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Characterization of a New Antifungal Chitin-Binding Peptide from Sugar Beet Leaves

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The intercellular washing fluid (IWF) from leaves of sugar beet (*Beta vulgaris* L.) contains a number of proteins exhibiting in vitro antifungal activity against the devastating leaf pathogen *Cercospora beticola* (Sacc.). Among these, a potent antifungal peptide, designated IWF4, was identified. The 30-amino-acid residue sequence of IWF4 is rich in cysteines (6) and glycines (7) and has a highly basic isoelectric point. IWF4 shows homology to the chitin-binding (hevein) domain of chitin-binding proteins, e.g. class I and IV chitinases. Accordingly, IWF4 has a strong affinity to chitin. Notably, it binds chitin more strongly than the chitin-binding chitinases. A full-length IWF4 cDNA clone was obtained that codes for a preproprotein of 76 amino acids containing an N-terminal putative signal peptide of 21 residues, followed by the mature IWF4 peptide of 30 residues, and an acidic C-terminal extension of 25 residues. IWF4 mRNA is expressed in the aerial parts of the plant only, with a constitutive expression in young and mature leaves and in young flowers. No induced expression of IWF4 protein or mRNA was detected during infection with *C. beticola* or after treatment with 2,6-dichloroisonicotinic acid, a well-known inducer of resistance in plants.

Plants produce a large number of defense-related proteins believed to be important in protecting them against pathogen infection. Many of the hitherto characterized proteins belong to the group of so-called pathogenesis-related proteins, a heterogeneous group separable into at least five different families and induced in many plants during infection with viral, bacterial, or fungal pathogens (Cutt and Klessig, 1992). A prominent and extensively studied family of pathogenesis-related proteins are the chitinases (EC 3.2.1.14), which have been shown to be capable of inhibiting fungal growth by the degradation of chitin in growing fungal hyphae (for a review, see Collinge et al., 1993). At least six classes of plant chitinases have been proposed on the basis of their primary structure (Meins et al., 1994). Of these, classes I and IV, as well as some members of class V, contain an N-terminal chitin-binding domain of approximately 40 amino acid residues containing 8 conserved Cys residues and separated from the catalytic domain by a variable hinge region. The chitin-binding class I chitinases show stronger specific activity on chitin, a β-1,4-linked polymer of GlcNAc, and possess significantly stronger in vitro antifungal activity than their non-chitin-binding counterparts, the class II chitinases (Legrand et al., 1987; Sela-Buurlage et al., 1993). These superior activities are presumably due to the ability of class I chitinases to both bind and hydrolyze the substrate.

The capability of binding chitin has been found for a wide array of otherwise unrelated plant proteins, including wheat germ agglutinin and other *Gramineae* lectins, hevein, wound-inducible proteins, stinging nettle lectin, and *Solanaceae* lectins (reviewed by Raikhel et al., 1993). These proteins contain one or more copies of the chitin-binding domain, the so-called "hevein" domain, with the eight conserved Cys residues involved in four intradomain disulfide bridges. Most, if not all, of these proteins may be involved in plant defense, and antifungal properties have indeed been demonstrated for hevein (Van Parijs et al., 1991) and stinging nettle lectin (Broekaert et al., 1989). Finally, two nearly identical antimicrobial proteins, Ac-AMP1 and Ac-AMP2, have been isolated from seeds of *Amaranthus caudatus* (Broekaert et al., 1992). These peptides of 29 and 30 residues, respectively, lack the C-terminal part of the full domain, including two Cys residues, and thus make up truncated versions of the chitin-binding domain, although they are still capable of binding chitin.

We are investigating the interaction between sugar beet and the fungal pathogen *Cercospora beticola* (Sacc.), which causes leaf spot disease. From sugar beet leaves infected with *C. beticola* we have isolated several chitinases belonging to four different classes, of which only the chitin-binding class IV chitinase Ch4 shows good in vitro antifungal activity against the fungus (Mikkelsen et al., 1992; Nielsen et al., 1993, 1994a, 1994b, 1994c; Berglund et al., 1995; Susi et al., 1995). In addition, we have isolated two small (46 amino acids) Cys-rich antifungal proteins, AX1 and AX2 (Kragh et al., 1995), which are related to the plant defensins (Broekaert et al., 1995). Presently we are engaged in isolating and characterizing antifungal proteins located in the intercellular space of sugar beet leaves. From this environment we have recently identified two proteins, IWF1 and IWF2, that show homology to the family of nonspecific lipid transfer proteins (Nielsen et al., 1996a). In the present paper we report the purification and characterization of a new highly potent antifungal peptide from the IWF. This peptide, designated IWF4, shows homology to the chitin-binding domains present in many plant proteins.

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Abbreviations: IC_{50}, concentration producing 50% growth inhibition; INA, 2,6-dichloroisonicotinic acid; IWF, intercellular washing fluid; RACE, rapid amplification of cDNA ends; RP, reverse-phase.

The highest level of homology is found with the Ac-AMP peptides from *Amaranthus*, which are the only hitherto reported truncated chitin-binding peptides. We also describe the cloning and characterization of a cDNA clone encoding IWF4. Furthermore, we report the tissue specificity and expression patterns of IWF4, which are strikingly different from those of the Ac-AMPs.

MATERIALS AND METHODS

Biological Materials and Bioassay

For the purification of antifungal proteins, plants of sugar beet (*Beta vulgaris* L. cv Monova) were grown in growth chambers as described previously (Nielsen et al., 1993). The inoculation with *Cercospora beticola* Sacc. isolate "FC573" (the gift of Dr. Earl G. Ruppel, U.S. Department of Agriculture, Fort Collins, CO) and chemical induction of resistance by treatment with INA (kindly supplied by Dr. Helmut Kessmann, Plant Protection Division, Ciba, Basel, Switzerland) were performed as described previously (Nielsen et al., 1993, 1994a).

