Purification, Characterization, and Molecular Cloning of Basic PR-1-Type Pathogenesis-Related Proteins from Barley

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Partial amino acid sequences of two proteins, purified from barley leaves reacting hypersensitively to the powdery mildew fungus, showed a high degree of amino acid identity to the PR-1 proteins originally described in tobacco. The proteins, subsequently designated HvPR-1a and HvPR-1b, show apparent pI values of approximately 10.5 and 11, respectively and apparent M_r 15,000. Independently, differential screening of a cDNA library prepared from barley leaves, exhibiting a compatible interaction with the powdery mildew fungus, resulted in isolation of cDNA species representing two PR-1 homologs. With the exception of one amino acid, the partial amino acid sequences of HvPR-1a and HvPR-1b are identical to internal sequences of the polypeptides derived from the two cDNA species. These derived polypeptides are each 164 amino acids long and both have putative N-terminal leader sequences of 24 amino acids. That these proposed leader sequences are functional is indicated by the observed occurrence of both proteins in the intercellular fluid. The proposed mature proteins (calculated M_r 14,490 and 15,204) share 91% identical amino acids with each other and 56 to 74% with other PR-1 proteins. Northern blot hybridization and immunoblotting, respectively, show that both transcripts and both proteins accumulate following inoculation of susceptible and hypersensitively resistant barley leaves with the powdery mildew fungus.

Additional keywords: cDNA clones, defense-related proteins, disease resistance, *Erysiphe graminis*, *Hordeum vulgare*, plant defense.

The defense responses of plants are physiologically complex, involving, for example, structural alterations of the cell wall, the production of antimicrobial secondary metabolites, as well as soluble proteins known as pathogenesis-related (PR) proteins (Dixon and Harrison 1990; Collinge *et al.* 1994). PR proteins are host proteins that accumulate in plants following attack by different types of pathogens (van Loon 1985). In tobacco, where most research concerning PR proteins has been conducted, five classes have been identified; each, as a generalization, comprise acidic, extracellular proteins and basic, intracellular isoforms (Linthorst 1991; Ryals *et al.* 1992; Melchers *et al.* 1993). The biochemical function of the PR-1 group proteins is as yet unknown. There are, however, indications that these possess antifungal activity (Niderman *et al.* 1992; Alexander *et al.* 1993). The PR-1 proteins are soluble in acidic buffers, present in acidic and basic isoforms, and have low molecular weight, i.e., 14–16 kDa. In tobacco, genes encoding three acidic PR-1 proteins (PR-1a, PR-1b, and PR-1c) and two basic proteins (cluster G-protein and prb-1b) are expressed upon infection, e.g., with tobacco mosaic virus (Jamet and Fritig 1986; Payne *et al.* 1989; Eyal *et al.* 1992). DNA sequences representing these five tobacco PR-1s have been isolated by several groups (see Linthorst 1991; Ryals *et al.* 1992; Eyal *et al.* 1992). In tomato, two basic PR-1 proteins have been isolated and characterized (Lucas *et al.* 1985; Joosten *et al.* 1990) and two PR-1 cDNAs have been isolated, of which one corresponds to an isolated protein (van Kan *et al.* 1992). Two PR-1 DNA sequences have been isolated from *Arabidopsis thaliana* (Metzler *et al.* 1991; Uknes *et al.* 1992). Our knowledge of PR-1 proteins in monocots is, however, more sparse. Casacuberta *et al.* (1991) isolated cDNA and genomic clones representing a basic PR-1 protein of maize seeds, and Gillikin *et al.* (1991) purified an acidic PR-1 protein from the same species. By using antisera against tobacco PR-1a, White *et al.* (1987) detected cross-reacting proteins in several other species, including barley.

We have previously reported the appearance of PR proteins in barley infected with the powdery mildew fungus (Bryngelsson *et al.* 1988; Bryngelsson and Collinge 1992). An acidic thaumatin-like protein, designated Hv-1, which belongs to the PR-5 class, was subsequently identified among PR proteins purified from powdery mildew-inoculated barley leaves (Bryngelsson and Gréen 1989). Shotgun screening of cDNA libraries by differential and subtractive hybridization techniques has yielded a number of clones representing barley

transcripts that accumulate in response to attack by the powdery mildew fungus (Gregersen *et al.* 1993). These include cDNAs encoding a peroxidase cDNA (Thordal-Christensen *et al.* 1992), a 14-3-3 protein (Brandt *et al.* 1992), and a GRP94 (endoplasmin) cDNA (Walther-Larsen *et al.* 1993). Using a similar cloning strategy, Scott and co-workers isolated six barley cDNAs representing transcripts accumulating after powdery mildew inoculation (see Scott 1992), which included a β-1,3-glucanase sequence (Jutidamrongphan *et al.* 1991).

In this study, we present the purification and characterization of basic barley leaf proteins induced after inoculation with the powdery mildew fungus (*Erysiphe graminis* f. sp. *hordei*). Partial amino acid sequencing revealed that two of the proteins were homologous to the PR-1 protein family. Independently, differential screening of a cDNA library from powdery mildew-infected barley leaves revealed two transcripts that correspond to the purified PR-1 proteins. This study represents the first purification of basic PR proteins from monocot leaves, and the first report of accumulating leaf transcripts for PR-1 proteins from monocots.

RESULTS

Protein purification.

When acid-soluble proteins from hypersensitively reacting cultivar Alva leaves are compared to proteins from control plants by isoelectric focusing, a number of accumulating basic and acidic proteins are visible (Bryngelsson *et al.* 1988). Purification of two of the accumulated basic proteins, Hv-4 and Hv-8, was initiated by extraction in an acidic buffer, ammonium sulphate precipitation and Sephadex G25 chromatography. Subsequently, all basic proteins were collected as the run-through of a weak anion exchanger (DEAE Sephacel). These basic proteins were applied to a cation exchange resin (CM Sephadex) and eluted with a NaCl gradient: Hv-4 was eluted at 0.015 M NaCl and Hv-8 at 0.03 M NaCl. The proteins were finally freed from contaminating polypeptides on a strong cation exchanger, mono S, where Hv-4 and Hv-8 were eluted as single peaks at roughly 0.013 and 0.03 M NaCl, respectively. The size was estimated by SDS-PAGE to be approximately 16 kDa for both proteins and the apparent pI values approximately 10.5 for Hv-4 and 11 for Hv-8, as extrapolated from isoelectric focusing in pH 8.0–10.5 gels (Fig. 1).

