

# Characterization and Localization of New Antifungal Cysteine-Rich Proteins from *Beta vulgaris*

Karsten M. Kragh, John E. Nielsen, Klaus K. Nielsen, Susanne Dreboldt, and Jørn D. Mikkelsen

Danisco Biotechnology, Langebrogade 1, P.O. Box 17, DK-1001 Copenhagen, Denmark

Received 19 September 1994. Accepted 8 February 1995.

Two novel antifungal proteins, AX1 and AX2, were isolated from leaves of sugar beet infected with *Cercospora beticola*. AX1 (MW = 5078 ± 3D) and AX2 (MW = 5193 ± 3D) were N-terminally sequenced and identified as monomeric, basic proteins consisting of 46 amino acid residues, of which eight are cysteines. Both AX proteins strongly inhibit growth of *C. beticola* and other filamentous fungi, but have little or no effect against bacteria. Based on primary sequence homology (24 to 46% identity), they are related to the superfamily of  $\gamma$ -thionins, which have been isolated recently from seeds of monocotyledons and Brassicaceae. Specific antibodies were raised against the AX proteins after conjugation to diphtheria toxoid. Using immunoblotting and immunohistology, we detected high concentrations of AX proteins extracellularly in cell walls and in globular bodies around necrotic lesions in sugar beet leaves infected with *C. beticola*, suggesting that AX proteins are involved in antifungal defense. Furthermore, AX proteins or serologically related proteins were detected in xylem, stomata, and stomatal cells as well as in sugar beet styles.

**Additional keywords:** conjugated antigen, intercellular, leaf spot disease, PR protein.

Dissecting the mechanisms of active plant defense and modifying them at the gene level to create more resistant crop plants are some of the great challenges for plant molecular biology. We have investigated the interaction of sugar beet and *Cercospora beticola*, which causes leaf spot disease, one of most destructive foliar disease of sugar beet (Smith and Ruppel 1973). The fungus enters into the leaf through open and closed stomata (Rathai 1976). After 8 to 10 days of growth in the intercellular space of the mesophyll visible lesions develop, which consist of masses of collapsed cells and fungal mycelium. The fungus produces a specific photosensitizing toxin, cercosporin, which causes degeneration of the membranes of mesophyll cells, with resultant cytoplasmic leakage and cell collapse, supplying the fungus with nutrients (Steinkamp et al. 1979). Tolerant sugar beet cultivars have been developed, which show less severe symptoms at a later time point, but no resistant cultivars are available (Smith and Ruppel 1979). Thus today the disease has to be controlled by intensive spraying with fungicides.

Previously, we have focused on the role of chitinases and  $\beta$ -1,3-glucanases of the host in this interaction (Fleming et al. 1991; Mikkelsen et al. 1992; Nielsen et al. 1993, 1994a, 1994b, 1994c). Protein extracts from sugar beet leaves infected with *C. beticola* contained high antifungal activity when applied in in vitro assays against this fungus. A substantial part of the antifungal activity could be attributed to chitinases and  $\beta$ -1,3-glucanases (Mikkelsen et al. 1992; Nielsen et al. 1994a, 1994b), but a high antifungal activity was still found after removal of chitin-binding proteins (including most of the chitinases) and acidic proteins. Within recent years, a wide range of new antifungal plant proteins have been identified, which include ribosome-inactivating proteins (Leah et al. 1991), chitin-binding proteins of the PR4 and hevein types (Hejgaard et al. 1992; van Parijs et al. 1991), thaumatin-like proteins (Vigers et al. 1991; Hejgaard et al. 1991), lipid transfer proteins (Monila et al. 1993), and numerous cysteine-rich proteins of low molecular weight. The latter group includes  $\alpha$ - and  $\beta$ -thionins from wheat and barley (Bohlmann et al. 1988; Garcia-Olmedo et al. 1992), chitin binding lectin-like peptides from amaranth seeds (Broekaert et al. 1992) and neurotoxin-like peptides from seeds of *Mirabilis jalapa* (Cammue et al. 1992). Recently, low molecular weight, cysteine-rich proteins from seeds of Brassicaceae have been demonstrated to have a strong antifungal activity (Terras et al. 1992 and 1993), and, prior to that, two then novel genes encoding similar proteins in pea pods were reported to be induced by fungal infection (Chiang and Hadwiger 1991). These proteins belong to a superfamily of small plant proteins, expressed in plant seeds, stems, leaves, and flowers, which we refer to as the  $\gamma$ -thionin family (Bloch and Richardson 1991; Chiang and Hadwiger 1991; Colilla et al. 1990; Gu et al. 1992; Mendez et al. 1990; Stiekema et al. 1988; Terras et al. 1992). In this investigation we have purified and characterized two novel antifungal and cysteine-rich proteins from extracts of *C. beticola*-infected sugar beet leaves. The AX proteins are related to the  $\gamma$ -thionins. Their localization in sugar beet leaves infected with *C. beticola* as well as in noninfected leaves and flowers strongly suggests that they have a protective role against fungi.

## RESULTS

### Purification.

After removal of chitin-binding and acidic proteins the remaining antifungal proteins were purified by hydrophobic interaction chromatography using Phenyl Sepharose and a buffer containing 1.0 M ammonium sulfate. The nonbound fraction

contained essentially all of the antifungal activity, suggesting that it was due to very hydrophilic proteins. After dialysis and subsequent concentration by cation exchange, the remaining proteins were separated by gel filtration on a Sephadex G-75 column. Fractions exhibiting high antifungal activity contained proteins of about 3 kDa according to the calibration of the column. These proteins were applied to cation exchange chromatography on a Mono S FPLC column. Two major and one minor UV-absorbing protein peaks designated AX1, AX2 and AX3 were resolved (Fig. 1).

A strong antifungal effect was exhibited by both AX1 and AX2, which were homogenous as apparent from SDS-PAGE followed by silver staining (Fig. 2) and reversed phase HPLC (data not shown). In contrast, AX3 was only weakly active in inhibiting growth of *C. beticola* and was therefore not examined further. Typical yields of 1 mg AX1 and 2 mg AX2 were obtained from 1 kg of *C. beticola*-infected leaf material.

#### Biochemical characterization.

From SDS-PAGE on High Density Phast gels (Pharmacia LKB) run without reducing agent, MWs of 5.4 and 5.3 kDa were determined for AX1 and AX2, respectively (Fig. 2). This agrees with MWs of about 3 kDa estimated from gel filtration during protein purification. Furthermore, MWs of  $5078 \pm 3$  D for AX1 and  $5193 \pm 3$  D for AX2 were revealed by electrospray mass spectrometry. Using SDS-PAGE on High Density Phast gels, a slight shift in the apparent MW from 5.4 to 4.6 kDa for AX1 and from 5.3 to 4.1 for AX2 was observed upon reduction with DTT (Fig. 2). The shift is probably due to unfolding of the proteins caused by disruption of intramolecular disulfide-bridges leading to enhanced binding of SDS and thus faster migration in comparison to the same proteins denatured by SDS in the absence of DTT. The relative high background staining in the lanes with DTT, which manifests itself in the

vicinity of the protein bands as an apparent smearing, is most probably due to partial oxidation of the proteins during electrophoresis, whereas the background staining at the top of the same lanes is probably due to artifactual staining of DTT. Finally, the MWs of reduced and nonreduced AX proteins determined by SDS-PAGE on High Density gels are in agreement with those apparent from SDS-PAGE on Tricine gels (see Fig. 6 below).

The consistency of the MWs determined for nonreduced AX1 and AX2 by SDS-PAGE, gel filtration and mass spectrometry together with the very small decrease in apparent MW from SDS-PAGE upon reduction with DTT indicates that AX1 and AX2 are monomeric proteins of 5.1 and 5.2 kDa.

After carboxymethylation and reversed-phase HPLC, N-terminal sequences of 45 amino acid residues were determined for both AX1 and AX2 (Fig. 3). The sequences were identical with those subsequently derived from cDNA clones encoding AX1 and AX2 (K. Bojsen et al., personal communication). Derived from the cDNA sequences, cysteine was identified to be the 46th and C-terminal residue of both AX1 and AX2. The MWs and pIs of AX1 and AX2 were calculated from their sequences using algorithms of the PCgene software (Intelligenetics). Assuming that all eight cysteines in both proteins form disulfide-bridges and that the methionine residue, found only in AX2, is oxidized the calculated MWs for AX1 and AX2 are  $(5086-8)$  D = 5078 D and  $(5185-8+16)$  D = 5193 D, respectively. Thus the calculated MWs are identical with those determined by mass spectrometry. Furthermore, isoelectric points of 10.3 and 10.5 were predicted for AX1 and AX2, respectively. Consistent herewith, the AX proteins could not be focused on isoelectric focusing Phast gels (Pharmacia LKB) in the pH 3-9 range (data not shown).

