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ORIGINAL PAPER

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NiaA, the structural nitrate reductase gene of *Phytophthora infestans*: isolation, characterization and expression analysis in *Aspergillus nidulans*

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Abstract The nitrate reductase (NR) gene *niaA* of the oomycete *Phytophthora infestans* was selected from a gene library by heterologous hybridization. *NiaA* occurs as a single-copy gene and its expression is regulated by the nitrogen source. The nucleotide sequence of *niaA* was determined and comparison of the deduced amino-acid sequence of 902 residues with NRs of higher fungi and plants revealed a significant homology, particularly within the three cofactor-binding domains for molybdenum, heme and FAD. The *P. infestans niaA* gene was used as a model gene to test whether oomycete genes are functional in the ascomycete *Aspergillus nidulans*, a fungus which is highly accessible for molecular genetic studies. The complete *niaA* gene was stably integrated into the genome of a *nia*$^-$ deletion mutant of *A. nidulans*. However, transformants containing one or more copies of the *niaA* gene were not able to complement the *nia*$^-$ mutant. This suggests that there is no functional expression of the introduced *niaA* gene in *A. nidulans*. In addition, the activity of two other oomycete gene promoters was analyzed in a transient expression assay. Plasmids containing chimaeric genes with the promoter of the *P. infestans* ubiquitin gene *ubi3R*, or the *Bremia lactucae ham34* gene, fused to the coding sequence of the *Escherichia coli* β-glucuronidase (GUS) reporter gene, were transferred to *A. nidulans* protoplasts. No significant GUS activity was detectable indicating that the *ubi3R* and *ham34* promoters are not active in *A. nidulans*. Apparently, the regulatory sequences which are sufficient for gene activation in oomycetes are not functional in the ascomycete *A. nidulans*.

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Introduction

The oomycete *Phytophthora infestans* (Mont.) de Bary is a destructive pathogen causing late blight on potato, tomato and several other Solanaceae. The oomycete class of fungi contains many economically important plant pathogens. To study the molecular and cellular processes involved in the interaction of oomycetous plant pathogens with their host plants, characterization of pathogenicity genes is an important step. We have isolated several *P. infestans* genes whose gene products might have a role in pathogenicity (Pieterse et al. 1991, 1993a,b, 1994). To study the function and regulation of these genes, DNA transformation is an essential tool. At present, while transformation of *P. infestans* is an established procedure (Judelson et al. 1991, 1993) the transformation efficiency is relatively low and, compared to some ascomycetes, *P. infestans* is slow growing. Moreover, for successful transformation, regulatory sequences of oomycete origin seem to be crucial. Regulatory sequences from genes of non-oomycetes, including ascomycete and basidiomycete fungi, are not functional in *P. infestans* (Judelson et al. 1992). In the present study we tested, in two different ways, whether oomycete regulatory sequences are functional in the ascomycete *Aspergillus nidulans*. If so, this would facilitate further molecular genetic studies on oomycete genes, such as promoter analysis or complementation experiments.

In this paper, we describe the isolation and characterization of the *P. infestans* nitrate reductase (NR)-encoding gene *niaA*. The *niaA* gene was subsequently used to test if oomycete regulatory sequences function in *A. nidulans*. This was done by transforming the *P. infestans niaA* gene to a *A. nidulans nia*$^-$ mutant and analyzing the transformants for functional complementation of the NR deficiency. It has been shown that NR deficiency in several filamentous fungi, including some plant pathogens,

can be complemented by the introduction of heterologous NR genes (Daboussi et al. 1989; Whitehead et al. 1989; Unkles et al. 1989a,b). In a second approach to test oomycete regulatory sequences in a non-oomycete we used constructs containing promoter-GUS fusions. The activity of the promoters of the *P. infestans* ubiquitin gene *ubi3R* (Pieterse et al. 1991) and the *Bremia lactucae ham34* gene (Judelson and Michelmore 1990) was analyzed by measuring GUS activity in transient expression assays in *A. nidulans*.

Material and methods

Culturing of P. infestans *and* A. nidulans. *P. infestans* strain 88069 was grown as described previously (Pieterse et al. 1991). The standard media and growth conditions for *A. nidulans* described by Cove (1966) were used.

Isolation and sequencing of the P. infestans niaA *gene.* A λEMBL3 genomic library of *P. infestans* was constructed as described previously (Pieterse et al. 1991). In total, 8×10^4 recombinant plaques comprising six times the *P. infestans* genome were plated in duplicate. Replica filters were hybridized for 16 h at low stringency (58°C) in a hybridization mix containing 5×SSC (750 mM NaCl, 75 mM Na_3-citrate), 5× Denhardt's solution [0.1% (w/v) Ficoll, 0.1% (w/v) polyvinylpyrrolidone, 0.1% (w/v) BSA (fraction V)], 0.5% SDS and 100 μg/ml of calf thymus DNA. As a probe the 1.5-kb *Bam*HI/*Bgl*II-fragment from pSTA10 was used, which covers a large part of the coding region of the NR gene (*niaD*) of *A. niger* (Unkles et al. 1989a). This probe was labelled with [α-^{32}P]dATP by random primer labelling as described by Feinberg and Vogelstein (1983). Filters were washed in 2×SSC/0.5% SDS at 58°C and exposed to Kodak X-OMAT S film. Positive plaques were purified by a second round of hybridization. DNA restriction analyses and subcloning of fragments from the selected lambda clones was performed according to standard procedures (Sambrook et al. 1989). Sequencing of DNA fragments was done on double-stranded DNA by the dideoxy chain-termination method (Sanger et al. 1977) using the Multiwell Microtitre Plate Sequencing System (Amersham) and [α-^{35}S]dATP as a label. Analyses of the sequence data and alignment of the amino-acid sequences were performed using the Sequence Analysis Software Package, Version 7.1, of the Genetics Computer Group (GCG) of the University of Wisconsin (Devereux et al. 1984).

