bZIP Transcription Factors in the Oomycete *Phytophthora infestans* with Novel DNA-Binding Domains Are Involved in Defense against Oxidative Stress

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Transcription factors of the basic leucine zipper (bZIP) family control development and stress responses in eukaryotes. To date, only one bZIP has been described in any oomycete; oomycetes are members of the stramenopile kingdom. In this study, we describe the identification of 38 bZIPs from the *Phytophthora infestans* genome. Half contain novel substitutions in the DNA-binding domain at a site that in other eukaryotes is reported to always be Asn. Interspecific comparisons indicated that the novel substitutions (usually Cys, but also Val and Tyr) arose after oomycetes diverged from other stramenopiles. About two-thirds of *P. infestans* bZIPs show dynamic changes in mRNA levels during the life cycle, with many of the genes being upregulated in sporangia, zoospores, or germinated zoospore cysts. One bZIP with the novel Cys substitution was shown to reside in the nucleus throughout growth and development. Using stable gene silencing, the functions of eight bZIPs with the Cys substitution were tested. All but one were found to play roles in protecting *P. infestans* from hydrogen peroxide-induced injury, and it is proposed that the novel Cys substitution serves as a redox sensor. A ninth bZIP lacking the novel Asn-to-Cys substitution, but having Cys nearby, was also shown through silencing to contribute to defense against peroxide. Little effect on asexual development, plant pathogenesis, or resistance to osmotic stress was observed in transformants silenced for any of the nine bZIPs.

The ability of organisms to grow, differentiate, and respond to the environment relies largely on their ability to modulate gene expression. Transcriptional regulators can be grouped into families of related proteins, many of which are conserved across kingdoms. One such family is the basic leucine zipper (bZIP) group, which has been described in fungi, metazoans, and plants (1–3). These transcription factors are defined by a DNA-binding region rich in basic amino acids, which forms an α-helix that contacts the major groove of DNA, followed by a leucine zipper, which forms a coiled-coil dimerization interface. Outside the DNA-binding and leucine zipper regions, bZIPs show little sequence conservation. Prior studies identified two sites in the DNA-binding domain that are invariant between bZIPs: an Asn at the position corresponding to residue 235 of the well-studied GCN4 transcription factor of *Saccharomyces cerevisiae* and Arg at position 243 (1–3). Mutations that alter these do not eliminate the ability of a bZIP to bind DNA but change the preferred target sequence (4–6).

In the case of transcription factor families that are conserved across kingdoms, key elements of their DNA-binding domains are usually preserved, but there are exceptions. For example, many basic helix-loop-helix (bHLH) transcription factors from plants diverge from the bHLH consensus for metazoans (7). A more extreme example of change are WRKY-GCM transcription factors, which have been identified from plants, fungi, slime molds, and some protists. These show limited sequence conservation but have maintained a DNA-binding fold (8). Changes in transcription factors are emerging as a major determinant of the morphological and physiological diversity underlying evolution (9–11).

In this study, we focused on bZIP transcription factors from *Phytophthora infestans*, the cause of the late blight diseases of potato and tomato (12). *P. infestans* is classified as an oomycete, a group that belongs to the stramenopile kingdom (13). Dynamic changes in transcription occur during the life cycle, which involves several spore stages that are important to pathogenicity (14, 15). Sporangia develop from hyphae and are important for movement to new hosts. Under cool and moist conditions, each sporangium cleaves into multiple biflagellate zoospores, which navigate to infection sites. Cysts formed from zoospores later germinate to make appressoria, which are used to penetrate the plant wall. bZIPs are candidates for regulators of these developmental transitions, considering their roles in differentiation in organisms of other kingdoms (1, 16). Indeed, in a prior study we showed that a bZIP from *P. infestans*, PiBZP1, is required for normal zoospore swimming and appressorium formation (17). In fungi and plants, bZIPs also regulate responses to stresses caused by starvation, osmotic imbalance, and reactive oxygen (18–21). The proteins also contribute to the virulence of fungal pathogens (22–24). It follows that bZIPs might be important for the pathogenicity of *P. infestans*, for example to resist the effects of reactive oxygen formed as part of the plant defense response (25).

