Plant, Cell and Environment (2004) **27**, 896–906

Constitutive and induced resistance to pathogens in *Arabidopsis thaliana* depends on nitrogen supply

R. DIETRICH, K. PLOß & M. HEIL

Max-Planck-Institute of Chemical Ecology, Department of Bioorganic Chemistry, Hans-Knöll-Str. 8, D-07745 Jena, Germany

ABSTRACT

Knowledge about the induced pathogen resistance of plants is rapidly increasing, but little information exists on its dependence on abiotic growing conditions. *Arabidopsis thaliana* plants that had been cultivated under different nitrogen regimes were treated with BION®, a chemical resistance elicitor. The activities of three enzyme classes functionally involved in resistance (chitinase, chitosanase and peroxidase) were quantified over 8 d following treatment as resistance markers. Constitutive levels of three markers and the induced level of peroxidase and chitinase activity were significantly lower under limiting nitrogen supply. Under such conditions the increase of chitosanase activity after resistance induction was severely delayed, although the induced maximum activity of chitosanase was not significantly affected. Total soluble protein content decreased during the first 12 h after resistance elicitation. Thereafter, the induced plants cultivated under high N conditions reached higher protein contents than controls, whereas N-limited induced plants continuously had reduced protein contents. A plant's investment in resistance-related compounds can be severely constrained under limiting nitrogen supply.

Key-words: benzothiodiazole; chitinase; chitosanase, disease resistance; induced systemic resistance; pathogenesis related proteins; pathogen resistance; peroxidase; resource availability; systemic acquired resistance.

INTRODUCTION

Plants are challenged by pathogens in virtually all natural and agronomic ecosystems. Disease resistance therefore represents a vitally important trait for successful survival and reproduction. Research during recent decades has greatly increased our knowledge on constitutive and induced resistance, the latter term describing resistance traits that are expressed only in response to a first 'challenging' attack (Kuc 1982). We follow the terminological discussion presented by (Heil 2001) and use ISR to describe all plant-wide resistance phenomena that are characterized by the synthesis of pathogenesis-related (PR) proteins and that depend on salicylic acid (SA) signalling – we thus use induced systemic resistance (ISR) and systemic acquired resistance (SAR) as exchangeable terms.

Many factors affecting the type and intensity of resistance are known, and much has been learned on the genetics and the biochemistry of the underlying signalling pathways and on the compounds being regularly correlated with – or even causally involved in – the expression of the resistant phenotype. In contrast, only few studies have analysed the influence of abiotic factors on the intensity of inducible resistance to pathogens (Cipollini 2002).

Nitrogen is one of the primary factors limiting plant growth (White 1993), and it strongly affects the expression of both constitutive and induced resistance traits (Johnson, Rigney & Bentley 1989; Bolter, Latoszek-Green & Tenuta 1998; Stout, Brovont & Duffey 1998). Several hypotheses have been formulated to understand the dependence of plant resistance on the availability of resources such as nitrogen (Rhoades 1979; Coley, Bryant & Chapin 1985; Herms & Mattson 1992; Hamilton *et al*. 2001). However, recent empirical studies on the effect of nitrogen availability on induced herbivore resistance have yielded mixed results. Some studies reported an increase of resistance markers with decreasing N supply (Wilkens, Spoerke & Stamp 1996; Stout *et al*. 1998), whereas others found the opposite (Baldwin *et al*. 1993; Bolter *et al*. 1998) or reported no detectable changes (Stout *et al*. 1998).

The mentioned theories and most of the empirical studies focused on plant–herbivore interactions (Berenbaum 1995), whereas experiments analysing the influence of physiological or ecological constrains on inducible pathogen resistance are scarce. Moreover, the outcome of studies determining the induction state of elicited resistance at one distinct time after elicitation, strongly depends on whether or not resistance detection has been conducted in a suitable time window. No information on the kinetics of resistance expression can be derived from such studies. Attenuation of the defence response may point to physiological constraints, as proposed by substrate flow-based theories (Reichardt *et al*. 1991).

The aim of the present study was to examine the kinetics of induced resistance expression to pathogens in *Arabidopsis thaliana* plants growing under different nitrogen conditions. We used an artificial resistance elicitor, BION®, which can be applied externally to the plants (Oostendorp *et al*. 2001). The active component of BION® (benzo (1,2,3) thiadiazole-carbothioic acid *S*-methylester: CGA 254–704)

Correspondence: Rüdiger Dietrich. E-mail: rdietrich@ice.mpg.de

acts in the disease-related signal pathway at or downstream of the site of salicylic acid (Ryals *et al*. 1996). The efficacy of biological resistance resulting from BION® treatment may vary among different plant–pathogen combinations (Achuo *et al*. 2002). However, the spectrum of resistance activation and the biochemical changes induced by BION® matched that of a biological induction in most cases for which such information is available (Friedrich *et al*. 1996; Görlach *et al*. 1996; Dann *et al*. 1998; Molina *et al*. 1999; Heil *et al*. 2000). Striking advantages of using chemical elicitors are that resistance can be induced: (1) at one exactly defined time; (2) in a dosage-controlled manner; and (3) without additional effects resulting from the presence of pathogens. All these factors lead to a higher degree of comparability among studies of induced resistance.

We induced resistance once and monitored the response of three resistance markers – chitinase, chitosanase, and peroxidase – during the following 8 d. Chitinases (EC 3.2.1.14) and chitosanases (EC 3.2.1.132) are abundant proteins, widely distributed in lower and higher plants (Punja & Zhang 1993; Neuhaus 1999; Elouakfaoui & Asselin 1992). Chitinases hydrolyse chitin (a polymer of *N*-acetylglucosamin) whereas chitosanases degrade chitosan (completely or mostly deacetylated chitin). In addition to the discrimination on substrate specificity, both enzyme activities can be distinguished by their molecular weight (Grenier & Asselin 1990) and their isoelectric point (Sharma *et al*. 1993). Nevertheless, differentiation between chitinases and chitosanases is sometimes complicated by the fact that some chitinases possess chitosanase activity, too (Moder *et al*. 1999). There is increasing evidence that these enzymes play an important role in the direct resistance of plants to fungal pathogens. Plants themselves contain neither chitin nor chitosan, but many plant pests do (Bartnicki-Garcia 1968). Several chitinases exhibit pronounced antifungal activity, particularly in combination with β-1,3-glucanases (Schlumbaum *et al*. 1986; Mauch, Mauch-Mani & Boller 1988) and gel-separated chitosanases from tomato are able to lyse different fungal spores (Grenier & Asselin 1990). Both enzymes have constitutive and – after wounding or biological infection – induced activities (Pan, Ye & Kuc 1991; Grenier & Asselin 1990). Moreover, plants overexpressing chitinase can show decreased susceptibility to infection by some fungi that have a chitin-containing cell wall (Broglie *et al*. 1991; Datta & Datta 1999). Chitinases and chitosanases have therefore been included in the group of 'pathogenesis-related' (PR) proteins (Grenier & Asselin 1990; van Loon 1997; Neuhaus 1999). Peroxidases (EC 1.11.1) occur constitutively in various isoforms in most plant tissues (Lagrimini *et al*. 1997). Their activity can be increased by several stimuli, many being related to defence responses. Peroxidases (PR9) are involved in the hypersensitive reaction (HR) of plant cells, which leads to local necrosis in cells surrounding an infected one, thereby often 'trapping' pathogens in a ring of dead cells. Peroxidases are required for the formation of barriers to infecting pathogens via the polymerization of cell wall components such as lignin, suberin and extensin (Cooper & Varner 1984). Furthermore, peroxidases protect against damage caused by active oxygen species, which dramatically increase during HR and other pathogen-elicited local defence responses, collectively called 'oxidative burst' (Croft, Voisey & Slusarenko 1990).

