



Structural and functional characterization of the *Colletotrichum lindemuthianum nit1* gene, which encodes a nitrate reductase enzyme

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ABSTRACT. *Colletotrichum lindemuthianum* is the causal agent of plant bean anthracnose, one of the most important diseases affecting the common bean. We investigated the structure and expression of the *nit1* gene (nitrate reductase) of *C. lindemuthianum*. The *nit1* gene open reading frame contains 2787 bp, interrupted by a single 69-bp intron. The predicted protein has 905 amino acids; it shows high identity with the nitrate reductase of *C. higginsianum* (79%) and *C. graminicola* (73%). Expression of *nit1* in *C. lindemuthianum* was evaluated in mycelia grown on different nitrogen sources under conditions of activation and repression. The gene was expressed after 15 min of induction with nitrate, reaching maximum expression at 360 min. The transcription was repressed in mycelia grown in media enriched with ammonia, urea or glutamine. Twenty *nit1*⁻ mutants were obtained in a medium treated with chlorate. Ten of these mutants were characterized by DNA hybridization, which identified point mutations, a deletion and an insertion. These rearrangements in the *nit1* gene in the different mutants may have occurred through activity of transposable elements.

Key words: *Colletotrichum lindemuthianum*; Nitrate reductase; Anthracnose; *Phaseolus vulgaris*

INTRODUCTION

Colletotrichum is one of the most important phytopathogenic fungi. The species belongs to the group ascomycetes, which contains several species capable of causing anthracnose on a wide variety of crops and ornamental plants. Many of these species have been used as models for the study of differentiation and plant-pathogen interaction. *Colletotrichum lindemuthianum* is the causal agent of anthracnose in the common bean (*Phaseolus vulgaris* L.). The frequent occurrence of moderate temperatures, combined with high humidity, favors disease development and may cause losses of up to 100% as well as depreciation in the quality of grains. This fungus exhibits a life cycle of 2 phases, a biotrophic and a necrotrophic one, and is therefore classified as hemibiotrophic (Perfect et al., 1999).

C. lindemuthianum has high genetic variability, manifested by the presence of numerous physiological races. This significant genetic variability is one of the most serious obstacles in combating this pathogen because it prevents the long-term use of resistant cultivars (Rodríguez-Guerra et al., 2003; Ansari et al., 2004). Many mechanisms have been proposed to explain the large pathogenic variability and genetic instability that exists in species of *Colletotrichum*. Among these mechanisms, we can highlight conidial anastomosis, the sexual cycle, the parasexual cycle, and mutations caused by the presence of transposable elements (Casela and Frederiksen, 1994; Ishikawa et al., 2010).

Several strategies have been used to identify active transposable elements in phytopathogenic fungi. One of them employs the principle of spontaneous inactivation of the *nit1* gene, which encodes the nitrate reductase enzyme involved in nitrate assimilation (Daboussi and Capy, 2003; Fávoro et al., 2007). In addition, transformation systems using the *nit1* gene can be easily developed (Pereira et al., 2004; Navarrete et al., 2009), and this gene can also be used as a trap to detect transposable elements (Langin et al., 1995). *nit1*⁻ mutants are obtained by positive selection in media containing chlorate and characterized further in culture media containing different nitrogen sources. Such mutants are widely used in determining vegetative compatibility groups (Oliveira and Costa, 2003) and obtaining recombinants through the parasexual cycle (Castro-Prado et al., 2007).

The last decade has witnessed a revolution in the genomics of the fungal kingdom. Since the sequencing of the first fungus in 1996, the number of available fungal genome sequences has increased by an order of magnitude. Moreover, many of these sequenced species form clusters of related organisms designed to enable comparative studies (Galagan et al., 2005). In 2006, the USDA/National Science Foundation Microbial Genome Sequencing Project was funded by the National Research Initiative to sequence the genome of *C. graminicola*, a major cause of stalk rot disease and one of the most economically important diseases of maize. *C. graminicola* strain M1.001 (also known as M2) is the first member of the *Colletotrichum* genus to be fully sequenced. The sequence data were generously donated to the public by DuPont in 2008 (*Colletotrichum* Sequencing Project, Broad Institute of Harvard and MIT [<http://www.broadinstitute.org/>]). Another species for which the genome has recently been released is the pathogenic ascomycetes *C. higginsianum* that causes anthracnose in many plants of the Brassicaceae family, including *Arabidopsis thaliana*. The *C. higginsianum* genome has been sequenced at the Max Planck Institute for Plant Breeding Research, Cologne, Germany, with funds from the Max Planck Society (*Colletotrichum* Sequencing Project, Broad Institute of Harvard and MIT [<http://www.broadinstitute.org/>]). In this study, we report the isolation and structural and functional characterization of the *nit1* gene in the phytopathogenic fungus *C.*

lindemuthianum, and provide a comparative analysis with the *nit1* sequences of *C. graminicola* and *C. higginsianum*.

MATERIAL AND METHODS

Microorganisms, culture conditions, and DNA isolation

We used isolated *C. lindemuthianum* fungus identified as 65-400, 72-801, 73-497, 81, 81-401, and 89. These isolates belong to the collection of the Laboratory Molecular Genetics of Plants, Department of General Biology, Federal University of Viçosa, Brazil. We also used the following strains of *Escherichia coli*: DH5 α (Promega[®], Brazil, One Shot[®] TOP10 (Invitrogen, Brazil), and XLI-Blue MRA (Agilent Technologies[®], Brazil).

Total DNA of *C. lindemuthianum* was isolated following the protocol established by Specht et al. (1982).

