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Immunohistological analysis of chemically induced proteins in sugar beet

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Abstract

Tissue-specific distribution of basic β-1,3-glucanase (Glu2), basic class II chitinase (Ch2), basic class IV chitinase (Ch4), and acidic class III chitinase (SE2) were examined both in leaves and roots of sugar beet treated with salicylic acid (SA), benzothiadiazole (BTH) and glycine betaine. Protein localization was monitored by immunohistological analysis using specific antibodies. BTH, SA as well as glycine betaine induced both Glu2 and chitinase isozymes in leaves and roots of treated plants. The enzymes were accumulated in extracellular space and cell walls. They were mostly deposited in parenchyma cells of leaves and cortex parenchyma and endodermis of roots. In leaf tissues, BTH and SA induced proteins more effectively than glycine betaine but the effect of glycine betaine in roots was as efficient as BTH and SA. Glycine betaine induced the formation of extracellular globuli containing Ch4. Induced proteins were spatially distributed over the whole plant regardless the site of the inducer application.

Additional key words: benzothiadiazole, *Beta vulgaris*, chitinase, β-1,3-glucanase, glycine betaine, immunolocalization, salicylic acid, sugar beet.

Introduction

Plants respond to pathogens by activation of the battery of defence mechanisms that often result in an enhanced resistance to subsequent infection. This status of activated resistance called systemic acquired resistance (SAR) (Kuć 1982, 1995, Ryals *et al.* 1996) or induced systemic resistance (ISR) (Van Loon *et al.* 1998) is mostly connected with the synthesis of the pathogenesis-related (PR) proteins. The roles of *PR* genes in disease resistance have been suggested by the tight correlation between expression levels of *PR* genes and the level of disease resistance. PR-proteins are currently classified in 14 independent families (Van Loon and Van Strien 1999), some of them display hydrolytic activities [*e.g.* (β-1,3-glucanases (PR-2) and chitinases (PR-3)] and are supposed to cleave fungal and bacterial cell walls resulting in pathogen suppression (Schlumbaum *et al.* 1986, Collinge *et al.* 1993, Neuhaus 1999). Transgenic plants expressing chitinase and β-1,3-glucanase genes exhibit enhanced protection against fungal pathogens (Broglie *et al.* 1991, Jongedijk *et al.* 1995). PR-proteins accumulate locally and systemically and deposit either extracellularly in cell walls or intracellularly in vacuoles.

Besides pathogens, a number of chemical compounds have been shown to induce similar defence responses (Kessmann *et al.* 1994). Probably the most extensively studied molecule in connection with SAR is salicylic acid (SA) as its endogenous level increases after pathogen infection as well as SA exogenous treatment. The role of SA in PR genes activation and SAR signal transduction was proved in many species (Delaney *et al.* 1994, Dempsey *et al.* 1999). The functional synthetic analogues of SA with similar effect, 2,6-dichloroisonicotinic acid (INA), and benzo[1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester (BTH) are, at the moment, another well characterized SAR inducers. They have been shown to induce resistance in plants against pathogens switching

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Abbreviations: BTH - benzo[1,2,3]thiadiazole-7-carbothioic acid-S-methyl ester, Ch2 - basic class II chitinase, Ch4 - basic class IV chitinase, Glu2 - basic β-1,3-glucanase, INA - 2,6-dichloroisonicotinic acid, SA - salicylic acid, SAR - systemic acquired resistance, SE2 - acidic class III chitinase.
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on a wide range of SAR genes including those encoding PR-proteins (Frierdich *et al.* 1996, Lawton *et al.* 1996, Šindelářová *et al.* 2002, Suo and Leung 2002). Interestingly, recent evidence suggests that these compounds prime endogenous plant defence system to respond more rapidly to invasion by a pathogen even if they are applied in the micromolar concentrations (Katz *et al.* 1998, Siegrist *et al.* 1998). For example, BTH was found to have potential for conditioning parsley cells for quicker coumarine secretion after elicitor treatment (Siegrist *et al.* 1998) or phytoalexin accumulation in cowpea seedlings (Latunde-Dada 2001).

Similarly to increased resistance to pathogens, enhanced tolerance to abiotic stresses can be achieved by treatment the plants with chemical compounds. Glycine betaine (betaine), a quarternary ammonium compound that occurs naturally in a wide variety of plants, animals and microorganisms (Rhodes and Hanson 1993, Sakamoto *et al.* 2002), accumulates in response to drought and salinity as well as to low and high temperatures. Betain is thought to protect the plant by acting as an osmolyte maintaining the water balance between the plant cell and the environment, and it might also play a role in protecting membranes and protein complexes. Increased production of betain resulted in improved tolerance to salinity and low temperature in transgenic tobacco plants (Holmstrom *et al.* 2000), and likewise to BTH, enhanced phenolic compound production in strawberry (Karjalainen *et al.* 2002).

In sugar beet, salicylic acid, BTH, chitosan, paraquat, and $AgNO_3$, were previously found to induce both acidic and basic extracellular proteins displaying chitinase and β-1,3-glucanase activities (Burketová *et al.* 1999). Additionally, an increased resistance to leaf pathogen *Cercospora beticola* following the treatment with INA was reported (Nielsen *et al.* 1994). In the present study we demonstrate histological localization of both basic and acidic β-1,3-glucanase and chitinase isozymes in sugar beet leaves and roots by means of immunohistology, and the ability of different SAR inducers to induce accumulation of these enzymes in plants.

Materials and methods

Plants: Sugar beet (*Beta vulgaris* L.) plants cv. Hilma were cultivated in pots filled with *Perlite*, and regularly watered with a half strength Steiner nutrient solution (Steiner 1984). They were grown at 16-h photoperiod (photon flux density of 60 μmol m^{-2} s^{-1}), and day/night temperature of 24/18 °C in a growth chamber.

