Laser microdissection of *Arabidopsis* cells at the powdery mildew infection site reveals site-specific processes and regulators

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Communicated by Frederick M. Ausubel, Harvard Medical School, Massachusetts General Hospital, Boston, MA, November 16, 2009 (received for review May 2, 2009)

To elucidate host processes and components required for the sustained growth and reproduction of the obligate biotrophic fungus *Golovinomyces orontii* on *Arabidopsis thaliana*, laser microdissection was used to isolate cells at the site of infection at 5 days postinfection for downstream global *Arabidopsis* expression profiling. Site-specific profiling increased sensitivity dramatically, allowing us to identify specific host processes, process components, and their putative regulators hidden in previous whole-leaf global expression analyses. For example, 67 transcription factors exhibited altered expression at the powdery mildew (PM) infection site, with subsets of these playing known or inferred roles in photosynthesis, cold/dehydration responses, defense, auxin signaling, and the cell cycle. Using integrated informatics analyses, we constructed putative regulatory networks for a subset of these processes and provided strong support for host cell cycle modulation at the PM infection site. Further experimentation revealed induced host endoreduplication occurred exclusively at the infection site and led us to identify MYB3R4 as a transcriptional regulator of this process. Induced endoreduplication was abrogated in *myb3r4* mutants, and *G. orontii* growth and reproduction were reduced. This suggests that, by increasing gene copy number, localized endoreduplication serves as a mechanism to meet the enhanced metabolic demands imposed by the fungus, which acquires all its nutrients from the plant host.

endoreduplication | cell cycle | obligate biotroph

Powdery mildews (PMs) are significant agronomic pathogens that infect cereals (e.g., barley); tomato; grapevine; and ornamentals, including roses (1). Although PM resistance has been long studied in cereal species, the genetic/genomic tractability and resources of *Arabidopsis* (2) allow for rapid systems-level data acquisition, hypothesis generation, and experimental validation. The reference (sequenced) strain of *Arabidopsis*, *Arabidopsis thaliana* Columbia-0 (*Col-0*), is susceptible to the adapted PM *Golovinomyces orontii* (3). *G. orontii* exclusively infects epidermal cells of *A. thaliana Col-0* with defined and microscopically visible stages of infection, including germination of the conidia, penetration of the epidermal cell, development of the haustorial complex (the feeding structure through which the fungus acquires its nutrients), and further growth and reproduction of the fungus on the leaf surface (3). The fungal (asexual) reproductive structures known as conidiophores contain the conidia (spores). In contrast, nonadapted PMs, such as the barley PM, *Blumeria graminis* f. sp. *hordei*, typically fail in the penetration phase of the interaction with the nonhost *Arabidopsis* (3). Instead of focusing on resistance mechanisms, we are interested in elucidating processes associated with the sustained growth and reproduction of an adapted PM. We would expect to uncover specific mechanisms associated with the sustained alteration of plant cellular processes and metabolism that allow for the continued accommodation of the haustorial complex and the associated fungal demand for nutrients and water, while limiting host defense responses, including cell death.

We previously performed whole-leaf expression profiling over the growth and reproduction phase of the *G. orontii*–*Arabidopsis* interaction (4). Here, we specifically profiled cells at the infection site to enhance our sensitivity at detecting responses localized to the infection site and to reduce complexity associated with differential infection site-specific and distal expression. We employed UV laser microdissection (LMD) to isolate epidermal and mesophyll cells of interest, because microcapillary methods do not readily allow one to examine mesophyll cells underlying the infected epidermal cell and protoplasting with cell sorting has an impact on cell wall integrity, which can affect defense responses. To use fragile mature *Arabidopsis* leaf tissue for LMD and downstream global RNA expression profiling, we developed/optimized methods of tissue preparation, RNA isolation, and amplification. This then allowed us to explore, at a global level, how the host transcriptional machinery is altered at the site of a compatible PM interaction and to identify known and previously unreported host responses associated with the sustained growth and reproduction of an adapted PM.

