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Resistance in barley against the powdery mildew fungus (*Erysiphe graminis* f.sp. *hordei*) is not associated with enhanced levels of endogenous jasmonates

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Abstract

Onset of acquired resistance of barley (*Hordeum vulgare*) chemically induced by 2,6-dichloroisonicotinic acid (DCINA) correlated with the accumulation of mRNA homologous to cDNA pHvJ256 which codes for a soluble leaf-thionin with a Mr. of 6 kDa [Wasternack *et al.*, 1994a]. In the present work, we extend this finding by showing that the thionin transcript also accumulated following treatment of barley with the resistance-inducing compounds 3,5-dichlorosalicylic acid (DCSA), salicylic acid (SA), and an extract from *Bacillus subtilis*. The polypeptide showed antifungal activity against the biotrophic cereal pathogens *Erysiphe graminis* f.sp. *hordei* and *Puccinia graminis* f.sp. *tritici* which may indicate a possible role in the mechanism of acquired resistance in barley. A thionin transcript hybridizing to pHvJ256 accumulated also in response to application of jasmonates, or treatments that elevated endogenous amounts of the plant growth substance, pointing to the possibility that signaling mediating defense responses in barley involves jasmonates. However, a topical spray application of jasmonic acid (JA) or jasmonate methyl ester (JM) did not protect barley leaves against infection by *E. graminis*. Performing a kinetic analysis by an enzyme immunoassay specific for (–)-JA, (–)-JM, and its amino acid conjugates, accumulation of jasmonates was detected in osmotically stressed barley but not at the onset of chemically induced or genetically based resistance governed by the powdery mildew resistance genes *Mlg*, Mla_{12}, or mlo_5. Furthermore, the jasmonate-inducible proteins JIP-23 and JIP-60 were strongly induced following JM- but not DCINA-treatment or inoculation with *E. graminis*. Hence, in barley, no indications were found in favour for the previously proposed model of a lipid-based signaling pathway via jasmonates mediating expression of resistance in plants against pathogens.

Abbreviations: Egh = *Erysiphe graminis* f.sp. *hordei*; ESH = elongated secondary hyphae; DCINA = 2,6-dichloroisonicotinic acid; DCSA = 3,5-dichlorosalicylic acid; HR = hypersensitive response; INA = isonicotinic acid, JA = jasmonic acid; JIP = jasmonate-induced protein; JM = jasmonate methyl ester; *Pgt* = *Puccinia graminis* f.sp. *tritici*; PR = pathogenesis related; SA = salicylic acid.

Introduction

In many higher plants an initial inoculation by a necrotizing microorganism can induce a defense-status that protects the plant against subsequent infection. This acquired resistance was first documented in 1901 and is thought to play an important role in the preservation of plants in nature [Chester, 1933; Métraux *et al.*, 1993]. The mechanism of this type of resistance is likely to be complex [Kessmann *et al.*, 1994]. The acquisition of resistance can last for weeks to months and

provides long lived protection against a broad spectrum of pathogens, thus performs demands for modern disease management strategies. The molecular mechanism of acquired resistance is still unclear. In dicots as well as in monocots, genes encoding pathogenesis-related (PR) proteins are expressed in resistant tissue following primary inoculation by pathogens or treatment with synthetic compounds [Ward *et al.*, 1991; Rebmann *et al.*, 1991; Uknes *et al.*, 1992; Kogel *et al.*, 1994]. The timing and the level of gene expression correlated with the onset and the degree of resistance to challenge inoculation, suggesting a causal role for the gene products in restricting fungal development *in vivo*.

We have chosen the powdery mildew disease of barley, caused by the fungus *Erysiphe graminis* f.sp. *hordei* (*Egh*), to study the mechanism of acquired resistance. Powdery mildew is a major disease of plants in temperate climates. As a research object, this system combines the advantage of an well known cytology and a well understood genetics of the disease [Ellingboe, 1972; Wiberg, 1974; Aist *et al.*, 1988; Sogaard and Jørgensen, 1988; Koga *et al.*, 1990]. Recently, the mechanism of acquired resistance induced in barley against *Egh* by the resistance-inducing compound DCINA was described as a phenocopy of the genetically based mechanism governed by the race-specific *Mlg* resistance gene [Kogel *et al.*, 1994]. This mechanism is characterized by the occurrence of a HR in attacked cells along with the accumulation of fluorescent material in papillae. This results in a reduced formation of fungal ESH, and finally in a diminution of fungal sporulation.

Among the proteins synthesized following treatment with DCINA, a leaf-specific, soluble thionin of 6 kDa has been identified [Wasternack *et al.*, 1994a] that is identical to the jasmonate-inducible thionin previously found in the vacuole [Andresen *et al.*, 1992; Hause *et al.*, 1994]. In the present paper, we show the accumulation of the same thionin transcript in response to treatments of barley with other resistance-inducing compounds. Because cell wall-bound thionins have been shown to be toxic to phytopathogenic microorganisms [Bohlmann *et al.*, 1988; Ebrahim-Nesbat *et al.*, 1993], we further examined the direct antifungal effects of the soluble thionin encoded by pHvJ256 on the biotrophic cereal pathogens *Egh* and *Pgt*.