Based on sequence alignments, AX1 and AX2 are related to seed proteins isolated from monocotyledonous and Brassicaceae species as well as to proteins encoded by cDNA clones of genes expressed in pea pod, potato tuber, and stem as well as in tobacco flower (Fig. 3) (Bloch and Richardson 1991; Chiang and Hadwiger 1991; Colilla et al. 1990; Gu et al. 1992; Mendez

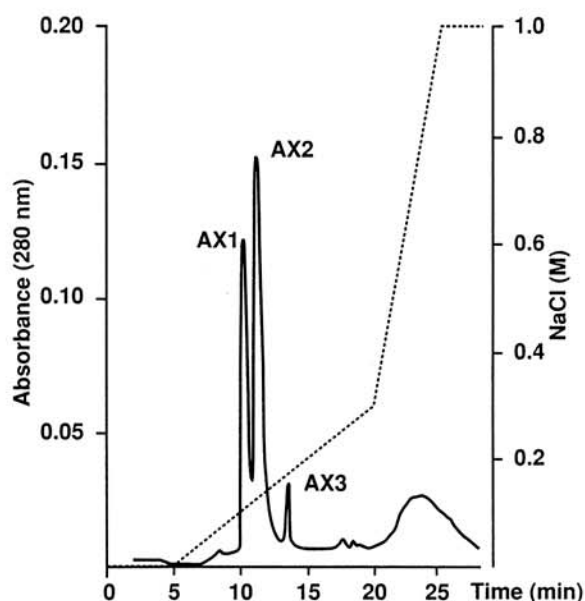


Fig. 1. Purification of AX proteins by cation exchange fast-performance liquid chromatography (FPLC) on a Mono S column. The proteins were eluted in a linear gradient of 0 to 0.3 M sodium chloride in 50 mM MES, pH 6.0 containing 5% (w/v) betaine and monitored by their UV absorbance at 280 nm. The flow rate was 1 ml/min.

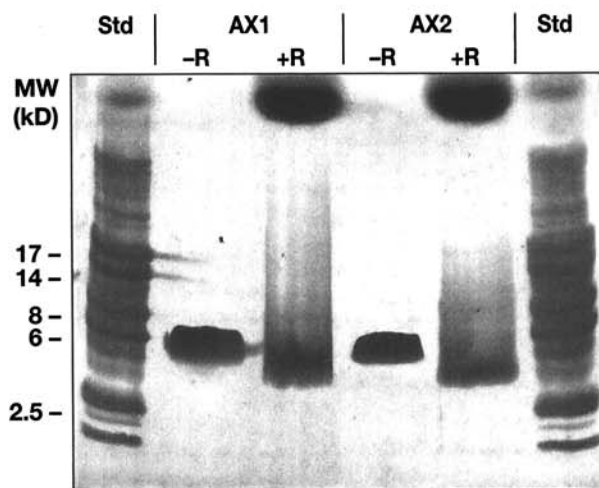


Fig. 2. SDS-PAGE analysis on High Density Phast gels of purified AX1 and AX2 treated without (-R) or with (+R) the reducing agent DTT. Proteins (0.6 µg) were electrophoresed together with standards of the indicated molecular weights and silver stained.

	10	20	30	40
AX1	AI-CKKPSKFFKGACGRDADCEKACDQ-ENWPGGVVCFPL--R-CECQRSC-			
AX2	*T-*r***My*S***FS*tn*Q***NR-*D**N*K*lvGF--k-*****P*-			
Barley	R*-*rrR*AG***P*VSNKN*AQV*M*-*G*G**N*DGP*--*R*K*M*R*-			
Wheat 1	K*-*rrR*AG***P*MSNKN*AQV*Q*-*G*G**N*DGP*--*R*K*I*Q*-			
Sorg. SI2	RV-*MGK*AG***L*M**QN*AQV*L*-*G*G**N*DGMV--*Q*K*I*Q*W			
Pot. P322	RH-*ESL*HR***P*T**sN*ASV*eT-*RfS**N*HG*R--*R*F*Tkp*-			
FST	RE-*TE*NT*P*I*ITKPP*R***IS-*KfTD*H*SKL*--*R*L*Tkp*-			
Pea PI39	NT-*EHLAdTyr*V*FTN*S*dDH*KnKAHLIS*T*HD-W--k-*F*TQN*-			
Pea PI230	NT-*ENLaGSy**V*F--GG*drH*RTQ*GAIS*R*RDDF--*--*W*Tkn*-			
RS-AFP1	QKL*Er**GTWS*V**NNNA*KNQ*InL*KARH*S*NYVFPahK*T*YFP*-			

Fig. 3. Alignment of amino acid sequences of AX1 and AX2 with sequences of similar proteins from seeds of barley (Mendez et al. 1990), wheat (Colilla et al. 1990), sorghum (Bloch and Richardson 1991), and radish (RS-AFP1; Broekaert et al. 1992; Terras et al. 1992) as well as sequences derived from cDNA clones expressed in potato tuber and stem (Stiekema et al. 1988), tobacco flowers (FST; Gu et al. 1992) and pea pods (Chiang and Hadwiger 1991). An asterisk (\*) shows identity with AX1, a dash (-) indicates an inferred deletion and letters in lower case indicate a conservative substitution compared to AX1 within one of the groups of similar amino acids (A,S,T; E,D; N,Q; R,K; I,L,M,V; F,Y,W).

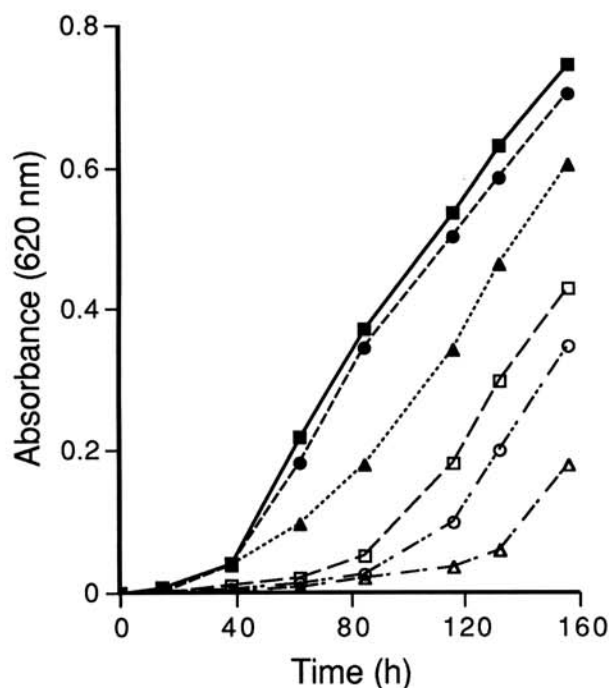


Fig. 4. Antifungal effect of AX2 and WIN N against *Cercospora beticola* measured in a microtiter plate bioassay. The assay mix contained 100 µl of potato dextrose broth, 40 µl of proteins sample in 100 mM Tris-HCl and 20 mM NaCl, pH 8.0, as well as about 400 spores in 100 µl water. The fungal growth was measured as increase in absorbance at 620 nm. The curves represent buffer control (filled squares), 28 µg/ml WIN N (filled circles), 2 µg/ml AX2 (filled triangles), 4 µg/ml AX2 (open squares), 8 µg/ml AX2 (open circles), and 8 µg/ml AX2 + 28 µg/ml WIN N (open triangles).

et al. 1990; Stiekema et al. 1988; Terras et al. 1992). AX1 and AX2 are 54% identical and show 24 to 46% identity to the other proteins. The cysteines, which constitute eight out of 45 to 51 amino acids in the proteins, are conserved in all of the sequences, as is glycine in positions 12 and 32 (Fig. 3). Furthermore, serine or alanine are conserved in position 7, an aromatic residue in position 10 as well as a basic residue in posi-

tion 38. In all but one of the sequences, glutamic acid is present in position 27, and, in 8 out of the 10 sequences, a basic residue is found in position 44. Not included in the alignment are three additional proteins from monocotyledonous seeds and eight additional proteins from seeds of Brassicaceae, all of which also show a similar N-terminal sequence (Bloch and Richardson 1991; Mendez et al. 1990; Terras et al. 1992, 1993).

#### Antifungal activity.

In the in vitro bioassay with *C. beticola*, AX1 and AX2 show a strong growth inhibiting effect which increases with increasing concentrations. As little as 2 µg/ml (0.4 µM) of AX2 is sufficient for 50% growth inhibition after 85 h of incubation (Fig. 4), whereas 4 µg/ml (0.8 µM) of AX1 is needed for 50% growth inhibition after 120 h (data not shown). Furthermore, the basic chitin-binding PR4 protein WIN N from barley seeds (Hejgaard et al. 1992) enhances the growth retarding effect of AX2, whereas WIN N alone only has a weak effect on the fungal growth (Fig. 4). The PR4 protein WIN N was tested because a serologically related protein of the same MW was found to be induced in infected sugar beet leaves (unpublished observation).