Chemical treatment: To induce protein synthesis, 3-week-old plants were either sprayed on leaves with inducer or their roots were dipped into inducer solution for 30 s and then rinsed with water. The compounds used were benzothiadiazole (1.2 mM) (*Bion®*, *Syngenta*, Basel, Switzerland), sodium salicylate (20 mM) and glycine betaine (0.3 M) (*Greenstim®*, *Finnsugar Bioproducts*, Helsinki, Finland).

Fixation and sections preparation: Collected root samples were fixed in 3 % paraformaldehyde and 0.5 % glutaraldehyde buffered with phosphate buffered saline (PBS), vacuum infiltrated and left for 2.5 h at 4 °C in fixative, after incubation the specimens were washed overnight in PBS, dehydrated in graded saccharose series (from 0.1 to 1.76 M in PBS) at 4 °C and frozen at -80°C. Dehydrated pieces of roots were cut to 6 μm tissue sections on *Cryotome-Cryostat* (*Shandon*, Pittsburgh, USA), collected sections attached to microscopic slides and rehydrated in declining saccharose series (from 1.76 to 0.1 M in PBS) followed by washing in PBS for 2 × 5 min. Then, the sections were transferred to Tris buffered saline (TBS) containing 1 % (v/v) Triton X-100 for 30 min.

Immunohistology: After immersion in blocking solution [TBS containing 20 mM glycine, 0.2 % gelatine (v/v), 0.1 % Tween 20 (v/v), 10 % goat preimmuneserum (v/v)] for 3 × 20 min, the sections were incubated in primary antibody in blocking solution overnight at 4 °C. Then, they were washed in TBS for 5 × 4 min, and incubated with the secondary goat anti-rabbit antibody coupled to alkaline phosphatase. Visualization was made following washing with TBS 5 × 4 min with NBT/BCIP substrate. Immunohistology controls were run in parallel and treated with preimmunoserum instead of primary antibodies.

To ease the interpretation of immunohistological observations using the light microscopy, control tissue structures were visualized using unspecific staining by Alcian blue and Kernechrot (Fig. 1). Cross-sections were immersed in Alcian blue 0.1 % (m/v) solution containing 3 % acetic acid and 0,5 % Triton X-100 for 2 h, rinsed with water and incubated 2-3 min in Kernechtrot 0.1 % (m/v) in distilled water containing 5 % $Al_2(SO_4)_3$.

Chemicals: Specific polyclonal antibodies raised against a basic β-1,3-glucanase (Glu 2), basic class II chitinase (Ch2), basic class IV chitinase (Ch4), and acidic class III chitinase (SE2) were kindly supplied by Dr. J. Kreiberg (*Danisco*, Copenhagen, Denmark). Unless stated otherwise, the chemicals were purchased from *Sigma Chemical Co.*, St. Louis, USA.

Results

All chemical inducers under study, SA, BTH and glycine betaine, did not cause any visible symptoms when applied both on leaves and roots up to the time of sample collection, however, prolonged cultivation of SA treated plants (more than 10 d after the treatment) resulted in slight chlorosis of leaves indicating the phytotoxicity of SA.

Immunohistological analysis of induced proteins in tissue cross-sections revealed differentially accumulated β-1,3-glucanase and chitinase isozymes. The proteins were deposited in extracellular spaces and cell walls depending on the particular inducer. The systemic effect of inducer treatment was evaluated on root cross-sections, which were prepared both from the plants treated with the inducers on leaves and roots. The foliar application of inducers resulted in the similar pattern of induced proteins in roots, but the signal was less pronounced than in direct treatment of roots (not shown).

In leaves (Fig. 2), the strongest staining for the basic β-1,3-glucanase (Glu2) was detected in BTH-treated plants. Glu2 accumulated mainly in the cell walls of parenchyma cells. SA and betaine induction of Glu2 was less evident. SA, BTH and betaine activated Glu2 synthesis in roots (Fig. 6) mainly in the endodermis and cortex parenchyma cells. Slight signal was also found in healthy control.

Similarly to Glu2, the basic class II chitinase (Ch2) was deposited prevalently in the cell walls of leaf parenchyma cells (Fig. 3). The most efficient inducer of the enzyme was BTH, SA activated Ch2 synthesis in a lesser extend and only traces of labelling were visible in betaine treated leaves. In untreated plants, no signal was found, indicating that the enzyme was not constitutively present in leaves. On the other hand, the staining of endodermis and xylem cells of root cross-sections of untreated plants suggests a constitutive presence of Ch2 in roots (Fig. 7). The synthesis of Ch2 was efficiently activated by SA and betaine in the cell walls of cortex parenchyma cells and endodermis.

Basic class IV chitinase (Ch4) was often found to be constitutively present both in leaves and roots of untreated plants. It was found mainly in leaf parenchyma and/or root endodermis cells (Figs. 4 and 8). Most potent inducer of Ch4 in leaves was BTH, however, in roots, besides BTH and SA, betaine induced accumulation of CH4 in endodermis and adjacent cortex cells, and in addition, typical extracellular globular bodies in-between cortical cells.

Comparing to the basic isozymes, the acidic class III chitinase (SE2) frequently accumulated in epidermis and in parenchyma cells (Fig. 5). It was more intensely induced by SA and betaine. Slight signal for SE2 was observed in endodermis of untreated control plants. Similarly to leaves, the deposition of SE2 was located rather to rhizodermis and outer parts of cortex (Fig. 9).

