Overexpression of a Novel Biotrophy-Specific *Colletotrichum truncatum* Effector, CtNUDIX, in Hemibiotrophic Fungal Phytopathogens Causes Incompatibility with Their Host Plants

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The hemibiotrophic fungus *Colletotrichum truncatum* causes anthracnose disease on lentils and a few other grain legumes. It shows initial symptomless intracellular growth, where colonized host cells remain viable (biotrophy), and then switches to necrotrophic growth, killing the colonized host plant tissues. Here, we report a novel effector gene, *CtNUDIX*, from *C. truncatum* that is exclusively expressed during the late biotrophic phase (before the switch to necrotrophy) and elicits a hypersensitive response (HR)-like cell death in tobacco leaves transiently expressing the effector. *CtNUDIX* homologs, which contain a signal peptide and a Nudix hydrolase domain, may be unique to hemibiotrophic fungal and fungus-like plant pathogens. *CtNUDIX* lacking a signal peptide or a Nudix motif failed to induce cell death in tobacco. Expression of *CtNUDIX*GFP in tobacco suggested that the fusion protein might act on the host cell plasma membrane. Overexpression of *CtNUDIX* in *C. truncatum* and the rice blast pathogen, *Magnaporthe oryzae*, resulted in incompatibility with the hosts lentil and barley, respectively, by causing an HR-like response in infected host cells associated with the biotrophic invasive hyphae. These results suggest that *C. truncatum* and possibly *M. oryzae* elicit cell death to signal the transition from biotrophy to necrotrophy.

*Colletotrichum truncatum* (Schwein.) Andrus & W. D. Moore is an ascomycete fungal pathogen that causes anthracnose on economically important pulse crops, including lentil (*Lens culinaris* Medik.), soybean (*Glycine max* L. Merr.), faba bean (*Vicia faba* L.), and pea (*Pisum sativum* L.) (1). The pathogen employs a two-stage hemibiotrophic infection strategy to invade host plants. Upon landing on the lentil leaflet surface, conidia of *C. truncatum* germinate to form appressoria, which breach the host cuticle and cell wall through penetration pegs that then develop into infection vesicles. These vesicles grow between the plant cell wall and the plasma membrane to form large, bulbous, invasive primary hyphae that are biotrophic in nature. The primary hyphae of *C. truncatum* are entirely confined to the first infected epidermal cells throughout the biotrophic phase (2). Thereafter, the fungus switches to a necrotrophic phase associated with the production of thin secondary hyphae that ramify intra- and intercellularly, killing and macerating host tissues via hydrolytic enzymes ahead of infection. Therefore, the transition from biotrophy to necrotrophy, known as the biotrophy-necrotrophy switch (BNS), is critical in anthracnose development.

To date, four genes have been shown to regulate the BNS: *CLTA1* and *CLNR1* (*Colletotrichum lindemuthianum* [3, 4]), *CPR1* (*Colletotrichum graminicola* [5]), and a gene encoding a putative importin-β2 protein (*Colletotrichum higginsianum* [6]). The *CLTA1* and *CLNR1* genes encode a GAL4-like transcriptional activator belonging to the fungal zinc cluster (Zn$^{2+}$/Cys$_6$) family and an AREA/NIT2-like global nitrogen regulator, respectively, whereas the *CPR1* gene encodes a subunit of the signal peptidase complex. Mutants harboring disrupted loci of these genes were indistinguishable from their wild-type strains until the formation of biotrophic primary hyphae. Thereafter, they rarely formed necrotrophic secondary hyphae, and their growth was restricted to primary hyphae occupying single epidermal cells, which remained alive even after prolonged incubation. More recently, a mitochondrial alternative oxidase from *Moniliophthora perniciosa*, the causal agent of witches’ broom disease in cacao, was identified as a regulator of the BNS (7). However, none of these proteins were secreted into the host. Plant pathogens secrete small proteinaceous and nonproteinaceous molecules (known as effectors) in the hosts to manipulate host cell structure and function, thereby either facilitating infection (virulence factors and toxins) or triggering host defense responses (avirulence factors and elicitors), or both (8–10). Nudix hydrolases, which are characterized by the presence of a conserved 23-amino-acid-residue Nudix (formerly MutT) motif, GX$_5$EX$_7$REVXEEXGU, where U is usually a hydrophobic amino acid (generally an isoleucine, leucine, or valine residue) and X is any amino acid residue, are widely distributed among organisms ranging from bacteria to mammals (11). The Nudix hydrolase domain-containing proteins (here called Nudix proteins) catalyze the hydrolysis of a variety of nucleoside triphosphates, nucleotide sugars, adenosine 5′-diphosphoribose (ADP-ribose), dinucleotide coenzymes, diadenosine oligophosphates (Ap$_n$A), and capped RNAs (12), as well as nonnucleotide derivatives like diphosphoinositol polyphosphates (inositol polyphosphates [IP$_n$]) (13–15). Some of these molecules play regulatory roles, whereas others are coenzymes and mutagenic or...
toxic components. Many Nudix hydrolases function as cellular surveillance enzymes to maintain physiological homeostasis by sensing and modulating the levels of their substrates (16). However, the role of secreted or nonsecreted Nudix proteins in phytopathogens is largely unknown.

