

Partial characterization and localization of a novel type of antifungal protein (IWF6) isolated from sugar beet leaves

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Abstract

An antifungal protein was isolated from the intercellular washing fluid (IWF) of leaves of sugar beet (*Beta vulgaris* L., cv. Monova) and purified to homogeneity. The protein, IWF6, comprising 37 amino acids with six cysteines, was able to inhibit the growth of the pathogen *Cercospora beticola* (Sacc.) in vitro, by 75% after 120 h of growth at a concentration of 20 µg ml⁻¹. The amino acid sequence data were used to generate a polymerase chain reaction (PCR) clone, employed for the isolation of a corresponding cDNA clone. The cDNA encodes a precursor protein with an N-terminal putative signal sequence of 45 amino acids, followed by the mature protein of 37 amino acids. Antibodies raised against a synthetic peptide covering the complete sequence of IWF6 were used in immunolocalization studies. The protein was recognized by the antibody in nearly all leaf cell types except epidermal cells. In necrotic tissue, the protein was mainly recognized on *C. beticola* hyphae growing in a 'pellet' (ball-like) structure. The hyphal 'pellets' are primarily located beneath the stomata. IWF6 shows less than 26% identity to any previously described protein. © 2000 Elsevier Science Ireland Ltd. All rights reserved.

Keywords: Antifungal protein; *Beta vulgaris*; *Cercospora beticola*

1. Introduction

Plants are constantly exposed to pathogenic attack by different microorganisms and in order to protect themselves from these attacks, they possess several defense mechanisms. One of these defense mechanisms is the production of proteins with antifungal activity. Several groups of antifungal proteins have been characterized so far: one group of antifungal proteins consists of hydrolytic enzymes like the chitinases [1–7] and the glucanases [8,9], while another group consists of small, cysteine-rich proteins [10–12].

We have investigated the production of small, cysteine-rich proteins in sugar beet which show good activity against the causal agent of the leaf spot disease, *Cercospora beticola*. Previously, we have identified a number of cysteine-rich proteins isolated from the intercellular washing fluid (IWF) of leaves from sugar beet. The proteins, belonging to the group of plant defensin-like proteins [13], non-specific lipid transfer proteins [14] and chitin-binding proteins [15], all showed strong antifungal activity in vitro against *C. beticola*. These proteins fall into three groups of antimicrobial proteins that vary with respect to size and cysteine-content. The plant defensins are 5 kDa proteins with eight cysteines, the chitin-binding proteins are 3.5 kDa proteins with six cysteines, while the non-specific lipid transfer proteins are 9–10 kDa proteins with

Abbreviations: FPLC, fast protein liquid chromatography; IWF, intercellular washing fluid; PAGE, polyacrylamide gel electrophoresis.

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eight cysteines [12]. They are all basic, with isoelectric points of 9 to 10, and inhibit the growth of various pathogens at low concentrations [10].

Here we report the isolation and characterization of a new type of antifungal protein. It is a 37 amino acid protein with six cysteines present and has an isoelectric point of 7. The protein shows no homology to any of the hitherto characterized antifungal proteins.

2. Materials and methods

2.1. Biological materials and bioassay

Plants of sugar beet (*Beta vulgaris* L., cv. Monova, Danisco Seed) were grown in growth chambers as described previously [1]. The *in vitro* inhibitory effects of protein fractions on the growth of *C. beticola* (Sacc.) (isolate 'FC573', Dr Earl G. Ruppel, USDA, Fort Collins, USA) were tested using the microtiter plate bioassay described previously [1]. In this assay, measuring the increase in absorbance at 620 nm follows the growth of submerged spore cultures. Cultures for microscopical analysis were grown likewise. The data presented are from one experiment, but the same growth curves and thus level of growth inhibition are observed in other experiments.

