Cyst Germination Proteins of the Potato Pathogen *Phytophthora infestans* Share Homology with Human Mucins

Birgit Görnhardt, Ila Rouhara, and Elmon Schmelzer

Max-Planck-Institut für Züchtungsforschung, Department of Biochemistry, Carl-von-Linné-Weg 10, D-50829 Köln, Germany

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We have cloned genes of *Phytophthora infestans*, the causal agent of potato late blight, that are activated shortly before the onset of invasion of the host tissue. The three genes isolated appear to be arranged in a genomic cluster and belong to a small polymorphic gene family. A conspicuous feature of the deduced proteins is an internal octapeptide repeat with the consensus sequence TTYAP TEE. Because of this structural motif, these novel *P. infestans* proteins were named Car (cyst-germination-specific acidic repeat) proteins. One of the genes, *car90*, codes for 1,489 amino acids including 120 octapeptide tandem repeats. Car proteins are transiently expressed during germination of cysts and formation of appressoria and are localized at the surface of germings. The structural motif of tandemly repeated oligopeptides also occurs in a prominent class of proteins, the mucins, from mammals. The *P. infestans* Car proteins share 51% sequence homology with the tandem repeat region of a subfamily of human mucins. According to the physiological functions ascribed to mucins, we suggest that Car proteins may serve as a mucous cover protecting the germling from desiccation, physical damage, and adverse effects of the plant defense response and may assist in adhesion to the leaf surface.

Additional keywords: catabolite repression, pathogenicity.

Since the first epidemic outbreaks of potato late blight in Europe in the middle of the last century, which caused the infamous Irish famine, this disease has posed a constant threat to the world's potato crop. Complete, durable resistance to the causal agent, *Phytophthora infestans*, has never been found. The disease is mainly controlled by intensive application of chemical fungicides. Besides fungicide resistance problems and profound effects on the environment and human health, this practice burdens farmers with enormous costs. The search for new strategies to prevent *P. infestans* epidemics is a great challenge for phytopathologists and requires more information about the biochemistry and molecular biology of the pathogenicity of *P. infestans*.

Corresponding author: Elmon Schmelzer; Fax: +49-221-5062-313; E-mail: schmelze@mpiz-koeln.mpg.de

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Since the discovery and first description of *P. infestans* as the cause of potato late blight (de Bary 1863, 1876) various aspects of the potato/*P. infestans* interaction have been intensively studied (Erwin et al. 1983; Ingram and Williams 1991; Lucas et al. 1991). As with many obligate biotrophic fungi, the hemibiotrophic oomycete *P. infestans* forms specialized structures enabling invasion and colonization of the plant tissue. The disease cycle is initiated by the release of biflagellate zoospores that develop within sporangia. Prior to germination, the motile, wall-less zoospore encysts. The cyst attaches to solid surfaces and rapidly germinates. At an appropriate site, the tip of the germ tube swells to a vesicular structure, the appressorium, from which an infection tube emerges that enters the first epidermal cell of the host (Pristou and Gallegly 1954).

Recently, as had been described earlier for rust fungi (Freytag et al. 1988; Staples et al. 1986), we have found that *P. infestans* is capable of developing infection structures on artificial membranes in the absence of the host plant (Krämer et al. 1997). Analysis of protein extracts of such in vitro grown fungal structures at several developmental stages (hyphae, cysts, germinating cysts, and appressoria) by two-dimensional sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) revealed large, stage-specific changes in protein synthesis. With germination of cysts, a complex program of differential gene expression is initiated that may control development of infection structures and invasion of the host and, thus, may determine pathogenicity.

However, the molecular mechanisms mediating pathogenicity of *P. infestans* are largely unknown. *P. infestans* belongs to the *Peronosporales*, notorious for being “a fungal geneticist's nightmare” (Shaw 1983). Molecular biological techniques have been successfully applied to *Phytophthora* spp. only recently. As a prerequisite for the functional analysis of genes, Judelson et al. (1991) established transformation of *P. infestans* and successfully applied the antisense transformation technique (Judelson et al. 1993). Only a limited number of *P. infestans* genes have been described up to now (Chen and Roxby 1996; Kamoun et al. 1997; Moon et al. 1992; Pieterse et al. 1991, 1995; Unkles et al. 1991). Some of them were obtained by differential cloning of genes that are expressed during the biotrophic phase after infection of a susceptible host plant (Pieterse et al. 1994a, 1993) and hence are possibly involved in pathogenicity.

We have used an alternative approach to identify putative pathogenicity genes of *P. infestans*. Taking advantage of in vitro grown infection structures (zoospores, germinating cysts, appressoria; Krämer et al. 1997), we cloned cDNAs from genes that are activated during germination of cysts and formation of appressoria. Since these two developmental stages directly precede penetration of the host cell wall, i.e., the onset of the actual infection process, genes activated during this time period may be related to initial stages of host cell invasion. Using this approach, we have found three novel *P. infestans* genes that are members of a multigene family. The encoded proteins possess conspicuous structural features that are similar to those of human mucins.

RESULTS

Identification of cDNAs specific for germinating cysts and appressoria.

First-strand cDNA from zoospores was used to subtract mRNAs common to zoospores and appressoria by repeated mRNA-cDNA subtractive hybridization (SH). After reverse transcription of the remaining mRNAs the resulting cDNAs were polymerase chain reaction (PCR) amplified and cloned into bacterial vectors. The library was then differentially screened with cDNAs from zoospores, germinating cysts, and appressoria.