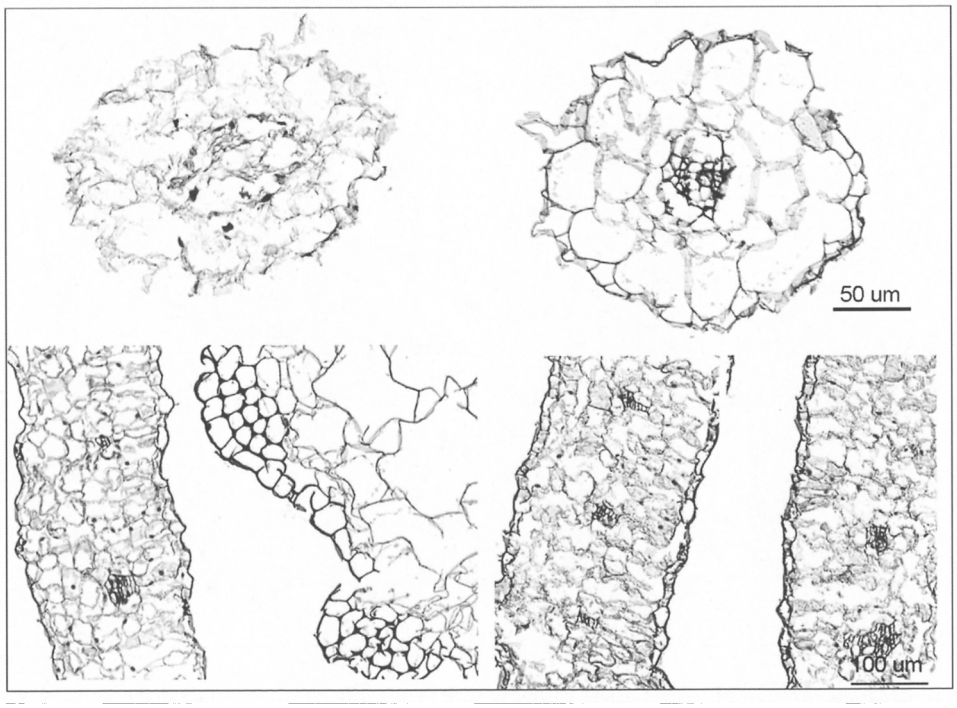

Fig. 1. Light micrograph of sugar beet root (upper) and leaf (lower) cross-sections of untreated plants stained by alcian blue and kernechrot to visualize tissue structures: cell walls are blue stained, nuclei of cut cells are red.

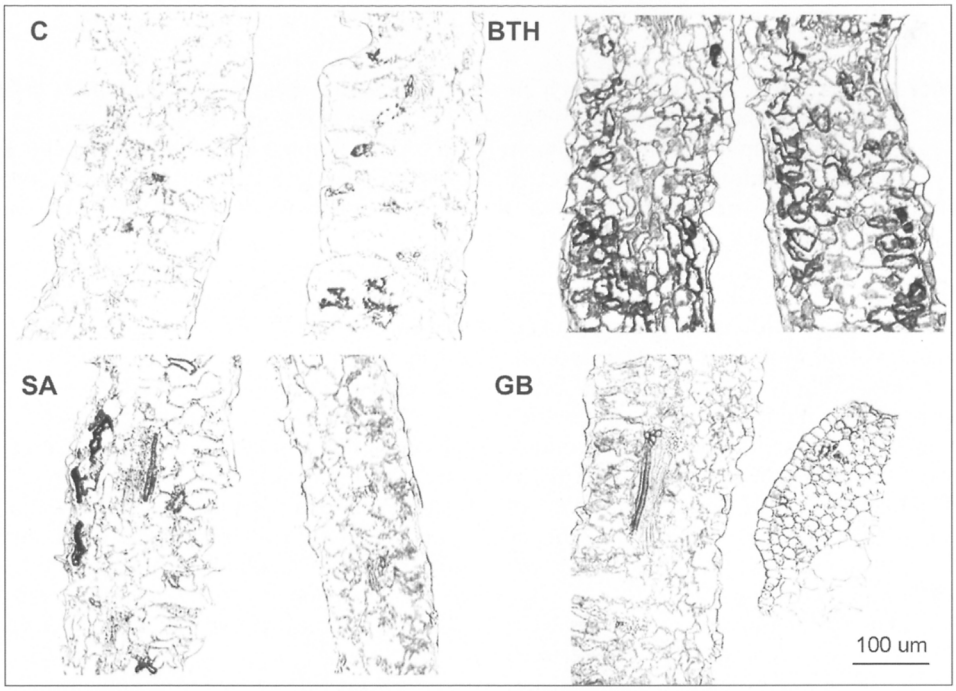

Fig. 2. Immunohistological localization of basic β-1,3-glucanase Glu2 in sugar beet leaves. Untreated control plant (C), plant treated with 1.2 mM benzothiadiazole (BTH), plant treated with 20 mM salicylic acid (SA), plant treated with 0.3 M glycine betaine (GB). Glu2 was strongly induced by BTH; the enzyme is deposited mainly in cell walls of parenchyma cells, some labelling is visible also in xylem vessels. Patterns were determined in at least 10 plants and were remarkably consistent.