2.2. Purification of antifungal protein IWF6 from the intercellular washing fluid of sugar beet leaves

The intercellular washing fluid (IWF) from 1 kg sugar beet leaves was isolated and fractionated as described previously [13]. In short, the IWF was applied to an ion exchange column containing 10 ml CM-Sepharose (Pharmacia LKB) equilibrated in 20 mM acetic acid, pH 4.5. Bound proteins were eluted by stepwise increasing the salt concentration in the starting buffer: 0.1, 0.3 and 0.5 M NaCl. The 0.3 M NaCl eluate comprised antifungal activity and was further purified by cation exchange fast protein liquid chromatography (FPLC) on a Mono S HR 5/5 column (Pharmacia LKB). The individual protein peaks from the Mono S column were further purified by reverse-phased HPLC on a Vydac C₄ silica column (The Separations Group, CA, USA). The solvent system was (A) 0.1% TFA in water and (B) 0.1%

TFA in acetonitrile. Proteins were eluted with a linear gradient from 5 to 45% of the B-buffer from 0 to 18 min after sample loading followed by 60% B-buffer for 2 min. Flow rate was 0.7 ml min⁻¹. Protein was detected at 214 and 280 nm. Discrete protein peaks were collected and freeze dried. After two washes with water followed by freeze drying, the protein peaks were resolved in 10 mM Tris-HCl, pH 8.0, for the analysis of purity (SDS-PAGE) and antifungal activity.

Prior to N-terminal sequencing, proteins were reduced and carboxymethylated and subjected to reversed-phase HPLC on a Vydac C₄ column. Amino acid sequencing was performed as described previously [1].

2.3. Polyacrylamide gel electrophoresis, antibodies and immunoblotting

Proteins were separated by SDS-PAGE on 16.6% Tricine gels as described [1,16]. Isoelectric focusing was performed using the Phast system (Pharmacia LKB) as described [2].

A full-length peptide covering the entire sequence of IWF6 was synthesized by Research Genetics (Alabama, USA) using the Fmoc technology [17]. The full length IWF6 peptide (37 amino acids) was conjugated to difteria toxoid before immunization as described [18].

Immunoblotting of proteins separated by Tricine SDS-PAGE was performed as outlined previously [1], using IWF6 antibodies diluted 1:100. Protein concentrations were determined using the Bio-Rad protein assay kit with γ -globulin as standard as described [13].

2.4. Immunolocalization of IWF6

For the immunolocalization studies of IWF6, the following sugar beet varieties were used: Monova, Marathon and +Tol (Danisco Seed). Infection with *C. beticola* was performed as described [1]. Samples were extracted from leaves of sugar beet (approx. 1 cm²) with necroses 12, 15 and 19 days after inoculation with *C. beticola*. Controls (non-infected) were included as well. After fixation in 2% paraformaldehyde and 0.25% glutaraldehyde, buffered with 50 mM sodium phosphate buffer pH 7.0, 3% sucrose was added. Fixation, paraffin embedding and sectioning was performed as previously described [3]. The only

deviation was the use of xylene instead of petroleum ether. Prior to the immune procedure, samples were reduced by treatment with 50 mM DTT in TBS (0.5 M Tris-HCl, 0.15 M NaCl, 0.1% Triton X-100, pH 7.6) for 20 min in order to obtain optimal detection by the antibodies (antibodies were raised against a synthetic form of IWF6, i.e. randomly coiled). Variations in exposure time to DTT (10, 20 and 30 min) gave no significant difference. The IWF6 antibodies and preimmune control antibodies, were used in a 1:50 dilution. The immune procedure previously described [3] was used.

2.5. Mass spectrometry

Matrix-assisted laser desorption mass spectrometry (MALDI MS) [17,18] was performed using a Voyager DE instrument (PerSeptive Biosystems, Framingham, MA) equipped with delayed extraction [20] in the linear configuration. All samples were analyzed in the positive mode using an acceleration voltage of 20 kV and externally calibrated. The matrix used was α -cyano 4-hydroxy cinnamic acid (Sigma) at a concentration of 15 mg ml⁻¹ in 70% CH₃CN, 0.1% TFA [19]. Sample preparation was the dried droplet method [21] and an average of at least 100 shots were collected and used for analysis.