Selection of recombinant phages from the genomic library

For the isolation of the *nit1* gene, we used the methodology described by Benton and Davis (1977), i.e., hybridization of DNA to single plaques *in situ*. Recombinant phages were isolated from the *C. lindemuthianum* genomic library, which was constructed by Soares (2007) using the Lambda EMBL3/*Bam*HI Vector Kit (Agilent Technologies[®]). We used the pNIT2.1 plasmid as a probe, courtesy of Thierry Langin (Institut de Biotechnologie des Plantes, Université Paris, France), which contains a genomic insert of part of the *nit1* gene of *C. lindemuthianum*. Hybridizations were performed at 65°C, and fragments of labeled DNA were detected using the Gene Images CDP-Star detection kit (Amersham Biosciences[®]).

Cloning and sequencing of the *nit1* gene

Fragments from the cleavage of phage DNA were cloned into the pZero vector (Invitrogen[®]). Clones were propagated in One Shot TOP10 *E. coli* (Invitrogen[®]), sequenced, and, when necessary, extracted and purified using the GeneJET[™] Plasmid Miniprep Kit (Life Tools Sciences[®]). The oligonucleotides used in this experiment are listed in Table 1.

Table 1. Description of oligonucleotides used in sequencing and study of *nit1* gene regulation.

| | Oligonucleotides | Sequencing (5'→3') |
|----|------------------|------------------------|
| 1 | Niac1 | GGTTATTATGGGAAACGAGC |
| 2 | Niac2 | GCAATTTGGGGTTATCTGG |
| 3 | Niac3 | CTATAAGACTGACCTCCCTC |
| 4 | Niac4 | CGCAGAAGCCAAGTTTCT |
| 5 | Niac3b | CCGTTAGTCGTATTAGTCTGG |
| 6 | Niacol2 | GTCAGTGAAGAGGGGTTCCAGG |
| 7 | Niacol1 | GGTACTCGGGCATCATCGGC |
| 8 | Clpcr1 | AAGATTTGGACAGGTCTCAAG |
| 9 | Clpcr2 | CGTCTTGGACAGGTCTCAAG |
| 10 | T7F | TAATACGACTCACTAATAGGG |
| 11 | M13R | GGATAACAATTTACACACAGG |
| 12 | EF1 | GAAGACTCACATCAAGTCG |
| 13 | EF2 | TTGAAGGAACCCCTTGCCGAG |

C. graminicola* and *C. higginsianum nit1* gene characterization and comparison with *C. lindemuthianum

For identification of the *nit1* gene in the *C. graminicola* and *C. higginsianum* database (*Colletotrichum* Sequencing Project, Broad Institute of Harvard and MIT [<http://www.broadinstitute.org/>]), we used the *C. lindemuthianum nit1* gene sequence and BLASTN. The structural characterization was performed by comparison with the *C. lindemuthianum* sequence and manual adjustments. The open reading frame (ORF) was translated using the DNAMAN program, and the amino acid sequences were aligned using the ClustalW program.

Phylogenetic analysis of the NIT1 protein

For phylogenetic analysis, the amino acid sequences of the NIT1 proteins from different groups of fungi were taken from GenBank and aligned using the ClustalW program. Phylogenetic inferences were performed by the neighbor-joining method using the PAUP 4.0b10 program, with bootstrap values using 5000 replicates.

Evaluation of *nit1* gene regulation

The methodology used to study the regulation of this gene was based on studies conducted by Haas et al. (1996) and Pereira et al. (2004). In experiments involving the activation of *nit1* gene expression, conidia of fungi were inoculated in potato dextrose (PD) broth. After the growth of mycelium, the samples were transferred to minimal medium (MM) (Pereira et al., 2004) without nitrate, containing 25 mM glutamine as the only source of nitrogen, and grown at 150 rpm, 24°C, for 36 h. The mycelium was transferred again to Erlenmeyer flasks containing MM, which was supplemented with 25 mM nitrate as the sole source of nitrogen, and the mycelium was collected at 0, 5, 15, 60, and 360 min.

In experiments involving the repression of *nit1* gene expression, the reverse order was followed. Mycelia of fungi grown in PD broth were transferred to MM containing 25 mM nitrate as the only source of nitrogen and grown at 150 rpm, 24°C, for 36 h. To each Erlenmeyer flask where mycelia were conditioned, a solution of glutamine was added to a final concentration of 25 mM, and samples were taken at 0, 5, 15, 60, and 360 min. In addition to these conditions, fungi grown in PD broth were also transferred to MM containing ammonium or urea as sole nitrogen sources to confirm the repression of the *nit1* gene under these conditions.

Total RNA was extracted using the TRIzol reagent (Invitrogen®), according to the instructions of the manufacturer. In addition, samples were treated with DNase RQI RNase-free (Promega®). cDNA synthesis was performed using the AMV reverse transcriptase (Promega®). Polymerase chain reactions (PCRs) were performed to detect cycles corresponding to the logarithmic phase of amplification and to determine how many cycles should be used in subsequent experiments. Primers EF1 and EF2 (which amplify a region of a ribosomal elongation factor of *Colletotrichum*) were used as endogenous controls in this experiment, while the Clpcr1 and Niacol2 oligonucleotides (Table 1) were used to evaluate gene expression.

Obtaining chlorate-resistant *nit1*⁻ mutants

To isolate chlorate-resistant mutants, 4 isolates of *C. lindemuthianum* (65-400,

72-801, 73-497, and 81-401) were inoculated in sterile test tubes containing pods set in agar:water and incubated at 22°C for 10 days. After growth, a suspension of 10⁷ conidia/mL in 0.1% Tween 80 solution was obtained, and 100 µL of this suspension was plated on Petri dishes containing minimal basal medium plus 1.5% KClO₃. These plates were incubated at 22°C for 20 days. Colonies resistant to chlorate were transferred to culture medium (PD agar), incubated for 7 days at 22°C, and stored at 4°C. Phenotypic classes of the obtained mutants were identified by growth on different nitrogen sources: 0.3% NaNO₃, 0.05% NaNO₂, 0.02% hypoxanthine, 0.02% uric acid, and 0.1% ammonium tartrate. The results were analyzed using the phenotypic classification for phytopathogenic fungi proposed by Yoder et al. (1986).