The goal of the present work was to learn more about the roles of bZIPs in *P. infestans* and how the proteins evolved in oomycetes. Since our prior study, the genome of *P. infestans* has been sequenced (26). Using that resource, we identified 38 bZIPs, of which half contain novel Cys, Val, or Tyr substitutions in the DNA-binding domain at the site previously reported to invariantly contain Asn. Interspecific comparisons indicated that these
atypical residues arose after the divergence of oomycetes from other stramenopiles. The majority of bZIPs showed strong differential expression during the life cycle, often being upregulated in sporangia, zoospores, or germinated zoospore cysts. Gene silencing experiments indicated that eight of the Cys-containing bZIPs contributed to resistance against hydrogen peroxide-induced damage but otherwise had little effect on growth or asexual development.

MATERIALS AND METHODS

Bioinformatics. Genome data for P. infestans, Phytophthora ramorum (version 1.1), Pythium ultimum, and Hyaloperonospora arabidopsidis (v. 8.3) were obtained initially from their respective genome projects websites and later from the fungidb.org website. Thalassiosira pseudonana data (v. 3) were from the Joint Genome Institute of the United States Department of Energy. P. infestans bZIP candidates were initially identified through BLAST searches and protein domain searches using PFAM, SMART, and INTERPRO (27–29), and their gene models were repaired as needed. By using the hmmbuild and hmmsearch programs from HMMER 3.0, bZIP domains from the candidates were used to develop a hidden Markov model (HMM), which was used to search the complete P. infestans proteome with an E-value cutoff of 10^{-10} (30). bZIP candidates from other oomycetes included proteins that matched this HMM and orthologs of the P. infestans proteins. T. pseudonana bZIPs included those matching the HMM and those containing bZIP domains based on PFAM, SMART, and INTERPRO. Alignments were performed using MUSCLE as implemented in SEAVIEW, and cladograms were generated using PhyML (31). Nuclear localization signals were identified using NLS Mapper (32). Phosphorylation sites were predicted using NetPhos 2.0 (33).

Manipulations of P. infestans. Experiments involved isolate 1306, an A1 strain isolated from tomato in California. Cultures were maintained at 18°C on rye-sucrose medium, and developmental stages were obtained essentially as described previously (17). In brief, sporangia were released from 9-day-old cultures by adding water and rubbing with a glass rod. To obtain nonsporulating mycelia, sporangia were inoculated into clarified broth and the resulting hyphae were harvested after 72 h. Sporangia were induced to initiate zoosporogenesis by incubation for 30 min in water at 10°C. Zoospores were released by incubating the sporangia for an additional 90 min, and cysts were obtained by adding CaCl₂ to 0.25 mM and vortexing for 1 min. Germinated cysts were obtained by incubating cysts for 6 h in water at 18°C. Transformations used the protoplast method with G418 selection (34).

Plant infections used tomato leaflets harvested from 4-week-old plants of cultivar New Yorker. Inoculations were performed using zoospores, which before use were passed through 15-µm nylon mesh to eliminate sporangia and hyphal fragments and adjusted to 5 x 10^4 per ml. As described in Results, leaflets were inoculated either by spraying or by placement of one drop on the upper surface of the leaf. Leaves were incubated at 18°C and >95% humidity with a 12-h light-dark cycle. Assays employed a minimum of six replicates.

Confocal microscopy was performed using a Leica TCS SP5 inverted microscope. Sequential scanning was performed with green fluorescent protein (GFP) and 4',6-diamidino-2-phenylindole (DAPI) filters. Staining with DAPI involved placing tissue in phosphate-buffered saline (pH 7.0) for 5 min and then 95% ethanol for 5 min. After the ethanol treatment had been repeated, the sample was placed in phosphate-buffered saline containing 50 to 75 nM DAPI and incubated at room temperature for 10 min in the dark. Samples were washed three times with water prior to microscopic examination.

Osmotic and oxidative stress assays were performed by measuring the radial growth of cultures on rye-sucrose agar, using as inoculum a plug from a 3-day-old nonsporulating culture. Medium was amended with either 0.3 M NaCl, 0.8 M sorbitol, 7.5 mM paraquat, or 0.75, 1.5, and 2.5 mM H₂O₂. Paraquat and H₂O₂ were added to medium just prior to agar solidification, and the resulting plates were used immediately. Growth was measured 3, 5, and 7 days postinoculation, using three replicates.