We decided to determine enzyme activity under different nitrogen conditions, as (1) Summermatter, Sticher & Metraux (1995) verified for *A. thaliana* plants induced by *Pseudomonas syringae pv syringae* that an increased resistance against this pathogen is linked with an increase of two resistance markers (chitinase and peroxidase); (2) at present, for *Arabidopsis thaliana* only mRNA data is available to describe the induction of pathogen resistance by BION® (Lawton *et al*. 1996), while apparently no study has quantified the level of enzyme activity (which gives a more direct information on functioning resistance); and (3) both markers have constitutive and inducible activities.

Such information will contribute to a better understanding of disease resistance under limiting conditions and allow a better understanding of its role in natural ecosystems and its applicability in agronomic plant protection.

MATERIALS AND METHODS

Plant material, growing conditions and induction of disease resistance

Seeds of *Arabidopsis thaliana* accession Col-0 (H. Zimmer, Botanical Institute, University of Cologne, Germany) were germinated on moistened filter paper for 5 d and then transferred to 0.4 L pots (two seeds per pot) containing commercially available soil without nitrogen (80% 'Nullerde' Archut Erzeugnisse GmbH, Vechta, Germany; 10% sand; 10% vermiculite 1–2 mm, Isola Mineralwollewerke, Sprockhövel, Germany). The conditions in the growth chamber (greenhouse chamber; York Industriekälte GmbH & CoKG, Mannheim, Germany) were 10 h photoperiod, 50% relative humidity and 21 °C. The plants were watered three times a week. The levels of nitrogen received by the plants in low (N1), medium (N3) and high (N10) nitrogen treatment groups were manipulated by fertilizing plants with mineral fertilizer containing different concentrations of N (10 mL every second day of an aqueous 1, 3 or 10 ammonium nitrate solution). After 10 d the plants were thinned to one plant per pot and grouped in pairs according to rosette diameter. After 25 d the plants were subjected to induction treatment. One plant per pair was randomly chosen as a control (sprayed only with water), the other one as induced (sprayed with an aqueous 300 mg L^{-1} BION® solution). BION® is distributed by Syngenta (formerly Novartis), Basel, Switzerland, and was kindly provided by K.-L. Nau, Novartis, Frankfurt, Germany. Both plants were sprayed twice until runoff with approx. 120 min in-between. At 12 h and 1, 2, 4 and 8 d after induction, leaf samples were collected randomly from seven selected pairs per N treatment and stored at –20 °C until enzyme analysis.

Extraction of (soluble) enzymes

Between 0.25 and 0.5 g of frozen leaf material 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 pH 5.0. After a 5-min centrifugation (Eppendorf centrifuge 5415D; Eppendorf, Hamburg, Germany) at 16 000 *g* the supernatants were subjected to gel filtration on NAP 10 columns (Amersham Biosciences, Freiburg, Germany) equilibrated with 50 mM Na-phosphate buffer at pH 5.0. After homogenization, the eluate protein concentration was determined as described by Bradford (Bradford 1976) with bovine serun albumin as a standard. Quantification of contents of total soluble proteins was conducted routinely in advance of all enzyme assays. In order to answer the question whether or not protein content is affected by elicitor treatment, we additionally quantified protein contents in treated and control plants at very high (N30, respectively, 10 mL every second day of an aqueous 30 g L^{-1} ammonium nitrate solution and the same treatment as indicated in the upper section) nitrogen supply.

Enzymatic assays

Chitinase

Assays based on a method of (Wirth & Wolf 1990) were conducted in 96-well microplates. A total volume of 100 μL reaction preparation contained 3.2 μg protein, 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 incubation at –20 °C the plate was centrifuged at 2100 *g* at 4 °C. One hundred microlitres of the supernatant were 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}.

Chitosanase

Chitosanase activities were quantified with a modified assay based on the one described by (Osswald *et al.* 1992). A total volume of 80 μL reaction preparation contained 6.2 μg protein, 0.2% (w/v) chitosan (Sigma; containing min. 86.5% glucosamin) and 25 mM Na-acetate buffer at a pH of 5.0. Each reaction including the control (no chitosan) was replicated four times. The assay was incubated at 37 °C overnight and stopped with 14.4 μL 0.25 M KOH. After 30 min incubation on ice the probes were subjected to centrifugation for 15 min at 18 000 *g* Fifty microlitres of the supernatant were transferred to a new tube and 200 μL of 0.5 M Na-phosphate buffer pH 8.4 was added. After mixing, 200 μL of the probe were transferred to a transparent 96-well microplate (Greiner, Frickenhausen, Germany) and 40 μL flourescamin (0.3% w/v in acetone) were added. The fluorescence was measured in a fluorometer 'Genios' (Tecan, Maennedorf, Switzerland) with an excitation filter at 390 nm and an emission filter at 460 nm. All values were reported as fluorescence units. Previous investigations of reaction progress curves with *Streptomyces* sp. chitosanase (Sigma) confirmed a linear time-response-relation between 0 and at least 40 000 fluorescense units.

Peroxidase

A total volume of 200 μL reaction contained 0.625 μg protein, 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, Nuckles & Kuc 1982).

Statistical analysis

Data evaluation was conducted with SPSS (10.0). Unless stated otherwise, we used the GLM procedure and conducted univariate, multifactorial analysis of variance (ANOVA) separately for all markers to detect putatively significant effects of elicitor treatment, nitrogen condition, and time after elicitation, on enzyme activity. ANOVA is the recommended type of data evaluation when different factors may affect a variable. This type of analysis allows us to sort out on a statistical basis which factors have an influence on the variable analysed (here: enzyme activity). One of the advantages of the ANOVA is to also indicate interactions among two or more factors, namely cases in which factor A determines whether or not another factor B has a significant effect on the variable. Graphical data presentation focuses on factors that have been proved to be significant in the statistical analyses. For analysis of 'induced maximum activities' of enzymes we used data of the 4 d after elicitor treatment. Sample sizes are given along with the results.

RESULTS

The activities of the presented enzyme classes were significantly affected by induction, nitrogen supply and time after induction (for all factors $P < 0.001$ according to univariate ANOVA, see Table 1 for peroxidase, Table 2 for chitinase and Table 3 for chitosanase). Treatment with BION® ('induction') therefore successfully and reliably increased the activity of chitinases, chitosanases and peroxidases, yet the activity of the enzyme classes was also strongly affected by nitrogen supply (significant effect of N supply). The activity of the resistance markers increased over several days (significant effect of time), and the time course of total soluble protein depended on the nitrogen regime under which the plants had been cultivated (significant nitrogen supply–time interaction) (Table 4).