2.6. Cloning of IWF6 cDNA

The cDNA sequence of IWF6 was obtained by 3' and 5' RACE essentially as described previously [14] using the following primers:

3' RACE Primers

Q_T: 5'-CCAGTGAGCAGAGTGACGAGGACTCGAGCTCAAGC(T)₁₇-3'

Q₀: 5'-CCAGTGAGCAGAGTGACG-3'

Q₁: GAGGACTCGAGCTCAAGC-3'

5' RACE primers

5'-Anchor: 5'-GGCCACGCGTCGACTAGT-ACGGGGGGGGGG-3'

5'-UNI: 5'-GGCCACGCGTCGACTAGTACG

2.7. 3' RACE

The amino acid sequence of the IWF6 protein was used to construct two degenerated oligonucleotide primers for the isolation of a partial cDNA clone by 3'RACE. Total RNA was purified from

sugar beet (cv. Monova) leaves 6 days after inoculation with *C. beticola* according to [22]. Reverse transcription followed by PCR was done with the RT-PCR kit from Perkin Elmer and according to their protocol. Briefly, 1 µg of total RNA and 2.5 pmol Q_T-primer was incubated at 42°C for 45 min with reverse transcriptase followed by incubations at 99°C for 5 min and 5°C for 5 min. In the first PCR 40 pmol of the primer Q₀ was used as downstream primer and the upstream primer was 150 pmol of the degenerated primer P1 (5'-GG(ACGT)TA(CT)TG(CT)AA(CT)AT(ACT)-(CT)T: position 297–313 in the IWF6 cDNA sequence). In the second nested PCR 50 pmol of the primer Q₁ was used as downstream primer and the upstream primer was 50 pmol of the degenerated primer P2 (5'-AA(CT)GT(ACGT)TG(CT)-TG(CT)GC(ACGT)GG: position 314–332 in the IWF6 cDNA sequence). The PCR conditions were: 1 min at 94°C, 2 min at 42°C, 1 min at 50°C and 5 min at 72°C for 1 cycle followed by 1 min at 94°C and 2 min at 42°C and 3 min at 72°C for 35 cycles followed by 10 min at 72°C. After the second PCR a single DNA product of 320 bp was obtained. The DNA product was cloned into the pT7Blue vector (Novagen) and sequenced using a Thermo Sequenase fluorescent cycle sequencing kit (Amersham) and an ALF DNA sequencer (Pharmacia).

2.8. 5' RACE

The sequence of the 5' end of IWF6 cDNA was obtained by 5'RACE using the 5'RACE system from Gibco BRL with 3 gene specific primers constructed from the partial cDNA sequence obtained by 3'RACE. Briefly, 1 µg of the same total RNA as used for 3'RACE and 2.5 pmol of a gene specific primer GSP6-1 (5'-CATCAAGAAGTC-CATAATTGTCTAG: position 508–532 in the IWF6 cDNA sequence) was incubated at 70°C for 10 min followed by the addition of reverse transcriptase and incubating at 42°C for 30 min, 70°C for 15 min and the addition of RNaseH and incubating further 10 min at 55°C. The cDNA was dC-tailed according to the protocol of Gibco BRL. The tailed cDNA was subjected to two rounds of PCR. In the first PCR 20 pmol of the 5'-Anchor primer was used as upstream primer and the downstream primer was 20 pmol of the gene specific primer GSP6-2 (5'-TGATCTT-TATTGACAAACAGACACGC: position 473-

498 in the IWF6 cDNA sequence). In the second PCR 50 pmol of the 5'-UNI primer was used as upstream primer and the downstream primer was 50 pmol of the gene specific primer GSP6-3 (5'-ACAGACACGCTAGTTAGATGACTAAGC:-position 456–482 in the IWF6 cDNA sequence). The condition for the first PCR was: 1 min at 94°C and 1 min at 51°C and 2 min at 72°C for 35 cycles followed by 10 min at 72°C. The condition for the second PCR was: 1 min at 94°C and 1 min at 55°C and 2 min at 72°C for 35 cycles followed by 10 min at 72°C. The single 510 bp DNA product was cloned into the pT7Blue vector (Novagen) and sequenced using a Thermo Sequenase fluorescent cycle sequencing kit (Amersham) and an ALF DNA sequencer (Pharmacia).

2.9. Southern blotting

Genomic DNA was isolated from sugar beet leaves according to [23], digested with appropriate restriction enzymes and separated on a 0.8% agarose gel. A DNA probe of IWF5 cDNA was ³²P-labelled by random priming using the Ready-To-Go Labelling Kit from Pharmacia. Southern transfer and hybridization was performed according to [24] using Hybond N+ membranes (Amersham) following the recommendations of the manufacturer.