The antifungal activity of protein fractions against spore cultures of *C. beticola* was assessed using the microtiter plate bioassay described previously, in which the growth of submerged fungal cultures is measured as an increase in A_{620} (Nielsen et al., 1993). The growth inhibition was also analyzed microscopically.

Purification of Extracellular Antifungal Protein IWF4

IWF was isolated from 500 to 700 g of leaves of 6-week-old sugar beet plants as described previously (Kragh et al., 1995) and purified as follows. In the initial step the IWF was fractionated by cation-exchange chromatography on a 10-mL CM-Sepharose column (Pharmacia LKB) equilibrated in 20 mM acetic acid, pH 4.5, at 4°C. The flow rate was 25 mL/h. After extensive washing with the Hac buffer, bound proteins were eluted in a stepwise manner by increasing the salt concentration in the starting buffer: 0.1, 0.3, and 0.5 M NaCl. Subsequently, discrete protein peaks detected at 280 nm were tested for antifungal activity against *C. beticola*. The 0.1 M fraction contained strong antifungal activity and was further fractionated by gel filtration on a Sephadex G-75 column (Pharmacia LKB; 2.5 × 70 cm) equilibrated in 50 mM Mes, pH 6.0, with a flow rate of 20 mL/h. Fractions of 10 mL were collected. Protein fractions containing antifungal activity were further purified by cation-exchange chromatography on a Mono-S fast protein liquid chromatography column (Pharmacia LKB; HR 5/5) equilibrated in 50 mM Mes, pH 6.0, containing 5% (w/v) betain (buffer A). After washing, bound proteins were eluted with a linear gradient from 0 to 0.5 M NaCl in 30 mL of the buffer A at a flow rate of 1 mL/min. Discrete protein peaks were subjected to a final purification by RP-HPLC on a Vydac C_4 column (The Separations Group, Hesperia, CA). The solvent system was: A, 0.1% (v/v) TFA in water; and B, 0.1% (v/v) TFA in acetonitrile. Proteins were eluted with a linear gradient from 5 to 45% of the buffer B from 0 to 18 min after sample loading, followed by 60% buffer B for 2 min. The flow rate was 0.7 mL/min. Protein was detected at 214 and 280 nm. Discrete protein peaks were freeze-dried, washed twice with water, freeze-dried again, and then resolved in water for the analysis of purity and antifungal activity.

Amino Acid Analyses, Protein Determination, and MS

Amino acid composition and protein concentration of purified IWF4 protein were determined according to the method of Barkholt and Jensen (1989) to obtain a correlation coefficient between UV absorbance (280 nm) and protein concentration, which would be used to estimate the IWF4 concentration in homogenous samples, as described previously (Kragh et al., 1995). Protein concentrations in nonhomogenous samples were determined according to the method of Bradford (1976), using a protein assay kit (Bio-Rad) with γ-globulin as the standard. Laser desorption MS was performed according to the method of Vorm and Roepstorff (1994). The IWF4 protein was carboxymethylated and subjected to RP-HPLC on a Vydac C_4 column, after which the entire amino acid sequence was determined by N-terminal sequencing as described previously (Nielsen et al., 1993).

SDS-PAGE, Antibodies, and Immunoblotting

Separation of proteins by Tricine SDS-PAGE (16.6% [w/v] gels; Schägger and von Jagow, 1987) on the "Mighty Small" system (Hoefer Scientific, San Francisco, CA) was performed as described previously (Nielsen et al., 1993). IEF was performed on the Phast system (Pharmacia LKB) as described previously (Nielsen et al., 1994b). For antibody production in rabbits, IWF4 was conjugated to diphtheria toxoid according to the method of Marcussen and Poulsen (1991) before immunization. Proteins separated by Tricine SDS-PAGE were immunoblotted using a semidry blotting system (JKA-Biotech, Copenhagen, Denmark) (Kyhse-Andersen, 1984) as described previously (Nielsen et al., 1993). Blots were incubated overnight with the rabbit anti-IWF4 antibodies (diluted 1:200) and visualized using alkaline phosphatase-conjugated secondary antibody (pig anti-rabbit IgG; Dakopatts, Copenhagen, Denmark) and nitroblue tetrazolium (Kyhse-Andersen, 1984).

Chitin-Binding Activity

The ability of purified IWF4 protein to bind chitin was investigated. Regenerated chitin was prepared as described by Molano et al. (1977) and modified by Kragh et al. (1990). A 2-mL chitin column was equilibrated in 10 mM Tris-HCl, pH 8. After 100 μg of homogenous IWF4 protein was loaded and incubated for 1 h at 4°C, the column was washed with the starting buffer and eluted with 20 mM acetic acid at stepwise, decreasing pH values: 3.2, 2.8, and 2.0, 10 mL of each. The eluates were subjected to RP-HPLC on a Vydac C_4 column as described above. Finally, the presence of IWF4 protein was analyzed by immunoblotting and MS.

Cloning of cDNA

A cDNA clone encoding IWF4 was generated by a combination of 3′ and 5′ RACE. The template was mRNA from sugar beet leaves infected with *C. beticola* at d 6 after

inoculation. For this, total RNA was isolated according to the method of Collinge et al. (1987).