Amino acid sequencing.

No amino acid derivatives were released by Edman degradation from the N-terminus of either Hv-4 or Hv-8 by direct sequencing. After cleavage of Hv-4 with endoproteinase Lys-C (which cleaves peptide bonds C-terminal to lysine), eight fragments were detected of which three—6, 13, and 17 amino acids long—were sequenced. Hv-8 was also cleaved with Lys-C, and sequences of 12, 17, and 20 amino acids were
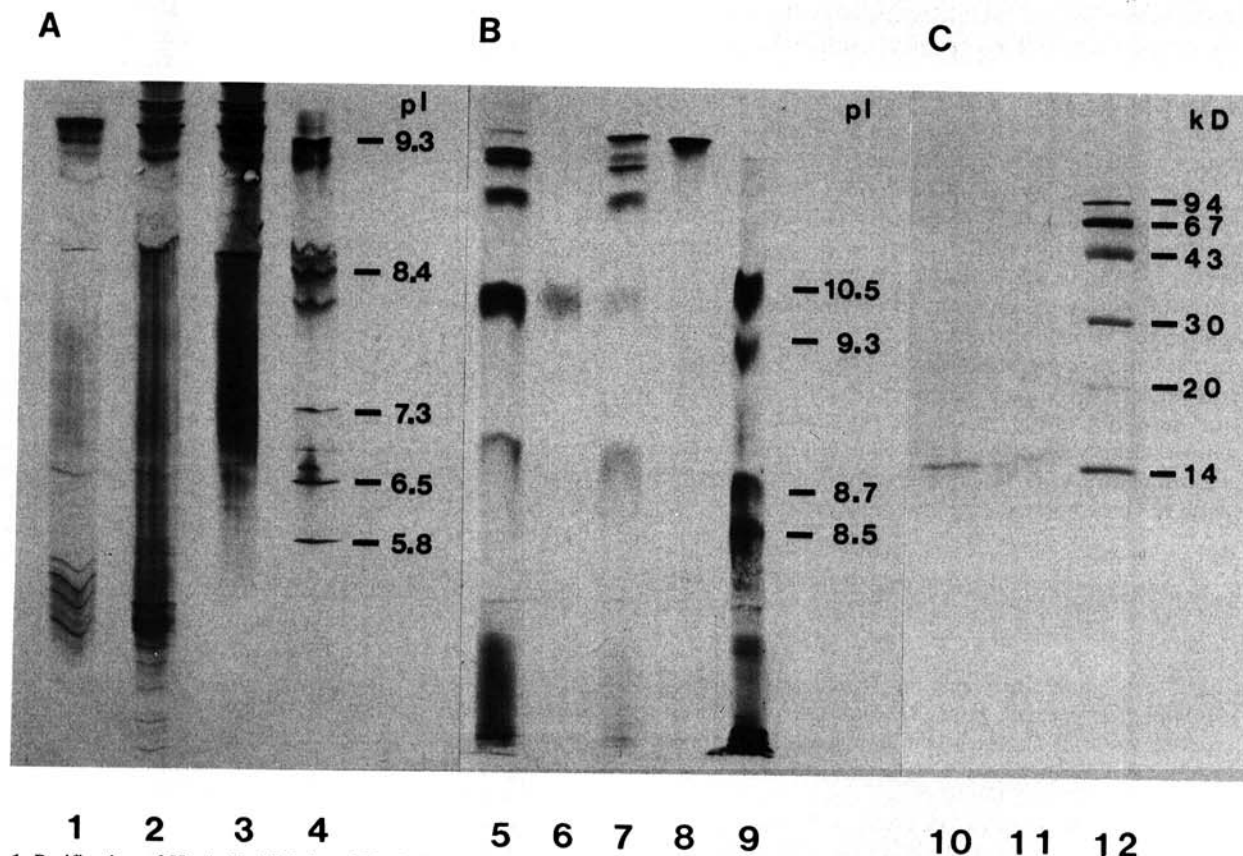

Fig. 1. Purification of Hv-4 (HvPR-1a) and Hv-8 (HvPR-1b) followed by isoelectric focusing in **A,** pH 3–9 and, **B,** pH 8–10.5 gels. Lane 1 shows the homogenate from noninoculated tissue, lane 2, from hypersensitively reacting barley leaves 6 days after inoculation with the powdery mildew fungus, lane 3, the run-through from a DEAE-Sephacel column, and, lane 4, pH marker. Basic proteins from lane 3 were fractionated on CM Sephadex and the fraction containing Hv-4 (lane 5) was purified on a mono S column in MES buffer pH 5.5 (lane 6). The fraction containing Hv-8 (lane 7) was purified on a mono S column equilibrated with NaAc, pH 5.0 (lane 8). A pH marker is shown in lane 9. The apparent molecular weights of Hv-4 and Hv-8 were determined by SDS-polyacrylamide gel electrophoresis **C,** Hv-4 (lane 10), Hv-8 (lane 11), and molecular weight markers (lane 12).

pHvPR-1a ------------------------------AGCTACGGCCTACACATACGGAGGCACAAAGAAATCAT 38
pHvPR-1b CGACAGCAACAGAATCCGTTGCTGTCGAGCT--GG--TATAGA---GCAGGCCCATAGAAATCAT 58

pHvPR-1a TTACACACCAAACCCAGAATGGAGACGCCCAAGCTGGCCATTTTGCTCGCCCTAGCCATGGCAGCC 104
                      M   E   T   P   K   L   A   I   L   L   A   L   A   M   A   A 16
                      M   Q   T   P   K   L   V   I   L   L   A   L   A   M   S   A 16
pHvPR-1b TTATACACCGAACCGAGAATGCAGACGCCCAAGCTAGTCATCTTGCTCGCCCTAGCCATGTCAGCC 124

pHvPR-1a GCCATGGTTAATCTTTCCCAAGCGCAAAACTCGCCTCAGGACTACCTTTCACCCCACAACGCCGCC 170
            A   M   V   N   L   S   Q   A ↑ Q   N   S   P   Q   D   Y   L   S   P   H   N   A   A 38
            A   M   V   N   L   S   Q   A ↓ Q   N   S   P   Q   D   Y   V   S   P   H   N   A   A 38
pHvPR-1b GCCATGGTTAACCTTTCCCAGGCGCAGAACTCGCCGCAGGACTACGTATCACCCCACAACGCTGCC 190