Genomic Southern-blot analysis. Genomic DNA of *P. infestans* strain 88069 and *A. nidulans* strain ANGW1103 was isolated as described by Pieterse et al. (1991). DNA was digested with various restriction enzymes and size-separated on 0.7% agarose/TBE gels. Following electrophoresis, DNA was blotted onto Hybond-N$^+$ membranes (Amersham) by capillary transfer (Sambrook et al. 1989) and hybridized at high stringency (65°C) in a hybridization mixture containing 0.5 M Na_2HPO_4/NaH_2PO_4 (pH 7.2), 7% SDS and 1 mM EDTA. Probes were labelled with [α-^{32}P]dATP by random primer labelling (Feinberg and Vogelstein 1983). Blots were washed in 0.1×SSC/0.1% SDS at 65°C and exposed to Kodak X-OMAT AR film.

RNA isolation and northern-blot analysis. Total RNA was isolated from a *P. infestans* mycelium grown in Henniger synthetic medium (Henniger 1959) with (1) 48 mM $NaNO_3$ and no ammonium or (2) 48 mM $(NH_4)_2SO_4$ and no nitrate. The RNA isolation was performed as previously described (Pieterse et al. 1991). For northern-blot analysis, 15 μg of total RNA was denatured in DMSO/glyoxal and electrophoresed on a 1.4% agarose gel (Sambrook et al. 1989). Following electrophoresis, the RNA was blotted onto a Hybond-N$^+$ membrane (Amersham) by capillary transfer and hybridized at high stringency (65°C) as described above using the random primer-labelled 2.2-kb *Nco*I-fragment from pNiaA-S as a probe. The blot was washed in 0.5×SSC/0.1% SDS at 65°C and exposed to Kodak X-Omat AR film. After de-probing, the blot was rehybridized with the 2.8-kb *Pst*I-insert from pSTA31 containing the constitutively expressed actin (*actA*) gene of *P. infestans* (Unkles et al. 1991).

Integrative transformation of A. nidulans. The plasmids pSTA10 (Unkles et al. 1989a) and pNiaA-H, containing the NR genes of *A. niger* and *P. infestans*, respectively, were transformed to *A. nidulans* as described by Wernars et al. (1985). As *A. nidulans* recipient strain the *nia*$^-$ deletion mutant ANGW1103, which is an *arg*$^+$ derivative of strain SAA1017 (*yA2*, *argB2*, *niaD26*) (T. Goossen, personal communication), was used. The *nia*$^-$ phenotype of SAA1017 is due to a deletion in the *niaD* gene (Tomsett and Cove 1979). $CaCl_2$-PEG-mediated transformation was performed using 2×10^7 protoplasts and 5 μg of plasmid DNA. Transformants were selected on minimal medium containing 50 mM $NaNO_3$ as a sole nitrogen source. For co-transformation, 2×10^7 protoplasts were transformed with a mixture of 5 μg pAN8-1 and 20 μg pSTA10 or pNiaA-H, respectively. Integration of plasmid pAN8-1 confers resistance to phleomycin (Mattern and Punt 1988). Transformants were selected on minimal medium containing 60 μg/ml phleomycin. The percentage of co-transformation was determined by transferring the transformants to minimal medium containing 50 mM $NaNO_3$ as a sole nitrogen source.

Transient expression assay. For the transient expression assays, in which the activity of various gene promoters was determined, transformation of *A. nidulans* strain ANGW1103 was performed as described above using 2×10^7 protoplasts and 5 μg of plasmid DNA. The plasmids used are listed in Table 2. After transformation, the protoplasts were allowed to regenerate for 4 h at 30°C in MMS medium (Wernars et al. 1985). Regenerated protoplasts were collected by centrifugation (5 min at 10 000 *g*) and homogenized in an equal volume of GUS-extraction buffer (50 mM $NaPO_4$, 10 mM Na_2EDTA, 0.1% Triton X-100, 10% N-laurylsarcosine, 10 mM β-mercaptoethanol, pH 7.0). After an incubation of 30 min on ice, the homogenate was cleared by centrifugation (2 min at 10 000 *g*) and protein concentrations were determined by the Biorad Protein Assay. GUS analyses were performed in a fluorometric assay as described by Gallagher (1992) using 5 μg of protein per assay and 4-methylumbelliferyl β-glucuronide (1 mM) as a substrate.

Results

Isolation of the *P. infestans niaA* gene

To isolate the gene encoding the nitrate reductase (NR) apoenzyme of *P. infestans*, a genomic library was screened under low-stringency conditions using a part of the coding region of the *A. niger niaD* gene as a probe. Initially eight clones were selected of which four were still positive in the second round of hybridization. Restriction-fragment analysis revealed no homology in restriction patterns indicating that the four clones were not overlapping. A Southern blot containing digested DNA of the four λ clones was hybridized with the same *A. niger niaD* probe and the hybridizing fragments were subcloned and partially sequenced. Several fragments from λnia2 showed significant sequence homology with parts of the *A. niger niaD* nt sequence whereas the sequenced fragments of the other three clones shared no homology at all. The latter were considered to be false positives. A partial restriction map of λnia2 was constructed (Fig. 1). The *P. infestans niaA* gene is located on an internal 3.6-kb *Sst*I-fragment. This fragment, and an overlapping 8.5-kb *Hin*dIII-fragment, were subcloned into pTZ19 U resulting in the plasmids pNiaA-S and pNiaA-H, respectively.

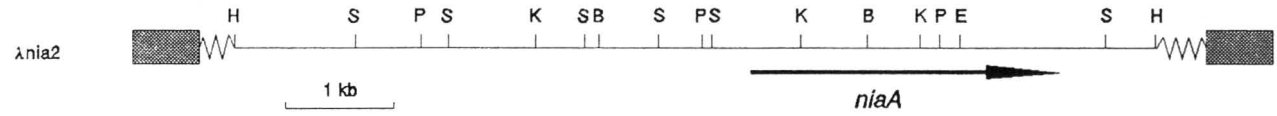

Fig. 1 Partial restriction map of the λnia2 clone containing a full length copy of the *P. infestans niaA* gene. The *arrow* indicates the position of the coding sequence. *H* = *Hin*dIII; *S* = *Sst*I; *P* = *Pst*I; *K* = *Kpn*I; *B* = *Bam*HI; *E* = *Eco*RI