When *C. beticola* is grown without antifungal proteins, the growth rate is almost constant after a lag phase of about 40 h. Approximately the same growth rate is reached upon addition of AX2 and/or WIN N, but only after a delay which increases with higher concentrations of the proteins. For 2 µg/ml AX2 and 28 µg/ml WIN N the onset of fungal growth is inhibited for about 4 days. The growth inhibition is most probably not overcome by breakdown of the antifungal proteins added, as no loss of AX2 applied in the bioassay was detected by immunoblotting after SDS-PAGE on Tricine gels (data not shown).

Morphologically, *C. beticola* grown for 6 days in the presence of AX2 with or without WIN N shows short, thick (5 µm diameter) and rarely branched hyphae extruding from condensed mycelial aggregates giving rise to a sea urchin like look compared to the buffer control with a much more extended mycelium with long, thin (1.5 µm diameter) and branched hyphae extruding from thick (5 µm diameter) and branched hyphae (Fig. 5). The mycelium grown for 6 days in the presence of WIN N alone shows few long, thin (1.5 µm diameter) hyphae but many thick (5 µm diameter) and very dark branched



hyphae compared to the control (Fig. 5). In conclusion, low micromolar concentrations of AX1 and AX2  $\pm$  WIN N markedly delay growth of *C. beticola* and, in addition AX2 changes the morphology of the fungal hyphae (AX1 was not examined).

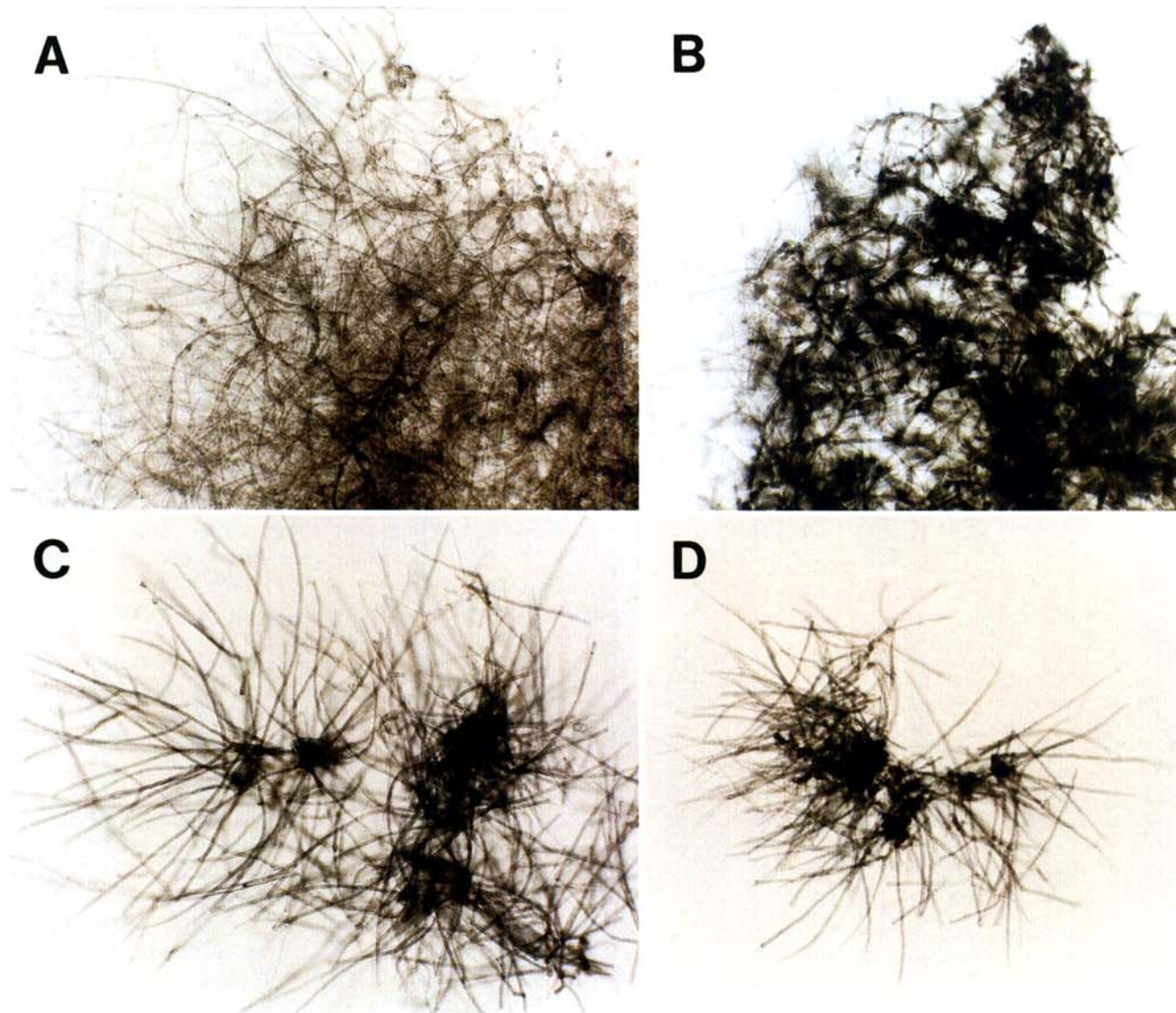
Furthermore, the effect of AX1 and AX2 on the growth of some additional microorganisms has been examined (data not shown). Concentrations of 40  $\mu\text{g/ml}$  of AX1 or AX2 effected a growth inhibition of 71 and 95%, respectively, on *Botrytis cinerea* after 145 h. Furthermore, AX1 and AX2 have shown strong inhibitory effects against *Fusarium graminearum* and *Bipolaris maydis* (D. Kendra, personal communication). In contrast, no effect of up to 50  $\mu\text{g/ml}$  of AX1 or AX2 on the growth of *Pseudomonas phaseolicola* (gramnegative) and *Bacillus subtilis* (gram positive) was observed, whereas a transient growth reduction of 20 to 27% was detected when *Escherichia coli* (gram negative) was incubated 24 h with 50  $\mu\text{g/ml}$  AX1 or AX2. Taken together, these results indicate that AX1 and AX2 are highly potent inhibitors of fungal growth, whereas they seem to have little or no effect on the growth of bacteria.

#### Generation of specific antibodies.

Two strategies were examined to generate specific antibodies against AX1 and AX2. Firstly, the native proteins were used for immunization of rabbits. No significant reaction with dot blotted AX proteins was observed when these antisera were tested (data not shown).

Secondly, AX1 and AX2 conjugated to diphtheria toxoid was used for immunization. Using immunoblotting after SDS-PAGE on Tricine gels, the obtained antisera react strongly with the non-reduced AX-protein which they have been raised against and cross-react at a lower intensity with the other AX protein (Fig. 6). The detection limit using nonreduced AX1 and anti-AX1 antiserum with alkaline phosphatase conjugated secondary antibodies is well below 2 ng of antigen (data not shown). As measured by mass spectrometry the MW of AX2 is only 115 Da higher than that of AX1, but on Tricine gels nonreduced AX1 and AX2 are clearly separated.

AX1 reduced with DTT prior to electrophoresis shows two bands when probed with anti-AX1 antiserum (Fig. 6A). Com-



**Fig. 5.** Morphology of *Cercospora beticola* (108 $\times$ ) grown for 6 days with buffer control (A), 28  $\mu\text{g/ml}$  WIN N (B), 8  $\mu\text{g/ml}$  AX2 (C), and 8  $\mu\text{g/ml}$  AX2 + 28  $\mu\text{g/ml}$  WIN N (D) as described for Figure 4.

pared to nonreduced AX1, one band has a slightly higher mobility, whereas the other has a slightly lower mobility and probably is due to partial oxidation. Both bands react less strongly than nonreduced AX1 with anti-AX1 antiserum, suggesting that some epitopes are lost or become inaccessible upon reduction. Anti-AX1 antiserum cross-reacts moderately with 2 bands in a nonreduced AX2 preparation and less strongly with 2 bands in a reduced AX2 preparation (Fig. 6A). The lower band in both preparations is probably due to a slight contamination with AX1 because this band is only weakly recognized by anti-AX2 antiserum and has the same mobility as AX1 (Fig. 6B). In raw extracts of *C. beticola*-infected cv. Rhizor leaves, which correspond to those used for purification, anti-AX1 antiserum reacts strongly with AX1 and weakly with AX2, whereas only a slight background staining is observed (Fig. 6A), indicating a high specificity of the antiserum.