Constructs for transformation. C-terminal fusions of PITG_11668 and GFP were generated using pGFPN (34). The bZIP gene was obtained by PCR from genomic DNA using primers containing Agel and NheI cloning sites and Invitrogen Platinum Taq DNA polymerase. Constructs were verified by restriction digestion and sequencing. Silencing constructs were based in pTOR, in which the ham34 promoter was used to drive transcription of a cassette containing about 500 nucleotides (nt) of sense sequences, the Ste20 intron, and antisense sequences (35, 36). The precise regions employed were nt 1 to 546 of PITG_11664, nt 200 to 700 of PITG_11668, nt 197 to 695 of PITG_11671, nt 255 to 833 of PITG_09198, and nt 214 to 724 of PITG_16038. The inverted repeat cassettes were first assembled in pBS-SK’+ from PCR-generated antisense and sense/intron fragments, with the latter being obtained by double-joint PCR (35), using EcoRI-XbaI and XbaI-SacI cloning sites, respectively. The cassette was then transferred as a EcoRI-SacI fragment to pTOR. Primers used for cloning are shown in Table S1 in the supplemental material.

Reverse transcription-PCR. RNA was extracted using the RNeasy plant minikit (Qiagen) from tissues ground under liquid nitrogen and treated with RNaseⅠ DNase (Promega). For silencing studies, RNA was extracted from chilled sporangia (PITG_11664 transformants), cysts (PITG_09198, PITG_11668, and PITG_16038), or germinating cysts (PITG_11671). cDNA was synthesized using a SuperScript III first-strand reverse transcription-PCR (RT-PCR) kit (Invitrogen). PCR was then performed using the primers shown in Table S1 in the supplemental material, which were targeted to the 3' end of the genes and designed using the Primer3 program. For quantitative PCR (RT-qPCR), primers were tested using a dilution series of templates and accepted if efficiencies were above 94%. Amplifications were performed using the Dynamo SYBR green qPCR kit (Thermo) with the following program: 95°C for 15 min, followed by 40 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s. At the end of the run, melting curves were generated to evaluate the fidelity of amplification. Expression levels were calculated using the ΔΔCT method, using a constitutive gene (ribosomal protein 53a, PITG_11766) as a control; prior studies demonstrated that this gene is expressed at similar levels during the life cycles of both P. infestans and Phytophthora parasitica (15, 37).

RESULTS

The P. infestans genome encodes bZIPs with canonical and novel DNA-binding domains. bZIPs from P. infestans were identified through an iterative process of searching its predicted proteome for bZIP domains using PFAM and INTERPRO, using the hits to re-search the proteome using BLASTP and checking any new matches for the bZIP domains using PFAM, SMART, and INTERPRO. Final candidates were selected by using a hidden Markov model based on the P. infestans bZIP domains to search its entire predicted proteome (26, 38). A total of 38 P. infestans proteins matching bZIP transcription factors (TFs) were thus identified (see Table S2 in the supplemental material). By comparison, the number of bZIPs were predicted at 21 for S. cerevisiae, 21 for Drosophila, and 81 for Arabidopsis (39). We also used ortholog and cDNA sequence data to check the accuracy of the P. infestans gene models, which resulted in the revision of 17 of the 38 models (see Table S2).

Selected features of the P. infestans bZIPs are shown in Fig. 1. Of the 38 proteins, 19 closely match the canonical bZIP definition, including having an Asn at the location corresponding to residue 235 of S. cerevisiae GCN4. This site was described previously as “invariant” due to its conservation in bZIPs from mammals, plants, and fungi and was shown by crystallographic studies to interact with DNA (5, 40, 41). The 19 remaining proteins had

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substitutions at position 235, with 15 containing Cys, three containing Val, and one containing Tyr. The bZIP previously identified from *P. infestans* (*PiBZP1*), fell into the Cys-containing class, although curiously, this locus had not been annotated as a gene in the reference genome. Representative bZIP domains from *P. infestans*, yeast, mammals, and plants are shown in Fig. 1A.