Molecular characterization of *nit1*⁻ mutants

Mycelia of *C. lindemuthianum* were obtained by inoculation of conidia on Petri dishes containing liquid GPYECH medium (Ansari et al., 2004) at 22°C for 7 days. Total DNA extraction of mutant and wild-type strains was performed as described by Specht et al. (1982). Aliquots of 5 µg total DNA were digested with the restriction enzyme *Bam*HI, for which there is no site in the *nit1* *C. lindemuthianum* gene. As a probe, the pNIT2.1 plasmid was used, which contains the *nit1* gene fragment from *C. lindemuthianum*. The probe was prepared using the Gene Images™ Random Prime Labeling Module kit (Amersham Biosciences®). Labeled DNA fragments were detected using the Gene Images CDP-Star detection kit (Amersham Biosciences®).

RESULTS

Isolation and characterization of the *C. lindemuthianum nit1* gene

A total sequence of 3690 bp was obtained from sequencing, corresponding to the *C. lindemuthianum nit1* gene. The structural region of this gene was characterized and consisted of 2787 bp (accession No. JF681041). The promoter region was also characterized and consisted of 640 bp as well as 230 bp corresponding to the 3'-flanking region, which contains the polyadenylation signal.

In the promoter region of the *C. lindemuthianum nit1* gene (Figure 1), we were able to identify regions that may function as *cis*-elements for gene expression. There are 3 possible *cis*-elements: a TATA box located at positions -105, -271, and -477 relative to the proposed ATG start codon and 2 GC boxes, located at positions -366 and -452. The promoter region also contains 4 TATC sequences located at positions -184, -189, -220, and -319. These *cis*-elements are binding sites for the positive regulator AREA of *Aspergillus nidulans*, NIT2 in *Neurospora crassa*, and NRE in *Penicillium chrysogenum* (Caddick et al., 1994; Haas and Marzluf, 1995; Marzluf, 1997). A possible binding region for NIT4 was found, located at position -340 (CTCCGTGT), which differs from the CTCCGHGG consensus described by Punt et al. (1995) only in its last base. In addition, partial palindromic sequences similar to the weak binding site of the NIT4 protein (TCCGTGGC) (Fu and Marzluf, 1993) were found, e.g., CCGCCGGC or CTTCCGTGT.

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-640   TCGTGAAAAG TATGTTCCAG ATTATGCCCG AGTCTCTTTG TCGTGAGCCT GCTACCCGCC
-580   GGCTCTATTC CATCTTCTGC TCTTTTGATG GAGGGAAGT GCTGGCTTAG TCTACGACTC
-520   TAGTGCCGTG CGGGTTCTGC GATGGAGATG CCGCATGGTA ATAGTGTGCT TTCCATCTCA
-460   TCCC GCCCCA TCCCGTCCCA TCCTGTCCCC ATGCCGGTCG GCACGTCTCC CTTCACGTC
-400   CTTTGCCCCC GTTCGGTGCG CTTCGCTTCC GCCCACACCA CAGACGTGAC AA CTCCGTTG
-340   CTAGGAAGTG CAGTTT TAT C CACGAGCAG GCCTTACTTA TTCGATTGTC ACCCCCAGCG
-280   TTTTCGTATAG GATCCTTCTC TTTCTCTCTGA ACTCTTCTGT CAAGCCTTCA TCCCA TATC
-220   GTTCTGTTCA TACGTCGAAC GGT TATC AGC CT TATC GGT CCACTGATT C GTCAACGCTC
-160   GTGTTTTGGA CTCACAAGTG ACGTCGTGG CACTCACTGC CAGACAACCT CTATACGGCA
-100   CAAGCCATCA TCCAACCCAC CCACCCACCT GCTTCAGAAC TAGAACATTT GGCTTCGCAT
-40    AGCACATTCT GGAATTTCGAC ACTGCTGCGC GACGTGA

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Figure 1. Nucleotide sequence of the *nit1* gene promoter. The possible start codon is in bold, possible *cis*-elements (TATA box and CG box) are underlined and possible binding sites of general (TATC) and specific (CTCCGTTG)-regulatory proteins are surrounded by rectangles.

Analysis of the structural region of the *nit1* gene identified a possible intron. This element starts at position 1808 (relative to the start codon ATG) and ends at position 1877, comprising 69 bp. This possible intron presents the 5' GT and 3' AG conserved sequences that are involved in splicing (Ballance, 1986), and an internal consensus sequence, CTGAC, is also present closer to the 3'-end. The sequence of the *nit1* gene of *C. lindemuthianum* was used to search for homology in the *C. graminicola* database to characterize the *C. graminicola nit1* gene. After screening, the sequence was identified in supercontig 1.8. By comparison and manual adjustments, the ORF of the gene was identified as 2772 bp. Similar to *C. lindemuthianum*, the *C. graminicola* gene contains only one intron, which starts at position 1803 and ends at position 1859, comprising 57 bp. The *nit1* ORF of *C. higginsianum* was identified in contig 336 and comprises 2715 bp. Similarly, the *C. higginsianum nit1* gene has only one intron, which starts at position 1802 and ends at position 1861, comprising 59 bp.