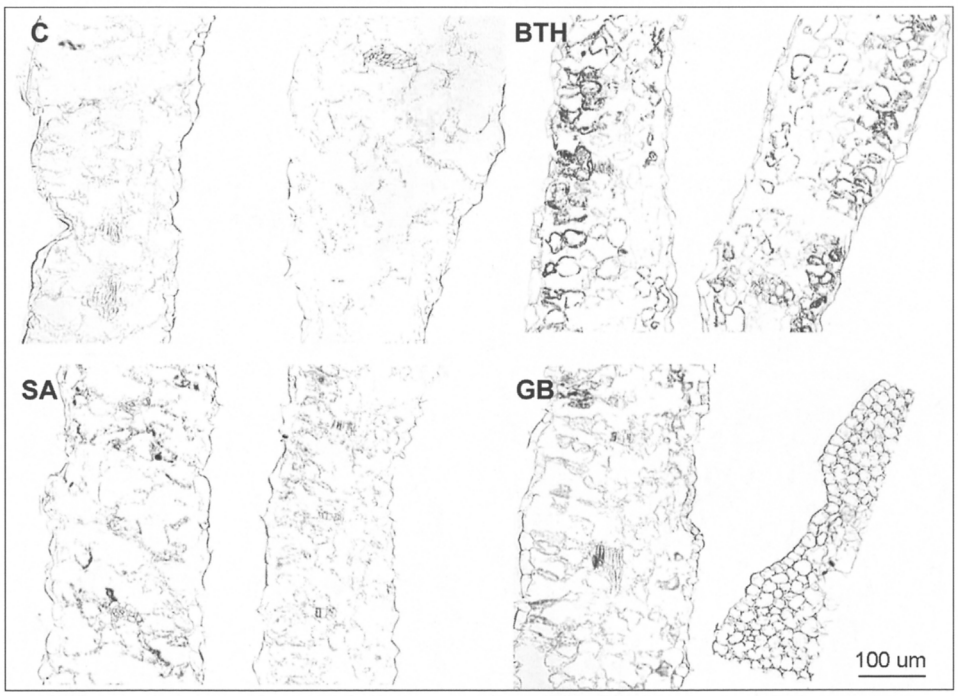

Fig. 3. Immunohistological localization of basic class II chitinase Ch2 in sugar beet leaves. Untreated control plant (C), plant treated with 1.2 mM benzothiadiazole (BTH), plant treated with 20 mM salicylic acid (SA), plant treated with 0.3 M glycine betaine (GB). Ch2 was induced by BTH, and slightly by SA; the enzyme is deposited mainly in cell walls of parenchyma cells. Patterns were determined in at least 10 plants and were remarkably consistent.

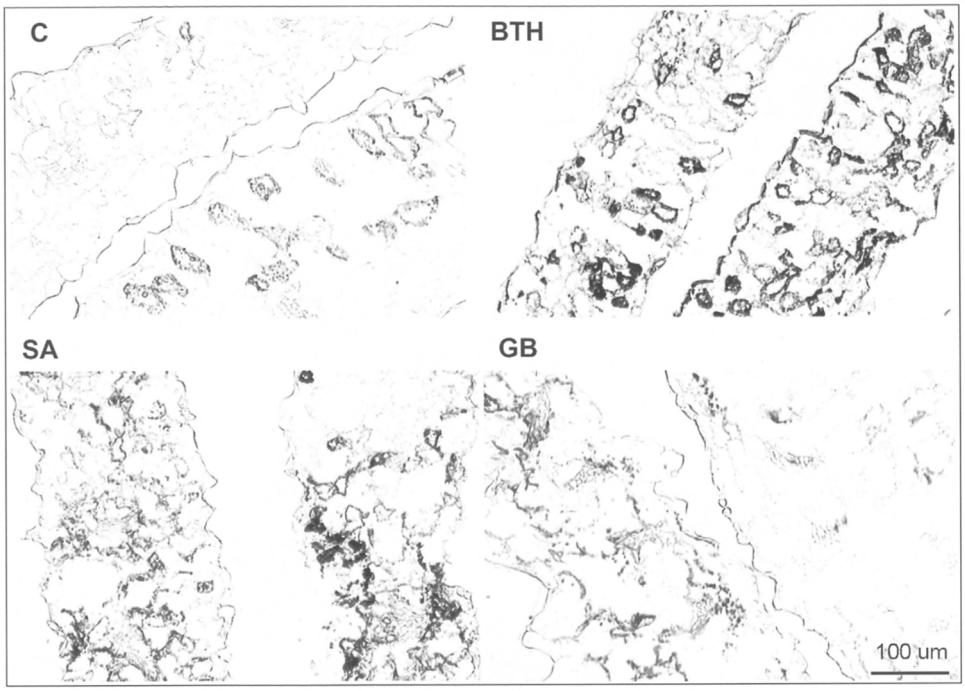

Fig. 4. Immunohistological localization of basic class VI chitinase Ch4 in sugar beet leaves. Untreated control plant (C), plant treated with 1.2 mM benzothiadiazole (BTH), plant treated with 20 mM salicylic acid (SA), plant treated with 0.3 M glycine betaine (GB). Ch4 was strongly induced by BTH in some parenchyma cells, and slightly by SA; Ch4 is present also in untreated control plants. Patterns were determined in at least 10 plants and were remarkably consistent.

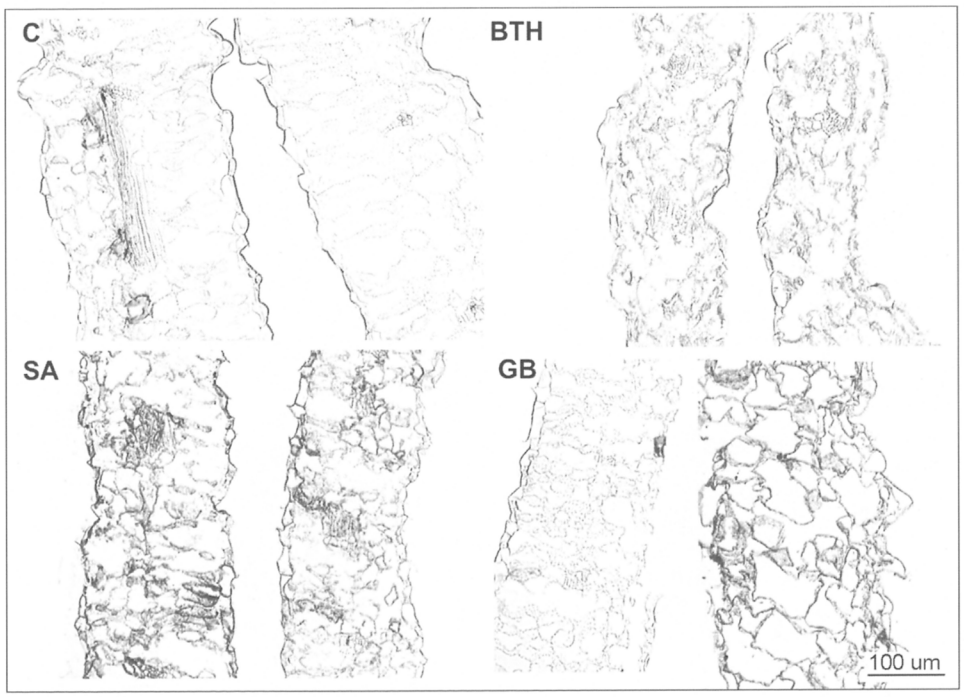

Fig. 5. Immunohistological localization of acidic class III chitinase SE2 in sugar beet leaves. Untreated control plant (C), plant treated with 1.2 mM benzothiadiazole (BTH), plant treated with 20 mM salicylic acid (SA), plant treated with 0.3 M glycine betaine (GB). SE2 was induced by SA, and in a lesser extend by BTH and GB. The signal for SE2 is visible in cell walls of parenchyma cells, as well as in epidermal cells.