Thionins belong to the large class of jasmonate-inducible proteins (JIPs) of barley [Andresen *et al.*, 1992]. All JIPs are inducible by application of jasmonate as well as treatments that rise endogenous levels of this plant growth substance. Therefore, induction of the thionin by DCINA raised the question as to whether onset of acquired resistance in barley, generally, is accompanied by elevated levels of endogenous jasmonates. Previously, increase of jasmonic acid (JA) followed by altered gene expression was shown in barley in response to osmotic stress like sorbitol-, mannitol-, or polyethylene-glycol-treatment, resulting in water deficit [Lehmann *et al.*, 1995; Reinbothe *et al.*, 1992], and upon wounding of tomato leaves [Peña-Cortés *et al.*, 1991]. This accumulation of jasmonates was mediated by action of a lipoxygenase [Peña-Cortés *et al.*, 1993; Wasternack *et al.*, 1994b]. A transient increase of endogenous jasmonates was also observed in various cell suspension cultures upon elicitor treatment. The increase was followed by *de novo* transcription of phenylalanine ammonia-lyase [Gundlach *et al.*, 1992] and other genes of the phenylpropanoid pathway [Dittrich *et al.*, 1992], that is activated in many plants upon microbial attack. Suggested by these facts, a lipid-based signaling pathway might exist, in which an elevated jasmonate level is a signal transducer switching on defense gene expression during plant pathogen interactions [Farmer and Ryan, 1992; Müller *et al.*, 1993]. In the present work, we tried to verify this model in the interaction of chemically induced barley and the powdery mildew fungus. This study was finally extended to different barley genotypes bearing genes Mla_{12}, *Mlg* and mlo_5 that confer resistance to *Egh*. However, using an enzyme immunoassay specific for jasmonic acid and its derivatives, and independently by comparing transcript accumulation of selected PR proteins in JM as well as DCINA-treated, or inoculated genetically resistant barley, no evidence for elevated endogenous jasmonate concentrations was found.

Materials and methods

Plants, pathogens, and inoculation. The barley cv. Pallas as well as Mla_{12}, *Mlg*, and mlo_5 back cross lines of Pallas were obtained from Lisa Munk, Copenhagen. Their generation has been described previously [Kølster *et al.*, 1986]. Plants were grown in a growth chamber at 18 °C, 80% relative humidity and a photoperiod of 16 h (100 μE s^{-1} m^{-2}). Inoculation experiments were carried out using conidia from *Erysiphe graminis* DC.: Fr. f.sp. *hordei* Em Marchal, race A6, expressing the Mla_{12} and *Mlg* avirulence function [Wiberg, 1974]. Inoculation was always performed at the beginning of the photoperiod.

Application of resistance-inducing compounds. DCINA (CGA 41396, Ciba-Geigy AG, Basel, Switzerland) or the respective dehalogenated compound INA (CGA 227519), was formulated as 25% active ingredient with a wettable powder carrier [Métraux *et al.*, 1991]. DCSA (Aldrich, Steinheim, Germany) and SA (Fluka, Buchs, Switzerland) were not formulated. The natural extract from *Bacillus subtilis* culture filtrates [Steiner, 1989] was generously supplied by U. Steiner and E. Oerke. Synthetic compounds were applied to 7d-old barley seedlings as a soil drench. The *B. subtilis* extract (lyophilizate) was diluted with water to a final concentration of 0.1 mg ml^{-1} and sprayed onto the leaves until water run-off.

Application of jasmonates and sorbitol. Segments (5 cm) of 7d-old primary leaves were floated on 45 μM of (±)-JM (Bedoukian Research Inc., Danbury, USA), 1 M sorbitol, or water (control) in petri dishes exposed to controlled light conditions (30 W/m^2) for 24 h at 25 °C. Segments were frozen at the indicated time points in liquid nitrogen.

Cytological investigations. In order to test the resistance-inducing activity of jasmonates, 7d-old plants were sprayed with 1 mg ml^{-1} of (±)-JM or (±)-JA in acetone. Subsequently, plants were placed in a plastic chamber and inoculated with *Egh*, race A6, keeping three different time ranges (4 h, 1 d, and 3 d) from treatment to inoculation. At the indicated time points, 6 cm segments were harvested, placed in a fixing solution (96% ethanol: chloroform, 4:1 v/v, containing 0.15% (w/v) TCA). Whole cell autofluorescence was observed by fluorescence microscopy (wavelength = 310 nm). For bright-field microscopy fixed segments were stained in Coomassie blue (0.6% methanolic Coomassie brilliant blue R-250: 15% TCA, 1:1 v/v) for 5 s, washed in water and mounted in 50% glycerol (v/v).

Extraction of RNA and northern blot analysis. Extraction of total leaf RNA (1.5 g fresh weight per sample), its electrophoresis, transfer onto nitrocellulose and northern blot analysis was performed according to Andresen *et al.* (1992). For each time point, primary leaf segments from 20 individuals were treated. If not indicated otherwise, fifteen micrograms of total RNA were loaded onto 1.2% denaturing formaldehyde-agarose gels and transferred onto nitrocellulose membranes (BA 85, Schleicher & Schüll, Darmstadt, Germany) after electrophoretic size separation. Hybridizations with ^{32}P-labeled cDNA probes were performed using standard conditions [Maniatis *et al.*, 1982]. In some experiments hybridizations were performed with digoxigenin-labeled RNA probes [Kogel *et al.*, 1994]. The cDNAs pHvJ3015, pHvJ256 [Andresen *et al.*, 1992] and pHvJ611 [Becker and Apel, 1992] were used to detect the corresponding mRNA of JIP-23, JIP-6, and JIP-60, respectively. Additionally, the peroxidase-cDNA clone pPOX381 [Rebmann *et al.*, 1991] and clone PRHv-1 [Hahn *et al.*, 1993] for a thaumatin-like protein was used in these experiments. Photographic UV inspection was used to check that equal amounts of total RNA were loaded onto the agarose gel. This was confirmed in some cases using a barley rDNA probe [Forde *et al.*, 1991].

Extraction and methylation of jasmonates. Jasmonates were extracted with 70% methanol from frozen leaf segments (approximately 1 g of fresh weight). After removal of the unsoluble material by centrifugation, supernatants were passed through C_{18} cartriges (Bakerbond spe Octadecyl RP, Baker, Groß-Gerau, Germany) to remove unpolar pigments and other lipophilic compounds. The effluents were reduced to the

aqueous phase by rotatory evaporation, adjusted to pH 3 with acetic acid, and partitioned three times against equal volumes of ethyl acetate. The organic part was reduced to dryness, redissolved in 0.5 ml of methanol and methylated with an excess of etherial diazomethane. After 5 min the methylated samples were reduced to dryness and dissolved in 0.5 ml of 50 mM Tris-HCL buffer pH 7.8, containing 5% (v/v) methanol.

