pH Response Transcription Factor PacC Controls Salt Stress Tolerance and Expression of the P-Type Na\(^+\)-ATPase Ena1 in *Fusarium oxysporum*

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Fungi possess efficient mechanisms of pH and ion homeostasis, allowing them to grow over a wide range of environmental conditions. In this study, we addressed the role of the pH response transcription factor PacC in salt tolerance of the vascular wilt pathogen *Fusarium oxysporum*. Loss-of-function pacC\(^{+-}\) mutants showed increased sensitivity to Li\(^+\) and Na\(^+\) and accumulated higher levels of these cations than the wild type. In contrast, strains expressing a dominant activating pacC allele were more salt tolerant and had lower intracellular Li\(^+\) and Na\(^+\) concentrations. Although the kinetics of Li\(^+\) influx were not altered by mutations in pacC, we found that Li\(^+\) efflux at an alkaline, but not at an acidic, ambient pH was significantly reduced in pacC\(^{+-}\) loss-of-function mutants. To explore the presence of a PacC-dependent efflux mechanism in *F. oxysporum*, we cloned ena1 encoding an orthologue of the yeast P-type Na\(^+\)-ATPase ENA1. Northern analysis revealed that efficient transcriptional activation of ena1 in *F. oxysporum* required the presence of high Na\(^+\) concentrations and alkaline ambient pH and was dependent on PacC function. We propose a model in which PacC controls ion homeostasis in *F. oxysporum* at a high pH by activating expression of ena1 coordinately with a second Na\(^+\)-responsive signaling pathway.

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Fungi are a versatile class of organisms that have successfully occupied numerous ecological niches, including those of plant and animal pathogenesis. A striking property of fungi and a major determinant of their evolutionary success is their capacity to adapt to an extremely wide range of environmental conditions. This ability depends crucially on the presence of cellular sensory networks that monitor the environment and mediate changes in gene expression in response to shifts in the external conditions. We use the vascular wilt pathogen *Fusarium oxysporum* as a model to understand how environmental signals regulate gene expression in fungi and how these regulatory mechanisms determine fungal virulence.

A key factor in fungal growth and development is ambient pH. Fungi grow over a wide range of pH conditions and must thus be able to tailor gene expression to the particular pH of their growth environment. A conserved signaling cascade integrated by the products of the *pal* genes, whose terminal component is the zinc-finger transcription factor PacC/Rim101p, regulates gene expression in response to ambient pH (18). Upon shift to alkaline pH, an inactive PacC precursor is posttranscriptionally activated by proteolytic processing into a shorter functional form that activates genes preferentially extracellularly (18). The pacC orthologue of *F. oxysporum* was recently cloned, and the encoded protein was shown to regulate pH-dependent gene expression and to function as a negative regulator of virulence on plants (11). Thus, pacC\(^{+-}\) loss-of-function mutants of *F. oxysporum* mimic growth at acidic ambient pH and exhibit increased virulence, whereas pacC\(^+\) strains expressing a dominant activating pacC allele mimic growth at alkaline pH and show significantly reduced virulence. At present, the downstream effector genes regulated by PacC in *F. oxysporum* remain largely unknown.

Yet another hallmark of fungal versatility is the capacity to grow over a wide range of salt concentrations. Generally, salt tolerance in living cells is conditioned by the capacity to maintain intracellular ion homeostasis. Fungi have developed exclusion systems to keep levels of intracellular sodium below concentrations toxic to the cell (9). In the best-studied system, that of *Saccharomyces cerevisiae*, the main Na\(^+\) efflux system is encoded by the *ENA* genes, a tandem array of four to five genes encoding nearly identical proteins. *ENA1*, the most important and the best-studied component of this system, is essential for ion homeostasis and salt tolerance in yeast (15, 23). The ENA1 protein works as a P-type Na\(^+\)-ATPase but can also mediate Li\(^+\) or K\(^+\) efflux (8, 14, 15, 23). Expression of *ENA1* in *S. cerevisiae* is tightly regulated by Na\(^+\) but also depends on alkaline ambient pH (for a review, see reference 20). Recent studies suggest that full expression of *ENA1* at alkaline pH requires *RIM101*, the *S. cerevisiae* orthologue of PacC, providing further evidence for a functional link between pH signaling and *ENA1* (17, 25).