We report here a novel effector gene, Ct21-1373, that is expressed exclusively during the late biotrophic phase of in planta infection by C. truncatum and appears to encode a Nudix hydrolase domain-containing protein. We designate it CtNUDIX (Colletotrichum truncatum NUCleoside Diphosphate linked to other moiety X). Agrobacterium tumefaciens-mediated transient expression of CtNUDIX in Nicotiana tabacum leaves induced severe hypersensitive response (HR)-like cell death, and the infection time course indicated that the accumulation of the effector occurred precisely before the BNS. Both results support the hypothesis that CtNUDIX may contribute to the BNS. To gain an understanding of CtNUDIX virulence activity, we used A. tumefaciens-mediated expression of CtNUDIX-GFP in leaves of N. tabacum. Transient expression of CtNUDIX fused to the N terminus of the enhanced green fluorescent protein (eGFP) reporter indicated localization of the fusion protein to the plasma membrane. Homologs of CtNUDIX may be unique to hemibiotrophic fungal and oomycete phytopathogens (here called hemibiotrophic phytopathogens), and its overexpression in C. truncatum and in a second hemibiotrophic phytopathogen, Magnaporthe oryzae, causes incompatibility with the hosts lentil and barley, respectively, by killing the host cells in the biotrophic phase of the infection process.

MATERIALS AND METHODS

Plant and fungal materials. Lentil plants of the Canadian cultivar Eston and the compatible C. truncatum CT-21 isolate were routinely maintained as described by Bhadauria et al. (17). Cell walls were extracted from 3-week-old lentil leaflets following the protocol described previously (18). M. oryzae strains generated in this study were cultured at 25°C on oatmeal tomato agar plates, as described previously (19).

Sequence analyses. An ORF finder algorithm (http://www.ncbi.nlm.nih.gov/orf) was employed to predict the coding region of CtNUDIX in all six frames ab initio; any sequence with a stop codon preceded by an in-frame ATG codon was translated into a protein sequence. The amino acid sequence was then scanned for potential signal peptides (SP) and transmembrane domains using SignalP version 3.0 with default settings and TMHMM server version 2, respectively. CtNUDIX and with a putative SP was queried against the NCBI nonredundant protein database using the BLASTP algorithm with the BIOSUM80 matrix without a low-complexity filter. N- and O-linked glycosylation sites were predicted using NetNGlyc 1.0 and NetOGlyc 2.0 servers (http://www.expasy.org), respectively. DnAMAN (Lynnnon, Pointe-Claire, Quebec, Canada) software was used to predict the molecular mass and pl of CtNUDIX and the MEGA version 4 program (20) for generating a multiple-sequence alignment and phylogenetic tree by the neighbor-joining method.

Infection time course and microscopic evaluation of the infection process. Leaflets from 3-week-old lentil plants were detached and inoculated in petri dishes lined with wet filter paper by droplet inoculation with a C. truncatum CT-21 isolate, as described previously (17). The inoculated leaflets were incubated with a 12-h photoperiod at room temperature. The progress of C. truncatum infection was microscopically assessed as described below, and the infection sites of leaflets were harvested at 3 time points: the appressorial penetration phase (16 h postinoculation [hpi]), biotrophic state (44 hpi), and necrotrophic state (68 hpi). These leaflet discs were then frozen in liquid nitrogen until required.

Leaflet discs from all time points were fixed in a fixation solution (60% methanol, 30% chloroform, 10% acetic acid). The fixed samples were rehydrated with decreasing ethanol gradients (100%, 80%, 70%, and 50% ethanol). Samples were then stained with 0.05% trypan blue (Harleco Parastains, Philadelphia, PA) in distilled water overnight and destained in distilled water. The stained leaves were then mounted in 30% glycerol on glass slides. The developmental stages were examined and photographed under an epifluorescence microscope (Zeiss Axioplan, Jena, Germany).

RNA extraction and Northern blot analysis. For isolating total mycelial RNA from CtNUDIX overexpression strains of C. truncatum and M. oryzae, 1 ml of conidial suspension (4 × 10^7 conidia ml^-1) was incubated in complete medium (0.6% yeast extract, 0.3% acid casein hydrolysate, 0.3% enzymatic casein hydrolysate, 1% sucrose) at 22 to 25°C for 48 h with constant shaking at 150 rpm. Mycelia were harvested by filtering through nylon mesh and rinsed with distilled water twice. The collected mycelia were frozen in liquid nitrogen and stored at −80°C until required. For cell wall-treated mycelia, collected mycelia were grown in vitro in minimal salt broth supplemented with lentil cell wall as the sole carbon source at 22 to 25°C for 8 h with constant shaking at 150 rpm. Mycelia were harvested and stored as described above. C. truncatum conidia were harvested from 10- to 14-day-old oatmeal agar culture plates, washed twice by centrifugation (3,000 rpm; 5 min), pelleted, and kept frozen until required. Total RNA isolation from conidia, mycelia, cell wall-treated mycelia, and C. truncatum-infected lentil leaflet tissues collected at various time points and Northern blot analyses were conducted as described previously (21). Probes for CtNUDIX and 60S ribosomal protein-encoding transcripts were amplified by reverse transcription (RT)-PCR using the gene-specific primer sets CNNUDIX/F/CNNUDIXR and C60S/F/C60S/R, respectively (see Table S1 in the supplemental material). Three independent biological replicates were used for RNA gel blot analyses.