Constitutive enzyme marker activities

Nitrogen supply affected the constitutive levels of the activities of all three investigated enzymes (Fig. 1), which were

Table 1. Results of univariate ANOVA conducted on putative effects of nitrogen supply, time after induction, and presence or absence of induction, on peroxidase activity

	SS	d.f.	*F*	*P*
N-supply	16.736	2	101.990	<0.001
Time	10563	4	7.838	<0.001
Induction	8.926	1	48.324	<0.001
N-supply × induction	2.115	2	18.105	<0.001
N-supply × time	1.372	8	4.084	<0.001
Time × induction	0.738	4	18.105	0.003
N-supply × induction × time	0.518	8	4.218	0.168

SS, sum of squares; d.f., degrees of freedom; *F*, test variable; *P*, level of significance, i.e. probability of error when an effect is assumed. Original data are presented in Figs 1a, 2a and 3.

Table 3. Results of univariate ANOVA conducted on putative effects of nitrogen supply, time after induction, and presence or absence of induction, on chitosanase activity

	SS	d.f.	*F*	*P*
N-supply	22101168	2	14.521	<0.001
Time	25087134	4	8.242	<0.001
Induction	54982692	1	72.252	<0.001
N-supply × induction	2631715	2	1.729	0.180
N-supply × time	15228453	8	2.501	0.013
Time × induction	17103013	4	5.619	<0.001
N-supply × induction × time	3268658	8	0.537	0.828

Original data are presented in Figs 1c, 2c and 5. See legend to Table 1 for acronyms.

significantly lower when plants were cultivated under nitrogen-poor conditions. Although the influence of N supply on peroxidases and chitinases (Fig. 1a & b) was much stronger than on chitosanases (Fig. 1c), the increase of constitutive activities of all three enzyme classes with increasing N supply was almost linear (Fig. 1a–c).

Induced enzyme marker activities

Induced maximum activities of chitinase and peroxidase were strongly and significantly affected by nitrogen supply ($P < 0.001$ for the nitrogen supply–induction interaction, see Tables 1 & 2). The increase of the induced maximum activities with increasing N-supply for peroxidase was stronger from 'N3' to 'N10' in comparison with 'N1' to 'N3' (Fig. 2a) whereas we measured an almost linear increase (from 'N1' to 'N10') for chitinases (Fig. 2b). Moreover, the relative increase of the induced compared to the control activity of chitinase was strongest under nitrogen-poor conditions (+ some 400% for 'N1'), and became weaker for the following treatments (+ some 200% for 'N3') and plus >100% for 'N10'. In contrast, induced maximum activities of chitosanase under 'N10' were only marginally (approximately 17%) higher than under 'N1' conditions. Although a slight increase of average (i.e. mean) chitosanase activities with increasing nitrogen supply could be observed (Fig. 2c), none of these differences proved significant, and no significant nitrogen supply–induction interaction could therefore be detected (Table 3). As the constitutive level of chitosanases was slightly but significantly lower at limiting N, the relative increase of chitosanases was highest at the 'N1' level (85%).

Expression kinetics

In response to elicitor treatment the activities of the three enzyme classes in induced plants in comparison with controls increased at least during the first 2–4 d after BION® treatment (see Figs 3–5). The velocity of this increase was strongly affected by the nitrogen regime under which the plants had been cultivated (significant nitrogen supply–time interaction for the selected markers, see Tables 1–3). This effect was particularly obvious in the case of induced chitosanase activity, for which almost the maximum effect of induction (i.e. the relative difference in enzyme activity between controls and induced plants) was already reached at 1 d after BION® treatment when plants received 'N10' nitrogen supply (46%; maximum 63% on the fourth day).

Table 2. Results of univariate ANOVA conducted on putative effects of nitrogen supply, time after induction, and presence or absence of induction, on chitinase activity

	SS	d.f.	*F*	*P*
N-supply	0.111	2	0.056	<0.001
Time	0.031	4	0.008	<0.001
Induction	0.101	1	0.101	<0.001
N-supply × induction	0.005	2	0.002	<0.001
N-supply × time	0.032	8	0.001	<0.001
Time × induction	0.022	4	0.005	<0.001
N-supply × induction × time	0.006	8	0.001	0.008

Original data are presented in Figs 1b, 2b and 4. See legend to Table 1 for acronyms.

Table 4. Results of univariate ANOVA conducted on putative effects of nitrogen supply time after induction, and presence or absence of induction, on the amount of total soluble protein

	SS	d.f.	*F*	*P*
N-supply	3157019	3	90.79	<0.001
Time	680238	4	14.67	<0.001
Induction	24628	1	2.12	0.146
N-supply × induction	151028	3	4.34	<0.001
N-supply × time	973397	12	14.67	0.005
Time × induction	180329	4	3.890	0.004
N-supply × induction × time	113431	12	0.816	0.634

Original data are presented in Fig. 6. See legend to Table 1 for acronyms.

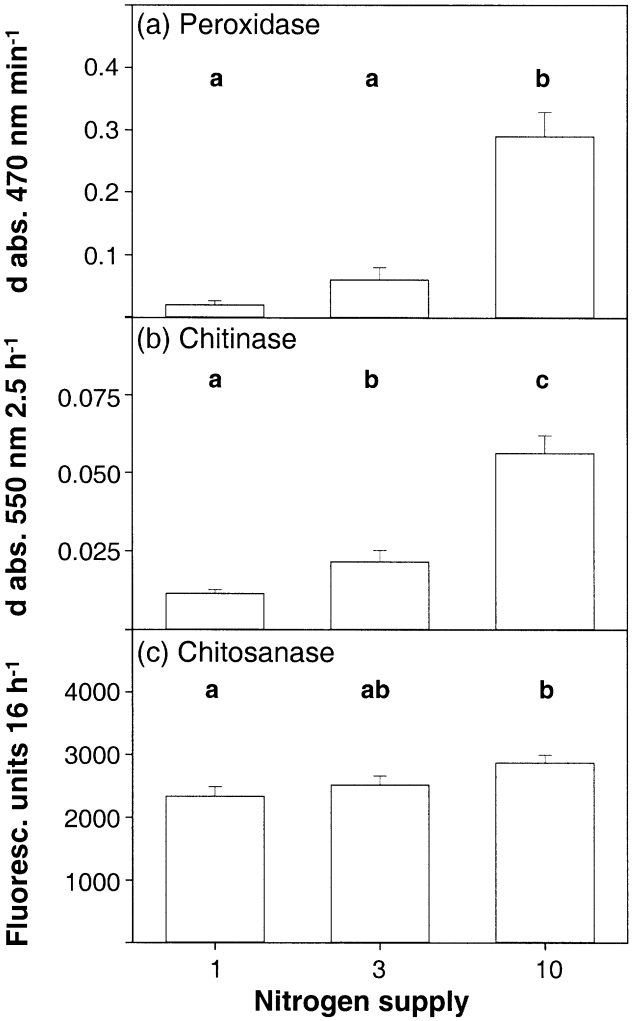

Figure 1. Effect of nitrogen availability on the constitutive activity of peroxidase (a) chitinase (b) and chitosanase (c) in *Arabidopsis thaliana*. Activities are expressed per 0.625 μg (a), per 3.2 μg (b) and per 6.2 μg (c) protein. Measurements summarize mean activities +SE at low (N1), medium (N3) and high (N10) nitrogen regimes. Letters indicate significant differences among fertilization regimes. Seven plants were collected at five different times. Data represent the averages of 35 plants per nitrogen regime.

