SHORT COMMUNICATION

Induced resistance enzymes in wild plants–do 'early birds' escape from pathogen attack?

Martin Heil · Kerstin Ploss

Abstract Systemic acquired resistance (SAR) of plants to pathogens is a well-defined phenomenon. The underlying signalling pathways and its application in crop protection are intensively studied. However, most studies are conducted on crop plants or on *Arabidopsis* as a model plant. The taxonomic distribution of this phenomenon and its dependence on life history are thus largely unknown. We quantified activities of three classes of resistance-related enzymes in 18 plant species to investigate whether plants with varying life histories differ in their investment in disease resistance. Enzyme activities were quantified in untreated plants, and in plants induced with BION, a chemical resistance elicitor. All species showed constitutive activities of chitinase, peroxidase, or glucanase. However, constitutive chitinase activities varied by 30 times, and peroxidase by 50 times, among species. Several species did not respond to the induction treatment, while enzyme activities in other species increased more than threefold after BION application. Plant species differ dramatically in the presence and inducibility of resistance enzymes. This variation could be related to life history: While all resistance enzymes were significantly induced in larger perennial plants that flower during summer, spring geophytes hardly showed inducible resistance. These plants grow in an environment that is characterised by a low-pathogen pressure, and thus may simply 'escape' from infection. Our study presents the first comparative data set on resistance-related enzymes in noncultivated plants. The current view on SAR—narrowed by the concentration on cultivated crops—is not sufficient to understand the ecological and evolutionary relevance of this widespread plant trait.

M. Heil · K. Ploss
Department of Bioorganic Chemistry,
Max-Planck Institute for Chemical Ecology,
Beutenberg Campus, Hans-Knöll-Str 8,
07745 Jena, Germany

M. Heil (✉)
Allgemeine Botanik/Pflanzenökologie, FB BioGeo,
Universität Essen,
Universitätsstr. 5,
45117 Essen, Germany
e-mail: Heil_Martin@web.de

Introduction

Plants are attacked by pathogens under virtually all natural environments. They can respond to infection by expressing a broad-spectrum disease resistance that is active against further pathogen attack. Several aspects of this response are expressed even in noninfected plant parts (i.e., systemically). This systemic acquired resistance (SAR) is characterised by (1) a local hypersensitive response (HR) leading to programmed cell death around the infected cells (Kombrink and Schmelzer 2001), (2) local changes in cell-wall structure and composition preventing the further penetration by pathogens, and (3) the local and systemic expression of pathogenesis-related (PR) proteins (van Loon 1997).

Although much has been learned on the biochemical and genetic aspects of resistance elicitation and signal transduction, surprisingly little is known on the ecology and evolution of this mechanism. For example, most of the plants studied represent one single life history, i.e., fast-growing annual crops. The purpose of our current study is a first screening of different species to answer the question: how widespread is SAR in plants with different life histories?

Ideally, this question should be studied by quantifying biological resistance, i.e., by quantifying how pathogens develop in the different plants after a standardized infection. However, the main goal of our study is interspecific comparison. This would require one pathogen that—with equal efficiency—infects all species, as differences in the plants' responses could otherwise result from any type of, for example, non-host resistance as well. Such a pathogen does not exist. We, therefore, chose artificial resistance elicitation and studied responses of enzymes that are generally known to be causally involved in SAR.

We used an artificial resistance elicitor, BION, to induce disease resistance in 18 plant species. Its active ingredient [benzo (1,2,3) thiadiazole-carbothioic acid S-methylester: CGA 254-704] acts in the disease-related signal pathway as a mimic of salicylic acid, the plant hormone involved in signal transduction. The spectrum of resistance activation and the biochemical changes induced by BION usually matches that of a biological induction (Friedrich et al. 1996; Görlach et al. 1996). BION can be applied externally to the plants and induces disease resistance in monocots as well as in dicots (Oostendorp et al. 2001). Using a chemical elicitor allowed us to induce putative resistance responses at one exactly defined time and in a dosage-controlled manner even in plant species for which no natural pathogens are known.

We quantified three resistance-related enzyme classes, chitinases, peroxidases, and glucanases. All three classes are PR proteins (van Loon 1997). Quantifying SAR on the level of enzyme activity allowed a focus on resistance-related phenotypic traits in plant species that are not genetically characterised and for which no natural pathogens have been described so far. Chitinases (EC 3.2.1.14) are widely distributed in plants (Punja and Zhang 1993) and are causally involved in pathogen resistance: purified chitinases exhibit pronounced antifungal activity, particularly in combination with β-1,3-glucanases (EC 3.2.1.6) (Mauch et al. 1988; Schlumbaum et al. 1986). Both enzymes have constitutive and—after wounding or biological infection—induced activities. Peroxidases (EC 1.11.1.) are involved in HR, but they are also required for the polymerization of cell-wall components such as lignin, suberin, and extensin leading to the formation of barriers for infecting pathogens (Cooper and Varner 1984).