Results and Discussion

Optimized Experimental and Quality-Control Procedures Allowed for High-Quality and Highly Correlated Global Expression Profiling at the Site of Infection. To identify specific host genes associated with the sustained growth and reproduction of *G. orontii*, we isolated groups of epidermal and mesophyll cells (~20 cells) surrounding *G. orontii*-infected epidermal cells from fixed serial leaf sections of WT *Arabidopsis Col-0* using LMD at 5 days postinfection (dpi) (Fig. 1). Samples of equivalent cell types from uninfected WT leaf sections were also isolated. With our experimental conditions, the growth and reproduction phase of *G. orontii* infection was well established at 5 dpi, yet individual colonies were well separated spatially, enabling quantitation of fungal growth and reproductive structures. To minimize cell type-associated variation, chosen infection sites did not include stomatal guard cells or vascular bundles. The phytohormone salicylic acid (SA) plays a significant role in limiting this stage of the *G. orontii*

Author contributions: D.C. and M.C.W. designed research; D.C. and N.I. performed research; D.C., G.H., C.K.K., and M.C.W. analyzed data; and M.C.W. wrote the paper.

The authors declare no conflict of interest.

Freely available online through the PNAS open access option.

Data deposition: The ATH1 GeneChip expression data reported in this paper have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/geo (accession no. GSE15680).

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This article contains supporting information online at www.pnas.org/cgi/content/full/0912492107/DCSupplemental.

Fig. 3. PM infection site-specific processes and predicted response networks. Functional classification of WT infected vs. uninfected LMD (A) and the *ics1* mutant vs. WT-infected LMD (B) samples. MAPMAN categories or subcategories ($P \leq 0.05$ when category was not significant) with bin numbers in parentheses. ABC, ATP-binding cassette; CHO, carbohydrate. Predicted site-specific response networks associated with photosynthesis (C) and cold/dehydration response (D). TFs with established roles (filled boxes) are connected to other TFs and genes in functional categories by lines with arrows (positive regulation) or bars (negative regulation) at the end. TFs in open boxes were identified as highly coexpressed, as indicated by dashed lines. Enhanced (red) and decreased (green) expression.

19-fold at the site of PM infection. AtCML9 acts as a negative regulator of ABA-regulated dehydration responses, with the *cml9* mutant exhibiting hypersensitivity to ABA and enhanced dehydration tolerance (23). Thus, because *CML9* expression is SA-dependent, it may integrate SA- and ABA-associated responses.

SA-mediated changes in redox status regulate the conformation of NPR1, a master regulator of SA-mediated defense genes, through thioredoxins such as TRXh5, allowing the active NPR1 monomer to translocate to the nucleus and activate the expression of pathogenesis-related genes such as *PR1* (24). We observed an SA-dependent enriched induction of both *TRXh5* and *PR* genes at the infection site at 5 dpi, emphasizing the localized nature of these redox-mediated responses (Fig. 3A and B and SI Appendix). However, these SA-dependent responses are not sufficient to result in resistance. Although enhanced SA synthesis and signaling have been associated with resistance to compatible PMs for a number of mutants, elevated SA and resistance were also correlated with cell death in these cases (3).

Site-Specific Profiling Suggests Modulation of the Cell Cycle at the PM Infection Site. The promoters of genes with altered expression at the site of infection are enriched in *cis*-acting regulatory elements associated with S- and M-phases of the cell cycle (SI Appendix). The site II element, an S-phase-associated element bound by TCP domain TFs, was enriched in the promoters of genes with altered expression at the PM infection site. TCP TFs are known to regulate the expression of RPs required for protein synthesis and to couple cell growth and division (25). Although we do not detect TCP TFs with altered expression at the infection site, nonplastidic RP expression is dramatically enhanced. We also observed enrichment in the cell cycle-1b *cis*-acting regulatory element. These elements are also associated with the S-phase of the cell cycle and are found in the promoters of histones, a number of which exhibited enhanced expression at the site of infection. In addition, we identified six TFs associated with the cell cycle-related processes transcription, translation, and cell elongation (Fig. 2B). For example, the transcription initiation factor IIF β -subunit and transcription elongation factor SPT42 exhibited enhanced expression at the site of infection, consistent with enhanced mRNA synthesis. Finally, the mitosis-specific activator (MSA) element was enriched in promoters of

genes with altered expression at the PM infection site, as was expression of *MYB3R4*, a TF that binds the MSA element and activates G₂/M progression (26).