In contrast, it took 2 d to reach the maximum effect of induction in plants receiving 'N3' nitrogen supply (40%, maximum 54% at the fourth day), and the earliest that the maximum effect of induction was reached was 4 d (75%, maximum 85% on the eighth day) after elicitation under the 'N1' nitrogen regime (see Fig. 5).

Changes in soluble protein content

The total soluble protein concentration of the plants was significantly affected by nitrogen availability and elicitor treatment (Table 4). As the interaction nitrogen supply × induction was significant, whereas the interaction time × induction was not, the overall effect of induction was only marginally significant ($P = 0.146$, see Table 4). Under all four nitrogen regimes, induced plants had a lower protein concentration in comparison with control plants 12 h after resistance elicitation (Fig. 6). The further response did, however, depend on nitrogen regime. Under 'N30' conditions, the protein contents of induced plants strongly increased and had already reached higher values than in untreated control plants at 1 d after BION® treatment. This pattern (higher protein concentrations in induced plants) remained stable over all the remaining observed time span. Induced plants growing under 'N3' and 'N10' conditions also compensated for the initial reduction in protein content and had higher protein contents than the controls at 8 d after resistance elicitation. Under low nitrogen supply ('N1'), there was also a continuous increase in the protein contents of induced plants in comparison with the low level that had been observed at 12 h after resistance elicitation.

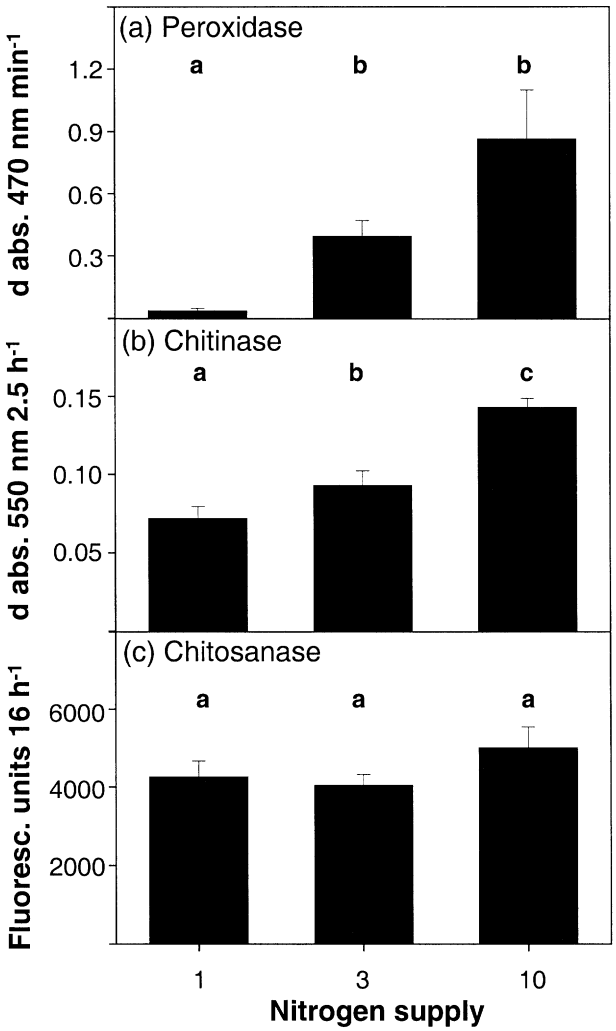

Figure 2. Effect of nitrogen availability on the induced maximum activity of peroxidase (a), chitinase (b) and chitosanase (c) in *Arabidopsis thaliana*. Activities are expressed per 0.625 μg (a), per 3.2 μg (b) and per 6.2 μg (c) protein. Measurements summarize activities of seven plants +SE on day 4 after elicitation with BION®. Different letters appearing at the top of the figure indicate significant differences among the various nitrogen supplies.

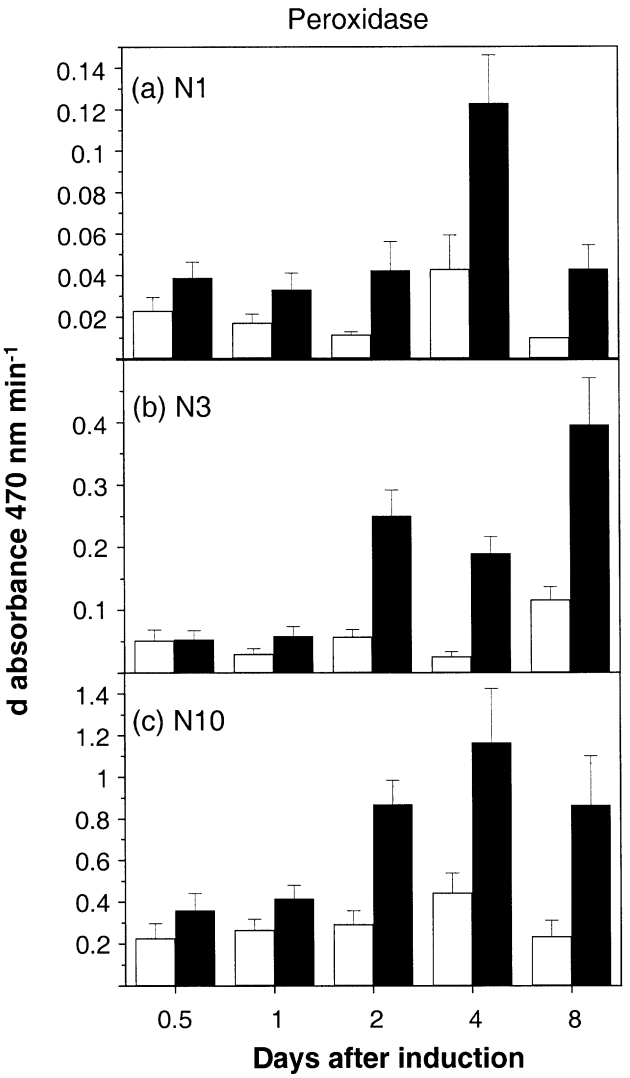

Figure 3. Dependence of the velocity of expression on the induced activity of peroxidase in *Arabidopsis thaliana*. Activities are expressed per 0.625 μg protein. Bars represent the mean activities +SE of induced activities (black bars, ■) compared with control activity (white bars, □) at low (N1), medium (N3) and high (N10) nitrogen supply at different times after elicitation with BION®. Bars stand for seven plants each.

However, protein contents of induced plants remained much lower and reached approximately the same level as in control plants at the earliest 8 d after elicitation (Fig. 6).

DISCUSSION

We investigated whether the activity and expression kinetics of three resistance-related enzymes are affected by resource limitation. The activities of chitinases, chitosanases and peroxidases in *Arabidopsis thaliana* Col 0 plants indeed strongly depended on nitrogen supply. Constitutive levels of the selected resistance markers were much lower in plants cultivated under limiting 'N1' than in plants grown under high 'N10' nitrogen conditions (Fig. 1). The same effect could be observed for maximum induced levels of peroxidase and chitinase activity (Fig. 2a & b). Interestingly, even the induction kinetics for the chitosanases were severely affected by nitrogen supply: the plants needed much more time to achieve maximum levels of chitosanase activity when suffering from a shortage of N (Fig. 5), although these maximum levels were only marginally influenced by N supply (Fig. 2c).