pHvPR-1a CGCGCTGCCGTCGGCGTGGGCGCAGTGAGCTGGAGCACGAAGCTGCAGGCGTACGCCCAGAGCTAC 236
            R   A   A   V   G   V   G   A   V   S   W   S   T   K   L   Q   A   Y   A   Q   S   Y 60
HvPR-1a   L   Q   A   Y   A   Q   S   Y
HvPR-1b   L   Q   A   F   A   Q   N   Y
            R   A   A   V   G   V   G   A   V   S   W   S   T   K   L   Q   A   F   A   Q   N   Y 60
pHvPR-1b CGCGCCGCCGTCGGCGTGGGCGCCGTGAGCTGGAGCACGAAGCTGCAGGCGTTCGCCCAGAACTAC 256

pHvPR-1a GCCAACCAGAGGATCGGCGACTGCAAGCTCCAGCACTCCGGCGGGGCCCTACGGGGAGAACATCTTC 302
            A   N   Q   R   I   G   D   C   K   L   Q   H   S   G   G   P   Y   G   E   N   I   F 82
HvPR-1a   A   N   Q   R   I   G   D   C   K
HvPR-1b   A   N   Q   R   I   N   D   C   K   L   Q   H   S   G   G   P   Y   G   E   N   I   F
            A   N   Q   R   I   N   D   C   K   L   Q   H   S   G   G   P   Y   G   E   N   I   F 82
pHvPR-1b GCCAACCAGAGGATCAACGACTGCAAGCTCCAGCACTCGGGCGGGGCCGTACGGGGAGAACATCTTC 322

pHvPR-1a TGGGGGTCGGCCGGCGCGGACTGGAAGGCGGCGGACGCGGTGAAGCTGTGGGTGGACGAGAAGAAG 368
            W   G   S   A   G   A   D   W   K   A   A   D   A   V   K   L   W   V   D   E   K   K 104
HvPR-1a   L   W   V   D   E   K
HvPR-1b   W   G   S   A   G   A   D   A   S   D   A   V   N   S   C   V   S   E   K
            W   G   S   A   G   A   D   W   K   A   S   D   A   V   N   S   W   V   S   E   K   K 104
pHvPR-1b TGGGGGTCGGCTGGCGCGGACTGGAAGGCATCGGACGCGGTGAACTCGTGGGTGAGCGAGAAGAAG 388

pHvPR-1a GACTACGACTACGGGTCCAACACCTGTGCAGGGGGGAAGGTGTGCGGGCACTACACGCAGGTGGTG 434
            D   Y   D   Y   G   S   N   T   C   A   G   G   K   V   C   G   H   Y   T   Q   V   V 126
HvPR-1a   D   Y   D   Y   G   S   N   T   C   A   G   G   K
HvPR-1b
            D   Y   D   Y   G   S   N   T   C   A   A   G   K   V   C   G   H   Y   T   Q   V   V 126
pHvPR-1b GACTACGACTACGGGCTCCAACACCTGCGCCGGCGGGGAAGGTGTGCGGGCACTACACTCAGGTGGTG 454

                         Sal I
pHvPR-1a TGGCGCGCGTCGACCAGCATCGGCTGCGCTCGCGTCGTCTGCAACAACAACGGCGGCGTCTTCATC 500
            W   R   A   S   T   S   I   G   C   A   R   V   V   C   N   N   N   G   G   V   F   I 148
            W   R   A   S   T   S   I   G   C   A   R   V   V   C   N   N   N   R   G   V   F   I 148
pHvPR-1b TGGCGCGCGTCGACCAGCATCGGCTGCGCTCGTGTAGTCTGCAACAACAACCGCGGCGTCTTCATC 520

pHvPR-1a ACCTGCAACTACGAGCCCGCCGGGAATGTTGTTGGACAGAAACCATACTAAACTGTGAT-TGC-TT 564
            T   C   N   Y   E   P   A   G   N   V   V   G   Q   K   P   Y   * 164
            T   C   N   Y   E   P   R   G   N   I   V   G   Q   K   P   Y   * 164
pHvPR-1b ACCTGCAACTACGAGCCCCGCGGGAACATCGTTGGACAGAAACCATACTAA-CCGTGATGCACGTC 585

pHvPR-1a TTCGCAGTACATATA--CATA-GTCCAG-TAGTCCCTCGTTCCTCACGTGCATGGGATTCATGCAC 626
pHvPR-1b TTCAGAGTACATACATGCATGCATACATATAGTCTCTCGTTCCT-A-----AT----CTCATGCAC 641

pHvPR-1a GCTCGTATCTATAATTAGCTAGAGGGGCTGGGCGAGTAAATAAAAGGAATGCTGTAATAAGCTATG 692
pHvPR-1b CTTTGTATTCCTAACTAGCTAGAGGGACCGAGCGAGTAAATAAAAGGAATGCCGTAATAAGCCATG 707

                                        polyadenyl.
pHvPR-1a TATTCTCCTCTCCATCATGGACAGTAATAAATAATGAGCTTT----------------- 734
pHvPR-1b TATTC-------CATCCTGGACTATAATAAATAATGAGCTCTTATATGAAAAAAAAAAA 759

Fig. 2. Translation of pHvPR-1a and pHvPR-1b, and comparison to amino acid sequences of HvPR-1a (Hv-4) and HvPR-1b (Hv-8) shown in bold. Nucleotides and amino acids that are identical in the two cDNA clones are shaded. The first amino acid of the predicted mature protein is indicated by an arrow.

obtained from three fragments (Fig. 2). Comparison with known protein and nucleic acid sequences revealed high homologies to the PR-1 family of pathogenesis-related proteins in different species (56-74%) and the two proteins, Hv-4 and Hv-8, were subsequently renamed HvPR-1a and HvPR-1b, respectively.

Screening of cDNA library for clones representing pathogen-induced genes.

In an independent study, approximately 6,000 cDNA clones of a λZAPII library were differentially screened using two first-strand cDNA probes, prepared from polyA⁺-RNA extracted from susceptible P02 leaves 72 hr after inoculation with the powdery mildew fungus and from noninoculated P02 control plants, respectively. One hundred and two clones were selected after two consecutive screenings. On a random basis, about nine out of 10 clones were confirmed to represent transcripts accumulating following inoculation, by hybridization to Northern blots of P02 barley RNA extracted at different time points after inoculation.