We quantified constitutive and induced activities of these enzymes in plants growing under natural conditions. The goals of our study were to (1) obtain first information on whether or not different plant species possess activities of these enzymes, (2) investigate whether or not the activities increase in response to chemical resistance elicitation, and (3) discuss putative differences among species in the context of their life history and taxonomic classification.

Materials and methods

Collection and treatment of plant material

All plants were collected in the field and represented native or common species growing in the wild in Germany. Plants were investigated at the beginning of their flowering period (see Table 1 for collection dates). Constitutive levels of enzyme activities were quantified in untreated plants. Induced activities were quantified after spraying plants once with an aqueous solution of BION (300 mg l^{-1}) until runoff. These plants were collected 4 to 8 days after spraying. Plant material was frozen at −20°C until extraction. Plants were collected mainly in 2003, but additional material was collected from Eranthis hyemalis in 2004. Sample sizes were five to seven plants per species and treatment.

Additional investigations of Eranthis hyemalis were conducted in 2004: Each seven plants were sprayed with water (spray control), an aqueous 1-mM solution of jasmonic acid, an aqueous 1-mM solution of salicylic acid, or with BION solution. Seven other plants were infiltrated with Chitosan (a colloidal solution of chitin) to mimic fungal infection. For this treatment, mock inoculation with an aqueous MgCl solution served as a control.

Table 1 Plant species used in the screening. Plant species were determined according to Oberdorfer (1990) and are listed below with family and collection date

Species	Family	Date
Alliaria petiolata (Bieb.) Cavara and Grande	Brassicaeae	02.05
Anemone nemorosa L.	Ranunculaceae	30.03
Anemone ranunculoides L.	Ranunculaceae	01.04
Cardamine pratensis L.	Brassicaceae	20.04
Cirsium arvense (L.) Scop.	Asteraceae	10.07
Corydalis cava L.	Papaveraceae	03.04
Crocus vernus (L.) Hill.	Iridaceae	26.03
Eranthis hyemalis Sal.	Ranunculaceae	04.03
Galanthus nivalis L.	Amaryllidaceae	26.03
Malus domestica L.	Rosaceae	13.05
Medicago sativa L.	Fabaceae	10.07
Potentilla reptans L.	Rosaceae	30.04
Primula veris L.	Primulaceae	24.03
Prunus sp.	Rosaceae	15.05
Ranunculus ficaria L.	Ranunculaceae	01.04
Syringa vulgaris L.	Oleaceae	05.05
Urtica dioica L.	Urticaceae	10.07
Viola sp.	Violaceae	30.03

Extraction of (soluble) enzymes

Frozen plant material 0.25–0.5 g was ground using a chilled mortar and a pestle in liquid N_2 and sea sand. The material was collected in 1.5 ml of 50 mM Na-phosphate buffer at pH 5.0. After 5 min of centrifugation (Eppendorf centrifuge 5415D) at 13,000 rpm, the supernatants were subjected to gel filtration on NAP 10 columns (Amersham Biosciences) equilibrated with 50 mM Na-phosphate buffer at pH 5.0. All enzyme activities are expressed per milligram of plant fresh weight. This is the activity a putative pathogen would have to cope with and thus appeared to be an ecologically relevant measure of unspecific disease resistance.

Chitinase activity

For the assays (based on a method of Wirth and Wolf 1990), a total volume of 100 μl reaction preparation contained 15 μl plant extract, 50 μl RBV-chitin (Loewe, München, Germany) and 25 mM Na-acetate buffer at a pH of 5.0. Each reaction was replicated four times, incubated 2.5 h at 37°C and stopped with 55 μl 0.05 M HCl. After 5 min of incubation at −20°C the plate was centrifuged at 3,800 rpm at 4°C. One hundred microliters of the supernatant was transferred to a new microplate and measured at 550 nm in a "Spectra Max 250" plate reader (MD, Ismaning, Germany). Previous investigations with a chitinase from *Streptomyces griseus* (Sigma, Taufkirchen, Germany) confirmed a linear time response between 0 and 0.4 OD_{550nm}.

