

PR-10 protein is induced by copper stress in roots and leaves of a Cu/Zn tolerant clone of birch, *Betula pendula*

M. UTRIAINEN,¹ H. KOKKO,¹ S. AURIOLA,² O. SARRAZIN^{1,3} & S. KÄRENLAMP¹

¹Department of Biochemistry and Biotechnology, ²Department of Pharmaceutical Chemistry, University of Kuopio, PO Box 1627, FIN-70211 Kuopio, Finland, and ³Department of Biochemistry, Université Paris 7, place Jussieu, 75251 Paris Cedex 05, France

ABSTRACT

The response of a Cu- and Zn-tolerant birch (*Betula pendula*) clone to copper stress was investigated. The plants were exposed to control and EC50 concentrations of Cu (0.3 and 30 μM CuSO_4 , respectively) for 7 d in hydroponic culture. Total proteins were extracted from the roots and leaves and separated by two-dimensional polyacrylamide gel electrophoresis. The differences in protein patterns on silver- or Coomassie-stained gels were analysed. The most apparent quantitative difference was the increase in the amount of a 17 kDa polypeptide caused by Cu stress in both roots and leaves. The protein was identified as Bet v 1-Sc3 (according to the current nomenclature PR-10c) using N-terminal amino acid sequencing and on-line high-performance liquid chromatography/electrospray ionization/ion trap mass spectrometry. The present results indicate that PR-10 is not only activated by pathogens but also by excessive amounts of copper ions. PR-10 proteins in birch have been reported earlier not to be induced by Ag, Li or Cd in birch suspension culture, but Cu has not been previously tested.

Key-words: birch pollen allergen; copper; Cu/Zn resistance; ESI-MS; heavy metals; pathogenesis-related protein; two-dimensional electrophoresis.

Abbreviations: CID, collision-induced dissociation; 2D, two-dimensional; EC50, the concentration which inhibits the root growth by 50%; HPLC-ESI-MS, high-performance liquid chromatography/electrospray ionization/ion trap mass spectrometry; IEF, isoelectric focusing; PVDF, polyvinylidene difluoride.

INTRODUCTION

Plants respond to copper stress through a variety of biochemical reactions (Van Assche & Clijsters 1990). Copper is a redox-active heavy metal which, when present in excessive amounts, causes uncontrolled redox reactions in the cells, resulting in the formation of damaging free radical species, peroxidative destruction of biomembranes and, finally, cell death (Hochstein *et al.*

1980; Sandman & Böger 1980a,b; Freedman *et al.* 1989). Toxic copper concentrations give rise to scavenging systems against oxidative stress, and stimulate catalase, peroxidase and ethylene production which, in turn, stimulate lipoxygenase capacity (Gora & Clijsters 1989; Weckx *et al.* 1993). Well-demonstrated responses to copper are potassium leakage due to destruction of biomembranes (McBrien & Hassall 1965) and induction of metal-binding polypeptides known as phytochelatins (Steffens 1990). The precise targets and primary toxic effects of copper within plant cells are not known for certain (Verkleij & Schat 1990). However, data on enzyme induction indicate that copper might act mainly at the root level (Van Assche & Clijsters 1990).

We have screened several micropropagated birch clones in hydroponics for their tolerance to copper and zinc (Utriainen *et al.* 1997). The aim of the present study was to characterize Cu-responsive proteins in the roots of the most tolerant clone. In order to understand metal tolerance in plants we need to identify the molecular mechanisms involved in the biochemical adaptation.

MATERIALS AND METHODS

Plant material and growth conditions

Clone 142 A is a seed-derived, Cu- and Zn-tolerant European white birch (*Betula pendula* Roth.). The parent tree originates from the spoil heaps of disused lead/zinc mines near Shrewsbury, central England. The EC50 value, i.e. the concentration which inhibits the root growth by 50%, for this clone is 30 μM Cu (Utriainen *et al.* 1997).

The details of the cultivation and copper exposure of micropropagated clone 142 A in hydroponics are described by Utriainen *et al.* (1997). The plants were exposed to the control and EC50 concentrations of Cu (0.3 and 30 μM CuSO_4 , respectively) for 7 d, after which all roots and most leaves (except for the youngest small leaves) were harvested.

Protein extraction

Total proteins were extracted from roots with the phenol partitioning method (Lopez *et al.* 1994), except that the frozen root material was homogenized in a hydraulic press

Correspondence: Sirpa O. Kärenlampi. Fax: + 3581 7 2811510; e-mail: Sirpa.Karenlampi@uku.fi

(HP-20, BAHCO, AB BIOX, Göteborg, Sweden), an additional washing with 80% acetone was carried out after washing with methanol/ammonium acetate solution, and the samples were solubilized in a mixture containing 95% (v/v) urea sample buffer [9.5 M urea, 2.0% (v/v) Triton X-100, 5% (v/v) β -mercaptoethanol, 1.6% (v/v) Bio-Lyte 5/7 ampholyte, 0.4% (v/v) Bio-Lyte 3/10 ampholyte] and 5% (v/v) sample solution A [1% SDS (w/v), 150 mM DTT]. Leaf proteins were isolated by the same method except that the leaf material was frozen in liquid nitrogen and homogenized in a mortar in the presence of sea sand. Total protein was analysed using Bio-Rad Protein Assay Dye reagent (Bio-Rad, Hercules, CA, USA).