When probed against nonreduced and reduced AX2, anti-AX2 antiserum shows the same intensity of reaction (Fig. 6B). In the latter case, three bands are detected probably because of partial oxidation during electrophoresis. This antiserum cross-

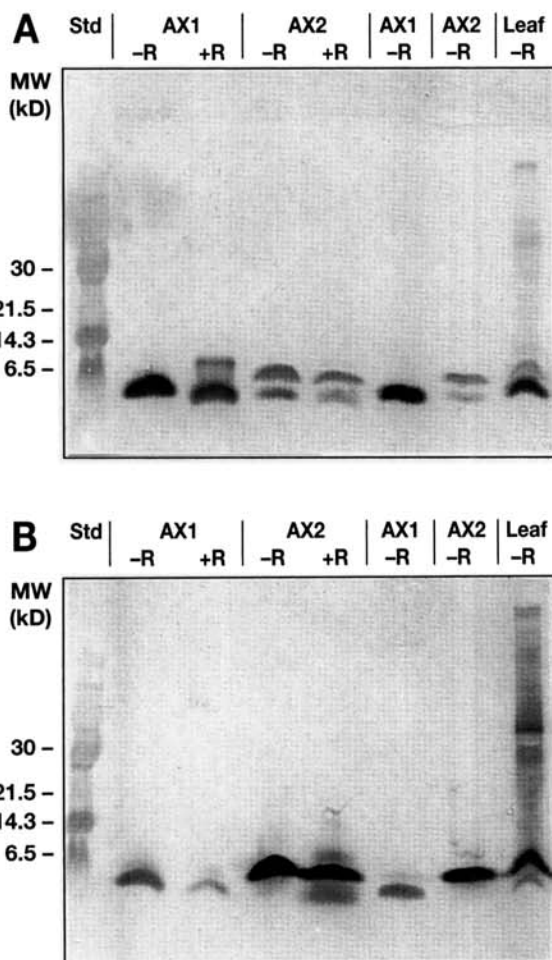
reacts with nonreduced AX1 and very weakly with reduced AX1. Using the anti-AX2 antiserum to probe against raw extracts of *C. beticola*-infected sugar beet leaves reveals a strong AX2 and a weak AX1 band but also a fairly high background reaction (Fig. 6B).

#### Localization studies.

Using immunoblotting after SDS-PAGE, high concentrations of AX1 and AX2 were detected in intercellular washing fluid (IF) from sugar beet leaves, whereas only very low concentrations of AX proteins were detected in the extract from the leaves homogenized after IF extraction (Fig. 7A). The identity of AX1 as well as AX2 purified by cation exchange from the IF was confirmed by N-terminal sequencing at least 40 residues from both proteins (data not shown). The leaf material used for this study had been sprayed with 2,6-dichloroisonicotinic acid (INA) which is known to induce systemic resistance against *C. beticola* (Nielsen et al. 1994c). The AX proteins were therefore initially expected to be induced. However, similar concentrations of AX proteins were found in IF from both untreated and INA sprayed leaves and likewise no induction of AX proteins was detected in total leaf extracts of INA treated leaves (data not shown). The localization of AX proteins in the IF is most probably not due to cell leakage, since the protein patterns detected by silver staining after SDS-PAGE in IF and in the subsequently homogenized leaves are totally different and the distribution of the extracellular chitinase Ch4 (Mikkelsen et al. 1992) in IF and subsequently homogenized leaves corresponds to that of the AX proteins (data not shown).

Using immunohistochemistry, AX proteins were localized on cross-sections of leaves of the susceptible cv. Monova, either infected with *C. beticola* and thus showing necrotic spots or noninfected (Fig. 8). No reactions were observed with preimmune serum (Fig. 8D and E) and identical patterns were detected when using anti-AX1 or anti-AX2 antiserum, indicating that both AX proteins are distributed similarly. In necrotic lesions of infected leaves, positive reactions with the antisera were detected primarily in epidermis and in xylem, whereas weak reactions were observed in between collapsed tissue (Fig. 8A). In the transition zone between necrotic and anatomically undisturbed tissue around lesions, a large number of globular bodies were observed (Fig. 8B). These bodies of about 10  $\mu$ m diameter were present in the intercellular space and reacted very strongly with anti-AX antiserum (Fig. 8A—C). In the transition zone, strong reactions were also observed at the intercellular surface of mesophyll cell walls (Fig. 8A—C). In both the transition zone and in anatomically undisturbed tissue of infected leaves, strong reactions were found in xylem (Fig. 8A), whereas some weak reactions were detected intracellularly in stomatal cells and intercellularly in substomatal cavities (data not shown). In noninfected sugar beet leaves strong positive reactions were found in xylem (Fig. 8F) and weak reactions were observed in stomatal cells (not visible in Fig. 8F). In conclusion, strong reactions with AX proteins and/or serologically related proteins are detected in cell walls and in globular bodies in the transition zone around necroses and strong xylem as well as weak stomata related reactions are found widespread in both infected and noninfected sugar beet leaves.

As shown in Figure 7B, we further detected a protein with a mobility similar to that of AX1 in an extract from sugar beet styles. In longitudinal sections of sugar beet styles anti-AX1



**Fig. 6.** Immunoblotting after SDS-PAGE on Tricine gels with anti-AX1 antiserum (A) and anti-AX2 antiserum (B). In lanes 2 to 5 from the left 200 ng of AX1 or AX2 treated without (–R) or with the reducing agent DTT (+R) were loaded, in lanes 6 and 7 fifty ng of AX1 or AX2 treated without DTT (–R). For comparison, leaf extract from heavily *Cercospora beticola*-infected 'Rhizor' leaves (16  $\mu$ g) and colored low molecular weight standards as indicated were electrophoresed.



antiserum (and also anti-AX2 antiserum) reacted strongly with an antigen that is localized intracellularly in stigma papillae and in cells composing the transmitting tract (Fig. 9). In old styles this antigen was only observed in the transmitting tract (data not shown).

## DISCUSSION

Based on amino acid sequence homology (24 to 46% identity), the AX proteins are related to the  $\gamma$ -thionin family of small cysteine-rich plant proteins. These include the classical  $\gamma$ -thionins from wheat and barley endosperm, found to inhibit translation in cell-free systems (Colilla et al. 1990; Mendez et al. 1990), three proteins isolated from seeds of sorghum and identified as inhibitors of insect  $\alpha$ -amylase (Bloch and Richardson 1991), a number of highly antifungal proteins from seeds of radish and other Brassicaceae species (Terras et al. 1992 and 1993), as well as gene products of cDNAs from pea pod, potato tuber, and stem as well as tobacco flower (Chiang and Hadwiger 1991; Gu et al. 1992; Stiekema et al. 1988).

By SDS-PAGE, gel filtration and electrospray mass spectrometry the AX proteins were found to exist as monomers in aqueous solution. In contrast, the antifungal proteins from Brassicaceae were found to be oligomeric proteins stabilized by disulfide bridges (Terras et al. 1992 and 1993). However, based on proton NMR analysis,  $\gamma$ -thionins from wheat and barley have been shown to exist as monomers with all four disulfide bridges being intramolecular (Bruix et al. 1993). The structures of these  $\gamma$ -thionins consist of a small triple-stranded antiparallel  $\beta$ -sheet linked to an  $\alpha$ -helix by three disulfide bridges thus forming a cysteine-stabilized  $\alpha$ -helical motif similar to those found in scorpion toxins and insect defensins (Bruix et al. 1993).

The AX proteins possess a strong growth inhibiting activity against *C. beticola* ( $I_{50}$  = 0.4 - 0.8  $\mu$ M) and other filamentous fungi, but little or no effect against bacteria. This agrees with the seed proteins from Brassicaceae species being active almost exclusively against filamentous fungi (Terras et al. 1992, 1993). In contrast, type 1 and 2 thionins from wheat and barley, which belong to a structurally different family of small, cysteine-rich proteins (Bohlmann et al. 1988; Mendez et al. 1990; Terras et al. 1992; Bruix et al. 1993), are generally toxic against bacteria and fungi as well as yeast and animal cells, with bacteria being more sensitive to these proteins than fungi (Terras et al. 1993; Garcia Olmedo et al. 1992). Because of their highly specific activity against fungi, the AX proteins and the seed proteins from brassicas are promising candidates for genetic engineering of antifungal disease resistance in plants, whereas the growth inhibiting effects of the other members of the  $\gamma$ -thionin family remain to be investigated.

The morphology of *C. beticola* is significantly changed from long, thin branched hyphae to thick rarely branched hyphae upon incubation with AX2. This is quite different from the effect of swelling and lysis of hyphal tips observed upon treating fungal pathogens with chitinases (Collinge et al. 1993; Nielsen et al. 1994b). Based on results which indicate that  $Ca^{2+}$  is involved in regulating hyphal extension and branching (Robson et al. 1991) and on the observation that *Pyricularia oryzae* displays a highly branched morphology upon treatment with one of the  $\gamma$ -thionins from Brassicaceae, it has been suggested that this  $\gamma$ -thionin interferes with morphogenic  $Ca^{2+}$  signalling and

thus deregulates hyphal growth (Terras et al. 1992). In this respect it is striking that scorpion toxins blocking membrane ion channels by binding via electrostatic interactions display a tertiary structure highly similar to those of the  $\gamma$ -thionins from wheat and barley (Bruix et al. 1993). It remains to be investigated whether the AX proteins and other  $\gamma$ -thionins have a similar 3-D structure and thus exhibit a similar action on ion channels.