cDNA synthesis and quantitative reverse transcription (qRT)-PCR. After eliminating genomic DNA using RNase-free amplification grade DNase I (Invitrogen, Carlsbad, CA), 2 μg of total RNA was reverse transcribed in a 20-μl reaction volume using 200 U SuperScript reverse transcriptase (Invitrogen, Carlsbad, CA) following the protocol of the supplier. The resulting cDNA was diluted 10-fold in sterilized ultrapure water. A real-time PCR detection platform, CFX96 (Bio-Rad, Hercules, CA), was used to quantify CtNUDIX expression in vegetative hyphae and conidia and in planta with the C. truncatum 60S ribosomal gene as an endogenous control (reference gene) using primer sets qCtNUDIX/F/R and C60S/F/R (see Table S1 in the supplemental material). Three biological repeats were used to obtain expression data. The 5-μl reaction mixture contained 2.5 μl of 2X Fast SYBR Green Master Mix (Applied Biosystems, Foster City, CA), 200 nmol of each primer, and 1 μl template. The following thermal conditions were used: 2 min of preheating at 95°C, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C. For normalization, the threshold cycle (C_T) values of reference genes were subtracted from the corresponding C_T values of CNNUDIX, yielding ΔC_T values. The relative expression of CNNUDIX transcripts was calculated by the 2^-ΔΔC_T method (22), using a ΔC_T value obtained for vegetative hyphae as a calibrator.

Binary constructs and agroinfiltration. For in planta expression of CNNUDIX, binary potato virus X (PVX) constructs were made in the vector pGR106 (23) and delivered in N. tabacum leaves via A. tumefaciens strain GV3101. All primers used in this study are described in Table S1 in the supplemental material. For in planta expression of CNNUDIX, binary PVX constructs were made in the vector pGR106. The C. truncatum ORF with (1 to 223) and without (25 to 223) SP were cloned into the pCR2.1T vector (Invitrogen), using primer sets CNNUDIX/F/CNNUDIXR and CNNUDIXΔ25-223/F/CNNUDIXR, respectively, and sequenced. Confirmed fragments were digested with Clal and NotI and ligated into pGR106 to generate the pGR106-CNNUDIX and pGR106-CNNUDIXΔSP vectors. For generating pGR106-CNNUDIXΔNudix motif (NM), plasmid pCR2.1-CtNUDIX was amplified using the primer set ΔnudimR/G to generate a pCR2.1-CtNUDIX variant lacking the entire 23-amino-acid (aa) NM (inverse PCR), self-ligated after phosphorylating the PCR product, and sequenced. Confirmed fragments were digested with Clal and NotI and ligated into pGR106. For subcellular localization of CNNUDIX, a
669-bp CtNUDIX ORF was amplified from the cDNA generated from total RNA isolated from C. truncatum-infected lentil leaflet tissues displaying the late biotrophic phase of the infection process with the primer pair CtNUDIXF/pKN170CtNUDIXR and cloned between the Clal and HindIII sites of PKNTG to generate a pKN170-CtNUDIX vector. pKN170 was constructed by cloning the eGFP-TrpC terminator sequence into pknG (24). The CtNUDIX ORF fused in frame with eGFP was amplified from pKN170-CtNUDIX using the primer set CtNUDIXF/eGFPPr and ligated into the Clal and NotI sites of pGRI06 to generate the pGRI06-CtNUDIX/eGFP vector. The constructs were then transformed to A. tumefaciens strain GV3101 carrying the pSoup helper plasmid. Infiltration assays with 0.3 optical density at 600 nm (OD600) units of recombinant A. tumefaciens strains were performed with 4- to 6-week-old N. tabacum plants as described previously (25). Responses were monitored from 3 to 7 days. For microscopic studies, leaflet tissues were collected at 7 dpi, fixed in a fixation solution (60% methanol, 30% chloroform, 10% acetic acid) until collected from droplet-inoculated areas at 10 dpi and fixed in a fixation postinoculation (dpi). For microscopic study, barley leaf tissues were collected 10 days after infiltration (dai). For autofluorescence detection, the edges of the infiltration zone (3 dai) were observed under a fluorescence microscope and photographed. For investigating the localization of CtNUDIX, N. tabacum leaf tissues infiltrated with recombinant A. tumefaciens strains carrying the pGRI06-CtNUDIX/eGFP construct were collected at 3 dai and infiltrated with 1 μg ml⁻¹ of propidium iodide (PI) (Invitrogen) solution. Following infiltration, leaf tissues were incubated in 0.8 M mannitol for 20 min prior to live-tissue imaging by confocal microscopy.

Confocal microscopy. Confocal laser scanning microscopy was performed on a Zeiss Confocor-2 LSM 510 (Carl Zeiss, Jena, Germany). GFP, PI, and FM4-64 were excited with an argon 488-nm laser. Fluorescence signals were captured through the band-pass emission filters (505 to 530 nm [eGFP]) and a long-pass barrier filter (650 nm [PI and FM4-64]).

