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Heterologous expression, isotopic-labeling and immuno-characterization of Cin1, a novel protein secreted by the phytopathogenic fungus *Venturia inaequalis*

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ABSTRACT

The phytopathogenic fungus *Venturia inaequalis* causes scab of apple. Once this fungus penetrates the plant surface, it forms a specialized body called a stroma between the inner cuticle surface and the epidermal cell wall. A novel *V. inaequalis* gene, *cin1*, is strongly up-regulated in the early stages of infection. This gene codes for a 523 residue secreted protein, containing eight imperfect repeats of ~60 amino acids. Cin1 was expressed in the methanolytic yeast *Pichia pastoris* using the pPICZ vector system. A protein of 57 kDa was secreted by these transformants and peptide fingerprinting indicated that it was the Cin1 protein product. Multiple angle laser light scattering confirmed the predicted mass of Cin1, showing it was not glycosylated by *Pichia* and was monomeric in solution. Through measurements of the hydrodynamic properties of Cin1, the experimental Stokes radius of Cin1 was calculated and corresponded to the theoretical value for a natively folded globular protein of size 57 kDa. The mobility of recombinant Cin1 on native PAGE was also consistent with that of a folded protein. To simplify future structural analyses, a two-domain truncated version, Cin1-2D, consisting of domains one and two, was also expressed using the same vector system. Both proteins were purified to homogeneity. Conditions for maximal (>98%) incorporation of ^{13}C and ^{15}N were determined. A mouse polyclonal antibody and three monoclonal antibodies (MAbs) were raised against the full-length version of Cin1. Analysis of the three MAbs using surface plasmon resonance indicated binding to distinct epitopes on the Cin1 protein. Western blots confirmed the different specificities of each MAb.

Ventura inaequalis (Cooke) Wint. causes black spot or scab of apple (*Malus pumila* (Mill.) Henry) [1]. *V. inaequalis* germinates on the apple leaf surface and forms an appressorium-like swelling prior to penetration of the cuticle. Once the cuticle has been breached, the fungal hyphae grow between the inner cuticle surface and the epidermal cell wall. Here the hyphae differentiate, producing a specialized body called a stroma that is made up of single or multiple layers of pseudoparenchyma, a laterally dividing cell-type which is distinct from the usual tubular mode of hyphal growth [1,2]. During the apple growing season, conidiophores are produced from the stromata through the perforated cuticle and are associated with the development of scab lesions.

Differentiation of sub-cuticular hyphae and stromata can be mimicked by growing *V. inaequalis in vitro* on cellophane discs [2]. *V. inaequalis* penetrates and forms stromata within the cellophane. A differential cDNA screen was performed on a library made from mycelia grown on cellophane. Two novel genes, *cin1* (EU189192) and *cin3* (EU189193), were found to be differentially expressed in the cellophane library. Full-length versions of these genes were found in an expressed sequence tag library from apple leaves infected with *V. inaequalis*, and quantitative real-time PCR (qRT-PCR) indicated that these genes were up-regulated over 1000-fold during the early stages of infection compared with *in vitro* growth [2]. Both genes encode putative secreted proteins with imperfect repeated domains, the number of which is variable and isolate dependent. One of these induced proteins, Cin1, codes for a 523 aa protein which has seven or eight repeats, depending upon the isolate, of between 52 and 65 aa each [2]. The predicted sequence of the processed protein has a preponderance of charged amino acids (18% acidic and 21% basic) and 32 cysteines (Fig. 1).

While homologs of Cin1 have not been identified, proteins with similar overall characteristics (leader peptide, repeated domain structure, high proportion of cysteines and charged residues) have been identified in the genomes of several fungi [3–6]. Some of these proteins have been shown to participate in cell-to-cell or

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Leader Seq      MQYSSLLATIVALSATTTSALPAAAA
Domain 1        DVFDPPTQYGYDG  KPLDASFCRTAGS  REKDCRKDVQACDKKYDDQGRETACAKGIREKYKP
Domain 2        AVV     YGYDG  KPLDLGFCTLAGI  REVDCRKDAQTCDKKYE SDK   CLNAIKEKYKP
Domain 3        VVDPNPPAYGYDG  KPLDASFCRSFGA  KENECRKDVLACDKKFDNEGRESACSKAIREKYKP
Domain 4        FYVAPPPVYGYDG  KPLDASFCRSFGA  KENECRKDILACDKKFDNEGRESACSKAIREKYKP
Domain 5        FYVAPPPVYGYDG  KPLDASFCRSFGA  KENECRKDILACDKKFDNEGRESACSKAIREKYKP
Domain 6        FYVA PPVYGYDG  KPLDASFCKGAGS  KENECKKDILACDKKYDNEGRETACAKAIREKYKT
Domain 7        STTTPVN YGYDG  KPLDASFCKKFPS  REAECRKDVENCDRKFDDQGRETKCSKDIKEKYK
Domain 8        EEKKADEKKEKDGKRQPTNADFCKRAGTSVLEAECRKAVKECDKKYQNIGHEKECSRDLEDKYTK
C-terminus      GSSSYGSGSGSYGPGGY
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Fig. 1. Amino acid sequence and domain structure of Cin1. The putative leader sequence is indicated in bold. Peptide fragments identified using tryptic digestion and ESI-FTMS are underlined. The sequence of Cin1-2D is highlighted in grey.

cell-to-substrate adherence, for instance during mating, flocculation or biofilm formation [7–11]. For example, deletion mutants of Qid74, a cell wall protein from *Trichoderma harzianum* exhibit increased sensitivity to cell wall degradation and reduced capacity for adherence [12]. In addition, several secreted effector proteins of phytopathogenic micobes possess repeat domains, and in some cases, variation in domain structure has been implicated in mediating host specificity [13–15]. Little structural information is available for these proteins, particularly from plant pathogenic fungi.