High concentrations of AX1 and AX2 were found in intercellular washing fluid from sugar beet leaves and both anti-AX antisera reacted strongly with cell walls and globular bodies in the intercellular space of leaves infected with *C. beticola* and with xylem in both infected and noninfected leaves. On this basis we conclude, that AX1 and AX2 are localized intercellularly.

Large numbers of extracellular globuli containing AX proteins were detected in the transition zone between the necrosis and anatomically undisturbed tissue in *C. beticola*-infected leaves of the susceptible cv. Monova. The extracellular sugar beet chitinase Ch4 likewise accumulates in these bodies (J. E. Nielsen et al., personal communication). Furthermore, extracellular "pocket-like" structures between mesophyll cells in TMV-infected tobacco have been reported to contain acidic chitinases,  $\beta$ -1,3-glucanases and thaumatin-like proteins (Dore et al. 1991).

Susceptible sugar beet leaves develop larger necroses than tolerant leaves upon infection with *C. beticola* (Feindt et al. 1981). In tolerant sugar beet leaves osmiophilic, electron-dense extracellular globuli have been reported to accumulate throughout *C. beticola*-infected areas, whereas similar bodies appear later and restricted to the borders of necrotized tissue in susceptible leaves (Lieber 1982; Feindt et al. 1981; Steinkamp et al. 1979). Furthermore fungal growth in susceptible leaves is restricted to the central part of the necrosis and is very seldom found in the transition zone between the necrosis and the anatomically undisturbed tissue where the globular bodies are present (Feindt et al. 1981). Taken together these results strongly suggest that intercellular globular bodies and proteins like AX1 and AX2 which they contain are involved in defense reactions, in particular against a fungus like *C. beticola*, which is almost entirely confined to the intercellular space. The finding of dictyosomes in cytoplasm very close to globular bodies in in-

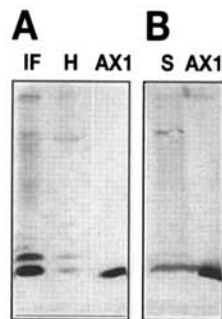
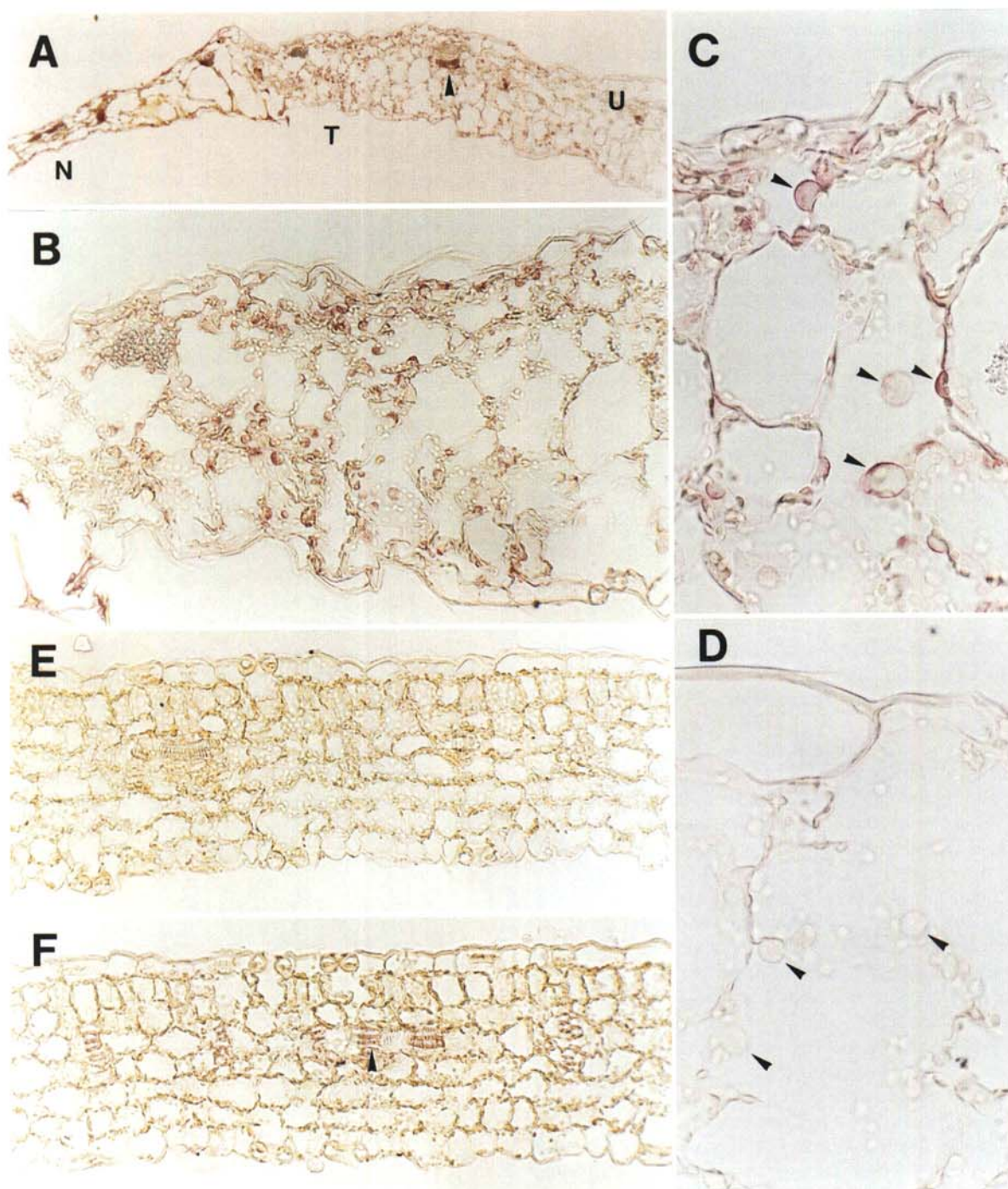


Fig. 7. Immunoblotting after SDS-PAGE with anti-AX1 antiserum. **A**, Detection of AX proteins in intercellular washing fluid (IF) and in an extract of leaves homogenized after extraction of IF (H). Samples of 10  $\mu$ g were loaded together with 10 ng of AX1, all being non-reduced. **B**, Detection of an antigen from sugar beet styles with anti-AX1 antiserum compared with purified AX1. Twentyfive micrograms of extract and 20 ng of AX1 were loaded.

infected sugar beet leaves (Feindt et al. 1981) suggests that chitinases, AX proteins and other proteins contained in the globuli, have been secreted, probably via the default pathway (Bednarek and Raikhel 1991; Denecke et al. 1990).

AX proteins and/or serologically related proteins were detected in xylem and intercellularly at cell walls lining the sub-

stomatal cavities as well as intracellularly in stomatal cells in noninfected leaves as well as in infected leaves. Stomata are the entry points for pathogens like *C. beticola* (Rathiah 1976), thus the cells around the stomata have a critical role for guarding the interior of the leaf. This could be the reason for the presence of AX proteins or serologically related proteins in



**Fig. 8.** Immunohistological localization of AX proteins in sugar beet leaves using anti-AX2 antiserum (1:50 diluted). **A**, Cross section of a leaf with necrosis (N), transition zone (T) around the necrosis, and anatomically undisturbed tissue (U) (76 $\times$ ). The arrow indicates xylem. **B**, Section of the transition zone from A with numerous globular bodies (294 $\times$ ). **C**, Transition zone at higher magnification (470 $\times$ ) with arrows indicating globular bodies. **D**, As C but incubated with preimmune serum. **E**, Healthy sugar beet leaf treated with preimmune serum (294 $\times$ ). **F**, As E but treated with anti-AX1 antiserum. Arrow indicates xylem.



these cells. Possibly for the same reason, xylem, another potential infection route for fungi, shows a strong reaction with the anti-AX antiserum.

Using immunohistology and immunoblotting, we also detected an intracellular antigen similar to AX1 in stigma papillae and in cells of the transmitting tract of sugar beet styles. Analogous to stomata, the style is an entry point into the reproductive tissue, and may thus contain AX-like defense proteins. By Southern blot analysis we have detected a multiple gene family encoding AX proteins (S. Madrid, personal communication) similar to the gene families for  $\gamma$ -thionins in pea, tobacco, and potato (Chiang and Hadwiger 1991; Gu et al. 1992; Stiekema et al. 1988). Interestingly, the tobacco  $\gamma$ -thionin cDNA is flower-specific (Gu et al. 1992).