In the present study we have addressed the role of pacC in salt tolerance of *F. oxysporum*. We provide evidence for the presence of a sodium efflux system based on an orthologue of the *S. cerevisiae* ENA1 gene. We further show that full transcriptional activation of the ena1 gene requires PacC and that both PacC and Ena1 play a fundamental role in the salt stress response of *F. oxysporum*.
MATERIALS AND METHODS

Fungal isolates and culture conditions. *F. oxysporum f.sp. lycopersici* strain 4287 (race 2) was obtained from J. Tello, Universidad de Almería, Almería, Spain, and stored at −80°C with glycerol as a microconidial suspension (13). Construction of the *pacC<sup>−−</sup>* loss-of-function mutant and the merodiploid strain carrying a dominant activating *pacC<sup>+</sup>* allele was described previously (11). For microconidia production, cultures were grown in potato dextrose broth (Difco, Detroit, Mich.) at 28°C as described previously (13).

For phenotypic analysis of colony growth, a 5-μl drop containing 2.5 × 10<sup>5</sup> freshly obtained microconidia was transferred on 1.5% (wt/vol) agar plates of synthetic medium (SM) (13) containing 1% (wt/vol) glucose, 0.1% NH₄NO₃, and different concentrations of NaCl or LiCl. Media were buffered with 100 mM Na₂HPO₄ at pH 4; 50 mM Na₂HPO₄–50 mM NaH₂PO₄, and 50 mM NaCl at pH 6; and 100 mM NaH₂PO₄ and 100 mM NaCl at pH 8.

Determination of cation accumulation, influx, and efflux. For determination of intracellular cation accumulation, microconidia were germinated in SM containing 1% (wt/vol) glucose and 0.1% NH₄NO₃, supplemented with 0.05, 0.1, or 0.15 mM LiCl or 0.5, 1, or 1.5 M NaCl. After 12 h, samples were filtered and processed for determination of intracellular ion content as previously reported (10, 21). Briefly, samples of cells were filtered, washed with 0.02 M MgCl₂, and treated with 0.2 M HCl, and the cations were analyzed by atomic absorption spectrophotometry.

For determination of lithium and rubidium influx (rubidium was used as a transport analog of potassium), microconidia were germinated in SM containing 1% (wt/vol) glucose and 0.1% NH₄NO₃ for 12 h. At time zero, 0.1 M LiCl or RbCl was added to the growth medium, and samples were taken at regular time intervals, filtered immediately, and processed for determination of intracellular ion content (21).

For determination of the lithium efflux rate, microconidia germinated in SM were supplemented with 0.1 M LiCl (wild-type and *pacC<sup>−−</sup>* strains) or 0.3 M LiCl (*pacC<sup>+</sup>*) strain. After 12 h, microconidia were filtered, washed with sterile 0.02 M MgCl₂, and resuspended in fresh SM buffered at pH 4.0 or 8.0 as described above. This medium was free of lithium and was supplemented with 0.05 M RbCl to trigger the efflux process. Samples were taken at regular time intervals, filtered, and processed for the determination of intracellular ion content (10, 21).

All experiments for determination of cation accumulation, influx, or efflux

![FIG. 1. *F. oxysporum* *pacC* mutants are affected in salt tolerance. (A) Wild-type strain 4287, loss-of-function mutant *pacC<sup>−−</sup>* and dominant activating mutant *pacC<sup>+</sup>* were grown for 3 days on plates with SM buffered at pH 6.0 and supplemented with the indicated compounds.](image1)

![FIG. 2. Mutations in *pacC* affect ion homeostasis. Intracellular accumulation of cations was determined in germlings of the *F. oxysporum* wild-type strain (■), loss-of-function mutant *pacC<sup>−−</sup>* (■), or dominant activating mutant *pacC<sup>+</sup>* (I) grown for 12 h in the presence of the indicated concentrations of Li⁺ (A and C) or Na⁺ (B).](image2)
were performed at least three times with similar results (the maximum standard deviations were <10%).

Nucleic acid manipulations, cloning, and analysis of the ena1 gene. For Northern analysis, microconidia were germinated for 12 h in SM without added Na+ and then transferred for different periods of time to SM buffered at the indicated pH values, with or without 0.5 M Na+. Total RNA was extracted from mycelium as reported elsewhere (12), and Northern analysis and probe labeling was performed as described previously (13) by using the nonisotopic digoxigenin labeling kit (Roche Diagnostic S.L, Barcelona, Spain). Southern and Northern analyses were carried out as described above.

