaler *et al.* (1999) showed that BTH treatment of tomato plants even increases the survivorship of the

beet armyworm (*Spodoptera exigua*). However, the assumed subtle sucking by a gall midge (Hatchett *et al.*, 1990) on the host tissue differs from the feeding of a chewing insect. Thus, it was not surprising that the negative effect of SA-application on insect performance was more pronounced in the *D. marginemtorquens/S. viminalis* system than in previously studied insect/plant systems.

The accumulation of SA in an attacked resistant *S. viminalis* genotype and negative effect of SA application to a susceptible genotype on *D. marginemtorquens* performance suggest that SA mediates resistance in the *S. viminalis/D. marginemtorquens* system. The timing of cell death, accumulation of phenolic compounds (I), and accumulation of SA are all consistent with present models of pathogen-induced HR (Rasmussen *et al.*, 1991; Nicholson & Hammerschmidt, 1992; Dangl *et al.*, 1996).

Resistance-related enzyme activities

Induction of the SA-dependent signal transduction pathway is known to trigger accumulation of pathogenesis-related (PR) proteins (*e.g.*, Van Loon, 1997). In the third study we investigated whether the previously observed gall midge-induced SA accumulation (II) in resistant *S. viminalis* plants correlates with enzymatic activity of certain PR proteins. We chose to investigate two types of PR proteins, peroxidase (PR protein family no. 9; Lagrimini *et al.*, 1987) and chitinase (PR protein families no. 3, 4, 8, and 11; van Loon, 1982; Métraux *et al.*, 1988; Melchers *et al.*, 1994). In addition, we tested if these enzyme activities were induced by environmental stress, or by exogenous SA application.

PR proteins are, by definition, proteins coded for by the plant but induced in the plant due to pathogen attack, or other stresses (Van Loon & Van Strien, 1999). Because PR proteins have been implicated as important in some plant/arthropod interactions (*e.g.*, Bronner *et al.*, 1991a, b; van der Westhuizen *et al.*, 1998), they deserve attention in studies on plant resistance to insect herbivores.

Peroxidases catalyse processes such as lignification (Lagrimini *et al.*, 1997a), cross-linking of cell-wall proteins (Bradley *et al.*, 1992), auxin catabolism (Lagrimini *et al.*, 1997b), and production of oxygen radicals (Bolwell & Wojtaszek, 1997). Furthermore, peroxidases are often up-regulated due to pathogen attack (*e.g.*, Svalheim & Robertson, 1990; Smit & Dubery, 1997). Local and systemic induction of various peroxidase isoforms usually accompany pathogen-induced HRs (Bestwick *et al.*, 1998; Hiraga *et al.*, 2000). Chitinases, (poly (1,4-(N-acetyl-β-D-glucosaminide)) glycanohydrolases), another group of PR proteins, are common among plants (Graham & Sticklen, 1994). Because chitin is a major structural component of both fungal cell walls (*e.g.*, Mauch *et al.*, 1988) and the arthropod exoskeleton (*e.g.*, Evans, 1984), chitinases have gained attention as being part of plant resistance to fungal pathogens and herbivorous arthropods. Similar to peroxidases, chitinases are induced in arthropod-resistant plant genotypes (Bronner *et al.*, 1991a), and confer resistance to arthropods when expressed in transgenic plants (Dowd & Lagrimini, 1997; Gatehouse & Gatehouse, 1998).

Because chitinases (Graham & Sticklen, 1994) and peroxidases (Bestwick *et al.*, 1998; Hiraga *et al.*, 2000) are induced in association with pathogen-induced HR, we investigated if these enzymes are associated with resistance in *S. viminalis* to *D. marginemtorquens*. Furthermore, because environmental factors had been indicated to influence the resistance (S. Larsson & S. Höglund, unpubl. data), we tested whether the activity of these enzymes vary with UV light irradiation and *Melampsora epitea* rust infection, similar to what has been found in other systems (Bestwick *et al.*, 1998; Jacobs *et al.*, 1999; Mercier *et al.*, 2000; Schmitz-Eiberger & Noga, 2001). In addition, because SA was found to mediate the resistance to *D. marginemtorquens* (II), the effects of exogenous SA application on peroxidase or chitinase activity in *S. viminalis* were investigated.