Enzyme immunoassay. The assay followed the protocol of Weiler [1986]. The properties of the antiserum were described by Knöfel *et al.* [1990]. The assay is highly specific for (–)-JM and its amino acid conjugates. Hence, methylation of the samples is required to determine concentration of all jasmonates. The measuring range of the standard curves lies between 0.05 and 25 pmol of (–)-JA, with a reliable detection limit of 0.1 pmol.

Assay for antifungal activity of the 6 kDa thionin. Intracellular, soluble thionin was partially purified from 5 cm leaf segments that were floated on 45 μM ± JM for 24 h using the procedure of Reimann-Philipp *et al.* [1989]: Leaf material (2.5 g) were ground under liquid N_2. The powder was resuspended in 40 ml of 50 mM sodium phosphate, pH 7.0, and stirred for 30 min on ice. After centrifugation for 10 min at 12,000 × g, 3.5 g of ammonium sulfate were dissolved in 10 ml of supernatant. The mixture was placed on ice for 30 min and centrifuged to remove the precipitate. The supernatant was mixed with 1.5 g of ammonium sulfate and stirred on ice for 30 min. The resulting precipitate was dissolved in 50 mM sodium phosphate buffer, pH 7.0, and desalted using a PD10 column (Pharmacia). Finally, the proteins were fractionated by FPLC using a superdex 75 column. Fractions corresponding to Mr of 3,000 to 10,000 were collected and used for toxicity assays.

The fraction was tested in 5, 10, 50, and 150 μM (protein) concentrations on 1% agar in petri dishes. Conidia of *Egh* and the wheat pathogen *Puccinia graminis* f.sp. *tritici* (*Pgt*) were freshly harvested from susceptible hosts and sprayed onto the agar. Germination of the conidia was microscopically evaluated after 24h.

Results

Resistance-inducing activity of DCINA, DCSA and a natural extract from Bacillus subtilis. We have previously shown that DCINA, but not the dehalogenated derivative INA induced resistance in barley to *Egh* [Kogel *et al.*, 1994]. This effect is dose-dependent in a concentration range of 0.1 to 2 ppm DCINA (Fig. 1A). Similarly, treatment of genetically susceptible barley seedlings by soil drench with DCSA results in the reduction of the number of powdery mildew colonies on primary leaves (Fig. 1B). However, as compared to DCINA, the DCSA concentration necessary for the induction of significant, comparable arrest of fungal development is approximately 50 times higher (2 ppm versus 100 ppm). SA has little effect on fungal development. To analyse the effect of the application method, DCINA and DCSA were sprayed onto primary leaves that were subsequently inoculated with *Egh*. As compared to soil drench application, spraying of these compounds was less effective and dose effect relationship was less pronounced (data not shown). In contrast, the extract from *B. subtilis* culture filtrate (0.1 mg ml^{-1}) was not effective as drench but in spray applications (up to approximately 30% of the effectiveness of DCINA, data not shown). Since the active agent in the extract has not yet been identified, no efforts were made to determine dose dependency.

Treatment of barley leaves with jasmonates. Because of a continued controversial discussion as to whether jasmonates are involved in putative plant defense pathways, we tested both, the effect of exogenously applied jasmonates on restricting fungal growth and, additionally, changes of endogenous growth substance concentrations during the establishment of resistance in barley seedlings against *Egh*. A topical spray application with JM or JA (1 mg ml^{-1} in acetone) did not protect barley against *Egh* within a time range from spraying to inoculation of 4 h to 3 d. No effect of the compounds on the formation of macroscopically visible mildew colonies was detected (Fig. 2). To analyse effects of jasmonates on early developmental stages, fungal growth was microscopically inspected through the analysis of ESH formation. Since timing of fungal establish-

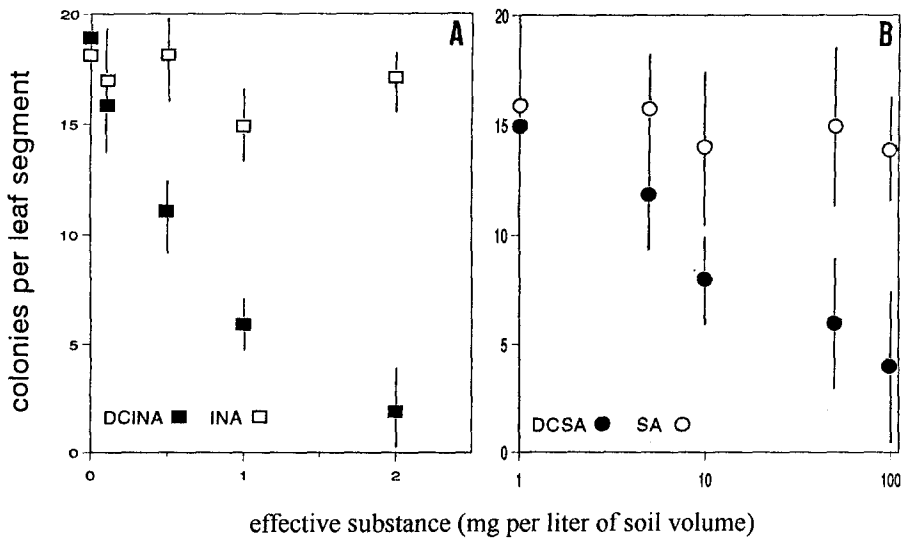

Fig. 1. Dose-dependence of the expression of resistance against *Erysiphe graminis* f.sp. *hordei*, race A6, in genetically susceptible barley cv. Pallas in response to treatment with (A) DCINA, INA and (B) DCSA, SA. The compounds were applied by soil drench treatment of 7-d-old seedlings. After 4 days, seedlings were inoculated with a spore concentration of 15 conidia per cm^2. The number of *Egh* colonies was counted 6 days after inoculation. Values are mean-values of 4 experiments each with 20 individuals per each concentration. Concentrations are calculated as milligram per liter of soil volume and given as ppm.