Peroxidase activity

A total volume of 200 μl reaction contained 10 μl plant extract, 36.6 mM H_2O_2, 40.25 mM guaiacol and 50 mM Na-phosphate buffer at pH 6.0. The oxidation of the substrate was measured spectrophotometrically at 470 nm as described previously (Hammerschmidt et al. 1982).

Glucanase activity

For the assays (based on a method of Wirth and Wolf 1990), a total volume of 100 μl reaction preparation contained 30 μl plant extract, 50 μl RBB-curdlan (Loewe, München, Germany) and 20 μl 50 mM Na-acetate buffer at a pH of 5.0. Each reaction was replicated four times, incubated 3 h at 37°C and stopped with 50 μl 2 M HCl. After 5 min incubation at −20°C, the plate was centrifuged at $2,100 \times g$ at 4°C. One hundred microliters of the supernatant was transferred to a new microplate and measured at 600 nm in a "Spectra Max 250" plate reader. Previous investigations with laminarinase from *Penicillum species* (Sigma, Taufkirchen, Germany) confirmed a linear time response between 0 and 0.3 OD_{600nm}.

Results

Constitutive activities

With two exceptions (*Eranthis hyemalis*, *Syringa vulgaris*), activities of chitinase, peroxidase, and glucanase were detected in all plant species investigated. In *Eranthys*, no constitutive activity of peroxidase could be detected (but see below), while no glucanase could be detected in *Syringa vulgaris*. Average activities differed by more than an order of magnitude among species (Fig. 1). No clear relations among the activities of the three enzyme classes within species occurred; for example, *Eranthys* almost completely lacked peroxidase activity, yet it showed the highest chitinase activity among all species tested. On the other hand, *Crocus vernus* exhibited highest activity of glucanase, but rather low activity of peroxidase (Fig. 1).

Furthermore, there was no clear relationship among constitutive enzyme activities and plant life histories. Highest glucanase activities were observed in *Crocus vernus*, an early flowering spring geophyte, and *Medicago sativa*, a larger perennial flowering in summer. Similarly, the highest and lowest activities of peroxidase and chitinase were equally distributed among plants flowering in spring and summer.

Induction effects

Although the type of life form did not determine absolute enzyme activities, it had a strong effect on whether or not resistance enzymes were inducible. Those five species that flowered later than mid of May (*Malus domesticus*, *Prunus* sp., *Urtica dioica*, *Medigaco sativa*, and *Cirsium arvense*) all exhibited inducible activities of all three resistance enzymes tested ($p<0.05$; Mann–Whitney U test comparing control and treated plants, see Fig. 1). Among the typical spring geophytes (*Eranthis hyemalis*, *Primula veris*, *Crocus vernus*, *Galanthus nivalis*, *Viola* sp., and *Anemone nemorosa*), in contrast, none exhibited inducible chitinase activity, and only two species each showed slight but significant induction effects on peroxidase and glucanase ($p<0.05$; Mann–Whitney U test, see Fig. 1).

Eranthyis hyemalis

As no peroxidase activity could be detected in *Eranthyis hyemalis* during the first measurements in 2003, an additional study was conducted in 2004. Extremely low activities of peroxidase could be detected (Fig. 2), which were not significantly affected by treatment ($F=1.875$; $df=5$, 37; $p=0.123$; univariate ANOVA). There was a significant treatment effect on the activity of chitinase ($F=10.353$; $df=5$, 37; $p<0.001$; univariate ANOVA) and glucanase ($F=129.172$; $df=5$, 37; $p<0.001$). Glucanase activity was
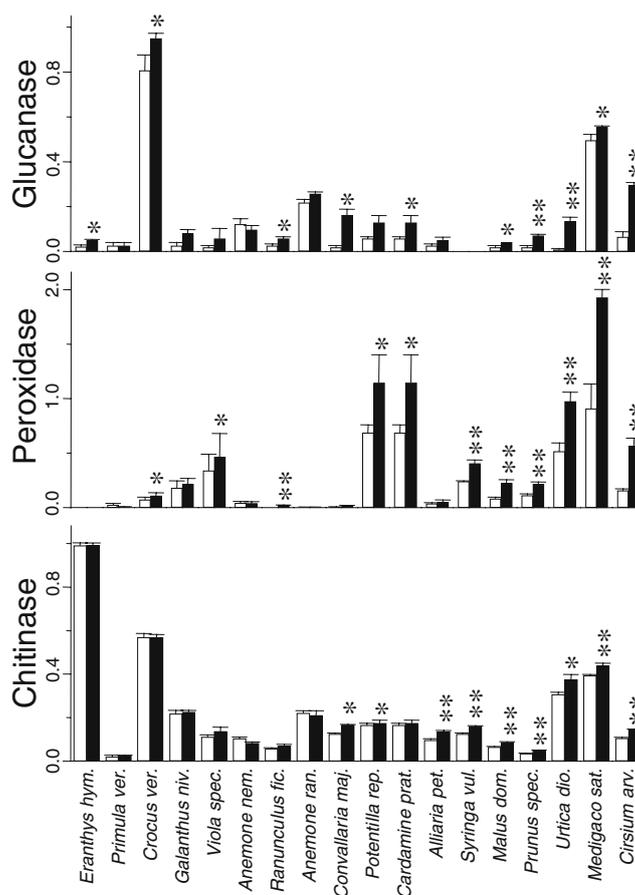
Fig. 1 Activities of three classes of resistance enzymes. Enzyme activities (mean+SE) are given as changes in specific absorptions (dA_{550} 2.5 h^{-1} 15 μl^{-1} plant extract for chitinase, dA_{600} 3 h^{-1} 30 μl^{-1} plant extract for glucanase and dA_{470} min^{-1} 10 μl^{-1} plant extract for peroxidase) separately for untreated controls (□) and for plants elicited with BION (▪). Significant differences between the treatments (according to Mann–Whitney U test) are indicated (*$p<0.05$, **$p<0.01$). Species order according to sampling date (see Table 1)

