A Functional Screen to Characterize the Secretomes of Eukaryotic Pathogens and Their Hosts In Planta

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Complex suites of proteins that are secreted by plants and phytopathogens into the plant apoplast play crucial roles in surveillance, assault, defense, and counter-defense. High-throughput genome-scale strategies are being developed to better understand the nature of these “secretomes” and the identity of pathogen-derived effector proteins that subvert plant defenses and promote pathogenicity. Although combined bioinformatic and experimental approaches recently have provided comprehensive coverage of secreted proteins from bacterial phytopathogens, far less is known about the secretomes and batteries of effectors of eukaryotic phytopathogens; notably fungi and oomycetes. The yeast secretion trap (YST) represents a potentially valuable technique to simultaneously target pathogen and host secretomes in infected plant material. A YST screen, using a new vector system, was applied to study the interaction between tomato (*Solanum lycopersicum*) and the oomycete *Phytophthora infestans*, revealing sets of genes encoding secreted proteins from both pathogen and host. Most of those from the oomycete had no identifiable function and were detectable in planta only during pathogenesis, underlining the value of YST as a tool to identify new candidate effectors and pathogenicity factors. In addition, the majority of the *P. infestans* proteins had homologs in the genomes of the related oomycetes *P. sojae* and *P. ramorum*.

Additional keywords: cell wall, signal peptide.

The plant cell wall represents the interface between the plant and the abiotic and biotic environments and provides a crucial defensive barrier against microbial pathogens that constantly seek to invade and exploit a new host. Therefore, the plant wall and apoplastic milieu can be viewed as a molecular battleground, a key element of which involves the co-evolution and interplay between plant- and pathogen-secreted proteomes, or secretomes, that are required for surveillance, assault, defense, and counterdefense.

Plant cells secrete a broad spectrum of constitutively expressed and inducible defense-related proteins and peptides whose functions include degradation of microbial cell wall polymers and the inhibition of proteins secreted by the patho-

gen (van der Hoorn and Jones 2004). Similarly, phytopathogens secrete suites of peptides and proteins during infection which can remain associated with the hyphal wall (Rast et al. 2003) or which are more mobile in the plant apoplast. Many of these proteins are broadly referred to as “effectors,” implying pathogen-derived molecules that alter host cell structure or function and influence pathogenicity or defense responses. They include enzymes involved in disassembly of the plant wall (Huitema et al. 2004; Vorwerk et al. 2004) and suppressors of extracellular plant defenses (Abramovitch and Martin 2004; Huitema et al. 2004; Rose et al. 2002). Depending on the nature of the interacting host and pathogen, specific secreted proteins can interact with the product of a corresponding host resistance (*R*) gene, in which case they are termed avirulence (*Avr*) proteins, and this interaction results in disease resistance (Dangl and Jones 2001), culminating in a hypersensitive response (HR).

As well as secreting proteins into the apoplast, gram-negative bacteria have evolved a type III secretion system (TTSS) to translocate virulence proteins in a specialized structure across the host plasma membrane and cell wall and inject them directly into the host cytosol (Alfano and Collmer 2004; Guttman et al. 2002). The translocated proteins, which can act as virulence and avirulence factors, also are termed effectors, playing a pivotal role in pathogenicity by suppressing or inducing defense responses such as the HR (Abramovitch and Martin 2004; Alfano and Collmer 2004; Chang et al. 2004). The identification of bacterial *Avr* proteins and effectors has been accelerated dramatically in the last few years by the combined use of computational and experimental strategies to characterize the TTSS-associated secretome (Chang et al. 2005; Greenberg and Vinatzer 2003). This has been particularly effective in the case of *Pseudomonas syringae*, resulting in essentially saturating coverage that will provide an excellent platform to determine translocated effector function, mechanism of action, and biological significance.

In contrast, far less is known about the secretome or the nature of potential effectors from eukaryotic microbial phytopathogens. For example, although most *Avr* proteins from eukaryotic phytopathogens that have been studied to date are apoplastic (Rep 2005), it recently has been shown that, as with bacterial phytopathogens, some *Avr* proteins from fungi and oomycetes are translocated into plant cells (Armstrong et al. 2005; Catanzariti et al. 2006; Rehmany et al. 2005). This new discovery further illustrates the complexity of the secretomes of plant pathogens and their hosts and, although individual components of these extracellular systems are being revealed through specific assays or molecular screens, progress is incre-

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mental and, clearly, much remains to be discovered. By analogy to the remarkable progress that has been made with profiling the TTSS secretome, a more extensive catalog of the secretomes of a eukaryotic phytopathogen–plant interaction would provide valuable new insights into apoplastic processes and the extent and nature of the suites of secreted effectors.

To date, the only reported large-scale secretome screen for a eukaryotic plant pathogen was a computational prediction of secreted proteins based on 1,000 unigenes from the oomycete *Phytophthora infestans* (Torto et al. 2003). As with the recent high-resolution studies of the bacterial TTSS secretome, an equivalent experimental screen for secreted proteins would provide an important complement to such a bioinformatic approach. In this regard, the yeast secretion trap (YST) functional screen represents an attractive option (Jacobs et al. 1997; Lee et al. 2004). This technique involves ligating a pool of heterologous cDNAs derived from infected plant tissue to a yeast invertase gene (*suc2*) and transforming the resulting library into an invertase-deficient yeast mutant, which then is grown on a medium with sucrose as the sole carbon source. Any yeast transformant containing a heterologous cDNA that directs secretion of the recombinant invertase fusion protein has the potential to rescue of the mutant. The resulting yeast transformants then can be isolated and the gene encoding the secreted protein identified.

This article describes the development and application of a new modified YST vector system (pYST-0 to pYST-2) to simultaneously identify secreted proteins involved in the interaction between tomato (*Solanum lycopersicum*) and the oomycete *P. infestans*. The temporal expression patterns of the resulting *P. infestans* genes are reported, together with detection of orthologous genes in the recently completed *P. sojae* and *P. ramorum* genome sequences.

RESULTS

Development of a pYST vector set and validation with plant pathogen-secreted proteins.