By this strategy we identified two clones that hybridized to the cDNA probes from germinating cysts and from appressoria, but not to cDNA probes from zoospores. These two cDNA clones, designated *car90* and *car59* (*car*: cyst-germination-

specific acidic repeat), contained 0.3 and 0.6 kb inserts, respectively. The two partial cDNAs display high DNA sequence similarity (70.8% sequence identity) and share an internal tandem repeat.

Genomic organization and DNA sequence of *car* genes.

A λ EMBL3 genomic library from *P. infestans* was screened with the *car90* and *car59* partial cDNAs. Three genomic clones, λ 1-2, λ 1-3, and λ 3-1, that hybridized with both the *car90* and *car59* cDNA fragments were isolated (Fig. 1). The nucleotide sequence of parts of these genomic clones was determined with a series of subclones from λ 1-2, λ 1-3, and λ 3-1 (GenBank accession numbers: AF061185, *car90*; AF061186, *carPs-car59*).

The three genomic clones apparently overlap and include three unique *car* genes: *car90*, *carpseudo* (*carPs*), and *car59*. Although sequence information for one of the genes is incomplete (*car59*, see below) it appears that all three genes share an internal, well-conserved, 24-nucleotide (nt) tandem repeat (TR) that is repeated with diverse frequencies, resulting in a marked length polymorphism of these genes. The 5'- and 3'-terminal coding regions are nonrepetitive (compare Figure 2). Two of the *car* genes are most probably the corresponding genomic DNA sequences of the *car90* and *car59* cDNA fragments. In the case of *car59*, complete sequence identity exists between the genomic DNA and the cDNA fragment. In the case of *car90*, two differences were found between the genomic DNA sequence and the cDNA fragment, which might result from incorrect PCR during the SH procedure or might be due to different *car* alleles. *carPs* has a length of 2,087 nt containing two frame-shift mutations at positions +275 and +1572 with respect to the predicted translational start codon and is therefore presumed to be a pseudogene. *car90* and *carPs* do not contain introns, as is typical for all except two (GenBank accession numbers U14405, *NiaA*, and U30474, *Piyp1*) of the more than a dozen *P. infestans* genes known so far. The *car90* open reading frame spans 4,540 nt, and is predicted to encode a 1,489 amino acid protein with a molecular mass of about 164 kDa (Fig. 2). The *carPs* and *car90* genes share 97% DNA sequence identity overall, but the TR region of *carPs* is substantially shorter than that of *car90*. For *car59*, sequence information of a major part of the internal 24-nt TR-region is missing, due to difficulties in designing specific primers for the repetitive region and because the TR region of *car59* lacks suitable restriction sites for subcloning. However, the N-terminal (1,682 nt) and C-terminal (273 nt) nonrepetitive regions of *car59* were sequenced completely and the *car59* TR region partly (1,164 nt or 48 TRs). From known sequence and restriction analysis data we suggest that *car59* has a single open reading frame longer than 7 kb, which would encode for a protein with a molecular mass of more than 240 kDa.

The promoter regions of all three genes contain a 16-nt sequence motif that is similar to the consensus sequence for transcription initiation in oomycetous genes (GCTCATTYYN CA(A/T)TTT) as suggested by Pieterse et al. (1994b). The position of this motif relative to the predicted start of translation is -63 in *car59* or -88 in *car90* and *carPs*. Directly downstream of this motif, the *car* genes contain a CT-rich region (-50 in *car59*, -65 in *car90*, -61 in *carPs*). This is a common structure found in the vicinity of transcription initiation sites of genes from filamentous fungi and appears to be

Fig. 1. Suggested genomic organization of three *Phytophthora infestans* *car* genes in a gene cluster. Upper bar: arrangement of *car* genes *car90*, *carPs*, and *car59*; arrows represent orientation and length of their coding regions (a frameshift mutation in *carPs* was disregarded). Sequence information was obtained from restriction and sequence analysis of three λ -EMBL3-phages (λ 1-2, λ 1-3, λ 3-1) indicated as narrow bars. The phages partially overlap with respect to the *P. infestans* genomic DNA insertions. λ -regions shown in light gray are assumed to be rearrangements generated during construction of genomic library. Hatched areas reflect position of clustered *car* genes with respect to the λ -phages.

associated with high-level gene expression (Unkles et al. 1991; Chen and Roxby 1997).

Although three rearrangements within the λ -clones must be postulated, known sequence, restriction, and Southern analysis data (see below) lead to the conclusion that the three *car* genes are arranged in a gene cluster spanning at least 20 kb, as shown in Figure 1. The following observations support this assumption: There is no rearrangement postulated for λ 3-1, which has a DNA insertion of more than 13 kb that includes both *carPs* and *car59*. There is also no rearrangement postulated for the major internal part of the λ 1-2 insert

(approximately 9 kb), which contains both *car90* and *carPs*. About 2 kb of the presumed overlapping region of λ 1-2 and λ 3-1 was sequenced in both clones. This region, which includes the *carPs* gene, shows absolute DNA sequence identity between the two clones including the two frameshifts of *carPs*. It is unlikely that 100% DNA sequence identity exists between two distinct pseudogenes. The sequence identity between λ 1-2 and λ 1-3 ends abruptly at a GATC sequence that is the recognition site of the restriction endonuclease *Sau3AI*. *Sau3AI* was used for cloning of the *P. infestans* DNA into the λ EMBL3 phages.