Genomic DNA of F. oxysporum isolate 4287 was extracted as previously reported (19) and used for PCR amplification on a Perkin-Elmer GeneAmp System 2400 with the primers ena3 (5'-TGACAAGCGACGATCTTTCTTCG-3') and ena4 (5'-GGTGATGCCCTTGTGCTTGAAGAC-3') derived from an F. oxysporum expressed sequence tag clone. The following PCR conditions were used: 35 cycles with denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. An initial denaturation step of 5 min at 94°C and a final elongation step at 72°C for 7 min were also performed. The amplified 250-bp DNA fragment was cloned into pGEM-T (Promega, Madison, Wis.), sequenced, and used to screen a lambda EMBL3 genomic library of F. oxysporum f.sp. lycopersici isolate 4287. Library screening, subcloning, and other routine procedures were performed as described in standard protocols (24). Sequencing of both DNA strands was performed at the Servicio Centralizado de Apoyo a la Investigación, University of Córdoba, by using the DyeDeoxy terminator cycle sequencing kit (PE Biosystems, Foster City, Calif.) on an ABI Prism 377 genetic analyzer apparatus (Applied Biosystems, Foster City, Calif.). DNA and protein sequence databases were searched by using the basic local alignment search tool (BLAST) algorithm (2) at the National Center for Biotechnology Information (Bethesda, Md.).

RESULTS

PacC controls salt tolerance and intracellular cation levels. To test the hypothesis that pacC may be involved in the salt stress response of F. oxysporum, 2 × 10^5 freshly obtained microconidia of the wild-type strain, a pacC+/− loss-of-function or a pacC−/− dominant activating mutant (11) were inoculated on plates containing SM (pH 6.0) supplemented with different concentrations of salts or sorbitol. The results depicted in Fig. 1 show that the pacC−/− loss-of-function mutant displayed a clear Li+ sensitivity phenotype and a somewhat more subtle Na+ sensitivity phenotype, whereas the dominant activating pacC+ allele conferred increased tolerance to these toxic cat-
ions. Importantly, pacC mutations did not affect growth at high concentrations of the nontoxic cation K\(^+\) or sorbitol, suggesting that PacC is specifically involved in the regulation of salt stress but not osmotic stress. Reintroduction of a functional pacC copy into the pacC\(^{-}\) mutant restored the wild-type growth phenotype, suggesting that the increased Li\(^+\) and Na\(^+\) sensitivity of the mutant was caused solely by the absence of a functional pacC allele (data not shown).

To determine whether the differences in sensitivity to Na\(^+\) or Li\(^+\) observed in the three strains were due to differential accumulation of these cations, we measured the intracellular levels of Li\(^+\) and Na\(^+\) in the different strains grown in liquid media.

FIG. 5. Amino acid sequence alignment of the predicted *F. oxysporum* ena1 gene product with fungal P-type Na\(^+\)-ATPases. Deduced Ena1 proteins from *F. oxysporum* (EMBL accession no. AY345588), *N. crassa* (AJ243520), and *S. cerevisiae* (U24069) are shown. Identical amino acids are highlighted on a shaded background. Dashes indicate gaps in the alignments. Nine predicted transmembrane domains are indicated by solid bars.
medium (pH 6.0) containing increasing levels of LiCl or NaCl. The results (Fig. 2) confirmed that increased sensitivity to Li\(^+\) and Na\(^+\) of the pacC\(^{+/-}\) mutant was related to higher internal levels of the two cations. Moreover, the increased tolerance observed in the pacC\(^+\) dominant activating mutant correlated with a lower intracellular accumulation of Li\(^+\) and Na\(^+\). Importantly, the three strains contained similar amounts of internal K\(^+\), ruling out the possibility that higher sensitivity or tolerance to Li\(^+\) and Na\(^+\) was caused by differences in the inhibition of K\(^+\) uptake by the toxic cations (Fig. 2C).

**pacC is required for efficient cation efflux at alkaline ambient pH.** Based on these initial results, we decided to explore the effect of PacC on cation fluxes. To determine Li\(^+\) influx in the wild-type strain and the pacC mutants, mycelia were transferred to 0.1 M LiCl, and the time course of Li\(^+\) uptake was monitored for several hours. During the first minutes, the three strains accumulated Li\(^+\) at a very similar rate, suggesting that the kinetics of Li\(^+\) influx was not altered in the pacC mutants (Fig. 3A). After a few minutes, the internal level of Li\(^+\) in the salt-tolerant pacC\(^+\) strain stabilized at ca. 10 nmol mg\(^{-1}\) and did not increase further, whereas levels continued rising in the wild-type strain and reached even higher levels in the salt-sensitive pacC\(^{+/-}\) mutant. Because it is generally assumed that most of the Na\(^+\) and Li\(^+\) influx in fungi under standard laboratory conditions takes place through the K\(^+\) influx system (22), we studied the time course of influx of the K\(^+\) transport analog Rb\(^+\). We found that even after 1 h in the presence of 0.1 M RbCl, the kinetics of uptake and the internal levels of Rb\(^+\) were highly similar in all of the strains (Fig. 3B). Taken together, these results support the view that mutations in pacC do not affect alkali cation influx, thus indicating that efflux of these toxic cations may be affected in the pacC mutants.