detectable in plants sprayed with water, jasmonic acid, and salicylic acid, yet not in plants sprayed with BION or infiltrated with chitosan or MgCl (Fig. 2). The activity of chitinase activity was high in all plants investigated, and the activities in plants sprayed with BION and infiltrated with MgCl or chitosan were significantly higher than in plants sprayed with jasmonic acid or salicylic acid ($p<0.05$; LSD post hoc test). However, control plants (sprayed with water only) exhibited intermediate activities and were not significantly different ($p>0.05$; LSD post hoc test) from any of the other treatments, confirming the result obtained in the year before (Fig. 2).

Discussion

We studied the activities of three enzyme classes that are related to pathogen resistance (chitinases, glucanases, and peroxidases) in a variety of wild plant species at their natural growing sites. All three enzyme classes are prominent "pathogenesis related" proteins whose induction in response to pathogen attack has been demonstrated in various plant–pathogen systems (van Loon and van Strien 1999). Several different lines of evidence (including transformation experiments) demonstrate that the expression of chitinases (particularly when co-expressed with glucanase) enhances plant pathogen resistance (Broglie et al. 1991; Datta and Datta 1999; Mauch et al. 1988; Neuhaus 1999; van der Westhuizen et al. 1998; Zhu et al. 1994). Plants that are characterized by reduced chitinase activity are significantly more vulnerable to attack by unspecific fungi under natural conditions (Heil et al. 1999), and plants apparently tend to reduce the activity of these enzymes when they are not required due to the presence of other defense mechanisms (Heil et al. 1999, 2000). We, therefore, assume that activities of these three enzyme classes give an ecologically relevant measure of a plant's overall resistance to pathogens.

Striking differences became obvious with respect to the constitutive activity of these enzymes (varying by 30 to 50 times among species) and to their inducibility by a chemical resistance elicitor, BION. Whether plants showed a detectable inducibility was related to their life history: only few of the perennial herbs flowering early in spring induced activities of resistance enzymes. In contrast, resistance enzymes in general were significantly induced in the larger perennials that flower later in spring or in summer. Plant species thus differ strongly in their expression of unspecific induced disease resistance.

'Do plants exist in nature being older than seedlings which have not been induced already?'(Heil 1999). The present data give first hints on the answer: many of the plant species investigated exhibited constitutive activities of resistance-related enzymes that were much higher than usually seen in control plants. In our study, natural influences might have had already induced the plants, at least partially. High 'constitutive' activities, thus, might result from previous, natural infections, or from a truly constitutive expression of these enzymes. Our data do not allow differentiation between these two explanations. However, they—apparently for the first time—allow an estimation of the average level of unspecific disease resistance that wild plants exhibit when growing under natural conditions, and that pathogens have to cope with when infecting these plants.

Whether or not plants responded to BION treatment depended both on plant species and on the enzyme regarded, but overall surprisingly few species responded. Chitinase activities showed a significant increase in nine of 18 species, peroxidase increased in nine species, and glucanase in 12 species. This result is contradictory to the general point of view regarding SAR as an almost omnipresent resistance mechanism. A methodical problem could be that different plant species might absorb BION with different efficiencies. However, BION has been developed to improve uptake rates of its active component and has been successfully used to induce resistance in species of families such as Brassicaceae (Godard et al. 1999; Lawton et al. 1996), Poaceae (Görlach et al. 1996), Chenopodiaceae (Mouhanna and Schlösser 1998), and Solanaceae (Thaler et al. 1999).

