Research Note

A Cysteine Protease Gene Is Expressed Early in Resistant Potato Interactions with *Phytophthora infestans*

Anna O. Avrova, Helen E. Stewart, Walter De Jong, Jacqueline Heilbronn, Gary D. Lyon, and Paul R. J. Birch

Departments of Fungal and Bacterial Plant Pathology and of Crop Genetics, Scottish Crop Research Institute (SCRI), Invergowrie, Dundee, DD2 5DA, U.K.

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A potato cysteine protease (*cyp*) cDNA expressed at an early stage of an incompatible interaction with *Phytophthora infestans* was isolated. Both the nucleotide and deduced amino acid sequences are highly homologous to those of a tomato cysteine protease, CYP1. Striking protein similarity to all known cathepsins in animals, particularly cathepsin K, was also observed. However, unlike cathepsins, a granulin binding domain is located near the carboxyl terminus of the putative CYP protein. In animals, granulins bind to receptors in the plasma membrane and signal cell growth and division. A ribonuclease protection assay demonstrated that the *cyp* gene is tightly regulated and is induced 15 h post inoculation with *P. infestans* in potato leaves either with high field resistance or in which a resistance (*R*) gene is activated. We conclude that a common signaling pathway is activated in each form of resistance.

Additional keywords: hypersensitive response, programmed cell death.

In recent years, evidence has suggested that many plant processes, including development, reproduction, xylogenesis, and senescence, involve programmed cell death (PCD) similar to apoptosis in animals (Greenberg 1996; Beers 1997). Moreover, PCD in plants occurs in response to pathogen attack, most demonstrably in the hypersensitive response (HR) that characterizes an “incompatible” interaction. Morphological features of apoptosis, such as membrane blebbing, chromatin condensation, and DNA fragmentation, can be observed in at least some examples of the HR (Pontier et al. 1998; Heath 1998). Pathogen recognition in plants may be mediated by receptors with domains that are similar to those found in pro-apoptotic adaptor proteins in animals (van der Biezen and Jones 1998), and signaling/signal transduction in both the HR and apoptosis involve

changes in protein phosphorylation, ion fluxes, and reactive oxygen species, and may involve lipid-based second messengers (Gilchrist 1997; Pontier et al. 1998).

Central to apoptosis in animal cells is the tightly regulated proteolytic cleavage of a limited number of cellular proteins. The main enzymes involved in this process are caspases, aspartate-specific cysteine proteases, although other cysteine proteases such as calpains and cathepsins, serine proteases such as granzymes, and the proteasome-ubiquitin pathway are all reported to be involved in apoptosis (reviewed in Solary et al. 1998). Cysteine protease genes have been shown to be up-regulated in plant PCD processes such as development (Xu and Chye 1999) and senescence (Drake et al. 1996; Kardailsky and Brewin 1996; Griffiths et al. 1997; Guerrero et al. 1998). Furthermore, cysteine protease activities appear to be involved in the HR (del Pozo and Lam 1998; D'Silva et al. 1998; Solomon et al. 1999). Nevertheless, caspase activity in these PCD processes remains to be conclusively proven. del Pozo and Lam (1998) reported cleavage of a short oligopeptide at the aspartate residue during infection of resistant tobacco by tobacco mosaic virus. However, with a longer synthetic peptide, no such activity was seen from soybean cysteine proteases purified from cells undergoing PCD in response to oxidative stress (Solomon et al. 1999). D'Silva et al. (1998) demonstrated the cleavage of poly (ADP-ribose) polymerase (PARP) by cysteine proteases activated during the HR of cowpea to cowpea rust fungus. PARP is normally a target for cleavage by caspases during apoptosis in animals (reviewed in Vaux and Korsmeyer 1999). Inhibition of cysteine proteases with cystatin blocked PCD in soybean triggered by either oxidative stress or an avirulent strain of *Pseudomonas syringae* pv. *glycinea* (Solomon et al. 1999). Both Solomon et al. (1999) and D'Silva et al. (1998) have suggested a regulatory role for cysteine proteases and their inhibitors during the HR in plants. As yet, the genes encoding these HR-associated cysteine proteases have not been isolated.