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GAGCTCACTTATCGACAACACATGTAGTTTTATTAGAGCTTCTAGAGCTTATCAATCGCTGTAATGCCGACACTCACGGCGTCACGTGCGGGGGAGGAGATTATTTAACGTCACGCGCAC  -248
TCGGTCACTTTCAAGCACGAGATCGTGAGTCAGCTTTAATGGCGAGTTTGAAGTTGTCCAGCAGCTCGAGTCTGCGATGTAGCGTTCAGAGATCACCCATCCTTTCGATGGCGTGCATGT  -128
ATTTCGATCTAACCCGCTTTCTTCTAGGTGCGACTTCTCTCGACGGGCGCTGGACTCCGACCTGAAAGAATTGTGACAAGCCGCGCGTGAGCAACGCGAACTTGGAACTGATTGGCCTCT    -8
CTCCACCATGACGCGTTTGAGATCTGCCGCCAGCACAATCACCAACCGGTCGACGCCGGCGTTGGCGCTAACGGCATCCCACGCGGGCTCGCATCCCGCCGTCATCGTGCCGATCCAACC  +113
      M  T  R  L  R  S  A  A  S  T  I  T  N  R  S  T  P  A  L  A  L  T  A  S  H  A  G  S  H  P  A  V  I  V  P  I  Q  P    38
GCTCTTGCTCACGCTCGAGCGCCGCCCATCATGCCCGCACCGTGTGAGTTATCCCGAAATCGAGGCATACCGCGGCATCAGTAGCTCAgtgcgttggaggcgacgtggtccaattaaaac  +233
 L  L  L  T  L  E  R  R  P  S  C  P  H  R  V  S  Y  P  E  I  E  A  Y  R  G  I  S  S  S                                    67
gcgtagtgaaaccggttgctcagggtgaaagccaaaggtatggacggctgagtaatcctaactcgcaggcgggcgcgctcttagCTGCGTTCTCACAATGCGATTGTCCGTGCAAGTGAC  +353
                                                                                    L  R  S  H  N  A  I  V  R  A  S  D     79
ATCATGGCGCAAATTGACCCCAGAGACGTTGGTACGCCCGATGAGTGGGTACCCCGGCACCCTGAGCTGATCCGGCTCACGGGACGACACCCGTTCAATAGTGAGCCCCCGCTGAAGTAC  +473
I  M  A  Q  I  D  P  R  D  V  G  T  P  D  E  W  V  P  R  H  P  E  L  I  R  L  T  G  R  H  P  F  N  S  E  P  P  L  K  Y   119
GCGAGCACCTTTATCACACCCATGGCATTGCACTACGTTCGCAACCACGGCCCCGTGCCGAGACTCGAGTGGGATACACATACGTTCTCGATCGACGGACTGGTGAAGAAGCCGCGTACG  +593
A  S  T  F  I  T  P  M  A  L  H  Y  V  R  N  H  G  P  V  P  R  L  E  W  D  T  H  T  F  S  I  D  G  L  V  K  K  P  R  T   159
TTCGGCATGAACGAGCTGGTCACCACTTTTGAACAGGAGACCGTGACGTTCCCGGTGTTGCTGGTATGTGCCGGTAATCGCCGTAAAGAACAGAATATGATCAAGAAGACTATCGGCTTT  +713
F  G  M  N  E  L  V  T  T  F  E  Q  E  T  V  T  F  P  V  L  L  V  C  A  G  N  R  R  K  E  Q  N  M  I  K  K  T  I  G  F   199
AGTTGGGGCGCGGCCGGATGCTCCACCGCTGAGTGGACAGGAGTCCCGCTTCACGTACTGCTGACGGCTTGCGGTGTTGATCGAGAGAAGGCACAGTGGGTGTGGTTTGAGGGCATCGAG  +833
S  W  G  A  A  G  C  S  T  A  E  W  T  G  V  P  L  H  V  L  L  T  A  C  G  V  D  R  E  K  A  Q  W  V  W  F  E  G  I  E   239
GACTTGCCACACGACAAGTACGGCACGTGCATTCGTGCCAGTACGGAGTTGGACCCGGAGTGCGATGTTCTGGTGGCGTGGAAAGCCAACGGTGAGCTACTCGGTCCTGATCATGGGTTC  +953
D  L  P  H  D  K  Y  G  T  C  I  R  A  S  T  E  L  D  P  E  C  D  V  L  V  A  W  K  A  N  G  E  L  L  G  P  D  H  G  F   279
CCTGTGCGCCTTATTGTCCCAGGTCACATCGGTGGACGCATGGTCAAGTGGCTCGAACGGATCCACGTGAGCGATCACGAGTCCTCCAATCACCATCACATTATGGACAATCGCGTACTA  +1073
P  V  R  L  I  V  P  G  H  I  G  G  R  M  V  K  W  L  E  R  I  H  V  S  D  H  E  S  S  N  H  H  H  I  M  D  N  R  V  L   319
CCGTCGCACGTCACTGCCGAGACGGCAACGGCGGAAGGTTGGTGGTCCAAGTCGCCGTACGCGATCATGAGGAGCTTAACGTCAATGCCGTGGTCATTTTACCGAACCATGACGACCTGTTG  +1193
P  S  H  V  T  A  E  T  A  T  A  E  G  W  W  S  K  S  P  Y  A  I  M  E  L  N  V  N  A  V  V  I  L  P  N  H  D  D  L  L   359
GCTCTCGGTGAAGACACCACGTTTAACGACATCGAGACGTACACGATCAAGGGGTACGCGTATTCAGGCGGTGGTCGGCGAGTTATTCGCGTAGAGGTGACGCTAGACGATGGCGCATCG  +1313
A  L  G  E  D  T  T  F  N  D  I  E  T  Y  T  I  K  G  Y  A  Y  S  G  G  G  R  R  V  I  R  V  E  V  T  L  D  D  G  A  S   399
TGGCAGCTAGCTCGCATCATTTACCATGAGAGGCCAAGCAAGTACGGCAAAATGTGGTGCTGGGTGCACTACGAGTTGGCCGCTCCGATGAGCAGTCTGCTTTGTGCTCGAGAAGTGTGC  +1433
W  Q  L  A  R  I  I  Y  H  E  R  P  S  K  Y  G  K  M  W  C  W  V  H  Y  E  L  A  A  P  M  S  S  L  L  C  A  R  E  V  C   439
GTTCGAGCTTGGGACAGCTCGATGAACCTCATGCCAGCGTTCCCTACATGGAATGTTATGGGCATGATGAACAACCCTTGGTACCGCGTCAAGATCCACCACGAACAAGACACGAACTCG  +1553
V  R  A  W  D  S  S  M  N  L  M  P  A  F  P  T  W  N  V  M  G  M  M  N  N  P  W  Y  R  V  K  I  H  H  E  Q  D  T  N  S   479