The amino acid sequence of the NIT1 protein was compared to the NIT1 of *C. higginsianum*, *C. graminicola* (Figure 2), and other fungi (Figure 3). The protein sequence has 905 amino acids with a predicted molecular mass of 101.1 kDa. In addition, it has 79% identity with the NIT1 of *C. higginsianum* and 73% identity with that of *C. graminicola*. Analysis of the amino acid sequences obtained from *C. lindemuthianum*, *C. higginsianum*, and *C. graminicola* allowed the identification of 3 major regions of NIT1: a binding domain for the molybdenum cofactor, a heme domain, and a binding domain for FAD. In the *C. lindemuthianum* NIT1, the binding domain for the molybdenum cofactor is located near the N-terminus of the protein and starts at Asp⁷⁷, extending to Val⁴⁵⁰. In this domain, a cysteine residue was found (Cys¹⁷⁴) that corresponds to the *A. nidulans* cysteine residue (Cys¹⁵⁰), which was demonstrated by Garde et al. (1995) as essential for binding of the molybdenum cofactor. The binding domain of heme starts at Arg⁵¹⁶ and ends at Asn⁵⁹³. This domain contains the intron as well as a histidine residue (His⁵⁷⁹) corresponding to the histidine residue of *A. nidulans* (His⁵⁴⁷), which was demonstrated by Garde et al. (1995) as essential for NIT1 functioning. Within the FAD domain, which extends from Glu⁶³⁸ to the end of the protein sequence, there are 2 amino acids important for its function, Trp⁶⁵¹ and His⁶⁸⁷. It was previously shown that replacing Trp⁶¹⁸ of the *A. nidulans* NIT1 protein, which corresponds to Trp⁶⁵¹ of *C. lindemuthianum*, with lysine resulted in transformants that were able to grow at 30°C but not at 37°C, when their sole source of nitrogen was nitrate. The His⁶⁵⁴ of the *A. nidulans* NIAD protein, which corresponds

to His⁶⁸⁷ of *C. lindemuthianum*, is not essential but has been demonstrated to be important for protein function (Garde et al., 1995). Using the amino acid sequences of these NIT1 proteins, a phylogenetic analysis was performed by aligning the NIT1 enzyme sequences of several fungi and plants. Figure 3 shows the consensus phylogenetic tree that was obtained, plus the bootstrap values utilizing 5000 replications.