Our results demonstrate that the ability of a plant to express defence compounds is strongly limited by abiotic growing conditions: limitations became obvious with respect to both the final activity of resistance markers and the time required for a full expression of this activity.

A central methodological issue of the present study is the use of biochemical assays to quantify enzymes on the level

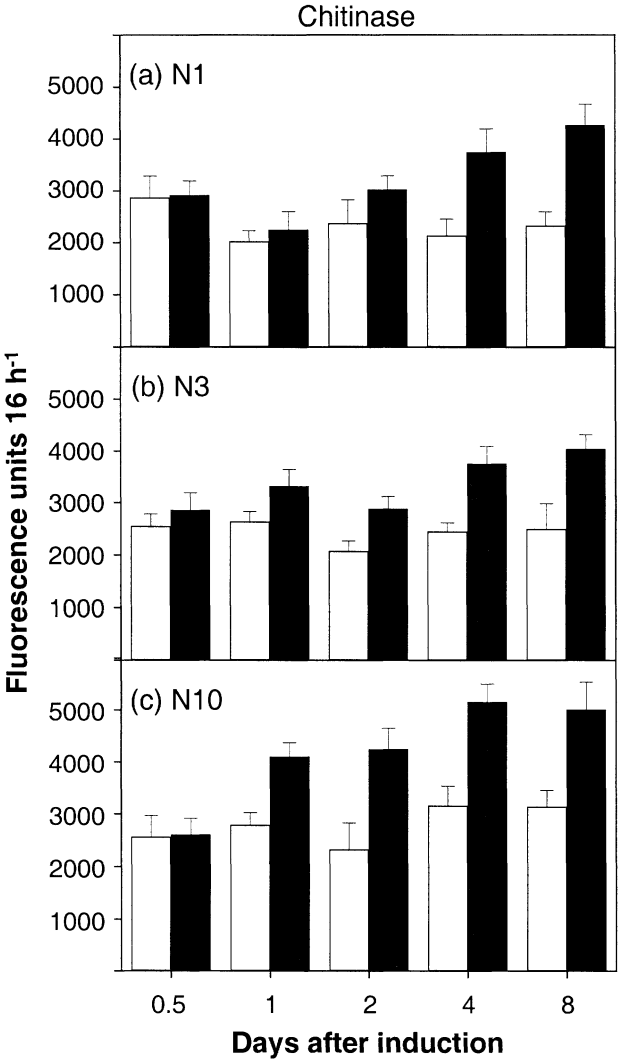

Figure 4. Dependence of the velocity of expression on the induced activity of chitinase in *Arabidopsis thaliana.* Activities are expressed per 3.2 μg protein. Bars represent the mean activities +SE of induced activities (black bars, ■) compared with control activity (white bars, □) at low (N1), medium (N3) and high (N10) nitrogen supply at different times after elicitation with BION®. Bars stand for seven plants each.

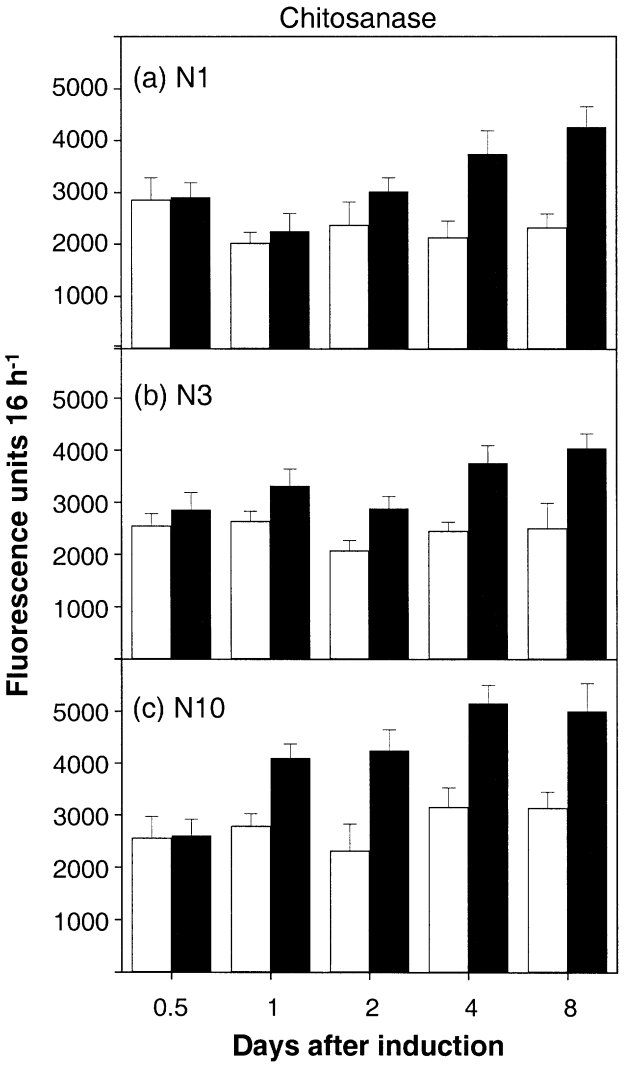

Figure 5. Dependence of the velocity of expression on the induced activity of chitosanase in *Arabidopsis thaliana*. Activities are expressed per 6.2 μg protein. Bars represent the mean activities +SE of induced activities (black bars, ■) compared with control activity (white bars, □) at low (N1), medium (N3) and high (N10) nitrogen supply at different times after elicitation with BION®. Bars stand for seven plants each.

of their activity. The activities of chitinases and peroxidases used in the present study correlates with the intensity and time course of the same resistance markers after biological infection (Summermatter *et al.* 1995). Moreover, the enzyme markers used are known to be causally involved in pathogen resistance (see Introduction section). We therefore assume these activities are relevant disease resistance markers. A second central issue of our study is that a chemical treatment (spraying an aqueous solution of BION®) was chosen to induce pathogen resistance. (Friedrich *et al.* 1996) reported BION® induced the same characteristic set of resistance genes, as did induction by biological agents or salicylic acid. Our study focused on protein function, whereas the data on molecular resistance marker after BION® induction presented so far has concerned the level of mRNA. Data on protein activity, as presented here, match the time courses reported by (Lawton *et al.* 1996) for mRNA expression in plants of the same ecotype and grown under similar conditions, pointing to a general comparability of both systems. Together with the benefits mentioned in the Introduction section all these observations demonstrate that chemical resistance elicitation with BION® was a suitable treatment for the questions asked in the present analysis.