CTACGGTTCGAGCATCCGACTCAAGCAGGAAACCAGAAAGGTGGCTGGATGACCAAGGAACGGATCATGACTAACGACGTCGACTCGATTAAGATGCTGCAAGTGGAGCCACTTGACACG  +1673
L  R  F  E  H  P  T  Q  A  G  N  Q  K  G  G  W  M  T  K  E  R  I  M  T  N  D  V  D  S  I  K  M  L  Q  V  E  P  L  D  T   519
AGCAGTGCTGCAACACCGAAGCCTGGTCTGACTGCAGACGAATTGAGTGAGCTGCCTCTGATTTTTGCAGACGAAGTTGCAAAACACAACTCGAAGAAAAGCTGCTGGTTTATTTGTCGC  +1793
S  S  A  A  T  P  K  P  G  L  T  A  D  E  L  S  E  L  P  L  I  F  A  D  E  V  A  K  H  N  S  K  K  S  C  W  F  I  C  R   559
GACCTGGTGTACGACGCAACACCGTTCCTGGACGAACACCCTGGTGGAGCCACAAGTATCTTGCTGTGTGGTGGCACAGACTGTACGGACGAATTCGAGAGCATCCACTCTACCAAAGCC  +1913
D  L  V  Y  D  A  T  P  F  L  D  E  H  P  G  G  A  T  S  I  L  L  C  G  G  T  D  C  T  D  E  F  E  S  I  H  S  T  K  A   599
TGGCAAATGCTGAAGAAATACTGTATCGGACGTTGTTCAAGCACAGAGGACGACACTGGAACCAGCGATACCTCGTCGGACCATGAAGAGACGGACGTGGCGCTCAAAGGCCGTACCAAG  +2033
W  Q  M  L  K  K  Y  C  I  G  R  C  S  S  T  E  D  D  T  G  T  S  D  T  S  S  D  H  E  E  T  D  V  A  L  K  G  R  T  K   639
GTGCCTATCGTACTGATCTCAAGAGAGGTGGTCAGCCACGATGCACGCATCTTTAAATTTGCTCTGCCTGCCAAGGATCTGAGACTCGGACTACCTATTGGCAACCACGTTTTCTTGTAC  +2153
V  P  I  V  L  I  S  R  E  V  V  S  H  D  A  R  I  F  K  F  A  L  P  A  K  D  L  R  L  G  L  P  I  G  N  H  V  F  L  Y   679
GCGAAGATCAACGGCAAGACCGCCGTGCGTGCGTACACACCGATATCGTCCGAGAACGACGAAGACCGAGGATTCGTGTCCTTCCTCATCAAAGTCTACTTTGCAGGCGACAACCCTGTG  +2273
A  K  I  N  G  K  T  A  V  R  A  Y  T  P  I  S  S  E  N  D  E  D  R  G  F  V  S  F  L  I  K  V  Y  F  A  G  D  N  P  V   719
CATCCAGAAGGAGGACTCTTTTCACAGTATCTCGATGGCTTGCATCTTGGTCAACAGATTCAGATCAAGGGACCGCTGGGCCACTTCACGTACTACGGAGACGGCAATTTCTCCCTCGAG  +2393
H  P  E  G  G  L  F  S  Q  Y  L  D  G  L  H  L  G  Q  Q  I  Q  I  K  G  P  L  G  H  F  T  Y  Y  G  D  G  N  F  S  L  E   759
ACGACCAATTTTCACGCCTACAAGTTCGGGTTCGTCGCCGGAGGGACAGGTATCACGCCCGTCTACCAGGTCATGCGCGCCATTCTTGAAGACGCAAAGGACCAGACCAAAGTTGCGCTC  +2513
T  T  N  F  H  A  Y  K  F  G  F  V  A  G  G  T  G  I  T  P  V  Y  Q  V  M  R  A  I  L  E  D  A  K  D  Q  T  K  V  A  L   799
ATCTATTGCGTGCGCTCGCAGCGTGACCTGCTGCTCCGAAAGGAACTTGAAACCCTACAAAAGCTGCGACCGGGTCAGTGCCGGATCTTCTACACTTTGTCAGACATGGAGCTGCTTGAT  +2633
I  Y  C  V  R  S  Q  R  D  L  L  L  R  K  E  L  E  T  L  Q  K  L  R  P  G  Q  C  R  I  F  Y  T  L  S  D  M  E  L  L  D   839
AGGAACGACCCTATTGTTCGAGGGTGGGCGTACGGTAAGAGTCGACTTAACTTTGCCATGGTGAAGAACATCATCGGCTCGGACGCCGAGGACGTTTGCATGTGCGGCCCCGAGGGTATG  +2763
R  N  D  P  I  V  R  G  W  A  Y  G  K  S  R  L  N  F  A  M  V  K  N  I  I  G  S  D  A  E  D  V  C  M  C  G  P  E  G  M   879
ATTGAGTACGCCTGCAAGCCAGCGCTACTTAAGCTGAACTACGACCTCAAAACCCAGACCACCGTGTTTTAATTGCACTATTTCGTGTAGCTAAGCTAAAAGTTTCAAAGAGAATCGTGA  +2873
I  E  Y  A  C  K  P  A  L  L  K  L  N  Y  D  L  K  T  Q  T  T  V  F  *                                                    902
TCTAAAAGAGTACATTGAGGTCCAGCCATCTTCACCTATAAAACGCGATGCTCCTTCTGCTTGGTTGCATAGGTAAGCAAAGGTACACGACCCAAGCACCCTGCACCCTTGGCTCCCGTC  +2993
TCAAGACTATATCCACCATTATCGTGTCAAGATACCTGGTGGTTCTGGCCACTTAACGATCTTGAATTTCGGATGACTCACTGCCAAGTGCCCTCTCGTCTGTTTGGTGACGCTACAGAC  +3113
AGGCGATGCTTTTTGTGAAGTCAGTGGTCGGATTGGATGGATAAAGCGCGTCACACTATCGTCTTGTCCAGCACTGCAAGCTGTGCACCATGCGTCATTTCGAGTGAGTCACCACTTTTT  +3233
CGAGCTC                                                                                                                    +3240
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Fig. 2 Nucleotide sequence of the *P. infestans niaA* gene and the deduced amino-acid sequence of the NR protein. Position +1 is the first nucleotide of the ATG start codon. The CT-rich regions in the promoter are *underlined*. *Lower case* letters indicate the 115-bp intron with the intron boundary nucleotides and the branch point consensus in *bold italics*. The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U14405