Total peroxidase activity was induced to a higher degree in resistant than in susceptible *S. viminalis* genotypes after attack by *D. marginemtorquens*. Induction of peroxidase activity may be harmful to the gall midge larva in several ways. First, the larvae might suffer from oxidative stress, *e.g.* due to hydrogen peroxide, generated by the peroxidases (Bi & Felton, 1995). Second, activation of the HR may be facilitated by hydrogen peroxide generation (I). Increased peroxidase activity often accompanies pathogen-induced HR (*e.g.*, Bestwick *et al.*, 1998; Hiraga *et al.*, 2000). Similarly, increased peroxidase activity accompanies some gall inducer-induced HRs (Bronner *et al.*, 1991b). Third, peroxidases might suppress the gall formation, for instance, by making the plant cell wall rigid to expansion (I) due to lignification and cross-linking of cell-wall components (Bradley *et al.*, 1992; Lagrimini *et al.*, 1997a).

Both UV light irradiation and rust infection induced peroxidase activity. Certain *S. viminalis* genotypes tend to be more resistant in the field than in the greenhouse (S. Larsson & S. Höglund, unpubl. data). Induced peroxidase activity was associated with resistance in *S. viminalis* to *D. marginemtorquens*. Thus, peroxidase activity seems to be a resistance factor that is partly regulated by environmental stresses (*i.e.*, UV and rust), and these stresses are most likely more pronounced in the field than in the greenhouse. This might explain why certain *S. viminalis* genotypes tend to be more resistant in the field compared with the greenhouse.

Exogenous SA application increased foliar total peroxidase activity. Attack by *D. marginemtorquens* induced accumulation of SA in resistant *S. viminalis* genotypes but not in susceptible genotypes (II). Resistance-related SA accumulation together with SA-induced peroxidase activity suggest that SA-induced peroxidase activity is essential to resistance in *S. viminalis* to *D. marginemtorquens*.

In contrast, no effect on chitinase isozyme pattern was found either after *D. marginemtorquens* larval attack, UV or rust treatment, or exogenous SA application. However, chitinase activity was present in all analysed plants, and it is possible that chitinases act in synergy with peroxidases in *S. viminalis* genotypes resistant to *D. marginemtorquens* similar to what has been found for other PR proteins in other systems (Arlorio *et al.*, 1992).

PR proteins have previously been reported as important in resistance against certain gall inducers (Bronner *et al.*, 1991a, b). The third study coupled a PR-protein (peroxidase) activity related to resistance to *D. marginemtorquens* to the SA-dependent signal transduction pathway. Furthermore, their study suggested that both abiotic and biotic stresses interact with the expression of resistance to the gall midge.

Interactions between resistance against the gall midge and feeding by other herbivores

The fourth study had a more ecological perspective than the three earlier studies. The herbivore species complex on willows in Swedish plantations consists of several arthropod groups such as Homoptera, Hymenoptera, Coleoptera, Lepidoptera, Diptera, (Forsberg *et al.*, 1991) and gall mites (Acari: Tarsonemoidea or Eriophyoidea) (pers. obs.). We investigated to what extent resistance to the gall midge might be influenced by feeding of a leaf beetle (*Phratora vulgatissima*), an aphid (*Aphis farinosa*), and a gall mite (*Aculops* sp.).

Herbivorous insects, and, in particular, sap feeders, often compete for food resources with the same, or another species, and competition is frequently mediated by the host plant (Denno *et al.*, 1995). Exploitative competition (*e.g.*, Futuyma, 1998) could either result from shortage of food or induction of defensive plant responses. Phytophagous organisms that induce the same defensive signal transduction pathway (*i.e.*, the SA- or JA-dependent pathway, *cf.* II) are likely to suffer from exploitative competition mediated by induced defensive responses in the plant. Because the plant is an important mediator of competition (Denno *et al.*, 1995), it is interesting to ask whether exploitative competition varies among host-plant genotypes. However, usually, genotype by environment (GxE) interactions have been studied in the context of abiotic factors (Maddox & Cappuccino, 1986; Rönnberg-Wästljung & Thorsén, 1988; Orians & Fritz, 1996; Rossi & Stiling, 1998).

Negative cross-talk (*e.g.*, Hunter, 2000), *i.e.*, suppression of one signal transduction pathway due to activation of a competing one, occurs at the molecular level, as detected by effects on the synthesis of PR proteins (Doares *et al.*, 1995; Niki *et al.*, 1998; but see Xu *et al.*, 1994). In addition, the existence of cross-talk has been reported in a field trial showing increased insect survival due to suppression of the JA-dependent pathway, after chemical induction of the SA-dependent pathway (Thaler *et al.*, 1999).