3′ RACE

First-strand cDNA synthesis followed by two consecutive PCR amplifications were performed as described previously (Nielsen et al., 1993). Oligonucleotide number 403 (5′-GAAGGATCCGTCGACATCGATAATACGACTGAATTCGGGA$(T)_{17}$-3′), comprising three restriction sites (*Bam*HI, *Sal*I, and *Cla*I) followed by an oligo(dT), was used as a primer in reverse transcription, whereas primer number 401 (5′-AAGGATCCGTCGACATC-3′; *Bam*HI and *Sal*I) and nested primer no. 402 (5′-GACATCGATAATACGAC; *Cla*I) were used as downstream primers in the first and second PCR amplification, respectively. The degenerated upstream primer UP1 (CGCTCTAGAGA(AG)TG(TC)AA-(CT)ATGTA(TC)GG, position 135 to 151 in the cDNA shown in Fig. 7), extended with nine bases containing an *Xba*I site, was used in the first PCR amplification and either of the two nested primers UP2A (CGCCTGCAGATG-TA(TC)GG(TCAG)CG(TCAG)TG(TC)CC, position 144–160, *Pst*I) and UP2B CGCCTGCAGATGTA(TC)GG(TCAG)AG-(GA)TG(TC)CC, same position, *Pst*I) was used in the second PCR amplification. The annealing temperature was 42°C in both amplifications. UP2 was split into the two primers A and B with 64 and 32 permutations, respectively, to increase the specificity of the reactions. After the second PCR, distinct DNA products were digested with *Cla*I/*Pst*I, cloned into pBluescript SK+, and sequenced according to the method of Sanger et al. (1977).

5′ RACE

The 5′ end of the IWF4 mRNA was obtained by 5′ RACE essentially as described previously (Nielsen et al., 1994b) using the 5′ RACE system from GIBCO-BRL. One microgram of total RNA and 2 pmol of a gene-specific downstream primer GSP1 (AAGCATGTAGCAAGCTAGGC, position 372–353 in the cDNA sequence in Fig. 7) were used for reverse transcription. Two PCR amplifications on the dC-tailed first-strand cDNA were performed as described previously (Nielsen et al., 1994b), using the nested gene-specific primers GSP2 (CTTATTAGGTAACCCATGCC, position 340–321) and GSP3 (TGCCATTACGATTACCT-GGC, position 324–305) in the first and second amplifications, respectively. The annealing temperature was 55°C in both amplifications. After the second PCR amplification, distinct DNA products were cloned into pT7Blue (Novagen, Madison, WI) and sequenced.

Southern and Northern Blotting Hybridization

From sugar beet leaves of cv Monova, genomic DNA was extracted according to the method of Dellaporte et al. (1983) and total RNA was extracted according to the method of Collinge et al. (1987). Southern and northern blotting hybridizations were performed as described previously (Nielsen et al., 1993) using a 324-bp ^{32}P-labeled IWF4 cDNA clone obtained by 5′ RACE as a probe in both hybridizations.

RESULTS

Purification and Biochemical Characterization of IWF4

The starting material for the purification of antifungal proteins from sugar beet leaves was the IWF exerting antifungal activity against *C. beticola*. Purification of the basic protein fraction was performed by chromatographic procedures and after each purification step antifungal subfractions were identified using the in vitro growth inhibition bioassay. The 0.3 M NaCl eluate from the CM-Sepharose column has previously been shown to contain the antifungal proteins IWF1 and IWF2 (Nielsen et al., 1996a). The 0.1 M NaCl eluate also possessed strong antifungal activity and was further purified by gel filtration followed by cation-exchange chromatography on a Mono-S column; here, all of the antifungal activity was confined to the split peak 1 (Fig. 1). In the final purification by RP-HPLC this peak was separating into two distinct closely migrating protein peaks, A and B (Fig. 1, inset). The subsequent analysis of peaks A and B by MS revealed that they were both homogenous proteins with molecular masses of 3185 ± 1 and 3201 ± 1 D, respectively. The homogeneity was also confirmed by SDS-PAGE, which showed no other protein bands following silver staining (Fig. 2, lane 1), and by amino acid sequencing.

After carboxymethylation and RP-HPLC the complete amino acid sequence of both protein peaks was determined by N-terminal sequencing. Proteins A and B proved to be identical peptides of 30 amino acid residues. Their slightly different mobility during chromatography and their difference in molecular mass of 16 D, as determined by MS, is presumably due to the presence of an oxidized residue in protein B, which is potentially the single Met in position 6 (Fig. 3). The sequence of this peptide, termed IWF4, was identical with that derived from the cDNA clone encoding IWF4 (see below, Fig. 7) and in full agreement with the

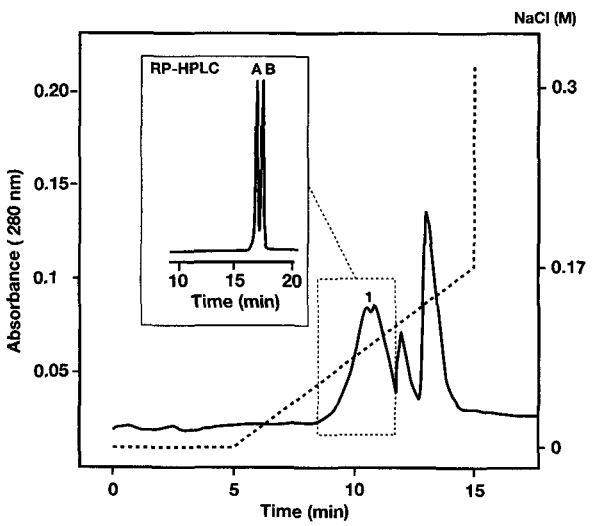

Figure 1. Purification of IWF4 by cation-exchange fast protein liquid chromatography followed by RP-HPLC. The split peak 1 in the eluate from the cation-exchange column making up IWF4 separates into isoforms A and B after RP-HPLC (inset).