It has been noted in several studies using the YST that the design of the cloning vector severely limits the number of cDNAs that potentially can be identified, given that it always has been used in a single vector system. Even though the anchored random primer used in the screen theoretically should anneal randomly to the transcripts and, thus, in all three reading frames, in practice the pYST one-vector system does not appear to provide full coverage. Indeed, the limited size of a one-vector-derived library suggests that priming does not occur randomly. If this is the case, there is as low as a one-in-three probability that a particular cDNA encoding a secreted protein will be ligated in frame with invertase and the necessary fusion protein secreted. Using the pSMASH vector (Goo et al. 1999) as a template, the pYST three-vector system was developed (pYST-0, pYST-1, and pYST-2) to allow cloning of any signal peptide (SP)-encoding cDNA to generate an in-frame fusion with the invertase, regardless of the reading frame at the 3' end of the heterologous cDNA and to compensate for any potential nonrandom annealing of the anchored random primer (Fig. 1A and B). We observed that the YST clones identified through the screen described here were present in equal proportions in the three YST vectors, thereby confirming the value of a three-vector set.

It also was noted that, when pSMASH was used in preliminary screens, yeast transformants were consistently recovered containing the *suc2* gene, with no additional heterologous sequence as a fusion (data not shown). The first encoded amino acid of the truncated *suc2* invertase without the SP is a methionine (Met²¹), and it was hypothesized that this might give

rise to an artifactual “SP-like” sequence, leading to a low-level secretion of the invertase protein alone and, consequently, false positives. A previous study using pSMASH to identify *Arabidopsis thaliana*-secreted proteins reported that 24% of the cDNA clones were false positives (Goo et al. 1999) and a similar proportion (17% of isolated clones) was described when using related YST vectors that contain Met²¹ and Thr²² (Jacobs et al. 1997). Accordingly, the YST vector set was redesigned to eliminate the first two amino acids (Met²¹ and Thr²²) of the mature protein; however, the frequency of false positives containing no heterologous insert using one vector of the three-vector YST alone was the same as that using the original pSMASH vector. When the pYST three-vector system was used in the current study, the frequency of such false positives was greatly reduced and only 5 of 157 cDNAs sequenced contained no heterologous sequence. Therefore, this increased efficiency was likely a consequence of the more effective capture of cDNAs in all reading frames, resulting in a higher ratio of SP-encoding sequences to false positives.

A single-vector YST system recently was used to confirm the secretion of specific fungal proteins (Catanzariti et al. 2006); however, to date, it has not been applied to screen libraries of

Fig. 1. Modified yeast secretion trap (pYST) cDNA library vectors and their use to identify oomycete and fungal secreted proteins. **A**, Schematic diagram of the three YST vectors (pYST-0, pYST-1, and pYST-2) used for cDNA library construction. Restriction enzyme sites for cDNA insertions and the positions of the promoter (PADH1) and terminator (TADH1) of the alcohol dehydrogenase gene are indicated. *suc2* represents a yeast invertase gene lacking its own signal peptide (SP) and initiator methionine. **B**, Nucleotide sequences of the pYST vector set. The variable C stretch in the pYST series of vectors is underlined. **C**, YST analysis of *P. sojae* *GIP1* and *Cochliobolus carbonum* *EXG2* with (panels 1 and 3, respectively) or without (panels 2 and 4, respectively) their SPs.

cDNAs from pathogen-infected plant tissues. To confirm the efficacy of the new modified pYST vector system as a means to identify secreted proteins from eukaryotic microbes, notably oomycetes and fungi, the full-length open reading frames (ORFs) of glucanase inhibitor protein 1 (PsGIP1) (Rose et al. 2002) from the oomycete *P. sojae* and exo-1,3- β -glucanase 2 from the ascomycete fungus *Cochliobolus carbonum* (CcEXG2) (Kim et al. 2001), two known secreted proteins, were cloned into pYST-0 and pYST-1, respectively. Yeast transformants expressing the PsGIP1¹⁻²⁵⁷:suc2 or the CcEXG2¹⁻⁴²²:suc2 fusions grew on sucrose, whereas yeast expressing the PsGIP1²²⁻²⁵⁷ or the CcEXG2¹⁹⁻⁴²² fusions, corresponding to the same proteins without the predicted SPs, did not grow (Fig. 1C). These results confirmed that the pYST vector system is a potentially valuable tool to identify new secreted proteins from oomycetous and fungal plant pathogens.

Yeast secretion trap library construction and screening of *P. infestans*-infected tomato leaf cDNAs.

Although the experiments with PsGIP1 and CcEXG2 demonstrated that the YST system can identify pathogen-derived secreted proteins, a critical question is whether it can be used to isolate both plant- and pathogen-secreted proteins that are expressed in planta during pathogenesis. A potential obstacle is

the high ratio of plant to microbial biomass, where the greater abundance of plant host cDNAs encoding secreted proteins might make the detection of pathogen-derived cDNAs relatively rare. A small-scale pilot study was performed using the pYST vector set to screen cDNAs derived from tomato leaves that were infected with a tomato-specialized isolate of the oomycete pathogen *P. infestans*, which resulted in a compatible interaction. To reduce the need to resequence redundant clones, heterologous cDNAs in the YST vectors derived from the first set of approximately 50 yeast colonies were used to screen subsequent sets of selected colonies by Southern blot analysis of the polymerase-chain reaction (PCR)-amplified inserts, in an iterative process. Using this approach, 625 separate yeast transformants were screened and 157 plasmids sequenced, resulting in the identification of 45 unique YST cDNAs, with an average insert size of approximately 400 bp.