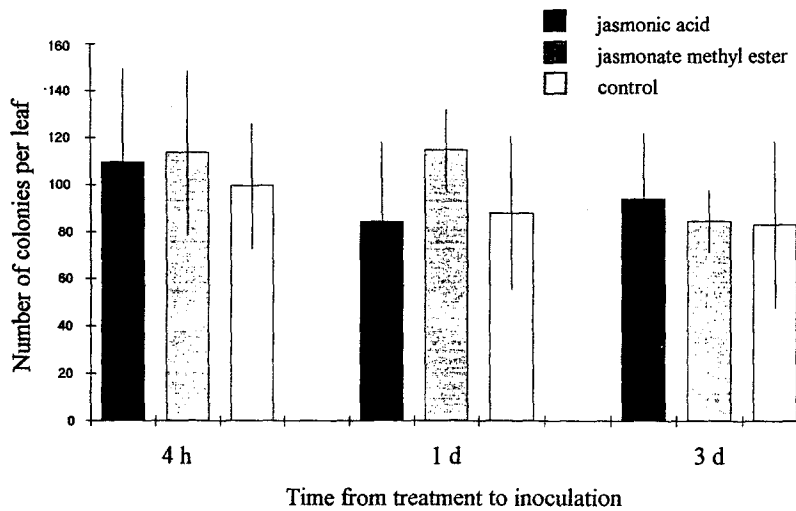

Fig. 2. Treatment of barley cv Pallas with different types of jasmonates. JA and JM (1 mg/ml in acetone, each) were applied to 7-d-old seedlings as a topical spray application. Inoculation with *Erysiphe graminis* f.sp. *hordei*, race A6 (25 conidia per cm^2), was done keeping a time range from treatment to inoculation of 4h, 1 d, and 3 d, respectively. The number of fungal colonies was counted 6 days after inoculation. Values are mean-values of 4 experiments each with 20 individuals per each compound. Control: topical spray with acetone.

ment on the host proceeds in a cell-type specific manner in short (< 450 µm in length) and long cells of the barley epidermis (Koga *et al.*, 1990), formation of ESH was independently evaluated on these cell types (Fig. 3). Regardless of the cell type infected and of the time range between treatment and inoculation, the number of ESH formed by the fungus was not reduced at 48 h after

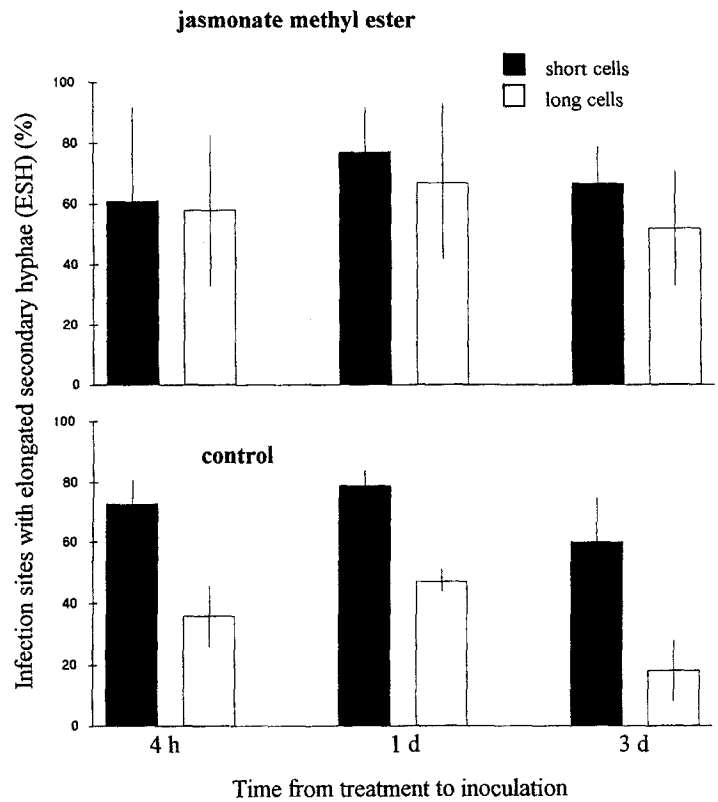

Fig. 3. Cell type-specific evaluation of the formation of ESH in the interaction of *Erysiphe graminis* f.sp. *hordei*, race A6, with barley cv Pallas treated with JM. The compound (1 mg/ml in acetone) was applied to 7-d-old seedlings as a topical spray application. Inoculation (3–5 conidia per mm^2) was done keeping a time range from treatment to inoculation of 4h, 1 d, and 3 d, respectively. Primary leaves were harvested 48 h after inoculation, and prepared for microscopy. Values are mean-values of 4 experiments each with 6 individuals (total number of inspected infection sites 4,000). Control: topical spray with acetone.

inoculation in leaves sprayed with 1 mg/ml JM compared to control leaves (acetone alone), indicating that JM does not exhibit direct toxic activity to the fungus.

To extend this analysis, plant responses to the invading pathogen were recorded in terms of occurrence of HR and formation of papillae. Since these prevailing reactions of barley to *Egh* also occur cell type specific in genetically based [Koga *et al.*, 1990; Görg *et al.*, 1993] and acquired resistance [Sahashi and Shishiyama, 1986; Kogel *et al.*, 1994], the evaluation was done separately in short and long cells. Fig. 4 shows that no increase in HR was detected in either cell type after JM-treatment compared to control leaves. In accordance with Koga *et al.* [1990], the total number of papillae formed in response to the attacking pathogen was different in short and long cell types (Fig. 4). However, no increase in the number of fluorescing papillae per infection site was observed following JM-treatment. When JA was used instead of JM, very similar data were obtained (not shown). These results indicate that, in barley, both types of jasmonates do not induce resistance nor seem to exhibit significant direct antifungal activity.