One of these clones hybridized to a transcript of approximately 0.8 kb, and preliminary sequence data showed a high sequence identity to the PR-1 gene family. This clone cross-hybridized to a further 50 of the clones selected after the initial differential screen.

Nucleotide sequences of PR-1 cDNAs.

Preliminary sequencing of 11 apparent full-length clones selected among the 51 putative PR-1 cDNAs indicated that these represent two different PR-1 genes: Four represent gene A and seven represent gene B. The longest representatives of each class were sequenced in both directions and designated pHvPR-1a and pHvPR-1b, respectively.

The nucleotide sequence of pHvPR-1a is 734 bp long, and contains one major open reading frame (positions 57–548) encoding a polypeptide of 164 amino acids (Fig. 2). No poly dA·dT tract is present in this clone. pHvPR-1b is 748 bp long,

and contains one major open reading frame (positions 77–568), also encoding a polypeptide of 164 amino acids (Fig. 2). This clone has a poly dA·dT tract extending beyond position 748. The two cDNA clones share 85% identical nucleotides. A putative polyadenylation signal (AAUAAA) is present in both clones in a conserved region at position 718 in pHvPR-1a and position 726 in pHvPR-1b.

Amino acid sequences of barley PR-1 proteins.

The polypeptides derived from pHvPR-1a and pHvPR-1b share 91% identical amino acids (Fig. 2). The sequences obtained from the purified proteins, in total 36 amino acids from pHvPR-1a and 49 from pHvPR-1b, are, with the exception of one amino acid, identical to internal sequences of the polypeptides derived from pHvPR-1a and pHvPR-1b, respectively. In addition, the lysine residues, predicted to occur proximally to each endoproteinase fragment, are indeed present in the translated sequences. Together, these endoproteinase fragments and the preceding lysine residues represent 65 amino acids (46% of mature protein), of which eight diverge between the two derived polypeptides (Fig. 2). This leads us to infer that the two cDNA clones indeed correspond to the two proteins isolated. A single base substitution in the HvPR-1b gene in the barley lines Alva and P02 can explain the amino acid difference at residue 99, i.e., cysteine versus tryptophan.

N-terminally, the two derived amino acid sequences apparently possess typical hydrophobic signal peptides for translocation into the endoplasmic reticulum. No N-terminal amino acid sequence data are available for the mature proteins, but computer-aided estimation of the cleavage site suggests a signal peptide of 24 amino acids for both proteins. The predicted N-terminal of the mature proteins is in agreement with the data of other PR-1 proteins where N-terminal amino acid sequence data is available from the mature proteins (Figs. 2 and 3), i.e., acidic maize PR-1 (Gillikin et al. 1991), basic PR-1 (p14) of tomato (Lucas et al. 1985), and PR-1a of tobacco (Payne et al. 1988) (Fig. 3). Thus, the calculated molecular

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pHvPR-1a        METPKLA I LLALAMAAAMVNLSQAQNSPQDYLSPHNAARAAVGVGAVSWSTKLQAYA  33
pHvPR-1b        MQTPKLV I LLALAMSAAMVNLSQAQNSPQDYVSPHNAARAAVGVGAVSWSTKLQAFA  33
Maize, MPR-1                          QNSPQDYVDPHNAARADVGVGPVSWDDTVAAYA  33
Tobacco, basic      MGFLTTI VACF ITFAI LIHSSKAQNSPQDYLNPHNAARRQVGVGPMTWDNRLAAYA  33
Tobacco, PR-1a MGFVLFSQLPSFLLVSTLLLFLVI SHSCRAQNSQQDYLDAHNTARADVGVEPLTWDDQVAAYA  33
Tomato, P6/p14      MGLFNI SLLLTCLMVLAI F-HSCEAQNSPQDYLAVHNDARAQVGVGPMSWDANLASRA  33

pHvPR-1a        QSYANQRI GDCKLQHSGGPYGENI FWGSAGADWKAADAVKLWVDEKKDYDYGSNTCAGGKVCG  96
pHvPR-1b        QNYANQRI NDCKLQHSGGPYGENI FWGSAGADWKASDAVNSWVSEKKDYDYGSNTCAAGKVCG  96
Maize, MPR-1    QSYAAQRQGDCKLI HSGGPYGENLFWGSAGADWSASDAVGSWVSEKQYYDHDTNSCAEGQVCG  96
Tobacco, basic  QNYANQRI GDCGMI HSHGPYGENLAAAFPQL--NAAGAVKMWVDEKRFYDYNSNSCVGG-VCG  93
Tobacco, PR-1a  QNYASQLAADCNLVHSHGQYGENLAEGSGDF-MTAAKAVEMWVDEKQYYDHDSNTCAQGQVCG  95
Tomato, P6/p14  QNYANSRAGDCNLI HSGA--GENLAKGGGDF--TGRAAVQLWVSERPSYNYATNQCVGGKKCR  92

pHvPR-1a        HYTQVVWRASTSI GCARVVCNNNGGVFI TCNYEPAGNVVGQKPY                    140
pHvPR-1b        HYTQVVWRASTSI GCARVVCNNNRGVFI TCNYEPRGNI VGQKPY                    140
Maize, MPR-1    HYTQVVWRDSTAI GCARVVCDNNAGVFI ICSYNPPGNVVGESPY                    140
Tobacco, basic  HYTQVVWRNSVRLGCARVRS-NNGWFFI TCNYDPPGNFI GGQRPFGDLEEQPFDSKLELPTDV 154
Tobacco, PR-1a  HYTQVVWRNSVRVGCARVQC-NNGGYVVSCNYDPPGNYRGESPY                    138
Tomato, P6/p14  HYTQVVWRNSVRLGCGRARC-NNGWWFI SCNYDPVGNWI GGQRPY                   135
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Fig. 3. Alignment of pHvPR-1a and pHvPR-1b derived polypeptides to the amino acid sequence of MPR-1 from maize (Gillikin et al. 1991), the derived polypeptide sequence of basic PR-1 (cluster G-protein) from tobacco (Payne et al. 1989), the amino acid and derived polypeptide sequence of the acidic PR-1a from tobacco (Payne et al. 1988), and the amino acid and derived polypeptide sequence of the basic p14 and P6 of tomato (Lucas et al. 1985; van Kan et al. 1992). Amino acids identical to the mature polypeptide of pHvPR-1a are shaded. The first amino acid of the mature protein is underlined where N-terminal amino acid sequences are available. Numbering starts at the first amino acid in the mature proteins.

masses of unprocessed and mature protein are 17,444 and 14,990 Da, respectively, for the pHvPR-1a derived sequence and for the pHvPR-1b sequence, these values are 17,703 and 15,204 Da. Furthermore, no C-terminal extension, as observed on the two basic PR-1s from tobacco, is present in the derived sequences.