The aims of the fourth study were to test whether *S. viminalis* genotypes are differently affected by aphid, gall mite or leaf beetle feeding with regard to resistance against *D. marginemtorquens*. Specifically, feeding by aphids or gall mites was hypothesised to increase resistance to *D. marginemtorquens* due to activation of the SA-dependent signalling pathway (Walling, 2000; Moran & Thompson, 2001). Leaf beetle feeding damage induces transcription of a proteinase inhibitor gene in *S. viminalis* (Saarikoski *et al.*, 1997). Because proteinase inhibitors are considered to be markers for the JA-dependent signal transduction pathway (Farmer *et al.*, 1992), leaf beetles were hypothesised to

suppress the resistance to *D. marginemtorquens* due to antagonistic cross-talk between the SA- and the JA-dependent pathways.

The effect of aphid feeding on resistance to *D. marginemtorquens* depended on *S. viminalis* genotype (only one resistant genotype was affected), providing an example of a plant genotype x biotic environment interaction. It is likely that the effect was caused by activation of similar defensive responses by the plant both to *D. marginemtorquens* and *A. farinosa* (Belefant-Miller *et al.*, 1994; Moran & Thompson, 2001; I; II). Thus, the suitability of a particular *S. viminalis* genotype as a host to *D. marginemtorquens* seems to, in part, depend on the feeding activity of other herbivores, in this case, the aphid *A. farinosa*. In contrast, JA-dependent responses due to leaf beetle feeding, or exogenous JA application, seemed negligible.

Gall mites had no significant effect on *D. marginemtorquens* larval survival, in spite of earlier observations of gall mite-induced HR (Bronner *et al.*, 1991a). However, the present experiment differed from the study by Bronner *et al.* (1991a), because they used a gall mite-resistant plant genotype. Unfortunately, we have no data on resistance to gall mites for the two *S. viminalis* genotypes that were used. Thus, it is possible that the *S. viminalis* genotypes were susceptible to the gall mite *Aculops* sp. and incapable of mounting resistance responses to the gall mite.

Leaf beetle feeding had negligible effects on *D. marginemtorquens* larval survival, but exogenous application of JA had a slightly negative effect on larval survival on a susceptible *S. viminalis* genotype. However, because the high-level JA treatment tended to suppress internode growth it could not be excluded that the reduced plant growth, rather than induction of defensive compounds, explained the marginal effect on larval survival; most gall inducers depend on vigorously growing plant tissue in order to develop optimally (*e.g.*, Price, 1991; Rohfritsch, 1992). Thus, it seemed as if the SA-mediated (II) resistance in *S. viminalis* to *D. marginemtorquens* is not seriously compromised by negative cross-talk with the JA-dependent pathway.

Other authors have reported effects on insect performance due to interaction between host-plant genotype and feeding by other insect species (*e.g.*, Cronin & Abrahamson, 1999). The fourth study suggested that the risk of some phytophagous insects to compete depends both on host-plant genotype and which signal transduction pathway(s) each insect induces.

Is the resistance in *S. viminalis* to *D. marginemtorquens* consistent with models of plant resistance to pathogens?

The interaction between *Salix viminalis* and *Dasineura marginemtorquens* shows interesting similarities with plant/pathogen interactions. For example, genetic variation in plant resistance, specificity of plant host species, relative immobility of the attacker, and demands of biochemical plant responses for successful colonisation are features that characterise both types of interactions.

From a mechanistic perspective, the resistance is highly consistent with models of resistance against certain pathogens. Incompatible plant/pathogen interactions often result in activation of the HR and induction of the SA-dependent signal transduction pathway (Rasmussen *et al.*, 1991; Martinez, *et al.*, 2000). This is exactly what happens in resistant *S. viminalis* genotypes after attack by *D. marginemtorquens* (I, II). *D. marginemtorquens* is a relatively sessile, obligate biotroph that depends on growing host tissue for survival, thus resembling biotrophic microbial pathogens. Resistance to biotrophic pathogens is typically signalled by the SA-dependent pathway (Rasmussen *et al.*, 1991; Shulaev *et al.*, 1997), whereas resistance to necrotrophic pathogens generally is signalled by the JA-dependent pathway (Penninckx, *et al.*, 1998; Vijayan *et al.*, 1998) although additional pathways may be involved (Vogel & Somerville, 2000). Furthermore, pathogen-induced HRs are often associated with increased peroxidase activity (Svalheim & Robertson, 1990; Smit & Dubery, 1997), another similarity with the resistance in *S. viminalis* to *D. marginemtorquens* (III). In addition, it has been suggested that SA mainly induces acidic apoplastic PR proteins whereas JA mainly induces basic vacuolar PR proteins (Niki *et al.*, 1998). The peroxidase isozyme set in *S. viminalis* seems to be consistent with this model because total peroxidase activity was induced by SA (III) and isoelectric focussing electrophoresis (IEF, pH 3-9) followed by activity staining revealed that all observed peroxidase isozymes were acidic (III). Thus, the resistance in *S. viminalis* to *D. marginemtorquens* is consistent with plant resistance to pathogens both with respect to the SA/biotroph-JA/necrotroph model (Rasmussen *et al.*, 1991; Shulaev *et al.*, 1997; Penninckx, *et al.*, 1998; Vijayan *et al.*, 1998) and the SA/acidic PR-proteins-JA/basic PR-proteins model (Niki *et al.*, 1998).