Recently, we reported the generation of a cDNA library enriched for potato sequences up-regulated at an early stage of an incompatible interaction with *Phytophthora infestans* (Birch et al. 1999). This library was generated by suppression

Corresponding author: Paul R. J. Birch; Telephone: +44 1382 562731; Fax: +44 1382 562426; E-mail: pbirch@scri.sari.ac.uk

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subtractive hybridization (SSH) of cDNA from leaves of resistant cv. Stirling (the "tester" cDNA population), 24 h post inoculation with an avirulent isolate of *P. infestans*, with cDNA from infected leaves of susceptible cv. Bintje as a driver. Sequence analysis of the library revealed two clones containing contiguous cDNA fragments that matched a number of cysteine proteases (Birch et al. 1999). Here, we describe the isolation of a full-length potato *cyp* cDNA, and demonstrate that it is up-regulated early in resistance responses to *P. infestans*.

To obtain a full-length cDNA sequence of the gene, cDNA of cv. Stirling 15 h after inoculation with an incompatible race of *P. infestans* was used as a template for 3' and 5' RACE (rapid amplification of cDNA ends; Marathon cDNA Amplification Kit, protocol PT1115-1; Clontech, Palo Alto, Ca). The cDNA was synthesized from RNA extracted with an RNeasy Plant minikit (Qiagen, Hilden, Germany). Double-stranded cDNA was synthesized with the First-Strand cDNA Synthesis Kit (Amersham Pharmacia Biotech, Little Chalfont, UK) and the Riboclonc cDNA Synthesis System (Promega, Madison, WI). Specific primers used for 3' and 5' RACE and for sequencing are given in Table 1. Amplification products were purified with the Promega Wizard PCR Preps kit, cloned with the Promega pGEM-T vector cloning kit, and transformed into Ultracompetent *Epicurian coli* XL2-blue MRF' cells (Stratagene, La Jolla, CA). Plasmid preparations (Qiagen Plasmid Miniprep kit) of clones derived from each amplification product were sequenced (ABI PRISM Dye Terminator cycle sequencing kit and ABI Model 377 DNA sequencer; Perkin Elmer, Warrington, U.K.) in both directions (with Promega M13 forward and reverse or with gene-specific primers; Table 1). In parallel to this work, a full-length cDNA matching the cysteine protease was independently identified at SCRI by random sequencing of a cDNA library from a doubled ($2n = 2x$) potato monoploid (851-5), cloned into pSPORT1 with the SuperScript plasmid system (GibcoBRL, Paisley, Scotland).

The complete cDNA sequence of *cyp* (EMBL accession number AJ245924) reveals an open reading frame encoding a putative polypeptide of 466 amino acids, with a molecular mass of approximately 50 kDa (Fig. 1). Both the DNA and deduced amino acid sequences show high levels of homology (93.7 and 95.7 %, respectively) to a tomato cysteine protease, CYP1 (accession no. AJ003137), and are also similar to gibberellin-induced oryzain α (accession no. D90406). Furthermore, the predicted amino acid sequence shows strong homology to all known cathepsins in animals, particularly cathepsin K (accession no. P43235) (Fig. 1). Two domains can be identified in the protein: a cysteine protease domain, pres-

ent in all plant and mammalian cysteine proteases, and also a C-terminal, granulinlike domain (Fig. 1). Detailed comparison of the cysteine protease domains between the potato CYP, tomato CYP1, rice oryzain α , and cathepsin K reveals that amino acids critical to the structure and function of cysteine proteases are conserved in all of them. Specifically, the catalytic cysteine-histidine-asparagine triad, Cys162, His298, Asn318 (Fig. 1, open circles) and cysteines forming disulfide bridges, Cys159-Cys201, Cys193-Cys234 and Cys292-Cys343 (Fig. 1, marked #) are conserved.

The granulin domain appears to be highly conserved among several plant cysteine proteases, including CYP1 and thiol protease C14 (accession no. M21444) from tomato, oryzain α and β (accession no. D90407) from rice, RD21A (accession no. P43297) from *Arabidopsis thaliana*, CP3 (accession no. Z99954) from *Phaseolus vulgaris*, thiol protease (accession no. X66061) from pea, and Mir3 (accession no. AF019147) from maize (Fig. 2). Other plant cysteine proteases, including CYS4 (accession no. P25251) from *Brassica napus*, See1 (accession no. X99936) and Ccp2 (accession no. D45403) from maize, aleurain (accession no. P05167), CYSP (accession no. P43156), EPB1 (accession no. U19359), and EPB2 (accession no. U19384) from barley, oryzain γ (accession no. D90408) from rice, papains (e.g., accession nos. P00784, X97789, X78056, X69877) and mammalian cathepsins (e.g., accession nos. M90696, P43235, P07711, P09668, P07858), lack such a domain.