Overexpression of CtNUDIX in C. truncatum and M. oryzae and infection assays. To generate the overexpression vector RP27:CtNUDIX, a 672-bp CtNUDIX ORF with a stop codon sequence was amplified from cDNA generated from total RNA isolated from C. truncatum-infected lentil leaflet tissues displaying the late biotrophic phase of the infection process with the primer pair CtNUDIXF/RP27CtNUDIXR and cloned between the Clal-HindIII sites of RP27-pKN170. The RP27-pKN170 vector was generated by cloning the RP27 promoter (26) into pKN170. The resulting RP27:CtNUDIX vector was transferred into the C. truncatum CT-21 isolate and M. oryzae wild-type strain P131 protoplasts using polyethylene glycol/CaCl₂, as described previously (27).

Conidia from wild-type P131 and CtNUDIX overexpression strains of M. oryzae were harvested from 10-day-old oatmeal agar culture plates and resuspended to 4 × 10⁸ conidia ml⁻¹ in sterile water supplemented with 0.025% Tween 20. Leaves of 10-day-old barley (Hordeum vulgare) seedlings of the susceptible cultivar CDC Silky were droplet inoculated with 15 μl of conidial suspension onto the adaxial surface without damaging it and allowed to develop blast lesions. Photographs were taken at 10 days postinoculation (dpi). For microscopic study, barley leaf tissues were collected from droplet-inoculated areas at 10 dpi and fixed in a fixation solution (60% methanol, 30% chloroform, 10% acetic acid) until required. Fixed samples were rehydrated with decreasing ethanol gradients as described above. Samples were then mounted in 30% glycerol on glass slides, examined under a compound light microscope, and photographed.

Similarly, conidia from CT-21 and CtNUDIX overexpression mutants were harvested by flooding the CT-21 and overexpression cultures with sterile water supplemented with 0.025% Tween 20. Three-week-old Eston plants were spray inoculated with conidial suspension (4 × 10⁷ conidia ml⁻¹) and allowed to develop water-soaked anthracnose lesions for up to 7 days. For microscopic studies, leaflet tissues were collected at 7 dpi, fixed in the fixation buffer as described above, visualized under a confocal microscope, and photographed.

RESULTS

Identification and analysis of the CtNUDIX gene. In a previous study, we constructed an in planta BNS-specific cDNA plasmid library from the susceptible Canadian lentil cultivar Eston infected with C. truncatum isolate CT-21 and identified 122 unique encoding proteins with putative signal peptides, including effectors (17). In this study, we characterized one of the effector proteins, Ct21-1373, now named CtNUDIX.

The CtNUDIX cDNA (GenBank accession no. [GB] HO663661) is 1,184-bp (bp) in size with an ORF of 669 bp, which encodes a 223-aa protein. An SP of 24 aa with a cleavage site between alanine-24 and glutamine-25 was predicted at the N terminus of the preprotein, giving rise to a 199-aa mature protein with a predicted molecular mass of 21.95 kDa and an isoelectric point of 9.79. Thus, CtNUDIX is a small, basic, secreted, soluble protein and lacks a transmembrane domain and an N- or O-glycosylation site in the deduced peptide sequence. A C-terminal Nudix hydrolase domain (psam00293) of 87 aa that contained a putative 23-aa Nudix hydrolase motif was also detected in the protein (Fig. 1A; see Fig. S1 in the supplemental material). With a cutoff E value of ≤10⁻³ using the BLASTP algorithm, CtNUDIX was matched only to two secreted Nudix domain-containing proteins, one belonging to C. graminicola (CtNUDIX; GB: EFBQ36857), the causal agent of anthracnose on cereals, and the other to M. oryzae (MoNUDIX), indicating that it is conserved among hemibiotrophic fungal phytopathogens. The M. oryzae 70-15 genome contains duplicate copies of MoNUDIX (MGG_14156 and MGG_14344). So far, neither has been functionally characterized. We also identified 3 putative Nudix effectors (containing a signal peptide and a Nudix domain) from Colletotrichum higginsianum and 7 from Phytophthora infestans, including 5 RxLR effectors, by searching the entire genomes of both pathogens. Both eukaryotes and prokaryotes contain Nudix proteins, though the secreted Nudix proteins are present only in hemibiotrophic fungi and oomycetes. Evolutionary relationships among the 13 Nudix effectors from five hemibiotrophic phytopathogen species were determined by bootstrap testing of phylogenies by the neighbor-joining method (Mega 4.1 [20]). In the phylogram, Nudix effectors separated into three phylogenetic lineages, or clades. Effectors of fungal origin clustered into one group that was separate from two groups of putative P. infestans Nudix effectors, indicating their divergent evolutionary patterns (Fig. 1B).

Pairwise alignment showed that CtNUDIX shares over 58% identity with Nudix effectors of fungal origin with sequence coverage of more than 55%, whereas with less than 50% sequence coverage, CtNUDIX aligns with a group of P. infestans effectors that include 5 RxLR effectors and an ADP-sugar pyrophosphatase with 58% or less identity (see Fig. S1 in the supplemental material).