Fig. 2. Complete deduced amino acid sequence of Car90. Sequence was deduced from the 4,470-nucleotide single open reading frame of *car90*. Car90 protein reveals a typical signal peptide (underlined) indicating secretion. A putative signal peptidase cleavage site is located between A₂₃ and D₂₄ (vertical arrow). Octapeptide with consensus sequence TTYAPTEE is tandemly repeated 120 times. Amino acid residues that deviate from consensus are in bold capitals. A 408 amino acid (aa)-long exact duplication within the tandem repeat (TR) is emphasized by a box; the first half (408 aa) is shaded. A short tetrapeptide TR stretch is overlined.

To investigate the complexity of the *car* gene family and to confirm the postulated rearrangements of λ 1-2 and λ 1-3 we performed genomic Southern blot analysis (Fig. 3). With a 2.1-kb *Sal*I DNA fragment derived from the TR region of *car90* as a probe (corresponding to amino acids 642–1448; Fig. 2), several bands were detected in each digest, indicating that the genome of *P. infestans* contains multiple copies of the *car* genes (Fig. 3A). Similar results were obtained when the gel blots were hybridized with DNA fragments derived from the 5' and 3' nonrepetitive region of *car90* (Fig. 3B, C), indicating that the ends of the *car* genes display strong sequence similarities as well. As 5' probe, an 833-nt long, PCR-derived subclone from λ 1-2 was used (corresponding to amino acids 1–277 of *car90*; Fig. 2). In this region, *car90* and *carPs* share 97% DNA sequence identity, while *car90* and *car59* display 77% DNA sequence identity. As 3' probe the 296-nt-long *car90* cDNA was used (corresponding to amino acids 1444–1489; Fig. 2). In this region, *car90* and *carPs* share 97% and *car90* and *car59* 71% DNA sequence identity.

Southern blot analysis would be expected to give a 3-kb *Eco*RI fragment that should hybridize to the 5' end of *car90* if no rearrangement exists in λ 1-3. Instead, a 6-kb *Eco*RI band was detected, as expected if *car90* and *carPs* are arranged as in λ 1-2 (Fig. 3B). Because recognition sites for the restriction endonuclease *Nde*I precede the TR region of both *carPs* and *car59* (+1,057 and +348 nt relative to the respective predicted translational start), Southern blot analysis would be expected to give a 3.7-kb *Nde*I fragment that should hybridize to all three probes tested, if *carPs* and *car59* are arranged as in λ 3-

1. This *Nde*I fragment should contain the TR and 3' region of *carPs* and the 5' end of *car59* (compare the suggested arrangement of *carPs* and *car59* in Figure 1). Indeed, an *Nde*I band of the expected size is detected with all three probes tested (Fig. 3A, B, and C).

Southern blot analysis of the λ -clones indicates that there are no further *car* genes localized within the suggested cluster covered by λ 1-2, 1-3, and 3-1.

Amino acid sequence of Car proteins.

The most remarkable feature of the primary structure of Car proteins is the very acidic internal octapeptide tandem repeat with the well-conserved consensus sequence TTYAPTEE. In Car90 the octapeptide is repeated 120 times, constituting two thirds of the protein (Fig. 3). The TR region contains an exact sequence duplication of 408 amino acids. The high content of glutamic acid residues and simultaneously low content of basic amino acids makes the Car proteins extremely acidic (calculated pI of Car90: 3.27; net charge: –322). Computer analysis predicts that Car proteins are very hydrophilic and flexible; there is no evidence for α -helix or β -sheet conformations.

The amino termini of the Car proteins have a typical hydrophobic secretion signal, with a putative signal peptidase cleavage site between Ala23 and Asp24, as predicted according to von Heijne (1986). The amino terminal nonrepetitive domains of Car90 and Car59, which stretch for approximately 390 residues, display about 61% sequence identity when gaps for optimal alignment are allowed. These amino terminal do-

Fig. 3. Southern blot analysis of the *car* gene family. Genomic DNA from *Phytophthora infestans* race 1-11 B was digested with the restriction endonucleases indicated. **A**, Hybridization with a 32 P-labeled, 2.1-kb *Sal*I DNA fragment from the tandem repeat (TR) region of *car90* (+1910 to +4044 nucleotides [nt] relative to the translational start or amino acids 642 to 1448; subclone was derived by partial digestion of λ 1-2 with *Sal*I). **B**, Hybridization with a 32 P-labeled, 0.84-kb, polymerase chain reaction (PCR)-derived DNA-fragment from the nonrepetitive 5' coding region of *car90* (+1 to +833 nt relative to translational start or amino acids 1 to 277; subclone was derived by PCR with primers specific for λ 1-2). **C**, Hybridization with the 32 P-labeled, 0.3-kb partial cDNA from the nonrepetitive 3' end of *car90* (+4329 to +4615 nt relative to the translational start or amino acids 1444 to 1489).

mains contain a short glycine-rich stretch and nine conserved cysteine residues. In the short carboxyl terminal, nonrepetitive domain (approximately 90 amino acids) Car59 and Car90 share six cysteine residues in identical positions. The sequence identity at the carboxyl terminus is about 63%. Car90 has an additional subdomain that is missing in Car59. It consists of a short tetrapeptide TR stretch with the consensus sequence DEPT that is repeated eight times and positioned 18 residues downstream of the octapeptide TR domain.