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C. graminicola      ---MEFVHSVPVTFRETFAKTDLGFPPSPATTVREGLYSVDGSSDCSVSGGLLDEPSV  57
C. higginsianum    --MDVLSASIPVAF--REPLAKAELSPFPSPATTVREGLYSVDGSSSEYVSVSGGLLDEPSV  57
C. lindemuthianum  MCLHLPSPSPRGMPCR---RRSCLFLLAQPLWGKVSLSIASNGGSD--SSLVAGLLDEPGA  56
      * * * * *
C. graminicola      EGFRSYPLPKSRTNKVLAEDLKTPDNHVTRDPRILRLTGHPNVEAPLSDLYDEGFIT  117
C. higginsianum    ECSRTPYLPKSRNKNVLAEDLKTPDNHVTRDPRILRLTGHPNVEAPLSDLYDEGFIT  117
C. lindemuthianum  EGERTYALPNSKANRVLPEDLRTPDSSHVPRDSRLRLTGHPNVEAPLSELYNEGFIT  116
      * * * * *
C. graminicola      TKDLHYVRNHGAVPKVDEDMFDWEFTIEGLVEKPKIMTLRDLISDYEQITYPVTLVCAG  177
C. higginsianum    TKDLHYVRNHGAVPKVDEDMFDWEFTIEGLVENPKIMTLRDLISDYEQITYPVTLVCAG  177
C. lindemuthianum  TRDLHYVRNHGAVPKCDDDDMFDWQFTVEGEVENPITMNLRDLISDYEQITYPVTLVCAG  176
      * * * * *
C. graminicola      NRRKEQNVVRKSKGFSWGAAGLSTALWTGVSIGDLLARAI P--KRGAKYVCFEGADKLPN  235
C. higginsianum    NRRKEQNVVRKSKGFSWGAAGLSTALWTGVSIGDLLARAMP--KRGAKYVCFEGADKLPN  235
C. lindemuthianum  NRRKEQNVVRKTKGFLGPHRLVGPPLWTGCAHRI PRLARPCPKRGARYVCFEGADKLPN  236
      * * * * *
C. graminicola      GYGTSLKLNWCMDPNRGVTVCYRMNGELLHPDHGKPVRIIPQIGGRSVKWLKRIIVT  295
C. higginsianum    GYGTSLKLNWCMDPNRGVTVCYRMNGELLHPDHGKPVRIIPQIGGRSVKWLKRIIVT  295
C. lindemuthianum  GYGTSLKLNWCMDRDRGVTVCYRMNGELLHPDHGKPVRIIPQIGGRSVKWLKRIIVT  296
      * * * * *
C. graminicola      KEPSNNWHIYDNRVLPPTVSPDASADLPEVWKDERYAIYDLNANSATCYPAHDEIVALV  355
C. higginsianum    KEPSNNWHIYDNRVLPPTVSPDASADLPEVWKDERYAIYDLNNSATCYPAHDEIVPLG  355
C. lindemuthianum  KEPSNNWHIYDNRVLPPTVSPDASADLPTWVKDERYAIYDLNNSVATPAHDEIVSLV  356
      * * * * *
C. graminicola      DGENSYKVRGYAYGGGGRI TRVELTLDRGKTWLLANIDY PEDIYRLAPEGETLYGGRVD  415
C. higginsianum    GLESETYKVRGYAYGGGGRI TRVELTLDRGKTWLLANVNY PEDIYRLAPEGETLYGGRVD  415
C. lindemuthianum  DGEKSYRIRGYAYGGGGRI TRVELTLDRGKTWLLANVNY PEDEYRFAPEGETLYGGRVD  416
      * * * * *
C. graminicola      MDWRET---SFCWCFWNLDIPIVQLADAADIMVRAMDESMMVQPRDMYWSVLGMNNPW  471
C. higginsianum    MDWRET---SFCWCFWLDIPIAQLADAADVIMVRAMDESMMVQPRDMYWSVLGMNNPW  471
C. lindemuthianum  MDWRETWLRGASGTLTQQV---AQLADAADVIMVRAMDEGMVQPRDMYWSVLGMNNPW  473
      * * * * *
C. graminicola      FRVVIHKEHSLRFEHPTQPALIAGGWMERVKKAGNLSNGFWGKEIEGEADNIVPEEIE  531
C. higginsianum    FRVVIHKEHSLRFEHPTQPALMAGGWMERVKKAGNLSNGFWGKEIEGEADNIVPEEIE  531
C. lindemuthianum  VRVVIHKEHSLRFEHPTQPALGGGWMERVKKAGNLSNGFWGKEIEGEADNIVSEEPF  533
      * * * * *
C. graminicola      KETCMTRDGVKRIITIEELRQHVGEEQPFVNVNGEYVDGTPFLEHHPGGAASIFGAAGQD  591
C. higginsianum    KIDICTKDTVKRVDITIEELRQHSEEEFVNVNGEYVDGTPFLEHHPGGAASIFGAAGQD  591
C. lindemuthianum  REICMTRSVKRVIAMDEL RQHGGEDTDFVNVNGEYVDGTPFLEHHPGGAASIFGAAGQD  593
      * * * * *
C. graminicola      VSEEFLTRNENAKMMPDYHIGTLDEAARKLLAEGEVPEDDDKTSRSVFLQSKSWTKG  651
C. higginsianum    VSEEFLTRNENAKMMPDYHIGTLDEAARKLLAEGEVPEDDDKTSRSVFLQSKSWTKG  651
C. lindemuthianum  VSEEFVTRNENAKMMPDYHIGSMDEAGNKTALGVVVEDDPEKRPVFLQSKAWTKG  653
      * * * * *
C. graminicola      ILSEKREASSDSKIPTFLEHPCQMVGLPVGQHLMLRLRDPVTRAIIRAYTPISEGTGK  711
C. higginsianum    ILSEKRVSSDSKIPTFLEHPCQMVGLPVGQHLMLRLRDPVTRAIIRAYTPISEGTGK  711
C. lindemuthianum  ILNARRMSSDTKIPTFSLQHPQSIGLPGQHLMLRLRDPVTRAIIRAYTPISEGSDK  713
      * * * * *
C. graminicola      GKLDVLIKIYYDTPDRKGGKMTQALNSIPIGHVDFKGFVKGFEYLGKGLCSIAGRSQRA  771
C. higginsianum    GKLVHLIKIYYSTPEREGKMTQALDSIPVGHVDFKGFVKGFEYLGKGLCSIAGKQRA  771
C. lindemuthianum  GKLVHLVKIYYDTPERKGGKMTQALNSIPIGHVDFKGFVKGFEYLGKGLCSIAGKQ--RR  772
      * * * * *
C. graminicola      IKRFIMICAGSGITPIFQVLRVAVMKDAADPTRCLLLYGNRAEDDILCRGELDAMAAANPD  831
C. higginsianum    VKRFIMICAGSGITPIFQVLRVAVMKNAADPTRCLVLDGNRVEEDI LCRELDVMAAAHPD  831
C. lindemuthianum  VKRFVIMICAGSGITPIFQVLRVAVKVNQDPTRCLVLDGNRVEEDI LCRELDVMAAANPD  832
      * * * * *
C. graminicola      RCRLLYSLTKPDASWTGFVGRMDG---KFFVQVGTYNENAGGDELVIVCGPEAFEACV  887
C. higginsianum    RCRLLYTLTKPDASWTGLKGRMDG---KFFEEHVQACENETGGDELVIVCGPEALEASV  887
C. lindemuthianum  RCRLLYLTKPASWTGLKGRIDAAFLTEVVGARRSDS---DGBELVIVCGPEPLEKSV  888
      * * * * *
C. graminicola      RTLLGGIGWKNDLVFF  904
C. higginsianum    RSFLTGLGWKDDLVFF  904
C. lindemuthianum  RSTLLDLGWKDDLVFF  905
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Figure 2. Aligned amino acid sequences of the *Colletotrichum lindemuthianum*, *C. graminicola* and *C. higginsianum* NIT1 proteins. The three main domains of nitrate reductase are highlighted in the figure: the binding domain of the molybdenum cofactor is shaded in gray, the heme domain is underlined, and the binding domain of FAD is highlighted in black.