To determine the species of origin of these clones, the sequences were used to search a number of DNA databases, taking advantage of the substantial expressed sequence tag (EST) collections for both tomato and *P. infestans*. In most cases, the cDNAs corresponded to a previously submitted EST, whereas others were new sequences. The percentage of GC content can be markedly different between tomato and *Phytophthora* spp. (Qutob et al. 2000) and, in theory, could be used to distinguish between the plant- and oomycete-derived genes. However, this did not always provide a clear indication; therefore, tomato or *P. infestans* genomic Southern blot analyses were performed using each cDNA that was not present in the databases. This proved to be definitive in all cases, and representative results are shown in Figure 2A and B, indicating that PiE2 (*P. infestans* Extracellular 2) (Table 1) cross-hybridizes only with *P. infestans* genomic DNA and showing the genomic organization of the genes encoding the *P. infestans*-secreted proteins PiE3, PiE4, PiE7, PiE9, and PiE12. These clones were selected arbitrarily from among those that are listed as unknown proteins and currently have no annotation to confirm their function. Despite the difference in relative biomass, it was determined that equal numbers of unique cDNAs were derived from the plant and the pathogen (Tables 1 and 2).

Classification of *P. infestans* and tomato YST clones.

Only 6 of the 23 *P. infestans* YST clones could be annotated based on sequence identity or similarity to previously identified genes (Table 1). Two (PiE1 and PiE5) corresponded to the elicitors ELI-INF1 and ELI-INF5, which are members of a family of evolutionarily related proteins that recently have been reclassified (Jiang et al. 2006). PiE1 (ELI-INF1, formerly INF1) is a well-characterized secreted elicitor of the hypersensitive response (Kamoun et al. 1997). Other annotated clones included an extracellular metalloproteinase (PiE17) and a cutinase (PiE18), classes of hydrolytic enzymes that commonly are secreted by phytopathogens and are believed to be pathogenicity determinants. Another previously reported *P. infestans* gene that was identified through the YST screen (PiE19) was *scr74*, which encodes a cysteine-rich species-specific elicitor-like protein (Liu et al. 2005), with sequence similarity to PcF, a necrosis-inducing protein secreted by *P. cactorum* (Orsomando et al. 2001) and a candidate *avr* gene *scr91* (Bos et al. 2003). Interestingly, the last annotated *P. infestans* YST clone (PiE16) has 46% amino acid sequence identity with a recently identified protein pheromone, termed temptin, from the sea hare *Aplysia brasiliana* (Cummins et al. 2004). Among the 23 PiE clones, all but 8 had homologs in the *P. sojae* and *P. ramorum* genomes.

Approximately half of the YST clones originating from tomato (Table 2, *SIE*, *S. lycopersicum* Extracellular) have an association with plant defenses, including previously reported

Fig. 2. Genomic DNA gel blot analysis of PiE clones. **A**, Representative genomic DNA gel blot screen of tomato and *Phytophthora infestans* (*P. i.*). DNA was digested with *EcoRI* (1), *EcoRV* (2), and *BamHI* (3) and hybridized with a PiE2 probe to confirm the species of origin. **B**, Genomic DNA gel blot analysis of the *P. infestans* genes. Genomic *P. infestans* DNA (10 μ g) was digested with *EcoRI* (1), *EcoRV* (2), *SacI* (3), *XbaI* (4), and *BamHI* (5) and hybridized with the indicated PiE clones. Molecular size markers are indicated (kb).

pathogenesis-related (PR) proteins (e.g., *SIE3* and *SIE17*), structural proteins such as extensin and related glycine-rich proteins, glycosyl hydrolases that target glycans that are typical constituents of microbial cell walls (e.g., *SIE2* and *SIE16*), and a putative peroxidase (*SIE15*). Other YST clones are pre-

dicted to encode proteins whose defensive role is less well defined, such as stellacyanin (*SIE11*), but that have been reported to be induced by wounding or elicitors (Takemoto et al. 2003). A third of the clones (*SIE4*, *SIE9*, *SIE13*, and *SIE20-22*) had no clear homology to known genes or obvious functional domains

Table 1. *Phytophthora infestans* yeast secretion trap clones isolated from *P. infestans*-infected tomato leaves^a

Clone (acc. no.) ^b	Database annotation				Homologue		PexFinder	SignalP 3.0 score	
	Protein name	GenBank ^c	E value ^d	Species	<i>P. sojae</i>	<i>P. ramorum</i>	HMM	HMM	NN
PIE1 (CV970777)	Elicitin ELI-INF1	AAB49807	1e-34	<i>P. infestans</i>	128080	78569	1.000	1.000	0.783
PIE2 (CV970779)	Unknown protein	CV933463	1e-58	<i>P. infestans</i>	134030	80982	0.134 (SA)	0.002 (SA)	0.168
PIE3 (CV970780)	Unknown protein	CV947495	2e-154	<i>P. infestans</i>	...	...	0.999	1.000	0.845
PIE4 (CV970781)	Unknown protein	CV945595	3e-100	<i>P. infestans</i>	130440	81685	0.997	0.999	0.785
PIE5 (CV970782)	Elicitin ELI-INF5	AAL16012	3e-29	<i>P. infestans</i>	108238	71633	0.997	0.997	0.642
PIE6 (CV970783)	Unknown protein	AF424687	0.0(n)	<i>P. infestans</i>	136058	81781	1.000	1.000	0.775
PIE7 (CV970784)	Unknown protein	CV969546	0	<i>P. infestans</i>	144427	78163	0.974	0.96	0.623
PIE8 (CV970785)	Unknown protein	CV953356	7e-104	<i>P. infestans</i>	...	...	0.106 (SA)	0.078 (SA)	0.686
PIE9 (CV970786)	Unknown protein	CV946996	0	<i>P. infestans</i>	...	...	0.995	0.996	0.623
PIE10 (CV970787)	Hypothetical protein	...	...	...	141791	82244	1.000	1.000	0.85
PIE11 (CV970788)	Unknown protein	XP_370456	6e-11	<i>Magnaporthe grisea</i>	...	...	0.998	0.997	0.745
PIE12 (CV970789)	Unknown protein	CV900695	0.0(n)	<i>P. infestans</i>	135684	82920	0.996	0.996	0.708
PIE13 (CV970790)	Unknown protein	CV899036	0.0(n)	<i>P. infestans</i>	...	...	0.993	0.994	0.754
PIE14 (CV970791)	Unknown protein	CV918188	1e-156	<i>P. infestans</i>	135689	86904	0.996	0.996	0.671
PIE15 (CV970792)	Unknown protein	XP_363174	3e-04	<i>M. grisea</i>	...	...	0.999	0.999	0.72
PIE16 (CV970793)	Temptin	AAS92605	1e-16	<i>Aplysia brasiliana</i>	143786	78188	0.999	0.999	0.804
PIE17 (CV970794)	Metalloproteinase	NP_639017	7e-16	<i>Xanthomonas campestris</i>	139557	74032	0.982	0.997	0.742
PIE18 (CV970795)	Cutinase	AAO92431	2e-50	<i>P. brassicae</i>	123647	39832	0.999	0.998	0.824
PIE19 (CV970796)	Elicitor-like SCR74	AAU21458	3e-37	<i>P. infestans</i>	...	43241	0.997	0.996	0.776
PIE20 (CV970797)	Unknown protein	CV965926	4e-140	<i>P. infestans</i>	...	...	1.000	1.000	0.773
PIE21 (CV970798)	Unknown protein	CV929998	0	<i>P. infestans</i>	121909	71256	0.968	0.415 (SA)	0.585
PIE22 (CV970799)	Unknown protein	CAE67074	1e-26	<i>Caenorhabditis elegans</i>	138595	81609	1.000	1.000	0.8
PIE23 (CV970800)	Unknown protein	CV948862	0	<i>P. infestans</i>	135685	82921	0.999	0.986	0.591