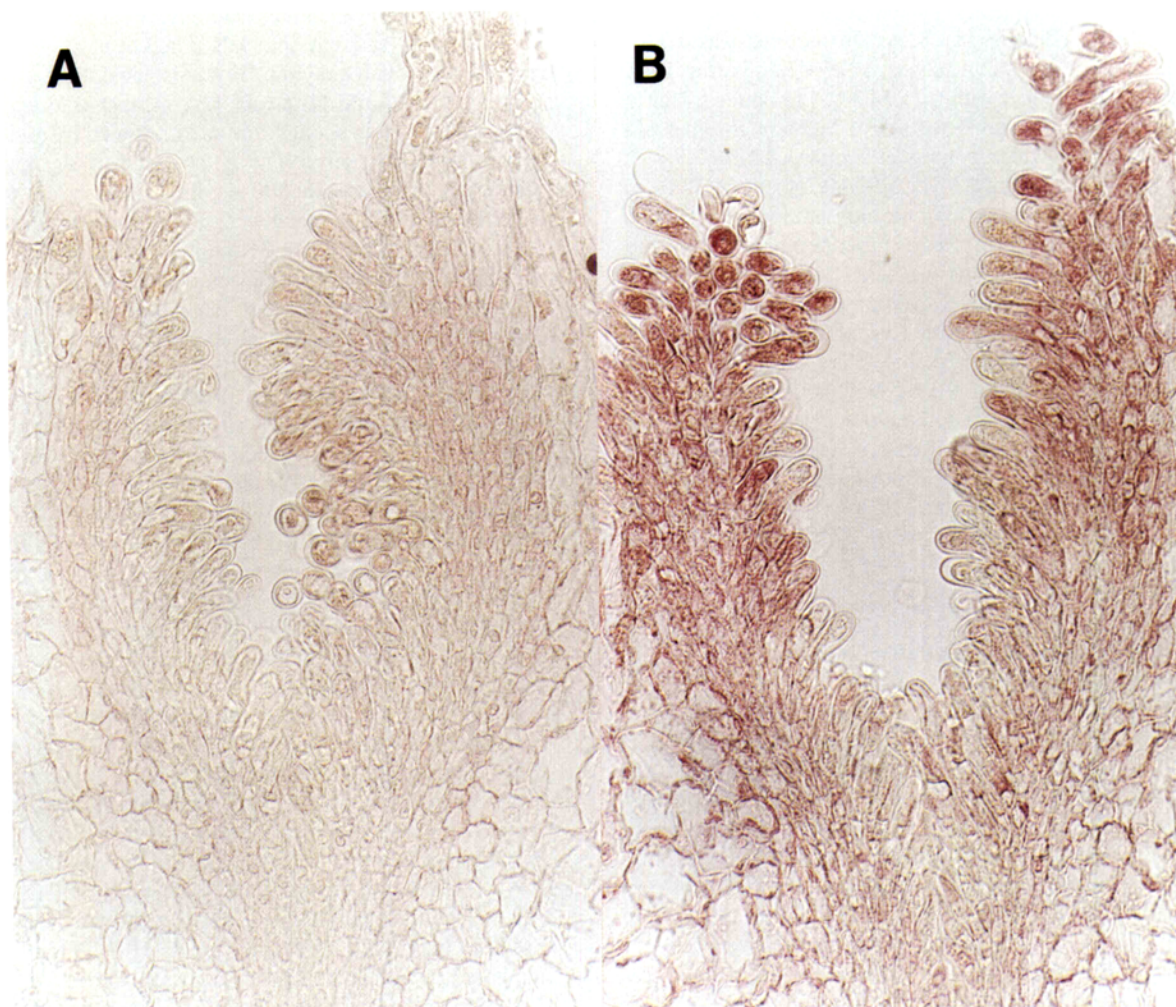
Preliminary studies using both anti-AX1 and anti-AX2 antiserum and immunoblotting after SDS-PAGE have shown little or no pathogen induction of AX proteins, when extracts of *C. beticola*-infected and noninfected leaves of the susceptible cv. Monova grown in growth chambers were analyzed (data not shown). In contrast, the two cDNAs encoding proteins of the  $\gamma$ -thionin family in pea pod were found to be strongly induced upon fungal infection (Chiang and Hadwiger 1991).

Apart from the lack of evidence for pathogen induction, the AX proteins display many of the characteristics of classical pathogenesis-related proteins (PR proteins) (van Loon 1985; Linthorst 1991), i.e., they are small proteins with a high pI, they are highly stable (active after reverse phase HPLC in water/acetonitril with 0.1% TFA), and extracellularly deposited. Furthermore, similar to chitinases, which constitute the PR-3 group of pathogenesis-related proteins (Collinge et al. 1993), members of the  $\gamma$ -thionin family are expressed in and probably involved in antifungal defense in plant leaves, seeds as well as in flowers.

## MATERIALS AND METHODS

### Purification of AX proteins.

For purification, leaves of sugar beet, cvs. Turbo and Rhizor, carrying 50 or more necrotic lesions due to natural infection with *C. beticola* were picked in a field in Italy (Maribo Italia, Bologna) and stored at 4°C until extraction. The purification was done at 4°C, except for the Phenyl Sepharose step. Two kilograms of *C. beticola*-infected leaves was homogenized using a Waring blender in 4 Liter of starting buffer: 0.1 M sodium



**Fig. 9.** Immunohistological examination of longitudinal section of sugar beet style using preimmune serum (1:50 diluted) (A) or anti-AX1 antiserum (1:50 diluted) (B) (294 $\times$ ).



citrate, pH 5.0, containing 1 mM DTT and 1 mM benzamidine, supplemented with 200 g of Dowex 1 × 2 (100 µm mesh size). The homogenate was squeezed through a double layer of 31 µm mesh nylon gauze before centrifugation at 20,000 × g. After heating the homogenate to 50°C for 20 min, precipitated proteins were removed by centrifugation, and solid ammonium sulfate was added to 90% saturation. After centrifugation, the precipitated proteins were redissolved in starting buffer (1 ml of buffer to 10 g of starting material) and dialyzed against 10 mM Tris, pH 8.0, containing 1 mM DTT and 1 mM benzamidine before loading onto a 50 ml of Fast Flow Sepharose Q (Pharmacia LKB) column and a 50 ml of chitin column (Kragh et al. 1990) equilibrated in the Tris buffer and connected in serie.

The nonbound protein fraction removed from the Sepharose Q and chitin columns by extensive washing with Tris buffer was supplemented to contain buffer H: 1 M ammonium sulfate, 10% (v/v) glycerol, 1 mM DTT, 0.1 M KH<sub>2</sub>PO<sub>4</sub>, pH 7.5 (200 ml/kg of leaf material extracted) and incubated with 50 ml of Phenyl Sepharose (Pharmacia LKB) for 2 h at RT. The slurry was then loaded on top of a column packed with additional 50 ml of Phenyl Sepharose equilibrated in buffer H. The flow through from the Phenyl Sepharose column (400 ml) was dialyzed extensively against 20 mM sodium acetate, pH 5.0, with 1 mM DTT and subsequently loaded onto a column of CM-CL6B Sepharose (Pharmacia LKB). This column was washed with buffer I: 50 mM sodium acetate, 10% (v/v) glycerol, 1 mM DTT, pH 5.0, and eluted with 0.25 M NaCl in buffer I. Half of the eluted fraction was subjected to gel filtration chromatography on a G-75 Sephadex column (Pharmacia LKB; 2.5 × 70 cm) equilibrated in 50 mM MES, pH 6.0. Fractions of 10 ml were collected. Fractions exhibiting high antifungal activity in the bioassay against *C. beticola*, as described below, were supplemented to contain 5% (w/v) betaine and subjected to ion-exchange FPLC on a Mono S cationic exchange column (HR 5/5; Pharmacia LKB) equilibrated in buffer A: 50 mM MES, pH 6.0 containing 5% (w/v) betaine. The bound proteins were eluted with a gradient of 0 to 0.3 M NaCl in 15 ml, buffer A at a flow rate of 1 ml/min. Prior to amino acid analyses and mass spectrometry, proteins were applied to reversed-phase HPLC on a Progel TSK Octadecyl-4 PW (Supelco INC.; 150 × 4.6 mm) or a RP-2 Spheri-10 (Brownlee; 30 × 4.6 mm) column and eluted with a gradient of acetonitrile in 0.1% TFA at a flow rate of 0.7 ml/min.

#### **Amino acid analyses, protein determination, and mass spectrometry.**

Amino acid compositions and exact protein concentrations were determined according to Barkholt and Jensen (1989). On the basis of protein concentrations and UV absorbance, the specific absorbance at 280 nm was found to be 1.36 and 1.57 per cm of 1 mg/ml solutions of AX1 and AX2, respectively. The specific absorbance was used to determine the concentrations of purified AX1 and AX2 samples, whereas protein concentrations in extracts were determined according to Bradford (1976) using the Bio-Rad protein assay kit with γ-globulin as standard. Carboxymethylation and amino acid sequencing was done as described elsewhere (Kragh et al. 1991). Electrospray mass spectrometry was performed as outlined by Nielsen et al. (1994a).