Structure of the *P. infestans niaA* gene

To enable sequencing of the entire *niaA* gene, overlapping restriction fragments from pNiaA-S were subcloned into pTZ19 U. Computer analysis of the nt sequence of the 3607-bp *Sst*I-fragment (as shown in Fig. 2) revealed a large continuous ORF which has significant regions of homology to the deduced amino-acid sequence of several other NR-encoding genes. However, this ORF does not contain an initiation codon at the 5′ end. Upstream of this ORF a putative intron of 115 bp is present and this intron is preceded by a smaller ORF encoding 67 aa with an initiation codon at the 5′ end. When the intron is removed a single ORF of 2706 nt with a coding capacity of 902 aa is found. The length of NR in other organisms is in the same range.

The putative intron begins with the nt sequence GTGCGT and ends with TAG which exactly matches the consensus sequences for 5′ and 3′ intron splice sites in filamentous fungi (GTPuNGPy and PyAG, respectively; Unkles 1992). The putative intron contains a 7-bp sequence, TCCTAAC, which is nearly identical to the yeast branch point consensus sequence TACTAAC. This sequence is known to be necessary for intron splicing and is specifically required for lariat formation. The TCCTAAC sequence in *niaA* is located at 23 bp from the 3′ splice junc-

tion which is consistent with the average location of branch point consensus sequences in filamentous fungi (Unkles 1992).

Analysis of the 367 nt upstream of the ATG start codon revealed that this region lacks the consensus 'core promoter' sequences TATAAA and CAAT, as well as the consensus sequence for the transcription initiation of oomycete genes, GCTCATTPyPyNCAWTTT (in which W is A or T; Pieterse et al. 1994). However, the upstream region contains some CT-rich regions which are commonly found in the vicinity of the transcription initiation sites of filamentous fungal genes and are thought to be important for determining the position of transcription initiation (Unkles 1992). The nt sequence surrounding the translation start codon (CCACCATG) follows the Kozak consensus sequence for translation initiation (Kozak 1984). In the promoter region of the *P. infestans niaA* gene no striking homology with promoters from other *nia* genes is detectable. The 3′ non-coding region lacks identifiable motifs for transcript processing. Although some short AT-rich stretches occur, no clear AATAAA polyadenylation signal is present.

Comparison of *P. infestans* NR with that of other organisms

In order to compare the predicted *P. infestans* NR aa sequence with other NRs, the aa sequences were aligned by computer and the alignment was refined by eye for maximum homology (Fig. 3). The overall aa sequence of *P. infestans* NR is fairly similar to the NR sequences of other filamentous fungi and plants. The similarity scores range from 58% to 63% and the highest degree of similarity is found with *Arabidopsis thaliana* NR.