Several studies investigated the dependence on nutrient supply of constitutive and induced defence traits, but general patterns are difficult to obtain. Among the studies that focused on constitutive resistance, those quantifying

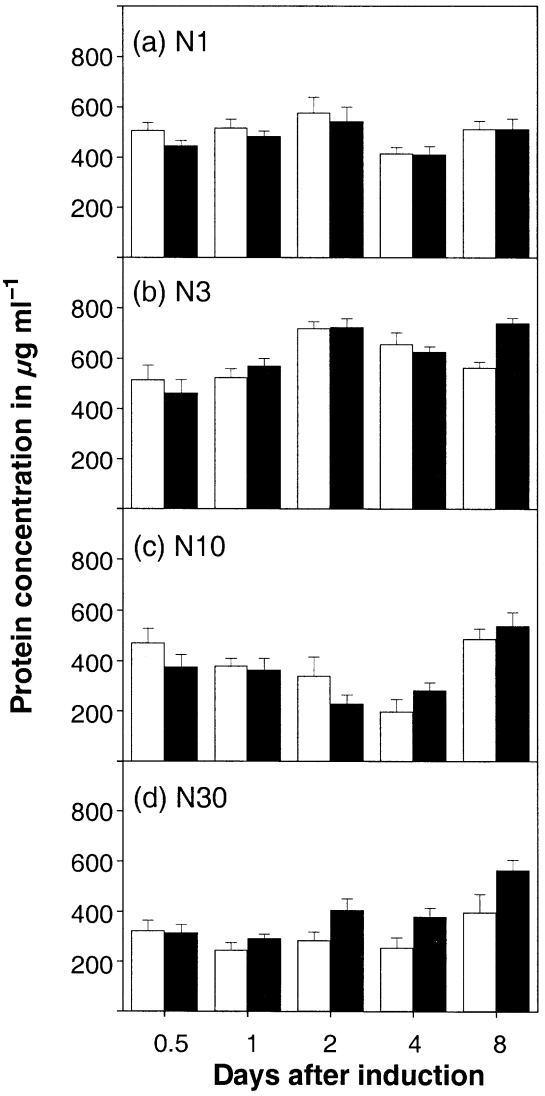

Figure 6. Time course of the amount of total soluble protein content in *Arabidopsis thaliana* after elicitation with BION®. Bars represent mean concentrations +SE of total soluble protein of induced plants (black bars, ■) compared to control plants (white bars, □) at low (N1), medium (N3) high (N10) and highest (N30) nitrogen supply at different times after elicitation with BION®. Bars represent seven plants each.

tannins and other phenolic compounds reported the most consistent picture: these compounds in general were more strongly expressed under N-limiting conditions (Waring *et al*. 1985; Larsson *et al*. 1986; Ruohomäki *et al*. 1996; Stout *et al*. 1998). For other compounds such as terpenes or alkaloids, an increase with decreasing nitrogen (Mihaliak & Lincoln 1985), no correlation (Muzika, Pregitzer & Hanover 1989) or a decrease with decreasing nitrogen (Johnson, Liu & Bentley 1987) could be demonstrated. Studies regarding constitutive enzymatic defences are even more limited, with only one study reporting an increase of polyphenol oxidase and proteinase inhibitor with decreasing N (Stout *et al*. 1998). Therefore, our study appears to be the first report of a lowered activity of resistance enzymes in plants growing under limiting nitrogen conditions, indicating that other ISR proteins could behave similarly. It remains to be investigated whether this reflects a general difference between resistance to herbivores (being covered by most studies conducted so far in the context of nitrogen availability) and resistance to pathogens (our study), or whether it is caused by the fact that defence proteins represent N-demanding enzymatic defences.

Only a few studies have focused on the dependence of induced resistance on nitrogen availability. For example, the production of damage-induced alkaloids was not affected by nitrogen in a study of Johnson *et al*. (1989), whereas Baldwin *et al*. (1993) found lower nicotine contents in tobacco plants cultivated under low N conditions. Only two studies besides the present one quantified induced enzymatic defences, but gained mixed results. (Bolter *et al*. 1998) reported papain inhibitor activity was induced in leaves of wounded potato plants directly proportional to the concentration of nitrogen supplied. In contrast (Stout *et al*. 1998) found no effect of varying N supply on proteinase inhibitors in tomato. The differences between these two studies might in part be explained by the use of different taxa or different growing conditions. However, our results demonstrate that, in the same plant individuals, induced enzymatic defence compounds are regulated differently. Whereas induced maximum activity of peroxidase and chitinase were directly correlated with nitrogen supply (Fig. 2a & b), induced maximum activities of chitosanase showed no detectable dependency on nitrogen conditions (Fig. 2c). This rules out all explanations such as, the C/N balance hypothesis (Bryant, Chapin & Klein 1983), which are based on major differences in chemical composition of defence compounds. We are currently lacking a convincing explanation of this phenomenon, although the different functions of the enzyme classes provide probable reasons for adaptive differences in the regulatory mechanisms controlling their expression. Peroxidases are not only involved in defence traits yet have other functions, too (Lagrimini *et al*. 1997). Chitosanases and chitinases, in contrast, have no known plant-internal substrate and therewith represent merely, or mainly, a resistance trait. They therefore depend not only on abiotic conditions but also on ecological conditions as is indicated by a stronger increase of induced maximum activities at poor nitrogen conditions.

The dependence of chitosanase expression on nitrogen supply is obvious when induction kinetics are regarded: although induced maximum activity of chitosanase was only marginally affected by N conditions, chitosanase expression was severely delayed when N was limiting (Fig. 5). To our knowledge so far only one study observed an attenuation of induction of a defensive compound (in this case: alkaloids) by limited nitrogen (Johnson *et al*. 1989). In our study the attenuation of chitosanase expression represents the only case in which allocation to resistance could be explained by a passive substrate flow or a 'using up' of overflow resources, as has been supposed by (Reichardt *et al*. 1991). All the other data, in contrast, clearly indicate that allocation of limited resources to resistance is a matter of fine regulation, ensuring an optimal adaptation to environmental stresses.

How dramatically both primary and secondary metabolism are affected during resistance expression – an intensive metabolic 're-programming' during resistance elicitation has been described by (Somssich & Hahlbrock 1998; Hahlbrock *et al*. 2003) – becomes obvious when regarding total soluble protein concentrations. All induced plants showed a decrease of total soluble protein during the first 12 h after elicitation, pointing to a need for amino acids for the synthesis of resistance compounds shortly after elicitation. This observation is in line with a variety of different studies reporting reductions in the expression of genes related to primary metabolism in response to resistance elicitation (Kombrink & Hahlbrock 1990; Logemann *et al*. 1995; Somssich & Hahlbrock 1998). This down-regulation of primary metabolism might be required to free up resources needed for *de novo* synthesis of defensive compounds (Heil 2002), an interpretation being in line with the fact that – in response to the decrease in protein content observed after 12 h – protein contents increased again in all induced plants. This effect led to much higher protein contents in induced as compared to control plants when plants were cultivated under highest 'N30' conditions. A general (up to eight-fold) increase in soluble protein content of intercellular washing fluid of *Phytophtora*-infected potato leaves has recently been described by (Hoegen *et al*. 2002) and was correlated with both decreases and increases in many different proteins.

In contrast, induced plants grown under limiting 'N1' conditions had continuously lower protein contents than the respective controls and reached approximately the same level as controls at the earliest 8 d after resistance elicitation. The plants apparently resynthesize the degraded proteins, reaching the original protein level plus resistance-related proteins under high N conditions, whereas under low N conditions trade-offs between proteins of the primary and secondary metabolism could occur.

Considering kinetics of resistance expression gives much more information on the plants' response and opens a wide field of interest. Fast responses to challenging pathogens appear evolutionarily important in order to avoid spread of the pathogen and, therewith, loss of plant material and resources due to spreading infection (Johnson *et al*. 1989;

Dicke & Bruin 2001; Heil & Baldwin 2002). However, the ability to respond quickly can be strongly limited by resource supply, and balanced expression regimes that may strongly differ among various defence traits can therefore be expected to have evolved.