Induction of a leaf-specific thionin by resistance-inducing compounds, but not by inoculation with Egh. Accumulation of gene transcripts homologous to the jasmonate-inducible thionin clone pHvJ256 was measured in cv Pallas treated with different resistance-inducing compounds. The clone encodes the Mr. 15,000 precurser polypeptide of a leaf-specific thionin containing the signal peptide, a thionin domain, and an acid polypeptide domain [Andresen *et al.*, 1992]. Fig. 5 shows

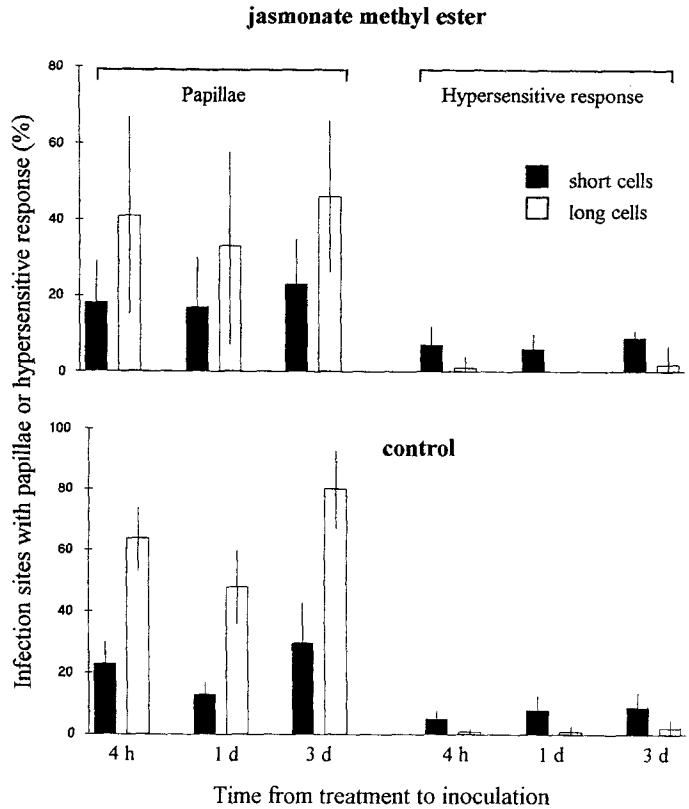

Fig. 4. Cell type-specific evaluation of the occurrence of papillae and HR in the interaction of *Erysiphe graminis* f.sp. *hordei*, race A6, with barley cv Pallas treated with JM. For experimental details see legend to Fig. 3.

accumulation of thionin-mRNA in primary leaves of seedlings 24 and 48 h after soil drench treatment with DCINA (2 ppm) or DCSA (100 ppm). A slight accumulation of thionin-mRNA was induced by SA and, to an even lower extent, by the extract from *B. subtilis*, which corresponds well with their weak resistance-inducing activity. No accumulation of the thionin was found in plants treated with water (not shown) or INA. The fungicide 'Opus' (BASF, Limburgerhof) containing the effective substance epoxiconazole strongly inhibited powdery mildew development on barley leaves at low dosages (spray solution: 0.31 mg/ml, data not shown). However, epoxiconazole did not induce the accumulation of the thionin-mRNA (Fig. 5), demonstrating the possibility to differentiate between the effect of a resistance-inducing compound and a fungicide using the thionin as probe.

The question arose as to whether pretreatment of barley with DCINA results in a further infection-related induction of thionin accumulation following inoculation. Figure 6 shows the kinetic analysis of thionin-mRNA accumulation in cv Pallas treated with 2 ppm of DCINA 4d before inoculation with *Egh*. Transcript accumulated with a maximum at 32 h after DCINA treatment. However, the profile of transcript accumulation remained monophasic, e.g., no infection-related induction of the thionin was observed. Additional experiments confirmed that thionin-mRNA homologous to pHvJ256 was not induced by *Egh* in various backcross lines of cv Pallas, irrespective of the presence of the functionally active dominant acting resistance alleles Mla_{12} and *Mlg*, conferring race-specific resistance, or the recessive acting allele mlo_5, conferring non-race-specific resistance to all races of *Egh* (data not shown).

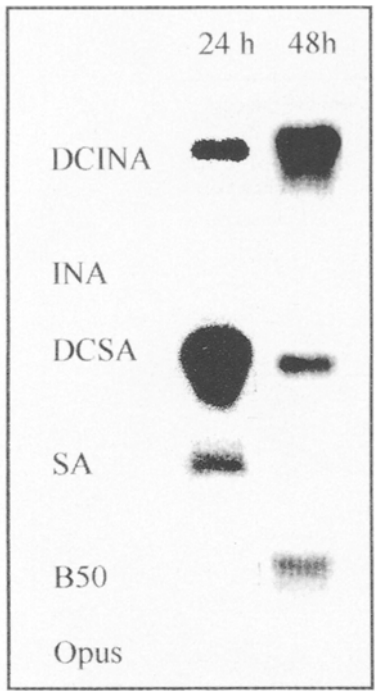

Fig. 5. Accumulation of leaf thionin-mRNA after treatment of 7-d-old seedlings cv Pallas with DCINA, INA, DCSA, SA, or treatment with a natural extract from *Bacillus subtilis* (B50), and the fungicide epoxiconazole. Data shown are representative of three replicate experiments. Each lane contains 20 µg of total RNA extracted from leaf segments from 20 individuals 24 and 48 h after application of compounds. Filters were subsequently hybridized with the cDNA probe pHvJ256 [Andresen *et al.*, 1992] encoding a leaf-specific thionin. DCINA and INA (2 ppm each) as well as DCSA and SA (100 ppm each) were applied as soil drench. The extract from *B. subtilis* (0.1 mg lyophilizate ml^{-1}) and epoxiconazole (0.31 mg ml^{-1}) were sprayed onto the seedlings until water run off. Wash stringency: 0.1xSSC at 65 °C.

Endogenous concentrations of jasmonates in chemically induced and genetically based resistance. To quantify endogenous amounts of jasmonates, an ELISA specific for (–)-JM and its amino acid conjugates was used. By this procedure, accumulation of jasmonates was detectable in cv Pallas following treatment with 1 M sorbitol but not after DCINA application (Fig. 7).