G. orontii-Induced Host Endoreduplication Is Observed at the PM Infection Site. Although modulation of the cell cycle had not been previously associated with the sustained growth of an adapted PM, our site-specific global expression profiling suggested this possibility. Therefore, we performed microscopic analyses to determine whether enhanced cell division or endoreduplication (progression through the growth and replication phases of the cell cycle without mitosis) occurred at the infection site. We did not observe microscopic evidence of enhanced cell division at the PM infection site or enhanced expression of cell division reporters (SI Appendix). However, we observed a clear visual difference in DAPI staining of DNA at the site of PM infection for both epidermal and underlying mesophyll cells (Fig. 4A). We further focused on quantifying these differences for the mesophyll cells directly underlying the *G. orontii*-infected (haustoria-containing) epidermal cell, because there is less natural variation in ploidy of mesophyll cells compared with leaf epidermal cells (27), and the observed differences were more dramatic for these underlying mesophyll cells. We found these underlying mesophyll cells were significantly larger, with increased ploidy and nuclear size compared with replicate uninfected samples of equivalent cell types and locale (Fig. 4A and B). In contrast, mesophyll cells distal to the infection site exhibited no increase in ploidy, nuclear size, or cell size compared with uninfected cells (SI Appendix). The mesophyll cells underlying the infection site exhibited increased DNA content up to 64C at 5 dpi with a median of 32C, whereas DNA content in mesophyll cells in parallel uninfected leaves or distal to the infection site had a median of 8C. In all cases, DNA content quantified by DAPI staining was proportional to nuclear size (SI Appendix).

The Arabidopsis TF MYB3R4 Regulates G. orontii-Induced Host Endoreduplication and the Extent of Sustained PM Growth and Reproduction. The enhanced expression of TFs involved in transcription, translation, and cell elongation at the site of infection is consistent with the observed endoreduplication. However, we did not observe statistically significant differences in expression

Fig. 4. PM-induced endoreduplication at the infection site is regulated by MYB3R4. Epidermal layer showing guard cell nuclei in uninfected (UI) leaves and germinated *G. orontii* conidium (c) in infected leaves at 5 dpi in WT *Col-0* (A) and *myb3r4* mutant (C) plants. Arrows point to a representative DAPI-stained nucleus in the guard cell or underlying mesophyll layer. (Scale bar, 10 μ m.) Nuclear DNA quantification by DAPI fluorescence of underlying mesophyll cells normalized to 2C guard cells for WT *Col-0* (B) and *myb3r4* mutant (D). Log₂ (RFU/RFU_{guard cell}) corresponds to C-values as follows: up to 0 is 2C, 0–1 is 4C; 1–2 is 8C; 2–3 is 16C; 3–4 is 32C, and 4–5 is 64C. RFU, relative fluorescence unit. Results are shown for *myb3r4-3*; similar results were obtained using *myb3r4-1*. (E) Reduced PM growth and reproduction on whole leaves was exhibited by *myb3r4* mutants at 10 dpi. The number of plants with disease ratings from 0 (no visible symptoms of infection) to 4 (100% coverage of infected leaves) is shown. (F) Reduced reproduction (cp/colony) was exhibited by *myb3r4* mutants at 5 dpi. c, initial germinated conidia; cp, conidiophore. (Scale bar, 100 μ m.) Independent experiments for A–F showed similar results. (G) Putative targets of MYB3R4 at the infection site were identified as mitosis-associated and/or NtMYBA2-activated genes with MSA core element(s) in 500-bp upstream promoter. Red (enhanced) and green (decreased) expression. Values in black fell just below stringent selection criteria. (H) Model for MYB3R4-mediated induced endoreduplication at the site of an adapted PM infection. Black indicates functionality, as does filled lines. P, phosphorylated.

for known TFs that mediate endoreduplication (28, 29). This could be because most of these TFs were identified in developmental studies of specialized cells with enhanced ploidy (e.g., trichomes) or because their activity is not regulated at the transcript level. The known transcriptional activator of mitosis,

MYB3R4, was, however, differentially induced with 2.5-fold higher expression in localized PM-infected cells.