CtNUDIX is expressed exclusively in the late biotrophic phase. Northern blot analysis revealed the absence of CtNUDIX transcripts in all in vitro-grown C. truncatum cell types, like mycelia, ungerminated conidia, and mycelia grown in minimal medium supplemented with lentil cell wall as a sole carbon source to mimic the in planta necrotrophic phase (here called cell wall-treated mycelia). Histochemical analysis using trypan blue staining of collected leaflet tissues suggested that appressorial penetration occurred at 16 hpi, following by the biotrophic phase (44 hpi), represented by large, intracellular primary hyphae (fully developed), and the necrotrophic phase, which coincided with the first visible symptoms of infection at 68 hpi (Fig. 2A). A strong hybridization signal was detected exclusively with RNA isolated from lentil tissues collected at 44 hpi (Fig. 2A and B). Thin secondary hyphae started to differentiate from the biotrophic primary hyphae in infected lentil tissues at 48 hpi, signaling the necrotrophic phase (17). CtNUDIX is therefore the first effector gene identified from any hemibiotrophic phytopathogen that shows explicit expression exclusively during and specific to the late biotrophic phase of
infection. Expression of CtNUDIX was also analyzed by qRT-PCR, where the transcript levels of CtNUDIX were normalized to a housekeeping 60S ribosomal gene and were expressed as relative values, with 1 corresponding to the expression level in mycelia. A 109-fold induction of CtNUDIX transcripts was observed with samples collected at 44 hpi, whereas during other stages, the expression level remained similar to that of mycelia (Fig. 2C), thereby corroborating the results of the Northern blot analysis. Using the same time course, we were able to identify appressorium penetration- and necrotrophy-specific genes encoding putative hydrolytic enzymes, β-1,6-galactanase (GB HO663724) and endo-1,4-β-mannosidase (GB HO663717), respectively (see Fig. S2 in the supplemental material). Putative NUDIX motifs were isolated by the NCBI conserved domain search server (see S4 in the supplemental material), suggesting that cell death caused by infiltrating A. tumefaciens strains carrying pGR106-CtNUDIX is attributable to CtNUDIX and that CtNUDIX is unlikely to act in cytoplasm. Furthermore, we tested the role of the Nudix motif in the CtNUDIX function. Tobacco leaves infiltrated with recombinant Agrobacterium strains carrying pGR106-CtNUDIXΔNM (lacking a Nudix motif) showed no macro- or microscopic cell death (Fig. 4; see Fig. S4 in the supplemental material), indicating that the Nudix motif is required for CtNUDIX function.

CtNUDIX is likely to function at the plasma membrane. To determine the location of CtNUDIX activity, a functional C-terminal eGFP fusion to CtNUDIX (with SP) (Fig. 4A) was constructed. The CtNUDIX:eGFP fusion protein accumulated at the cell periphery when expressed in N. tabacum leaves via agroinfiltration 72 hours after infiltration (hai) (Fig. 4B). The green fluorescence signal remained associated with the plasma membrane after mannitol-induced plasmolysis of the epidermal cells labeled with either PI (Fig. 4C) or an endocytotic tracker, FM4-64 (Fig. 4D). PI stained cell walls, as it could not leak through the plasma membrane (indicative of an intact plasma membrane). Some cells that had lost their membrane integrity showed red staining (PI) of

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we selected seven transformants based on the neomycin resistance gene, NEO. These transformants expressed the 

CtNUDIX cDNA fragment, all 7 transformants showed varying transcript levels in mycelia 

(CtNUDIX/Ct strains) and 6.0 ± 0.5 mm/day (CtNUDIX/Mo strains)). (see Fig. S6 and S7 in the supplement-

mental material), conidiogenesis (~21 × 10⁶ conidia/Ø 5.2-cm petri dish [CtNUDIX/Ct strains] and ~4 × 10⁶ conidia/Ø 5.2-cm petri dish [CtNUDIX/Mo strains]), conidium germination, and appressorium formation for expression analyses. The expression of CtNUDIX in overexpression mutants of C. truncatum was quantitatively determined by qRT-PCR, and all seven transformants showed varying transcript levels in mycelia grown in a complete medium (3- to 707-fold increases, considering the expression of CtNUDIX in wild-type mycelia as a calibrator) (Fig. 5B). For analyzing mRNA levels in M. oryzae overexpression strains, Northern blot analysis was conducted. When probed with a 672-bp CtNUDIX cDNA fragment, all 7 transformants showed an ~1.2-kb band, whereas total RNA isolated from strain P131 mycelia did not hybridize, indicating that these M. oryzae transformants expressed the C. truncatum effector gene, CtNUDIX (Fig. 6A).

In order to assess the ability to cause anthracnose and blast disease, two overexpression strains of each species, CtNUDIX/Ct-7 and CtNUDIX/Ct-10, and CtNUDIX/Mo-9 and CtNUDIX/Mo-17, showing higher expression of CtNUDIX than others, were selected for pathogenicity testing with the susceptible lentil cultivar Eston and the barley (H. vulgare) cultivar CDC Silky. Eston plants sprayed with CT-21 conidia collapsed at 7 dpi (Fig. 5C), and barley seedlings droplet inoculated with P131 conidia showed typical eye-shaped gray or whitish blast lesions with a reddish-brown margin at 10 dpi. On the other hand, overexpression strains failed to induce anthracnose or blast symptoms, i.e., were nonpathogenic. However, host cells penetrated by overexpression strains exhibited light-brown discoloration, which was suspected to be due to HR-like cell death of the infected cells (Fig. 5D and 6C).