Comparison of the Car90 amino acid sequence with data bank sequences revealed no marked similarities to known

proteins for the nonrepetitive domains, while several proteins that contain tetra- or octapeptide TRs showed partial sequence similarities to the Car90 TR region (Fig. 4). Striking similarities were found between Car proteins and a subfamily of human mucins (MUC5) (Guyonnet Duperat et al. 1995). MUC5 proteins also contain a conserved octapeptide TR with the consensus sequence STTSAPTT. Consequently, the repeating units of MUC5 mucins and Car proteins (TTYAPTEE) share five amino acids out of eight that are identical in type and position (Fig. 4). Furthermore, as with the Car proteins, mucins contain several conserved cysteine residues in the carboxyl

TTYAPTEE ET TYAPTEE	Car-octapeptide-consensus (2x)
T <u>T</u> SAP T <u>T</u> S T <u>T</u> SAP T <u>T</u> S	Muc5AC-octapeptide-consensus ^a (2x)
T <u>E</u> T <u>P</u> T <u>E</u> T <u>P</u> T <u>E</u> T <u>P</u>	270-tetrapeptide-consensus ^b (3x)
E <u>P</u> P <u>T</u> E <u>P</u> P <u>T</u> E <u>P</u> P <u>T</u>	EPPT-tetrapeptide-consensus ^c (3x)
K <u>E</u> P <u>A</u> P <u>T</u> T <u>P</u> K <u>E</u> P <u>A</u> P <u>T</u> T <u>P</u>	MSF-octapeptide-consensus ^d (2x)

Fig. 4. Comparison of repeating units in proteins with sequence similarity to the Car consensus sequence. Underlined amino acids are also part of the Car consensus sequence. Bold and underlined amino acids are found in the identical position in the Car consensus sequence. a = Guyonnet Duperat et al. 1995; b = Giorda et al. 1990; c = accession X74358; d = Merberg et al. 1993.

Fig. 5. *Car* transcript accumulation. **A**, Northern (RNA) analysis of the *car* gene family shows developmentally regulated expression of *car* genes. Poly A⁺ RNA from different developmental stages and from two different *Phytophthora infestans* isolates (88069 and 1-11 **B**) was hybridized with ³²P-labeled DNA from the tandem repeat (TR) region of *car90*. **B**, Same filter as in **A** hybridized with ³²P-labeled *actA*-DNA for quantification of fungal mRNA. **C**, Polymorphism of *car* transcripts in germinating cysts of two different *P. infestans* isolates (Northern filter identical to **A**). Arrowheads = position of *car* transcripts (< for 88069; > for 1-11 **B**). **D**, Bright-light micrograph of a *P. infestans* germling at appressorial stage. Cytoplasm is accumulated within appressorium and adjacent part of germ tube, separated from rest of germling by a false septum. **E**, Whole mount in situ hybridization of *P. infestans* at appressorial stage. In situ-hybridization performed with ³⁵S-dUTP-labeled sense or antisense transcripts of the *car90* TR region as indicated above. Ap = appressorium; Cy = cyst; fs = false septum; gC = germinating cysts; gt = germ tube; Zoo = zoospores; My = mycelium; iP = infected potato leaves 28 h after inoculation with *P. infestans* zoospores; *P. i.* = *P. infestans*. White bars = 10 µm.

and amino terminal nonrepetitive domains and they too have a signal peptide for secretion.

Expression patterns of *car* genes.

To confirm that the expression of *car* genes is specifically induced during cyst germination and to examine their temporal and spatial expression at further developmental stages of *P. infestans*, Northern (RNA) blot and whole mount in situ hybridization experiments were performed (Fig. 5). The results showed that *car* gene transcripts accumulated to high amounts in germinating cysts and appressorium-forming fungal structures, but were absent at all other developmental stages analyzed.

For Northern blot analysis, mRNA was isolated from zoospores, germinating cysts, and mycelium, and from infected leaves of a susceptible potato plant 28 h post inoculation. Gel blots were hybridized with a 2.2-kb DNA fragment derived from the TR region of the *car90* gene (Fig. 5A). While there was no hybridization to mRNAs from zoospores, mycelium, or infected plant material, several hybridizing bands (5 to 7) were found with the mRNA isolated from germinating cysts, concurring with the results of the Southern analysis. Northern blot hybridization always resulted in a polydisperse signal in the range of about 1 to 10 kb. *actA*, an actin gene that is constitutively expressed in *P. infestans* (Unkles et al. 1991) was used as an internal standard for quantification of fungal mRNA (Fig. 5B). As demonstrated previously, under high stringency washing conditions *actA* does not cross-hybridize with potato RNAs (Pieterse et al. 1994b). Hybridization of the Northern blots with *actA* led to detection of a single band, suggesting that the polydisperse signals did not originate from degraded mRNA. Interestingly, in Northern blot analysis, the mRNAs of mucins show very similar polydisperse signals (Bergeron et al. 1996). Besides the possibility that due to their considerable length the transcripts are unstable (Gum 1992), we suggest that the TR regions of mucin and *car* transcripts may tend to form stable secondary structures, leading to shifts in electrophoretic mobility.

Sequence analysis already indicated a high degree of polymorphism of gene lengths, ranging from about 2.1 kb (*carPs*)

and 4.5 kb (*car90*) to presumably 7.5 kb (*car59*). This is reflected by the length of transcripts ranging from about 2.2 kb to more than 12 kb as detected in Northern blots. Northern blot comparison between mRNA derived from germinating cysts of different *P. infestans* isolates suggested that transcript-length polymorphism is present not only within one organism, but also between organisms (Fig. 5C). Although the differences between the two strains could be due to experimental variation or expression of different members of the *car* gene family at slightly different times, the mRNA used for Northern analysis originated from germinating cysts that were not exactly synchronous, taking slight expression variations into account.