Using a genetic approach, we therefore examined the role of MYB3R4 in the adapted PM–*Arabidopsis* interaction. We assessed two independent transferred DNA (T-DNA) insertion mutants of MYB3R4 (see *Materials and Methods*) and WT plants for differences in PM growth and reproduction and in host endoreduplication at the site of infection. The *myb3r4* mutants exhibited reduced visible PM symptoms (Fig. 4E and *SI Appendix*), with fewer conidiophores per germinated conidium compared with WT (Fig. 4F). These differences were not attributed to gross changes in plant or leaf size, morphology, or development (*SI Appendix*), and, similar to Haga et al. (26), we did not observe significant cytokinesis-associated defects in *myb3r4* leaf cells. A number of *Arabidopsis* mutants conferring enhanced PM resistance exhibit constitutive or PM-induced cell death (3). We, however, did not observe either constitutive or induced cell death in the *myb3r4* mutants (*SI Appendix*). We next determined whether PM-induced endoreduplication was altered in *myb3r4* plants. We found host endoreduplication in the mesophyll cells underlying the infected epidermal cells was abrogated in *myb3r4* plants with a median DNA content equivalent to 16C for both infected and uninfected samples (Fig. 4C and D).

Model for MYB3R4 Function in PM-Induced Endoreduplication at the Site of Infection. MYB3R4 is one of five *Arabidopsis* TFs containing three tandem imperfect repeat sequences of ~50 amino acids in the MYB domain similar to mammalian c-MYB proteins. Like mammalian c-MYB proteins, MYB3R4 regulates the cell cycle. It is a known transcriptional activator of cytokinesis that binds the G₂/M-phase-specific MSA element (26). Orthologous MYBs, such as tobacco NtMYBA2 (30), contain a C-terminal domain that allows for posttranslational control of NtMYBA2 activity. For NtMYBA2, full activation requires phosphorylation of the C-terminal domain of the protein by specific cyclin (CYC)/cyclin-dependent kinase (CDK) complexes (30). This also appears to be the case for MYB3R4 because it contains this C-terminal domain with multiple CDK consensus phosphorylation sites, and coexpression of CYCB1 with MYB3R4 enhanced its transactivation of specific target genes (26).

The fact that PM-induced endoreduplication was abrogated in *myb3r4* plants indicates that the functional MYB3R4 protein is required for establishment of the endocycle. Furthermore, it suggests that unphosphorylated (or under-phosphorylated) MYB3R4 acts as a repressor of mitosis required for induced endoreduplication (Fig. 4H). To examine this possibility further, we identified putative targets of MYB3R4 with altered expression in our LMD dataset (Fig. 4G and *SI Appendix*). These genes exclusively exhibited decreased expression at the PM infection site and include the known MYB3R4 target *CYCB1:2* (26). MYB3R4 activator vs. repressor function is likely controlled by localized concentrations of specific CYCs (e.g., CYCBs) and CDKs (CDKA or CDKB) required for the C-terminal phosphorylation and mitosis-activating function of MYB3R4 (26, 30). Therefore, localized regulated proteolysis and/or reduced expression of these CDKs and/or CYCs could modulate MYB3R4 function. *CDKB1:2*, *CYCB1:2*, and *CYCB1:5* expression was reduced at the infection site, suggesting they might play this role (Fig. 4H). An alternative possibility is that a PM-induced inhibitor of the relevant CDK(s) could prevent phosphorylation of MYB3R4, thereby promoting endoreduplication. A number of known and putative CDK inhibitors are induced in response to pathogen, including SIAMESE, a small nuclear protein that interacts with CDKA to suppress mitosis as part of the switch to endoreduplication in trichomes (31).