Two-dimensional gel electrophoresis

Electrophoresis in the first dimension (isoelectric focusing, IEF) was performed as described by O'Farrell (1975) with some modifications. The 160×1.5 mm tubes contained 2% Triton X-100 instead of Nonidet P-40. Ampholytes pH 5–7 and 3–10 (Bio-Rad) at concentrations of 1.6 and 0.4%, respectively, were used. The upper reservoir was filled with 0.02 M NaOH, and the lower reservoir contained 0.01 M H_3PO_4 . Electrofocusing (Protean II xi 2-D Cell, Bio-Rad) was performed at room temperature: 200 V for 2 h, followed by 500 V for 2 h, and then 800 V for 16 h. The IEF gels were extruded and equilibrated by gently shaking in 3 mL of a modified SDS-sample buffer [0.0625 M Tris-HCl, pH 6.8, 2.3% (w/v) SDS, 10% (v/v) glycerol, 0.002% (w/v) bromophenol blue] for 5 min to remove most of the ampholytes and urea and to facilitate the diffusion of SDS and dye into the gel. For the second dimension, the gels were transferred on top of a 4% acrylamide stacking gel (Protean II xi 2-D Cell Instruction Manual) and sealed with modified SDS-equilibration buffer without β -mercaptoethanol but including melted 1% agarose. After running the stacking gel with 25 mA and the separating gel (12% acrylamide) with 35 mA, the polypeptides were identified by staining with silver nitrate using the procedure described by Blum *et al.* (1987), with the modifications reported by Flengsrud & Kobro (1989); 1.25% glutaraldehyde instead of 5% was used in the pre-treatment. Alternatively, the gels were stained with Coomassie Brilliant Blue.

Computer-based analysis of 2D gels

The silver- or Coomassie-stained 2D gels were evaluated by a computer-based system, Cream™ (Kem-En-Tec Software Systems, Denmark).

N-terminal amino acid sequence analysis with protein sequencer

The proteins were transferred from the 2D gels to polyvinylidene difluoride membrane (PVDF, Immobilon-P, Millipore) at 90 V for 30 min using 10 mM CAPS buffer, 10% methanol, pH 9. The membrane was stained

with 0.1% Coomassie Brilliant Blue and destained with 50% methanol. The stained protein of interest was excised from six membranes and was allowed to dry at room temperature. The Applied Biosystems 477 A Pulsed Liquid Phase protein sequencer was used to determine the N-terminal amino acid sequence of the protein from the pooled sample.

In-gel trypsin digestion of the protein

The protein spot was excised from the Coomassie Brilliant Blue-stained gel and digested with trypsin as described by Clauser & Andrews (1996).

HPLC-electrospray ionization mass spectrometry

Positive ion mass spectra were acquired with on-line HPLC-ESI-MS. The measurements were carried out using an LCQ quadrupole ion trap mass spectrometer equipped with an ESI source (Finnigan MAT, San Jose, CA, USA), a Rheos 4000 pump (Flux Instruments, Danderyd, Sweden) and a Rheodyne 7725 injector with a 20 μ L loop (Cotati, CA, USA).

The tryptic digests (20 μ L) were injected into the reversed-phase column (SynChrom RP-8, 5 μ m, 100×2.1 mm I.D., Lafayette, IN, USA). The peptides were separated using a gradient from 2 to 70% acetonitrile in 40 min, + 23 °C. Eluent A was 50 mM formic acid, and eluent B was 70% acetonitrile containing 50 mM formic acid. The total eluent flow of 0.2 mL min⁻¹ was directed to the ESI source, which is designed to cope with flow rates up to 1 mL min⁻¹. The system was tuned by infusion of peptide MRFA from a T-split to the eluent flow. The spray needle potential was set to 5 kV. The spray was stabilized using a nitrogen sheath flow, with the value set to 85. The stainless steel inlet capillary was heated to 225 °C. The capillary voltage was 32 V and the tube lens offset was 40 V.

The full scan mass spectra from m/z 300 to m/z 2000 were measured using 400 ms for collection of the ions in the trap; three microscans were summed. Collision-induced dissociation (CID) MS-MS spectra of the peptides were obtained using the dependent scan mode of the instrument. Full scan MS data were collected until the base peak ion exceeded a pre-set intensity threshold (2×10^4). This triggers acquisition of a CID MS-MS product ion spectrum of the ion. The collision energy was 35% and isolation width was 2 mass units. The scan range was dependent on the parent mass ion.

RESULTS

Polypeptides related to Cu stress from the Cu- and Zn-tolerant birch clone 142 A grown in hydroponic culture in the presence of 0.3 μ M or 30 μ M Cu for 7 d were studied. The polypeptide composition was analysed by two-dimensional gel electrophoresis. Our previous results showed that the exposure of the birch clone 142 A for 7 d to 30 μ M

Cu did not affect the Cu content in the leaves but increased the Cu content of the roots almost 30-fold (Utriainen *et al.* 1997). Therefore, only root protein fractions were initially analysed. The two-dimensional polypeptide patterns obtained from the roots of clone 142 A are shown in Fig. 1. The between-gel reproducibility was high. A good resolution of the polypeptides was achieved in the molecular mass range of 14–80 kDa.