#### **Bioassays.**

The in vitro bioassay with *C. beticola* (isolate FC573, Earl G. Ruppel, USDA, Fort Collins, Colorado) was carried out by following the growth of submerged spore cultures in microtiter wells by measuring the absorbance at 620 nm as described by Nielsen et al. (1994b). Samples for microscopical analysis were grown likewise for 6 days. The same assay was also used with spores of *Botrytis cinerea* (KVL 1727, Plant Pathology Dept., Agric. Univ. Copenhagen, Denmark). For detection of antibacterial activity against *Pseudomonas phaseolicola* (KVL 2169), *Bacillus subtilis* (KVL 2202), and *Escherichia coli* (DH5α, BRL Gibco) overnight cultures of these bacteria grown in LB medium (Sambrook et al. 1989) were adjusted to OD<sub>620nm</sub> = 0.5 and diluted 500× in LB medium. In microtiter plates 80 µl of the diluted cultures were incubated with 20 µl of test sample at 25°C with shaking (150 rpm). The change in absorbance at 620 nm was monitored at specified times for 48 h.

#### **Sample preparation, SDS-PAGE, antibodies, and immunoblotting.**

For extraction of intercellular washing fluid (IF), leaves of 6-week old sugar beet plants of cv. Monova grown in a growth chamber (Nielsen et al. 1993) were sprayed four times with 25 ppm 2,6-dichloroisonicotinic acid (INA) suspended in 0.05% (v/v) Tween 20 at 2-day intervals (Nielsen et al. 1994c). Two days after the final spraying, IF was isolated. The leaves were submerged in 20 mM HAc (pH 4.5), incubated *in vacuo* in an exicator for 5 min at about 4 torr and infiltrated by releasing the vacuum. Following air-drying of the leaf surface, IF was collected by centrifugation at 500 × g for 15 min in 500-ml centrifuge tubes. The leaf material was subsequently ground in liquid nitrogen, extracted with 0.1 M sodium citrate, pH 5.0, containing 1 mM DTT and 1 mM benzamidine (2 ml g<sup>-1</sup>), and centrifuged at 20,000 × g for 10 min. An extract of sugar beet styles prepared according to Singh et al. (1991) was provided by S. Guldager Petersen.

Sugar beet leaf extracts and IF were desalted on PD-10 or NAP-5 columns (Pharmacia LKB) and concentrated using Centricon 3 microconcentrators (Amicon). Protein samples were subjected to SDS-PAGE using 16.5% Tricine gels (Schägger and von Jagow 1987) on a Mighty Small system (Hoefer) or using precast High Density gels on a Phast System (Pharmacia LKB). The samples were boiled for 2 min in 1/5 volume of 5× sample buffer containing 60 mM Tris-HCl (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, and 0.1% (w/v) bromophenol blue with or without 50 mM DTT as specified. Low molecular weight standards (2.5 to 17 kDa) from Pharmacia LKB and coloured molecular weight standards (2.3 to 46 kDa) from Amersham were used. The High Density Phast gels were silver stained according to the manufacturer's instruction. Proteins separated on Tricine gels were blotted to Immobilon PVDF membranes (Millipore) using a semidry blotting system (JKA-Biotech, Copenhagen, Denmark) (Kyhse-Andersen 1984). The blot was incubated overnight with anti-AX1 or anti-AX2 antiserum (diluted 1:500) raised in rabbits (Statens Seruminstitut, Denmark) after conjugation of AX1 and AX2 to diphtheria toxoid according to Marcussen and Poulsen (1991). The blots were visualized using alkaline phosphatase-conjugated secondary antibodies (pig anti-rabbit IgG; Dakopatts, Denmark) and nitroblue tetrazolium (Kyhse-Andersen 1984).

## Immunohistology.

For infection, 6-week-old plants of cv. Monova were inoculated with conidia of *C. beticola* (isolate FC573) and incubated in a mist chamber (30°C, 100% RH) as described previously (Nielsen et al. 1993). Noninfected leaves from growth chambers (25/18°C, 70% RH) (Nielsen et al. 1993) were used as controls. Young and old styles were isolated from the top and lower (20 cm from the top) flowers, respectively, of two sugar beet breeding lines (Maribo Seed, Denmark) grown in a greenhouse. All tissues were fixed in 2% (v/v) paraformaldehyde, 0.25% (v/v) glutaraldehyde, and 3% (w/v) sucrose buffered with 50 mM sodium phosphate, pH 7.0. After incubation for 2 h at 25°C followed by 15 h at 5°C, the specimens were washed 3 x 20 min in 50 mM sodium phosphate, pH 7.0. Dehydration of the samples was carried out using series of 30-min washes with 50, 70, 80, 96% ethanol followed by three washes in 99% ethanol. After further incubations in petroleum (2 x 2 h) (Shellsol D70k, Q7712) and in paraffin with 7% beeswax (2 x 2 h), the samples were embedded in paraffin. Cross sections of 12.5 µm thickness were made on a Supercut 2050 pyramitome (Reichert Jung).

For immunohistological investigations, tissue sections were preincubated with 20% swine serum in TBS (0.5M Tris/HCl, pH 7.6, and 0.15 M NaCl, 0.1% (v/v) Triton X-100) for 30 min, before treatment for 1 h with anti-AX antiserum diluted 1:50 in TBS. Excess antibodies were removed by washing with TBS (5 x 5 min). Thereafter the sections were incubated for 30 min with alkaline phosphatase coupled secondary antibodies (pig anti-rabbit IgG D306; Dakopatts) diluted 1:20 in TBS. Surplus of secondary antibodies was removed by washing with TBS as described above. After incubating the sections with 30 mM veronal acetate buffer, pH 9.2, for 5 min, they were stained with Fast Red (Sigma) in veronal acetate buffer supplied with 10 µM levamisole (Sigma) for about 20 min. Excess reagent was removed by washing with water. Controls were run in parallel but incubated with preimmune serum.

## ACKNOWLEDGMENTS

The excellent assistance of Jette Skovsgaard, Bo Lindberg, and Clive Phipps Walter is gratefully acknowledged. We also want to thank Ib Svendsen for performing amino acid sequencing, Peter Roepstorff for performing mass spectrometry, Steen Guldager Petersen for kindly providing a protein extract from laboriously isolated sugar beet styles, Jan Marcussen for advice about antigen conjugations, Dave Kendra, Kirsten Bojsen, and Susan Madrid for providing unpublished observations as well as David B. Collinge for critically reading the manuscript.