^a *P. sojae* and *P. ramorum* genome sequences at the JGI website; HMM and NN = hidden Markov model and neural network methods, respectively; scores in bold indicate clones with no predicted signal peptide; (n) = nucleotide-nucleotide BLAST and (SA) = predicted signal anchor.

^b Clone name and *P. infestans* expressed sequence tag accession number.

^c Accession number.

^d E value from translating BLAST or protein-protein BLAST.

Table 2. Tomato yeast secretion trap clones isolated from *Phytophthora infestans*-infected tomato leaves

Clone (acc. no.) ^b	Database annotation					SignalP 3.0 score ^a	
	Protein name	SGN ^c acc. no.	GenBank acc. no.	E value ^d	Species	HMM	NN
SIE1 (CV970753)	Glycine-rich protein	SGN-U220422	AAP83840	0.018	<i>Solanum lycopersicum</i>	0.992	0.876
SIE2 (CV970754)	Glycosyl hydrolase family protein 17	SGN-U223104	NP_564059	5e-27	<i>Arabidopsis thaliana</i>	1.000	0.875
SIE3 (CV970755)	Pathogenesis-related (PR) protein PR-1	SGN-U212923	P04284	4e-39	<i>S. lycopersicum</i>	1.000	0.945
SIE4 (CV970756)	Expressed protein	SGN-U216346	BG127004	6e-147	<i>S. lycopersicum</i>	1.000	0.882
SIE5 (CV970757)	Extensin	SGN-U285067	X55683	2e-35(n)	<i>S. lycopersicum</i>	0.998	0.742
SIE6 (CV970758)	Glycine-rich protein	SGN-U217408	CAB67122	2e-08	<i>Nicotiana tabacum</i>	0.973	0.874
SIE7 (CV970759)	Glycine-rich protein	SGN-U212549	T07013	5e-09	<i>S. lycopersicum</i>	0.997	0.893
SIE8 (CV970760)	Glycine-rich protein	...	P09789	0.27	<i>Petunia × hybrida</i>	0.993	0.784
SIE9 (CV970761)	Unknown protein	SGN-U225752	NP_174546	2e-11	<i>Arabidopsis thaliana</i>	0.000	0.063
SIE10 (CV970762)	Phosphate/PEP translocator	SGN-U214131	T03836	8e-95	<i>Nicotiana tabacum</i>	0.006	0.241
SIE11 (CV970763)	Stellacyanin-like protein	SGN-U215639	AAG01535	3e-34	<i>Capsicum annuum</i>	0.989	0.824
SIE12 (CV970764)	GDSL-motif lipase	SGN-U214390	T02229	7e-45	<i>N. tabacum</i>	1.000	0.909
SIE13 (CV970765)	Expressed protein	SGN-U213711	AAL16012	...	...	0.999	0.89
SIE14 (CV970766)	Glycine-rich protein	SGN-U238191	AAP83840	1e-09	<i>S. lycopersicum</i>	0.994	0.905
SIE15 (CV970767)	Putative peroxidase	SGN-U219648	AAK59478	2e-34	<i>A. thaliana</i>	1.000	0.798
SIE16 (CV970768)	β-1,3-Glucanase-like protein	SGN-U215533	CAB80165	2e-79	<i>A. thaliana</i>	0.998	0.795
SIE17 (CV970769)	PR protein P2	SGN-U214985	CAA41439	3e-57	<i>S. lycopersicum</i>	0.994	0.749
SIE18 (CV970770)	Glycine-rich protein	SGN-U216000	BP905591	0.27	<i>S. lycopersicum</i>	0.994	0.892
SIE19 (CV970771)	Protease inhibitor/seed storage/LTP family	SGN-U218425	BT014512	3e-30	<i>S. lycopersicum</i>	0.997	0.778
SIE20 (CV970772)	Unknown protein	SGN-U217114	CV966669	3e-08	<i>N. tabacum</i>	0.978	0.852
SIE21 (CV970773)	Wound-induced protein	SGN-U214651	CAA31852	3e-37	<i>S. lycopersicum</i>	0.999	0.916
SIE22 (CV970774)	Harpin-induced protein	SGN-U217180	NP_198513	5e-32	<i>A. thaliana</i>	0.000	0.02

^a HMM and NN = hidden Markov model and neural network methods, respectively; scores in bold indicate clones with no predicted signal peptide.

^b Clone name and *Solanum lycopersicum* expressed sequence tag accession number.

^c SGN = SOL Genomics Network hosted at Cornell University.

^d E value from translating BLAST or protein-protein BLAST; (n) = nucleotide-nucleotide BLAST.

and motifs. Three clones (*SIE9*, *SIE10*, and *SIE22*) had no predicted SP (Table 2, bold and underlined) with either hidden Markov model (HMM) or neural network (NN) methods.