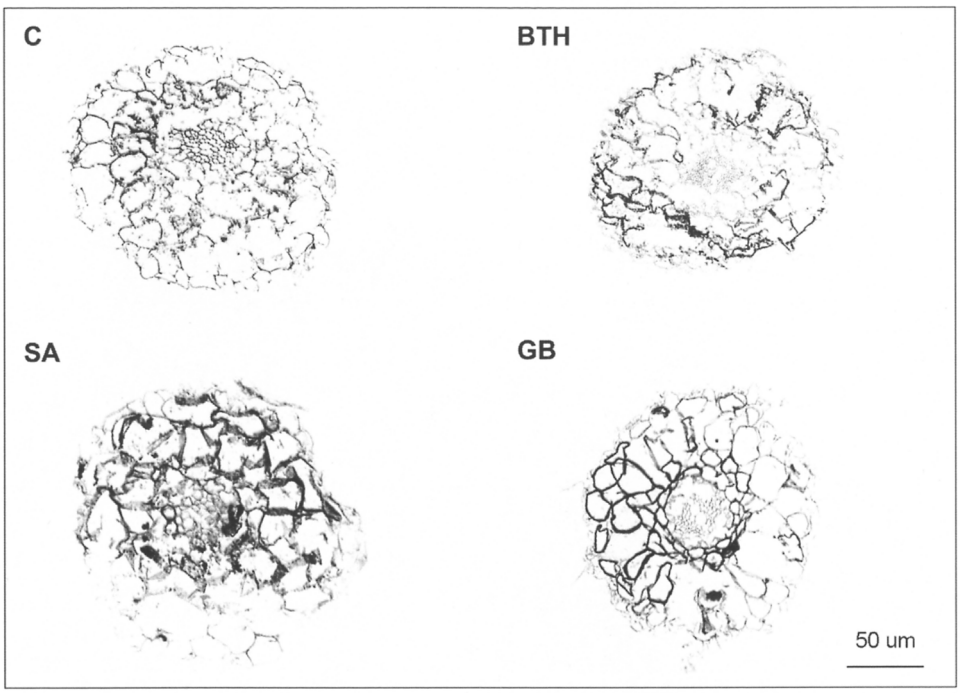

Fig. 6. Immunohistological localization of basic β-1,3-glucanase Glu2 in sugar beet roots. Untreated control plant (C), plant treated with 1.2 mM benzothiadiazole (BTH), plant treated with 20 mM salicylic acid (SA), plant treated with 0.3 M glycine betaine (GB). Glu2 was strongly induced mainly by SA, the other inducers induced the accumulation of Glu2 as well. The enzyme is deposited in cortex parenchyma cell walls and endodermis (GB).

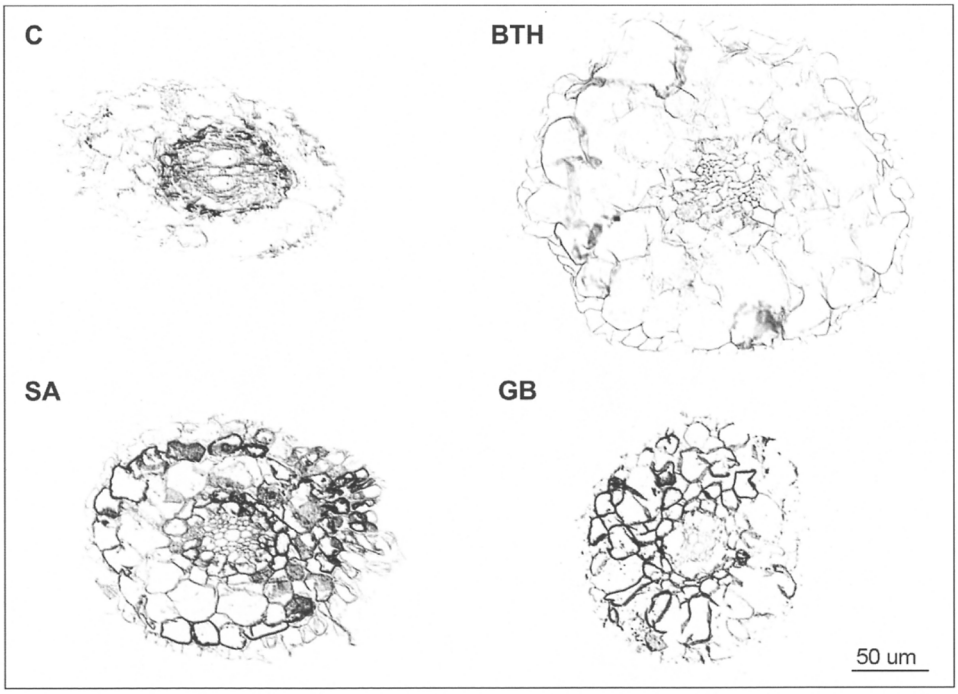

Fig. 7. Immunohistological localization of basic class II chitinase Ch2 in sugar beet roots. Untreated control plant (C), plant treated with 1.2 mM benzothiadiazole (BTH), plant treated with 20 mM salicylic acid (SA), plant treated with 0.3 M glycine betaine (GB). Ch2 was induced by SA and GB; the enzyme is deposited mainly in cell walls of cortex parenchyma and endodermis (GB). Slight signal is visible also in untreated control plants.