To extend this finding, endogenous jasmonates were analysed in compatible and genetically incompatible powdery mildew – barley combinations including genotypes bearing the alleles *Mlg*, Mla_{12} and mlo_5. Fig. 7 demonstrates that, within a time range between onset and establishment of resistance (0 to 48 h of inoculation, Freialdenhoven *et al.*, 1994; Görg *et al.*, 1993), no accumulation of endogenous jasmonates was detectable.

In order to provide additional, independent evidence for jasmonates not being involved in resistance responses, profiles of DCINA, *Egh* (incompatible interaction with Pallas-*Mlg*), and JM-induced transcript accumulation of PR proteins were compared. The different treatments did not result in the accumulation of the same transcripts: Peroxidase-mRNA was accumulated by either treatment, whereas thionin transcript accumulated only in response to chemical treatment. Most importantly, JM but not DCINA or *Egh* induced accumulation of the transcripts for JIP-23, the most abundant protein of the JIP-class as well as the transcripts for JIP-60 and a thaumatin-like protein (Fig. 8). Additional experiments showed that JIP-23 and JIP-60 were also not induced by *Egh* in backcross lines of cv. Pallas bearing the specific resistance genes Mla_{12} and mlo_5 (data not shown).

Antifungal activity of the leaf specific thionin. To demonstrate the antifungal activity of the leaf specific thionin, the polypeptide was isolated from leaf segments (cv. Salome) floated on JM. Salome is known to contain high amounts of soluble thionin upon this treatment. Success of the purification protocol was checked by gel electrophoresis revealing a major band at 6 kDa corresponding to the thionin [Wasternack *et al.*, 1994a]. Half-maximal inhibition of germination of uredospores from *Pgt* was observed at a protein concentration of 10 µM and germination of conidia from *Egh* at a concentratium of 25 µM. No inhibitory activity was detected in extracts from control leaves (treatment: water instead of JM) up to protein concentrations of 200 µM.

Discussion

In this report, we confirmed the resistance-inducing activity of the chemicals DCINA and DCSA, showed the close correlation between accumulation of a transcript for a soluble leaf-thionin and expression of acquired resistance, and provided strong evidence that jasmonates are not

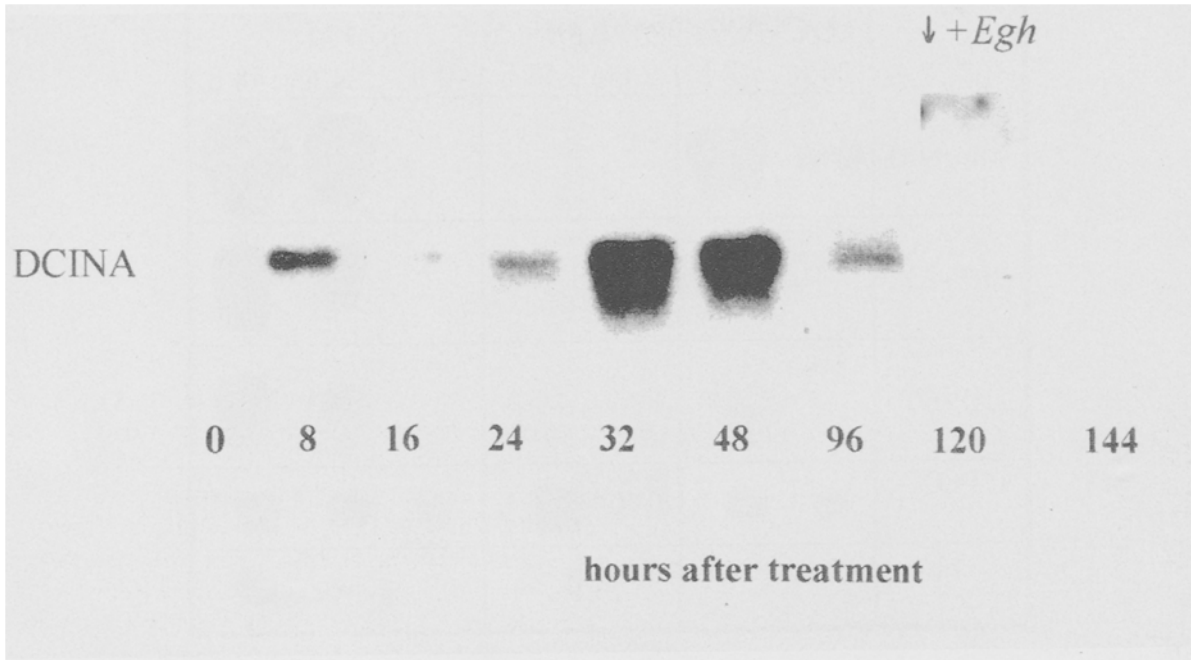

Fig. 6. Time course of leaf thionin-mRNA accumulation after soil drench treatment of 7-d-old seedlings cv Pallas with DCINA (2 ppm) and inoculation with *Erysiphe graminis* f.sp. *hordei*, race A6, 4 days after application of the compound. Data shown are representative of three replicate experiments. Each lane contains 20 µg of total RNA extracted from leaf segments from 20 individuals. Filters were subsequently hybridized with the cDNA probe pHvJ256 [Andresen *et al.*, 1992]. Wash stringency: 0.1xSSC at 65 °C.

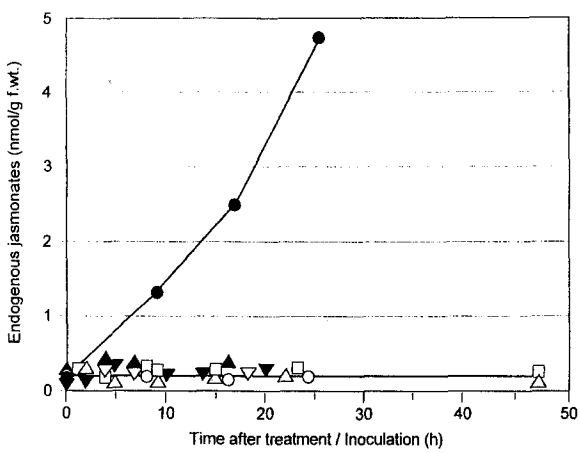

Fig. 7. Endogenous jasmonates in different genotypes of barley in response to osmotic stress, chemical induction of resistance, or inoculation with *Erysiphe graminis* f.sp. *hordei*, race A6. Treatment of cv Salome with 1 M sorbitol (●) and water (○), respectively. Treatment of cv Pallas with 2 ppm DCINA (△). Inoculation of cv Pallas (□) and the respective backcross lines P-*Mlg* (▲), P-*Mla*$_{12}$ (▼), and P-*mlo* (▽) with *Egh* (3–5 conidia per mm^2). Extraction of jasmonates and ELISA performance is described in methods.

involved in the chemically-induced or genetically based resistance reactions of barley against the powdery mildew fungus.