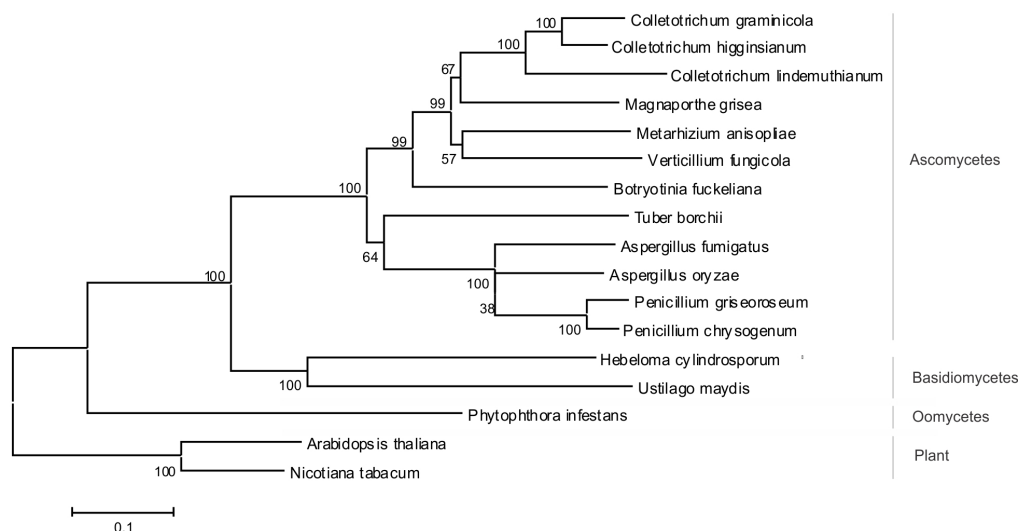


Figure 3. Phylogenetic analysis of the nitrate reductase protein. The rooted tree was based on a comparison of the *Colletotrichum lindemuthianum* nitrate reductase amino acid sequence with that of other filamentous fungi. The values of the bootstrap percentage are shown in each branch and based on 5000 replicates. GenBank accession Nos.: *Nicotiana tabacum* (CAA32217.1); *Arabidopsis thaliana* (NP_177899.1); *Phytophthora infestans* (AAA86681); *Ustilago maydis* (71019527); *Hebeloma cylindrosporum* (CAB60010.1); *Penicillium chrysogenum* (AAB03900.1); *Penicillium griseo-roseum* (AAP12556.1); *Aspergillus fumigatus* (AAL85636.1); *Aspergillus oryzae* (BAA08551.1); *Tuber borchii* (AAN64993.1); *Botryotinia fuckeliana* (AAC02633.1); *Verticillium fungicola* (AAO63560.1); *Metarhizium anisopliae* (CAA04554.1); *Magnaporthe grisea* (XP_369402.1); *C. lindemuthianum* (JF681041); *C. graminicola* and *C. higginsianum* (*Colletotrichum* Sequencing Project, Broad Institute of Harvard and MIT (<http://www.broadinstitute.org/>)).

The tree branches were well sustained with, in most clades, bootstrap values above 99%, indicating a consistent and robust phylogenetic tree. A high degree of tree consistency indicates that the analyzed genes coding for NIT1 probably had a common origin and can, therefore, be considered counterparts of each other. A clear division between different groups directly correlated with the taxonomic category to which they belong could be noticed, reflecting their possible evolutionary history.

In addition, a correlation was found between the characteristics of each individual and group division observed in the tree. Zhou and Kleinhofs (1996) reported that the number of introns was an important correlation in the group ascomycetes. Interestingly, all individuals in the branch containing *Aspergillus fumigatus* have 6 introns in conserved regions and belong to the same taxonomic order. Similarly, the branch containing *Botryotinia fuckeliana* is characterized by fungi whose *nit1* genes contain none or only one intron. Although *Hebeloma cylindrosporum* and *Ustilago maydis* differ regarding the number of introns, with 12 and none, respectively, the identity of the NIT1 protein sequence was high enough for them to be grouped in the same class, reflecting that they belong to the same taxon. *C. lindemuthianum*, *C. graminicola*, and *C. higginsianum* have high identity in their NIT1 protein sequence and cluster close in the tree, as expected.

Study of *C. lindemuthianum nit1* gene regulation

To verify the regulation of nitrate assimilation by *C. lindemuthianum*, induction and repression experiments of the *nit1* gene were performed, and semi-quantitative reverse transcription-PCR (RT-PCR) using 23 amplification cycles was used to evaluate the presence of *nit1* gene transcripts under different cultivation conditions. The results of the sequence analysis of the promoter region of this experiment indicated that the *nit1* gene could be regulated in a positive way, as *cis*-elements specific for positive regulators were found.

The results showed that *C. lindemuthianum nit1* gene transcription is activated by NaNO_3 and repressed by glutamine, ammonium, and urea (Figure 4). Repression experiments were performed by growing mycelia in MM containing nitrate as the sole source of nitrogen and adding glutamine at 0, 5, 15, 60, and 360 min (Haas et al., 1996). Under these conditions, no difference was found in the cDNA of *nit1* at 0 and 5 min. Reduced *nit1* cDNA was found at 15 min after glutamine addition, and *nit1* cDNA was undetectable at 60 and 360 min after glutamine addition (Figure 4A).

Activation experiments were performed by growing mycelia in the presence of glutamine for 36 h and transferring to MM containing nitrate as the nitrogen source at 0, 5, 15, 60, and 360 min (Haas et al., 1996). At time zero, no *nit1* cDNA was detected. *nit1* cDNA was first observed at 15 min after transfer to MM, and at 60 min after transfer, *nit1* gene transcripts were observed at near maximal levels. Maximal levels of gene transcription were obtained after 360 min in MM (Figure 4B).

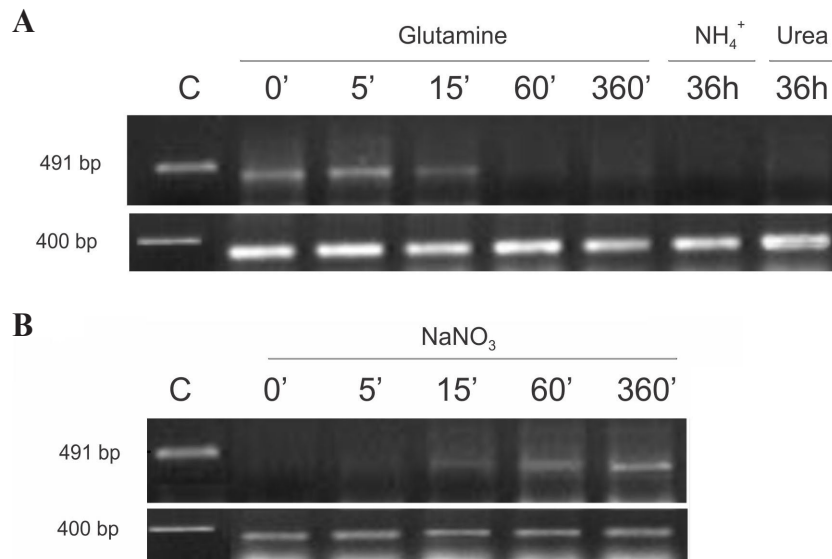


Figure 4. Analysis of *Colletotrichum lindemuthianum nit1* gene transcription by RT-PCR. **A.** Analysis of gene repression from mycelia cultivated in media containing nitrate as the nitrogen source and collected after the addition of glutamine at the times indicated. Suppression was also examined in mycelia cultivated in NH_4^+ and urea. **B.** Analysis of gene activation in mycelia cultivated on glutamine and collected after transfer to medium containing nitrate as the nitrogen source at the times indicated. Internal positive controls for detection of cDNA in each real-time reaction were the amplification of cDNA with EF1 and EF2 oligonucleotides, which amplify a region of approximately 400 bp of a constitutively expressed gene that codes for the ribosomal elongation factor. Lane C = positive control.