CONCLUSIONS

As to our knowledge, this study presents the first data on the dependence of induced and constitutive disease resistance markers on nitrogen supply. Limiting nitrogen supply had in general detrimental effects on the expression of resistance compounds. We observed no case in which plants grown under 'lower N' conditions had higher values of a defensive compound than plants receiving 'high N'. Resistance elicitation is obviously a resource-demanding process, which may take place at the cost of existing compounds, and which can be limited by restricted resource supply.

A plant's ability to respond to pathogen infection therewith can be constrained by physiological demands and resource limitation. Some of these limitations became obvious only when regarding induction kinetics rather than defining induced stages at one time after resistance elicitation. More studies quantifying resistance at different times after elicitation and using: (1) several defence compounds as well as 'biological' measures of resistance; (2) plants of different taxa; and (3) a range of resource conditions, are required in order to gain a better understanding of the ability of plants to defend themselves against pathogens under different growing conditions.

ACKNOWLEDGMENTS

The authors thank J. Gershenzon, B. Mauch-Mani and two anonymous referees for helpful comments on the manuscript. This study gained significant benefits from many discussions with L.C. van Loon, K. Hahlbrock, J.-P. Métraux and B. Mauch-Mani. Special thanks go to Wilhelm Boland for providing us with all facilities required for conducting this study. Practical support came from Ulrike Preiß, Tamara Krügel, Iro Lange and Andreas Weber. Financial support by the German Research Foundation (DFG grant HE 3169/2) and the Max-Planck-Society is gratefully acknowledged.

REFERENCES

Achuo A.E., Audenaert K., Meziane H. & Hofte M. (2002) The SA-dependent defense pathway is active against different pathogens in tomatoe and tobacco. *Mededelingen Faculteit Landbouwkundige En Toegepaste Biologische Wetenschappen Universiteit Gent*, 67, 149–157.

Baldwin I.T., Oesch R.C., Merhige P.M. & Hayes K. (1993) Damage-induced root nitrogen metabolism in *Nicotiana sylvestris*: testing C/N predictions for alkaloid production. *Journal of Chemical Ecology* **19,** 3029–3043.

Bartnicki-Garcia S. (1968) Cell wall chemistry, morphogenesis and taxonomy of fungi. *Annual Review of Microbiology* **22,** 87–108.

Berenbaum M.R. (1995) The chemistry of defence: theory and practice. *Proceedings of the National Academy of Sciences USA* **92,** 2–8.

Bolter C., Latoszek-Green M. & Tenuta M. (1998) Dependence of methyl jasmonate- and wound-induced cysteine proteinase inhibitor activity on nitrogen concentration. *Journal of Plant Physiology* **152,** 427–432.

Bradford M.M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72,** 248–254.

Broglie K., Chet I., Holliday M., Cressman R., Biddle P., Kowlton S., Mauvais C. & Broglie R. (1991) Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. *Science* **254,** 1194–1197.

Bryant J.P., Chapin III F.S. & Klein D.R. (1983) Carbon/nutrient balance of boreal plants in relation to vertebrate herbivory. *Oikos* **40,** 357–368.

Cipollini D. (2002) Does competition magnify the fitness costs of induced resistance in *Arabidopsis thaliana*? A manipulative approach. *Oecologia* **131,** 514–520.

Coley P.D., Bryant J.P. & Chapin III F.S. (1985) Resource availability and plant antiherbivore defense. *Science* **230,** 895–899.

Cooper J.B. & Varner J.E. (1984) Cross-linking of soluble extensin in isolated cell-walls. *Plant Physiology* **76,** 414–417.

Croft K.P.C., Voisey C.R. & Slusarenko A.J. (1990) Mechanism of hypersensitive cell collapse: correlation of increased lipoxygenase activity with membrane damage in leaves of *Phaseolus vulgaris* (L.) inoculated with an avirulent race of *Pseudomonas syringae* pv. *phaseolicola*. *Physiological and Molecular Plant Pathology* **36,** 49–62.

Dann E., Diers B., Byrum J. & Hammerschmidt R. (1998) Effect of treating soybean with 2,6-dichlorisonicotinic acid (INA) and benzothiadiazol (BTH) on seed yields and the level of disease caused by *Sclerotonia sclerotiorum* in field and greenhouse studies. *European Journal of Plant Pathology* **104,** 271–278.

Datta K.N. & Datta S.K. (1999) Expression and function of PR-protein genes in transgenic plants. In *Pathogenesis-Related Proteins in Plants* (eds S. Datta & S. Muthukrishnan), pp. 261–277. CRC Press, Boca Raton, FL, USA.

Dicke M. & Bruin J. (2001) Chemical information transfer between plants: back to the future. *Biochemical Systematics and Ecology* **29,** 981–994.

Elouakfaoui S. & Asselin A. (1992) Multiple forms of chitosanase activities. *Phytochemistry* **31,** 1513–1518.

Friedrich L., Lawton K., Ruess W., *et al.* (1996) A benzothiadiazole derivative induces systemic acquired resistance in tobacco. *Plant Journal* **10,** 61–70.

Görlach J., Volrath S., Knauf-Beiter G., *et al.* (1996) Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *Plant Cell* **8,** 629–643.

Grenier J. & Asselin A. (1990) Some pathogenesis-related proteins are chitosanases with lytic activity against fungal spores. *Molecular Plant–Microbe Interactions* **3,** 401–407.

Hahlbrock K., Bednarek P., Ciolkowski I., *et al.* (2003) Non-self recognition, transcriptional reprogramming, and secondary metabolite accumulation during plant/pathogen interactions. *Proceedings of the National Academy of Science of the USA* **100,** 14569–14576.

Hamilton J.G., Zangerl A.R., DeLucia E.H. & Berenbaum M.R. (2001) The carbon-nutrient balance hypothesis: its rise and fall. *Ecology Letters* **4,** 86–95.

Hammerschmidt R., Nuckles E.M. & Kuc J. (1982) Association of enhanced peroxidase-activity with induced systemic resistance

of cucumber to *Colletotrichum lagenarium*. *Physiological Plant Pathology* **20,** 73–82.

Heil M. (2001) The ecological concept of costs of induced systemic resistance (ISR). *European Journal of Plant Pathology* **107,** 137–146.

Heil M. (2002) Ecological costs of induced resistance. *Current Opinion in Plant Biology* **5,** 345–350.

Heil M. & Baldwin I.T. (2002) Fitness costs of induced resistance: emerging experimental support for a slippery concept. *Trends in Plant Science* **7,** 61–67.

Heil M., Hilpert A., Kaiser W. & Linsenmair K.E. (2000) Reduced growth and seed set following chemical induction of pathogen defence: does systemic acquired resistance (SAR) incur allocation costs? *Journal of Ecology* **88,** 645–654.

Herms D.A. & Mattson W.J. (1992) The dilemma of plants: to grow or to defend. *Quarterly Review of Biology* **67,** 283–335.

Hoegen E., Strömberg A., Pihlgren U. & Kombrink E. (2002) Primary structure and tissue-specific expression of the pathogenesis-related protein PR-1b in potato. *Molecular Plant Pathology* **3,** 329–345.