A cytological analysis of the DCINA-induced resistance revealed the hypersensitive cell death (HR) of infected epidermal cells along with the accumulation of fluorescent material in papillae to be closely related to fungal arrest [Kogel *et al.*, 1994]. Whether this mechanism holds true for the effect induced by DCSA, SA and the extract from *B. subtilis* must be object for further investigations. No protection of barley seedlings against *Egh* was obtained by a topical spray application with jasmonates. This was shown by a macroscopic evaluation of the number of mildew colonies on leaves treated with JM and JA, respectively (Fig 2). A more detailed microscopic analysis of single infection sites at early stages of the interaction supported this finding: Inspection of the incidence of fungal ESH (Fig. 3) – a measure commonly used to determine whether functional haustoria have become established in host epidermal cells – and the occurrence of plant

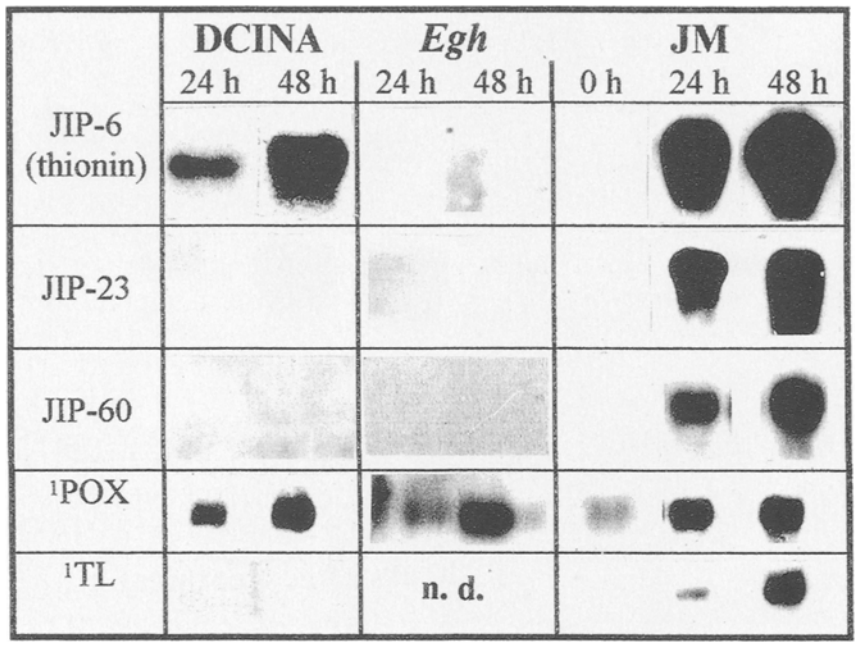

Fig. 8. Accumulation of mRNA encoding PR proteins in barley cv Pallas-*Mlg* 24 and 48 h after treatment with DCINA, inoculation with *Erysiphe graminis* f.sp. *hordei*, race A6 (3–5 conidia mm^2), or application of JM. Data shown are representative of three replicate experiments. Each lane contains 15 µg of total RNA extracted from leaf segments from 20 individuals. Filters were subsequently hybridized with the following probes: barley leaf thionin (JIP-6) cDNA clone pHvJ256, barley JIP-23 cDNA clone pHvJ3015 [Andresen *et al.*, 1992], barley JIP-60 cDNA clone pHvJ611 [Becker and Apel, 1992], wheat peroxidase (POX) cDNA clone pPOX381 [Rebmann *et al.*, 1991], and barley thaumatin-like protein (TL) cDNA clone pRHv-1 [Hahn *et al.*, 1993]. DCINA (2 ppm) was applied as soil drench to 7-d-old seedlings. For jasmonate application, leaf segments from 7-d-old primary leaves were floated on 45 µM of JM. No accumulation of mRNAs was observed in the respective water controls. [1] Hybridization with digoxigenin-labeled pPOX381 and PRHv-1 RNA transcripts. Wash stringency: 0.1xSSC at 65 °C.

responses in terms of HR and papillae formation (Fig. 4) at different time points from treatment to inoculation, revealed no significant differences in fungal development in jasmonate-treated as compared to control leaves. Thus, jasmonates neither exhibited a direct toxic activity to the fungus nor induced a host defense response. Interestingly, the number of ESH was somewhat enhanced and the number of papillae somewhat reduced in JM-treated as compared to control leaves, indicating that plants were even more weakened in their defense against *Egh*. Hence, in accordance with Schweizer *et al.* [1993], no resistance-inducing activity could be attributed to jasmonates, but in contrast to these authors, we could not find indication for a direct toxic activity of jasmonates on the fungus.

Onset of DCINA-induced acquired resistance of barley correlated with the accumulation of mRNA coding for a soluble thionin. The polypeptide had a Mr. of 6 kDa as demonstrated by western blot analysis using thionin antibodies [Wasternack *et al.*, 1994a]. In the present work, we extended this finding by showing that the thionin transcript also accumulated following treatment of barley seedlings with the resistance-inducing compound DCSA as well as, to a minor extent, with SA and the extract from *B. subtilis*. This indicates a close correlation between accumulation of the thionin and expression of acquired resistance. Since the fungicide epoxiconazole did not induce thionin accumulation, the thionin may be used to differentiate between a chemically induced resistance status of the plant and the toxic effects of a fungicide.