In the silver-stained gels, the Cream computer program detected 431 and 413 proteins from the Cu-exposed and control root samples, respectively. From these, 358 proteins were present in both exposed and control gels, as judged by their location in the gels. Fifty-five proteins were found only in the control sample and 73 only in the exposed sample. Because the silver staining of proteins is affected not only by the protein concentration but also by

their characteristics, the intensities of the protein spots from these gels were not analysed.

In the Coomassie Brilliant Blue-stained gels, the Cream computer program detected 333 and 226 proteins from the Cu-exposed and control sample, respectively. From these, 204 proteins were present in both exposed and control gels, as judged by their location in the gel. Twenty-two proteins were found only in the control sample and 129 only in the exposed sample. When comparing the intensities of the proteins, a 5-fold enhancement was observed in a 17 kDa protein in the exposed sample compared with the control. The concentration of this protein was $\approx 0.7\%$ of the total protein concentration in the exposed sample. In addition to roots, this protein was induced by copper in birch leaves (Fig. 2). The induction was ≈ 50 -fold as calculated from one gel.

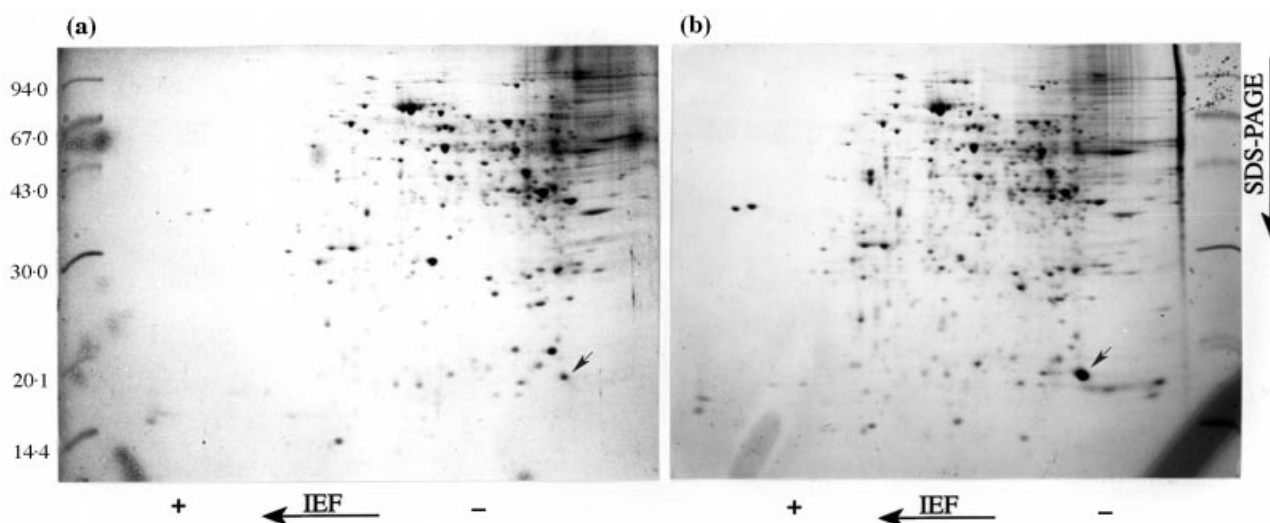


Figure 1. Two-dimensional electrophoretogram of Coomassie Brilliant Blue-stained proteins from roots of *Betula pendula* clone 142 A exposed to (a) $0.3 \mu\text{M}$ (control) and (b) $30 \mu\text{M}$ of CuSO_4 for 7 d.

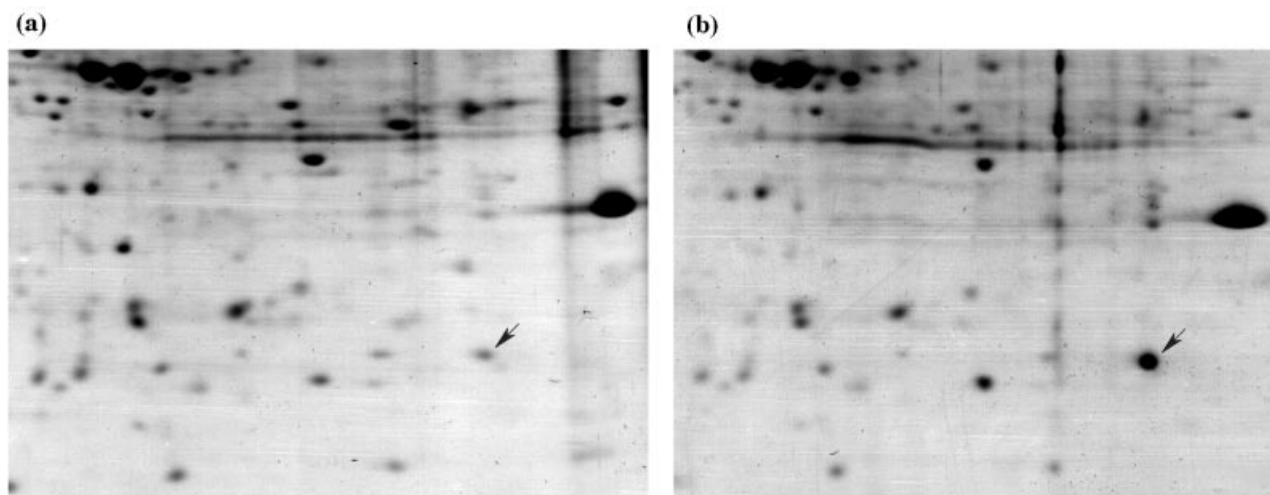


Figure 2. Partial 2D electrophoretogram of Coomassie Brilliant Blue-stained proteins from leaves of *Betula pendula* clone 142 A. The roots were exposed to (a) $0.3 \mu\text{M}$ (control) and (b) $30 \mu\text{M}$ of CuSO_4 for 7 d.