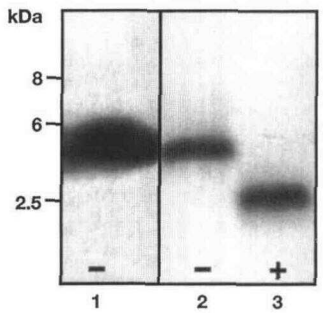

Figure 2. SDS-PAGE analysis and immunodetection of the purified IWF4. *Lane 1, Silver-stained gel; lanes 2 and 3,* immunoblotting using antibody against IWF4. + and −, With and without reduction by DTT treatment. One microgram (lane 1) or 250 ng (lanes 2 and 3) of protein was loaded in each lane.

amino acid compositional analysis (data not shown). The molecular mass of IWF4 was calculated from the sequence using the algorithms of the PC/Gene software (IntelliGenetics, Mountain View, CA). If we assume that all six Cys residues in IWF4 form disulfide bridges and that the Met is oxidized in isoform B, the calculated molecular masses for the two isoforms are (3190 − 6) = 3184 D (A) and (3190 − 6 + 16) = 3200 D (B). Thus, the calculated molecular masses coincide with those determined by MS.

By SDS-PAGE, the IWF4 protein (both isoforms) showed an apparent mass of 2.5 kD under reducing conditions (Fig. 2, lane 3), which is in agreement with the MS data. On the other hand, under nonreduced conditions a higher molecular mass band of approximately 4.5 kD was observed (Fig. 2, lanes 1 and 2).

It was not possible to focus IWF4 on an IEF gel with a pH range between 3.0 and 9.0. This result is in agreement with a predicted pI of 9.7 calculated from the sequence data (PC/Gene), assuming that all Cys residues participate in disulfide linkages.

The antibody raised against IWF4 after conjugation of the protein to diphtheria toxoid recognized the protein in its reduced as well as nonreduced stage (Fig. 2, lanes 2 and 3).

No or very little IWF4 protein could be detected on western blots of crude leaf homogenates or IWFs loading 20 μg of protein, either in unstressed control plants or during infection with *C. beticola* or treatment with INA (data not shown). In agreement with this apparent very low, noninducible expression of IWF4 protein, the total amount of IWF4 protein that could be purified from the IWF of 700 g of leaves never exceeded 100 μg.

Homology to Chitin-Binding Proteins

The amino acid sequence of IWF4 showed the highest level of homology to the two antimicrobial proteins Ac-AMP1 and Ac-AMP2, previously isolated from seeds of *A. caudatus* (Broekaert et al., 1992). Ac-AMP1 differs from the Ac-AMP2 of 30 residues by only the lack of the C-terminal Arg. When a gap of one amino acid was allowed in Ac-AMP2 for optimal alignment, 66% identity was found between IWF4 and Ac-AMP2 (Fig. 3). Notably, the 6 Cys and the 7 Gly residues were conserved in these two proteins. IWF4 also showed homology to Cys-rich domains of chitin-binding proteins, e.g. the N-terminal chitin-binding domain of class I and IV chitinases. The highest degree of similarity, 55% identity, was found for a basic class I chitinase from tobacco (Shinshi et al., 1990), in which 4 of the Cys and 5 of the Gly residues in IWF4 could be aligned (Fig. 3).

Chitin-Binding Activity

Because of its homology to chitin-binding proteins the ability of IWF4 to bind chitin was investigated. When IWF4 was applied to the chitin column at pH 8.0, none of the protein was found in the run off/wash fractions or in the eluate at pH 3.2, as analyzed by RP-HPLC. Less than 50% of the IWF4 protein was eluted at pH 2.8, whereas the residual fraction was detected in the eluate at pH 2.0 (Fig. 4). Thus, IWF4 bound very strongly to chitin. The identity of the IWF4 protein peaks was confirmed by MS and immunoblotting (data not shown).

Antifungal Activity

A strong growth-inhibiting activity of IWF4 was found against spore cultures of *C. beticola* using the in vitro bioassay, and the inhibitory effect increased with increasing protein concentrations. As measured by the increase in A_{620}, detectable growth started approximately 40 h after the start of incubation in the control culture, whereas the addition of IWF4 caused a delay in detectable growth start and subsequently a somewhat slower initial growth rate (Fig. 5). Two micrograms per milliliter of IWF4 (0.7 μM) was sufficient to give ≥50% growth inhibition (IC_{50}) after 80 h of incubation time.

The morphology of the restricted fungal hyphae was also altered upon treatment with IWF4. Compared with the control culture, shorter and somewhat more branched hyphae were observed when IWF4 was included in the growth medium (Fig. 6). In the assay shown in Figures 5 and 6, the nonoxidized form of IWF4 was used. However, in comparative assays oxidized and nonoxidized IWF4 showed the same level of activity (data not shown). The growth-inhibiting activity of IWF4 was affected by the presence of calcium ions. When $CaCl_2$ was added to the growth medium (final concentration 5 mM), a significantly weaker inhibition was observed, whereas the presence of similar concentrations of NaCl had no adverse effect on the antifungal potential of IWF4 (results not shown).

The antifungal potential of IWF4 was also tested in dual combinations with other sugar beet antifungal proteins:

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IWF4          SGECNMYGRCPPGYCCSKFGYCGVGRAYCG                    30
Ac-AMP2       V***VR-****S*M***Q*****K*PK***R                   30
CHIT-TOB   EQC*SQAGGA***S*L******W**NTND***PGNCQSQCP...         41
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Figure 3. Comparison of the amino acid sequence of IWF4 with Ac-AMP2 from seeds of *A. caudatus* (Broekaert et al., 1992) and the N-terminal Cys-rich domain of class I tobacco chitinase (Shinshi et al., 1987). To optimize the alignment one gap (–) was introduced in Ac-AMP2. Amino acids identical with those in IWF4 are indicated by asterisks.