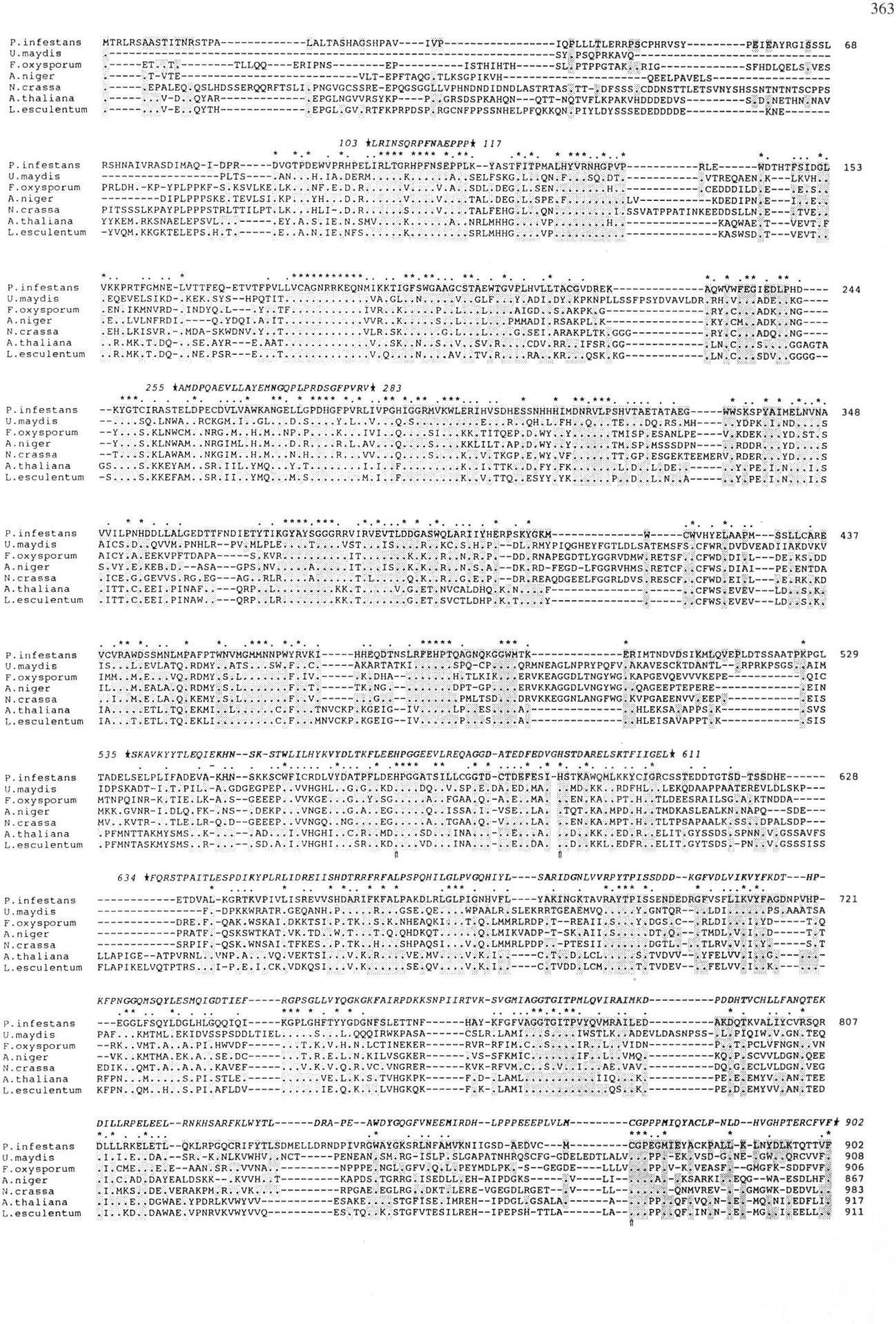
NRs contain three functional domains which are required for enzyme activity, i.e., the molybdenum cofactor domain, the heme domain and the FAD domain (Crawford et al. 1988). This so-called three-redox-centre protein is thought to have evolved from gene fusions between sequences coding for one-redox-centre proteins. Examples of the latter are rat liver sulphite oxidase (molybdenum-cofactor-containing protein; Crawford et al. 1988), bovine liver cytochrome *b5* (heme-binding protein; Ozols and Strittmatter 1969) and human NADH-cytochrome *b5* reductase (FAD-containing protein; Yubisui et al. 1984). The molybdenum cofactor domain in *P. infestans* NR is positioned in the N-terminal part of the protein. Two short peptides from sulphite oxidase show significant homology to amino-acid sequences 103–117 (47% identity) and 255–283 (48% identity). The precise extent of this domain is unknown. The heme domain of *P. infestans* NR is located at amino-acid positions 535–611. This region shows 42% identity to the bovine liver cytochrome *b5* catalytic domain. The histidine residues at positions 572 and 595 are conserved in all NR sequences as well as in all members of the cytochrome *b5* superfamily (Guiard and Lederer 1979) in which they are considered to function as heme-ligands. Amino acids 634–902 show 41% identity to the catalytic domain of human NADH-cytochrome *b5* reductase indicating that the FAD domain is situated at the C-terminal part of the protein. The lysine residue at position 710 and the cysteine residue at position 874, both of which are essential in cytochrome *b5* reductase (Hackett et al. 1986), are conserved in all NR sequences and are thought to be binding sites for NADPH. The peptide sequences representing the three domains are located in relatively highly conserved regions of the NR protein.

Nitrate reductase in *P. infestans* is encoded by a single-copy gene

On a genomic Southern blot, a probe derived from the coding region of the *niaA* gene hybridized to single DNA fragments in lanes containing *P. infestans* DNA digested with *Eco*RI, *Hin*dIII, *Kpn*I, *Pst*I and *Sst*I (Fig. 4). The sizes of the hybridizing *Hin*dIII-, *Kpn*I-, *Pst*I- and *Sst*I-fragments correspond to the sizes of the fragments in λnia2. In the *Bam*HI digest, two fragments of 2.1 and 20 kb in length hybridize. The 1.1-kb *Kpn*I fragment which was used as a probe, has an internal *Bam*HI restriction site. The 2.1-kb *Bam*HI fragment coincides with the 2.1-kb *Bam*HI fragment in λnia2. Neither the size of the hybridizing 20-kb *Bam*HI fragment nor that of the 19-kb *Eco*RI fragment can be verified as they are not completely represented in λnia2. These data demonstrate that the *P. infestans* genome contains a single copy of the *niaA* gene.

To determine whether the *P. infestans niaA* gene is transcribed, expression of the *niaA* gene was analyzed by northern-blot hybridization. The blot contained total RNA isolated from mycelium which was grown for 5 days in liquid Henniger synthetic medium containing a high concentration of $NaNO_3$ (48 mM) and no ammonium, and total RNA isolated from mycelium grown on liquid Henniger synthetic medium with 48 mM $(NH_4)_2SO_4$ and no nitrate. After hybridization with the *niaA* probe a single transcript of approximately 3200 nt was detected (Fig. 5a). The length of the *niaA* mRNA is in agreement with the length calculated from the nt sequence. The *niaA* mRNA was present in mycelium which was grown in $NaNO_3$-containing medium but not in mycelium grown in medium supplemented with ammonium. Hybridization of the same blot

Fig. 3 Comparison of the predicted amino-acid sequence of *P. infestans* NR with the NRs of four other filamentous fungi, i.e., *Ustilago maydis* (Banks et al. 1993), *Fusarium oxysporum* (Diolez et al. 1993), *Aspergillus niger* (Unkles et al. 1992) and *Neurospora crassa* (Okamoto et al. 1991), and two plants, i.e., *Arabidopsis thaliana* (Crawford et al. 1988) and *Lycopersicon esculentum* (Daniel-Vedele et al. 1989). Identical amino-acids are indicated by *shaded dots*. Spaces introduced for optimal alignment are indicated by a *dash* (–). In the line directly above the *P. infestans* sequence, amino-acid residues which are identical and similar in all seven NRs are indicated by *asterisks* and *dots*, respectively. The consensus sequences for the molybdenum cofactor domain at position 103–117 and 255–283, the heme domain at position 535–611, and the FAD domain at position 634–902 are shown in *bold italics*. *Arrows* (⇑) indicate conserved functionally essential residues