3. Results

3.1. Purification and biochemical characterization of IWF6

The 0.3 M NaCl eluate from the CM-Sepharose column possessed strong antifungal activity. When this fraction was subjected to cation exchange chromatography, six major and a number of smaller protein peaks were eluted. All peaks display antifungal activity, and have been shown to contain a number of chitinases [2–4], plant defensins [13] and non-specific lipid transfer proteins [14]. Strong antifungal activity was also found in peak 4 and SDS-polyacrylamide gel electrophoresis revealed that this protein peak contained a number of different proteins. When peak 4 was further separated by RP-HPLC on a Vydac C₄ column, it separated into 9–10 distinct protein peaks on the RP-HPLC column (data not shown). One of these exhibited strong antifungal activity and was subsequently re-chromatographed by reversed-phase HPLC using a Vydac C₄ column (Fig. 1). Only the major peak was collected and used in the subsequent work. The quality of the purification was evaluated by MALDI MS (Fig. 1, inset) and N-terminal amino acid sequencing, and only one protein was present. The complete N-terminal amino acid sequence of the protein was

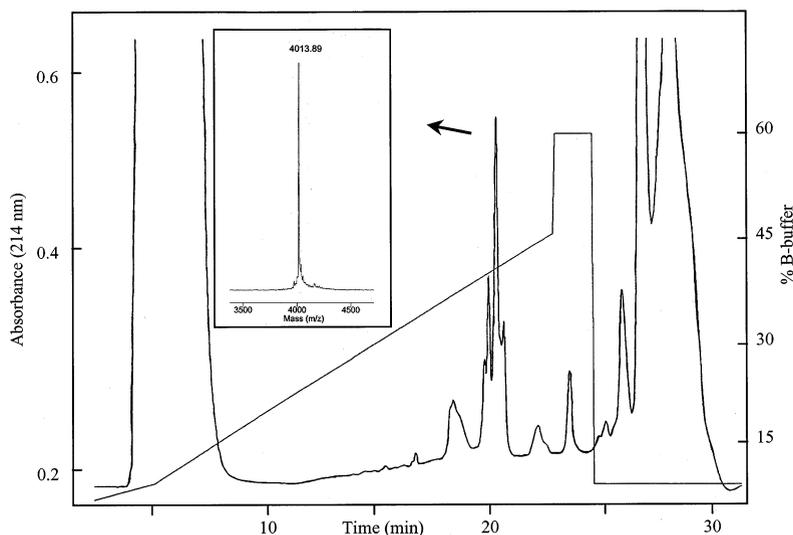


Fig. 1. RP-HPLC purification of IWF6. The fraction was checked for purity by SDS-PAGE analysis (not shown) and by mass spectrometry (inset).

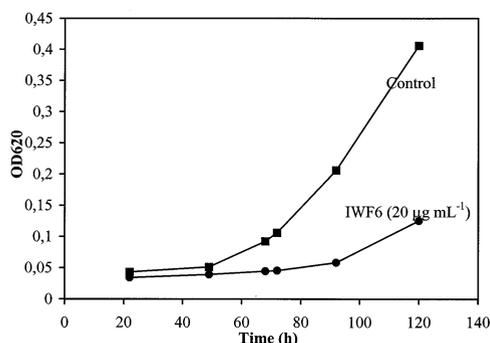


Fig. 2. Biological assay showing the growth of spore cultures of *C. beticola*. The fungal growth was measured as increase in A_{620} in the absence (rectangles) or presence (circles) of $20 \mu\text{g ml}^{-1}$ IWF6.

obtained by automated Edman degradation. The protein consisted of 37 amino acids, six of which were cysteines. The SDS-PAGE analysis indicated an apparent molecular mass of 3.5 kDa which is in agreement with the observed molecular weight (Mr) of 4012.89 as determined by MALDI MS.

An isoelectric point of 7.0 was deduced when IWF6 was subjected to isoelectric focusing using IEF Phast gels with a pH range of 3–9 (not shown). This result was in agreement with a predicted isoelectric point (pI) of 7.5 (when all cysteines take part of disulfide bonds) as calculated from the amino acid sequence (PC/Gene, version 6.85).