To investigate which stage of fungal development was impaired by CtNUDIX, we collected leaf tissues infected by overexpression strains, along with their wild-type isolates, and fixed them in a fixation buffer, as described in Materials and Methods. The fixed tissues were examined under a light microscope and

FIG 2 CtNUDIX transcripts are exclusively accumulated in the in planta late biotrophic phase of the infection. (A) Trypan blue-stained in planta fungal structures. Lentil leaflets were droplet inoculated with C. truncatum isolate CT-21 and harvested at 16, 44, and 68 hpi, which represent appressorium penetration and the in planta biotrophic and necrotrophic phases of C. truncatum, respectively. M, mycelia; C, ungerminated conidia; M + CW, cell wall-treated mycelia; A, appressorium; PP, penetration peg; PH, primary hyphae; SH, secondary hyphae. Scale bars, 10 μm. (B) Northern blot analysis of C. truncatum gene expression (CtNUDIX and 60S ribosomal protein-encoding genes) in the infection time course and in vitro-grown cell types. Total RNA samples (20 μg/lane) were fractioned on a formaldehyde-agarose denaturing gel and transferred to a nylon membrane. The blots were then probed with a 593-bp 60S ribosomal gene fragment (as a marker to evaluate the in planta biomass of C. truncatum) and a 672-bp CtNUDIX cDNA fragment. An ethidium bromide-stained agarose gel prior to transfer to a nylon membrane is shown as a loading control. (C) Quantitative RT-PCR analysis of CtNUDIX transcript levels in fungal cell types of C. truncatum, such as mycelia and conidia, and an infection time course. The transcript levels of CtNUDIX were normalized to a housekeeping gene, the 60S ribosomal gene. All relative expression values of genes are reported as means ± standard errors (calculated from 3 biological replications and 3 technical replications/biological replication) on a log2 scale.
spore masses were visible on collapsed lentil leaf tissues (Fig. 5D), indicating necrotrophic invasive hyphae. Acervuli with black setae and the binary vector pGR106 (negative control), pGR106-observed on tobacco leaves following the infiltration of recombinant INF1 (positive control) at 5 dai. The assay was replicated independently 10 times. The presence (+) or absence (−) of macroscopic cell death observed in the infiltration zones is shown.

FIG 3 Transient expression of CnNUDIX in tobacco. Shown are symptoms observed on tobacco leaves following the infiltration of recombinant A. tumefaciens strains carrying the binary vector pGR106 (negative control), pGR106-CnNUDIX, pGR106-CnNUDIXASP, pGR106-CnNUDIXΔNM, and pGR106-INF1 (positive control) at 5 dai. The assay was replicated independently 10 times. The presence (+) or absence (−) of macroscopic cell death observed in the infiltration zones is shown.

photographed. By 7 and 10 dpi, respectively, strains CT-21 and P131 had macerated leaf tissues (Fig. 5C and 6B) caused by forming necrotrophic invasive hyphae. Acervuli with black setae and spor masses were visible on collapsed lentil leaf tissues (Fig. 5D), whereas conidiophores producing conidia in the droplet-inoculated areas of barley leaves became evident at this time (Fig. 6C). Under the same conditions, conidia of overexpression strains formed melanized appressoria and penetrated leaf tissues via penetration pegs to form infection vesicles that differentiated into initial biotrophic invasive hyphae (BIH) as efficiently as their wild-type strains. However, these BIH, unlike those of the wild type, could neither switch to the necrotrophic phase (CnNUDIX/Ct strains) nor traverse to neighboring cells (CnNUDIX/Mo strains) and were trapped in the first infected epidermal cells, which turned brown (Fig. 5D and 6C). The brown staining of epidermal cells corresponds to the accumulation of phenolic compounds after cell death (29). This suggested that CnNUDIX induced incompatibility of C. truncatum and M. oryzae with their respective host plants by causing cell death in the biotrophic phase of the infection process, as a result of which no necrotrophic invasive hyphae developed. By histochemical analysis of rice cells infected by GFP-expressing M. oryzae, Kankanala and associates (30) showed that host cells had lost viability after the fungus moved to the adjacent cells. In contrast, it is obvious here that CnNUDIX had killed the host cells well before moving to neighboring cells and blocked the transition to the necrotrophic invasive phase.

**DISCUSSION**

Hemibiotrophic phytopathogens pose a serious threat to a sustainable global food supply by causing notorious plant diseases, like late blight of potato and tomato, rice blast, and anthracnose, in several crops of economic significance. The morphological, genetic, and physiological transition of the intracellular hyphae of these pathogens from biotrophy to necrotrophy plays a critical role in disease development. Despite being a decisive factor in the manifestation of disease, only a few studies published to date have addressed this important biotrophy-necrotrophy transition (3–7). More recently, Kleemann et al. (31) identified a necrosis- and ethylene-inducing peptide 1-like protein from C. higginsianum ChnLP1 that was exclusively expressed in biotrophic hyphae with nascent necrotrophic hyphae and caused cell death in N. benthamiana transiently expressing the effector.