Granulins are a family of evolutionarily ancient proteins that are involved in regulating cell growth in animals (Battman and Bennett 1998). Antisense expression of granulin in a cancerous animal cell line results in inhibition of tumorigenicity, suggesting a role for granulins in stimulating cell division (Zhang and Serrero 1998). They are structurally organized as multiple repeats of the cysteine-rich module shown in Figure 2 (Trinh et al. 1999) and have been shown to bind to a specific cell surface receptor in animals (Xia and Serrero 1998). The presence of a granulin domain at the C terminus of the putative potato CYP protein raises the possibility of such receptors in plants. If they exist, and they have a role in promoting cell growth, binding a cysteine protease to such receptors may lead to their inactivation, and to the consequent activation of a cell death pathway. If this is so, one might expect the *cyp* gene to be up-regulated at an early stage of hypersensitive cell death in response to pathogen attack. In addition, one would expect CYP to be secreted into the apoplast. A classic hydrophobic leader sequence (amino acids 1 to 24) and a signal peptide cleavage site between amino acids 24 and 25 (according to Nielsen et al. 1997) suggest that CYP may indeed be a secreted protein.

To assess the transcriptional regulation of the *cyp* gene we performed ribonuclease protection assays (RPA II Kit, 108K0438; Ambion, Austin, TX) with RNAs isolated from different time points (15, 48, and 72 h) after inoculation of a variety of potato cultivars with *P. infestans*. To allow for variation in response, five glasshouse-grown plants of each cultivar were sprayed with a suspension of zoospores (5×10^4 per ml), as described by Stewart et al. (1983), and leaf material pooled prior to RNA extraction. In each case, RNAs from uninfected leaves were prepared as controls. Chosen were cv. Bintje, which is free of resistance (*R*) genes and which has a field resistance rating of only 2 according to the International

Table 1. Oligonucleotide primers used for 3' and 5' RACE (rapid amplification cDNA ends) and for sequencing

Primer name	Sequence	Annealing temp.
CYPR1	5'-CAATAGGCTGTGCAAGAATGCG-3'	65°C
CYPR3	5'-ATAAGTCGTACAATGAAGGTTGC-3'	65°C
CYPR4	5'-CAAAGGCGTAGTCCATAAGACC-3'	62°C
CYPF1	5'-GCAGCTCACAGCTCAACTCTC-3'	62°C
149RT1	5'-CAGCAGAAAGGTGTAGAGACAG-3'	64°C
149RT2	5'-TGAAGAAGGACTATTGTTCTAC-3'	60°C
149D	5'-CTGTCTCTACACCTTCTGCTG-3'	64°C