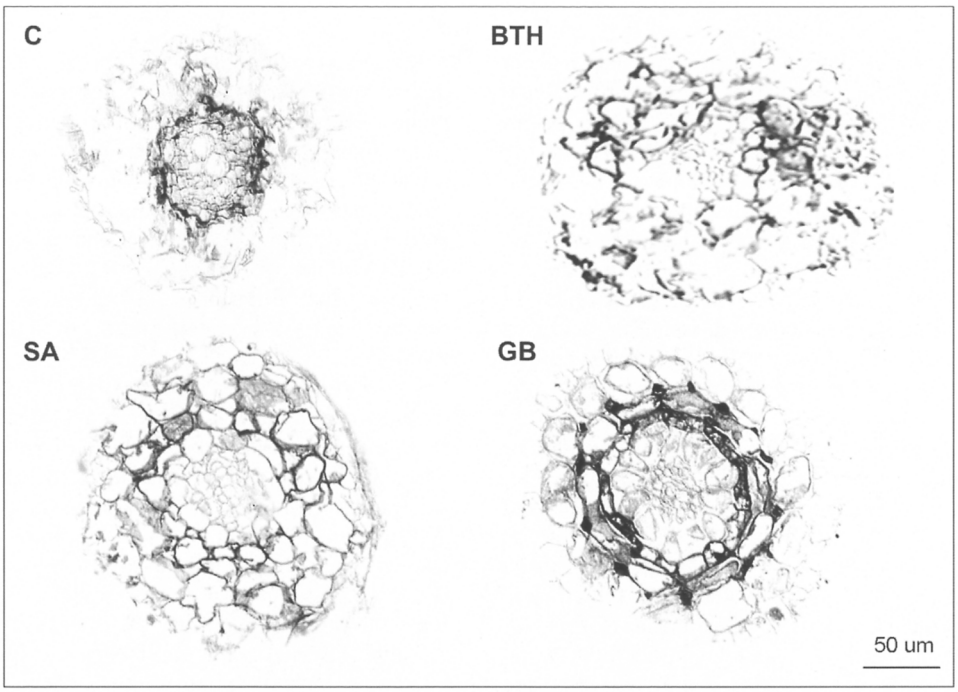

Fig. 8. Immunohistological localization of basic class VI chitinase Ch4 in sugar beet roots. Untreated control plant (C), plant treated with 1.2 mM benzothiadiazole (BTH), plant treated with 20 mM salicylic acid (SA), plant treated with 0.3 M glycine betaine (GB). Ch4 was strongly induced by all inducers, Ch4 is deposited mainly in cell walls of cortex parenchyma and endodermis, GB induced the formation of extracellular globuli.

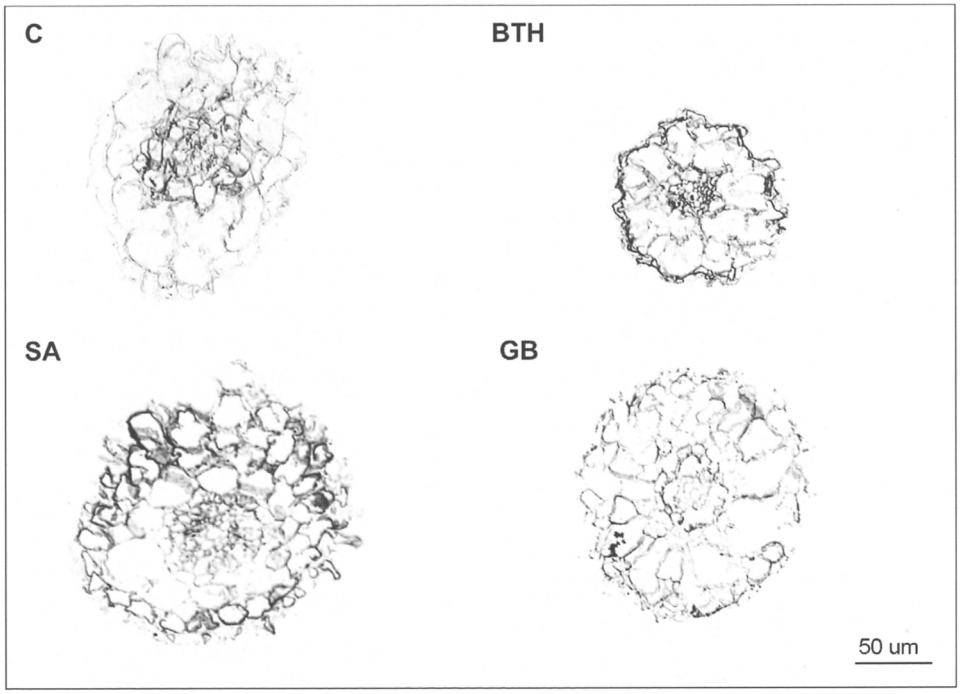

Fig. 9. Immunohistological localization of acidic class III chitinase SE2 in sugar beet roots. Untreated control plant (C), plant treated with 1.2 mM benzothiadiazole (BTH), plant treated with 20 mM salicylic acid (SA), plant treated with 0.3 M glycine betaine (GB). SE2 was induced by BTH and SA. The signal for SE2 is visible mainly in cell walls of cortex parenchyma and rhizodermis.