The predicted proteins derived from all the YST clones were searched for the presence of an N-terminal SP using SignalP (version 3.0) (Bendtsen et al. 2004) and Tables 1 and 2 show the scores using either an HMM or NN method (those scores indicating no predicted SP are in bold and underlined). In addition, the equivalent scores generated using an earlier version of SignalP (SignalP, v2.0.b2) that formed the basis of PexFinder, an algorithm designed to predict extracellular proteins from EST collections and previously used in an analysis of *P. infestans* ESTs (Torto et al. 2003), are shown in Table 1. Of the PiE proteins, 20 were predicted to have an N-terminal SP and 3 (PiE2, PiE8, and PiE21) an N-terminal signal anchor (SA), which is defined as an uncleaved SP. An NN D score of 0.48 or greater is an indication of the presence of an SP, and all PiE proteins had a positive score apart from PiE2. It was noted that the older version of SignalP used in PexFinder gave predictions similar to those of the newer version 3.0, with the exceptions of PiE21, which was given a high probability score by PexFinder but a low score or an indicated SA using version 3.0 (Table 1). In contrast, three SIE proteins (*SIE9*, *SIE10*, and *SIE22*) had no predicted SP or SA with either the NN or HMM (Table 2).

Transcriptional dynamics

of *P. infestans* YST clones during infection of tomato.

A key objective of this study was to determine whether the improved YST vector system could identify new potential effectors and pathogenicity factors that are secreted specifically by eukaryotic pathogens in planta during pathogenesis. The expression patterns of a selection of the PiE clones were compared by Northern blot analysis of RNA from axenically

Fig. 3. RNA gel blot analysis of several PiE clones. Total RNA (20 µg per lane) was isolated from noninfected tomato leaves (Ninf), leaves that had been wounded (Wnd) or infected with *P. infestans* (Pinf), and axenically grown *P. infestans* mycelia (Mycl). Ethidium bromide-stained rRNAs are shown to evaluate loading.

grown *P. infestans* mycelia, control or wounded tomato leaves, or leaves 4 days post inoculation (dpi) with *P. infestans* (Fig. 3), focusing mostly on new genes with no predictable function. As a positive control, the expression of the well-characterized elicitor ELI-INF1 (Kamoun et al. 1997) was monitored (PiE1) and, although transcripts were detected in cultured mycelia, mitochondrial (m)RNA levels were substantially higher during pathogenesis. This result is different from that of Kamoun and associates (1997), who reported that ELI-INF1 was downregulated during infection of potato, although they used a tomato-specialized isolate of *P. infestans* and, therefore, this difference may simply reflect the differences in plant host. It also is noteworthy that the rate of infection of potato was significantly slower than that observed in this current study; therefore, the dynamics of infection also might contribute to the differing expression patterns. In contrast with the abundance of ELI-INF1 transcript in vitro, all the transcripts tested that encoded unknown or hypothetical *P. infestans*-secreted proteins were detected only in infected leaves and not in in vitro-grown mycelia. The presence of plant mRNAs with sequence homology to PiE6 and PiE7 was indicated by crossreacting bands of a different size in noninfected or wounded leaves (Fig. 3); however, crosshybridizing bands were not seen by Southern analysis of tomato genomic DNA at the same hybridization stringency, suggesting a low level of sequence similarity.

Fig. 4. RNA gel blot analysis of PiE clones expressed in tomato leaves over an infection time course. Total RNA (20 µg) was isolated from axenically grown *Phytophthora infestans* mycelia (Mycl), noninfected tomato leaves (Ninf), or *P. infestans*-infected tomato leaves at 0, 1, 2, 3, and 4 days post inoculation (dpi). The blots were hybridized with the indicated PiE clones and a specific probe corresponding to *P. infestans* actin (*actA*). Ethidium bromide-stained rRNAs are shown to evaluate loading.

Given that *P. infestans* is a hemibiotrophic organism, it is likely that different subsets of proteins are secreted into the host apoplast during the biotrophic or necrotrophic phases of the life cycle. To provide more insight into the temporal dynamics of the transcripts underlying the secretome, Northern blot analysis was used to monitor the abundance of a selection of the *PiE* mRNAs in a time course of infection from 1 to 4 dpi, again focusing on new hypothetical proteins (Fig. 4). The expression of a *P. infestans* actin gene (*actA*) used as a constitutively expressed control (Unkles et al. 1991) first was detected at 2 dpi and its abundance increased thereafter, providing an indication of the relative oomycete biomass. Most of the *PiE* genes were detected again only in infected tissues rather than in axenically grown mycelia, and they exhibited a range of expression patterns in terms of the timing of onset and maximal levels of transcript accumulation. Some genes, such as *PiE2* and *PiE13*, appeared to be expressed constitutively over the 4-day time course, in that mRNA abundance paralleled the equivalent relative abundance of *actA* mRNA. Others were expressed predominantly early during infection and showed a decrease in signal intensity on the Northern blot (e.g., *PiE6* and *PiE16*), or a relative decrease in expression in proportion to the accumulation of hyphal biomass (e.g., *PiE20* and *PiE22*), as judged by *actA* mRNA levels.

Analysis of *PiE12*, *PiE14*, and *PiE23* protein structure.

To gain insight into putative functions for the unidentified *PiE* candidates, translated sequences were submitted to Motif Scan for prediction of functional domains. In addition to the N-terminal SP, *PiE12*, *PiE14*, and *PiE23* were predicted to contain bipartite nuclear localization signals (NLSs) (data not shown).