Both the canonical bZIPs and those with the Cys, Val, and Tyr substitutions otherwise had good matches with bZIP definitions in the domain databases. For example, all had several positively charged amino acids within the DNA-binding domain, including Arg at the position corresponding to residue 243 of GCN4, which is highly conserved in other eukaryotes. The leucine zippers in...
cluded three to five heptad repeats. Such features are illustrated in Fig. 1A and in Table S2 and Fig. S1 in the supplemental material, which compares sequence logo plots of the bZIP domains of *P. infestans* with those modeled in the SMART database. Like most bZIPs from other eukaryotic taxa, no other functional domains were detected within the proteins, which ranged in size from 167 to 724 amino acids. In a few *P. infestans* proteins, some leucines within the zipper were replaced with other bulky hydrophobic amino acids such as Ile, Val, Phe, or Met. Such residues can also stabilize the dimeric coiled-coil structure of the zipper, albeit not to the same extent as leucine (42). Such substitutions may impair dimerization, although GCN4 of yeast proved to be relatively tolerant of such changes (43).

In phylogenetic analyses based on the bZIP domain, the canonical (Asn-235) and novel bZIPs formed separate clades (Fig. 1B). Many of the genes are very similar to each other in sequence and cluster within the genome. The most striking cluster includes the Cys-containing proteins encoded by PITG_09198, PITG_09199, PITG_09200, and PITG_09201, which are near each other on subcontig 14 of the assembly, and nearby gene PITG_09190. These five show a minimum of 93% and 96% identity at the amino acid and nucleotide levels, respectively.

Several other predicted proteins from *P. infestans* showed significant matches in BLASTP against members of our final list of 38 bZIPs yet lacked convincing bZIP domains. Their genes were often physically close to genes encoding proteins judged to be authentic bZIPs. One example is PITG_10799, which is adjacent to bZIP PITG_10798; their proteins share 51% identity. A second example is PITG_13584, which resides near bZIP PITG_13587; their proteins have 46% identity. Such bZIP-like proteins are proposed to have arisen through gene duplication from an authentic transcription factor but to have accumulated mutations in their functional domains. PITG_10799 nevertheless has homologs in *P. ramorum* and *Phytophthora sojae*, suggesting that it has a function.

The novel bZIPs are oomycete specific. An analysis of other eukaryotic taxa indicated that bZIPs with atypical amino acids at the site matching position 235 of GCN4 are an oomycete-specific innovation. In *P. ramorum*, 21 canonical bZIPs and 28 with Cys, Val, or Tyr substitutions were identified (Fig. 2; also, see Table S3 in the supplemental material). Similar atypical forms were also observed but at lower frequencies in two more divergent oomycetes, *Pythium ultimum* (10 of 40 predicted bZIPs) and *H. arabidopsidis* (9 of 27). We also examined the diatom *T. pseudonana*, which like oomycetes is a stramenopile. None of its predicted 21 bZIP proteins contained amino acids other than Asn at the site.

Another novel feature of bZIPs in *P. infestans* is that one protein, encoded by PITG_00964, contains two bZIP domains. Although not shown in Fig. 1B, these domains clustered together in phylogenetic analyses. We also identified similar proteins with two bZIP domains in *P. ramorum*, *Pythium ultimum*, and *H. arabidopsidis*. This has appeared to originate prior to the divergence of stramenopiles, as Rayko et al. (44) also found proteins with two bZIP domains in *T. pseudonana*, although these do not appear to be orthologs of PITG_00964.

Phylogenetic analysis of bZIP domains from three stramenopiles (*P. infestans*, *Pythium ultimum*, and *T. pseudonana*) suggested that most of the Asn types have orthologs in each species, although some oomycete-specific groups were detected (see Fig. S2 in the supplemental material). Orthologous pairs of Val- and Tyr-type bZIPs were observed in *P. infestans* and *Pythium ultimum* but not *T. pseudonana*. Application of phylogenetics to other eukaryotes provided little evidence for the clustering of any particular *P. infestans* bZIP with any animal, fungal, or plant bZIP, regardless of whether the bZIP domain or full protein was employed in the analysis (data not shown). This result is similar to that of prior studies, which found little affinity in bZIPs across kingdoms and proposed that only a single bZIP existed prior to the animal/fungus/plant separation (45).

A novel bZIP is nucleus localized. To help assess if the novel bZIPs are authentic transcription factors, we demonstrated that one of the Cys forms (PITG_11668) resides in nuclei. This was accomplished by examining three independent transformants of *P. infestans* in which a C-terminal fusion of PITG_11668 with green fluorescent protein (GFP) was expressed from the constitutive *ham34* promoter. As shown in Fig. 3, the protein localized to nuclei in hyphae, sporangia, cysts, and germinated cysts. No shifts between nucleus and cytoplasm were observed during development, unlike with some bZIPs, which are regulated by subcellular localization (46, 47). Similar results were observed in each transformant, which showed no significant differences from the wild-type in growth, development, or virulence on tomato.