For the N-terminal amino acid sequence analysis of the 17 kDa protein, six identical unstained gels were blotted onto PVDF membranes. The membranes were stained with Coomassie Brilliant Blue and the 17 kDa protein was excised and pooled to obtain sufficient amount of protein for sequence analysis. Fifteen amino acids were determined from the N-terminus. The first amino acid from the N-terminal sequence remained unclear. Comparison of this sequence with the sequences deposited in the EMBL protein sequence data bank revealed a 100% identity with the Bet v 1-Sc3 protein (PIR: locus S 47249) from the European white birch (Swoboda *et al.* 1995).

Mass spectrometry of the tryptic peptides of the 17 kDa protein was used to confirm the identity of the protein with Bet v 1-Sc3. The peptides were separated using a reverse-phase HPLC column and an acetonitrile gradient. Figure 3 shows the full scan HPLC-MS base peak ion chromatogram of the tryptic digest of the protein from a leaf sample. The full scan HPLC-ESI mass spectra of the tryptic peptides show singly or doubly protonated molecules as base peaks (data not shown). The charge state of the peaks and the monoisotopic molecular weights were measured using a 10 mu scan range (zoom scan function). The measured masses of these peaks found in the leaf sample match the masses of the following six expected tryptic peptides from Bet v 1-Sc3: ISYEIK (measured monoisotopic mass 752.1, calculated monoisotopic mass 752.4), IVAAPGGGSILK (1082.4,

1082.6), ITFPEGSHFK (1162.4, 1162.6), AVENYLVAHPNAYN (1575.3, 1574.8), GVFNYEDEATSVIAPAR (1838.51838.9) and SFVLDADNLIPK (1331.4, 1331.7). The structures of these peptides were verified using CID of the $[M + H]^+$ ions; in the case of GVFNYEDEATSVIAPAR, the $[M + 2H]^{2+}$ ion at m/z 920. The fragment ions were labelled using the system developed by Biemann (1988). The full scan MS-MS spectra of these peptides showed mainly Y', B and A type ions, which is typical for low-energy collision mass spectra (Fig. 4). In the leaf sample, two additional peptides were found, where the MS-MS spectra were of poor quality: VDEIDHANFK (1187.4, 1187.6) and GDISLNEEEEIK (1246.4, 1246.6).

From the root sample run in 2D gel, the following five tryptic peptides were found by HPLC-ESI-MS: ISYEIK, IVAAPGGGSILK, GDISLNEEEEIK, GVFNYEDEATSVIAPAR and SFVLDADNLIPK.

Figure 5 shows the complete amino acid sequence of Bet v 1-Sc3. Also shown are the identified tryptic peptides and the N-terminal sequence obtained by protein sequencer.

DISCUSSION

Two-dimensional gel electrophoresis combined with mass spectrometric identification is the single most powerful analytical method currently available for separating complex protein mixtures such as cell or tissue extracts (Shevchenko *et al.* 1996). Two-dimensional gel elec-