In fungi, two Na\(^+\) and Li\(^+\) efflux systems have been described, a sodium/proton antiporter working at acidic pH and a P-type sodium ATPase functioning at a higher pH (20). To test whether one or both of these efflux systems was affected in the pacC mutants, microconidia of the three strains were germinated for 12 h in liquid medium containing 0.1 M LiCl (wild type and the pacC\(^{+/-}\) strain) or 0.3 M LiCl (pacC\(^+\) strain), transferred to lithium-free medium buffered either at pH 4.0 or 8.0, and the time course of Li\(^+\) efflux was determined. The results shown in Fig. 4 clearly indicate that, although at pH 4.0 the three strains extruded lithium with the same efficiency (Fig. 4A), at pH 8.0 the pacC\(^{-/-}\) loss-of-function mutant displayed a strongly reduced Li\(^+\) efflux compared to the wild type and the pacC\(^+\) strain (Fig. 4B). These results are in agreement with the increased Li\(^+\) accumulation and sensitivity of the pacC\(^{+/-}\) loss-of-function mutant. Although we did not detect differences between the wild type and the pacC\(^+\) strain in terms of kinetics of Li\(^+\) efflux, the fact that absolute concentrations of internal Li\(^+\) in the pacC\(^+\) strain were significantly lower than those of the wild type (see Fig. 2A) suggests that Li\(^+\) efflux was more efficient in the pacC\(^+\) strain. In summary, our results indicate that PacC controls salt tolerance in *F. oxysporum* by regulating an efflux system functional at an alkaline pH but not at an acidic pH. In support of this view, differences in salt tolerance between the wild type and the pacC mutants were almost negligible when strains were grown on plates at pH 4.0 (results not shown).

**PacC controls transcriptional activation of ena1 encoding a P-type Na\(^+\)-ATPase of *F. oxysporum*.** The results shown in Fig. 4 suggest that, like *S. cerevisiae*, *F. oxysporum* has at least two different Na\(^+\) (Li\(^+\)) efflux systems: one functioning at pH 4.0 that is still present in the pacC\(^{+/-}\) loss-of-function mutant and the other one working at pH 8.0 that is not present in the mutant. Because the main alkaline Na\(^+\) and Li\(^+\) efflux system in yeast and filamentous fungi is based on P-type Na\(^+\)-ATPases encoded by the *ENA* genes (1, 3, 6, 7, 15), we decided to clone the *ena1* gene, which encodes a P-type Na\(^+\)-ATPase.
the ENA1 orthologue in *F. oxysporum*. PCR amplification with degenerate primers derived from highly conserved regions of the *Neurospora crassa ena1* gene produced a fragment of the expected size (250 bp) that was cloned and sequenced. After we confirmed its homology with *N. crassa ena1*, we used the fragment to probe a λEMBL3 genomic library of *F. oxysporum* isolate 4287. Sequencing of a hybridizing genomic clones revealed the presence of an open reading frame of 3,261 nucleotides encoding a predicted protein of 1,087 amino acids. Sequence alignment of *F. oxysporum ena1* with fungal *ena1* genes in the databases suggested the presence of three introns 215, 51, and 49 nucleotides in length, respectively. The 5′-flanking sequence of the *ena1* gene contains four copies of the PacC consensus binding sequence 5′-GCCARG-3′ (26) at positions −549, −474, −382, and −321. The sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AY345588. The deduced *F. oxysporum Ena1* protein has 70 and 46% amino acid identity with the *Ena1* proteins of *N. crassa* and *S. cerevisiae*, respectively, and contains nine putative transmembrane regions that are highly conserved between fungal P-type Na⁺-ATPases (Fig. 5). Southern analysis of genomic DNA digested with different restriction enzymes suggested that *ena1* is present as a single copy in the *F. oxysporum* genome. Moreover, a BLAST search of the *F. graminearum* complete genome sequence with *ena1* only produced one significant match (results not shown).

To study the mechanisms controlling expression of *ena1* in *F. oxysporum*, Northern hybridization analysis was performed with total RNA from mycelia of the wild-type strain transferred for 2 h to SM buffered at different pH values and containing either 0.5 M Na⁺ or no added Na⁺. No *ena1* transcript was detected in mycelia grown in the absence of Na⁺ (Fig. 6A). In contrast, *ena1* expression was strongly induced in the presence of Na⁺ at pH 8.0 but not at pH 4.0. Thus, a combination of Na⁺ and alkaline pH is required to trigger the expression of *ena1* gene.