The IWF6 protein was present in the intercellular washing fluid (IWF) at extremely low concentrations (typically 100 µg in 2 kg leaves), and several IWF isolations and purifications were required in order to obtain sufficient amounts of protein (µg quantities) for amino acid sequencing and (limited) assaying for biological activity. Furthermore, it proved very difficult to determine the exact protein concentration as different methods gave different values. The stated concentrations are thus maximum values and the actual concentration is probably lower than stated.

Antibodies were raised against a synthetic 37-mer comprising the entire sequence of IWF6. In order to obtain maximum detection by the antibody it proved necessary to reduce the protein with DTT prior to Western blotting.

3.2. Antifungal activity

IWF6 showed a strong growth inhibiting effect against spore cultures of *C. beticola*. In the control culture, detectable growth (measured by increase in

A_{620}) started 40–50 h after incubation start (Fig. 2). When $20 \mu\text{g ml}^{-1}$ IWF6 was added to the spore cultures, culture start was strongly delayed, and detectable growth not observed until 70–80 h after growth start. Furthermore, the growth rate of the culture was substantially slower (Fig. 2). Microscopical examination revealed that also the morphology of the IWF6 treated fungal hyphae was affected: the restricted hyphae were shorter and somewhat more branched (not shown), an effect similar to that exerted by another antifungal sugar beet peptide, IWF4 [15]. The difficulties in obtaining reproducible concentration values make the actual concentration uncertain and probably much lower than stated (the indicated concentrations are the maximum values obtained by protein determination). The antifungal activity of IWF6 was confirmed in repeated experiments.

3.3. Localization of IWF6

IWF6 was detected in all leaf cell types except epidermal cells [Fig. 3(A)], in various paraffin sections of non-infected sugar beet leaves by pre-treatment with DTT before employing the normal immune procedure. Without treatment with DTT, the same plants show only a faint positive reaction in xylem and stomata. In sugar beet leaves infected with *C. beticola*, IWF6 was only found in significant amounts in tissue probably about to disintegrate due to infection, as well as in the established necrosis (Fig. 3(C) and (D)). Almost no IWF6 was found in the anatomical normal tissue surrounding the necroses. In the established necrosis (Fig. 3(C) and (D)), the IWF6 was detected to *Cercospora* hyphae present in a ball-like structure, 'pellet' [25] and not to single hyphae. The hyphal 'pellets' are mainly placed under stomata. These reactions on *C. beticola* could also be seen without DTT treatment, but not as intensively stained. No significant difference was observed in the distribution of IWF6 in the necroses from day 12–19. Preimmune controls were all negative.

3.4. Sequence homology

A database search was performed (BLAST vers. 2.0.10) in order to identify potential homology to other published protein sequences.

IWF6 shows no significant similarity to any protein sequence in the database. Only, the C-ter-