Discussion

Previous experiments demonstrated that sugar beet represents a sensitive plant model for the study of defence response activation by inducers of diverse origin (Fleming *et al.* 1991, Burketová *et al.* 1999). In the present study, BTH, SA as well as betaine were capable of inducing both β-1,3-glucanase and chitinase isozymes in leaves and roots of treated plants. The fashion of the protein induction and the deposition was dependent on the particular inducer, *e.g.* BTH was most efficient in Glu2 and Ch4 induction in leaves, whereas SA increased the accumulation of SE2. BTH and SA, induced synthesis of the protein in a similar extend, whereas betaine was less efficient on leaves. All the hydrolases under study accumulated extracellularly in the cell walls or extracellular globuli, which is in accordance with previous finding of Nielsen *et al.* (1993, 1996) and Gottschalk *et al.* (1998), who found Glu2 and Ch4 in extracellular globules in the vicinity of necroses caused by *C. beticola.* Extracellular deposition of these basic isozymes is surprising since the basic forms of PR-proteins are typically located intracellularly in vacuoles (Boller and Vogeli 1984, Boller and Metraux 1988). Moreover, Nielsen *et al.* (1993, 1996) and Gottschalk *et al.* (1998) reported strictly localized induction of Glu2 and Ch4 in *C. beticola* infected leaves, whereas in our experiments, the enzymes were detected also in the tissues distant from the site of inducer application, indicating the systemic effect of chemical inducers.

Although both SA and BTH are involved in the establishment of induced resistance to pathogens, their mode of action is different. While exogenously applied SA was shown to directly activate *PR* genes resulting in the accumulation of PR-proteins in plant tissues (Antoniw and White 1980, Delaney *et al.* 1994), BTH frequently only "potentiates" plants for future pathogen attack without instant PR-proteins synthesis (Katz *et al.* 1998, Latunde-Dada and Lucas 2001). Dissimilarly, in our experiments, sugar beet plants responded to BTH treatment by the accumulation of β-1,3-glucanase and chitinases in extracellular spaces. Our findings are in accordance with the results in tobacco (Friedrich *et al.* 1996) and *Arabidopsis* (Lawton *et al.* 1996), where BTH has been shown to activate the expression of a wide range of *PR* genes including those encoding glucanases and chitinases. The inducibility of defence genes by BTH or other chemical compounds is dependent on a particular plant species. In sugar beet, BTH induced accumulation of the same set of β-1,3-glucanase and chitinase isozymes as did the pathogen *C. beticola.* On the other hand, in wheat and barley, BTH, sodium salicylate and $NiCl_2$ activated different *PR* genes than pathogens and vice versa (Yu and Muehlbauer 2001, Kragh *et al.* 1993) that suggests the difference in chemical inducibility of defence genes between monocotyledons and dicotyledons.

Although the genes *Glu2, Ch4,* and *SE2* in sugar beet were switched on by *C. beticola* (Nielsen *et al.* 1994), INA, a similar compound to BTH, did not activate any of them when applied alone. Nevertheless, INA primed sugar beet plants to rapid expression of this genes in subsequent infection. However, our previous study proved the ability of BTH to induce extracellular β-1,3-glucanase and chitinase isozymes directly after the BTH treatment without the necessity of pathogen infection (Burketová *et al.* 1999). Our present immunohistological study supports these data. Thus, it remains unclear whether this difference is due to a low INA concentration used in experiments or diverse mode of action of the compounds.

Surprisingly, glycine betaine, the osmoprotectant compound, induced in root the same hydrolases as did SA and BTH. Moreover, a typical extracellular bodies containing Ch4 were observed in root cross-sections. Up to our knowledge, this is the first report on the induction of chitinase and β-1,3-glucanase isozymes by glycine betaine in plants. The data dealing with the exogenous application of glycine betaine are rather rare. Recently, the comparison of the effect of glycine betaine and BTH application has been investigated in strawberry plants (Karjalainen *et al.* 2002). Both compounds induced the accumulation of several phenolic compounds with the protective effect against pathogens.

In summary, the results presented here show that BTH, SA and glycine betaine directly induce chitinase and β-1,3-glucanase isozymes in leaf and root tissues. Regarding the accumulation of the induced proteins in extracellular space and their antifungal activity, these chemically induced proteins may be involved in resistance to leaf as well as root pathogens of sugar beet.

References

Antoniw, J., White, R.F.: The effects of aspirin and polyacrylic acid on soluble leaf proteins and resistance to virus infection in five cultivars of tobacco. - Phytopathol. Z. **98**: 331-341, 1980.

Boller, T., Vogeli, U.: Vacuolar localization of ethylene induced chitinase in bean leaves. - Plant Physiol. **74**: 442-444, 1984.

Boller, T., Metraux, J.P.: Extracellular localization of chitinase in cucumber. - Physiol. mol. Plant Pathol. **33**: 11-16, 1988.

Broglie, K., Chet, I., Holliday, M., Cressman, R., Diddle, P., Knowlton, S., Mauvais, C.J., Broglie, R.: Transgenic plants with enhanced resistance to fungal pathogen *Rhizoctonia solani.* - Science **254**: 1194-1194, 1991.

Burketová, L., Šindelářová, M., Šindelář, L.: Benzothiadiazole as an inducer of β-1,3-glucanase and chitinase isozymes in sugar beet. - Biol. Plant. **42**: 279-287, 1999.

Collinge, D.B., Kragh, K.M., Mikkelsen, J.D., Nielsen, K.K.,

Rasmunssen, U., Vad, K.: Plant chitinases. - Plant J. **3**: 31-40, 1993.

Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney T., Gutrella, M., Kessmann, H., Ward, E., Ryals, J.: A central role of salicylic acid in plant disease resistance. - Science **266**: 1247-1250, 1994.

Dempsey, D.A., Shah, J., Klessig, D.F.: Salicylic acid and disease resistance in plants. - Crit. Rev. Plant Sci. **18**: 547-575, 1999.

Fleming, T.M., McCarthy, D.A., White, R.F., Antoniw, J.W., Mikkelsen, J.D.: Induction and characterization of some of the pathogenesis-related proteins in sugar beet. - Physiol. mol. Plant Pathol. **39**:147-160, 1991.

Friedrich, L., Lawton, K., Ruess, W., Masner, P., Specker, N., Rella, M.G., Meier, B., Dincher, S., Staub, T., Uknes, S., Metraux, J.P., Kessmann, H., Ryals, J.: A benzothiadiazole derivative induces systemic acquired resistance in tobacco. - Plant J. **10**: 61-70, 1996.