Monopartite importin-dependent nuclear localization signals were predicted in 29 of the 38 *P. infestans* bZIPs, including PITG_11668. Seventeen of these signals reside near or within the predicted DNA-binding domain. Sequences that determine nuclear localization are not well defined in oomycetes, but some plant and animal bZIPs have also been reported to bear a localization motif within the DNA-binding domain (48, 49).
Many bZIPs are expressed preferentially in spore stages. mRNA levels were determined for 34 of the 38 P. infestans bZIPs in five asexual life stages (Fig. 4; also, see Table S2 in the supplemental material). These were nonsporulating hyphae, sporangia, sporangia chilled to induce zoosporogenesis, zoospores released from sporangia (including zoospore cysts), and germinated cysts (including appressoria). The data presented in Fig. 4 combine results from RT-qPCR (19 genes, from this study) and microarray analysis (22 genes, mined from the data described by Judelson et al. [15]). Seven genes were measured by both methods, which yielded similar results (correlation coefficient $R = 0.85$). Expression of the remaining four genes was not detected.

Many of the genes were upregulated at one or more stages of development. The most common pattern was induction in zoospores or germinated cysts. For example, PITG_09198 and PITG_09199 displayed low levels of mRNA in hyphae, sporangia, and chilled sporangia but about 10-fold-larger amounts in zoospores and germinated cysts. Others, such as PITG_07437 and PITG_11671, were not induced until the germinated cyst stage. A few genes, such as PITG_09280 and PITG_16038, were induced in sporangia compared to hyphae, with mRNA levels falling to basal levels in zoospores and germinated cysts. About 10 genes, such as PITG_02733, showed only modest changes throughout development.

Both canonical bZIPs and those with the novel substitutions included members with stage-induced and more constitutive patterns of transcription. A greater proportion of atypical than canonical bZIPs were expressed preferentially in the zoospore or germinated cyst stages (50% versus 23%, respectively, counting genes expressed $>2$-fold more highly than average in those stages). Some of this could be attributed to the tandemly repeated genes in the PITG_09198 cluster, which presumably had inherited both coding and promoter sequences from their ancestor.

Silencing of eight novel bZIPs indicates that they are not essential for development. To probe the cellular roles of the Cys-containing bZIPs, gene silencing was applied to several that are transcribed differentially during asexual development. This entailed expressing hairpin constructs in stable transformants, using about 500 nt of coding sequences from the target gene. Two prior studies in P. infestans have shown that single constructs can silence closely related genes (50, 51). We were successful at obtaining silenced strains with each construct.
struct, with an average of about 25% of transformants being either fully or moderately silenced. Transformants were screened first by semiquantitative RT-PCR using tissue extracted from the stage at which the targeted gene was expressed at the highest level. Promising lines were retested by RT-qPCR, using three biological replicates (Fig. 5). With PITG_11671, each silenced strain showed >95% suppression of expression compared to the progenitor strain 1306 and a transformant obtained using an empty vector. Most silenced transformants recovered for PITG_11664 and PITG_11668 showed >99% suppression. Silenced transformants obtained using the PITG_09198 construct showed full or partial silencing of all genes in the group, based on RT-qPCR performed using a primer pair specific for PITG_09190, a pair specific for PITG_09198, and a pair designed to amplify PITG_09199, PITG_09200, and PITG_09201.

It should be noted that while PITG_11664, PITG_11668, and PITG_11671 are close to each other in the genome, their proteins are highly divergent, with only about 47% amino acid identity. This is unlike members of the PITG_09198 cluster, which are at least 93% identical. Semiquantitative RT-PCR confirmed that the inverted repeat constructs used for PITG_11664, PITG_11668, and PITG_11671 did not cause cross-silencing (see Fig. S3 in the supplemental material).

Assessments of growth rate, sporangium production, zoospore release, and the fraction of zoospore cysts forming germ tubes and appressoria were performed to test the role of the eight genes in asexual development (Table 1). Changes in phenotype were not observed. The only significant difference ($P < 0.05$) between the wild type and the silenced lines involved PITG_11668, where zoospore release was impaired by 40%. Although not examined quantitatively, zoospore mobility appeared normal.