Whole mount in situ hybridization was used to investigate the accumulation of *car* transcripts during appressorium formation. The results showed that *car* transcripts were present in the cytoplasmic part of appressorium-forming germings (Fig. 5D, E). During formation of the appressorium the cytoplasm accumulates in the appressorium where it becomes separated from the empty cyst and distal part of the germ tube by formation of a so-called false septum (Fig. 5D; Krämer et

Fig. 6. Nutrient- and stage-dependent expression of Car proteins shown by Western (immunoblot) analysis. CarMAP4 affinity purified antibodies were used to detect Car proteins in crude protein cell extracts from sporangia (Sp), zoospores (Zoo), and germinating cysts (gC). Cysts were germinated for 2 h in tap water (w), nutrient rich pea broth (pb), or 2% sucrose (S).

Fig. 7. Immunocytochemical localization of Car proteins. Cysts of *Phytophthora infestans* were sprayed on gelatin-covered slides and incubated for 5 h, enabling cyst germination and appressoria formation. After fixation, slides were used for immunolocalization studies with antibodies raised against the Car octapeptide tandem repeat (TR). **A**, Confocal optical section through an appressorial structure of *P. infestans*. **B**, Overlay of serial confocal optical sections at a step size of 2 μm through the same fungal structure as in **A**. **C**, Confocal optical section at the appressorial surface. Ap = appressorium; Cy = cyst. **A–C**, White bars = 10 μm.

al. 1997). In situ hybridization with the antisense but not the sense probe of the *car* TR led to the accumulation of silver grains within the appressorium and apical part of the germ tube (Fig. 5E), exactly where the cytoplasm is located.

Protein synthesis and localization.

Crude protein extracts from sporangia, zoospores, and germinating cysts were examined by Western blot (immunoblot) analysis with affinity purified antibodies raised against the acidic octapeptide TR region of the Car proteins. Very few or no immunologically cross-reacting components were detectable in sporangia or zoospores. In germinating cysts several proteins of different size cross-reacted with the Car antibodies. The apparent molecular masses of these proteins were 200 kDa or more (Fig. 6) and did not correspond to the molecular masses of Car proteins as deduced from their transcript lengths. This phenomenon is well known for other proteins with extremely low pI values. Most likely, the proteins migrate more slowly within the polyacrylamide gel due to reduced binding of SDS molecules, resulting in an increase of their apparent molecular mass (Burton et al. 1981; Kaufmann et al. 1984).

As shown in Figure 6, the accumulation of Car proteins is inhibited by the presence of carbohydrates. Car proteins were detected in protein extracts from cysts that germinated in tap water (Fig. 6, 'w'), whereas in extracts from cysts that germinated in nutrient-rich pea-broth ('pb') or tap water containing 2% sucrose ('S') no proteins were detected with the Car antibodies. These results suggest that the expression of *car* genes is controlled by the availability of nutrients, in particular carbon.

To check whether Car proteins are actually secreted, as suggested by the presence of a typical signal peptide, cysts of *P. infestans* germinating on gelatin-coated microscopic slides were stained with indirect immunofluorescence with the Car antibodies and analyzed by confocal laser microscopy. From optical cross-sections, as shown in Figure 7A, it is apparent that Car proteins are localized at the cell wall. In optical sections at the surface of appressorial fungal structures a non-homogenous distribution of staining was visible (Fig. 7C), indicating that the Car proteins may form viscous patches. However, Figure 7B implies that the whole fungal structure is covered by at least a thin film of Car proteins.

DISCUSSION

The capability of (hemi)biotrophic pathogens to grow and multiply on living plants, designated as basic pathogenicity, results from the combined action of many fungal genes that control the developmental and metabolic processes resulting in infection and colonization of the host (Knogge 1998). A particularly critical phase of the life cycle of a (hemi)biotrophic fungus is reached just before entering the biotrophic stage inside the tissue, when, at the plant surface, the young germling develops and initiates penetration.

With an SH approach, we detected a novel gene family that is activated during this critical phase of the *P. infestans* life cycle. Expression of the *car* gene family appears to be subjected to strict developmental and metabolic control. We conclude from our Northern and Western data and from the in situ RNA hybridization studies that the *car* genes are active during

the short time period between onset of cyst germination and completion of appressoria formation. We assume that expression is repressed as soon as *P. infestans* has invaded the first host cell and has access to nutrients, particularly carbon sources. Similarly, Pieterse et al. (1994a) have discovered that the members of two other putative pathogenicity-related gene families of *P. infestans*, *ipiO* and *ipiB*, are activated during cyst germination and the *ipiO* genes are repressed by glucose. Interestingly, the *ipiO* gene promoters as well as our *car* gene promoters contain a sequence motif highly homologous to (A/T)₄T(C/G)(C/T)GGGG that functions as a glucose repression element in yeast (Nehlin et al. 1991; Lundin et al. 1994) (*car90* and *carPs* 5'T₄ACTGGGG-, *car59* 5'A₄GTGGGT-). Thus, this or a similar motif might also be involved in regulation of *P. infestans* genes. Catabolite repression may represent a common phenomenon of pathogenicity-related genes since it has been observed in other phytopathogens. For instance, *mpgI*, a hydrophobin gene from *Magnaporthe grisea* needed for appressoria development (Talbot et al. 1993), and a cutinase from *Fusarium solani* f. sp. *pisi* (Kamper et al. 1994), are repressed by carbon and nitrogen or carbon alone, respectively.