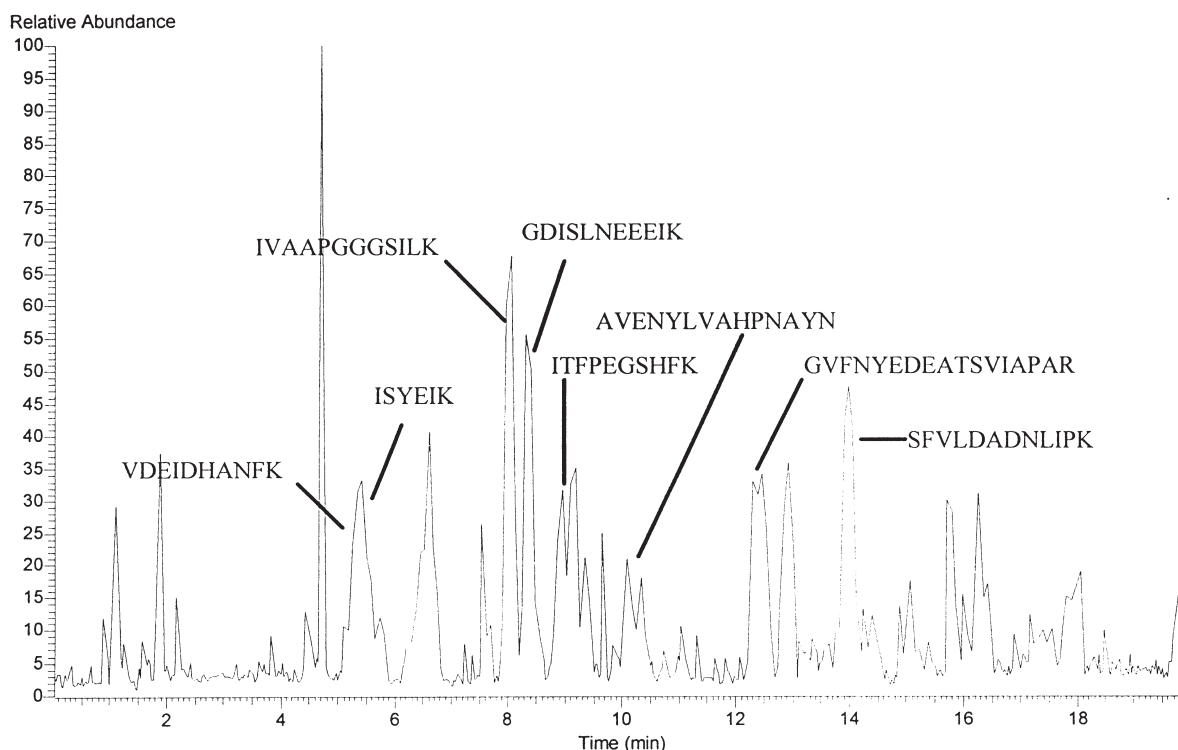


Figure 3. HPLC-ESI-MS base peak ion chromatogram of the tryptic digest from the 17 kDa protein spot extracted from the Coomassie Brilliant Blue-stained gel shown in Fig. 1.

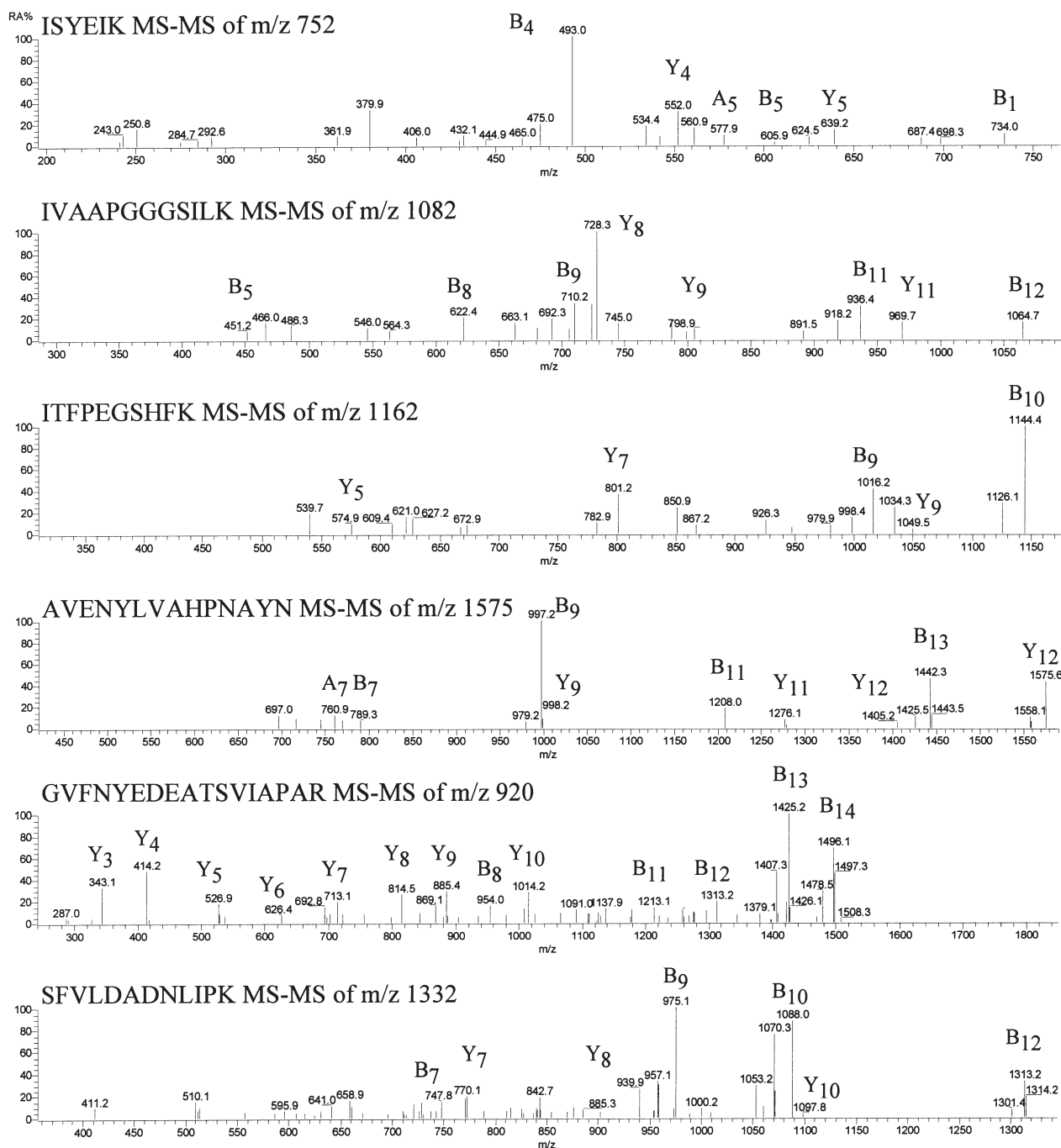


Figure 4. HPLC-ESI-MS-MS spectra of six peptides found in the leaf sample. The conditions were as described in Fig. 3. The peptides were separated using a SynChrom RP-8 column 100 × 2.1 mm I. D. with a gradient from 2 to 70% acetonitrile (50 mM formic acid) in 40 min.

trophoresis has been used to analyse and compare the expression of proteins during plant development or in response to environmental changes (e.g. Zurfluc & Guilfoyle 1980; Renaudin *et al.* 1991; Lopez *et al.* 1994). To compare 2D gels, it is essential that the proteins are well resolved, the gels lack artifacts due to proteolysis and the protein patterns are reproducible from gel to gel. In our experience, a modified phenol extrac-

tion method resulted in the best resolution of proteins in 2D gels, probably because this method is efficient in removing the non-protein components which interfere with the isoelectric focusing step.