Isolation and characterization of *nit1*⁻ mutants

Selection in medium containing chlorate allowed the isolation of 102 mutants (Table 2), which were then classified phenotypically by growth in media containing different nitrogen sources. Four physiological races of *C. lindemuthianum* produced spontaneous mutants, although at very different frequencies. Race 72-801 had the largest number of *nit1*⁻ mutants.

Table 2. Selection of chlorate-resistant mutants.

| Mutants | Physiological races | | | |
|--------------------------|---------------------|--------|--------|--------|
| | 65-400 | 72-801 | 73-497 | 81-401 |
| Total | 9 | 59 | 21 | 13 |
| <i>nit1</i> ⁻ | 2 | 20 | 3 | - |

Values for *nit1*⁻ mutants were obtained after phenotypically classification.

For molecular characterization, we selected 10 mutants (race 72-801). Total DNA was extracted, digested with the *Bam*HI enzyme, and hybridized with the *C. lindemuthianum nit1* gene (Figure 5).

Although the vast majority of mutants showed hybridization profiles similar to the wild-type strain, possibly indicating the occurrence of point mutations in the *nit1* gene, some mutants had interesting profiles. Mutants 17A, 21A, and 26A presented a band significantly smaller than the wild-type strain, indicating a deletion event. Mutant 32A presented a band with a fragment size greater than that of the wild-type strain, indicating the insertion of a transposable element in the *nit1* gene.

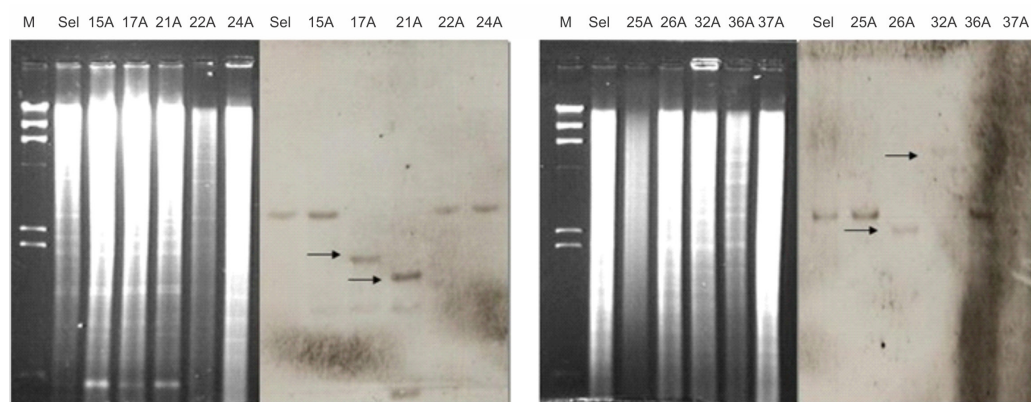


Figure 5. Analysis of different *nit1*⁻ mutants. Lane M = DNA of λ bacteriophage digested with enzyme *Hind*III; lane Sel = a wild strain (physiologic race 72-801); other numbers represent different *nit1*⁻ mutants phenotypically classified in media containing different nitrogen sources. As probe, we used a gene fragment of *Colletotrichum lindemuthianum nit1*.

DISCUSSION

Screening of the *C. lindemuthianum* genomic library enabled the isolation and characterization of a nucleotide sequence of 3690 bp corresponding to the *nit1* gene, which encodes the enzyme NIT1.

In the promoter region of the *C. lindemuthianum nit1* gene (Figure 1), in addition to TATA box and GC box *cis*-elements, 4 TATC sequences were identified, located at positions -184, -189, -220, and -319. These *cis*-elements are binding sites of the general positive regulators AREA of *A. nidulans*, NIT2 of *N. crassa*, and NRE of *P. chrysogenum* (Caddick et al., 1994; Haas and Marzluf, 1995; Marzluf, 1997). Elements with at least 2 copies of GATA sequences, separated by approximately 30 bp and either oriented in the same or opposite directions, are considered strong binding sites for NIT2 (Haas and Marzluf, 1995). In *P. chrysogenum*, strong binding sites of NRE were described by Haas and Marzluf (1995) as regions with at least 2 GATA sequences separated by 5 and 27 bp, configured either in the same direction or in opposite directions. GATA elements separated by 74 or 96 bp did not provide strong binding sites for NRE (Haas and Marzluf, 1995). In *C. lindemuthianum*, 2 TATC regions, located at positions -184 and -220, are separated by 32 nucleotides and, therefore, may constitute a strong binding region of a regulatory general nitrogen assimilation protein. Another possible binding site for this protein would be between the *cis*-elements located at positions -189 and -220, which are separated by 23 nucleotides. Strong binding of the *P. chrysogenum* NRE to 2 GATA factors may be related to functioning of the protein in a dimeric form, which has also been suggested for NIT2 in *N. crassa* (Feng et al., 1993; Haas and Marzluf, 1995).

The analysis of the structural region of the *C. lindemuthianum nit1* gene enabled full characterization of a sequence of 2787 bp, which is interrupted by a single intron. Similarly, the analysis of the structural region of the *C. higginsianum* and *C. graminicola nit1* genes enabled the identification of sequences of 2772 and 2715 bp, respectively, both interrupted by a single intron.