The most remarkable feature of the Car proteins is their structural composition, which is characterized in the case of Car90 by a highly conserved octapeptide, TTYAPTEE, that is repeated in tandem more than 100 times. This octapeptide repeat constitutes the large, central part of the primary sequence to which at the N- and C-termini substantially shorter, non-repetitive stretches are added. The repetitive part determines the unusual properties of the proteins such as the extremely low pI and the high hydrophilicity. It is also largely responsible for the high molecular masses of Car proteins. Individual members of this protein family may reach more than 300 kDa, as estimated from the longest transcripts (9 to 12 kb) detected on Northern blots. Such large Car proteins may contain 300 to 400 tandemly repeated octapeptides, provided that the N- and C-terminal, nonrepetitive regions are of similar length, as in Car90 and Car59.

Tandemly repeated oligopeptides as major structural elements are well known from a prominent class of proteins, the mucins of mammals. As described above, high homology exists between the consensus sequence of the repeats of the Car proteins and the MUC5AC subfamily of human mucins (Guyonnet Duperat et al. 1995). Mucins are a large family of high-molecular-weight glycoproteins (Devine and McKenzie 1992; Rose 1992). They are secreted by epithelial cells from the gastrointestinal, respiratory, and reproductive tracts and cover the epithelia as a thick, mucous coat. Important physiological functions have been ascribed to mucins, such as maintenance of pH, protection of epithelial cells from desiccation, pathogenic microorganisms, or proteolytic enzyme digestion, and protection from chemical or mechanical damage (Guyonnet Duperat et al. 1995).

The protein backbone of mucins consists of a repetitive part of variable length that is framed by non-repetitive N- and C-terminal domains. The repetitive part is densely glycosylated predominantly via O-glycosidic bonds (Carraway and Hull 1989). The mucin glycans usually contain anionic sugars giving rise to an acidic net charge and high hydrophilicity. The large size (up to 10⁷ Da) and the extreme hydrophilicity of the molecules appear to account for the viscoelastic and sticky consistency of the mucus.

Whether or not Car proteins are glycosylated remains to be determined. Signal motifs for N-glycosylation are missing from the Car sequences so far analyzed. However, the high proportion of threonine residues could account for massive O-linked glycosylation as is the case for mucins. Our attempts to determine O-glycosylation, with a glycan detection kit or by enzymatic deglycosylation, did not lead to clear-cut results (data not shown). Notably, in contrast to the protein backbones of mucins, the Car proteins by themselves are highly negatively charged and hydrophilic; therefore, side chains of anionic sugars are not needed to confer these features. Similarly, the fact that our antibodies, raised against the repetitive part of Car proteins, do have access to the protein moiety argues against extensive glycosylation.

In conclusion, we have discovered a class of proteins that is novel to microorganisms. Preliminary genomic Southern analysis suggests that other *Phytophthora* spp., for instance *P. sojae*, may also possess *car* genes (B. Görnhardt and E. Schmelzer, *unpublished results*). The structural features of Car proteins and the surprising homology with mucins of mammals imply important functions similar to those ascribed to mucins (Devine and McKenzie 1992). Our data indicate that the Car proteins are secreted by *P. infestans* germlings during development on the leaf surface and cover at least parts of the cell wall. The extreme hydrophilicity, flexible shape, and presence of a number of conserved cysteine residues within the nonrepetitive N- and C-terminal parts, potentially resulting in intermolecular crosslinking and oligomerization, suggest that Car proteins may form a viscous, mucous layer, as described for mucins. Interestingly, mucins also contain cysteine-rich subdomains at their ends (Gum 1992). This mucous cover may serve important protective functions during the most vulnerable stage of the pathogen's life cycle. It may shelter the germling from desiccation, physical damage, and adverse effects of the plant defense response. In addition, because of their potential viscous and sticky consistency, Car proteins may participate in adhesion to the leaf surface. This is a common feature of fungal infection structures and is apparently required to enable penetration of the host surface (Epstein and Nicholson 1997). According to a description of the physical properties of adhesives and adhesive performance by Eagland (1988), adhesives typically spread in a liquid state and polymerize thereafter. Such prerequisites may be fulfilled by Car proteins.

Potato-*P. infestans* interactions have been extensively studied but detailed knowledge about cellular and molecular aspects is mostly restricted to the plant side. Concentrating on the pathogen, we have found novel genes encoding for proteins with peculiar features. Expression of these genes exclusively in infection structures of *P. infestans* indicates that they may play an important role in pathogenicity.

MATERIALS AND METHODS

Cultivation of *P. infestans* and inoculation of potato leaves.

The *P. infestans* (Mont.) de Bary strain 88069 (race 1.3.4.7) was obtained from F. Govers in Wageningen, The Netherlands, and two different isolates of race 1-11 (A, B) were obtained from Biologische Bundesanstalt, Braunschweig, Germany. The fungus was maintained on vegetable juice agar (Rohwer et al. 1987) and grown at 17°C in the dark.

To induce sporangia formation the fungus was grown on rye-sucrose agar (RSA) (Caten and Jinks 1968). Liquid cultures in pea broth (Tooley and Carras 1992) were initiated from zoospores obtained from 11- to 14-day-old cultures on RSA. Cultures were incubated for about 10 days at 17°C to obtain mycelium for nucleic acid and protein extractions.