The novel bZIPs are important for defense against oxidative stress. In other organisms, bZIP TFs are known to participate in the perception and/or response to various stresses, including oxidative and osmotic insults (19–21). We also hypothesized that as in several other classes of proteins, the Cys residues that substituted for Asn-235 in the $P. infestans$ bZIPs might be redox sensors (52–54). Therefore, we tested the effect of silencing the novel bZIPs on the ability of $P. infestans$ to survive oxidative stress. To establish conditions for the experiment, we first assessed the effect of several concentrations of H$_2$O$_2$ and paraquat against $P. infestans$. Little effect was noted for paraquat, but reproducible sensitivity to H$_2$O$_2$ was observed.

Assessments of the effect of silencing revealed that PITG_11664, PITG_11668, and the PITG_09198 cluster contributed to the ability of $P. infestans$ to resist H$_2$O$_2$ treatment. This is shown in Fig. 6, which illustrates the growth of the 1306 progenitor strain, empty vector controls, silenced transformants, and nonsilenced transformants resulting from the hairpin constructs on rye-sucrose medium amended with two concentrations of H$_2$O$_2$. In each case, the silenced strains exhibited much less growth in the presence of H$_2$O$_2$ than the controls. It may be noted that this assay was performed on hyphae, even though the genes are upregulated in spore stages. However, each of the genes is also expressed in hyphae. In contrast to the above genes, strains silenced for PITG_11671 did not show impaired growth in the presence of H$_2$O$_2$, even at concentrations up to 2.5 mM.

We also evaluated the effect of silencing PITG_11664,

![FIG 5 Silencing of bZIP genes using inverted repeats targeting PITG_11671, PITG_11664, PITG_11668, or the PITG_09198 cluster. Shown are RT-qPCR data using RNA from silenced transformants (prefix “T”), wild-type progenitor strain 1306, and an empty vector transformant (EV). Data were obtained using primers specific to the genes named in each panel, from three biological replicates with two technical replicates each, and normalized to the gene for ribosomal protein S3a. mRNA levels are expressed relative to that in strain 1306.](ec.asm.org)
TABLE 1 Effect of silencing on asexual development

| Gene                  | Strain | Growth rate (mm/day) | No. of sporangia/plate (10^5) | No. of cysts/plate (10^5) | Cyst germination and appressorium formation (%) |
|-----------------------|--------|----------------------|-------------------------------|---------------------------|-----------------------------------------------|
| PITG_09198 cluster    | 1306   | 12 ± 1               | 18 ± 2                        | 81 ± 6                    | 78 ± 1                                        |
|                       | EV     | 12 ± 1               | 24 ± 2                        | 42 ± 7                    | 77 ± 3                                        |
|                       | Silenced | 13 ± 0              | 22 ± 3                        | 48 ± 8                    | 77 ± 6                                        |
| PITG_11664            | 1306   | 13 ± 0               | 9 ± 2                         | 54 ± 19                   | 69 ± 2                                        |
|                       | EV     | 12 ± 0               | 14 ± 3                        | 30 ± 6                    | 82 ± 3                                        |
|                       | Silenced | 13 ± 1              | 15 ± 2                        | 47 ± 9                    | 77 ± 7                                        |
| PITG_11668            | 1306   | 13 ± 1               | 42 ± 5                        | 81 ± 6                    | 88 ± 5                                        |
|                       | EV     | 12 ± 0               | 36 ± 3                        | 75 ± 14                   | 89 ± 3                                        |
|                       | Silenced | 12 ± 2              | 34 ± 2                        | 48 ± 7^a                  | 89 ± 2                                        |
| PITG_11671            | 1306   | 12 ± 1               | 30 ± 4                        | 97 ± 6                    | 90 ± 0                                        |
|                       | EV     | 11 ± 1               | 24 ± 2                        | 120 ± 84                  | 81 ± 3                                        |
|                       | Silenced | 12 ± 1              | 19 ± 14                       | 62 ± 51                   | 84 ± 2                                        |

^a Data for the wild type (strain 1306) are from three independent cultures. Also tested were three independent empty vector transformants (EV) and the silenced transformants shown in Fig. 5. Values are means ± standard deviations. Separate experiments involving strain 1306 and the EV strains were performed along with each group of silenced transformants. Cultures grown on 100-mm rye sucrose plates were scored for radial growth, sporulation, the ability to form zoospores and cysts, and appressorium formation, as described in Materials and Methods. Values are from the linear stage of colony expansion, typically between 3 and 6 days after inoculation.