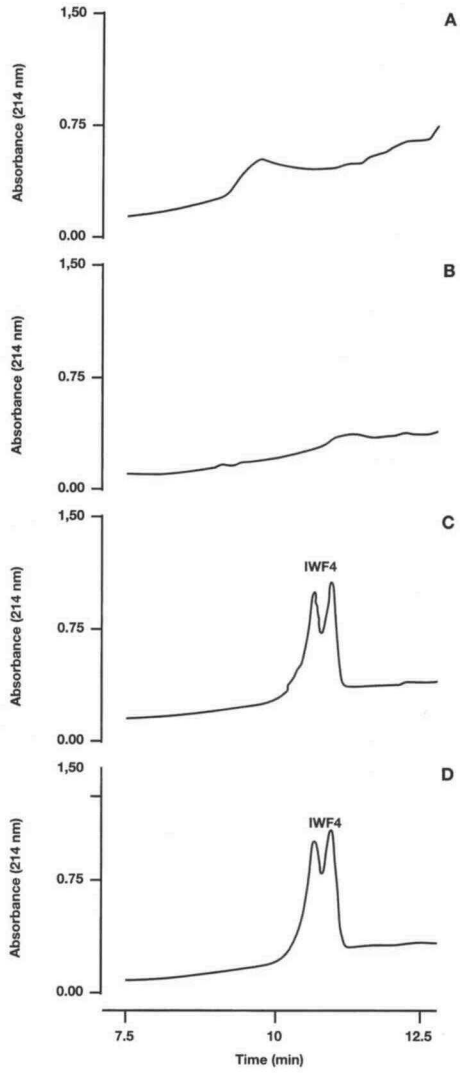

Figure 4. Chitin-binding activity of IWF4 analyzed by RP-HPLC. Homogenous IWF4 protein (100 μg) was loaded onto a 2-mL chitin column at pH 8.0, followed by elution at stepwise decreasing pHs. The eluates were subjected to RP-HPLC. A, 10 mM Tris-HCl, pH 8.0 (run off/wash); B to D, 20 mM acetic acid at pH 3.2 (B), pH 2.8 (C), and pH 2.0 (D).

AX2, IWF1, and IWF2 (Kragh et al., 1995; Nielsen et al., 1996a). These assays demonstrated additive but not synergistic effects in all combinations (results not shown).

Isolation of IWF4 cDNA

A full-length IWF4 cDNA was obtained by 3′ RACE followed by 5′ RACE on mRNA isolated from *Cercospora*-infected sugar beet leaves.

3′ RACE

A single, distinct DNA product of approximately 420 bp was visualized on an agarose gel after the first PCR amplification using the degenerated upstream primer UP1 derived from the amino acid sequence of the IWF4 protein,

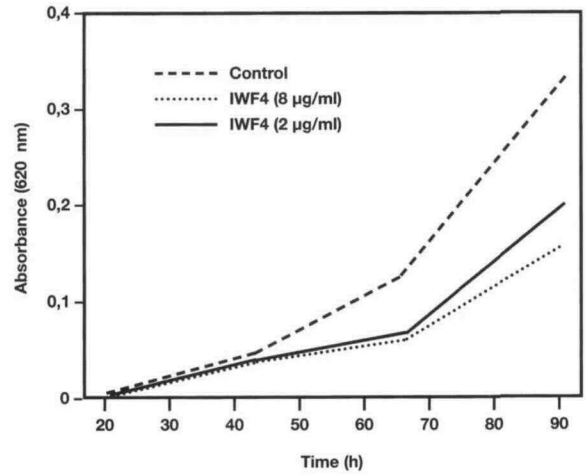

Figure 5. Antifungal activity of IWF4 against spore cultures of *C. beticola*. The fungal growth was measured as an increase in A_{620}.

and this product was further amplified in the second PCR using either of the nested primers UP2A and UP2B. The product obtained using UP2A was subsequently digested, cloned into pBluescript SK+, and completely sequenced in both directions. The translated amino acid sequence of this 408-bp clone, comprising nucleotides 144 to 551 of the IWF4 cDNA sequence (Fig. 7), was identical with the sequence of the IWF4 protein. Furthermore, the amplified cDNA clone contained a stop codon for mRNA translation and a polyadenylation site, confirming that the complete 3′ end of the IWF4 cDNA had been obtained.

5′ RACE

The two successive PCR amplifications on the 5′ RACE products, using IWF4-specific downstream primers located outside the stop codon identified in the 3′ RACE clone, generated one distinct DNA product. This product was cloned into pT7 Blue and sequenced in both directions. The product (Fig. 7, nucleotides 1–324) contained 143 bp up-

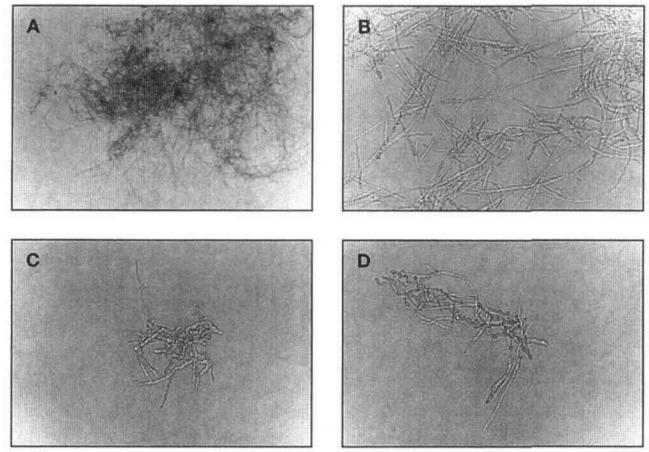

Figure 6. Morphology of *C. beticola* grown for 4 d with buffer (A and B) or 8 μg/mL IWF4 (C and D). Magnification: A, ×76; and B to D, ×294.