Gottschalk, T.E., Mikkelsen, J.D., Nielsen, J.E., Nielsen, K.K., Brunstedt, J.: Immunolocalization and characterization of a β-1,3-glucanase from sugar beet, deduction of its primary structure and nucleotide sequence by cDNA and genomic cloning. - Plant Sci. **132**: 153-167, 1998.

Holmstrom, K.O., Somersalo, S., Mandal, A., Palva, T., Welin, B.: Improved tolerance to salinity and low temperature in transgenic tobacco producing glycine betaine. - J. exp. Bot. **51**: 177-185, 2000.

Jongedijk, E., Tigelaar, H., Van Roekel, J.S.C., Bres Vloemans, S.A., Dekker, I., Elzen, P.J.M., Van den Cornelissen, B.J.C., Melchers, L.S.: Synergistic activity of chitinases and β-1,3-glucanases enhances fungal resistance in transgenic tomato plants. - Euphytica **85**: 173-180, 1995.

Karjalainen, R., Lehtinen, A., Hietaniemi, V., Pihlava, J.M., Tiilikkala, K., Keinanen, M., Julkunen-Titto, R., Jokinen, K.: Benzothiadiazole and glycine betaine treatments enhance phenolic compounds production in strawberry. - Acta Hort. **567**: 353-356, 2002.

Katz, V.A., Thulke, O.U., Conrath, U.: A benzothiadiazole primes parsley cells for augmented elicitation of defense responses. - Plant Physiol. **117**: 1333-1339, 1998.

Kessmann, H., Staub, T., Hofmann, C., Maetzke, T., Herzog, J., Ward, E., Uknes, S., Ryals, J.: Induction of systemic acquired resistance in plants by chemicals. - Annu. Rev. Phytopathol. **32**: 439-459, 1994.

Kragh, K.M., Jacobsen, S., Mikkelsen, J.D., Nielsen, A.: Tissue specificity and induction of class I, II and III chitinases in barley (*Hordeum vulgare*). - Physiol. Plant. **89**: 490-498, 1993.

Kuć, J.: Induced immunity to plant disease. - Bioscience **32**: 854-860, 1982.

Kuć, J.: Induced systemic resistance. - In: Hammerschmidt, R., Kuć, J. (ed.): Induced Resistance to Disease in Plants. Pp. 169-175. Kluwer Academic Publishers, Dordrecht 1995.

Latunde-Dada, A.O., Lucas, J.: The plant defence activator acibenzolar-S-methyl primes cowpea [*Vigna unguiculata* (L.) Walp.] seedlings for rapid induction of resistance. - Physiol. mol. Plant Pathol. **58**: 199-208, 2001.

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

Neuhaus, J.-M.: Plant chitinases (PR-3, PR-4, PR-8, PR-11). - In: Datta, S.K., Muthukrishnan, S. (ed.): Pathogenesis-related Proteins in Plants. Pp. 49-76. CRC Press, Boca Raton - London - New York - Washington 1999.

Nielsen, K.K., Bojsen, K., Collinge, D.B., Mikkelsen, J.D.: Induced resistance in sugar beet against *Cercospora beticola*: induction by dichloroisonicotinic acid is independent of chitinase and β-1,3-glucanase transcript accumulation. - Physiol. mol. Plant Pathol. **45**: 89-99, 1994.

Nielsen, J.E., Nielsen, K.K., Mikkelsen, J.D.: Immunohistological localization of a basic class IV chitinase in *Beta vulgaris* leaves after infection with *Cercospora beticola*. - Plant Sci. **119**: 191-202, 1996.

Nielsen, K.K., Mikkelsen, J.D., Kragh, K.M., Bojsen, K.: An acidic class-III chitinase in sugar beet – induction by *Cercospora beticola*, characterization, and expression in transgenic tobacco plants. - Mol. Plant-Microbe Interact. **6**: 495-506, 1993.

Rhodes, D., Hanson, A.D.: Quarternary ammonium and tertiary sulfonium compounds in higher plants. - Annu. Rev. Plant Physiol. Plant mol. Biol. 44: 357-384, 1993.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., Hunt, M.D.: Systemic acquired resistance. - Plant Cell **8**: 1809-1819, 1996.

Sakamoto, A., Murata, N.: The role of glycine betaine in the protection of plants from stress: Clues from transgenic plants. - Plant Cell Environ. **25**: 163-171, 2002.

Schlumbaum, A., Mauch, F., Vogeli, U., Boller, T.: Plant chitinases are potent inhibitors of fungal growth. - Nature **324**: 365-367, 1986.

Siegrist, J., Muhlenbeck, S., Buchenauer, H.: Cultured parsley cells, a model system for the rapid testing of abiotic and natural substances as inducers of systemic acquired resistance. - Physiol. mol. Plant Pathol. **53**: 223-238, 1998.

Šindelářová, M., Šindelář, L., Burketová, L.: Glucose-6-phosphate dehydrogenase, ribonucleases and esterases upon tobacco mosaic virus infection and benzothiodiazole treatment in tobacco. - Biol. Plant. **45**: 423-432, 2002.

Steiner, A.A.: The universal nutrient solution. - In: Proceedings of the Sixth International Congress on 'Soilless Culture'. Pp. 633-650. Pudoc, Wageningen 1984.

Suo, Y., Leung, D.W.M.: BTH-induced accumulation of extracellular proteins and blackspot disease in rose. - Biol. Plant. **45**: 273-279, 2002.

Van Loon, L.C., Bakker, P.A.H.M., Pieterse, C.M.J.: Systemic resistance induced by rhizosphere bacteria. - Annu. Rev. Phytopathol. **36**: 453-483, 1998.

Van Loon, L.C., Van Strien, E.A.: The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. - Physiol. mol. Plant Pathol. **55**: 85-97, 1999.

Yu, G.-Y., Muehlbauer, G.J.: Benzothiadiazole-induced gene expression in wheat spikes does not provide resistance to *Fusarium* head blight. - Physiol. mol. Plant Pathol. **59**: 129-136, 2001.