Comparison of the derived amino acid sequences to various other PR-1 proteins is presented in Figure 3. In the calculations of identities to the pHvPR-1a derived sequence, the putative signal peptides are disregarded. The highest amino acid identity, approximately 74%, is found to the acidic maize sequence, while there is 67% identity to the basic tobacco PR-1 sequence, 60% to PR-1a and 59% to P6/p14 from tomato.

Induction by the powdery mildew fungus.

The accumulation of HvPR-1a and HvPR-1b transcripts and proteins was followed by Northern and IEF-immunoblotting in a time course study over 6 days following inoculation of resistant Alva plants and susceptible P02 plants.

Although use of the 3' ends of the cDNAs (distal to the SalI site; see Fig. 2) as probes on Southern blots can discriminate the cDNA sequences from each other to a high degree, it is not possible to unequivocally distinguish between the two transcripts on Northern blots after washing at high stringency (see Fig. 4). Thus, no differences in the accumulation patterns of the two transcripts have been observed (data not shown). The transcripts accumulate in hypersensitively reacting plants as well as in susceptible plants 3 days after inoculation, and more heavily 6 days after inoculation. After 6 days, there is considerably more transcript present in susceptible P02 plants than in resistant Alva plants. These results have been confirmed in several independent time course experiments. We have also demonstrated accumulation of HvPR-1 transcripts in barley leaves inoculated with several other fungal pathogens, and following treatment with UV light, methyl jasmonate, and salicylate, but not following wounding by crushing the leaves (data not shown).

As HvPR-1b has a higher pI value than HvPR-1a, immunoblotting was performed on basic IEF gels in order to distinguish between the proteins in crude extracts. In Figure 5, HvPR-1a and HvPR-1b antisera were applied on crude protein extracts from the same time course material used for extraction of the RNA presented in Figure 4. Antiserum produced against the two proteins had slightly different affinities. The antiserum against HvPR-1a reacts approximately to the same
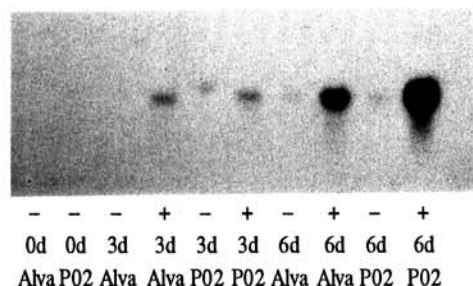
extent to HvPR-1b and accumulated proteins are clearly visible 3 days after inoculation in both reaction types, and the level increases throughout the experiment. The antiserum against HvPR-1b, on the other hand, has comparatively low affinity for HvPR-1a but cross-reacts clearly with a third highly basic protein with a pI value close to that of HvPR-1b. This indicates either the presence of at least one more basic isoform of barley PR-1 proteins or a proportion of the protein population exhibiting a different conformation or posttranslational modification.

Protein localization in leaf tissue.

The presence of a hydrophobic putative leader sequence and absence of a C-terminal extension for organelle targeting indicates export to the intercellular space. To test this prediction, Alva plants (6 days after inoculation) were vacuum infiltrated with buffer and the intercellular fluid was collected. After a second "wash" of the intercellular space, the remain-
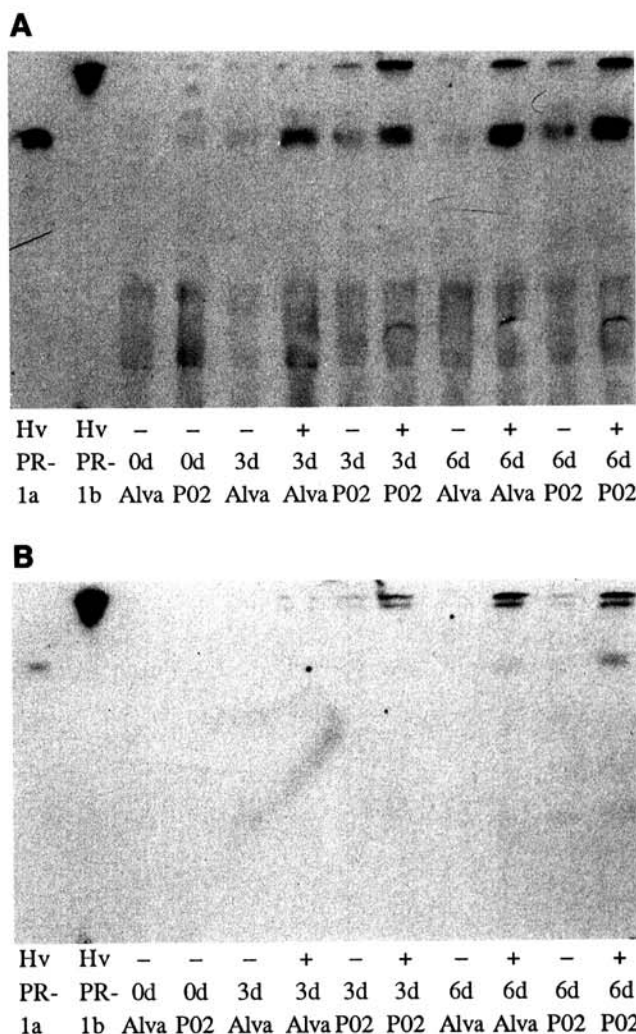
Hv	Hv	−	−	−	+	−	+	−	+	−	+
PR-	PR-	0d	0d	3d	3d	3d	3d	6d	6d	6d	6d
1a	1b	Alva	P02	Alva	Alva	P02	P02	Alva	Alva	P02	P02

Hv	Hv	−	−	−	+	−	+	−	+	−	+
PR-	PR-	0d	0d	3d	3d	3d	3d	6d	6d	6d	6d
1a	1b	Alva	P02	Alva	Alva	P02	P02	Alva	Alva	P02	P02

Fig. 5. Accumulation of HvPR-1a and HvPR-1b after inoculation of cultivars Alva (hypersensitive reaction) and P02 (susceptible) with the powdery mildew fungus (+) and noninoculated controls (−). Acid-soluble proteins (3 μg) extracted at different time points after inoculation were separated on pH 8–10.5 IEF gels and transferred to nitrocellulose membranes. PR-1-type proteins were identified with antiserum against **A,** HvPR-1a and, **B,** HvPR-1b.