How does the genotypic variation in resistance in *S. viminalis* to *D. marginemtorquens* fit to genetic models of plant resistance to pathogens? The gene-for-gene (GFG) model (Flor, 1971) was formulated to describe pathogen race-specific plant resistance. According to the GFG model an interaction between the products of an avirulence (Avr) gene carried by the pathogen and a resistance (R) gene carried by the plant results in resistance to the pathogen and the interaction is referred to as incompatible. Such incompatible interactions often culminate in the HR (Hammond-Kosack & Jones, 1997). Furthermore, compatible interactions, *i.e.*, successful infections, occur when the pathogen is not recognised by the plant due to a lack of an Avr gene in the pathogen or a lack of an R gene in the plant (Flor, 1971). The GFG model is reported as valid for another gall midge

species and its host, the Hessian fly (*Mayetiola destructor*) on wheat (Stuart *et al.*, 1998). However, resistance in the *S. viminalis* genotypes where *D. marginemtorquens* mortality is higher than 90 % does not seem to depend on gall midge genotype; gall midges from different populations have not proven to vary in virulence (S. Larsson & S. Höglund, unpubl. data). Thus, the GFG model does not seem to hold for the *D. marginemtorquens*/*S. viminalis* system and the resistance would be classified as race-non-specific rather than race-specific (Agrios, 1997). In consistence with this classification, the observed HR-like necrosis (I) should be triggered by a general elicitor present in all genotypes of the gall midge (Vleeshouwers *et al.*, 2000).

Strong *et al.* (1993) classified 240 *S. viminalis* genotypes, used in a field trial, with respect to number of galled leaves per plant and found that the genotypes displayed a bimodal distribution. One "bell" of the bimodal distribution consisted of a group of extremely resistant genotypes (less than 10 % larval survival), from which genotypes used in most of the studies in this thesis have been selected (I; II; III). These strongly resistant genotypes were all descendants of one particular pollen parent, and thus, it was concluded that the resistance was mono- or oligogenic (Strong *et al.*, 1993). Hence, this type of mono- or oligogenic resistance shows consistence with what in plant pathology is referred to as vertical resistance (Agrios, 1997). However, the parallel is not complete because, in contrast to vertical resistance, the resistance in *S. viminalis* seems to be independent of *D. marginemtorquens* genotype (S. Larsson & S. Höglund, unpubl. data). The other bell of the distribution of *S. viminalis* genotypes described by Strong *et al.* (1993) consisted of genotypes that contained a quantitative genetic source of resistance, suggesting a polygenic resistance type similar to horizontal resistance in plant pathology (Agrios, 1997). In two of the studies (III; IV) we investigated genotypes expressing such resistance to *D. marginemtorquens*. The term horizontal resistance seems to apply well to this type of resistance because it is most likely polygenic (Strong *et al.*, 1993) and varies to some degree with environmental variables (III; IV) (Agrios, 1997). Thus, resistance in *S. viminalis* to *D. marginemtorquens* seems to consist both of horizontal and vertical components.

In conclusion, we have shown that plant responses, generally associated with plant resistance to pathogens, such as hypersensitive cell death (I), SA-accumulation (II), and increased PR protein activity (III) are involved in the resistance in *S. viminalis* to *D. marginemtorquens*. Thus, models developed to describe plant resistance to microbial pathogens on the cellular and molecular levels (Rasmussen *et al.*, 1991; Shulaev *et al.*, 1997; Niki *et al.*, 1998; Penninckx, *et al.*, 1998; Vijayan *et al.*, 1998) are relevant to the *S. viminalis*/*D. marginemtorquens* system. Furthermore, because the resistance seems to include a strong mono- or oligogenic type of resistance (studied in papers I & II) as well as a more variable polygenic resistance (studied in papers III & IV) (Strong *et al.*, 1993), models of inheritance and expression of plant resistance to pathogens (Agrios, 1997) are applicable to this system.

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