We next studied the kinetics of *ena1* activation in the wild type and the *pacC* mutants upon transfer of mycelia to SM buffered at pH 8.0 containing 0.5 M Na⁺. In the wild-type strain, the *ena1* transcript was detectable after 10 min and increased further until 20 min after transfer (Fig. 6B). In the *pacC*⁻⁻ mutant, *ena1* transcript levels increased much earlier and reached higher levels than in the wild-type strain. In both strains, transcript levels remained high until 60 min after transfer and declined slightly after 90 min (data not shown). In contrast, in the *pacC*⁺⁺⁻⁻ mutant *ena1* expression was induced much later, only 60 to 90 min after the transfer, and remained at much lower levels. These results suggest that PacC positively controls transcriptional activation of *ena1* in response to Na⁺ at alkaline pH.

**DISCUSSION**

The transcription factor PacC regulates expression of alkaline- and acid-expressed genes in *F. oxysporum* (11). We show here that this factor plays an essential role in salt tolerance of *F. oxysporum* because strains that lack PacC are highly sensitive to Na⁺ and Li⁺. Our data strongly suggest that salt sensitivity in these mutants is caused by a defect in the Na⁺ and Li⁺ efflux process. Conversely, dominant activating *pacC*⁻⁻ mutants show increased salt tolerance, correlating with rapid and increased expression of a sodium efflux system in this strain. In a previous study, we proposed (10) the existence of an Na⁺/(Li⁺)-ATPase in *F. oxysporum* as the main system involved in the efflux of these cations (10). We identify here *ena1*, an *F. oxysporum* orthologue of the *ENA1* genes from yeast and *N. crassa* (3, 6, 7, 15, 23). We show that expression of *ena1* requires a combination of high Na⁺ concentrations and alkaline pH. Although the presence of Na⁺ alone is sufficient to induce expression of P-type Na⁺-ATPases in certain cases (1), our results are in
agreement with most previous studies demonstrating that full expression of \textit{ENa} genes requires a combination of Na$^+$ and high pH (1, 3).

The changes in salt tolerance and intracellular Na$^+$ and Li$^+$ levels observed in the \textit{pacC} mutants suggested a functional link between ambient pH, PacC, and \textit{ena1} expression in \textit{F. oxysporum}. Our data support a positive role of PacC in the regulation of \textit{ena1}, since \textit{pacC}$^{+/-}$ loss-of-function mutants show slightly delayed and reduced expression of \textit{ena1}, whereas \textit{pacC} strains expressing a dominant activating \textit{pacC} allele induce \textit{ena1} expression more rapidly and to higher levels than did the wild type. Our results are similar to those reported in \textit{S. cerevisiae}, where the \textit{pacC} orthologue \textit{RIM101} was shown to control the expression of \textit{ENA1} (17, 25). Further supporting this view, we found that the 5'-flanking sequence of the \textit{ena1} gene of \textit{F. oxysporum} contains four copies of the PacC consensus binding sequence 5'-GGCCARG-3'. In Fig. 7 we present a model summarizing the regulation of \textit{ena1} expression by PacC in \textit{F. oxysporum}. According to this model, PacC activates \textit{ena1} expression at alkaline ambient pH coordinately with a second factor. According to this model, PacC activates

expression of \textit{fl}, with the consequent effects on cation ef

after prolonged times of exposure, absolute concentrations of mutants could be explained by differences in

of \textit{ENa}, a plasma membrane Na$^+$/H$^+$ antiporter from fungi and plants (Z. Caracuel et al., unpublished data).

The results of the present study suggest a major role for PacC in salt tolerance of \textit{F. oxysporum}. Although most of the changes in Na$^+$ sensitivity and accumulation observed in the \textit{pacC} mutants could be explained by differences in \textit{ena1} expression with the consequent effects on cation efflux, such a function for \textit{ena1} in \textit{F. oxysporum} remains to be demonstrated. Moreover, a role of additional regulatory mechanisms controlled by PacC cannot be ruled out. Thus, the fact that even after prolonged times of exposure, absolute concentrations of internal Li$^+$ in the \textit{pacC}$^{+/-}$ and \textit{pacC} mutants stabilized at significantly higher and lower levels than in the wild-type strain, respectively, suggests that mutations in \textit{pacC} may not only affect cation efflux but also intracellular cation sensing and homeostasis, although the underlying mechanisms remain to be elucidated.

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