−	−	−	+	−	+	−	+	−	+
0d	0d	3d	3d	3d	3d	6d	6d	6d	6d
Alva	P02	Alva	Alva	P02	P02	Alva	Alva	P02	P02

Fig. 4. Accumulation of the transcript for HvPR-1a after inoculation of cultivars Alva (hypersensitive reaction) and P02 (susceptible) with the powdery mildew fungus (+) and noninoculated controls (−) as shown on northern blots with 15 μg total RNA per lane. Wash stringency: 0.1× SSC at 70° C.

ing proteins in these leaves were extracted in the acidic buffer. Immunoblotting on basic IEF gels shows that most of the HvPR-1 proteins, including the putative third species, can be collected in the intercellular fluid, based on comparison with the amount of PR-1 proteins in nonwashed plants (Fig. 6).

Genomic organization.

Genomic DNA of the two PR-1 genes can be distinguished by band intensity on Southern blots after high-stringency wash (Fig. 7A). Although none of the restriction enzymes used for the genomic DNA recognize sites in the cDNAs, hybridization results in two bands of equal intensity when using HindIII, XbaI, and EcoRV. This indicates either presence of an intron approximately in the middle of each of the processed transcripts or that the genes represented by pHvPR-1a

A

B
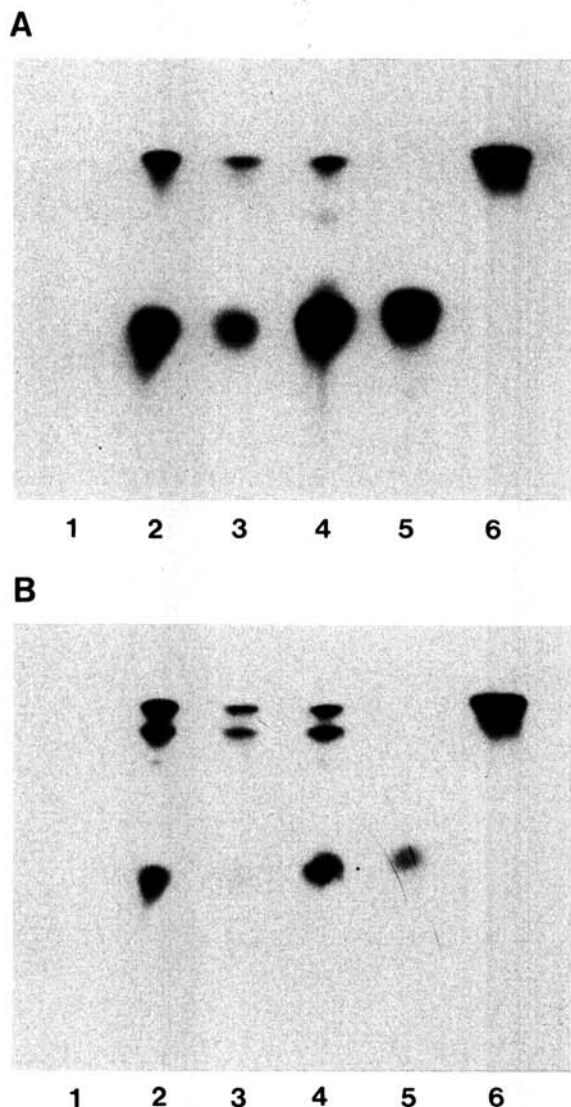
Fig. 6. Localization of basic PR-1 proteins in barley leaves demonstrated by immunoblotting on pH 8-10.5 isoelectric focusing gels using antiserum against **A,** HvPR-1a and, **B,** HvPR-1b. Lane 1 shows the homogenate from noninoculated tissue, lane 2, from hypersensitively reacting barley leaves 6 days after inoculation with the powdery mildew fungus, lane 3, barley leaves devoid of intercellular fluid, lane 4, intercellular fluid, lane 5, pure HvPR-1a, and lane 6, pure HvPR-1b.
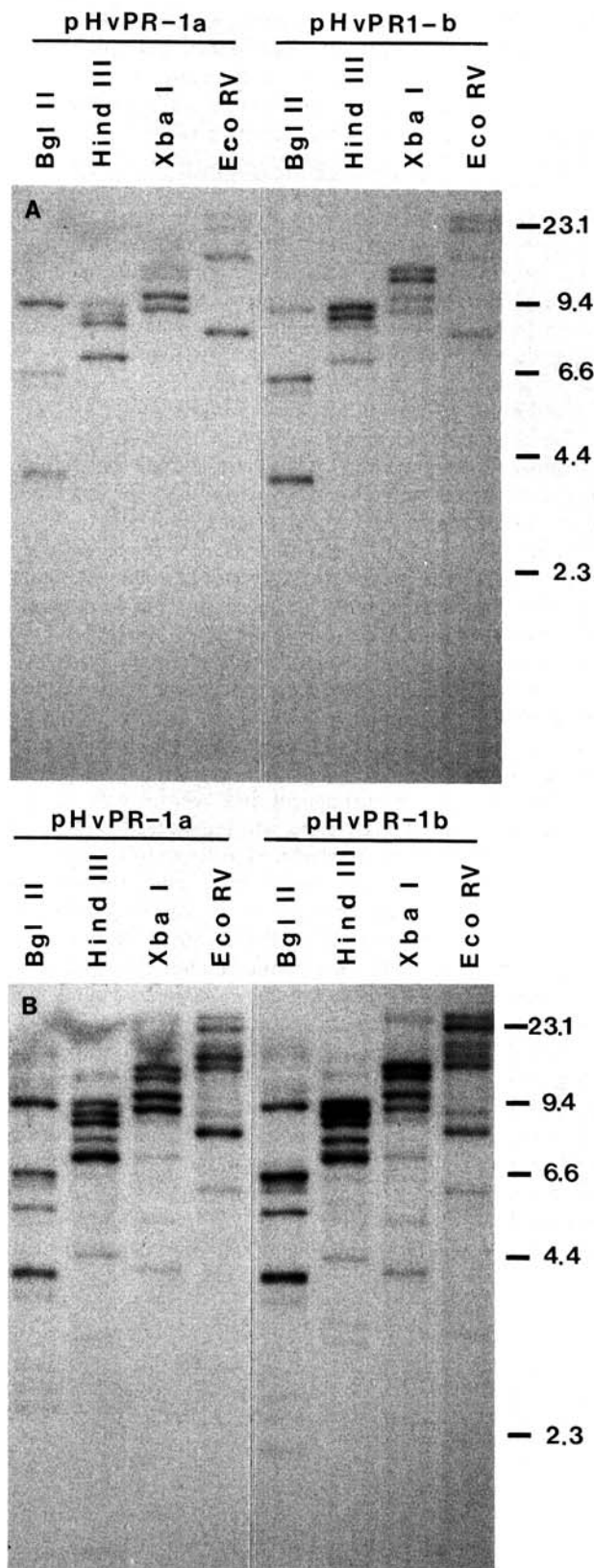
Fig. 7. Southern blot of genomic barley (P01, isogenic line of Pallas containing the *Ml-a* gene) DNA, digested with different restriction enzymes and hybridized with the insert of pHvPR-1a and pHvPR-1b. **A,** After high stringency wash (0.1× SSC at 68° C). **B,** After medium stringency wash (1× SSC at 65° C).