## LITERATURE CITED

- Barkholt, V., and Jensen, A. L. 1989. Amino acid analysis: Determination of cysteine plus half-cysteine in proteins after hydrochloric acid hydrolysis with a disulfide compound as additive. *Anal. Biochem.* 177:318-322.
- Bednarek, S. Y., and Raikhel, N. V. 1991. The barley lectin carboxyl-terminal propeptide is a vacuolar protein sorting determinant in plants. *Plant Cell* 3:1195-1206.
- Bloch Jr., C., and Richardson, M. 1991. A new family of small (5 kDa) protein inhibitors of insect  $\alpha$ -amylases from seeds of sorghum (*Sorghum bicolor* (L.) Moench) have sequence homologies with wheat  $\gamma$ -purothionins. *FEBS Lett.* 279:101-104.
- Bohlmann, H., Clausen, S., Behnke, S., Giese, H., Hiller, C., Reimann-Philipp, U., Schrader, G., Barkholt, V., and Apel, K. 1988. Leaf-specific thionins of barley—a novel class of cell wall proteins toxic to plant-pathogenic fungi and possibly involved in the defense mechanism of plants. *EMBO J.* 7:1559-1565.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Broekaert, W. F., Mariën, W., Terras, F. R. G., de Bolle, M. F. C., Proost, P., van Damme, J., Dillen, L., Claeys, M., Rees, S. B., Vanderleyden, J., and Cammue, B. P. A. 1992. Antimicrobial peptides from *Amaranthus caudatus* seeds with sequence homology to the cysteine/glycine-rich domain of chitin-binding proteins. *Biochemistry* 31:4308-4314.
- Broekaert, W., Cammue, B. P. A., Osborn, R. W., Rees, S. B., Terras, F. R., and Vanderleyden, J. 1993. Biocidal proteins. PCT Patent Application: WO 93/05153.
- Bruix, M., Jiménez, M. A., Santoro, J., Gonzáles, C., Colilla, F. J., Mendéz, E., and Rico, M. 1992. Solution structure of  $\gamma$ 1-H and  $\gamma$ 1-P thionins from barley and wheat endosperm determined by  $^1$ H-NMR: A structural motif common to toxic arthropod proteins. *Biochemistry* 32:715-724.
- Cammue, B. P. A., De Bolle, M. F. C., Terras, F. R. G., Proost, P., Van Damme, J., Rees, S. B., Vanderleyden, J., and Broekaert, W. F. 1992. Isolation and characterization of a novel class of plant antimicrobial peptides from *Mirabilis jalapa* L. seeds. *J. Biol. Chem.* 267:2228-2233.
- Chiang, C. C., and Hadwiger, L. A. 1991. The *Fusarium solani*-induced expression of a pea gene family encoding high cysteine content proteins. *Mol. Plant-Microbe Interact.* 4:324-331.
- Colilla, F. J., Rocher, A., and Mendez, E. 1990.  $\gamma$ -Purothionins: Amino acid sequence of two polypeptides of a new family of thionins from wheat endosperm. *FEBS Lett.* 270:191-194.
- Collinge, D. B., Kragh, K. M., Mikkelsen, J. D., Nielsen, K. K., Rasmussen, U., and Vad, K. 1993. Plant chitinases. *Plant J.* 3:31-40.
- Denecke, J., Botterman, J., and Deblaere, R. 1990. Protein secretion in plant cells can occur via a default pathway. *Plant Cell* 2:51-59.
- Dore, I., Legrand, M., Cornelissen, B. J., and Bol, J. F. 1991. Subcellular localization of acidic and basic PR proteins in tobacco mosaic virus-infected tobacco. *Arch. Virol.* 120:97-107.
- Feindt, F., Mendgen, K., and Heitefuss, R. 1981. Feinstruktur unterschiedlicher Zellwandreaktionen im Blattparenchym anfälliger und resistenter Rüben (*Beta vulgaris* L.) nach Infektion durch *Cercospora beticola* Sacc. *Phytopathol. Z.* 101:248-264.
- Fleming, T. M., McCarthy, D. A., White, R. F., Antoniw, J. F., and Mikkelsen, J. D. 1991. Induction and characterization of some of the pathogenesis-related proteins in sugar beet. *Physiol. Mol. Plant Pathol.* 39:147-160.
- Garcia-Olmedo, F., Carmona, M. J., Lopez-Fando, J. J., Fernandez, J. A., Castagnaro, A., Molina, A., Hernandez-Lucas, C., and Carbonero, P. 1992. Characterization and analysis of thionin genes. Pages 283-302 in: *Genes Involved in Plant Defense*. T. Boller and F. Meins, eds. Springer-Verlag, New York.
- Gu, Q., Kawata, E. E., Morse, M.-J., Wu, H. M., and Cheung, A. Y. 1992. A flower-specific cDNA encoding a novel thionin in tobacco. *Mol. Gen. Genet.* 234:89-96.
- Hejgaard, J., Jacobsen, S., and Svendsen, I. 1991. Two antifungal thaumatin-like proteins from barley grain. *FEBS Lett.* 291:127-131.
- Hejgaard, J., Jacobsen, S., Bjørn, S. E., and Kragh, K. M. 1992. Antifungal activity of chitin-binding PR-4 type proteins from barley grain and stressed leaf. *FEBS Lett.* 307:389-392.
- Kragh, K. M., Jacobsen, S., and Mikkelsen, J. D. 1990. Induction, purification and characterization of barley leaf chitinase. *Plant Sci.* 71:55-68.
- Kragh, K. M., Jacobsen, S., Mikkelsen, J. D., and Nielsen, K. A. 1991. Purification and characterization of three chitinases and on  $\beta$ -1,3-glucanase accumulating in the medium of cell suspension cultures of barley (*Hordeum vulgare* L.). *Plant Sci.* 76:65-77.
- Kyhse-Andersen, J. 1984. Electrophoretic blotting of multiple gels: A simple apparatus without buffer tank for rapid transfer of proteins from polyacrylamide to nitrocellulose. *J. Biochem. Biophys. Methods* 10:203-209.
- Leah, R., Tommerup, H., Svendsen, I., and Mundy, J. 1991. Biochemical and molecular characterization of three barley seed proteins with antifungal activity. *J. Biol. Chem.* 266:1564-1573.
- Lieber, H. 1982. Untersuchungen Zur Erfassung Der Resistenz Der Zuckerrübe, *Beta vulgaris* L., Gegenüber *Cercospora beticola* Sacc. Ph. D. thesis, Institut für Pflanzenpathologie und Pflanzenschutz, Georg-August Universität Göttingen, Germany.
- Linthorst, H. J. M. 1991. Pathogenesis-related proteins of plants. *Crit.*



- Rev. Plant Sci. 10:123-150.
- Marcussen, J., and Poulsen, C. 1991. A nondestructive method for peptide bond conjugation of antigenic haptens to a diphtheria toxoid carrier, exemplified by two antisera specific to acetolactate synthase. *Anal. Biochem.* 198:318-323.
- Mendez, E., Moreno, A., Colilla, F., Palaez, F., Limas, G. G., Mendez, R., Soriano, F., Salinas, M., and de Haro, C. 1990. Primary structure and inhibition of protein synthesis in eukaryotic cell-free system of a novel thionin,  $\gamma$ -hordothionin, from barley endosperm. *Eur. J. Biochem.* 194:533-539.
- Mikkelsen, J. D., Berglund, L., Nielsen, K. K., Christiansen, H., and Bojsen, K. 1992. Structure of endochitinase genes from sugar beets. Pages 344-353 in: *Advances in Chitin and Chitosan*. C. J. Brine, P. A. Sandford, J. P. Zikakis eds. Elsevier Applied Science, London and New York.
- Monila, A., Segura, A., and Garcia-Olmedo, F. 1993. Lipid transfer proteins (nsLTPs) from barley and maize leaves are potent inhibitors of bacterial and fungal plant pathogens. *FEBS Lett.* 316:119-122.
- Nielsen, K. K., Mikkelsen, J. D., Kragh, K. M., and Bojsen, K. 1993. 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.
- Nielsen, K. K., Bojsen, K., Roepstorff, P., and Mikkelsen, J. D. 1994a. A hydroxyproline-containing class IV chitinase of sugar beet is glycosylated with xylose. *Plant Mol. Biol.* 25:241-257.
- Nielsen, K. K., Jørgensen, P., and Mikkelsen, J. D. 1994b. Antifungal activity of sugar beet chitinase against *Cercospora beticola*: An autoradiographic study on cell wall degradation. *Plant Pathol.* 43:979-986.
- Nielsen, K. K., Bojsen, K., Collinge, D. B., and Mikkelsen, J. D. 1994c. Induced resistance in sugar beet against *Cercospora beticola*: Induction by dichloroisonicotinic acid is independent of chitinase and  $\beta$ -1,3-glucanase transcript accumulation. *Physiol. Mol. Plant Pathol.* 45:89-99.
- Rathaiah 1976. Infection of sugar beet by *Cercospora beticola* in relation to stomatal condition. *Phytopathology* 66:737-740.
- Robson, G. D., Wiebe, M. G., and Trinci, A. P. J. 1991. Involvement of  $\text{Ca}^{2+}$  in the regulation of hyphal extension and branching in *Fusarium graminearum* A 3/5. *Exp. Mycol.* 15:263-272.
- Sambrook, J., Fritsch, E. F., and Maniatis, T. 1989. *Molecular Cloning. A Laboratory Manual*. 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
- Schägger H., and von Jagow, G. 1987. Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* 166:368-379.
- Singh, A., Ai, Y., and Kao, T. 1991. Characterization of ribonuclease activity of three S-allele-associated proteins of *Petunia inflata*. *Plant Physiol.* 96:61-68.
- Smith, G. A., and Ruppel, E. G. 1979. Registration of FC 606 and FC 606 CMS sugar beet germplasm. *Crop Sci.* 19:300.
- Steinkamp, M. P. Martin, S. S., Hoefert, L. L., and Ruppel, E. G. 1979. Ultrastructure of lesions produced by *Cercospora beticola* in leaves of *Beta vulgaris*. *Physiol. Plant Pathol.* 15:13-26.
- Stiekema, W. J., Heidekamp, F., Dirkse, W. G., van Beckum, J., de Haan, P., ten Bosch, C., and Louwerse, J. D. 1988. Molecular cloning and analysis of four potato tuber mRNAs. *Plant Mol. Biol.* 11:255-269.
- Terras, F. R. G., Schoofs, H. M. E., De Bolle, M. F. C., van Leuven, F., Rees, S. B., Vanderleyden, J., Cammue, B. P. A., and Broekaert, W. F. 1992. Analysis of two novel classes of plant antifungal proteins from radish (*Raphanus sativus* L.) Seeds. *J. Biol. Chem.* 267:15301-15309.
- Terras, F. R. G., Torrekens, S., van Leuven, F., Osborn, R. W., Vanderleyden, J., Cammue, B. P. A., and Broekaert, W. F. 1993. A new family of basic cysteine-rich plant antifungal proteins from Brassicaceae species. *FEBS Lett.* 316:233-240.
- van Loon, L. C. 1985. Pathogenesis-related proteins. *Plant Mol. Biol.* 4:111-116.
- van Parijs, J., Broekaert, W. F., Goldstein, I. J., and Peumans, W. J. 1991. Hevein: an antifungal protein from rubber-tree (*Hevea brasiliensis*) latex. *Planta* 183:258-264.
- Vigers, A. J., Roberts, W. K., and Selitrennikoff, C. P. 1991. A new family of plant antifungal proteins. *Mol. Plant-Microbe Interact.* 4:315-323.