A conserved host-targeting signal for translocation of proteins from *Plasmodium* spp. (malaria parasites) into host cells has been described recently (Hiller et al. 2004; Marti et al. 2004) and a similar motif, termed RXLR, is present in some secreted oomycete proteins (Armstrong et al. 2005; Rehmany et al. 2005). Recently, it has been reported that the *Phytophthora* RXLR motif indeed acts as a host-targeting signal in *Plasmodium* (Bhattacharjee et al. 2006). Visual inspection of the *PiE12*, *PiE14*, and *PiE23* translations revealed an RXLR-like

motif (where X is represented by Q/NLGAGGG) within 60 amino acids of the N-terminus, preceded by an SP and followed by NLSs, indicating that they represent an alternative form of the classical RXLR motif containing proteins. Homologous sequences of *PiE12*, *PiE14*, and *PiE23* were obtained by searching the sequence contigs in *Phytophthora sojae*. The protein sequences of *PiE12*, *PiE14*, and *PiE23* showed 65, 54, and 61% homology to Ps_135684, Ps_135689, and Ps_135685, respectively, at the amino acid level. All the homologous sequences contained SPs, RXLR-like motifs, and predicted NLSs and were found to be clustered in a 20-kb sequence contig in the genome of *P. sojae*, further suggesting that they may represent members of a gene family (Fig. 5).

DISCUSSION

The YST screen effectively targets both host and pathogen secretomes that are expressed in planta during pathogenesis.

The excellent progress that has been made recently in characterizing the TTSS-related secretomes of phytopathogenic bacteria has been achieved by coupling bioinformatic and high-throughput experimental approaches. For example, this has provided a near to complete coverage of the suites of effector proteins that are translocated into plant cells (Alfano and Collmer 2004; Chang et al. 2005). In contrast, far less is known about the secretomes of eukaryotic phytopathogens, notably fungi and oomycetes. In part, this may be because many of these organisms are obligate biotrophs and, therefore, more difficult to study, but it is striking that no coupled bioinformatic and high-throughput functional secretome screens analogous to the bacterial secretome studies have been described to date.

Several computational tools have been developed to predict the presence of N-terminal SPs that direct secreted proteins to the endoplasmic reticulum (ER) or Golgi-dependent, secretory pathway. For example, SignalP version 2.0 was used effectively to screen 1,000 *P. infestans* unigenes for secretory proteins (Torto et al. 2003). Computational prediction is an attractive approach, particularly in the context of large-scale evaluations, due to its relative ease and speed. However, as with all prediction-

Fig. 5. Sequence alignment and genomic organization of *PiE12*, *PiE14*, and *PiE23*. **A**, Alignment of the N-terminal sequences of *PiE12*, *PiE14*, *PiE23*, and homologs from *Phytophthora sojae* (Ps). The N-terminal signal peptide is indicated in italics and the conserved, expanded RXLR-like motif is underlined. **B**, Clustering of homologs of *PiE12*, *PiE14*, and *PiE23* in *P. sojae*. Arrows indicate homologs of *PiE12*, *PiE14*, and *PiE23* and their orientations. A thin horizontal line represents a DNA sequence contig.

based tools, there is a consistent error rate and both false positive and negative predictions should be expected; for example, Torto and associates (2003) reported a 17% false positive error rate, which is not unusual for such programs. Therefore, as was illustrated with the bacterial secretome analyses, experimental screens provide an important complement to bioinformatic analyses by functionally validating or refuting the predictions.

The YST has been used to identify genes encoding secreted and plasma membrane-localized proteins from a range of eukaryotes (Lee et al. 2004), including a study of secreted plant proteins from *P. parasitica*-resistant tobacco leaves (Hugot et al. 2004). However, to date, no reports exist of its use to screen a library of pathogen-infected plant tissues. The objective of this study was to determine whether the YST screen represents an effective tool that ultimately could be used in combination with existing bioinformatic approaches to examine the secretomes of both host and eukaryotic phytopathogen in planta during pathogenesis.

The efficiency of the YST approach is largely dependent on optimizing the proportion of heterologous cDNA sequences that can be generated in frame with invertase and on minimizing the background level of false positives. The new pYST three-vector system (Fig. 1B) was developed to address these criteria and resulted in significant improvements over the existing YST vector on both counts. Moreover, it was demonstrated that it could identify both oomycetous and fungal-secreted proteins (Fig. 1C). Another potential obstacle to this approach was the possibility that *P. infestans* sequences would not be detectable due to the high ratio of plant to oomycete biomass in the infected tissue; however, comparable numbers of plant and pathogen clones were identified (Tables 1 and 2). Because the majority of the PiE clones have homologs in the *P. sojae* and *P. ramorum* genomes (Table 1), it is likely that these three species secrete similar suites of proteins during infection of their respective hosts.

Many *P. infestans*-secreted proteins are expressed only in planta and exhibit temporal differences in expression.

Of the tomato *SIE* genes that were identified, half had some relationship with defense responses, including some of the well-studied hydrolases that degrade the microbial hyphal walls or proteins involved in wall reinforcement. Others were annotated based on induction by wounding or an elicited response, but no known biochemical action. Interestingly, three clones (*SIE9*, *SIE10*, and *SIE22*) clearly had no predicted SP (Table 2) and, indeed, had entirely hydrophilic rather than characteristic hydrophobic N-termini (data not shown). One explanation for the identification of SP-less sequences is the existence of a “non-classical” (ER or Golgi independent) secretory pathway, as has been described in mammals and a lower eukaryote (Nickel 2005). A recent review also cataloged more than 40 secreted but SP-less proteins in yeast (Nombela et al. 2006), presenting several alternative protein secretory routes. We are aware of no demonstrated examples in the literature of plant proteins that are secreted by nonclassical routes, although studies in our lab recently have demonstrated their existence (data not shown). This further emphasizes the value of the YST screen, because it potentially identifies all secreted proteins, as opposed to computational prediction that currently is adequate only for proteins with classical SPs. Nevertheless, we cannot exclude the possibility that the YST screen yielded false positives.

Most of the *PiE* genes had no predictable function, but those that were annotated included known or putative effectors, such as elicitor (*PiE1*), cutinase (*PiE18*), and a protein with homology to a necrosis-inducing phytotoxin (*PiE19*). An intriguing exception was *PiE16*, which has sequence homology with a mollusk protein pheromone. Sexual reproduction in *P. infestans*

involves the interaction between two distinct mating types, which is mediated by attractants and, although a recently identified *Phytophthora* mating hormone was nonproteinaceous (Qi et al. 2005), it would be interesting to see whether *PiE16* has a related function. It was noted that, of the 23 *PiE* clones, only three (*PiE1*, *PiE5*, and *PiE6*) were previously reported in the Torto and associates (2003) bioinformatics analysis.