The *myb3r4* mutants exhibited abrogated PM-induced endoreduplication, with reduced growth and reproduction of *G. orontii*. This suggests that induced endoreduplication at the site of

infection facilitates the sustained growth and reproduction of *G. orontii* on *Arabidopsis*, which acquires all its nutrients from the plant host (Fig. 4H). Localized endoreduplication increases gene copy number, and thus could enhance the metabolic capacity of host cells at the infection site. Although it is not always the case, endoreduplication has been associated with both elevated metabolic activity and larger cell sizes (28, 32). Consistent with this role at the site of PM infection at 5 dpi, we observed the enhanced expression of genes required to increase metabolic capacity (e.g., involved in transcription, translation, energy generation, and cofactor synthesis; *SI Appendix*). In addition to a general role in increasing metabolic capacity, endoreduplication at the site of PM infection might allow for the dramatically enhanced expression of genes specifically required to facilitate the growth and reproduction of the fungus such as those involved in localized nutrient (e.g., hexose) accumulation and transport. Indeed, we observed a dramatic increase in expression of these genes (*SI Appendix*). In the case of nutrient transporters, the combination of enhanced expression of the transporters and the larger surface area of the higher ploidy cells could allow for dramatically enhanced transport rates. Although defense-associated genes (e.g., *PR* genes) may also be more highly expressed as a consequence of endoreduplication, in our system, it appears this enhanced host defense is outweighed by the enhanced capacity to provide nutrients required by the fungus. This strategy, employing induced localized endoreduplication to meet enhanced metabolic demands, has also been observed for other intimately associated obligate plant biotrophs that establish a defined site of nutrient uptake/exchange. For example, root knot and cyst nematodes establish metabolically active specialized nematode feeding sites of enhanced ploidy (33). In addition, endoreduplication plays a crit-

ical role in the *Sinorhizobium-Medicago* symbiosis, with reduced endoploidy in nodules of *Medicago* sp. resulting in inefficient nitrogen fixation (34). Therefore, we propose that induced endoreduplication at the interaction site may be an important common strategy used in this type of intimate interaction and that targets identified in our system may have broader applications.

Materials and Methods

A. thaliana Col-0, *ics1-2*, and *myb3r4* mutants in the *Col-0* background were grown in environmental chambers, with a subset infected with *G. orontii* at 4 weeks and assayed for PM phenotypes as in the article by Chandran et al. (4) and *SI Appendix*. Homozygous *myb3r4-1* (SALK_059819c) and *myb3r4-3* (SALK_116974c) T-DNA insertion lines were obtained from the Arabidopsis Biological Resource Center. The *myb3r4-1* was previously characterized as a lack-of-function T-DNA mutant with an insertion in exon 2 (26); the *myb3r4-3* T-DNA insertion is located in exon 1. For PM expression profiling, fully expanded mature leaves were harvested at 5 dpi. Sample preparation, RNA extraction and amplification, and microarray analysis are described in the text and further detailed in *SI Appendix*. TAIR9 gene functional descriptions were employed (2), with MAPMAN (35) used to identify statistically enriched processes ($p \leq 0.05$) and the ATTED-II *Arabidopsis* expression database (36) used for coexpression analyses. In brief, for nuclear DNA quantitation, fluorescence values from 30 DAPI-stained mesophyll cell nuclei of WT and mutant leaves underlying PM-infected and uninfected sites were quantified and normalized to those of nuclei of reference diploid guard cells from the same image after subtraction of background from each measurement (*SI Appendix*). All experiments were repeated at least twice.

ACKNOWLEDGMENTS. We thank Drs. Denise Schichnes, Steven Ruzin, and Terence Speed for technical advice and Dr. Shauna Somerville for valuable comments on the manuscript. This work was supported by the National Science Foundation (NSF) (Grant Arabidopsis 2010 MCB-0420267) and Winkler Family Foundation awards (to M.C.W.). C.K.K. participated in the Deutsche Forschungsgemeinschaft-NSF 2010 Exchange program.

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