Recent reports (32, 33) presented a conceptual model describing the molecular mechanism underpinning the BNS in which phytopathogens secrete distinct classes of effector proteins that first suppress plant defense responses and associated programmed cell death (PCD) during the biotrophic phase and later induce large-scale necrosis during the necrotrophic phase of infection. The authors characterized an effector gene, SNE1 (suppression of necrosis 1) from P. infestans that was specifically expressed during the biotrophic colonization of its host plant, tomato (Solanum lycopersicum). Using agroinfiltration assays, Kelley and colleagues (32) demonstrated that SNE1 suppresses the activity of cell death-inducing effectors, such as PiNPP1.1 from P. infestans (34) and PsojNIP from Phytophthora sojae (35), that are secreted during necrotrophy and the transition from biotrophy to necrotrophy, respectively. SNE1 also suppressed PCD mediated by the Avr-R protein interactions from a broad spectrum of pathosystems. Thus, SNE1 and PiNPP1.1 are coordinately expressed by P. infestans during the biotrophic and necrotrophic phases of S. lycopersicum colonization and act antagonistically, as SNE1 blocked the activity of PiNPP1.1 to avert cell death and to maintain the biotrophic phase before transition to necrotrophy (32).

In the current study, we have characterized a novel effector, CnNUDIX (containing a 23-aa Nudix motif) from C. truncatum. Northern blot analysis revealed the abrupt accumulation of CnNUDIX transcripts exclusively at 44 hpi, where lentil epidermal cells were completely occupied by thick biotrophic hyphae of C. truncatum. Using agroinfiltration assays, we have demonstrated that CnNUDIX induces severe cell death, similar to HR in tobacco leaves at 5 dai, which, together with the expression pattern, suggests that C. truncatum secretes CnNUDIX precisely before the transition to necrotrophy and therefore may possess a highly sophisticated mechanism to facilitate the BNS. In addition, the presence of a Nudix motif in CnNUDIX is essential to induce HR-like cell death. The CnNUDIX homologs are well conserved in other hemibiotrophic phytopathogens, including C. graminicola and M. oryzae. By searching the entire genomes available in the public database of the Broad Institute (http://www.broadinstitute.org), we identified three proteins from C. higginsianum and eight pro-
teins from *P. infestans* that contained a putative SP and a Nudix domain, indicating that NUDIX effectors are well conserved across the hemibiotrophic phytopathogens. However, none of these proteins has been functionally characterized. Biotrophic and necrotrophic phytopathogens like *Puccinia graminis* f. sp. *tritici* (black stem rust of wheat), *Puccinia triticina* (brown rust of wheat), *Puccinia striiformis* f. sp. *tritici* (stripe rust of wheat), *Ustilago maydis* (corn smut), *Sclerotinia sclerotiorum* (multihost rot disease), *Pyrenophora tritici-repentis* (tan spot of wheat), *Verticillium dahliae*, and *Verticillium albo-atrum* (vascular wilt disease) lack secreted *CtNUDIX* homologs, suggesting that Nudix effectors like *CtNUDIX* may be unique to hemibiotrophic phytopathogens. Recently, an RxLR effector, *Avr3b*, encoding an ADP-ribose/NADH pyrophosphorylase with C-terminal W and Nudix hydrolase motifs, identified from the soybean root rot pathogen *P. sojae* was shown to enhance susceptibility of tobacco (after transient expression) to *Phytophthora capsici* and *Phytophthora parasitica*. *Avr3b*-like RxLR Nudix effectors are conserved in *Phytophthora* spp. The authors hypothesize that *Avr3b* might be delivered into host cells to impair host immunity (36).

We hypothesized that the overexpression of *CtNUDIX* in hemibiotrophic phytopathogens would transform a compatible interaction into an incompatible one by blocking the pathogen’s growth before switching to a destructive necrotrophic phase. To test this hypothesis, *CtNUDIX* was expressed in *C. truncatum* and *M. oryzae* under the control of the strong constitutive expression promoter RP27 (26). *CtNUDIX* did not affect vegetative growth, conidiogenesis, infection-related appressorium morphogenesis, and initial *in planta* biotrophic colonization (penetration pegs, infection vesicles, and early biotrophic invasive hyphae), indicating that *CtNUDIX* was dispensable for vegetative and asexual development and initial infection by the fungus. However, even after prolonged incubation (10 dpi), no secondary hyphae were visible in lentil and barley epidermal cells infected by *CtNUDIX* overexpression strains. After the same length of incubation, wild-type strains invaded host tissues with secondary hyphae and developed typical disease symptoms (anthracnose or blast lesions on leaves) (Fig. 5C and 6B). Taken together, our data suggest that *CtNUDIX* may contribute to the hemibiotrophic parasitism.

![FIG 4](https://www.journals.aps.org/ec/article-pdf/3/22/5487/548701/548701.pdf)

**FIG 4** Localization of *CtNUDIX:eGFP* fusion protein in *N. tabacum*. (A) *CtNUDIX:eGFP* fusion construct pGR106-AtNUDIX:eGFP. The expression of the *CtNUDIX:eGFP* fusion construct was under the control of the cauliflower mosaic virus 35S promoter (35S::*CtNUDIX:eGFP*). (B) Leaf cells transiently expressing *CtNUDIX:eGFP* fusion proteins were visualized under a confocal microscope using an argon laser. Scale bar, 50 μm. DIC, differential interference contrast. (C and D) PI-labeled (C) and FM4-64-labeled (D) plasmolyzed *N. tabacum* leaf tissues expressing a 35S::*CtNUDIX:eGFP* construct were examined under a confocal microscope for GFP (green) and PI (red) or FM4-64 (red) fluorescence detection. The arrows and arrowheads indicate the cell wall and plasma membrane, respectively. The areas outlined by dashed lines represent dead cells. Scale bar, 25 μm.