Cin1 is expressed *in planta* but not produced *in vitro* in liquid culture, thus the native protein cannot be easily purified. To facilitate the structural characterization of this novel protein, we expressed Cin1, and a simplified two-domain truncated version Cin1-2D, in a heterologous system using *Pichia pastoris*. Both proteins were purified to homogeneity, and conditions were optimized for maximal isotope labeling of Cin1-2D. Measurement of the hydrodynamic parameters [16–18] of full length recombinant Cin1 and its mobility on native PAGE indicated that it resembled that of a natively folded globular protein. Three monoclonal antibodies (MAbs) with distinct specificities were raised against Cin1 and characterized using surface plasmon resonance and Western blotting.

Materials and methods

Materials

Bovine serum albumin (BSA), Roswell Park Memorial Institute (RPMI) and Dulbecco's Modified Eagle's Medium culture media (DMEM), hypoxanthine aminopterin thymidine selection additive, L-glutamine, sodium pyruvate, Tween-20, horseradish peroxidase, peroxidase-labeled goat anti-mouse IgG (Fc specific), and *o*-phenylenediamine (OPD) were obtained from Sigma–Aldrich Chemical Co. (Milwalkee, WI). Fetal calf serum was obtained from Hybrimax, Discovery Technology International (Sarasota, FL). Cell culture plasticware and Maxisorb microELISA flat bottom F16 modules were obtained from Nunc (Roskilde, Denmark). Avidin (AV)[1], Biotin-PEO-*N*-hydroxysuccinimidyl ester (B-NHS), was from Pierce Chemicals (Rockford, IL). Mouse monoclonal antibody (MAb) isotyping kit was obtained from Bio-Rad Laboratories (Hercules, CA). All other reagents were AnalR grade or better.

Strains, media and vectors

pGEMT-EASY (Promega, Madison, WI) and *Escherichia coli* DH5α (Invitrogen, Carlsbad, CA) were used for all cloning experiments. *Pichia pastoris* strain KM71H (methanol utilizing slow, Mut^S) and the pPICZαB expression vector were purchased from Invitrogen. Yeast extract–peptone–dextrose medium (YPD), buffered minimal glycerol-complex medium (BMGY), buffered methanol minimal media (BMM) and buffered minimal methanol-complex medium (BMMY), were as described in the easy-select manual (Invitrogen). Zeocin (Invitrogen) was added to a final concentration of 100 μg mL^{-1}.

Construction of expression plasmids

PCR primers, pPICZCin1-5′ (5′-TAT-CTC-TCG-AGA-AAA-GAG-AGG-CTG-CCG-ACG-TTT-TCG-ACC-CTC-C) and pPICZCin1-3′ (5′-AAA-AGG-AAA-AAA-GCG-GCC-GCT-CTA-GTA-TCC-TCC-TGG-ACC), were designed to create a fusion of the mature Cin1 protein with the α-mating factor leader sequence in the pPICZαB vector. The primers incorporated XhoI and NotI restriction sites (underlined) in the 5′ and 3′ primers, respectively, to facilitate cloning. Included in the 5′ primer was the KEX recognition site and a single copy of the EA dipeptide (double underlined). PCR was carried out under standard conditions using Expand Long-Template *Taq* polymerase (Roche, Basel, Switzerland). A truncated version of Cin1 containing only the first two domains (Fig. 1) was amplified using pPICZCin1-5′ and pPICZCin1-2D (5′-AAA-AGG-AAA-AAA-GCG-GCC-GCT-CTC-TAG-GCA-GGT-GGT-TTA-GGG-TCG-ACG-AC) and cloned into pPICZαB as above to produce pPICZαBCin1-2D. All constructs were resequenced to verify the fidelity of the PCR amplification.

Transformation of Pichia pastoris

pPICZαBCin1 and pPICZαBCin1-2D were linearized with Sac1 and BstXI, respectively. Cells of *P. pastoris* KM71H were transformed by electroporation with 5 μg linearized DNA. Colonies that grew on YPDS agar medium containing 100 μg mL^{-1} Zeocin were examined for the secretion of Cin1, using SDS–PAGE, and the clone expressing the highest amount of Cin1 was chosen for production and purification steps. Integration at the AOX promoter was confirmed by PCR analysis of genomic DNA from each transformant chosen for expression work.

Protein determination

Recombinant Cin1 was separated on SDS–PAGE gels and stained with Coomassie Brilliant blue R. Cin1 concentration was estimated,

[1] *Abbreviations used:* AV, avidin; B-NHS; biotin-PEO-*N*-hydroxysuccinimidyl ester; BMGY, buffered minimal glycerol-complex medium; BMM, buffered minimal methanol medium; BMMY, buffered minimal methanol-complex medium; BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; ESI-FTMS, electrospray Fourier transform mass spectrometry; MAb, monoclonal antibody; MALDI-ToF–MS, matrix-assisted laser desorption ionization time of flight mass spectrometry; MALLS, multiple angle laser light scattering; OPD, *o*-phenylenediamine; PB, 0.1 M potassium phosphate pH 6.0; PBS, phosphate buffered saline; PBST, phosphate buffered saline containing 0.1% Tween-20; qRT-PCR, quantitative real-time PCR; RPMI, Roswell Park Memorial Institute culture media; R_S^D, experimentally determined Stokes radius; R_S^{NF}, Stokes radius for a native folded globular protein; R_S^{MG}, Stokes radius for molten globular state; R_S^{PMG}, Stokes radius for pre-molten globular state; R_S^{Coil}, Stokes radius for coiled state; R_S^{Urea}, Stokes radius for completely denatured state in urea; YPD, yeast peptone dextrose medium.