Northern blot analysis of the *PiE* clones with no known function revealed that most were expressed only in planta during pathogenesis. Moreover, the temporal variation in transcript expression patterns of the *PiE* genes (Fig. 4) supports the idea that different subsets of proteins are secreted into the plant apoplast at various times during infection; in this case, perhaps in association with distinct phases of *P. infestans* biotrophy or necrotrophy. These data further suggest that a YST screen of infected plant tissues is a valuable means to identify the crucial subset of effectors and pathogenicity determinants that are expressed in planta, as opposed to computational prediction based on EST collections from cultured organisms, particularly for “nonmodel” phytopathogens that are not currently targeted by large-scale sequencing projects. This certainly is also the case for obligate biotrophs that cannot be grown axenically and microbial symbionts, such as mycorrhizal fungi.

MATERIALS AND METHODS

P. infestans strain, culture conditions, and inoculum production.

The tomato-specialized *P. infestans* isolate US970001 (ATCC #MYA-2350), a member of the US-17 clonal lineage (Smart et al. 2003), was cultured in rye B agar (Caten and Jinks 1968) at 15°C with 18 h of light per day. To generate zoospores for subsequent inoculation of tomato plants, detached tomato leaflets were inoculated with a *P. infestans* sporangial suspension and incubated in moist chambers for 5 days as described in Smart and associates (1998). Sporangia then were collected in water, diluted to 20,000 sporangia ml⁻¹, and incubated at 4°C for at least 2 h to induce zoospore release.

Plant material and infection by *P. infestans*.

Tomato (*S. lycopersicum* cv. Rio Grande) plants were grown in a greenhouse at Cornell under conditions of 16 h of light and 8 h of dark. Attached leaves from 4-week-old plants were spray inoculated with *P. infestans* zoospore suspensions in a humid chamber at 15°C with 16 h of light and 12 h at 100% relative humidity, as described by Smart and associates (2003). Symptoms were monitored daily and leaves were harvested at the indicated times for RNA extraction and cDNA library construction. For the wounded leaf material, young attached leaves were stabbed with a block of needles and left on the plant for 6 h before harvesting. All plant tissue was frozen in liquid nitrogen immediately after collection and stored at -80°C.

Construction of the pYST yeast signal sequence trap library vectors.

The pYST-0, pYST-1, and pYST-2 vectors for construction of YST libraries were derived from the pSMASH plasmid (Goo et al. 1999) (a generous gift from Dr. O.K. Park), using PCR to generate variants of the pYST vector that allow the cloning of cDNA inserts in all three reading frames in frame with the *suc2* invertase gene. The PCR primers used were *suc2*-5 (5'-GCGCGCGCGGCCGCC(0-2)TTGGATAAAAGGTACCCAAACGAAACTAGC-3') and *suc2*-3 (5'-CATGTTTG GTGATATCATCTCCGATGAAGCCTCCGTTATCCCA-3'). The PCR products were subcloned as *NotI*-*EcoRV* fragments into pSMASH that previously had been digested with *NotI* and *EcoRV* and sequenced. The *suc2*-5 primer was designed such

that the first two amino acid (Met²¹ and Thr²²) of the *suc2* mature protein were eliminated, unlike the original pSMASH vector.

Construction of a *P. infestans*-infected tomato leaf cDNA library and control plasmid DNAs.

To create the *P. infestans*-infected tomato leaf YST cDNA library, total RNA was isolated as described by Rose and associates (1996) from a pooled sample of tomato leaves 1, 2, 3, and 4 dpi with *P. infestans*. Poly(A) mRNA was purified from total RNA using an Oligotex mRNA Midi Kit (Qiagen, Valencia, CA, U.S.A.) according to the manufacturer's instructions. The first-strand cDNAs were synthesized from mRNA using the N6-*NotI* primer (5'-GAGAGAGAGAGAGAGAACC GCGCGGCCNNNNNN-3'; the underlined bases correspond to the *NotI* site) and a cDNA Synthesis Kit (Stratagene, La Jolla, CA, U.S.A.). After second-strand synthesis, the cDNAs were prepared for unidirectional cloning by ligation with *EcoRI* adapters according to the manufacturer's instructions (Stratagene), followed by *NotI* digestion. The cDNA fragments (0.3 to 1.0 kb) were recovered following agarose gel electrophoresis with a Gel Extraction Kit (Qiagen) and ligated to a mixture of the three vectors (pYST-0, pYST-1, and pYST-2) that previously had been digested with *EcoRI* and *NotI*. Ligations were transformed into electrocompetent TOP10 *Escherichia coli* (Invitrogen, Carlsbad, CA, U.S.A.) by electroporation and the transformants selected on an ampicillin-containing medium. cDNA library plasmid DNA was purified using Perfectprep Midi spin columns (Brinkmann Instruments Inc., Westbury, NY, U.S.A.) from a pool of the transformed bacterial colonies.

To generate control plasmids to test the YST system with genes encoding known secreted proteins from eukaryotic plant pathogens, cDNAs encoding the open reading frames of *P. sojae* *PsGIP1* (Rose et al. 2002) and *C. carbonum* *CcEXG2* (Kim et al. 2001), with or without the region corresponding to the predicted SP, were amplified by PCR from genomic DNA of *P. sojae* (race 1) or the *CcEXG2* cDNA (a generous gift from Dr. J. Walton). The following gene-specific primers were used that incorporated *EcoRI* and *NotI* restriction sites for subsequent cloning into the pYST-0 or pYST-1 vectors for *PsGIP1* or *CcEXG2*, respectively: *PsGIP1*¹⁻²⁵⁷ 5'-GCGCGCG AATTCATGAAGGTTTCCCGCC-3' and 5'-GCGCGCGC GGCCGCCTTCTTGATGACAGAGTT-3'; *PsGIP1*²²⁻²⁵⁷ (5'-GCGCGCGAATTCATGAACAGTGCAGCGCTCC-3' and 5'-GCGCGCGCGCGCCCTTCTTGATGACAGAGTT-3'; *CcEXG2*¹⁻⁴²² 5'-GCGCGCGAATTCATGATTCTCACCAAAC TC-3' and 5'-GCGCGCGCGCGCCGCGCAGGCAGCGCCAA ACTT-3'; and *CcEXG2*¹⁹⁻⁴²² 5'-GCGCGCGAATTCATGGCTC CTGCCAGCAGAAG-3' and 5'-GCGCGCGCGCGCCGCGC AGGCAGCGCCAACTT-3'.