A difficulty in using 2D gels for N-terminal sequencing with a protein sequencer is that the protein yield is usually small and many gels have to be run in order to obtain a sequence from a protein. In this work, six identical

```

1  GVFNYEDEAT SVIAPARL LFK SFVLADADNLI PKVAPENVSS AENIEGNGGP 50
51 GTIKKITFPE GSHFKYMKHR VDEIDHANFK YCYSIIIEGGP LGDTLEKISY 100
101 EIKIIVAAPGG GSILKITSKY HTKGDISLNE EETKAGKEKG AGLFKAVENY 150
151 LVAHPNAYN

```

Figure 5. Amino acid sequence of Bet v 1-Sc3 protein induced by copper in birch roots and leaves. The sequence obtained by N-terminal amino acid sequencing is shown as a shaded box. The sequences found in MS-MS spectra are shown in open boxes.

unstained gels were blotted onto PVDF membranes; with this material, not more than 15 amino acids were uncovered. In addition, the first N-terminal amino acid remained unidentified because the first cycle gave an unclear result. On the other hand, using mass spectral analysis even 5 ng of protein may be sufficient for the identification of the protein (Wilm *et al.* 1996). We were successful in the identification of Bet v 1-Sc3 from a single gel, covering $\approx 58\%$ of the total amino acid sequence. The identified N-terminal peptide (monoisotopic mass 1838.9) confirmed the presence of glycine in the N-terminus and the removal of methionine which was predicted from the cDNA sequence of Bet v 1-Sc3 (Swoboda *et al.* 1995). The removal of methionine is in agreement with an earlier finding on the major birch pollen allergen Bet v I which belongs to the same protein family as Bet v 1-Sc3 (Ferreira *et al.* 1993). It is not known whether Bet v 1-Sc3 is expressed in pollen. So far we have found it in the roots and leaves of birch. Bet v I-Sc1, Sc2 and Sc3 have been classified by Van Loon *et al.* (1994) in the PR-10 family of intracellular defence-related proteins that have a ribonuclease-like structure. According to the current nomenclature, Bet v 1-Sc1, Sc2 and Sc3 are now called PR-10a, b and c, respectively. There are three previous reports on the localization of PR-10 in root tissue: from the root hairs of garden pea (Mylona *et al.* 1994), carrot (Yamamoto *et al.* 1997) and rice (Moons *et al.* 1997).

The PR proteins are induced by many biotic (e.g. fungi, bacteria, viruses, viroids and hormonal shock) and abiotic (e.g. heat shock, wounding, anaerobiosis, and a large number of chemicals including ozone) stresses (Bol *et al.* 1990; Linthorst 1991; Kärenlampi *et al.* 1994). PR-10 has been shown to be induced by elicitors (Walter *et al.* 1990), pathogens (Swoboda *et al.* 1995) and wounding (Warner *et al.* 1992). We have recently found that PR-10 is induced by ozone in birch (Pääkkönen *et al.* 1998). Mercuric chloride induces PR proteins, i.e. 1,3- β -glucanases and chitinases in maize (Didierjean *et al.* 1996). Bet v 1-Sc3 has been reported not to be induced by Ag, Li and Cd in birch (*Betula verrucosa*) suspension culture (Swoboda *et al.* 1994) but Cu was not tested. The present results show that this protein is induced by Cu in birch roots and leaves. It is of interest that the induction was much higher in the leaves (35-fold compared to controls) than in the roots (5-fold compared to controls), although the leaf Cu content was not increased (Utriainen *et al.* 1997).

The systemic induction of PR-10c in birch leaves may be explained as follows. Redox active metals like copper may directly generate active oxygen species (AOS) such as superoxide anion, hydrogen peroxide and hydroxyl radicals in the Cu-exposed roots. AOS initiate the peroxidation of lipids. Linoleic acid, which is the most important lipid component in plant roots (De Vos *et al.* 1993), is known to be metabolized to jasmonic acid, one of the intermediates being a peroxide (Creelman & Mullet 1997). Jasmonic acid may serve as a systemic signal molecule which migrates to the leaves. It is known to induce PR-10 transcripts and protein in rice roots (Moons *et al.* 1997). Evidence for the involvement of AOS in PR-10 induction is also provided by studies on oxidative stress caused by ozone; ozone can induce PR-10 transcripts (Eckey-Kaltenbach *et al.* 1997; Pääkkönen *et al.* 1998, Korhonen *et al.* 1998). Furthermore, the PR-10 promoter from asparagus (AoPR1) is responsive to hydrogen peroxide (Bi *et al.* 1995).

AOS can be highly toxic since they attack several cell constituents such as amino acids, proteins, carbohydrates, nucleic acids and lipids. The roots exposed to Cu may suffer from oxidative stress more than the leaves, where the Cu content is not increased. Jasmonic acid may mediate a stronger induction of PR-10 in the leaves than in the roots where the overall synthesis of new protein may be impaired.