Potato_CYP	M A A H S S T L T I	S L L L M L I F S T	L S S A S D M S I I	S Y D E T H I H R R	40
Tomato_CYP1	M A A H S S T L T I	S I L L M L I F S T	L S S A S D M S I I	S Y D E T H I H R R	40
Rice_ORYA	M R I . S M A L A A	A A L L L L L . S .	L . A A A D M S I I	S Y G E R	31
Human_CATK	M W G L K .	V L L L P V V . . .	. S F A L Y P E E I	L . D . T H	25
Potato_CYP	S D D E V S A L Y E	S W L I E H G K S Y	N A L G E K D K R F	Q I F K D N L K Y I	80
Tomato_CYP1	T D D E V S A L Y E	S W L I E H G K S Y	N A L G E K D K R F	Q I F K D N L R Y I	80
Rice_ORYA	S E E E A R R L Y A	E W K A E H G K S Y	N A V G E E E R R Y	A A F R D N L R Y I	71
Human_CATK	. . W E . . . L . .	. W K K T H R K Q Y	N N K V D E I S R R	L I W E K N L K Y I	57
Potato_CYP	D E Q N . . . S V P	N Q S Y K L L G L T K	F A D L T N E E Y R	S I Y L G T K S S G	117
Tomato_CYP1	D E Q N . . . S V P	N Q S Y K L L G L T K	F A D L T N E E Y R	S I Y L G T K S S G	117
Rice_ORYA	D E H N A A A D A G	V H S . R L G L N R	F A D L T N E E Y R	D T Y L G L . N K P	111
Human_CATK	S I H N L E A S L G	V H T Y E L A M N H	L G D M T S E E V V	Q K M T G L K V P L	97
Potato_CYP	D R R K L S K N K S	D R Y L P K V G D S	L P E S V D W R D K	G V L V G V K D Q G	157
Tomato_CYP1	D R K K L S K N K S	D R Y L P K V G D S	L P E S . D W R E K	G V L V G V K D Q G	157
Rice_ORYA	R R E . . R K . V S	D R Y L A A D N E A	L P E S V D W R T K	G A V A E . K D Q G	148
Human_CATK	S H . . . S R S N D	T L Y . P E W E G R	A P D S V D . R K K	G Y V T P V K N Q G	134
<div>#</div> <div>o</div> <div>#</div>					
Potato_CYP	S C G S C W A F S A	V A A M E S I N A I	V T G N L I S L S E	Q E L V D C D K S Y	197
Tomato_CYP1	S C G S C W A F S A	V A A M E S I N A I	V T G N L I S L S E	Q E L V D C D R S Y	197
Rice_ORYA	G C G S C W A F S A	A A V E D I N Q I	V T G D L I S L S E	Q E L V D C D T S Y	188
Human_CATK	Q C G S C W A F S S	V G A L E G Q L K K	K T G K L . N L S P	Q N L V D C V . S E	173
Potato_CYP	N E G C D G G L M D	Y A F E F V I N N G	G I D T E E D Y P Y	K E R N D V C D Q Y	237
Tomato_CYP1	N E G C D G G L M D	Y A F E F V I K N G	G I D T E E D Y P Y	K E R N G V C D Q Y	237
Rice_ORYA	N E G C N G G L M D	Y A F D F . I N N G	G I D T E D D Y P Y	K G K D E R C D V N	228
Human_CATK	N D G C G G G Y M T	N A F Q Y V Q K N R	G I D S E D A Y P Y	V G Q E E S C M . Y	212
Potato_CYP	R K N A K V V K I D	S Y E D V P V N N E	K A L Q K A V A H .	Q P V S I A I E A G	276
Tomato_CYP1	R K N A K V V K I D	S Y E D V P V N N E	K A L Q K A V A H .	Q P V S I A I E A G	276
Rice_ORYA	R K N A K V V T I D	S Y E D V T P N S E	T S L Q K A V R N .	Q P V S V A I E A G	267
Human_CATK	N P T G K A A K C R	G Y R E I P E G N E	K A L K R A V A R V	G P V S V A I D A S	252
Potato_CYP	G R D L Q H Y K S G	I F T G K . C G T A	. V D H G V V A A G	Y G S E N G M D Y W	314
Tomato_CYP1	G R D F Q H Y K S G	I F T G K . C G T A	. V D H G V V I A G	Y G T E N G M D Y W	314
Rice_ORYA	G R A F Q L Y S S G	I F T G K . C G T A	. L D H G V A A V G	Y G T E N G K D Y W	305
Human_CATK	L T S F Q F Y S K G	V Y Y D E S C N S D	N L N H A V L A V G	Y G I . K G N K H W	292
Potato_CYP	I V R N S W G A K W	G E K G Y L R V Q R	N V A S S S G L C G	L A T E P S Y P V K	354
Tomato_CYP1	I V R N S W G A N W	G E N G Y L R V Q R	N V A S S S G L C G	L A I E P S Y P V K	354
Rice_ORYA	I V R N S W G K S W	G E S G Y V R M E R	N I K A S S G K C G	I A V E P S Y P L K	345
Human_CATK	I . N S W G E N W	G N K G Y I L M A R	N K N N A . . C G	I A N L A S P . K	328
Potato_CYP	T G A N P P K P A P	S P P S P V K P P T	E C D E Y S Q C P V	G T T C C C V L E F	394
Tomato_CYP1	T G P N P P K P A P	S P P S P V K P P T	E C D E Y S Q C A V	G T T C C C I L Q F	394
Rice_ORYA	K G E N P P N P G P	T P P S P T P P P T	V C D N Y Y T C P D	S T T C C C I Y E Y	385
Human_CATK	M ~ ~ ~ ~ ~ ~ ~ ~	~ ~ ~ ~ ~ ~ ~ ~	~ ~ ~ ~ ~ ~ ~ ~	~ ~ ~ ~ ~ ~ ~ ~	329
Potato_CYP	R R S C F S W G C C	P L E G A T C C E D	H S S C C P H D Y P	V C N V R Q G T C S	434
Tomato_CYP1	R R S C F S W G C C	P L E G A T C C E D	H Y S C C P H D Y P	I C N V R Q G T C S	434
Rice_ORYA	G K Y C Y A W G C C	P L E G A T C C D D	H Y S C C P H E Y P	I C N V Q Q G T C L	425
Human_CATK	~ ~ ~ ~ ~ ~ ~ ~	~ ~ ~ ~ ~ ~ ~ ~	~ ~ ~ ~ ~ ~ ~ ~	~ ~ ~ ~ ~ ~ ~ ~	329
Potato_CYP	M S K G N P L G V K	A M K R I L A Q P I	G A F G N G . G K K	S S S ~ M A	468
Tomato_CYP1	M S K G N P L G V K	A M K R I L A Q P I	G A F G N G . G K K	S S S ~ M R	468
Rice_ORYA	M A K D S P L A V K	A L K R T L A K P N	L S F L F G N G K K	S S A ~ M W G	461
Human_CATK	~ ~ ~ ~ ~ ~ ~ ~	~ ~ ~ ~ ~ ~ ~ ~	~ ~ ~ ~ ~ ~ ~ ~	~ ~ ~ ~ ~ ~ ~ ~	329