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ACTCAACAAATTCAGAAAAAAACAGAAGCAAAAAAAGTTTATTGAAAGAGTAAGTTGAGGTGAAA   65

ATGATGAAAAGCTTTGTGATAGTTATGTTGGTCATGTCCATGATGGTGGCTACATCTATGGCA  128
 M  M  K  S  F  V  I  V  M  L  V  M  S  M  M  V  A  T  S  M  A   21
    UP1→          ---------UP2A/B→-----
AGTGGTGAATGCAATATGTATGGTCGATGCCCCCCAGGGTATTGTTGTAGCAAGTTTGGCTAC  191
 S  G  E  C  N  M  Y  G  R  C  P  P  G  Y  C  C  S  K  F  G  Y   42

TGTGGTGTCGGACGCGCCTATTGTGGCGATGCTGAGCAGAAGGTTGAAGATCATCCATCTAAT  254
 C  G  V  G  R  A  Y  C  G  D  A  E  Q  K  V  E  D  H  P  S  N   63
                                                ←GSP3
GATGCTGATGTTCCTGAGTTTGTTGGAGCTGGTGCCCCATGATGCTCGAAGCCAGGTAATCGT  317
 D  A  D  V  P  E  F  V  G  A  G  A  P                           76
                                  ←GSP1
AATGGCATGGGTTACCTAATAAGTAAACTCATTGTGCCTAGCTTGCTACATGCTTATCCACTA  380
←GSP2
TAAATAAGCTCCTACAGGAGTTGTGTTTTTCTTTTAATTTTGTAATCAAGGGTTTGACTTTAA  443

TTAATGAGACCAATGTATACTTGCATGTCGGATAAATATATGAACTAAGCCACTCGTATTGGT  506

TTATTATAAAACTACTATAAAAAAAAAAAAAAAAAAAAAAAAAAA                    551
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Figure 7. cDNA sequence and deduced amino acid sequence of IWF4. Start and stop codons for mRNA translation and putative polyadenylation signal are marked by bold letters. The amino acid sequence identical with the mature IWF4 protein is underlined. The locations of the degenerate primers used for 3′ RACE (UP1 and UP2) and the gene-specific primers used for 5′ RACE (GSP1-3) are marked with arrows indicating polarity 5′ to 3′.

stream of the IWF4 sequence obtained by 3′ RACE, followed by 181 bp, showing 100% identity with the 3′ RACE clone. The 5′ RACE product was inferred to contain the entire IWF4 cDNA sequence due to the presence of ATG start (Fig. 7, position 66) and TGA stop codons (position 294) for mRNA translation, as well as a characteristic adenosine-rich sequence upstream of the start codon (Kozac, 1984; Lütcke et al., 1987). In agreement with this, the translated amino acid sequence contained the entire IWF4 protein.

Characterization of the IWF4 cDNA and Deduced Amino Acid Sequence

The obtained full-length IWF4 cDNA contained 551 bp, with an open reading frame of 228 bp. The 5′ adenosine-rich region of 65 bp was followed by an ATG start codon at position 66, a TGA stop codon at position 294, and a 3′ noncoding region of 254 bp, containing a polyadenylation signal at position 383 and a polyadenylation site at position 525 (Fig. 7).

A polypeptide chain of 76 amino acid residues was encoded by the IWF4 cDNA. From this it is inferred that IWF4 is encoded as a preproprotein containing an N-terminal putative signal peptide of 21 residues processing all the characteristics of signal sequences (von Heijne, 1983), followed by the mature IWF4 protein of 30 residues and a C-terminal extension of 25 residues. The C-terminal prosequence differed from the mature IWF4 protein by the lack of Cys residues (6 in IWF4) and by its acidic nature, with a predicted pI of 3.8 (pI 9.7 for IWF4).

The primary structure of the IWF4 preproprotein deduced from the cDNA sequence was comparable to that of Ac-AMP2 (De Bolle et al., 1993). Both sequences contain three domains: the putative N-terminal signal peptide, followed by the mature protein domain, and a C-terminal peptide. The entire IWF4 preprosequence was 10 amino acid residues shorter than Ac-AMP because of shorter N-terminal signal peptide and C-terminal peptide domains (25 and 31 amino acid residues, respectively, in Ac-AMP2). No sequence similarities were found between the N-terminal signal peptide and C-terminal peptide domains of the two proteins. Notably, however, the N-terminal signal peptide of both IWF4 and Ac-AMP2 are rich in Met residues (7 in both). The calculated pIs of the mature protein domains are comparable, showing pIs of 9.7 and 10.9 for IWF4 and Ac-AMP2, respectively, whereas the C-terminal peptide domain of Ac-AMP2 differs from the IWF4 C-terminal peptide in having a slightly basic pI of 7.8, in contrast to the highly acidic pI of the IWF4 C-terminal peptide. Finally, a putative N-glycosylation site present in the C-terminal peptide of Ac-AMP2 (De Bolle et al., 1993) was not found in IWF4.

Genomic Diversity

Genomic DNA of sugar beet cv Monova was digested with the three restriction enzymes *Hin*dIII, *Eco*RI, and *Xba*I, of which only *Hin*dIII cuts within the IWF4 cDNA (Fig. 7, position 74). Southern blots using these digests were probed with the 324-bp IWF4 cDNA clone obtained by 5′ RACE, followed by a high-stringency wash. Two to three strongly hybridizing fragments were present in all three digests, and the *Eco*RI digest also contained a few weakly hybridizing fragments (Fig. 8). The smallest fragment (800 bp) was found in the *Hin*dIII digest, confirming the presence of a recogni-

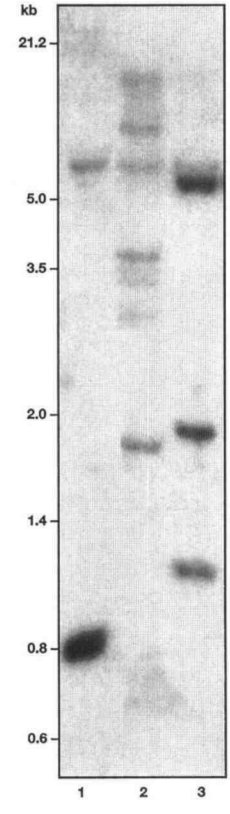

Figure 8. Southern blots. Genomic DNA from sugar beet cv Monova was digested with *Hin*dIII (lane 1), *Eco*RI (lane 2), and *Xba*I (lane 3), probed with a 324-bp IWF4 cDNA clone, and washed at high stringency.