P.infestans
U.maydis
F.oxysporum
A.niger
N.crassa
A.thaliana
L.esculentum
103 ↓LRINSQRPFNAEPPP↓ 117
255 ↓AMDPQAEVLLAYEMNGQPLPRDSGFPVRV↓ 283
535 ↓SKAVKYYTLEQIEKHN--SK-STWLILHYKVYDLTKFLEEHPGGEEVLREQAGGD-ATEDFEDVGHSTDARELSKTFIIGEL↓ 611
634 ↓FQRSTPAITLESPDIKYPLRLIDREIISHDTRRFRFALPSPQHILGLPVGQHIYL----SARIDGNLVVRPYTPISSDDD--KGFVDLVIKVYFKDT---HP-
KFPNGGQMSQYLESMQIGDTIEF-----RGPSGLLVYQGKGKFAIRPDKKSNPIIRTVK-SVGMIAGGTGITPMLQVIRAIMKD----------PDDHTVCHLLFANQTEK
DILLRPELEEL--RNKHSARFKLWYTL------DRA-PE--AWDYGQGFVNEEMIRDH---LPPPEEEPLVLM---------CGPPPMIQYACLP-NLD--HVGHPTERCFVF↓ 902

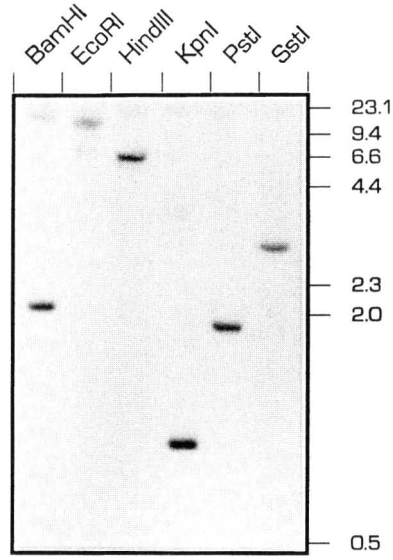


Fig. 4 Autoradiograph of a Southern blot containing genomic DNA of *P. infestans* digested with various restriction enzymes (as indicated) and hybridized with a 1.1-kb *Kpn*I fragment from the coding region of *niaA* gene. Molecular-size markers are indicated in kb

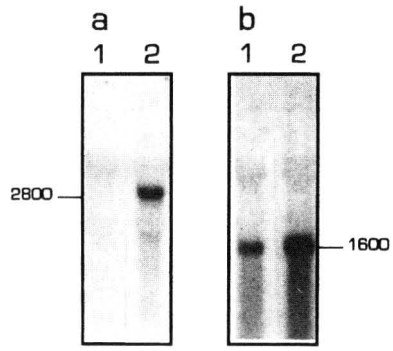


Fig. 5 **a** Autoradiograph of a N=northern-blot containing total RNA isolated from a *P. infestans* mycelium grown in liquid synthetic Henniger medium with 48 mM $(NH_4)_2SO_4$ (1) or 48 mM $NaNO_3$ (2) as a nitrogen source and hybridized with a 2.2-kb *Nco*I fragment from the coding region of the *niaA* gene. **b** Autoradiograph of the same blot which, after de-probing, was hybridized with a probe derived from the *P. infestans* actin gene, *actA*

with an actin probe demonstrated that there was sufficient RNA present on the blot (Fig. 5b). It can thus be concluded that the *niaA* gene in *P. infestans* is a transcriptionally active gene and that expression of the *niaA* gene is dependent on the nitrogen source provided in the medium.

Transcriptional activation of the *niaA* promoter and other oomycete promoters in *A. nidulans*

NR genes from various fungi can complement NR deficiency in *A. nidulans*. Conversely, the *niaD* gene of *A. nidulans*, can complement NR-deficient strains of several other ascomycetous fungi (Daboussi et al. 1989). The enzyme is strongly conserved through evolution and, as shown above, NR encoded by the *P. infestans niaA* gene contains all the conserved domains which are essential for enzyme activity. It is anticipated, therefore, that the enzyme derived from the *P. infestans niaA* gene should be able to complement a NR-deficient strain of another species. Therefore we used the *P. infestans niaA* gene as a model gene to test whether or not the promoter of an oomycete gene can be activated in the non-oomycete *A. nidulans*.

Plasmid pNiaA-H, which contains the intact *P. infestans niaA* gene with 4.4 kb upstream of and 0.8 kb downstream from the *niaA* coding region, was transformed to ANGW1103, a NR-deficient *A. nidulans* strain. In a control experiment plasmid pSTA10 (Unkles et al. 1989a) containing the *A. niger niaD* gene was transformed to the same recipient strain. Numerous pSTA10 transformants grew on medium containing 50 mM of $NaNO_3$ as a sole nitrogen source demonstrating that the *A. niger niaD* gene can functionally complement the deletion in ANGW1103. However, upon transformation with pNiaA-H no colonies appeared on selective medium and apparently in this case complementation did not occur. Co-transformation of ANGW1103 with pAN8-1 in combination with pSTA10 or pNiaA-H resulted in many phleomycin-resistant transformants. When the percentage co-transformation was determined by analyzing growth of the transformants on minimal medium with 50 mM $NaNO_3$ as a sole nitrogen source it appeared that co-transformation of pSTA10 occurred in 37% of the cases (Table 1). Integration of pNiaA-H in the pAN8-1 + pNiaA-H co-transformation experiment was determined by Southern-blot analyses and it appeared that from ten randomly selected transformants at least four were co-transformed with pNiaA-H (Table 1). All co-transformants contained full-length copies of the *P. infestans niaA* gene integrated in the genome (data not shown). Nevertheless, these stable transformants were not able to complement the nia^- phenotype. These results strongly suggest that the *P. infestans niaA* gene is not expressed in *A. nidulans*. However, it can not be ruled out that the protein is not functional.