Johnson N.D., Liu B. & Bentley B.L. (1987) The effects of nitrogen fixation, soil nitrate and defoliation on the growth, alkaloids and nitrogen level of *Lupinus succulentus* (Fabacaea). *Oecologia* **74,** 425–431.

Johnson N., Rigney L. & Bentley B. (1989) Short-term induction of alkaloid production in lupines. Differences between N_2-fixing and nitrogen-limited plants. *Journal of Chemical Ecology* **15,** 2425–2434.

Kombrink E. & Hahlbrock K. (1990) Rapid, systemic repression of the synthesis of ribulose 1,5-bisphosphate carboxylase small-subunit mRNA in fungus-infected or elicitor-treated potato leaves. *Planta* **181,** 216–219.

Kuc J. (1982) Induced immunity to plant disease. *Bioscience* **32,** 854–860.

Lagrimini L.M., Gingas V., Finger F., Rothstein S. & Liu T.-T.Y. (1997) Characterization of antisense transformed plants deficient in the tobacco anionic peroxidase. *Plant Physiology* **114,** 1187–1196.

Larsson S., Wiren A., Lundgren L. & Ericsson T. (1986) Effect of light and nutrient stress on leaf phenolic chemistry in *Salix dasyclados* susceptibility to *Galerucella lineola* (Coleoptera). *Oikos* **47,** 205–210.

Lawton K.A., Friedrich L., Hunt M., Weymann K., Delaney T., Kessmann H., Staub T. & Ryals J.A. (1996) Benzothiadiazole induces disease resistance in Arabidopsis by activation of the systemic acquired resistance signal transduction pathway. *Plant Journal* **10,** 71–82.

Logemann E., Wu S.-C., Schröder J., Schmelzer E., Somssich I.E. & Hahlbrock K. (1995) Gene activation by UV light, fungal elicitor or fungal infection in *Petroselium crispum* is correlated with repression of cell-cycle-related genes. *Plant Journal* **6,** 865–876.

van Loon L.C. (1997) Induced resistance in plants and the role of pathogenesis-related proteins. *European Journal of Plant Pathology* **103,** 753–765.

Mauch F., Mauch-Mani B. & Boller T. (1988) Antifungal hydrolases in pea tissue II. Inhibition of fungal growth by combination of chitinase and β-1,3 glucanase. *Plant Physiology* **88,** 936–1042.

Mihaliak C.A. & Lincoln D.E. (1985) Growth pattern and carbon allocation to volatile leaf terpens under nitrogen-limiting conditions in *Heterotheca subaxillaris* (Asteraceae). *Oecologia* **66,** 423–426.

Moder W., Bunk A., Albrecht A., Doostdar H., Niedz R.P., McDonald R.E., Mayer R.T. & Osswald W.F. (1999) Characterization of acidic chitinases from culture medium of sweet orange callus tissue. *Journal of Plant Physiology* **154,** 296–301.

Molina A., Görlach J., Volrath S. & Ryals J. (1999) Wheat genes encoding two types of PR-1 proteins are pathogen inducible, but do not respond to activators of systemic acquired resistance. *Molecular Plant–Microbe Interactions* **12,** 53–58.

Muzika R.M., Pregitzer K.S. & Hanover J.W. (1989) Changes in terpen production following nitrogen fertilization of grand fir (*Abies grandis* (Dougl.) Lindl.) seedlings. *Oecologia* **80,** 485–489.

Neuhaus J.M. (1999) Plant Chitinases (PR-3, PR-4, PR-8, PR-11). In *Pathogenesis-Related Proteins in Plants* (eds S.K. Datta & S. Muthukrishnan), pp. 77–105. CRC Press, Boca Raton, FL, USA.

Oostendorp M., Kunz W., Dietrich B. & Staub T. (2001) Induced resistance in plants by chemicals. *European Journal of Plant Pathology* **107,** 19–28.

Osswald W.F., McDonald R.E., Niedz R.P., Shapiro J.P. & Mayer R.T. (1992) Quantitative fluorometric analysis of plant and microbial chitosanases. *Analytical Biochemistry* **204,** 40–46.

Pan S.Q., Ye X.S. & Kuc J. (1991) A technique for detection of chitinase, β-1,3-glucanase, and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectrofocussing. *Phytopathology* **81,** 970–974.

Punja Z.K. & Zhang Y.-Y. (1993) Plant Chitinases and their roles in resistance to fungal diseases. *Journal of Nematology* **25,** 526–540.

Reichardt P.B., Chapin III F.S., Bryant J.P., Mattes B.R. & Clausen T.P. (1991) Carbon/nutrient balance as a predictor of plant defense in Alaskan balsam poplar: potential importance of metabolic turnover. *Oecologia* **88,** 401–406.

Rhoades D.F. (1979) Evolution of plant chemical defense against herbivores. In *Herbivores: Their Interaction with Secondary Plant Metabolites* (eds G.A. Rosenthal & D.H. Janzen), pp. 4–53. Academic Press, New York and London.

Ruohomäki K., Chapin III F.S., Haukioja E., Neuvonen S. & Suomela J. (1996) Delayed inducible resistance in mountain birch in response to fertilization and shade. *Ecology* **77,** 2302–2311.

Ryals J., Neuenschwander U., Willits M., Molina A., Steiner H. & Hunt M. (1996) Systemic acquired resistance. *Plant Cell* **8,** 1809–1819.

Schlumbaum A., Mauch F., Vögeli U. & Boller T. (1986) Plant chitinases are potent inhibitors of fungal growth. *Nature* **324,** 365–367.

Sharma P., Borja D., Stougaard P. & Lönneborg A. (1993) PR-proteins accumulating in spruce roots infected with a pathogenic *Pytium* sp. isolate include chitinases, chitosanases and β-1,3-glucanases. *Physiological and Molecular Plant Pathology* **43,** 57–67.

Somssich I.E. & Hahlbrock K. (1998) Pathogen defence in plants – a paradigm of biological complexity. *Trends in Plant Science* **3,** 86–90.

Stout M.J., Brovont R.A. & Duffey S.S. (1998) Effect of nitrogen availability on expression of constitutive and inducible chemical defenses in tomato, *Lycopersicon esculentum*. *Journal of Chemical Ecology* **24,** 945–963.

Summermatter K., Sticher L. & Metraux J.P. (1995) Systemic responses in *Arabidopsis thaliana* infected and challenged with *Pseudomonas syringae* pv *syringae*. *Plant Physiology* **108,** 1379–1385.

Waring R.H., McDonald A.J.S., Larsson S.S., Ericsson T., Wiren A., Arwidsson E., Ericsson A. & Lohammer T. (1985) Differences in chemical composition of plants grown at constant relative growth rates with stable mineral nutrition. *Oecologia* **66,** 157–160.

White T. (1993) *The Inadequate Environment – Nitrogen and the Abundance of Animals*. Springer, New York, USA.

Wilkens R., Spoerke J. & Stamp N. (1996) Differential responses of growth and two soluble phenolics of tomato to resource availability. *Ecology* **77,** 247–258.

Wirth S.J. & Wolf G.A. (1990) Dye-labelled substrates for the assay and detection of chitinase and lysozyme activity. *Journal of Microbiological Methods* **12,** 197–205.

Received 13 January 2004; received in revised form 1 March 2004; accepted for publication 3 March 2004