tion site for this enzyme within the gene. The presence of more than one strongly hybridizing fragment in all digests may indicate either that the fragments carry the IWF4 gene in alternate loci or that they make up a small gene family encoding two or a few closely related isoforms. A parallel genomic Southern blot using DNA from another sugar beet cultivar, +Tol (Danisco Seed; Nielsen et al., 1993), gave the same hybridization pattern as seen for cv Monova (not shown), demonstrating that no polymorphism exists between the two cultivars with respect to this gene.

Expression of IWF4 mRNA

The expression of IWF4 mRNA in different organs and tissues of sugar beet plants of cv Monova was analyzed using RNA blots. The analysis included leaves and petioles (young and mature), young and mature flowers, and central and outer root tissue, all tissues being isolated from nonstressed 6-week-old plants. A high accumulation of IWF4 mRNA was found in the leaf tissue, both in young and mature leaves (Fig. 9, lanes 4 and 7), whereas in the petioles a very low level of mRNA expression was observed in young petioles only (Fig. 9, lane 1). Expression of IWF4 mRNA was also found in flower tissue, with a fairly high level of transcript accumulation in young flowers (Fig. 9, lane 6) and a very low, barely detectable accumulation in mature flowers (Fig. 9, lane 5). No expression of IWF4 mRNA was detected in root tissue (Fig. 9, lanes 2 and 3).

Time-course analyses were performed to investigate the accumulation of IWF4 transcript in mature leaves of 6-week-old plants after treatment with INA and/or during infection with *C. beticola*. In these experiments a constitutive level of mRNA accumulation was found in nontreated leaves, whereas no increased expression was observed following inducer treatment or fungal infection (results not shown).

To summarize, IWF4 transcripts accumulated only in the aerial parts of the plant. A strong, constitutive, noninducible mRNA accumulation was found in the leaves regardless of age, and additional expression was observed in young flowers.

DISCUSSION

IWF4 was purified to homogeneity from the IWF of sugar beet leaves. IWF4 shows homology to the Cys-/Gly-rich domain of chitin-binding proteins, of which the majority contain single or multiple chitin-binding domains of 40 to 43 residues, with 8 Cys residues involved in four intradomain disulfide bridges. Compared with these, the 30-amino-acid residue sequence of IWF4 lacks the C-terminal part of the "full" domain, including the two terminal Cys residues that form a separate disulfide bridge (Beintema, 1994). Furthermore, the distance between the two N-terminal Cys residues is smaller in IWF4. In spite of these deletions IWF4 shows a strong chitin-binding activity. Indeed, the present study demonstrated that IWF4 binds even more strongly to chitin than does the class IV sugar beet chitinase Ch4, which is readily elutable at pH 3.2 (Mikkelsen et al., 1992). Other chitinases, in both classes I and IV, can be released from the chitin affinity column at pH 3.2 (Boller et al., 1983; Broekaert et al., 1988; Jacobsen et al., 1990; Rasmussen et al., 1992; Kragh et al., 1993; Sela-Buurlage et al., 1993).

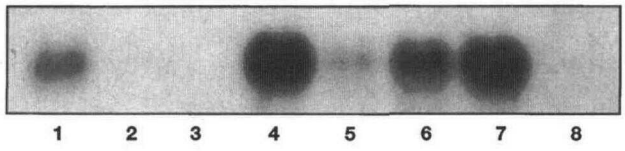

Figure 9. RNA blots. Fifteen micrograms of total RNA, isolated from different sugar beet tissues, was loaded in each lane. The blot was probed with a 324-bp IWF4 cDNA clone and washed at high stringency. Tissue: Lane 1, petiole (young leaf); lanes 2 and 3, root (central and outer tissue, respectively); lane 4, young leaf; lanes 5 and 6, flower (mature and young, respectively); lane 7, mature leaf; and lane 8, petiole (mature leaf).

IWF4 shows the highest degree of similarity to the antimicrobial proteins Ac-AMP1 and Ac-AMP2 of 29 and 30 amino acid residues, respectively, isolated from seeds of amaranth (Broekaert et al., 1992). The Ac-AMPs are the only previously reported antimicrobial peptides consisting of a sole truncated chitin-binding domain (Raikhel et al., 1993). Recently, an antifungal protein Pn-AFP of 41 amino acids was isolated from seeds of *Pharbitis nil* (Koo et al., 1995). Pn-AFP represents a nontruncated version of the chitin-binding domain containing all 8 Cys residues and is thus more similar to the hevein of 43 amino acid residues (Van Parijs et al., 1991).

The apparent mass of IWF4 determined by SDS-PAGE, 4.5 kD in the nonreduced versus 2.5 kD in the reduced stage, may indicate that IWF4 is a dimeric protein. However, the result of the MS analysis, as well as the strong homology to thoroughly characterized chitin-binding domains, strongly indicates that all Cys residues are involved in intramolecular disulfide bridge formation, making dimer formation less likely. The low mobility of the unreduced protein in SDS-PAGE is probably instead due to the erratic behavior of such a small, compact, and basic molecule in the SDS-gel. A similar difference in mobility of unreduced versus reduced proteins was observed in the Ac-AMPs (Broekaert et al., 1992).