^b Significantly different from the wild-type value (P < 0.05).

PITG_11668, PITG_11671, and the PITG_09198 cluster on growth during osmotic stress. This involved propagating the silenced transformants and controls on rye-sucrose medium amended with either 0.3 M NaCl or 0.8 M sorbitol, both of which slow the growth of the wild type by about 50%. The silenced transformants grew normally under such conditions (see Fig. S4 in the supplemental material).

The eight silenced novel bZIPs are not required for pathogenic development. We also measured the effect of silencing on plant infection by inoculating tomato leaves with zoospore suspensions. For all silenced lines (PITG_11664, PITG_11668, PITG_11671, and the five genes in the PITG_09198 cluster), no differences were observed between controls and the silenced strains in the rate of lesion expansion, latent period, or extent of host necrosis (see Fig. S5 in the supplemental material).

To help interpret this result, we measured the expression of these bZIPs during plant infection (Fig. 7). All were expressed at one or more stages. PITG_11664 and PITG_11668 showed relatively low levels of expression during the first 3 days of infection, which corresponds to the biotrophic phase of the interaction, but high levels starting on day 4, which is when host necrosis begins. In contrast, the other genes exhibited either relatively constitutive patterns or somewhat lower levels of expression between 1 and 3 days postinoculation (dpi) compared to early (0.5 dpi) or late (4 to 5 dpi) stages. The absence of an effect of silencing on pathogenicity therefore cannot be attributed to the lack of expression of the native genes during plant colonization.

A Cys-containing canonical bZIP also contributes to peroxide resistance. As stated above, we hypothesize that the novel Asn-235-to-Cys substitution in the DNA-binding domains of many P. infestans bZIPs is involved in redox sensing. This was proposed previously for Cys in a different location of human bZIP clun, at the site aligning with amino acid 242 of GCN4 (54). Examination of the DNA-binding domains of P. infestans bZIPs showed

![FIG 6 Effect of silencing bZIP genes on the response of P. infestans to oxidative stress.](image-url)

Growth was measured by placing mycelial plugs from cultures onto rye-sucrose medium with or without the indicated concentrations of H₂O₂; pictures were taken 5 days postinoculation. Strains were the parental isolate 1306, an empty vector transformant (EV), silenced transformants (prefix “T” with “s” in parentheses), or nonsilenced strains from the same transformation (prefix “T” with “ns” in parentheses). Strains silenced for PITG_11664, PITG_11668, and the PITG_09198 cluster show inhibition of growth in the presence of H₂O₂.
that PITG_16038, PITG_16183, and PITG_18417 contained Cys at that position; all belong to the canonical Asn-235 class.

To test if this type of protein contributes to the oxidative stress response of *P. infestans*, we silenced PITG_16038 using an inverted repeat construct. Two of 56 transformants tested showed strong silencing (Fig. 8A). Both exhibited impaired growth in the presence of H$_2$O$_2$, compared to controls on rye-sucrose medium (Fig. 8B). Growth *in planta* appeared normal (Fig. 8C), and no significant alterations were observed in growth, sporulation, zoospore release, encystment, or cyst germination (data not shown). PITG_16038 is expressed in all tissues, with slightly higher mRNA levels in spore stages or late stages of plant infection (Fig. 4 and 7).