The induction of PR-10 by copper was studied here in a Cu- and Zn-tolerant birch clone. It will be of interest to know whether the expression of this protein is linked to the heavy metal tolerance of birch. There is some evidence to suggest that the manipulation of antioxidant capacity may be a valuable route to obtain stress-tolerant, and perhaps also metal-tolerant, plants.

Specific probes (antibodies) are needed for a more detailed characterization of the expression of this protein. We are presently producing specific antibodies and developing an ELISA test for a more in-depth characterization of Bet v 1 Sc3 from birch clones of varying metal tolerance and metal exposure.

ACKNOWLEDGMENTS

This work was supported by the Academy of Finland, Ecotoxicology Programme, and by the European Commission (Contract no: ENV4-CT95-0083). O.S. was supported by the Erasmus student exchange programme. Ms Rauni Kärenlampi and Ms Outi Virtaharju are gratefully acknowledged for further processing of the 2D data.

REFERENCES

- Bi Y.-M., Kenton P., Mur L., Darby R. & Draper J. (1995) Hydrogen peroxide does not function downstream of salicylic acid in the induction of PR protein expression. *the Plant Journal* **8**, 235–245.
- Biemann K. (1988) Contributions of mass spectrometry to peptide and protein structure. *Biomedical and Environmental Mass Spectrometry* **16**, 99–111.
- Blum H., Beier H. & Gross H.J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8**, 93–99.
- Bol J.F., Linthorst H.J.M. & Cornelissen B.J.C. (1990) Plant pathogenesis-related proteins induced by virus infection. *Annual Review of Phytopathology* **28**, 113–138.
- Clauser K. & Andrews L. (1996) In-gel digestion of proteins for MALDI-TOF. <http://www.ccc.uab.edu/shared/massproto.htm>.
- Creelman R.A. & Mullet J.E. (1997a) Biosynthesis and action of jasmonates in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **48**, 355–381.
- De Vos C.H.R., Ten Bookum W.M., Vooijs R., Schat H. & De Kok L.J. (1993) Effect of copper on fatty acid composition and peroxidation of lipids in the roots of copper tolerant and sensitive *Silene cucubalus*. *Plant Physiology and Biochemistry* **31**, 151–158.
- Didierjean L., Frendo P., Nasser W., Genot G., Marivet J. & Burkard G. (1996) Heavy-metal-responsive genes in maize: identification and comparison of their expression upon various forms of abiotic stress. *Planta* **199**, 1–8.
- Eckey-Kaltenbach H., Kiefer E., Grosskopf E., Ernst D. & Sandermann H. Jr (1997) Differential transcript induction of parsley pathogenesis-related proteins and of a small heat shock protein by ozone and heat shock. *Plant Molecular Biology* **33**, 343–350.
- Ferreira F.D., Hoffmann-Sommergruber K., Breiteneder H., Pettenburger K., Ebner C., Sommergruber W., Steiner R., Böhle B., Sperr W.R., Valent P., Kungl A.J., Breitenbach M., Kraft D. & Scheiner O. (1993) Purification and characterization of recombinant Bet v I, the major birch pollen allergen. Immunological equivalence to natural Bet v I. *Journal of Biological Chemistry* **268**, 19574–19580.
- Flengsrud R. & Kobro G. (1989) A method for two-dimensional electrophoresis of proteins from green plant tissues. *Analytical Biochemistry* **177**, 33–36.
- Freedman J.F., Ciriolo M.R. & Peisach J. (1989) The role of glutathione in copper metabolism and toxicity. *Journal of Biological Chemistry* **264**, 5598–5605.
- Gora L. & Clijsters H. (1989) Effect of copper and zinc on the ethylene metabolism in *Phaseolus vulgaris* L. In *Biochemical and Physiological Aspects of Ethylene Production in Lower and Higher Plants* (eds H. Clijsters, M. De Proft, R. Marcelle & M. Van Poucke), pp. 219–228. Kluwer Academic Publishers, Dordrecht.
- Hochstein P., Kumar K.S. & Forman S.J. (1980) Lipid peroxidation and the cytotoxicity of copper. *Annals of the New York Academy of Sciences* **355**, 240–248.
- Kärenlampi S.O., Airaksinen K., Miettinen A.T.E., Kokko H., Holopainen J.K., Kärenlampi L.V. & Karjalainen R.O. (1994) Pathogenesis-related proteins in ozone-exposed Norway spruce [*Picea abies* (Karst) L.]. *New Phytologist* **126**, 81–89.
- Korhonen M.-S., Pellinen R., Kiiskinen M. & Kangasjärvi J.S. (1998) Cellular-molecular diagnostics of ozone injury in birch (*Betula pendula* Roth) In *Responses of Plant Metabolism to Air Pollution* (eds L. J. De Kok and I. Stulen), pp. 351–354. Backhuys Publishers, Leiden, the Netherlands.
- Linthorst H.J.M. (1991) Pathogenesis related proteins of plants. *Critical Reviews of Plant Science* **10**, 123–150.
- Lopez F., Vansuyt G., Fourcroy P. & Casse-Delbart F. (1994) Accumulation of a 22-kDa protein and its mRNA in the leaves of *Raphanus sativus* in response to salt stress or water deficit. *Physiologia Plantarum* **91**, 605–614.
- McBrien D.C.H. & Hassall K.A. (1965) Loss of potassium by *Chlorella vulgaris* after contact with toxic amounts of copper sulphate. *Physiologia Plantarum* **18**, 1059–1065.
- Moons A., Prinsen E., Bauw G. & Van Montagu M. (1997) Antagonistic effects of abscisic acid and jasmonates on salt stress-inducible transcripts in rice roots. *the Plant Cell* **9**, 2243–2259.
- Mylona P., Moerman M., Yang W.-C., Gloudemans T., Van De Kerckhove J., Van Kammen A., Bisseling T. & Franssen H.J. (1994) The root epidermis-specific pea gene RH2 is homologous to a pathogenesis-related gene. *Plant Molecular Biology* **26**, 39–50.
- O'Farrell P.H. (1975) High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* **250**, 4007–4021.
- Pääkkönen E., Seppänen S., Holopainen T., Kokko H., Kärenlampi S., Kärenlampi L. & Kangasjärvi J. (1998) Induction of genes for the stress proteins PR-10 and PAL in regulation to growth, visible injuries and stomatal conductance in birch (*Betula pendula*) clones exposed to ozone and/or drought. *the New Phytologist* **138**, 295–305.
- Renaudin J.-P., Tournaire C. & de Teyssendier la Serve B. (1991) Quantitative analysis of protein changes during meristem initiation and bud development in protoplast-derived *Petunia hybrida* callus. *Physiologia Plantarum* **82**, 48–56.
- Sandman G. & Böger P. (1980a) Copper deficiency and toxicity in *Scenedesmus*. *Zeitschrift für Pflanzenphysiologie* **98**, 53–59.
- Sandman G. & Böger P. (1980b) Copper-mediated lipid peroxidation processes in photosynthetic membranes. *Plant Physiology* **66**, 797–800.
- Shevchenko A., Jensen O.N., Podtelejnikov A.V., Sagliocco F., Wilm M., Vorm O., Mortensen P., Shevchenko A., Boucherie H. & Mann M. (1996) Linking genome and proteome by mass spectrometry: Large-scale identification of yeast proteins from two dimensional gels. *Proceedings of the National Academy of Sciences, USA* **93**, 14440–14445.
- Somssich I.E., Schmelzer E., Bollmann J. & Hahlbrock K. (1986) Rapid activation by fungal elicitor of genes encoding 'pathogenesis-related' proteins in cultured parsley cells. *Proceedings of the National Academy of Sciences, USA* **83**, 2427–2430.
- Steffens J.C. (1990) The heavy metal-binding peptides of plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **41**, 553–575.
- Swoboda I., Scheiner O., Heberle-Bors E. & Vicente O. (1995) cDNA cloning and characterization of three genes in the Bet v 1 gene family that encode pathogenesis-related proteins. *Plant, Cell and Environment* **18**, 865–874.
- Swoboda I., Scheiner O., Kraft D., Breitenbach M., Heberle-Bors E. & Vicente O. (1994) A birch gene family encoding pollen allergens and pathogenesis-related proteins. *Biochimica et Biophysica Acta* **1219**, 457–464.
- Urtiainen M.A., Kärenlampi L.V., Kärenlampi S.O. & Schat H. (1997) Differential tolerance to copper and zinc of micropropagated birches tested in hydroponics. *the New Phytologist* **137**, 543–549.
- Van Assche F. & Clijsters H. (1990) Effects of metals on enzyme activity in plants. *Plant, Cell and Environment* **13**, 195–206.
- Van Loon L.C., Pierpoint W.C., Boller T. & Conejero V. (1994) Recommendations for naming plant pathogenesis-related proteins. *Plant Molecular Biology Reporter* **12**, 245–264.
- Verkleij J.A.C. & Schat H. (1990) Mechanisms of metal tolerance in higher plants. In *Heavy Metal Tolerance in Plants: Evolutionary Aspects* (ed. A.J. Shaw), pp. 21–38. CRC Press, Boca Raton.