Leaves of potato (*Solanum tuberosum*) cv. Isola, which carries the R-4 gene for late blight resistance, were inoculated with *P. infestans* by submerging them in a zoospore/water suspension (1×10^6 zoospores per ml). Leaves were then incubated at 17°C at 95 to 100 % humidity in plastic boxes on wet filter paper in the dark.

Enrichment of fungal developmental stages.

Sporangia were harvested from 11- to 14-day-old RSA cultures. The mycelium on the agar plate was washed with 10 ml of deionized water pre-cooled to 4°C and sporangia were pelleted by centrifugation. Release of zoospores was induced by incubating the RSA cultures submerged with water as described above at 4°C for 1.5 to 4 h. The zoospore suspension was collected from the dishes and filtered through a 20- μ m nylon net. Cysts were isolated by vigorous shaking of the filtered zoospore suspension for 2 min before sedimentation of the cysts by centrifugation. The cysts were resuspended in sterile tap water, pea broth, or 2 % sucrose in tap water, respectively, and the titer was adjusted to 1×10^6 cysts per ml. Germination of cysts was achieved by incubation of a cyst suspension at 17°C for 2 to 3 h.

For appressoria formation, cysts resuspended in tap water were sprayed on polypropylene foil (PLASTIBRAND, Brand, Germany) and incubated for 4 h at 17°C in plastic containers with humidity near 100%. After incubation, foils were washed gently with deionized water to enrich for fungal structures that adhere strongly to the plastic surface via appressoria. The foils with remaining fungal structures and about 4 ml of deionized water were frozen in liquid nitrogen and then ice pieces containing the fungal structures were quickly crumbled off the foils and lyophilized.

Isolation of genomic DNA and construction of a genomic library.

Genomic DNA from *P. infestans* was isolated from mycelium grown in liquid culture. Mycelium was ground in liquid nitrogen to a fine powder and DNA was extracted according to Pieterse et al. (1991).

A genomic library was constructed in the replacement vector λ EMBL3 (Frischauf et al. 1983). For this purpose *P. infestans* 1-11 (A) genomic DNA was partially digested with the restriction endonuclease *Sau3AI*. DNA fragments (9 to 23 kb) were cloned into the pre-digested vector Lambda EMBL3 *Bam*HI (Stratagene, Heidelberg, Germany). Cloning and packaging (packaging extract Gigapack II Gold; Stratagene) were performed according to the manufacturer's instructions.

Isolation of RNA and poly A⁺ RNA.

Total RNA was prepared from various in vitro enriched developmental stages of *P. infestans* and from colonized potato leaflets by the guanidine hydrochloride RNA extraction method according to Logemann et al. (1987). Poly A⁺ RNA was isolated with the help of magnetic beads (Oligo(dT)₂₅-

Dynabeads; Deutsche DYNAL, Hamburg, Germany) according to the manufacturer's instructions.

Construction of a subtracted cDNA library and differential screening.

To enrich for germinating cysts and appressoria-specific cDNAs of *P. infestans*, a PCR-based SH method according to Sharma et al. (1993) was used with minor modifications.

Starting with 300 µg of zoospore total RNA, a first-strand cDNA-library was synthesized on Oligo(dT)₂₅-Dynabeads with SuperScript reverse transcriptase (GIBCO BRL, Eggenstein, Germany). Poly A⁺ RNA derived from 75 µg of total RNA from in vitro enriched appressorial structures was hybridized to the zoospore first-strand cDNA library in three consecutive cycles. *Escherichia coli* 5S RNA (1.6 µg; Boehringer Mannheim, Mannheim, Germany) was used as carrier. Subtractive mRNAs were reverse transcribed, and the resulting double-stranded cDNAs ligated to adapters and PCR amplified. PCR products were purified via adsorption on QIAquick silica gel membranes (Qiagen, Hilden, Germany) and subcloned into the pT7-Blue vector (Novagen, Heidelberg, Germany).

Colony hybridizations with single radiolabeled SH inserts were performed to reduce the number of colonies to be analyzed in a differentially dot blot screening.

For differential screening, plasmid DNA from 200 SH clones was prepared, heat denatured, and dotted on rastered NC membranes (Schleicher and Schüll, Dassel, Germany). Six replica filters were generated. The SH clones were differentially hybridized in duplicate at high stringency in hybridization mix containing 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 0.1% (wt/vol) N-lauroylsarcosine-Na-salt, 0.02% (wt/vol) SDS, 1% (wt/vol) blocking reagent (Boehringer Mannheim) at 65°C for 16 h. As probes, cDNA derived from poly A⁺ RNA of in vitro enriched zoospores, germinating cysts, or appressoria of *P. infestans* 1-11 A was used and radioactively labeled according to Sambrook et al. (1989). The filters were washed twice with 1× SSC, 0.5% SDS at 65°C.

Screening of genomic library, subcloning, and sequencing.

Approximately 3 × 10⁵ recombinant plaques of the genomic λEMBL3 *P. infestans* 1-11 library were screened for hybridization to the isolated, partial, SH cDNAs *Car59* and *Car90*. Hybridization conditions were as described above. The cDNA probes were ³²P-labeled with the Ready to Go DNA Multiprimer labeling Kit (GIBCO BRL, Eggenstein, Germany) according to the manufacturer's instructions. Plaques giving a positive hybridization signal were carried through two purification rounds.

DNA from selected lambda clones was digested with several restriction enzymes, and DNA fragments ranging from 0.15 to about 5.8 kb were subcloned into the pUC18 vector. In addition, two PCR products (primer: *Nco*I/5941 [CGACCTTCCATGGTTTCCAGG/TCTTCACGTGTGCCGGCATCG] and gZ90Eco/λArmUni [CTGTAATACGGAGGAATTCAGT/GCGACCTGCAGGTCAACGGATC]) were cloned into the pT7Blue vector.