Found in almost all forms of life from bacteria to mammals, Nudix proteins participate in a wide range of crucial housekeeping functions, including the hydrolysis of mutagenic nucleotides, the modulation of the levels of toxic and signaling molecules, and the monitoring of metabolic intermediates (12). The *Arabidopsis thaliana* genome encodes 24 Nudix proteins (AtNUDT1 to -24 [37]). Little is currently known about the biological functions of...
One of the well-characterized NUDT genes, AtNUDT7, has been identified as a negative regulator of basal defense. AtNUDT7 loss-of-function mutation leads to enhanced basal defense against Pseudomonas syringae, attributed to nonrepressor of pathogenesis related genes 1 (NPR1) and salicylic acid (SA)-dependent and -independent defense signaling pathways. Furthermore, the authors demonstrated that ADP-ribose and NADH are preferred substrates of NUDT7 and that the hydrolysis activity of NUDT7 is essential for its biological function (38). Phytopathogens are likely to possess two sets of Nudix proteins; one set of proteins is required for housekeeping functions, whereas others (Nudix effectors) might be delivered into the host plant cell to facilitate pathogenesis. For example, plant-pathogenic bacteria like Ralstonia solanacearum (bacterial wilt pathogen) and Xanthomonas campestris pv. vesicatoria (the causative agent of bacterial spot disease on pepper and tomato) possess a

![FIG 6](image_url)

**FIG 5** Overexpression of CtNUDIX in C. truncatum causes incompatibility with its host, lentil. (A) An overexpression construct, RP27::CtNUDIX. The expression of CtNUDIX was under the control of the strong constitutive expression promoter RP27 and the TrpC terminator. (B) qRT-PCR analysis of CtNUDIX overexpression transformants of C. truncatum. (C) Three-week-old susceptible L. culinaris cv. Eston plants were sprayed with conidial suspensions (4 × 10⁴ conidia ml⁻¹) of CT-21, CtNUDIX/Ct-7, and CtNUDIX/Ct-10 and photographed at 7 dpi. (D) Microscopic evaluation of lentil leaf cells infected by CT-21 and overexpression mutants at 7 dpi. Ac, acervulus; PH, primary hyphae; SH, necrotrophic hyphae. The asterisks represent appressoria. Scale bars, 25 μm.

![FIG 6](image_url)

**FIG 6** Heterologous expression of CtNUDIX in M. oryzae causes incompatibility with its host, barley. (A) Northern blot analysis of CtNUDIX-expressing M. oryzae transformants. RNA gel blots prepared from total mycelial RNA of transformants were hybridized with a 672-bp CtNUDIX cDNA fragment. Ethidium bromide staining of total RNA is shown as a loading control. (B) Ten-day-old leaves of susceptible H. vulgare cv. CDC Silky were droplet inoculated with P131, CtNUDIX/Mo-9, and CtNUDIX/Mo-17. Fifteen microliters of conidial suspension (4 × 10⁴ conidia ml⁻¹) were spotted onto the adaxial sides of leaves without damaging the surface and photographed at 10 dpi. The arrowheads mark typical necrotic-blast lesions on leaves inoculated with P131, whereas there is discoloration (light brown) on leaves inoculated with CtNUDIX overexpression strains. (C) Microscopic evaluation of barley cells infected by P131 and overexpression mutants CtNUDIX/Mo-9 and CtNUDIX/Mo-17 (10 dpi). Scale bars, 10 μm. A, appressorium; BIH, biotrophic invasive hyphae; NIH, necrotrophic invasive hyphae.
novel class of type 3 secretion system (T3SS) effectors that contain a Nudix motif. These effectors are delivered into the host cell cytoplasm via the T3SS to promote pathogenesis, presumably by modulating the levels of nucleoside diphosphate derivatives in host cells (39–41). However, fungal phytopathogens lack a T3SS, and therefore, it is likely that these pathogens secrete Nudix effectors into the plant extracellular space, where they might target apoplastic or host cell plasma membrane components, or are translocated from the apoplast into the host cytoplasm. The mechanism by which CitNUDIX induces host cell death in the late biotrophic phase of *in planta* infection to facilitate fungal proliferation is likely associated with its function at the plasma membrane (Fig. 4), though further experiments using the native system are required to confirm this hypothesis. This perturbation may cause leakage, which in turn allows flooding of the plant cell with a nonnative protein(s) that is recognized by the general surveillance systems in the host cell and induces cell death.

We speculate that CitNUDIX may hydrolyze extracellular energetic pyrophosphate bonds of IPs, which are attached to the phospholipid bilayer of the plant cell surface or AP_A. This hydrolysis might perturb the plant cell plasma membrane dynamics, which results in loss of membrane integrity and eventually triggers cell death. Therefore, it is likely that the expression of the NUDIX effector shuts down the biotrophic phase and facilitates switching the lifestyle of phytopathogens from the biotrophic mode of parasitism to necrosis. The exact targets or substrates of CitNUDIX and the mechanism by which CitNUDIX elicits HR-like cell death remain to be clarified. In addition, targeted deletion of the CitNUDIX gene in *C. truncatum* is required to confirm the function of this late-biotrophic-specific effector as a regulator of the BNS.

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