IWF4 exerts a strong growth-inhibiting activity against *C. beticola*, with an IC_{50} of ≤ 2 $\mu g/mL$ (0.7 μM) after 80 h of incubation time. This activity is comparable to that of the Ac-AMPs, with IC_{50} values varying between 1 and 10 $\mu g/mL$ (0.3–3 μM), depending on the fungal species (Broekaert et al., 1992). The Pn-AFP shows IC_{50} values in this range, varying from 0.6 to 18 $\mu g/mL$ (0.1–4 μM) when tested against eight different fungi (Koo et al., 1995). Notably, the antifungal potential of hevein is significantly weaker, showing IC_{50} values of 90 to 1250 $\mu g/mL$ (20–280 μM; eight fungi; Van Parijs et al., 1991). In addition the stinging nettle lectin, consisting of two chitin-binding domains, shows lower antifungal activity than the truncated, single-domain peptides, with IC_{50} values of 20 to 150 $\mu g/mL$ (2–18 μM; seven fungi; Broekaert et al., 1989). The observed calcium salt sensitivity of IWF4 is in agreement with observations in other families of small, Cys-rich antifungal proteins (including the Ac-AMPs), all showing reduced antifungal activity when tested in growth medium supplemented with $CaCl_2$ (Cammue et al., 1994).

The IWF4 cDNA encodes a preproprotein with N- and C-terminal sequences flanking the mature protein. The 21-amino-acid N-terminal sequence shows the characteristics of signal peptides facilitating protein secretion, which is in agreement with the presence of IWF4 in the IWF. The acidic C-terminal extension of 25 residues shows no homology to any published protein sequence (Swiss-Prot, release 31). The Ac-AMP2 cDNA encodes a C-terminal prosequence that shows little homology to that of IWF4. Moreover, the Ac-AMP2 C-terminal peptide is longer (31 amino acid residues), has a slightly basic pI, and contains an *N*-glycosylation site not present in the IWF4 C-terminal sequence. The functional roles of these prosequences are not known. Acidic C-terminal propeptides are also present in the family of type 1 thionins, which are small, Cys-rich antimicrobial proteins present in the endosperm of various monocotyledons (Florack and Stiekema, 1994). Florack et al. (1994) studied the expression of a hordothionin in tobacco using different constructs, some of which lacked the sequence encoding the acidic C-terminal peptide. They found that inclusion of the peptide enhanced the protein expression significantly but had no effect on the subcellular (intracellular) localization of the protein. The study indicated that the acidic peptide was not involved in sorting and suggested that it might facilitate transport through membranes (Florack et al., 1994). Thus, these propeptides may increase the stability of the mature protein during transport and processing. However, C-terminal prosequences of some other proteins have been shown to facilitate the transport of the proteins to the vacuole (Bednarek and Raikhel, 1992; Nakamura and Matsuoka, 1993).

Whereas the N- and C-terminal peptides of IWF4 show little homology to those of Ac-AMP2, the mature IWF4 and Ac-AMP proteins resemble each other in size, pI, amino acid sequence, chitin-binding ability, and antifungal potentials. However, striking differences exist with respect to tissue specificity and expression patterns. Our studies demonstrated a strong expression of IWF4 in vegetative, aerial tissue, i.e. leaves and flowers, whereas no root expression was observed. Furthermore, no pathogen- or INA-induced expression of IWF4 was found, either on mRNA or on the protein level. In contrast, the Ac-AMPs have been shown by northern blotting experiments to be seed-specific; they are expressed in near-mature seeds of amaranth only and have no homologs that are expressed in vegetative tissue, i.e. leaves and roots (De Bolle et al., 1993). Moreover, the expression of the Ac-AMPs is induced by pathogen infection and other stress factors. The genes encoding the hevein-like Pn-AFPs in *P. nil* show expression patterns very similar to the Ac-AMPs in that they are expressed during seed maturation and germination but not in the aerial vegetative parts of the plant (Koo et al., 1995).

Because of the apparent lack of induction by infection with *C. beticola* IWF4 cannot be regarded as a "classical" pathogenesis-related protein. We have not attempted to estimate the concentration of the IWF4 protein in the IWF. However, there is no doubt that the peptide is present in cv Monova at concentrations below those required for good antifungal activity, which is consistent with the high susceptibility of this cultivar to *C. beticola*.. Furthermore, IWF4 is not induced upon treatment with INA, suggesting that this peptide is not important for the resistance mediated by this chemical.

In spite of the low, noninducible levels of IWF4 found in the sugar beet leaves, the strong antifungal potential of the protein suggests that it has a role in plant defense. Moreover, immunohistochemical studies of *Cercospora*-infected leaf tissue indicated that IWF4, like AX2 (Kragh et al., 1995) and the antifungal chitinase Ch4 (Nielsen et al., 1996b), is primarily present in extracellular "protein bodies," presumably involved in defense (J. Nielsen, unpublished data). Whether, as has been suggested for the Ac-AMPs (Raikhel et al., 1993), IWF4 acts by simply "sticking" to nascent chitin fibers in the apices of growing fungal hyphae, thereby disturbing cross-linking and assembling of the fungal cell wall, still remains to be demonstrated. The strong chitin-binding affinity observed for IWF4, compared with the chitin-binding chitinases, may support this hypothesis. On the other hand, the sensitivity of both IWF4 and the Ac-AMPs to calcium salt does not point to an interaction with chitin for their antifungal action. We have investigated the influence of calcium on the chitin-binding activity of IWF4. At $CaCl_2$ concentrations 10 times higher than required for the complete abolishment of the growth-inhibiting activity of IWF4 the protein showed no reduced affinity to chitin (K.K. Nielsen, unpublished data). Finally, the strikingly different expression patterns of IWF4 and the Ac-AMPs may suggest different roles for these proteins in the plant. We are now in the progress of producing transgenic sugar beet plants overexpressing IWF4 to test its potential as an efficient agent for increasing resistance to *C. beticola*.

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