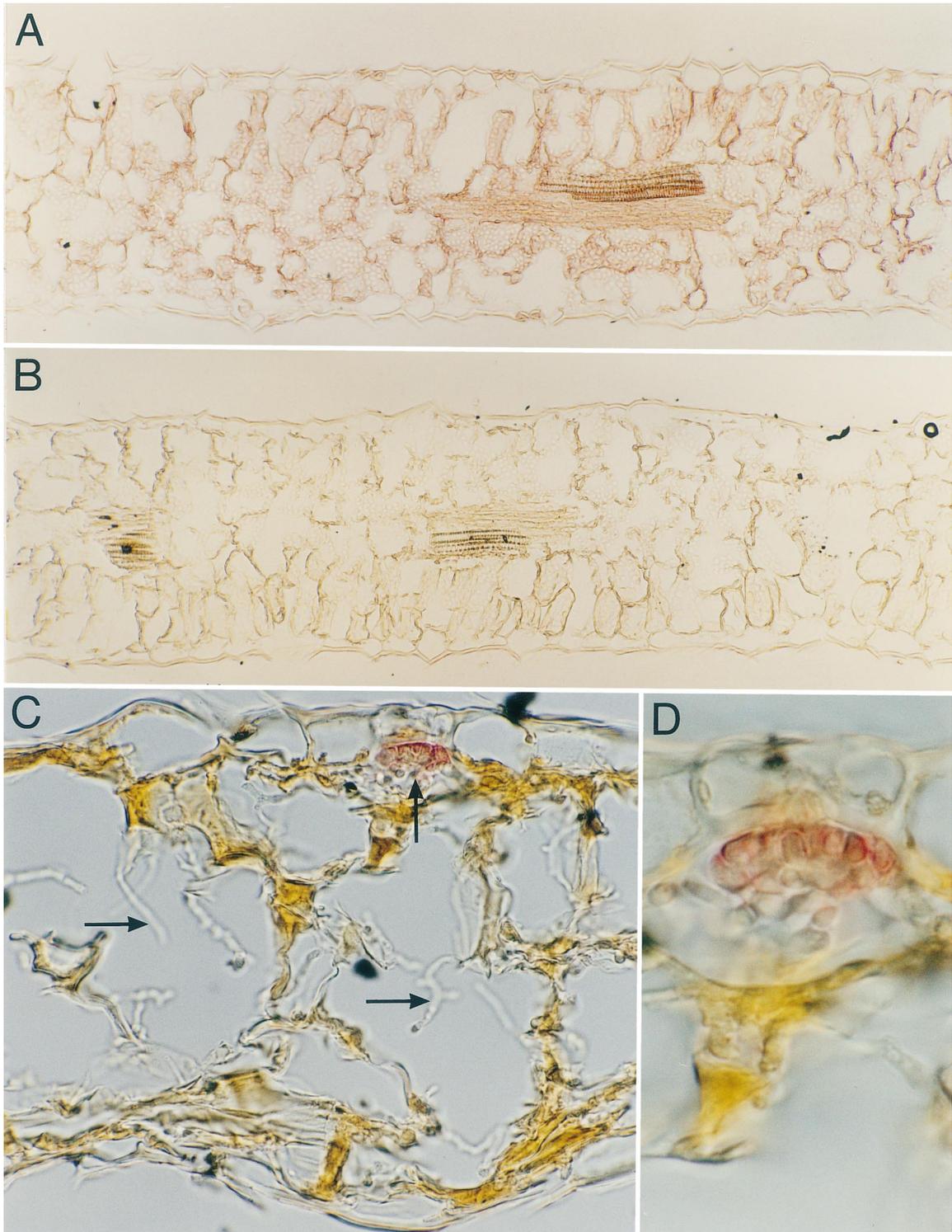


Fig. 3. IWF6 immuno-visualized (red) on cross sections of sugar beet leaves after pretreatment with DTT (20 min) prior to immunolocalization with alkaline phosphatase-coupled secondary antibodies and Fast Red development. (A), (C) and (D) IWF6 antiserum (1:50). (B) control (pre-immune serum 1:50). Fig. 3(A) show a broad distribution of IWF6 in the leaf except in the epidermal cells. Fig. 3(C) and (D) Necrose infected with *Cercospora*, IWF6 is detected on the hyphal 'pellets' typically found beneath stomata (vertical arrow). No IWF6 was detected on separate hyphae (horizontal arrows). (A) and (B) 108 ×, (C) 470 × and (D) 1180 × magnification. The hyphae are pale white and the necrotic sugar beet tissue is yellow (natural colors).

minal part of IWF6 shows a low level of homology to agelenin, a neurotoxin of 35 amino acid residues isolated from the venom of the spider *Agelena opulenta* [26]. The optimal identity is obtained by introducing a gap of two residues in IWF6, by which the six cysteines present in both proteins can be aligned. Hence, an identity of 35% (or 26% without gap) is found between the two proteins on the amino acid level (Fig. 4).

The RPRPRP-motif present in the N-terminal of IWF6 has not been observed in any of the hitherto characterized antifungal proteins. A similar motif is present in the C-terminal part of a presumed *Mycobacterium leprae* antigen [27]. In addition, the hinge region of some chitinases is rich in proline, thus giving this region its flexible structure [4].