**DISCUSSION**

Remarkably, half of bZIPs from *P. infestans* contain a striking difference compared to bZIPs examined from eukaryotes to date, namely, the replacement of the “invariant” Asn with Cys, Val, or Tyr. This novelty appears to be specific to oomycetes, as other eukaryotes, including another stramenopile, exclusively utilize Asn. The diversification of bZIPs in oomycetes compared to those in other fungi and plants is consistent with their separation from other eukaryotes, and supports that bZIPs evolved to function as oxidative stress sensors in diverse taxa. Indeed, many bZIPs from fungi play similar roles (19, 21, 56). While many of the *P. infestans* bZIPs mediate this stress response, including Yap1, and orthologs in other fungi play similar roles (19, 21, 56). While many of the *P. infestans* bZIPs are needed for protection against H$_2$O$_2$, they did not appear to be induced by that compound (data not shown). This suggests that the genes may be regulated by posttranslational processes, like many bZIPs from other taxa. For example, Yap1 is controlled by a mechanism that redistributes the protein from cytoplasm to nucleus upon exposure to reactive oxygen (57). As PITG_11668 resided exclusively in nuclei in the absence of oxidative stress, its control may involve a strategy that more resembles that of Atf1 of *Aspergillus nidulans*, which is always nuclear and activated by a mitogen-activated kinase that moves into nuclei upon H$_2$O$_2$ treatment (58). PITG_11668 contains 19 predicted serine/threonine phosphorylation sites, although this is not significantly higher than the number in the average *P. infestans* protein. It may not be a coincidence that the most frequent amino acid substitution in the atypical *P. infestans* bZIPs was Cys. This acts as an oxidative stress sensor in some proteins, where the breakage of disulfide bonds or oxidation of Cys to its sulfenic acid alters the conformation of the protein (52, 53). Indeed, such events are part of how Yap1 and Swi6 of yeast, and cJun of humans, are controlled. In the case of Yap1, a nuclear export signal is masked by a disulfide bond, but its reduction exposes the signal which redistributes the transcription factor to the cytoplasm (46). Oxidation of a Cys in Swi6 is also used to control the stress response (58).

Regulation of cJun by stress has been suggested to involve glutathiolation of a Cys in its DNA-binding domain (position 242 in Swi6) (59). Thus, it is proposed that the novel Asn→Cys substitution present in nearly half of *P. infestans* bZIPs evolved to function in redox sensing, like some Cys residues in other transcription factors. Silencing a *P. infestans* bZIP containing the canonical Asn residue would help solidify this hypothesis, although Asn-containing bZIPs might also participate in the stress response. It is conceivable that several subclasses of bZIPs, regulated through multiple mechanisms, contribute to the protection of *P. infestans* from oxidative and other stresses. Indeed, several *P. infestans* bZIPs with the canonical Asn, such as PITG_16038, contain the Cys residue used by cJun to sense oxidative stress (59).

One of our initial hypotheses was that many bZIPs would serve as developmental regulators, since many are expressed differentially during the life cycle. This does not appear to be the case for most of the *Cys*-class bZIPs silenced in this study, although one seemed to partially influence zoospore release. The limited number of developmental abnormalities in the silenced strains could...

**FIG 7** Expression of selected bZIP genes *in planta* based on RT-qPCR. Tomato leaflets were spray-inoculated with zoospores from wild-type *P. infestans* and sampled at 0.5, 1, 2, 3, 4, and 5 days postinfection. Normalization was performed using the gene for ribosomal protein S3a (PITG_11766) and then transformed to per-gene normalized values. Expression levels at 5 dpi may be overstated 2-fold based on results for PITG_11766 reported by Haas et al. (26).

**FIG 8** Silencing of PITG_16038. (A) RT-qPCR of mRNA from strain 1306, an empty vector transformant (EV), and two silenced transformants (prefix “Te”). (B) Sensitivity of strains to hydrogen peroxide. (C) Pathogenicity of strains.
be due to redundancy between transcription factors. Alternately, the stage-specific upregulation of many bZIPS may have evolved to accommodate stage-specific stress. For example, sporangia, zoospores, and germinated cysts are prone to stresses that likely involve the release of reactive oxygen, such as exposure to UV light and desiccation (60, 61).

Another feature of bZIP evolution in the family’s expansion through gene duplications, which is a common theme in oomycete genomes (62–64). In P. infestans, genes encoding 6 Asn-class and 12 atypical bZIPS cluster with at least one other bZIP. If one subtracts such duplications from the total, the number of bZIPS, 22, is nearly identical to that predicted for Caeno- rhabditis elegans, S. cerevisiae, and Drosophila melanogaster (39). The largest cluster of Cys-containing bZIPS in P. infestans is located on supercontig 14. Two canonical bZIPS, PITG_09279 and PITG_09278, are also encoded by genes on that supercontig.

In fungi, bZIP knockouts have been used to help unravel the network involved in oxidative stress. Genes regulated by fungal bZIPS that help detoxify the cell include catalases, peroxidases, oxidoreductases, and glutathione synthases (20, 21, 65, 66). Genome-wide expression analysis of the P. infestans strains with silenced bZIPS will help understand the mechanisms that govern oxidative stress response in P. infestans.

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