and pHvPR-1b are each present in two copies. However, when the 3' ends of the cDNAs are used (distal to the *SalI* site; see Fig. 2) as probes on the genomic Southerns, the same two bands hybridize for each of the two clones (data not shown). This demonstrates that at least two gene copies, located on not completely identical DNA segments, must be present for both HvPR-1a and HvPR-1b.

After medium-stringency wash, 8–10 bands are visible, and the three to four bands that are present after high-stringency wash are generally the most predominant with both probes (Fig. 7B). This indicates the presence of at least four PR-1 genes in the barley genome, and that the genes represented by pHvPR-1a and pHvPR-1b are more closely related to each other than to any of the other hypothetical PR-1 genes.

DISCUSSION

This paper presents the first detailed characterization of barley homologs of the PR-1 family of pathogenesis-related proteins, originally described in tobacco. Leaf accumulation of two transcripts and each of the encoded proteins have been demonstrated in response to attack by the powdery mildew fungus. A similar type of induction of a PR-1 gene has been shown to occur in germinating maize seedlings after inoculation with the fungal pathogen *Fusarium moniliforme* (Casacuberta *et al.* 1991).

From the sequence and hybridization data, it appears that the two genes are very closely related: The cDNAs exhibit 85% identical nucleotides, no difference in transcript accumulation pattern has been observed, and both sequences hybridize more strongly to each other than to any other sequence in the barley genome. The predicted mature proteins differ in only 12 of their 140 amino acids, and in particular, it is striking that only three out of 24 amino acids differ in the putative signal peptide. Nevertheless, a clear difference is observed on IEF gels, where the apparent pI values are 10.5 and 11. The substitutions D→S (acidic to polar, position 77 in Fig. 3) and G→R (neutral to basic, position 120) apparently account for the observed difference in pI.

One of the results provided by the genomic Southern (Fig. 7B) is the strong indication of further PR-1 genes in addition to the two identified. Although protein purification and cDNA cloning were performed independently, the proteins and cDNAs obtained nevertheless originate from the same two genes, indicating that these two PR-1 genes are induced more strongly in response to powdery mildew attack than other PR-1 genes. The fact that amino acid no. 99 of HvPR-1b is tryptophan in P02 (and all other published PR-1s) and cysteine in cv. Alva, is most likely due to cultivar variation rather than, for example, cloning artefacts, reflected in a single base pair substitution, and does not lead to the conclusion that a third gene is in question. An alternate explanation, suggested by the genomic Southern in Figure 7A, is that the HvPR-1b gene, as well as the HvPR-1a gene, is present in at least two copies. This, in turn, may relate to the third band on the immunoblot of crude extracts (Figs. 5 and 6). Although it cannot be ruled out that other basic PR-1 proteins exist in barley, particularly in view of the recognition of the two IEF bands at ~pI 11 by the antiserum raised against HvPR-1b, a search for additional PR-1s should perhaps be made among low pI proteins.

Presence of the two PR-1 proteins in the intercellular fluid confirms the inference, based on the presence of putative leader sequences and lack of C-terminal extensions, that the proteins are exported. The model, based on results from tobacco (see, e.g., Melchers *et al.* 1993), implying that basic PR proteins have vacuolar localization, seems to hold neither for basic PR-1 protein from other species, nor, as demonstrated here, for basic barley PR-1s.

MATERIALS AND METHODS

Plant material and inoculation.

Hordeum vulgare L. 'Alva' (Svalöf-Weibull, Svalöv, Sweden) and an isogenic line, P02, of cv. Pallas (Kølster *et al.* 1986) were grown in a growth chamber at a 16:8 hr light/dark regime (420 µmol s^{-1} m^{-2}, 60% RH, 18° C during the day, 12° C during night) for 8 days. Plants were inoculated with the powdery mildew fungus (*Erysiphe graminis* DC. ex Mérat f. sp. *hordei* Em Marchal; syn. *Brumeria graminis* (DC.) Speer f. sp. *hordei* Em Marchal) isolate C15 (Kølster *et al.* 1986) by shaking conidia of heavily infected plants over the material. Alva (*Ml-La*) is resistant to C15, reacting with a hypersensitive response, whereas P02 is susceptible. At defined time points after inoculation, leaves were harvested and immediately frozen in liquid nitrogen prior to storage at –70° C.

Protein purification and preparation of antibodies.

Plant material, harvested 8 days after inoculation, was extracted with citrate buffer, pH 2.8, and the fraction precipitated with 30–90% ammonium sulphate was passed over Sephadex G25 after resuspension in 1 mM Tris-HCl, pH 6.8, as described in Bryngelsson and Greén (1989). The protein fraction was loaded onto a DEAE-Sephacel column equilibrated with 1 mM Tris-HCl, pH 6.8. The run-through was concentrated by ultra filtration and passed over CM Sephadex equilibrated in 25 mM morpholinoethanesulfonic acid (MES), pH 5.5. The column was eluted with a linear 0–0.5 M NaCl gradient and fractions containing two basic proteins, designated Hv-4 and Hv-8, were pooled and concentrated. The protein solution was filtered through a 0.22 µm filter and injected onto a mono S HR 5/5 cation exchange column (Pharmacia, Sweden). Elution was carried out under the control of the programmer of an fast protein liquid chromatography system applying a 0–0.25 M NaCl linear gradient in 5 mM MES, pH 5.5 for Hv-4 and in 50 mM NaAc, pH 5.0, for Hv-8.