3.5. Characterization of IWF6 cDNA

The nucleotide sequence of the full-length IWF6 cDNA clone, obtained by a combination of 3' and 5' RACE, and the deduced amino acid sequence is shown in Fig. 5. The IWF6 cDNA contains 593 nucleotides including a polyadenylation site (AATAAA) located 82 nucleotides upstream of a

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IWF6      RPRPRPCIRAGGYCNIL--NVCCAGLTCEEHDIQDAACV - 37
          |         |         |         |         |         |
Agelenin  GGCLPHNRFNCNALS GPRCCSGLKCKELSIWDSRCL - 35
  
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Fig. 4. Comparison of the amino acid sequence of IWF6 with agelenin, a neurotoxin from the venom of the spider *A. opulenta* [26]. In order to optimize the alignment, one gap has been introduced in IWF6. The character to show that two aligned residues are identical is '|'. Identity: 35.1% (with gap) or 25.7% (without gap).

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ATAAGTGGCAAGCAAAAAGAAATCATTTCACTAAAAAAGCAAAAAGAAAAAGTGGAGAAGTCATCAT 66
GCTTTAAGCTGGTTTTCTTAGTCTTTCTGTCTCAACATTTTCAGCTTCAACACTTGAAGAAGATGC 132
                                     M 1
AACCAACTATAATGGAAGAAGTACAAAGGAACAGTCTTGAAGAAGTGGTGTCTTACAAGCAGA 198
Q P T I M E E L T K E Q V L E E L G A Y K Q 23
TAATAGCATCAGCAGCAACAAGAGACCGCGGCGAGTTATTAAGAACGGTGATAGAAAGCGGTTGGCG 264
I I A S A A T R D R G E L L R T V I E A V A 45
GACCGCGACCGCGACCGTGCATAGGGGCTGGGCGCTACTGTACACTTTTGAACGCTATGTTGTGCTG 330
R P R P R P C I R A G G Y C N I L N V C C A 67
GGTTGACTTGTGAGAACATGATATACAAGACGCGCTCTGCGTCTAAATCTTGGACTTGTCTTAAAA 396
G L T C E E H D I Q D A V C V -
AAATGCTTATTATAAGTGTATTTGGCCTTTTAGGCTTGTATCTTCAATTTTCGTCTTTAAGCTTAGT 462
          GSP6-2          GSP6-1
CATCTAAGTACGGTCTGTTTGTCAATAAAGATCATTTGCTTCTCTAGACAATTTGGACTTCTTT 528
GATGCTTTTATTTTAAATATAAATAATTTCCCGCTTTCAAAAAATAAAAAAAAAAAAAAAAAAAAA 593
  
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Fig. 5. cDNA sequence and deduced amino acid sequence of IWF6. The amino acid sequence of the putative N-terminal signal sequence is in italic letters. The position of the primers used to generate the RACE cDNA clones are indicated with arrows.

poly(A) tail of 24 nucleotides. The sequence contains an open reading frame encoding 82 amino acid residues with the first in frame ATG at position 129 and a stop codon at position 375. In comparison with IWF6, the cDNA encode a precursor protein with an N-terminal putative signal sequence of 45 amino acid residues followed by the mature protein of 37 amino acid residues. In accordance herewith, the preprotein contains a putative cleavage site between the alanine at position 45 and the arginine at position 46 [28]. The cDNA derived sequence is identical to the amino acid sequence of the isolated IWF6 except for the amino acid Ala to Val change at position 35 of the mature protein. This amino acid change was observed in both the 3' as well as the 5' RACE derived clones, thus making it unlikely that it is due to a PCR introduced error.

3.6. Southern blotting

Genomic DNA from sugar beet was digested by the restriction enzymes *Bam*HI or *Xba*I and subjected to Southern hybridization analysis using IWF6 cDNA as probe. Each restriction enzyme digest resulted in only one hybridizing band, indicating that only one gene (or a small number of genes) for IWF6 is present in the genome of sugar beet (data not shown).

4. Discussion

A novel type of antifungal protein was isolated from the IWF of sugar beet leaves using cation exchange chromatography and reverse-phased HPLC. The protein, IWF6, was present in very low amounts and several purifications were necessary to obtain sufficient material for sequence determination and growth inhibition assays. The complete amino acid sequence of 37 residues was obtained by N-terminal sequencing. The calculated molecular weight (M_r) of IWF6 (based on the amino acid sequence) is 4012.59 assuming the six cysteines present form disulfide bridges. This correlates well with the MALDI MS data (observed molecular weight: 4012.89 ± 0.4) and indicates that all six cysteines take part in disulfide bonds.