Two other oomycete promoters, the *ham34* and the *ubi3R* gene promoters, were tested for activity in *A. nidulans*. The *ham34* gene of *B. lactucae* is highly expressed in germinating spores (Judelson and Michelmore 1990) and its promoter is frequently used to drive the expression of antibiotic genes in chimaeric constructs employed for transformation of *P. infestans* (Judelson et al. 1991, 1992, 1993). The *P. infestans ubi3R* gene is expressed in mycelium grown in vitro and at a ten-fold higher level during colonization of potato leaves (Pieterse et al. 1991). Plasmids containing the *ham34* promoter and the *ubi3R* promoter fused to the β-glucuronidase (GUS) reporter gene, pHAMT35G and pPUB-HAM respectively (Judelson et al. 1992, 1993), were transformed to *A. nidulans*. Subsequently, transient expression was determined by measuring GUS activity. Compared to the control plasmid pNOM102 in which the GUS gene is driven by the *A. nidulans gpd* promoter (Roberts et al. 1989), pHAMT35G and pPUB-HAM gave hardly any GUS activity in the tran-

Table 1 Co-transformation frequencies

Plasmids	Percentage co-transformation determined by	
	Growth on $NaNO_3^-$ containing medium[a]	Southern-blot analysis[b]
pAN8-1 + pSTA10	37 (52)	ND[c, d]
pAN8-1 + pNiaA-H	0 (47)	40 (10)

[a] Indicated in parentheses are the number of phleomycin-resistant colonies that were tested for growth on medium containing $NaNO_3$ as a sole nitrogen source
[b] Indicated in parentheses are the number of phleomycin-resistant colonies of which the DNA was analyzed by Southern-blot hybridizations
[c] ND = not determined
[d] From the 19 transformants that grew on medium containing $NaNO_3$ as a sole nitrogen source, two were analyzed by Southern-blot hybridizations. Both transformants appeared to contain one or more complete copies of plasmid pSTA10

Table 2 Comparison of promoter activities in transient expression assays in *A. nidulans*

Plasmid[a]	Source of promoter fused to GUS	GUS activity[b]
pNOM102[c]	*gpd* from *A. nidulans*	100
pHAMT35G[d]	*ham34* from *B. lactucae*[e]	4
pPOB-HAM[f]	*ubi3R* from *P. infestans*[g]	2
pCF181[h]	*ecp2* from *C. fulvum*	40
pMOG18[i]	35*S* from CaMV	36

[a] The plasmids contain the indicated promoters fused to the coding region (1870 bp) of the GUS reporter gene encoding β-glucuronidase
[b] Expressed as relative activity in percentages compared to pNOM102 (100%)
[c] Roberts et al. 1989
[d] Judelson et al. 1992
[e] Judelson and Michelmore 1990
[f] Judelson et al. 1993
[g] Pieterse et al. 1991
[h] Wubben et al. 1994
[i] Sijmons et al. 1990

sient-expression assay (Table 2). Although the activity of the *ham34* promoter was consistently a fraction higher than that of the *ubi3R* promoter, it never reached more than 4% of the activity of the *A. nidulans gpd* promoter. In contrast, the promoters of the *ecp2* gene of *Cladosporium fulvum*, a plant pathogenic ascomycete, and the CaMV 35*S* gene, a very active plant virus promoter, have significantly higher activities than the two oomycete gene promoters (Table 2). These data show that the two oomycete gene promoters are not functional in *A. nidulans*.

Discussion

In this paper we describe the isolation and characterization of the *P. infestans niaA* gene encoding NR. The gene contains an ORF encoding a protein of 902 amino-acids. Comparison of the amino-acid sequence with other NRs shows that, except for the amino-terminal sequence, the overall structure of NR proteins is highly conserved among species. Based on comparisons with amino-acid sequences from one-redox-centre proteins, the approximate boundaries of the molybdenum cofactor domain, the heme domain and the FAD domain were identified. All conserved residues which have been shown to be functionally essential are present in *P. infestans* NR.

The *niaA* gene is the first known *P. infestans* gene which appears to have an intron. All other *P. infestans* genes studied to-date, ten in total, lack introns (Pieterse et al. 1991, 1993, 1994; Unkles et al. 1991; Moon et al. 1992). So far, only one other oomycetous gene with introns has been described, i.e., the *Phytophthora parasitica trp1* gene (Karlovsky and Prell 1991).

P. infestans contains a single copy of the *niaA* gene per haploid genome. If, as in other filamentous fungi or plants, inactivation of this gene results in insensitivity to chlorate, the *niaA* gene could be a potentially powerful tool to trap mobile elements in *P. infestans* (Grandbastien et al. 1989; Daboussi et al. 1992). The *niaA* gene is transcriptionally active and expression is dependent on the nitrogen source present in the medium. In the absence of sufficient levels of favourable nitrogen sources, such as ammonium, the *niaA* gene is expressed. In the presence of ammonium expression of the *niaA* gene is not detectable. Whether regulation of the *P. infestans niaA* gene is based on nitrogen catabolite repression and nitrate induction, as has been observed in other fungi (Marzluf et al. 1992), needs to be investigated. The hexameric sequence TAGATA, which is present in the promoters of several nitrogen-regulated structural genes and functions in a similar manner as the binding site for the NIT-2 trans-acting regulatory protein in *Neurospora crassa* (Fu and Marzluf 1990), is not found in the promoter region of the *niaA* gene. This suggests that in filamentous fungi the mechanism of regulation of expression of nitrogen-regulated structural genes in not conserved and might be one of the reasons that the *P. infestans niaA* gene is not functional in *A. nidulans*.

The NR enzyme has been strongly conserved throughout evolution, and since NR encoded by the *P. infestans niaA* gene contains all the conserved domains which are essential for enzyme activity the enzyme derived from the *P. infestans niaA* gene ought to be able to complement a NR-deficient strain of another species. Nevertheless, in our experiments such complementation did not occur. This may be due to the possibility that the protein is not functional in *A. nidulans*. However, the most likely explanation for this observation is that the enzyme is absent simply because the *P. infestans niaA* gene is not activated in *A. nidulans*. The latter explanation is supported by the finding that two other oomycete gene promoters, those of *ubi3R* and *ham34*, are not functional in *A. nidulans* and leads to the general conclusion that oomycete gene promoters are not recognized in *A. nidulans*. Similar observations were reported by Judelson et al. (1992) who were unable to find activation of oomycete gene promoters in *Saccharomyces cerevisiae*, *N. crassa* and *Ustilago maydis*. This suggests

that oomycete genes need specialized components for transcription which are absent in non-oomycete fungi.

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