The PCR conditions were 94°C for 3 min; 35 cycles of 94°C for 30 s, 55°C for 60 s, and 72°C for 90 s; and finally 72°C for 10 min. PCR products were digested with *EcoRI* and *NotI* and cloned into the appropriate vectors, and the resulting plasmids *PsGIP1*¹⁻²⁵⁷:*suc2*, *PsGIP1*²²⁻²⁵⁷:*suc2*, *CcEXG2*¹⁻⁴²²:*suc2*, or *CcEXG2*¹⁹⁻⁴²²:*suc2* were sequenced to confirm an in-frame fusion with *suc2*.

Yeast transformation and selection.

cDNA library plasmids were transformed into the yeast (*Saccharomyces cerevisiae*) strain DBY α 2445 (Mata, *suc2* Δ -9, *lys2-801*, *ura3-52*, *ade2-101*) (Goo et al. 1999) (a generous gift from Dr. O.K. Park) using the YEASTMAKER Yeast Transformation System 2 (BD Biosciences, San Jose, CA, U.S.A.). Yeast transformants appeared on a sucrose agar plate (1% yeast extract, 2% peptone, 2% sucrose, and 2% agar) after

incubation at 30°C for 6 to 10 days. The colonies were restreaked on sucrose agar plates, and plasmid DNA was electroporated into XL1Blue *E. coli* cells and subsequently sequenced using a primer corresponding to ADH1 promoter of the pYST vectors (5'-TCCTCGTCATTGTTCTCGTTCC-3'). To eliminate redundant YST clones, the first set of approximately 50 unique cDNA inserts, obtained by digesting a mixed pool of plasmid DNAs with *EcoRI* and *NotI*, were gel purified, labeled with [α -³²P]-dCTP, and used to probe a DNA gel blot of the inserts from the next set of positive yeast colonies, obtained by colony PCR using the primers Y-5 (5'-TCCTCGTCA TTGTTCTCGTTCC-3') and Y-3 (5'-CCTTTTATCCAAGCGG CC-3'). Colony PCR was performed using a program of 35 cycles at 94°C for 30 s, 55°C for 45 s, and 72°C for 60 s, with an initial denaturing step at 94°C for 3 min and a final extension at 72°C for 10 min. Hybridization and washing conditions were as described below for the genomic DNA gel blot analysis. Plasmid DNA from each nonhybridized individual yeast colony was recovered for the second round of sequencing analysis. To eliminate the redundant clones completely from all the positive yeast colonies, several rounds of the screening were performed as described above. In all cases, cDNAs encoding proteins lacking a predicted SP were retransformed into the yeast strain to confirm the ability to allow growth on a sucrose agar plate.

Sequence analysis.

Sequences of the *PiE* and *SIE* clones were used to search GenBank, the TIGR Tomato Gene Index, the Solanaceae Genomics Network, and the *Phytophthora* Functional Genomics Database. Signal peptides and putative cleavage sites were predicted by SignalP (version 3.0). Sequences were aligned using ClustalW software and adjusted manually. Prediction of nuclear localization signals was performed using MotifScan. *P. sojae* and *P. ramorum* homologs of *PiE12* were identified in the microbial genome database and subjected to motif analysis as described above.

DNA gel blot analysis.

Genomic DNA was isolated from *P. infestans* mycelia and from young tomato leaves as described previously (Rose et al. 1997, 2002). The genomic DNA samples were digested with the indicated restriction enzymes, separated by electrophoresis on a 0.8% agarose gel, denatured, and blotted onto nylon membranes (Amersham Biosciences, Piscataway, NJ, U.S.A.). Full-length cDNA probes were labeled with [α -³²P]-dCTP using the Ready-To-Go DNA Labeling Kit (Amersham Biosciences) and purified with ProbeQuant G-50 Micro Columns (Amersham Biosciences). Hybridization and washing procedures were as described by Catalá and associates (2001) with a final wash in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% sodium dodecyl sulfate (SDS) at 65°C for 1 h. After washing, membranes were exposed to X-ray film.

RNA gel blot analysis.

Total RNA was isolated as described previously (Rose et al. 1996) from *P. infestans* mycelia and from infected tomato leaves 1, 2, 3, and 4 dpi with *P. infestans*. Aliquots (20 μ g per lane) were fractionated on 1.0% (wt/vol) agarose/10% (vol/vol) formaldehyde denaturing gels, visualized with ethidium bromide to confirm equal loading and transferred onto Hybond N nylon membranes (Amersham Biosciences). Hybridization was performed as described by Catalá and associates (2001) and washes were carried out twice in 2× SSC/0.1% SDS at 65°C and twice in 0.1× SSC/0.1% SDS at 65°C for 15 min each.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

Cornell University's Solanaceae Genomics Network: solddb.cit.cornell.edu or www.sgn.cornell.edu

European Bioinformatics Institute (EBI) ClustalW website: www.ebi.ac.uk/clustalw

Joint Genome Institute (JGI) Eukaryotic Genomics website: genome.jgi-psf.org/euk_home.html

National Center for Biotechnology Information (NCBI) GenBank: www.ncbi.nlm.nih.gov

Phytophthora Functional Genomics database: www.pfgd.org

Prosite's Motif Scan: myhits.isb-sib.ch/cgi-bin/motif_scan

SignalP server: www.cbs.dtu.dk/services/SignalP

TIGR Tomato Gene Index: www.tigr.org/tdb/tgi/tgi