The isoelectric point of 7.0, as determined by isoelectric focusing of IWF6 and in agreement

with the predicted pI of 7.5, is very different from that of all other antifungal proteins isolated from sugar beet so far which are all basic of nature and have isoelectric points of 9 or above [13–15].

When spore cultures of *C. beticola* were grown in the presence of 20 $\mu\text{g ml}^{-1}$ (highest estimated concentration, see results) of IWF6, the growth of the fungus was strongly inhibited. After 90 h of growth, a 90% growth inhibition is observed and after 120 h the fungus is still more than 70% inhibited. Although assay conditions not being directly comparable, the antifungal potency of IWF6 is judged to be at the level of other classes of antifungal peptides, e.g. the plant defensins and the chitin-binding proteins, for which the level of protein needed for 50% growth inhibition is in the range of 5–10 $\mu\text{g ml}^{-1}$, depending on the fungal species investigated [12]. These levels are found when assaying in low ionic strength media, whereas when adding divalent cations the activity of the proteins is severely decreased [10]. Due to the limited amount of material, it was not possible to test the effect of divalent cations on the antifungal potency of IWF6.

Antibodies raised against a synthetic 37-mer comprising the entire sequence of IWF6 were used to localize the native protein in paraffin embedded sections of sugar beet leaves. To obtain maximum labeling, the sugar beet leaves had to be reduced by DTT before recognition by the antibody. This is most likely because the antibody was raised against a randomly folded, synthetic peptide. The recognition of IWF6 in all leaf cell types (except epidermal cells in non-infected leaves) with no obvious induction during fungal infection, may suggest that IWF6 does not play a major role in the plant defense response. However, it cannot be excluded that the present plants were stressed by other factors (i.e. handling procedure) and thus reacted by expressing the protein in many cell types.

In the established necrosis, the IWF6 seemed to be localized only on the surface of *C. beticola* when the hyphae grow in ‘pellet’ but not to single hyphae. This is an interesting observation since the antifungal proteins IWF4 and especially AX2 in previous investigations have been shown to reduce the growth of *C. beticola* in vitro and induce morphological changes resulting in hyphal structures resembling the ‘pellet’ of hyphae observed in the necroses [13,15]. Although various sugar beet

antifungal proteins have been localized to the hyphae present in necroses [3,14], the IWF6 localization is different because it was found only in the ‘pellet’ of hyphae. Intensive staining for IWF6 was observed in some uninfected leaves (possibly stressed) and in infected leaves with possible initiation of infection, whereas almost no IWF6 was observed proximal to the necroses. These findings in combination with the intensive IWF6 detection on hyphal pellets beneath the stomata, could suggest that IWF6 participates in an early defense response as *C. beticola* is known to invade through stomata.

IWF6 is a new type of antifungal protein and shows no homology to other known antifungal proteins. A low level of identity (26%) is observed to agelenin, a neurotoxin isolated from the venom of the spider *A. opulenta* [26]. Agelenin belongs to the group of cysteine-rich neuropeptides and is an irreversible presynaptic toxin [2]. This resembles the actions of ω -toxins [29] which act by blocking P-type (high-threshold) calcium channels [29–31]. Members of the plant defensin family have been shown to induce rapid membrane responses in fungi possibly via a receptor mediated response or by direct insertion of the protein in the membrane thus forming an ion channel [32].

Although the biological role of the different antifungal proteins isolated so far is still unknown, growing evidence suggest a role in plant defense mechanism against invading pathogens [12]. This is supported by the fact that they are mostly predominant in the peripheral cell layers or cells exposed to infection (stomata and stigma) or cells exposed to spreading infection (xylem) [10,14,33]. This ‘peripheral’ localization could be important in forming a primary antifungal defense against pathogens. The broad distribution of IWF6 in sugar beet leaf cells except epidermis suggest that it takes part of a second line of defense mechanism against invading pathogens, although it seems to disappear from the anatomical normal tissue quite early in the infection and is only found in the necroses in combination with *C. beticola* hyphae displaying reduced growth.

The low degree of homology to agelenin, a neurotoxin possibly acting by blocking specific calcium channels, could suggest a similar mode of action of IWF6. Whether the biological role of IWF6 also includes the action on calcium channels remains to be elucidated.

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