DNA sequence analysis.

DNA sequences were determined on PE/Applied Biosystems 377 and 3700 sequencers (Weiterstadt, Germany) with

BigDye-terminator chemistry. A series of partially overlapping subclones and the following specific primers to bridge restriction sites were used:

140 (GACAGCGACTCAAGGTCACGCAAC)
16un2 (GCTACTACTACAATCGTGGTATC)
16un3 (AATCTCTGATTCTGTTGTCTACG)
-406 (GTCACGCTGCACGGCATGGC)
5921 (CAGAAGATATGGCTGGCAGTCG)
5941 (TCTTCACGTGTGCCGGCATCG)
5C59 (CGTCGTCATTGCTGACAGAAGTC)
9016 (GTGAAGTTCAAGCCACAACATATAC)
C90P2 (CGATGACTCATTTCCATAACACG)
C59P (GTTAGATCCTCACTATTAGTAGCG)
C59P2 (CGAGTAAGTAAACGAGACGATC)
C90P (CCACGTGCTAGTACGTCATTTAC)
gZ90Eco (CTGTAATACGGAGGAATTCAGT)
KpnP (GATGTCGTGAAGGTACCATAG)
gZ90 (CCCTGTCAGAAGTCGACGTCGTC).

Oligonucleotides were purchased from MWG Biotech, Ebersberg, Germany.

Sequence data were analyzed with the Wisconsin Sequence Analysis Package of the Genetic Computer Group (GCG, Madison, WI). Additionally, BLASTP & BEAUTY (NCBI's gapped BLASTP with BEAUTY; BCM Search-Launcher) was used for protein sequence comparison.

The GenBank accession numbers for the *car* genes are AF061185 (*car90*) and AF061186 (*carPs*, *car59*), respectively.

Southern and Northern analysis.

For Southern analysis, genomic DNA was digested with restriction enzymes, subjected to electrophoresis on a 1% agarose gel, and transferred to Nylon membranes (Hybond N; Amersham Buchler, Braunschweig, Germany) as described previously (Sambrook et al. 1989). Prehybridization was at 65°C for at least 8 h in 5× SSC, 0.1% N-lauroylsarcosine-Na-salt, 0.02% SDS, 1% blocking reagent. For hybridization, DNA fragments, ³²P-labeled by random primed labeling (Ready to go DNA Multiprimer Labeling Kit; GIBCO BRL) were added. The membranes were washed twice with 0.1% SDS, 0.5× SSC at 65°C.

For Northern analysis, poly A⁺ RNA was subjected to electrophoresis on a 1.2% agarose gel containing 2% formaldehyde and blotted on Nylon membranes as described previously (Sambrook et al. 1989). The conditions for hybridization and washing were the same as those described for the DNA blot.

Isolation of proteins.

Crude protein extracts from various in vitro enriched developmental stages of *P. infestans* were prepared by grinding the fungal structures in liquid nitrogen to a fine powder and suspending it in extraction buffer (40 mM phosphate buffer pH 7.0, 5 mM EDTA, 20% glycerol, 10 mM β-mercaptoethanol, 5 mM PMSF [phenylmethylsulfonyl-fluoride]). Cell debris was removed by centrifugation at 13,000 × g for 10 min. The supernatant was concentrated with 100K microconcentrators (MICROSEP; Filtron, Karlstein, Germany).

Production of antiserum against the Car TR and Western analysis.

To produce antiserum against the Car proteins, a MAP4-peptide was synthesized (BIOGENES; Berlin). Therefore, a

16mer peptide (TTYAPTEE)₂ reflecting the Car TR consensus sequence was coupled four times and via a polylysine linker covalently bound to a synthetic bead. Two rabbits were immunized against this antigen. Antisera were combined and affinity purified against the 16mer peptide.

For Western blot analysis, aliquots of protein extracts (20 µg) from various in vitro enriched developmental stages of *P. infestans* were subjected to SDS-PAGE (Laemmli 1970), blotted on nitrocellulose membranes, and detected by reaction with CarMAP4 antiserum with ECL Western blotting detection reagents from Amersham Buchler (Braunschweig, Germany).

Immunocytochemical staining and in situ hybridization.

To get germinating cysts as well as appressorial structures for immunolocalization and in situ hybridization, cysts in tap water were sprayed on gelatin-covered microscopic slides and incubated for 3 to 5 h at 17°C and 95 to 100% humidity. Fixation was performed in 5% dimethyl sulfoxide (DMSO), 3.7% formaldehyde in MSB (50 mM MES [morpholineethanesulfonic acid], 50 mM KCl, 2 mM MgCl₂, 10 mM EGTA, 1 mM EDTA, pH 6.7] for 2 h on ice. Slides were washed in MSB and fungal structures were dehydrated stepwise in ethanol (20, 40, 60, 80, and 96%, 5 min each). Car proteins were visualized at the surface of *P. infestans* germings by indirect immunofluorescence staining with appropriate dilutions of primary (anti Car-TR) and secondary (Cy3-conjugated AffiniPure Goat Anti-Rabbit IgG; dianova, Hamburg, Germany) antibodies.

Car transcripts were detected by in situ hybridization according to Schmelzer et al. (1988) with [³⁵S]UTP labeled antisense transcripts of a 2.2-kb *car90* TR DNA fragment.

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