- Walter M.H., Liu J.-W., Grand C., Lamb C.J. & Hess D. (1990) Bean pathogenesis-related (PR) proteins deduced from elicitor-induced transcripts are member of a ubiquitous new class of conserved PR proteins including pollen allergens. *Molecular and General Genetics* **222**, 353–360.
- Warner S.A.J., Scott R. & Draper J. (1992) Characterisation of a wound-induced transcripts from the monocot asparagus that shares similarity with a class of intracellular pathogenesis-related (PR) proteins. *Plant Molecular Biology* **19**, 555–561.
- Weckx J., Vangronsveld J. & Clijsters H. (1993) Heavy metal induction of ethylene production and stress enzymes. I. Kinetics of the responses. In *Cellular and Molecular Aspects of the Plant Hormone Ethylene* (eds J.C. Pech, A. Latché & C. Balagué), pp. 238–239. Kluwer Academic Publishers, Dordrecht.
- Wilm M., Shevchenko A., Houthaeve T., Breit S., Schweigerer L., Fotsis T. & Mann M. (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**, 466–469.
- Yamamoto M., Torikai S. & Oeda K. (1997) A major root protein of carrots with high homology to intracellular pathogenesis-related (PR) proteins and pollen allergens. *Plant Cell Physiology* **38**, 1080–1086.
- Zurfluc L.L. & Guilfoyle T.J. (1980) Auxin-induced changes in the patterns of protein synthesis in soybean hypocotyl. *Proceedings of the National Academy of Sciences, USA* **77**, 357–361.

Received 11 December 1997; received in revised form 15 April 1998; accepted for publication 15 April 1998