In filamentous fungi, sequence analysis of genes encoding NIT1 reveals variations in the presence and size of introns. The positions of introns tend to be conserved within kingdoms but differ between the fungi and plant kingdoms (Zhou and Kleinhofs, 1996). For the fungi *U. maydis* (Banks et al., 1993) and *B. fuckeliana* (Levis et al., 1997a), there is no report of introns in their *nit1* genes, while the *nit1* gene of the fungus *H. cylindrosporum* (Jargeat et al., 2000) contains 12 introns. In *P. camemberti*, 6 introns were located in conserved regions, although they were of different size (Navarrete et al., 2009). The *nit1* genes of *Beauveria bassiana*, *Fusarium oxysporum*, *Gibberella fujikuroi*, *Metarhizium anisopliae*, and *Verticillium fungicola* also contain a single intron, and its location is restricted to the heme domain of the protein, which appears to be an ancestral feature of the protein for these organisms. In general, in fungi, most introns within the *nit1* genes are located in conserved regions that correspond to the binding domain for the molybdenum cofactor. In plants such as *Lycopersicon esculentum*, *Nicotiana tabacum*, *Oryza sativa*, and *P. vulgaris*, the 3 introns present in these species occupy the same position. In general, these introns tend to occupy regions within and not between functional domains (Zhou and Kleinhofs, 1996).

Translation of the sequence of the characterized structural region allowed for the calculation of a protein comprising 905 amino acids with a molecular weight of 101.1 kDa, which corresponds to the *C. lindemuthianum* NIT1 protein (Figure 2). This is also the approximate

size of proteins calculated for *C. higginsianum* and *C. graminicola*, which comprise 904 amino acids and have a molecular weight of approximately 100.7 kDa. The amino acid sequence of the *C. lindemuthianum* NIT1 protein showed high identity with those of other fungi, which indicates that this protein is evolutionarily conserved. In addition, it has been reported that because of the conservation of NIT1 protein domains, high identity can be observed between the proteins of fungi and plants and functionally related proteins of mammals, e.g., sulfite oxidase, cytochrome b5, and NADH-cytochrome b5 reductase (Campbell and Kinghorn, 1990).

Filamentous fungi generally have strong regulatory mechanisms for the assimilation of nitrate by means of positive and negative regulators that ensure metabolic repression when primary sources of nitrogen such as ammonium or glutamine are present. We also performed a study of the induction and repression of the *C. lindemuthianum nit1* gene by cultivating the fungus on different nitrogen sources. As shown in Figure 4, transcripts of the *nit1* gene could not be detected when the fungus was cultured in media containing ammonium or urea as the sole nitrogen source. We also observed that after 15 min of growth in media containing glutamine, the transcription level decreased to approximately half.

Similar results were obtained by Haas et al. (1996) and Pereira et al. (2004) when studying the *nit1* gene regulation of the fungi *P. chrysogenum* and *P. griseoroseum*. In *N. crassa*, the estimated half-life of NIT3 mRNA was only 5 min (Okamoto et al., 1991). This sharp decrease in the NIT1 mRNA level is a mechanism that allows the cell to respond quickly to the nutritional condition of the environment and ensures great economy in terms of energy because assimilation of a more metabolically available nitrogen source can occur. In *N. crassa*, the enzymatic activity of NIT3 decreases relatively quickly in mycelia growing in primary nitrogen sources, with a half-life of about 15 min (Okamoto et al., 1991). According to these authors, this relatively rapid decline of the enzymatic activity helps to ensure the preferential use of primary sources of nitrogen.

Finally, it took 15 min until *nit1* gene transcripts could be detected during induction of the gene with NaNO₃. A quick response to this condition is also reported for *N. crassa* (Okamoto et al., 1991), *P. chrysogenum* (Haas et al., 1996), and *P. griseoroseum* (Pereira et al., 2004). In *N. crassa*, the NIT3 mRNA rises to maximal levels after just 15 min of induction by nitrate, and the activity of the NIT3 protein increases rapidly in the first 15-20 min, reaching a peak at 60 min (Okamoto et al., 1991).

In addition to its participation in metabolic pathways of nitrogen assimilation, the gene that encodes NIT1 has been highlighted in recent studies for the use in the detection and isolation of transposable elements. It has been suggested that stress conditions, e.g., the presence of chlorate, can trigger the movement of transposable elements, resulting in a high frequency of spontaneous mutations. Transposition in response to environmental stress has been proposed as an adaptive response of the genome and has been detected in various organisms, including fungi, yeasts, and plants (McClintock, 1984; Capy et al., 2000). In this study, we successfully used a system for positive selection of spontaneous mutations in the *nit1* gene, allowing the isolation of 20 *nit1* mutants. Ten of them were analyzed by DNA hybridization to assess the nature of the mutations. The different profiles of hybridization (Figure 5) suggest the presence of transposable elements in *C. lindemuthianum*. In fact, this system has been used very successfully for the isolation of several transposable elements in fungi. The *fol1*, *impala*, *Ant1*, *Vader*, *Flipper*, and *bupfer* elements are examples of transposons cloned by selection of spontaneous mutations in the gene that encodes NIT1 in *F. oxysporum*, *Aspergillus niger*, *A.*

fumigatus, *Botrytis cinerea*, and *B. bassiana*, respectively (Daboussi et al., 1992; Langin et al., 1995; Glayzer et al., 1995; Amutan et al., 1996; Levis et al., 1997b; Maurer et al., 1997). However, for a better understanding of the genetic mechanisms responsible for the different profiles obtained by hybridization of the mutant *C. lindemuthianum nit1*, our future studies will focus on determining the nature of this source of variation among strains, investigating to what extent this variation can be explained by the presence of active transposable elements.

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