Crude extracts for the time course study were obtained by extracting leaves with citrate buffer, pH 2.8, centrifugation to remove cell debris and dialysis against 0.01 M Tris-HCl, pH 6.8. Intercellular fluid from barley leaves were obtained using the method of Kragh *et al.* (1990).

For the preparation of antibodies, purified Hv-4 and Hv-8 proteins (0.15 mg in 0.01 M sodium phosphate buffer, pH 7.5) were each mixed with Freund's complete adjuvant and injected intradermally into two rabbits. A second injection using the same amount of protein in incomplete adjuvant was injected a month later. The rabbits were subsequently bled every second week, and the serum was clarified by centrifugation (10,000 *g* for 10 min). The antiserum was partially purified by precipitation with ammonium sulphate (33%) and dialysis against 0.01 M Tris, pH 6.8.

Protein electrophoresis and immunoblotting.

Electrophoretic analyses were performed on 15% SDS-polyacrylamide gels essentially using the system of Laemmli (1970) and Coomassie brilliant blue staining. Isoelectric focusing (IEF) in pH 3–10 gels was performed as described previously (Bryngelsson and Greén 1989). Basic isoelectric focusing gels were produced by prerunning 5% polyacrylamide slabs, 0.5 mm thick, containing Pharmalyte pH 8–10.5. Samples were focused at 25 W constant power for 1 hr at 10° C and stained with silver (Heukeshoven and Dernick, 1985). Protein molecular weight markers and IEF markers were obtained from Pharmacia.

Gels were transferred in 0.025 M Tris, pH 8.3, 0.192 M glycine, 20% methanol onto nitrocellulose filters (Hybond-ECL, Amersham, UK) for 1 hr in a mini trans-blot electrophoretic transfer cell (Bio-Rad, Richmond, CA) at 100 V. Filters were blocked for 1 hr in 0.02 M Tris, pH 7.6, 0.137 M NaCl, 0.1% Tween 20 (TBST) containing 5% skimmed milk powder and then incubated for 1 hr with diluted antiserum (1:2,000). Filters were washed three times in TBST and incubated for 1 hr in horseradish peroxidase-linked anti-rabbit antibodies from donkey (Amersham) diluted 1:2,000 in TBST. Detection was performed using the ECL system (enhanced chemiluminescence) as recommended by the supplier (Amersham).

Chemical amino acid sequencing.

The purified protein was desalted by reverse-phase HPLC and lyophilized. The material was taken up in 6 M guanidine-HCl, incubated at 56° C for 15 min, and diluted to 2 M guanidine-HCl, 0.1 M Tris, pH 8.5. Endoproteinase Lys-C from *Acrymobacter lyteii* (WAKO, Japan) was added and incubated overnight at 37° C. Sulfhydryl groups were modified by the addition of 0.1% (v/v) 2-mercaptoethanol and incubation at 37° C for 30 min followed by the addition of 4-vinylpyridine (0.3% v/v) and incubation at room temperature for 60 min. Peptides were isolated by reverse-phase HPLC on a C4 Aquapore column (Brownlee 2.1 × 30 mm with Beckman gold 126 pumps, 100 µl/min) and eluted with a linear gradient of 0–60% acetonitrile in 0.1% TFA over 60 min. Sequencing was performed using a 470A Applied Biosystem gas phase sequencer equipped with an automatic analyzer for amino acid phenylthiohydantoin derivatives.

RNA and DNA purification and preparation of cDNA library.

Total RNA was obtained from frozen leaf material essentially according to Collinge et al. (1987). Poly A⁺-RNA was isolated on oligo-dT-cellulose according to Martin and Northcote (1981). Barley genomic DNA was isolated according to Ausubel et al. (1987).

A cDNA library was constructed in λZAPII (Stratagene, La Jolla, CA) using poly A⁺-RNA isolated from P02 leaves 72 hr after inoculation with *E. g.* f. sp. *hordei* as described for "library 2," in Thordal-Christensen et al. (1992). Isolated clones were converted to pBluescript SK(−) using the *in vivo* excision procedure recommended by the manufacturer (Stratagene).

Blotting and hybridization techniques.

Recombinant bacteriophage were transferred to nitrocellulose membranes (Millipore, MA). Southern blots of cloned DNA and northern blots following formaldehyde electrophoresis were prepared on Zeta-Probe nylon membranes (Bio-Rad). Genomic Southern blots were prepared on Hybond N⁺ (Amersham). All transfers were made according to Sambrook et al. (1989). First-strand cDNA probes for differential hybridization to plaque-lifts were prepared according to Thordal-Christensen et al. (1992). Probes were prepared from cloned DNA using the "Megaprime" random primer labeling kit (Amersham). Prehybridization to plaque-lifts, Southern blots of cloned DNA and Northern blots utilized 4× SSC, 0.5% SDS, 4× Denhardt, 200 µg/ml salmon sperm DNA, 1 µg/ml polyadenylic acid. Hybridization was made in the same solution with the exceptions that Denhardt was used at 2× and dextran sulphate was included to 7% (w/v) final. Genomic Southern blot hybridizations were performed in an aqueous hybridization solution essentially according to Anderson and Young (1985). For washing conditions, see figure legends.

DNA sequencing.

The dideoxynucleotide-chain termination method of Sanger et al. (1977) was applied using the "Sequenase" DNA sequencing kit (USB, Cleveland, OH) according to the manufacturers' recommendations, using [³⁵S]-dATP (Amersham) as label. The reactions were resolved on 8 M urea, 6% polyacrylamide, buffer gradient gels (1–5× TBE). Nucleotide sequences were determined in both directions using the KS, SK, T7, and T3 primers on double-stranded plasmid DNA of the cDNA clones and derived subclones, all in pBluescript vectors SK(−) and KS(−) (Stratagene). Computer-assisted analysis of sequence data was performed using either DNASIS (version 5.02) and PROSIS (version 1.07, Pharmacia) software or the GCG package (Devereux et al. 1984).

Nucleotide sequence accession numbers.

The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence databases under the accession numbers X74939 for pHvPR-1a and X74940 for pHvPR-1b.

NOTE ADDED IN PROOF

A cDNA clone encoding a barley PR-1 homologue has been identified recently (Muradov et al., Plant Mol. Biol. 23:439-442, 1993.) The derived polypeptide sequence diverges from HvPR-1b by only three amino acids. However, the sequence represents another gene as larger deviations appear in the untranslated regions.

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