Fig. 1. Amino acid sequence alignment of the predicted potato CYP polypeptide with sequences of cysteine proteases from tomato (accession no. AJ003137), rice (accession no. D90406), and human cathepsin K (accession no. P43235). Likely site of cleavage to generate the mature protein is indicated with solid triangle. Solid black line indicates cys-protease domain; dashed line indicates granulin domain. Catalytic cysteine-histidine-asparagine triad is marked with open circles; cysteines forming disulfide bridges are marked #. Proteins were aligned with PILEUP and LINEUP (Wisconsin Genetics Computer Group software package), and are displayed with the use of PRETTYBOX. Black shading shows identical amino acids; gray shading indicates conserved substitutions.

1–9 scale (NIAB 1999), cv. Stirling, with high field resistance (rating 8; NIAB 1999) and an uncharacterized *R* gene (Milbourne 1999), and two genotypes of Black's differential series (Black et al. 1953), Pentland Ace and 1512 c (16) (containing the *R3* and *R2* genes, respectively) that, when included in SCRI field trials of breeder lines, have been allocated a field resistance rating of 2 (averaged over 10 years; H. E. Stewart, *unpublished data*). In all cases, *P. infestans* inoculations were made with either a race 1,4 isolate, which gave a clear incompatible interaction with Stirling, Pentland Ace, and 1512 c (16), or with a race 1,2,3,4,6,7 isolate, which gave a compatible interaction with Pentland Ace and 1512 c (16) and very slowly developing lesions with Stirling. The races had been isolated from potato crops in Scotland during the previous growing season, and maintained on RyeA agar over winter and on detached potato leaflets prior to use. Both races gave a clear compatible interaction with Bintje. Additional plants (inoculated and uninoculated) kept as controls for 5 days after inoculation showed similar levels of infection on Bintje, but a clear difference between Stirling plants inoculated with the different races (results not shown).

15-h time points in incompatible interactions for Pentland Ace, 1512 c (16), and Stirling (Fig. 3). Interestingly, a similar pattern of *cyp* up-regulation was observed at the 15-h time point in the so-called "compatible" interaction between Stirling and *P. infestans* (Fig. 3C, lane 2). Lesions only slowly develop in this interaction, as there are appreciable levels of foliar blight resistance. This suggests that a common signaling pathway, leading to the expression of *cyp*, is being activated in each of these resistance responses. In a recent review, Kamoun et al. (1999) suggest that resistance responses to oomycetes, whether *R* gene mediated or quantitative (field), invariably involve the HR. If this is so, then the *cyp* gene may be a marker for hypersensitive cell death.

Fig. 3. Ribonuclease protection assay with RNA from leaves of potato cvs. (A and B) Bintje, (C and D) Stirling, and (E and F) Pentland Ace. In each panel RNA is from uninfected plant (lane 1), from plant 15, 48, and 72 h after inoculation with a compatible race of *P. infestans* (lanes 2–4, respectively), and 15, 48, and 72 h after inoculation with an incompatible race of *P. infestans* (lanes 5–7, respectively). ³²P-labeled riboprobe corresponding to the 3' UTR of the cysteine protease (clone STL2; Birch et al. 1999) (A, C, E) was, in each case, hybridized to 30 µg of total RNA, and ³²P-labeled riboprobe corresponding to a fragment of potato 25S rRNA (B, D, F) was hybridized, in each case, to 10 µg of total RNA.

which are the main effectors of apoptosis, may be activated by adaptors, such as Apaf1, or by other, regulatory, caspases (e.g., Vaux and Korsmeyer 1999). In addition, cathepsin B has been shown to activate caspases (Vancompernelle et al. 1998). It is thus intriguing that the *cyp* gene encodes a cathepsin-like cysteine protease. It will be interesting to discover whether this protease possesses caspase-like regulatory functions by activating and inactivating proteins, and whether the granulin binding domain is involved in directing this activity to specific protein targets.

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