Thionin transcript that is induced by treatments resulting in acquired resistance was also jasmonate-responsive [Wasternack *et al.*, 1994a; Fig. 8]. However, jasmonates did not show resistance-inducing activity in our experiments.

Therefore, expression of the thionin following treatment with jasmonates obviously is not sufficient for inducing resistance in barley. The thionin transcript accumulating in acquired resistant leaves is homologous to pHvJ256. Since thionins homologous to pHvJ256 are located in the plant vacuole [Andresen *et al.*, 1992; Hause *et al.*, 1994], it is tempting to speculate that the fungitoxic property of the soluble thionin, shown here against the biotrophic fungi *Egh* and *Pgt*, is manifested only in case the resistance-inducing compounds simultanously induce cell collaps.

Thionin-mRNA homologous to pHvJ256 was not accumulated in response to inoculation, even when plants were pretreated with DCINA (Fig. 6). Additional experiments clearly showed that the thionin is also not induced in genetically based resistance in the presence of functionally active resistance loci *Mlg*, Mla_{12}, and mlo_5 (see Fig. 8 for *Mlg*), indicating that the vacuolar located, soluble thionin homologous to pHvJ256 is not a factor in the mechanisms governed by these types of genes. In seeming contrast, it was previously shown that specific members of the thionin family are induced in barley upon infection with powdery mildew. However, thionin clones different to pHvJ256 were used as probes in these experiments [Bohlmann *et al.*, 1988]. Similarly, the barley cultivar Midas carrying the resistance allele *Mla6* that leads to non-hypersensitive resistance, responded with a fluctuating thionin transcript accumulation upon infection with the avirulent or virulent near-isogenic isolates of *Egh* [Boyd *et al.*, 1994]. From these findings, it was claimed that thionins are part of a genetically based resistance mechanism [Bohlmann and Apel, 1991]. This concept is based on i. the fungitoxic properties of thionins, demonstrated using bacteria and several phytopathogenic fungi [Bohlmann *et al.*, 1988 Fernandez de Caleya *et al.*, 1972], ii. their preferential accumulation in the papillae and cell wall close to the infection sites on resistant but not susceptible barley leaves [Ebrahim-Nesbat *et al.*, 1989], iii. that leaf thionins are members of a multigene family of 50 to 100 genes per haploid genome [Bohlmann *et al.*, 1988] which could cause diversity of leaf thionin variants [Bohlmann and Apel, 1991]. Interestingly, this diversity is not given randomly, and conserved regions containing cysteine residues were found only within the thionin domain, that is characterized by its toxic properties [Bunge *et al.*, 1992], and may differ among different thionins. The recently evaluated PCR-based fingerprint method may offer the possibility to analyse in more detail different thionin mRNA subclasses involved in defense reactions [Bohl and Apel, 1993].

Thionins are members of the class of Jasmonate-Inducible Polypeptides [JIPs, Andresen *et al.*, 1993]. JIPs are relatively stable polypeptides with molecular masses of 6 kDa, 10/11 kDa, 23 kDa, 37 kDa, and 66/68 kDa [Weidhase *et al.*, 1987; Müller-Uri, 1988]. The presence of the thionin in seedlings with acquired resistance could be interpreted as an indication that jasmonates are involved in the signaling pathway regulating the onset of defense reactions. This consideration gained relevance by earlier reports on jasmonates being powerful inducers of wound-responsive proteinase inhibitor II of tomato and potato [Farmer and Ryan, 1992; Hildmann *et al.*, 1992] and an endogenous rise of jasmonates followed by phytoalexin synthesis in elicited plant cell suspension cultures of different genera [Müller *et al.*, 1993]. Furthermore, pathogen induced increase of lipoxygenase, an important enzyme in the pathway of jasmonate biosynthesis might be interpreted in favour of this hypothesis. However, a more careful evaluation of pathogen-induced lipoxygenase activity showed that the final products were not jasmonates but hexanals and related compounds, being of direct antimicrobial activity [Croft *et al.*, 1993]. In addition, indications were found that gene expression upon wounding, well known to be mediated by the signal jasmonate [Farmer and Ryan, 1992; Hildmann *et al.*, 1992], was clearly distinguishable from a defense response to pathogen infection [Choi *et al.*, 1994]. As demonstrated by our results, no accumulation of jasmonates was detected in barley leaves at the onset or the development of acquired resistance chemically induced by DCINA. Furthermore, there was no increase of the amount of jasmonates following inoculation with *Egh*, race A6, in different resistant genotypes of cv. Pallas (Fig. 7). Although local rise of jasmonates at the site of infection cannot be ruled out completely, it must be noted that the reliable detection limit of the ELISA used for the determination of jasmonates lies at 0.1 pmol. Thus, the average amounts of endogenous

jasmonates in untreated barley clearly were above the detection limits of the assay.

Our assumption that jasmonates are not involved in acquired resistance is also supported by the fact that different types of mRNAs coding for PR proteins accumulated in response to the avirulent fungus and treatments with DCINA or JM (Fig. 8). Most remarkably, JIP-23 and JIP-60 were not induced by *Egh* or DCINA. Since the jasmonate-inducible polypeptides were shown to be induced only by treatment resulting in enhanced endogenous jasmonate levels [Lehmann *et al.*, 1995; Feussner *et al.*, 1994], a lack of accumulation of these polypeptides must be interpreted by a lack of jasmonate accumulation, and therefore supports the ELISA data (Fig. 7). Together, the data presented here strongly suggest that signaling in resistance does not proceed via jasmonates.

Recently, JIP-60 was identified as a ribosome inactivating protein [Chaudhry *et al.*, 1994] known to impair protein synthesis. The suggestion that JIP-60 might be a putative plant defense protein via this function [Reinbothe *et al.*, 1994a; Reinbothe *et al.*, 1994b] is not supported by our data because DCINA-mediated and genetically based resistance in barley was not accompanied by expression of JIP-60. Furthermore, barley leaves containing JIP-60 upon jasmonate treatment were not protected against subsequent infection by *Egh* [Schweizer *et al.*, 1993].

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