All but one of the species tested in our study responded to BION treatment with significant changes in at least one enzyme class (Fig. 1) or in their content of soluble proteins (data not shown). BION, obviously, was successfully taken up and recognized by all these species and, thus, could have led to increases in all resistance markers. Finally, *Eranthys hyemalis* received additional treatments, comprising the application of two known, resistance-related plant hormones and of a chitosan-preparation that has successfully been used to mimic fungal infection in several different plant species. However, none of these applications caused a significant response in peroxidase activity, and chitinase activity in plants sprayed with water as a control also did not differ significantly from any other treatment. All these considerations demonstrate that the patterns observed are more likely to represent true differences in the plants' strategies.

SAR obviously is less common than is generally assumed: SAR represents only one among many different strategies of disease resistance. Plants characterized by different life histories apparently have evolved different strategies to defend themselves against pathogens: plants that flower early in spring grow and reproduce under low pathogen pressure. Moreover, the growing period of these species may be too short as to make use of a response that
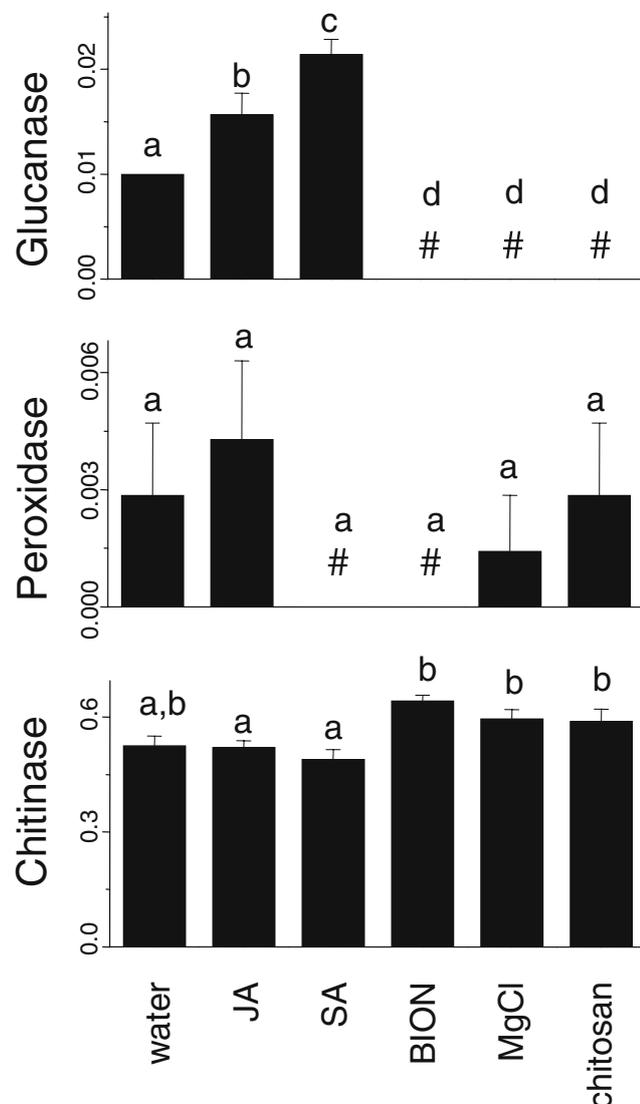

Fig. 2 Activities of resistance enzymes in *Eranthis hyemalis*. Plants received three spraying treatments (jasmonic acid, *JA*; salicylic acid, *SA*, and BION), for which water spraying served as a control, and an infiltration with chitosan (control: infiltration of MgCl solution). Enzyme activities differing significantly among treatments ($p<0.05$ according to LSD post hoc test) are indicated by *different letters*; activities below the detection level are marked #

firstly has to be induced. A constitutive disease resistance at a given species-specific level that cannot be further induced appears a more successful strategy under such circumstances. In contrast, the larger perennials flowering in late spring or summer and having a vegetative and reproductive period that covers a large part of the vegetation period are likely to be attacked by different pathogens during a large part of their growing period. This growing period, however, is long, and, thus, allows coping with the time lag between infection and resistance induction that characterize any induced resistance mechanism (Heil and Baldwin 2002). Further studies widening the spectrum of life forms beyond the ones covered by the present study and using natural pathogens both for resistance elicitation and quantification are required for a better understanding of the ecological and evolutionary relevance of induced systemic resistance of plants to pathogens.

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